Mycobacterial Diseases of Animals

Guest Editors: Mitchell V. Palmer, Michael D. Welsh, and Jesse M. Hostetter

Mycobacterial Diseases of Animals

Mycobacterial Diseases of Animals

Guest Editors: Mitchell V. Palmer, Michael D. Welsh, and Jesse M. Hostetter

Contents

Editorial
Mycobacterial Diseases of Animals

Mitchell V. Palmer,[1] Michael D. Welsh,[2] and Jesse M. Hostetter[3]

[1] *National Animal Disease Center, Agricultural Research Service, USDA, Ames, IA, USA*
[2] *Veterinary Sciences Division, Agri-Food and Biosciences Institute, Belfast BT4 3SD, UK*
[3] *College of Veterinary Medicine, Iowa State University, Ames, IA 50010, USA*

Correspondence should be addressed to Mitchell V. Palmer, mitchell.palmer@ars.usda.gov

Although *Mycobacterium tuberculosis* and *Mycobacterium leprae* are the most notable mycobacterial human pathogens, *Mycobacterium bovis*, *Mycobacterium avium* subsp. *avium*, *Mycobacterium avium* subsp. *paratuberculosis*, *Mycobacterium ulcerans*, and other mycobacteria are the etiology of important diseases in humans and a wide range of animal species including, cattle, sheep, goats, deer, possums, badgers, elephants, dogs, cats, birds, amphibians, and fish. Moreover, species such as *M. bovis* represent serious zoonotic pathogens and have become important agents at the interface of humans, domestic livestock, and wildlife.

This special issue on mycobacterial diseases of animals contains 26 papers comprising 6 reviews, 3 case reports, and 17 original research papers on various topics including animal models, immunology, epidemiology, microbiology, pathology, environment, and history. Authors from 13 different countries provide a diverse examination of mixed topics. The first 6 papers are reviews; M. Munyeme and H. M. Munang'andu discuss numerous anthropogenic factors that impact wildlife, livestock, and humans in the habitat of the endangered lechwe antelope. Bovine tuberculosis eradication efforts are impeded by the presence of an established wildlife reservoir of *M. bovis*. Accordingly, M.V. Cunha et al. describe the current status of bovine tuberculosis in Portugal, discussing interspecies transmission and the impact of infected wildlife on the status of tuberculosis in cattle. Many mycobacterioses occur in nonmammalian hosts and K. Dhama et al. provide an in-depth review of avian mycobacterioses.

In many species, pathogenic mycobacteria undergo a prolonged asymptomatic, or latent period, after which disease is reactivated in a subset of infected hosts. Containment of disease and latency likely coincide with shifts in host immune response. Accordingly, B. L. Plattner and J. M. Hostetter thoroughly review the role of gamma/delta T-lymphocytes in mycobacterial diseases of humans, cattle, and mice.

The last 2 reviews are historical in nature. First, M. Good and A. Duignan discuss tuberculin, the mainstay of TB testing. Their review emphasizes the origins, properties, limitations, and use of tuberculin in control programs, leading to the final review in which M. V. Palmer and W. R. Waters describe the genesis of the US bovine tuberculosis eradication effort in 1917. The authors describe laudable research conducted by veterinarians and other scientists, decades before an eradication program existed.

The next 3 papers are best described as case reports, discussing mycobacterioses in wildlife. M. Pate et al. provide the first description of *Mycobacterium celatum*-induced disease in heretofore-unrecognized hosts in Slovenia. W. R. Waters and colleagues describe herds of fallow deer and elk with unusually high disease prevalence. The authors demonstrate the apparent misdiagnosis of numerous *M. bovis*-infected deer and elk and show the usefulness of novel serology based diagnostic tests. M. Carstensen and M. W. DonCarlos detail the uncovering of *M. bovis* in deer and cattle in Minnesota and their efforts to identify deer to cattle transmission, determine the prevalence of disease in free-ranging deer, and methods used to prevent the establishment of a persistent wildlife reservoir.

Animal models of tuberculosis are extremely useful, particularly when conservation or ethics prevent experimental infection of the host of interest. This special issue's original research papers begin with L. McCallan et al.'s

thorough description of ferrets infected with *M. bovis* via aerosol as a model for evaluation of tuberculosis vaccines. Research papers continue with a focus on epidemiology. S. Barandiaran et al. reminds us of often-overlooked hosts of bovine tuberculosis such as swine, examining transmission of *M. bovis* from cattle to swine using spoligotyping. Disease transmission between livestock and wildlife is of great interest. The next 2 papers focus on that interface. C. C. Okafor et al. examine deer to cattle transmission of *M. bovis* in northern Michigan. From Canada, T. K. Shury and D. Bergeson describe the use of various diagnostic strategies, as well as lesion distribution, and epidemiology of tuberculosis in elk and white-tailed deer in southwestern Manitoba.

Switching pathogens, host, and geography, A. A. Rita et al. examine the prevalence of ovine paratuberculosis in Italy using both serology and fecal culture. The suggested link between *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) and Crohn's disease raises questions of public health significance. To that end, H. Okura and colleagues document a low prevalence of *Map* in muscle from *Map*-infected cattle, suggesting a bacteremic phase in bovine paratuberculosis. Emphasizing the broad host range of the myriad mycobacterial species, L. Durnez and colleagues discuss the presence of various mycobacteria in insectivores and rodents on cattle farms in Tanzania and compare findings to mycobacteria isolated from cattle.

Both *M. bovis* and *Map* are known to persist in the environment, which facilitates interspecies and intraspecies transmission. As a result, A. E. Fine et al. detail the survivability of *M. bovis* on various feedstuffs and organic matter. Prolonged survival in soil is a feature of *Map*. However, E. A. Raizman and collaborators show that not only can *Map* survive in soil, but also can pass through soil to ground water potentially finding its way to local watersheds. For researchers of paratuberculosis, the survivability of samples in the laboratory is important. E. A. Raizman et al. examine the effect of prolonged frozen storage on *Map* viability.

Great effort and immense resources have been expended to study the diagnosis of mycobacterial diseases. Further complicating diagnosis can be concurrent infection by multiple mycobacteria in the same host. Using a gamma interferon release assay, C. Barry et al. examine the cell-mediated immune response of cattle experimentally infected with both *Map* and *Mycobacterium avium* subsp. *avium*. It is believed that exposure to nontuberculosis mycobacteria, such as *Map*, can result in falsely positive tuberculin skin test reactions. Consequently, S. D. Fitzgerald et al. document a low number of false-positive tuberculin test reactors in Michigan among cattle with confirmed *Map* infection. J. A. Fernández-Silva et al. use molecular epidemiological tools to characterize *Map* isolates from cattle in Colombia, while F. Delgado et al. describe the use of in situ PCR for detection of *Map* in formalin-fixed samples.

The host range of *M. bovis* includes most mammals including wild species such as African Cape Buffalo. Tuberculosis in buffalo on public and private lands is of concern not only from an animal health and conservation perspective, but also from an economic perspective due to the negative impact on ecotourism. Consequently, H. M. Munang'andu et al. describe the use of tuberculin testing in the formation of a tuberculosis-free herd of buffalo in Zambia's Kafue Basin.

Diagnosis of mycobacteriosis is accomplished either by confirming the pathogen's presence or examination of host immune response to the pathogen. Investigation of host immune response is foundational to the development of novel diagnostics. A. Jolly et al. examine the effects of antibodies, induced by the highly immunogenic lipoarabinomannan of *Map*, on phagocytosis and pathogen survival in bovine macrophages.

In humans, (multidrug resistant) MDR and (extensively drug resistant) XDR strains of *M. tuberculosis* are of serious concern. Emphasizing the zoonotic nature of *M. bovis*, S. D. Fitzgerald and colleagues examine *M. bovis* isolates from deer in the endemic region of Michigan for evidence of drug resistance similar to MDR and XDR patterns seen in *M. tuberculosis*.

Acknowledgment

Review Article

Tuberculosis in Birds: Insights into the *Mycobacterium avium* Infections

Kuldeep Dhama,[1] Mahesh Mahendran,[2] Ruchi Tiwari,[3] Shambhu Dayal Singh,[1] Deepak Kumar,[4] Shoorvir Singh,[5] and Pradeep Mahadev Sawant[6]

[1] *Avian Diseases Section, Division of Pathology, Indian Veterinary Research Institute (IVRI), Izatnagar 243 122, India*
[2] *Avian Disease Diagnostic Laboratory, Animal Husbandry Department, Government of Kerala, Thiruvalla, Kerala 689 105, India*
[3] *Division of Bacteriology and Mycology, Indian Veterinary Research Institute (IVRI), Izatnagar 243 122, India*
[4] *Division of Animal Biotechnology, Indian Veterinary Research Institute (IVRI), Izatnagar 243 122, India*
[5] *Central Institute for Research on Goats, Makhdoom, Mathura 281 122, India*
[6] *Immunology Section, Division of Animal Biotechnology, Indian Veterinary Research Institute (IVRI), Izatnagar 243 122, India*

Correspondence should be addressed to Kuldeep Dhama, kdhama@rediffmail.com

Tuberculosis, a List B disease of World Organization for Animal Health, caused by *M. avium* or *M. genavense* predominantly affects poultry and pet or captive birds. Clinical manifestations in birds include emaciation, depression and diarrhea along with marked atrophy of breast muscle. Unlike tuberculosis in animals and man, lesions in lungs are rare. Tubercular nodules can be seen in liver, spleen, intestine and bone marrow. Granulomatous lesion without calcification is a prominent feature. The disease is a rarity in organized poultry sector due to improved farm practices, but occurs in zoo aviaries. Molecular techniques like polymerase chain reaction combined with restriction fragment length polymorphism and gene probes aid in rapid identification and characterization of mycobacteria subspecies, and overcome disadvantages of conventional methods which are slow, labour intensive and may at times fail to produce precise results. *M. avium subsp. avium* with genotype *IS901+* and *IS1245+* causes infections in animals and human beings too. The bacterium causes sensitivity in cattle to the tuberculin test. The paper discusses in brief the *M. avium* infection in birds, its importance in a zoonotic perspective, and outlines conventional and novel strategies for its diagnosis, prevention and eradication in domestic/pet birds and humans alike.

1. Introduction

Avian tuberculosis is one of the most important diseases that affect domestic and pet birds. Several mycobacterial species can be involved in the aetiology of avian tuberculosis. The disease is most often caused by *Mycobacterium avium* belonging to serotypes 1, 2, 3, and 6 (genotype *IS901+* and *IS1245+*) and *M. genavense* [1–3]. Other species, such as *M. intracellulare, M. scrofulaceum, M. fortuitum, M. tuberculosis,* and *M. bovis* can also cause avian tuberculosis, but the incidences are rare [2, 4–6]. *M. avium* causes avian tuberculosis in probably all avian species, especially in waterfowl, galliformes, columbiformes, passerines, psittacines, raptors, and ratites [1, 7–10]. The disease has a worldwide distribution but is seen most frequently in the North Temperate Zone [11–14]. Susceptibility to disease varies from species to species. Hejlicek and Treml [15] broadly classified bird species into four groups according to their susceptibility to avian tuberculosis as highly susceptible: domestic fowl, sparrows, pheasants, and partridges; less susceptible: guinea fowl and domestic turkeys; moderately resistant: domestic goose and duck, highly resistant: the domestic pigeon. In any avian species, stress factors appear to enhance the development of the disease and this is particularly noteworthy in case of birds living in captivity [4]. Infected birds and contaminated water and soil are the main source of infection as the *Mycobacteria* can survive for several months in the environment [2, 5]. The disease is more prevalent in places with high population density and poor sanitation and hygienic conditions. The practices of allowing birds to roam freely and keeping the

breeders for several years are highly conducive to the spread of tuberculosis [11]. In a flock if once established, TB induces unthriftiness, decreased egg production, and increased mortality, which culminates into severe economical losses.

Mycobacterium avium complex (MAC), comprising *M. avium subsp. avium, M. avium subsp. paratuberculosis, M. avium subsp. Silvaticum,* and *M. intracellulare*, may also infect different animal species like swine, cattle, deer, sheep, goat, horses, cats, dogs, and exotic species besides causing infection in immunocompromised human beings [3, 4, 16, 17]. *M. genavense* has also been reported in a dog and an immunocompromised cat. *M. intracellulare* is a closely related pathogen of birds with a lower prevalence [18]. Although successful experimental infections with *M. a. paratuberculosis* in poultry have been reported [19], however, this subspecies, known to cause Johne's disease (paratuberculosis) in ruminants and other mammals, has not been encountered during any of the cases of avian tuberculosis till date. *M. avium subsp. avium* (MAA) is considered as the most important pathogen causing tuberculosis in domestic birds [2, 20]. On the basis of genetic and phenotypic differences it has been proposed to categorize MAA into two subspecies, namely, *M. a. hominissuis* for human and porcine isolates and *M. a. avium* for bird-type isolates [21]. In humans, *M. avium* is capable of inducing a progressive disease that is refractory to antibiotic treatment and is recognized as localized primary lymphadenitis, pulmonary disease, and a disseminated form of infection [4, 22]. Hence the handling of infected birds in farms or live cultures of *M. avium* in laboratories should be carried out with adequate care.

2. Etiology

M. avium, the causative agent of avian tuberculosis, considered as "atypical mycobacteria", comprises aerobic, nonspore-forming and nonmotile rod shaped bacteria that vary in length from $1-3\,\mu m$ and cords are not formed, unlike *M. tuberculosis* [2]. They are weakly Gram-positive and stained specifically by acid fast (Ziehl-Neelsen) staining method, due to high levels of lipids in mycobacterial cell wall. *M. avium* is highly resistant to environmental challenges and can survive in soil for up to 4 years, and this makes eradication of the organism difficult [1, 2, 23]. *M. avium* is resistant to high and low temperatures, dryness, pH changes, and many commonly used disinfectants. However, the unprotected organism is killed by direct sunlight. In contrast to *M. tuberculosis* and *M. bovis*, *M. avium* grows at temperatures ranging from 25–45°C, the most favorable range being 29–45°C and for primary isolation, growth can be enhanced with 5–10% CO_2 tension [3, 23]. Strains of *M. avium* can be identified by serological procedures. To date, 28 MAC serotypes have been identified from which the serotypes 1–6, 8–11, and 21 belong to *M. avium* subsp. *avium* (MAA). Serovars 7, 12–20, and 25 have been ascribed to *M. intracellulare*. However, no consensus was achieved on other serovars, and some isolates cannot be typed [3, 24]. Serotypes 1, 2, and 3 are considered virulent for chickens (Table 1) [2, 11, 23]. Serotypes 1 and 2 are most commonly isolated from domestic birds, and serovar 3 is

TABLE 1: Serotypes of *M. avium* complex (MAC) and their susceptibility to various species of certain birds and mammals [2, 9].

Species	MAC serotypes	Susceptibility
Domestic fowl (*Gallus domesticus*)	1, 2	High
Turkey	1, 2	Moderate
Pheasants	1, 2	High
Wild birds	2, 3	High
Cattle	1, 2	Moderate
Swine	1, 2, 4, 8	High
Rabbit	1, 2	High
Man	1, 4 to 20; 23, 25	Low (in healthy individuals); High (in immunocompromised)

recovered sporadically from wild birds. Serotypes 1 and 2 can affect animals, whereas 4–20 are mainly found in humans. Serovar-1 is the most common organism isolated from birds and from human beings. Distinguishing serovars can help provide a means for studying origin and distribution of specific strains. According to the current taxonomy, *M. avium* contains four subspecies, namely, *M. avium* subsp. *avium*; *M. avium. hominissuis*; *M. avium. Paratuberculosis; M. avium. silvaticum,* which is diagnosed rarely in birds [3, 21]. It is well established that most *M. a. avium* isolates from birds have a repetitive sequence *IS901* in their genome and also produce a characteristic three band pattern in *IS1245* restriction fragment length polymorphism (RFLP) [25]. It has been postulated that the presence of *IS901* correlates with pathogenicity in birds [25–27]. Other than *M. a. silvaticum*, IS901 has only been detected in *M. avium* strains with serotypes 1, 2, and 3 [5].

M. avium is the most significant cause of poultry disease. Disease onset in birds is normally more rapid with *M. genavense* than with *M. avium*. In wild birds, though the disease is uncommon, TB may develop when they are in contact with infected chickens. *Mycobacterium avium* complex and *M. intracellulare* can also infect an extensive range of different animal species. *M. tuberculosis* is less commonly the cause of infection in birds, often as a result of transmission from pet bird owners, and also clinical signs differ from those caused by the more commonly occurring species of mycobacteria. In case of psittacine birds, apart from this, tuberculosis due to *M. tuberculosis* or *M. bovis* has also been reported. In canaries, tuberculosis may be caused frequently by *M. tuberculosis* [2].

3. Transmission

The main source of infection is infected birds as they shed large amounts of organism into the environment. The bacilli are exuded from ulcerated lesions of the intestine and are voided in droppings. The most common route of infection for susceptible birds is the alimentary tract [1, 2]. Respiratory tract is also suggested as a potential source of infection. The disease gets transmitted to the susceptible birds by ingestion and inhalation of aerosolized infectious organisms.

Persistence within flocks is associated with keeping older stocks without following adequate cleanliness and hygiene [2]. Further, maintaining birds closely confined under stressful conditions provide favorable ways for the spread of the disease. The ability of the organism to persist in the environment for many years, especially in soil and litter favor the disease transmission to a great extent [5]. Litter, pens, equipment, and pasture contaminated with excreta of infected domestic birds and the hands, feet, and clothing of attendants play an important role in disease transmission. Wild birds, pigs, and some mammals may also act as significant reservoirs of infection [2, 11]. Wild birds, such as sparrows, crows, and pigeons may be infected with *M. avium* and may spread it to poultry flocks [7]. Also, rats and other rodents are known to act as mechanical carriers in transmission of the disease. The agent can also be disseminated by infected carcasses and offals. Occasionally, skin invasion and spread via infected eggs may occur. *M. avium* has been isolated from eggs of naturally infected chickens, but hatched chicks have not developed the disease [2]. The bacilli does not survive in eggs after proper boiling.

4. The Disease and Manifestations

Avian tuberculosis is a contagious disease which occurs in chickens, pheasants, quail, guinea fowl, turkeys, parrots, budgerigars, ducks, goose, doves, partridges, pigeons, and other captive and wild game birds and has also been reported in ostriches, emus, and rheas in many zoological parks. Tuberculosis in birds is most prevalent in chickens and in wild birds raised in captivity. In poultry, the disease follows a slow course through the flocks. The classical presentation is characterised by chronic and progressive wasting and weakness. Avian tuberculosis in domestic birds is primarily an intestinal and hepatic disease with dissemination to other organs including the lungs, air sacs, spleen, bone marrow, and skin [2, 6, 11, 23]. Similarly, avian tuberculosis reported in free living birds including raptors were presented with the disseminated form involving the digestive tract, liver and spleen [8, 28, 29]. The disease has a long incubation period and a protracted course and if appreciable, the symptoms can prolong for weeks or months. Because of the chances to become established through a longer exposure, the disease is less prevalent in young fowls and lesions are less severe in them when compared to adult birds. Usually the losses are experienced more in older stocks of age group 18–20 months. The disease process can be divided into three phases: latency, lesion development, and period of cachexia [2, 5, 11]. During cachexia, massive tubercles with large numbers of bacilli develop. In the classic form of infection the tubercles or granulomas develop in multiple organs; a second form is manifested with lesions in the intestinal tract; a third type of infection often experienced as a nontuberculous one, mainly seen in finches, canaries, and psittacines [2, 5]. Some birds show respiratory signs and sudden death may occur, dyspnoea is less common, and granulomatous ocular lesions [30] and skin lesions have been reported.

Clinical signs are not pathognomonic in avian TB and vary depending on the organs involved. Birds with the intestinal form of tuberculosis often present with chronic wasting disease. In majority of cases of tuberculosis in birds, especially in the initial phase of infection, clinical signs are not grossly observable. However, in advanced cases, birds may develop symptoms like progressive weight loss, depression, white diarrhea with soiled feathers, increased thirst, respiratory distress, fatigue, and decreased egg production [11, 23, 31–33]. Feathers are often dull or ruffled and comb, wattle, and earlobes often appear pale, thinner and dry. Birds eventually become lethargic and emaciated with marked atrophy of breast muscles manifested as "knife edged" keel [2, 5]. In extreme cases, the body fat disappears, and the face of the bird appears smaller than normal. If a jerky hopping gait is observed due to unilateral lameness then it should be assumed that there could be the presence of tubercular lesions in bone marrow of the leg bones or joints. Some birds may adapt a sitting position. Tuberculous arthritis can even lead to paralysis. Fatal results often occur due to massive hemorrhage caused by ruptured liver or spleen. In this case, occasionally birds may die suddenly in good bodily condition and yet show advanced lesions of tuberculosis. The body temperature of the affected bird remains normal, even in severe cases. In most cases, an infected bird without overt clinical signs may serve as carrier that result in the persistence of infection in flocks. In commercial broiler production units, generally avian tuberculosis is uncommon primarily due to the short life span and in layers and breeders, the infection is a matter of much concern. Mortality over a short period may be insignificant, but the intermittent loss of adult birds in valuable breeding stock and decreased egg production in layers are detrimental. Occasionally, heavy losses may occur in pullets on multiage sites where the infection is endemic and the hygienic standards are poor.

After entering the host, *M. avium* prevents the fusion of phagosomes with lysosome and the subsequent bacteremia provides a generalized distribution of lesion. The gross lesions are characterized by the presence of epithelioid cells containing large numbers of organisms that may either diffusely infiltrate the organ or form discrete granulomas [6]. There is presence of tubercular nodules in intestine, liver, spleen, ovaries, testes, and bone marrow but the pulmonary lesions, which are a striking feature of tuberculosis in other species, are rarely observed in birds [2, 5]. Pulmonary avian tuberculosis is only seen occasionally as in case of tuberculosis of pigeons and water fowl [1, 2]. The principal lesions of tuberculosis in birds are seen in intestine, where affection often presents with studded greyish-white to greyish-yellow nodules. Before the intestinal tract is opened, the ulcerated areas appear as tumour-like masses attached to the gut wall, but when the intestine is opened, the true nature of the mass becomes evident. The nodules bulge from the serosal surface of the intestine and can be palpated. Due to this, spleen takes irregular "knobbly" appearance. Lesions evident as deep ulcers filled with caseous material discharges the organism into the intestinal lumen and get excreted via the droppings. Typical caseous lesions, without calcification, are always found in the liver and spleen, with considerable enlargement of the organs [2, 5]. Nodules are firm but can be incised easily since mineralization is rare in avian TB (this is
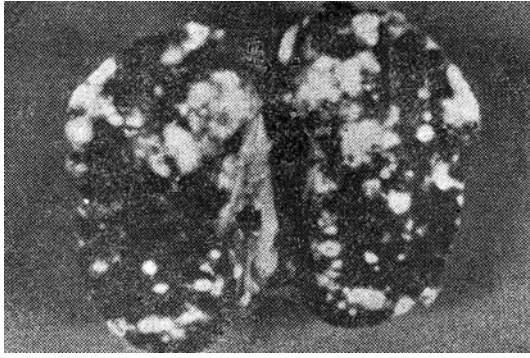
FIGURE 1: Spleen of Demoiselle cranes (Anthropoides virgo) showing caseous nodules, measuring 1–5 mm in size, on the cut surfaces of the organ.
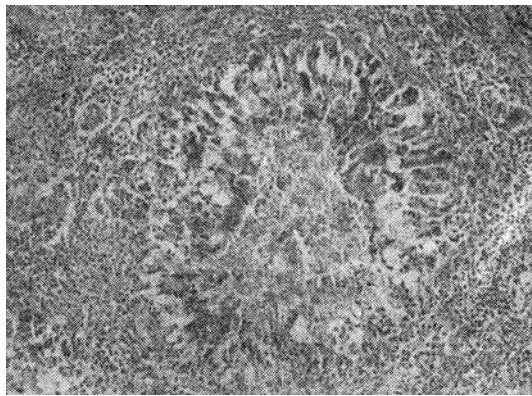
FIGURE 2: Section of spleen showing a granuloma with associated inflammatory cells, H&E × 120.

in contrast to leucosis, in which lesions cannot be enucleated from the surrounding tissue). The bone marrow of the long bones frequently contains tubercular nodules. Some exotic bird species may have lesions in the liver and spleen without intestinal involvement. Microscopically, lesions consist of granulomas with a central necrosis, either coagulative or caseous, and multinucleate giant cells. Acid fast bacilli are numerous in the central or necrotic zone of the tubercle [2, 11]. Gross and microscopic lesions in spleen of Demoiselle cranes (*Anthropoides virgo*) are depicted in Figures 1 and 2.

The incidence of avian tuberculosis in pet birds kept in captivity appears to exceed the prevalence in poultry [22, 34]. Some of the reasons of the incidence of the infection in pet birds are age of the host, population density, and the ability of organism to survive environmental inclemency [2]. Contact with contaminated water, soil, or feed predisposes to infection [22]. In case of pet birds, the etiology of avian tuberculosis is rarely identified due to the difficulty in isolating some mycobacterial species [23]. Weight loss, diarrhea, dyspnea, lameness, and poor feathering are the usual signs in pet birds. Earlier, most cases of infection were assumed to be caused by *Mycobacterium avium* complex (MAC). However, the use of molecular techniques brought to light the prominent role of fastidious mycobacteria,

primarily *M. genavense*, in avian tuberculosis of pet birds [22]. *M genavense* is responsible for the majority of avian mycobacterial infections (up to 80%) in pet birds while the MAC was found responsible for 5% to 10% of mycobacterial infections [22]. In pet birds, *M. genavense* causes a disseminated disease with clinical and histopathological features indistinguishable from infection caused by members of the MAC [34].

Recently, avian tuberculosis in domestic poultry have declined due to changes in poultry husbandry practices, namely, integrated poultry farming, emphasizing all-pullet flocks rather than older hens and maintaining one-age flocks, all in all out farming system, along with better hygiene, disinfection, and biosecurity practices. However, the occurrence of avian TB in birds in zoo aviaries is still an economically important affair since certain species of exotic birds are of high value and most of these birds will be in endangered or near extinction categories. Avian TB is more common in zoological parks, perhaps because of inadequate cleaning and disinfection of pens. Caged birds are reported to soon succumb to avian TB.

M. avium can infect and cause disease in some domesticated mammals but lesions usually are localized and less severe. It multiplies in tissue for a considerable period and induces sensitivity to tuberculin. Swine, rabbit, and mink are readily infected; infection has been reported in cattle and horse; monkey is also susceptible; while goat, guinea pig, rat, and mouse are relatively resistant to infection, cat and dog are highly resistant to *M. avium* infection [16, 17].

5. Infection and Immunity

The cellular arm of the immune system is more important than the humoral arm in preventing and controlling mycobacterial infections [2, 5]. Delayed type of hypersensitivity (DTH), judged by the thickness of wattle, is evident at 2 days after infection and increases as the disease progresses. The organism after entry when phagocytosed by nonactivated macrophages is able to downregulate its killing mechanism by preventing normal fusion of the phagosome with lysosomes. Macrophages that lack microbicidal components are destroyed by the intracellular growth of the organism, and a lesion develops. Also, during infections, thymus is consistently colonized by *M. avium* and as the T-cell differentiation depends on the antigens encountered within the thymus, infection of this organ can alter the immune response to infection [35]. However, if activated, the macrophages can readily destroy and degrade phagocytosed mycobacteria [11, 36]. They have usually good killing potential against the invading mycobacterial species [37, 38]. This is augmented by the release of lymphokines like tumor necrosis factor (TNF) and interleukin-2 (IL-2), which helps in killing *M. avium*. Macrophage activation is also performed by interferon gamma (IFN-γ), which is released by a subset of CD4+ T lymphocytes and natural killer (NK) cells on stimulation by the interleukins released by the macrophage during its encounter with the mycobacteria. The T lymphocytes also stimulates B cells to produce antibodies against mycobacteria but these antibodies do not appear to have a major protective

effect for the host against infection and high antibody titers can be correlated with serious infections [6, 36, 39]. Recently, it has been identified that lipoarabinomannan, an important outer cell wall component of mycobacteria, are highly potent nonpeptidic molecules which can be used to modulate the host immune response [40].

6. Diagnosis of Infection

The diagnosis of M. avium infection is based on clinical signs, postmortem gross lesions, and by demonstrating the acid-fast bacilli in crushed lesions using microscopy, which is sufficient for a positive diagnosis [2–5]. If acid-fast bacilli are not found, but typical signs or lesions are present in the birds, culture of the organism must be attempted. In necropsy, liver or spleen is usually the best organ to use, but if the carcass is decomposed, bone marrow may prove more satisfactory as it could be less contaminated. In live birds, cultural examination using feces or tracheal swabs is necessary to isolate and identify the etiological agent [23, 41]. But usually a definitive diagnosis is performed by culturing the organism in suitable media,namely, Dorset's or Herrold's egg yolk medium, Lowenstein-Jensen medium, Middlebrook 7H10 and 7H11, or Coletsos medium, with 1% sodium pyruvate [3–5, 23]. For growth of M. avium, media containing whole egg or egg yolk is desirable and the incubation temperature should be 37–40°C. Growth may be confined to the edge of the water of condensation. Cultures should be incubated for at least 8 weeks. Typically M. avium produces "smooth" colonies, within 2–4 weeks; rough variants do occur; smooth transparent colonies are virulent for chickens while variants with smooth domed or rough colonies are avirulent. The colonies, observed only after 10–21 days of incubation, are small, slightly raised, discrete, and grayish white in appearance [2, 11]. Colonies are larger if the medium contains glycerin. Shorter incubation times can be achieved using the liquid culture BACTEC system. Some strains of M. avium have been identified to have special requirement of mycobactin as a growth factor.

Recently, comparison of the different methods, namely, the conventional culture method (solid Herrold's and Stonebrink media and liquid Sula medium) and newly developed liquid culture systems, the manual mycobacteria growth indicator tube (M-MGIT), and the fully automated BACTEC MGIT 960 system (A-MGIT), for the detection of M. avium subsp. avium (MAA) in naturally infected hens revealed overall detection rates to be 60, 70, and 76%, with the mean time of mycobacteria detection being 32.6, 17.6, and 14.6 d, respectively [42].

In live birds, during life time, besides the culture and isolation techniques, immunological tests, namely, tuberculin test; whole blood agglutination test and enzyme-linked immunosorbent assay (ELISA) are also valuable diagnostic tools; and nowadays various molecular tools are also being employed for identification of the causative agent at subspecies level and epidemiological studies (Table 2). Using standard purified protein derivative (PPD) of heat-treated culture of M. avium, tuberculin test can be performed in the wattle, which is considered as the test of choice in domestic fowl/poultry. This test is less useful in other species of bird. Birds are tested by intradermal inoculation of 0.05 mL or 0.1 mL tuberculin (2000 IU) and the test is read after 48 hours [2]. A positive reaction is identified as a hot and oedematous swelling at the site or by the presence of a small firm nodule of approximately 5 mm in diameter [5, 11]. It serves as a means of identifying birds infected with or sensitized to the same species of tubercle bacillus. Tuberculin test has 80% accuracy in detecting infective birds relative to gross lesions but in an advanced stage of infection birds may give no reaction. In whole blood agglutination test, a drop of antigen (M. avium stained with 1% malachite green) is mixed with a drop of blood and a positive reaction is indicated by agglutination within few minutes [5, 48]. It is a better test, especially for waterfowl. Advantage of this test is that stock has only to be handled once, but false positive reaction is a disadvantage which makes the test a less specific one. The tuberculin test or the haemagglutination (stained antigen) tests are most frequently used for export testing of poultry. However, neither the tuberculin test nor the agglutination test is likely to be of any value in cases of M. tuberculosis infection in caged birds. ELISA which is reported to be less specific than tuberculin test can detect specific antibodies and thereby help determine exposure to M. avium. However, false positives may be common in ELISA. The identification of immunogenic proteins of M. avium may favor the development of more precise serodiagnostic tools [49]. Tuberculin test and serological tests are normally used to determine the prevalence of disease in a flock, or to detect infected birds. When used to detect the presence of tuberculosis in a flock they should be supported by the necropsy of any birds that give positive reactions. IFN-γ assay used to diagnose human tuberculosis may also be useful in diagnosing the infection in birds.

Species and subspecies level typing of mycobacteria requires a specialised laboratory. Conventional biochemical tests for species identification are lengthy and fail to distinguish between M. avium and M. intracellulare. Classification of MAC organisms into 28 serovars has been made by seroagglutination [3]. The MAC colonies can be identified using high performance liquid chromatography (HPLC) for detecting mycolic acid [4]. HPLC and use of monoclonal antibodies to major serovars in ELISA also facilitates typing of mycobacteria. In the past decade, biotechnological tools, exploiting nucleic acid detection methodology, like DNA probes, polymerase chain reaction (PCR), and PCR-restriction fragment length polymorphism (RFLP) are being widely employed for specific detection of the etiological agent [3, 4, 50, 51]. Commercial nucleic acid hybridisation probes have become a "gold standard" for distinction between M. avium, M. intracellulare, and M. genavense [2, 5, 41, 52]. For intraspecies genotyping, pulsed-field gel electrophoresis of large DNA restriction fragments has proved to be highly sensitive [53]. The PCR approach, using species-specific primers is also capable of specifically detecting DNA fragments of M. avium genome, thus acting as a diagnostic alternative to the conventional procedures [41, 54–57]. Also, a multiplex PCR method has been developed for the determination of the subspecies within M. avium species,

TABLE 2: Diagnostic methods and tests used in birds [2, 3, 5, 23, 43–47].

Type of test	Performed in	Time required	Merits	Demerits
Observing gross lesions	Dead birds	1 hour	Easy diagnosis	Only presumptive diagnosis
Acid fast staining	Dead birds	1 hour	Easy definitive diagnosis	Less sensitive, Not able to distinguish amongst species
Isolation/Culture	Dead birds	About 4 weeks	Definitive diagnosis	Time consuming
Tuberculin test	Live birds	48 hours	Easy to perform Definitive diagnosis	Time consuming, Test is not very sensitive, Possibility of false positive and false negative results
Agglutination test	Live birds	Few minutes	Can differentiate serotypes. Useful for screening large flocks for immediate culling	Occasionally false positive reactionsNot reliable in caged birds
ELISA	Live birds	2 hours	Definitive diagnosis Can be used for exotic and pet birds	Less specific than tuberculin test False positives may be there
DNA probes	Bacterial cultures	4–6 hours	Highly sensitive and specific	Probe may react with isolates that genetically or biochemically do not fit within the MAC
PCR	Dead/live birds/cultures	4 hours	Highly sensitive and specific	Requires specialized laboratory and trained personnel
RFLP	Bacterial cultures, clinical samples	1 day	Differentiates mycobacteria to the species level Discriminative for the analysis of strain relatedness	Insufficient quantities of gene makes visualization of digested fragments difficult
Multiplex PCR	Bacterial cultures/clinical samples	5–8 hrs	Rapid and inexpensive technique for subspecies identification and differential diagnosis of the MAC complex	Requires specialized laboratory
Sequencing of the 16S rRNA gene	Bacterial cultures	2 days	Powerful technique for differentiating species	Labor-intensive and difficult to implement in routine diagnosis
HPLC	Bacterial cultures	1 day	Can identify *Mycobacterium* isolates to the species level	Uses costly equipment and requires substantial amounts of the test organism.
Real-Time PCR	Bacterial cultures/clinical samples	4–6 h	Low risk of sample contaminationOffers the possibility to quantify bacterial load	Sensitivity could be affected by the initial volume of DNA present
MIRU-VNTR/MATR-VNTR typing	Bacterial cultures/clinical samples	1 day	Improves RFLP discrimination Useful for determination of genotypic diversity of *M. avium* subspecies	Requires specialized laboratory
Pathogenicity tests	Live young birds	5–6 weeks	Likelihood of the etiological agent can be knownUseful in cases where the typing facilities are not available	Time consuming and concerned to ethical issues

and for differentiating *M. avium* from *M. intracellulare* and *M. tuberculosis* complex [56, 58, 59]. Efficient differentiation of MAC species and subspecies by use of five-target multiplex PCR, designed to amplify a 16S rRNA gene target common to all Mycobacterium species, has been proved to be rapid, reliable, and simple [60]. Lappayawichit et al. [61] reported the differentiation of species of mycobacteria by amplifying 16-23S ribosomal DNA and further digesting with restriction enzyme like *Hae* III, *Msp* I, and *Bst* XI.

Likewise, for differentiation of various mycobacterial species, insertion sequences (IS) in DNA molecule have been identified. *IS 901* and *IS 1245*, which are virtually *M. avium* specific, has been shown to be the most discriminative for the analysis of various strains based on PCR-RFLP [1, 5, 25, 62]. Generally, the PCR-RFLP analysis of suspected tissue samples like liver, spleen, and gonads can be performed targeting 16S-rRNA gene for *Mycobacterium* spp., IS6110 for *M. tuberculosis*, IS1245 for MAC, IS901 for *M. avium* subsp.

Avium, and hsp65 for *M. genavense* [34, 58, 63–66]. Utility of PCR-RFLP of hsp65 has been reported for the identification of *M. avium* [67]. O'Grady et al. [43] performed RFLP investigation using probes derived from IS901, IS1245 and IS1311 to study the molecular epidemiology of *M. avium,* and *M. intracellulare* infection, in particular to gain an understanding of the sources of infection in humans. 16S rRNA and hsp65 sequencing may also be used to differentiate between mycobacterial strains and for distinguishing the *M. avium* subsets [68–72]. The real-time TaqMan PCR assay targeting the hsp65 gene of *M. genavense* and MAC subsp. may provide a useful tool for evaluating clinical samples for DNA from mycobacteria species that most commonly infect birds [44]. Slana et al. [45] has recently developed a real-time quantitative PCR for the identification and quantification of *Mycobacterium avium* subsp. *avium* and *M. a. hominissuis.* Other novel tests like IFN-γ assay, GenoType assay, and DNA microarrays that are used to diagnose human tuberculosis may also be useful in diagnosing the infection in birds [22, 73, 74]. More recently, the use of molecular techniques for species identification brought to light the prominent role of nonculturable mycobacteria, primarily *M. genavense,* in several cases of avian tuberculosis in pet birds [5, 34].

Utilization of new variable-number tandem-repeat markers (VNTRs) of genetic elements called mycobacterial interspersed repetitive units (MIRUs) for typing *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* strains has been reported to be fast typing method, and which in combination with other methods, might prove to be optimal for PCR-based molecular epidemiological studies [46]. More recently, the usefulness of a MIRU-VNTR typing has been described for determination of genotypic diversity of *M. avium* subspecies (*M. avium* subsp. *avium,* *M. avium* subsp. *hominissuis,* and *M. avium* subsp. *Silvaticum*) from human and animal origins [47]. Inagaki et al. [75] reported MATR (*Mycobacterium avium* tandem repeat—MATR)-VNTR typing method (MATR-VNTR) to be having excellent discriminatory power compared with MIRU-VNTR and IS1245-RFLP typing; and its concomitant use with IS1245-RFLP typing increases the discriminatory power. MATR-VNTR typing is inexpensive and easy to perform and thus could be very useful for epidemiological studies.

In case the typing facilities are not available, pathogenicity tests are performed for knowing the likelihood of the etiological agent, which should be carried out on the species of bird being investigated, but failing that, domestic fowl or Japanese quail may be used. Young adult birds are best, and when inoculated intravenously with 1 mL of the suspension (culture at 0.1 mg/mL) the bird will die in 5–6 weeks if the organism is virulent, or, by that time, the bird will have extensive lesions filled with acid-fast bacilli [3].

Avian tuberculosis should be differentially diagnosed from those diseases that are known to develop tumorous or granulomatous lesions in gastrointestinal (GI) tract and other visceral organs. Diseases that are to be differentially diagnosed are pseudotuberculosis (common in ducks and turkeys caused by *Yersinia pseudotuberculosis*), Coligranuloma (Hjarre's disease-*Escherichia coli*), neoplasia due to lymphoid leucosis (Retrovirus) or Marek's disease (Herpes virus), fowl cholera (*Pasteurella multocida*), Pullorum disease (*Salmonella* Pullorum), and enterohepatitis (Black head, *Histomonas meleagridis*) [9].

7. Therapy in Avian Tuberculosis

Generally, mycobacterium infections caused by *M. tuberculosis* and *M. bovis* are treated with antibiotics such as isoniazid, ethambutol, rifampicin, and pyrazinamide in human beings [6]. Treatment of infected animals is rarely attempted because of the high cost and prolonged time. Moreover it is considered illegal in some countries. *M. avium,* on the other hand, is resistant to these antituberculosis drugs [2]. Due to this fact and also because of the economical considerations, treatment is not considered a viable option, particularly in poultry sector. However, in case of *M. avium* infection of exotic pet birds or birds maintained in zoo aviaries, treatment against *M. avium* has to be considered and therapy duration can go up to 12–18 months. In avian therapeutics related to mycobacterial infections, the major difficulty is that the pharmacokinetics in birds for most of the antimycobacterial drugs is unknown [6]. Also, the relative hydrophobicity of the mycobacterial cell wall acts as a barrier that restricts the activity of many hydrophilic antibiotics like the aminoglycosides, fluoroquinolones, and macrolides [6]. Besides, the slow growth and intracellular location of mycobacteria necessitate the need for extended periods of therapy.

There are clinical reports documenting the apparent successful treatment of parrots with mycobacterial infections, but no studies to date investigate the treatment of mycobacterial infections in birds [6]. *M. avium* has been reported to respond to trimethoprim-sulfamethoxazole, sulfisoxazole, amikacin, gentamicin, and kanamycin, during *in vitro* studies [76]. *M. avium* infections in pet birds have been treated with isoniazid, rifampin, rifabutin, ethambutol, clofazimine, ciprofloxacin, enrofloxacin, streptomycin, and amikacin and successful therapy of *M. genavense* infections with clarithromycin in humans has been reported [6]. The apparent effectiveness of the newer macrolides like clarithromycin and azithromycin against both *M. avium* and *M. genavense* make them suitable for treating mycobacterial infections in birds. However, the initial therapeutic regimen should include rifabutin and ethambutol, and later azithromycin or clarithromycin can be administered concurrently. Birds that respond poorly to therapy should have either a fluoroquinolone or an aminoglycoside added to the regimen. An alternative or additional drug that may prove useful, especially in birds with a marked inflammatory response, would be clofazamine. All these drugs may be curative at a total daily dose of 85 mg/kg for clarithromycin, 43 mg/kg for azithromycin, 56 mg/kg for rifabutin, 56 to 85 mg/kg for ethambutol, and 6 to 12 mg/kg for clofazamine as per the reports of VanDerHeyden [6]. In another study, to augment the potential of existing drugs, a mycobacteriolytic preparation called "stazyme" has been developed from the *Staphylococcus* strain Clavelis. Stazyme was able to break the permeability barrier of *M. avium* isolates, significantly enhancing the activity of anti-tuberculous drugs like ethambutol, rifampicin, and amikacin [77].

8. Preventive Measures

The eradication of M. avium infection is difficult due to the chronic carrier state and intermittent shedding of organisms by the infected birds. Measures to eliminate disease and establishing/maintaining TB-free flock should be followed. Gill and Blandy [78] and Dhama et al. [11, 79] described measures like sacrificing the affected flocks, abandoning the equipments and housing materials, removal of litter and contaminated soil, elimination of older flocks, following of strict biosecurity procedures besides regular monitoring with tuberculin and agglutination tests. Stress is a key factor as it causes an increase in the rate of shedding to precipitate outbreaks. The best way to control this disease is to remove infected ones and carriers and also to reduce stress factors by improving the environmental parameters [2, 11]. Prevention is best done by minimizing overcrowding, providing proper ventilation and supplementing adequate amounts of vitamins and minerals in diet. In case of avian TB in a farm, birds in other flocks in the same farm should be quarantined and tested at 6–12 week intervals. Neither the tuberculin nor the agglutination tests can be depended upon for the detection of every infected bird, therefore, as long as one infected bird remains in a flock, dissemination of disease is possible. So entire flock needs to be depopulated and repopulation on noninfected soil and fresh litter may be the best approach. Frequent removal of fecal material is the single most important factor in preventing transmission. Disinfection of the poultry houses should be done frequently. The practice of managing poultry in free-range system should not be followed. Provide proper biosecurity measures to prevent unrestricted movement of chickens, thus preventing exposure from previously infected premises or from wild birds, pigs, and other mammals. For exotic birds prevent contact with infected birds or the premises and housing previously used by them. Monitoring for infection and disposing of positive reactors should be followed along with practicing all reasonable hygienic precautions to prevent entry of the infection. During the import of exotic or domestic birds, tuberculin testing must be mandatory in order to identify the presence of M. avium and the newly introduced birds should be quarantined for a minimum of 2 months [2, 5]. The difficulty of tuberculin testing of all chickens or even a majority of flocks makes it impossible to obtain exact data on the incidence of *M. avium* infections of chickens. No vaccines are available for use in birds. Experimental vaccines with killed and/or live mycobacteria for protecting chickens against TB have been evaluated. Satisfactory protection was obtained when M. avium serovar 6 was given orally [5, 9, 11]. Combination of inactivated and live M. avium serovars 7 and 19 can also give protection to a limited extent. Nucleic acid-based vaccines may also be experimentally tried using M. avium genes that can generate proteins to elicit cell mediated immunity in birds [80, 81]. Simple, whole cell or lysate vaccines and combinations of vaccine preparations were identified that led to high levels of protection [82].

9. *Mycobacterium avium*: The Zoonotic Implications

Mycobacterium avium subsp. *avium* (MAA) represent veterinary and economic risks in birds (mainly poultry) as well as mammals (pigs, etc.). Infected animals and their products (mainly eggs) often come from small household production and pose a risk for human health [83]. Exposure of humans to infected birds with the MAA microorganism may cause a zoonotic infection, particularly in those with immunocompromised diseases such as HIV/AIDS [84]. In addition, the situation worsens due to the spread of HIV infection in developing countries [85]. Unlike *M. tuberculosis*, human beings are generally resistant to *M. avium* infection but occasionally they can get infected. Human pulmonary tuberculosis due to avian tubercle bacilli has been reported during the early 1940's [86]. High incidence of sensitivity to avian tuberculin in man has also been identified [87, 88]. It is essential to bear in mind that *M. avium*, *M. intracellulare,* and *M. genavense* are of public health concern mostly in immunocompromised hosts. Infections of humans and animals caused by this *M. intracellulare* agent are expected to rise. The *M. avium* infection, seen in many AIDS patients, is a progressive disease that is refractory to treatment [65, 89–91]. This is especially true in cases of exposure to large numbers of organisms [6]. In humans, *M. avium* is capable of inducing localized primary lymphadenitis, pulmonary disease, and a disseminated form of infection particularly in case of immunosuppressed individuals or patients under transplant therapy [4, 22, 92, 93]. *M. avium* also causes local wound infections with swelling of regional lymph nodes. In adults, the organism frequently affects the lungs, producing respiratory signs and in children, the cervical lymph nodes are often involved. Eccles and Ptak [93] reported that *M. avium* causes a serious disseminated bacterial infection in up to 40% of patients with advanced HIV infection. In AIDS patients, the main route for *M. avium* infection is the gastrointestinal tract and *M. avium* is naturally tolerant to the low pH that exists in stomach [94]. The transmission occurring via aerosols results in pulmonary infections as the organism frequently affects the lungs with endobronchial lesions [95]. During the infection, *M. avium* can be demonstrated *in vivo* in lymph nodes, bone marrow, urine, and sputum [87, 91, 95, 96]. Primarily, the serotype-1 of *M. avium* subsp. *avium* has been isolated from such individuals, clearly pointing role of birds in acquiring infection [4]. It should also noteworthy that *M. avium* is a pathogen that infects several hosts including birds, humans, cattle, and pigs [45, 97, 98]. They are also encountered in environmental sources like soil and water, having considerable ability to overcome adverse and competitive conditions thanks to a major preprotein translocase subunit that is coded by *secA* gene of the species [99].

During a study from 1953 to 1968, in cattle and swine of Great Britain, 13% of the total tubercle bacilli were typed as *M. avium* and in pig population it was an astonishing 81%. This should be correlated with the ever increasing number of recorded cases of tuberculosis in man caused by *M. avium* [64, 100]. Contaminated food originating from pig or other
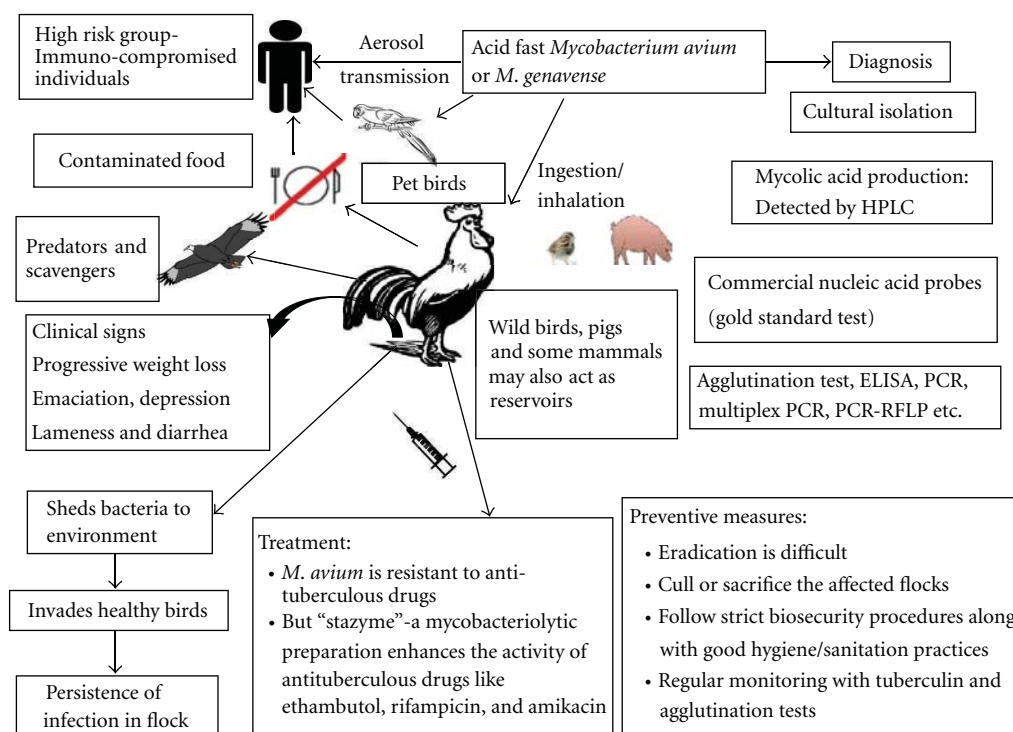
FIGURE 3: Salient features of avian tuberculosis.

livestock is identified as potential source of human infection. *M. avium* can infect and cause disease in some domestic mammals but lesions usually are localized and less severe. When domestic farm animals are infected, particularly cattle and pigs, tuberculous lesions are commonly found localized to the head and intestinal lymph nodes [1, 101]. As reported by some workers, *M. avium* isolates from swine represent the major threat to human beings. The similarity of the IS1245 RFLP patterns of the human and porcine isolates indicates close genetic relatedness, suggesting that *M. avium* is transmitted between pigs and humans [64, 102].

Regarding the therapeutics, *M. avium* is of special concern because drug regimens commonly used for treating tuberculosis in humans are not effective [22]. *M. avium* strains are notorious in being resistant to isoniazid, the most popular anti-TB drug [103, 104]. However the infection was found subsided when treated with azithromycin or clarithromycin together with ethambutol [6, 105]. Further, the *M. avium* isolates have been demonstrated to get inhibited by sufficient concentrations of sulfamethoxazole in serum [106]. Rifabutin prophylaxis may also help in controlling the disseminated infection [93]. Dunne et al. [107] suggested the use of azithromycin as a safe, effective, and convenient option during disseminated infection in HIV-infected patients. As per the findings of Horgen et al. [108], rifampin-clarithromycin and rifampin-amikacin are the most potent two-drug combinations, while rifampin-amikacin-clarithromycin has been identified as a potent three-drug combination. Likewise, Saito et al. [109] suggested the use of benzoxazinorifamycin in combination with clofazimine to be highly efficacious in the therapy of

M. avium infections. In case of infection with *M. genavense*, clarithromycin is the better choice when compared to azithromycin [6].

Besides therapeutic interventions, there have been numerous attempts to check the *M. avium* isolates in both environment and in host. Lin et al. [110] suggested that copper-silver ionization of drinking water is a better option for the effective control of *M. avium*. David et al. [111] proposed the use of synthetic macrocyclic compounds as a new family of compounds that are capable of acting against *M. avium* infections. The use of recombinant cytokine molecules for the effective killing of *M. avium* especially interleukin-4 (IL-4) has been well studied [112–114]. The use of adjunctive immunomodulatory therapy by using recombinant granulocyte-macrophage colony-stimulating factor has also been reported [115]. Salem et al. [116] reported that by encapsulating antibiotics like amikacin, streptomycin, ciprofloxacin, sparfloxacin, and clarithromycin, their effect against *M. avium* can be enhanced. Iron accumulation has been suggested to contribute to an increase of the susceptibility to mycobacterial infections. Iron deprivation, by the use of iron chelators, restricts *M. avium* growth and this offers a novel approach in controlling infections in man [117].

So it is considered prudent to keep infected birds away from humans, particularly the elderly, and individuals with poor immune status. Hence the handling of infected birds in farms should be carried out with adequate care, and manipulation of material from infected birds or open live cultures of *M. avium* in laboratories must be performed with appropriate biohazard containment [3]. Healthy individuals with normally functioning immune system have a high

resistance to this infection. However, it is recommended to take proper precautions and avoid contact or exposure to infected birds or their carcasses.

Salient features of avian tuberculosis are presented in Figure 3.

10. Conclusion and Future Perspectives

Members of the *Mycobacterium avium* complex (MAC) are ubiquitous bacteria that can be found in water, food, and other environmental samples and are considered opportunistic pathogens for numerous animal species, mainly birds and pigs, as well as for humans. Infections caused by the MAC are on the rise in both human and veterinary medicine. Avian tuberculosis is an important disease which affects companion, captive exotic, wild, and domestic bird, and has public health significance too. The most significant cause of poultry disease is *M. avium*. In recent years, the incidence of avian tuberculosis in domestic poultry have declined due to introduction of novel poultry husbandry practices, namely, maintaining one-age flocks, all in-all outfarming system; provision of better hygiene and sanitation; stringent implementation of biosecurity practices. But the inevitable occasional stress and production demands in the poultry sector could create dynamics similar to those that occur in immune-compromised individuals. Also, *M. avium* pose a significant threat in layer and breeder farms, where high age groups are maintained. Unless eliminated from the domestic birds, tuberculosis will remain an economic burden on the swine industry too and the role of pigs in transmitting the disease to humans has been well documented. Disseminated form of infections with *M. avium* is seen in increasing numbers in immunocompromised individuals. *M. avium* subsp. *avium* may have wild birds as major reservoirs that are responsible for its shedding into environment and facilitating its spread for years. The diversity of strain types indicates that infections are acquired not from a single reservoir alone. This is in contrary to the belief that existent infected birds are the primary sources of infection through fecal contamination of the environment. Under these perspectives more studies should be performed on identifying avian reservoirs and environmental sources of *M. avium*. RFLP analysis and multiplex PCR methods can further discriminate between different isolates, which is particularly useful for epidemiological studies. Identification of the MAC members based on culture examination followed by biochemical testing, can take up to several weeks, as opposed to molecular biology methods that provide fast and accurate identification to the species level, which is important in diagnosis and treatment of avian tuberculosis. A means of effectively discriminating among closely related yet pathogenically diverse members of the MAC would enable better diagnosis and treatment as well as further our understanding of the epidemiology of these pathogens. Moreover, viewing the importance, the advanced diagnostic tools and novel prevention strategies that are employed against *M. tuberculosis* and *M. avium* in man needs to be standardized for *M. avium* infections in birds and animals as well.

References

[1] L. Dvorska, L. Matlova, W. Y. Ayele et al., "Avian tuberculosis in naturally infected captive water birds of the Ardeideae and Threskiornithidae families studied by serotyping, IS901 RFLP typing, and virulence for poultry," *Veterinary Microbiology*, vol. 119, no. 2–4, pp. 366–374, 2007.

[2] R. M. Fulton and C. O. Thoen, "Tuberculosis," in *Diseases of Poultry*, Y. M. Saif, H. J. Barnes, J. R. Glisson, F. M. Fadly, L. R. Mc Dougald, and D. E. Swayne, Eds., pp. 836–844, Iowa State University Press, Ames, IA, USA, 2003.

[3] "Manual of Diagnostic Tests and Vaccines for Terrestrial Animals," Avian tuberculosis. Chapter 2.3.6. pp. 497–508, 2010, http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online.

[4] A. Aranaz, E. Liibana, A. Mateos, and L. Domínguez, "Laboratory Diagnosis of Avian Avian tuberculosis," *Seminars in Avian and Exotic Pet Medicine*, vol. 6, no. 1, pp. 9–17, 1997.

[5] L. A. Tell, L. Woods, and R. L. Cromie, "Avian tuberculosis in birds," *Review Science and Technology Office Internationale des Epizooties*, vol. 20, pp. 180–203, 2001.

[6] N. VanDerHeyden, "Mycobacterial infections: new strategies in the treatment of avian tuberculosis," *Seminars in Avian Exotic Pet Medicine*, vol. 6, no. 1, pp. 25–33, 1997.

[7] K. Dhama, M. Mahendran, and S. Tomar, "Pathogens transmitted by migratory birds: threat perceptions to poultry health and production," *International Journal of Poultry Science*, vol. 7, no. 6, pp. 516–525, 2008.

[8] J. J. Heatley, M. M. Mitchell, A. Roy, D. Y. Cho, D. L. Williams, and T. N. Tully Jr., "Disseminated avian tuberculosis in a bald eagle (*Haliaeetus leucocephalus*)," *Journal of Avian Medicine and Surgery*, vol. 21, no. 3, pp. 201–209, 2007.

[9] C. O. Thoen, "Tuberculosis," in *Diseases of Poultry*, B. W. Calnek, Ed., pp. 167–178, London, UK, Mosby-Wolfe, 1997.

[10] R. L. Sah, S. D. Singh, and S. C. Arya, "Tuberculosis in some captive zoo birds: case records," *Indian Journal of Veterinary Pathology*, vol. 9, pp. 84–87, 1985.

[11] K. Dhama, M. Mahendran, and S. Tomar, "Avian tuberculosis: an overview," *Poultry Punch*, vol. 24, no. 3, pp. 38–52, 2007.

[12] B. B. Mallick, R. L. Chakraborty, and S. K. Chattopadhyay, "Some observations on the naturally occurring cases of tuberculosis in ducks," *Indian Journal of Animal Health*, vol. 9, pp. 171–173, 1970.

[13] D. N. Mohanty and G. M. Patnaik, "Pathology of tuberculosis in a silver pheasant," *Indian Journal of Animal Science*, vol. 41, pp. 196–198, 1971.

[14] G. Singh, T. P. Joshi, J. M. Lall, and P. K. Iyer, "Tuberculosis in ducks," *Indian Journal of Veterinary Science*, vol. 38, pp. 424–430, 1968.

[15] K. Hejlicek and F. Treml, "Comparison of pathogenesis and epizootiology signification of avian avian tuberculosis in different sorts of domestic and free living synanthropic fowl," *Veterinary Medicine Czechoslovakia*, vol. 40, pp. 187–194, 1995.

[16] M. F. Thorel, H. Huchzermeyer, R. Weiss, and J. J. Fontaine, "*Mycobacterium avium* infections in animals," *Veterinary Research*, vol. 28, no. 5, pp. 439–447, 1997.

[17] M. F. Thorel, H. Huchzermeyer, and A. L. Michel, "*Mycobacterium avium* and *M. intracellulare* infection in mammals," *Review Science and Technology Office Internationale des Epizooties*, vol. 20, pp. 204–218, 2001.

[18] M. Schrenzel, M. Nicolas, C. Witte et al., "Molecular epidemiology of *Mycobacterium avium* subsp. *avium* and

Mycobacterium intracellulare in captive birds," *Veterinary Microbiology*, vol. 126, no. 1–3, pp. 122–131, 2008.

[19] A. B. Larsen and H. W. Moon, "Experimental *Mycobacterium paratuberculosis* infection in chickens," *American Journal of Veterinary Research*, vol. 33, no. 6, pp. 1231–1235, 1972.

[20] M. F. Thorel, M. Krichevsky, and V. V. Levy-Frebault, "Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov," *International Journal of Systematic Bacteriology*, vol. 40, no. 3, pp. 254–260, 1990.

[21] W. Mijs, P. De Haas, R. Rossau et al., "Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* to bird-type isolates and *M. avium* subsp. *hominissuis* for the human/porcine type of *M. aviumavium*," *International Journal of Systematic and Evolutionary Microbiology*, vol. 52, no. 5, pp. 1505–1518, 2002.

[22] R. K. Hoop, "Public health implications of exotic pet avian tuberculosis," *Seminars in Avian Exotic Pet Medicine*, vol. 6, no. 1, pp. 3–8, 1997.

[23] C. O. Thoen, "Tuberculosis," in *A Laboratory Manual for the Isolation and Identification of Avian Pathogens*, D. E. Swayne, J. R. Gilson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, Eds., pp. 69–73, American Association of Avian Pathologists, Philadelphia, Pa, USA, 1998.

[24] C. B. Inderlied, C. A. Kemper, and L. E. M. Bermudez, "The *Mycobacterium avium* complex," *Clinical Microbiology Review*, vol. 6, no. 3, pp. 266–310, 1993.

[25] V. Ritacco, K. Kremer, T. Van Der Laan, J. E. M. Pijnenburg, P. E. W. De Haas, and D. Van Soolingen, "Use of IS901 and IS1245 in RFLP typing of *Mycobacterium avium* complex: relatedness among serovar reference strains, human and animal isolates," *International Journal of Tuberculosis and Lung Disease*, vol. 2, no. 3, pp. 242–251, 1998.

[26] I. Pavlik, P. Svastova, J. Bartil, L. Dvorska, and I. Rychlik, "Relationship between IS901 in the *Mycobacterium avium* complex strains isolated from birds, animals, humans, and the environment and virulence for poultry," *Clinical and Diagnostic Laboratory Immunology*, vol. 7, no. 2, pp. 212–217, 2000.

[27] L. Dvorska, T. J. Bull, M. Bartos et al., "A standardised restriction fragment length polymorphism (RFLP) method for typing *Mycobacterium avium* isolates links IS901 with virulence for birds," *Journal of Microbiological Methods*, vol. 55, no. 1, pp. 11–27, 2003.

[28] M. Lairmore, T. Spraker, and R. Jones, "Two cases of tuberculosis in raptors in Colorado," *Journal of Wildlife Diseases*, vol. 21, no. 1, pp. 54–57, 1985.

[29] J. Millan, N. Negre, E. Castellanos et al., "Avian tuberculosis in free-living raptors in majorca Island, Spain," *Avian Pathology*, vol. 39, no. 1, pp. 1–6, 2010.

[30] A. M. Pocknell, B. J. Miller, J. L. Neufeld, and B. H. Grahn, "Conjunctival mycobacteriosis in two emus (Dromaius novaehollandiae)," *Veterinary Pathology*, vol. 33, no. 3, pp. 346–348, 1996.

[31] E. Boughton, "Tuberculosis caused by *Mycobacterium avium*," *Veterinary Bulletin*, vol. 39, pp. 457–465, 1969.

[32] R. Chandra, V. D. P. Rao, J. C. Gomez-Villamandos, S. K. Shukla, and P. S. Banerjee, "Avian tuberculosis," in *Diseases of Poultry and their Control*, pp. 93–98, IBD Book Company, Bombay, India, 2001.

[33] C. M. Kahn, "Avian tuberculosis," in *Merck Veterinary Manual*, pp. 2267–2268, National Publishing, Philadelphia, Pa, USA, 2005.

[34] G. Manarolla, E. Liandris, G. Pisoni et al., "Avian tuberculosis in companion birds: 20-year survey," *Veterinary Microbiology*, vol. 133, no. 4, pp. 323–327, 2009.

[35] C. Nobrega, P. J. Cardona, S. Roque, P. P. Oa, R. Appelberg, and M. Correia-Neves, "The thymus as a target for mycobacterial infections," *Microbes and Infection*, vol. 9, no. 14-15, pp. 1521–1529, 2007.

[36] F. M. Collins, "Antituberculous immunity: new solutions to an old problem," *Reviews of Infectious Diseases*, vol. 13, no. 5, pp. 940–950, 1991.

[37] K. Dhama, M. P. Bansal, and G. C. Ram, "*In vitro* evaluation of nitrite production and intracellular killing activities of bovine peripheral blood monocytes pulsed with *M. bovis* BCG," *Indian Veterinary Journal*, vol. 75, no. 12, pp. 1075–1078, 1998.

[38] K. Dhama, M. P. Bansal, and G. C. Ram, "*In vitro* study of the role of vitamin D_3 in activating peripheral bovine blood monocytes pulsed with *Mycobacterium bovis* BCG," *Indian Veterinary Medical Journal*, vol. 23, pp. 225–228, 1999.

[39] R. L. Cromie, N. J. Ash, M. J. Brown, and J. L. Stanford, "Avian immune responses to *Mycobacterium avium*: the wild-fowl example," *Developmental and Comparative Immunology*, vol. 24, no. 2-3, pp. 169–185, 2000.

[40] S. Colavecchia, A. Jolly, A. Stempler, E. Fernandez, and S. Mundo, "Lipoarabinomannans from *Mycobacterium avium* subsp. *avium affect* bovine immune responses to different antigens," *Veterinary Immunology and Immunopathology*, vol. 128, no. 1–3, p. 317, 2009.

[41] H. Saito, H. Tomioka, K. Sato, H. Tasaka, and D. J. Dawson, "Identification of various serovar strains of *Mycobacterium avium* complex by using DNA probes specific for *Mycobacterium avium* and *Mycobacterium intracellulare*," *Journal of Clinical Microbiology*, vol. 28, no. 8, pp. 1694–1697, 1990.

[42] E. J. Shitaye, V. Beran, J. Svobodová, M. Morávková, V. Babák, and I. Pavlík, "Comparison of the conventional culture, the manual fluorescent MGIT system and the automated fluorescent MGIT 960 culture system for the detection of *Mycobacterium avium* subsp. *avium* in tissues of naturally infected hen," *Folia Microbiology (Praha)*, vol. 54, no. 2, pp. 137–141, 2009.

[43] D. O'Grady, O. Flynn, E. Costello et al., "Restriction fragment length polymorphism analysis of *Mycobacterium avium* isolates from animal and human sources," *International Journal of Tuberculosis and Lung Disease*, vol. 4, no. 3, pp. 278–281, 2000.

[44] L. A. Tell, C. M. Leutenegger, R. S. Larsen et al., "Real-time polymerase chain reaction testing for the detection of *Mycobacterium genavense* and *Mycobacterium avium* complex species in Avian samples," *Avian Diseases*, vol. 47, no. 4, pp. 1406–1415, 2003.

[45] I. Slana, M. Kaevska, P. Kralik, A. Horvathova, and I. Pavlik, "Distribution of *Mycobacterium avium* subsp. *avium* and *M. a. hominissuis* in artificially infected pigs studied by culture and IS*901* and IS*1245* quantitative real time PCR," *Veterinary Microbiology*, vol. 144, no. 3-4, pp. 437–443, 2010.

[46] V. C. Thibault, M. Grayon, M. L. Boschiroli et al., "New variable-number tandem-repeat markers for typing *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* strains: comparison with IS900 and IS1245 restriction fragment length polymorphism typing," *Journal of Clinical Microbiology*, vol. 45, no. 8, pp. 2404–2410, 2007.

[47] N. Radomski, V. C. Thibault, C. Karoui et al., "Determination of genotypic diversity of *Mycobacterium avium* subspecies from human and animal origins by mycobacterial interspersed repetitive-unit-variable-number tandemrepeat and IS1311 restriction fragment length polymorphism typing methods," *Journal of Clinical Microbiology*, vol. 48, no. 4, pp. 1026–1034, 2010.

[48] M. Rozanska, "Preparation of antigen for whole blood rapid agglutination test and its specificity for diagnosis of avian tuberculosis," *Bulletin of Veterinary Institute Pulawy*, vol. 9, no. 1, pp. 20–25, 1965.

[49] E. Amador, L. Lloret, A. I. Castillo, and Y. Lopez, "Identification of immunogenic proteins of *Mycobacterium avium* with diagnostic potential," *International Journal of Infectious Diseases*, vol. 14, no. 1, p. 129, 2010.

[50] J. T. Crawford, "Development of rapid techniques for identification of *M. avium* infections," *Research in Microbiology*, vol. 145, no. 3, pp. 177–181, 1994.

[51] J. W. U. Fries, R. J. Patel, W. F. Piessens, and D. F. Wirth, "Genus- and species-specific DNA probes to identify mycobacteria using the polymerase chain reaction," *Molecular and Cellular Probes*, vol. 4, no. 2, pp. 87–105, 1990.

[52] H. Soini, E. Enrola, and M. K. Viljanen, "Genetic diversity among *Mycobacterium avium* complex AccuProbe-positive isolates," *Journal of Clinical Microbiology*, vol. 34, no. 1, pp. 55–57, 1996.

[53] G. H. Mazurek, S. Hartman, and Y. Zhang, "Large DNA restriction fragment polymorphism in the *Mycobacterium avium M. intracellulare* complex: a potential epidemiologic tool," *Journal of Clinical Microbiology*, vol. 31, no. 2, pp. 390–394, 1993.

[54] D. Thierry, P. Matsiota-Bernard, C. Nauciel, and J. L. Guesdon, "Comparison of polymerase chain reaction and nonradioactive hybridization techniques for the identification of *Mycobacterium avium* strains," *Molecular and Cellular Probes*, vol. 8, no. 6, pp. 469–471, 1994.

[55] T. Yamamoto, T. Shibagaki, S. Yamori et al., "Polymerase chain reaction for the differentiation of *Mycobacterium intracellulare* and *Mycobacterium avium*," *Tubercle and Lung Disease*, vol. 74, no. 5, pp. 342–345, 1993.

[56] T. Yamamoto, T. Shibagaki, S. Yamori et al., "Strategy for the detection and differentiation of *Mycobacterium avium* species in isolates and heavily infected tissues," *Research in Veterinary Science*, vol. 85, no. 2, pp. 257–264, 2008.

[57] B. Springer, L. Stockman, K. Teschner, G. D. Roberts, and E. C. Bottger, "Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods," *Journal of Clinical Microbiology*, vol. 34, no. 2, pp. 296–303, 1996.

[58] M. Bartos, P. Hlozek, P. Svastova et al., "Identification of members of *Mycobacterium avium* species by Accu-Probes, serotyping, and single IS900, IS901, IS1245 and IS901-flanking region PCR with internal standards," *Journal of Microbiological Methods*, vol. 64, no. 3, pp. 333–345, 2006.

[59] D. Cousins, B. Francis, and D. Dawson, "Multiplex PCR provides a low-cost alternative to DNA probe methods for rapid identification of *Mycobacterium avium* and *Mycobacterium intracellulare*," *Journal of Clinical Microbiology*, vol. 34, no. 9, pp. 2331–2333, 1996.

[60] S. J. S. J. Shin, B. S. Lee, W. J. Koh et al., "Efficient differentiation of *Mycobacterium avium* complex species and subspecies by use of five-target multiplex PCR," *Journal of Clinical Microbiology*, vol. 48, no. 11, pp. 4057–4062, 2010.

[61] P. Lappayawichit, S. Rienthong, D. Rienthong et al., "Differentiation of Mycobacterium species by restriction enzyme analysis of amplified 16S-23S ribosomal DNA spacer sequences," *Tubercle and Lung Disease*, vol. 77, no. 3, pp. 257–263, 1996.

[62] D. O'Grady, O. Flynn, E. Costello et al., "Restriction fragment length polymorphism analysis of *Mycobacterium avium* isolates from animal and human sources," *International Journal of Tuberculosis and Lung Disease*, vol. 4, no. 3, pp. 278–281, 2000.

[63] J. Ikonomopoulos, E. Fragkiadaki, E. Liandris, K. Sotirakoglou, E. Xylouri, and M. Gazouli, "Estimation of the spread of pathogenic mycobacteria in organic broiler farms by the polymerase chain reaction," *Veterinary Microbiology*, vol. 133, no. 3, pp. 278–282, 2009.

[64] P. Mobius, P. Lentzsch, I. Moser, L. Naumann, G. Martin, and H. Kohler, "Comparative macrorestriction and RFLP analysis of *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *hominissuis* isolates from man, pig, and cattle," *Veterinary Microbiology*, vol. 117, no. 2–4, pp. 284–291, 2006.

[65] D. Van Soolingen, J. Bauer, V. Ritacco et al., "IS1245 restriction fragment length polymorphism typing of *Mycobacterium avium* isolates: proposal for standardization," *Journal of Clinical Microbiology*, vol. 36, no. 10, pp. 3051–3054, 1998.

[66] J. E. Shitaye, L. Matlova, A. Horvathova et al., "*Mycobacterium avium* subsp. *avium* distribution studied in a naturally infected hen flock and in the environment by culture, serotyping and IS901 RFLP methods," *Veterinary Microbiology*, vol. 127, no. 1-2, pp. 155–164, 2008.

[67] A. S. Bannalikar and R. Verma, "Detection of *Mycobacterium avium* & *M. tuberculosis* from human sputum cultures by PCR-RFLP analysis of hsp65 gene & pncA PCR," *Indian Journal of Medical Research*, vol. 123, no. 2, pp. 165–172, 2006.

[68] A. Devallois, M. Picardeau, K. K. Goh, C. Sola, V. Vincent, and N. Rastogi, "Comparative evaluation of PCR and commercial DNA probes for detection and identification to species level of *Mycobacterium avium* and *Mycobacterium intracellulare*," *Journal of Clinical Microbiology*, vol. 34, no. 11, pp. 2756–2759, 1996.

[69] P. Kirschner, P. A. Meier, and E. C. Bottger, "Genotypic identification and detection of mycobacteria," in *Diagnostic Molecular Microbiology*, D. H. Persing, T. F. Smith, F. C. Tenover, and T. C. White, Eds., pp. 173–190, American Society for Microbiology, Washington, DC, USA, 1993.

[70] A. Telenti, F. Marchesi, M. Balz, F. Bally, E. C. Bottger, and T. Bodmer, "Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis," *Journal of Clinical Microbiology*, vol. 31, no. 2, pp. 175–178, 1993.

[71] F. Trueba, M. Fabre, and P. Saint-Blancard, "Rapid identification of *Mycobacterium genavense* with a new commercially available molecular test, INNO-LiPA MYCOBACTERIA v2," *Journal of Clinical Microbiology*, vol. 42, no. 9, pp. 4403–4404, 2004.

[72] C. Y. Turenne, M. Semret, D. V. Cousins, D. M. Collins, and M. A. Behr, "Sequencing of hsp65 distinguishes among subsets of the *Mycobacterium avium* complex," *Journal of Clinical Microbiology*, vol. 44, no. 2, pp. 433–440, 2006.

[73] N. Fatima, "Newer diagnostic techniques for tuberculosis," *Respiratory Medicine*, vol. 2, no. 4, pp. 151–154, 2009.

[74] I. K. Neonakis, Z. Gitti, E. Krambovitis, and D. A. Spandidos, "Molecular diagnostic tools in mycobacteriology," *Journal of Microbiological Methods*, vol. 75, no. 1, pp. 1–11, 2008.

[75] T. Inagaki, K. Nishimori, T. Yagi et al., "Comparison of a variable-number tandem-repeat (VNTR) method for typing *Mycobacterium avium* with mycobacterial interspersed repetitive-unit-VNTR and IS1245 restriction fragment length polymorphism typing," *Journal of Clinical Microbiology*, vol. 47, no. 7, pp. 2156–2164, 2009.

[76] D. E. Davis, J. L. Carpenter, S. Trevino, J. Koch, and A. J. Ognibene, "In vitro susceptibility of *Mycobacterium avium* complex to antibacterial agents," *Diagnostic Microbiology and Infectious Diseases*, vol. 8, no. 3, pp. 149–155, 1987.

[77] N. Rastogi, K. S. Goh, and S. Clavel-Seres, "Stazyme, a mycobacteriolytic preparation from a *Staphylococcus* strain, is able to break the permeability barrier in multiple drug resistant *Mycobacterium avium*," *FEMS Immunology and Medical Microbiology*, vol. 19, no. 4, pp. 297–305, 1997.

[78] I. Gill and M. L. Blandy, "Control of avian tuberculosis in a commercial poultry flock," *Australian Veterinary Journal*, vol. 63, no. 12, pp. 422–423, 1986.

[79] K. Dhama, J. M. Kataria, B. B. Dash, M. Mahendran, and S. Tomar, "Biosecurity-the first line of defense in poultry disease control," in *Proceedings of the Souvenir of National Symposium on Emerging and Exotic Diseases of Poultry*, pp. 73–77, IVRI, Uttarpradesh, India, 2005.

[80] K. Dhama and M. Mahendran, "Technologies and advances in diagnosis and control of poultry diseases: safeguarding poultry health and productivity," *Poultry Technology*, vol. 2, no. 12, pp. 13–16, 2008.

[81] K. Dhama, M. Mahendran, P. K. Gupta, and A. Rai, "DNA vaccines and their applications in veterinary practice: current perspectives," *Veterinary Research Communications*, vol. 32, no. 5, pp. 341–356, 2008.

[82] J. O. Falkinham III, W. B. Gross, and F. W. Pierson, "Effect of different cell fractions of *Mycobacterium avium* and vaccination regimens on *Mycobacterium avium* infection," *Scandinavian Journal of Immunology*, vol. 59, no. 5, pp. 478–484, 2004.

[83] P. Kríz, M. Slaný, J. E. J. E. Shitaye, and I. Pavlík, "Avian mycobacteriosis in humans remains a threat in the Czech Republic," *Klin Mikrobiol Infekc Lek*, vol. 16, no. 1, pp. 10–17, 2010.

[84] G. Martin and D. Schimmel, "*Mycobacterium avium* infections in poultry—a risk for human health or not?" *Dutch Tierarztl Wochenschr*, vol. 107, pp. 53–58, 2000.

[85] Y. Une and T. Mori, "Tuberculosis as a zoonosis from a veterinary perspective," *Comparative Immunology, Microbiology and Infectious Diseases*, vol. 30, no. 5-6, pp. 415–425, 2007.

[86] F. C. S. Bradbury and D. P. H. Belf, "Human pulmonary tuberculosis due to an avian tubercle bacilli: report of a case," *The Lancet*, vol. 247, no. 6386, pp. 89–91, 1946.

[87] G. K. Crompton, M. E. Schonell, and A. Wallace, "Disseminated infection with *Mycobacterium avium*: part III-sensitivity to avian tuberculin among contacts," *Tubercle*, vol. 49, no. 1, pp. 38–41, 1968.

[88] M. P. Flynn, "Comparative testing with human tuberculin and avian tuberculin in county Westmeath," *Tubercle*, vol. 43, no. 1, pp. 64–75, 1962.

[89] F. Biet, M. L. Boschiroli, M. F. Thorel, and L. A. Guilloteau, "Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium-intracellulare* complex (MAC)," *Veterinary Research*, vol. 36, no. 3, pp. 411–436, 2005.

[90] A. M. Dhople, A. A. Dhople, and M. A. Ibanez, "In vitro activities of 2,2′-bipyridyl analogues against *Mycobacterium avium* and *M. tuberculosis*," *Tubercle and Lung Disease*, vol. 76, no. 2, pp. 136–140, 1995.

[91] G. A. Falk, S. J. Hadley, F. E. Sharkey, M. Liss, and C. Muschenheim, "*Mycobacterium avium* infections in man," *The American Journal of Medicine*, vol. 54, no. 6, pp. 801–810, 1973.

[92] L. E. Bermudez, M. Wu, E. Miltner, and C. B. Inderlied, "Isolation of two subpopulations of *Mycobacterium avium* within human macrophages," *FEMS Microbiology Letters*, vol. 178, no. 1, pp. 19–26, 1999.

[93] R. N. E. Eccles and R. N. J. Ptak, "*Mycobacterium avium* complex infection in AIDS: clinical features, treatment, and prevention," *Journal of the Association of Nurses in AIDS Care*, vol. 6, no. 5, pp. 37–47, 1995.

[94] T. Bodmer, E. Miltner, and L. E. Bermudez, "*Mycobacterium avium* resists exposure to the acidic conditions of the stomach," *FEMS Microbiology Letters*, vol. 182, no. 1, pp. 45–49, 2000.

[95] K. Fukuoka, Y. Nakano, A. Nakajima, S. Hontsu, and H. Kimura, "Endobronchial lesions involved in *Mycobacterium avium* infection," *Respiratory Medicine*, vol. 97, no. 12, pp. 1261–1264, 2003.

[96] P. R. J. Gangadharam, V. K. Perumal, B. T. Jairam, N. R. Podapati, R. B. Taylor, and J. F. LaBrecque, "Virulence of *Mycobacterium avium* complex strains from acquired immune deficiency syndrome patients: relationship with characteristics of the parasite and host," *Microbial Pathogenesis*, vol. 7, no. 4, pp. 263–278, 1989.

[97] C. O. Thoen, "*Mycobacterium avium* infections in animals," *Research in Microbiology*, vol. 145, no. 3, pp. 173–177, 1994.

[98] O. F. Thoresen and F. Saxegaard, "Comparative use of DNA probes for *Mycobacterium avium* and *Mycobacterium intracellulare* and serotyping for identification and characterization of animal isolates of the *M. avium* complex," *Veterinary Microbiology*, vol. 34, no. 1, pp. 83–88, 1993.

[99] A. Limia, F. J. Sangari, D. Wagner, and L. E. Bermudez, "Characterization and expression of *secA* in *Mycobacterium avium*," *FEMS Microbiology Letters*, vol. 197, no. 2, pp. 151–157, 2001.

[100] I. W. Lesslie and K. J. Birn, "*Mycobacterium avium* infections in cattle and pigs in Great Britain," *Tubercle*, vol. 51, no. 4, pp. 446–451, 1970.

[101] J. Van Ingen, H. J. Wisselink, C. B. van Solt-Smits, M. J. Boeree, and D. van Soolingen, "Isolation of mycobacteria other than *Mycobacterium avium* from porcine lymph nodes," *Veterinary Microbiology*, vol. 144, no. 1-2, pp. 250–253, 2010.

[102] T. Tirkkonen, J. Pakarinen, A. M. Moisander, J. Makinen, H. Soini, and T. Ali-Vehmas, "High genetic relatedness among *Mycobacterium avium* strains isolated from pigs and humans revealed by comparative IS*1245* RFLP analysis," *Veterinary Microbiology*, vol. 125, no. 1-2, pp. 175–181, 2007.

[103] A. E. Suzuki and J. M. Inamine, "Genetic aspects of drug resistance in *Mycobacterium avium*," *Research in Microbiology*, vol. 145, no. 3, pp. 210–213, 1994.

[104] M. Tsukamura, "In vitro bacteriostatic and bactericidal activity of isoniazid on the *Mycobacterium avium-Mycobacterium intracellulare* complex," *Tubercle*, vol. 71, no. 3, pp. 199–204, 1990.

[105] S. D. Shafran, "Prevention and treatment of disseminated *Mycobacterium avium* complex infection in human immunodeficiency virus-infected individuals," *International Journal of Infectious Diseases*, vol. 3, no. 1, pp. 39–47, 1998.

[106] W. V. Raszka Jr., L. P. Skillman, and P. L. McEvoy, "In vitro susceptibility of clinical isolates of *Mycobacterium avium* and *M. intracellulare* to folate antagonists," *Diagnostic*

Microbiology and Infectious Disease, vol. 18, no. 3, pp. 201–204, 1994.

[107] M. W. Dunne, G. Foulds, and J. A. Retsema, "Rationale for the use of azithromycin as *Mycobacterium avium* chemoprophylaxis," *American Journal of Medicine*, vol. 102, no. 5, pp. 37–49, 1997.

[108] L. Horgen, E. Legrand, and N. Rastogi, "Postantibiotic effects of rifampin, amikacin, clarithromycin and ethambutol used alone or in various two-, three- and four-drug combinations against *Mycobacterium avium*," *FEMS Immunology and Medical Microbiology*, vol. 23, no. 1, pp. 37–44, 1999.

[109] H. Saito, H. Tomioka, K. Sato, S. Kawshara, T. Hidaka, and S. Dekio, "Therapeutic effect of KRM-1648 with various antimicrobials against *Mycobacterium avium* complex infection in mice," *Tubercle and Lung Disease*, vol. 76, no. 1, pp. 51–58, 1995.

[110] Y. E. Lin, R. D. Vidic, J. E. Stout, C. A. McCartney, and V. L. Yu, "Inactivation of *Mycobacterium avium* by copper and silver ions," *Water Research*, vol. 32, no. 7, pp. 1997–2000, 1998.

[111] S. David, V. Barros, K. P. Guerra, and R. Delgado, "Exploring *Mycobacterium avium* inhibition by macrocyclic compounds," *Research in Microbiology*, vol. 156, no. 9, pp. 904–910, 2005.

[112] L. E. Bermudez and L. S. Young, "Killing of *Mycobacterium avium*: insights provided by the use of recombinant cytokines," *Research in Microbiology*, vol. 141, no. 2, pp. 241–243, 1990.

[113] L. Chinen, I. M. Cipriano, R. S. de Oliveira, S. C. Leao, M. Mariano, and C. W. Carneiro, "Recombinant interleukin-4-treated macrophages, epithelioid cell surrogates, harbor and arrest *Mycobacterium avium* multiplication in vitro," *Microbes and Infection*, vol. 8, no. 4, pp. 965–973, 2006.

[114] K. Dhama, M. Mahendran, R. S. Chauhan, and S. Tomar, "Cytokines: their functional roles and prospective in veterinary practice-A review," *Journal of Immunology and Immunopathology*, vol. 10, no. 2, pp. 79–89, 2008.

[115] T. I. Silvaa, A. Copea, J. Goepelb, and J. M. Greiga, "The use of adjuvant granulocyte-macrophage colony-stimulating factor in HIV-related disseminated atypical mycobacterial infection," *Journal of Infection*, vol. 54, no. 4, pp. 207–210, 2007.

[116] I. I. Salem, D. L. Flasher, and N. Duzgunes, "Liposome-encapsulated antibiotics," *Methods in Enzymology*, vol. 391, pp. 261–291, 2005.

[117] S. S. Fernandes, A. A. Nunes, A. R. Gomes et al., "Identification of a new hexadentate iron chelator capable of restricting the intramacrophagic growth of *Mycobacterium avium*," *Microbes and Infection*, vol. 12, no. 4, pp. 287–294, 2010.

Review Article

Perspectives on the History of Bovine TB and the Role of Tuberculin in Bovine TB Eradication

Margaret Good and Anthony Duignan

Department of Agriculture, Fisheries and Food, Agriculture House, Kildare Street, Dublin 2, Ireland

Correspondence should be addressed to Margaret Good, margaret.good@agriculture.gov.ie

Tuberculosis remains a significant disease of animals and humans worldwide. Bovine tuberculosis is caused by Mycobacteria with an extremely wide host range and serious, although currently probably underdiagnosed, zoonotic potential. Where bovine tuberculosis controls are effective, human zoonotic TB, due to *Mycobacterium bovis* or *M. caprae*, is uncommon and clinical cases are infrequent in cattle. Therefore, the control and ultimate eradication of bovine tuberculosis is desirable. Tuberculin tests are the primary screening tool used in bovine eradication. The choice of tuberculin test is dependent on the environment in which it is to be used. Tuberculin potency is critical to test performance, and the accurate determination of potency is therefore particularly important. The design of a control or eradication programme should take into consideration the fundamental scientific knowledge, the epidemiological profile of disease, the experience of other eradication programmes, and the presence, in the same ecosystem, of maintenance hosts, in which infection is self-sustaining and which are capable of transmitting infection. A control or eradication programme will necessarily require modification as it progresses and must be under constant review to identify the optimal desirable goals, the efficacy of policy, and constraints to progress.

1. Introduction

All members of the closely related phylogenic grouping of Mycobacteria known collectively as the *M. tuberculosis* complex may cause tuberculosis in a range of species including man. Some members of this group are predominantly human (*M. tuberculosis*, *M. africanum*, *M. canetti*) or rodent pathogens (*M. microti*), whereas others have a wide host spectrum (*M. bovis, M. caprae*) [1, 2]. Hewinson et al. recently expanded the "phylogenetic analysis of strains of the *M. tuberculosis* complex to include single nucleotide mutations and deletions of spoligotype units" and concluded that "this group of organisms might best be described as a series of host adapted ecotypes, each with a different host preference representing different niches" [3]. Originally *M. caprae* had been considered to be a subspecies of either *M. tuberculosis* or *M. bovis*; however, it is now apparent that phylogenetically it preceded *M. bovis* and it is only since the development of genotyping techniques allowing greater discrimination that its existence became apparent [2].

2. Bovine Tuberculosis

In cattle the most important causes of tuberculosis—bovine TB (bTB)—are *M. bovis* and *M. caprae*, both of which cause infectious disease that may result in significant productivity problems due to ill health [2, 4–6]. *M. bovis* has one of the broadest host ranges of all known pathogens and has been diagnosed worldwide. O'Reilly and Daborn citing various authors list the species in which the disease has been reported as domesticated and feral cattle, goat, pig, sheep, horse, cat, dog, fennec fox, deer, bison, buffalo, badger, possum, hare, ferret, wild and feral pig, antelope, Arabian Oryx, camel, llama, alpaca, man, humans, and nonhuman primates [7]. *M. bovis* has also been detected in lion, hyena, kudu, baboon, leopard, cheetah, warthog and bushpig, elk, coyotes, meerkats, black rhinoceros, aoudad (Barbary sheep), and Lynx [8–14]. Tuberculosis due to *M. bovis* or *M. caprae* is a zoonotic disease with a complex epidemiological pattern which includes the transmission of infection within, and between, man, domestic animals, and wildlife. The

occurrence of *M. caprae* has been reported in many European countries such as Austria, France, Germany, Hungary, Italy, Slovenia, and the Czech Republic but to date it has not been detected in Ireland (see [2], Department of Agriculture, Fisheries and Food (DAFF) records unpublished). Disease caused by *M. caprae* is not considered to be substantially different from that caused by *M. bovis* and the same tests can be used for its diagnosis [15].

3. Zoonotic Implications

It is estimated that 1.5–2 M people die each year from tuberculosis of the approximately 2 billion infected persons worldwide [16]. *M. bovis* infection currently accounts for only a small percentage of reported cases but it was a major public health problem in Europe and elsewhere, when this organism was transmitted to man in milk from infected cows, prior to the advent of pasteurization of milk and milk products [7]. Thoen et al. and de la Rua-Domenech provide several reasons why *M. bovis* in humans is underdiagnosed even in developed countries [17, 18]. The consumption of unpasteurised milk or milk products still remains a risk for infection in countries where bTB has not been eradicated where ethnic populations present significantly different epidemiological profile or where HIV is prevalent [19–23]. Zoonotic TB was originally considered primarily as a disease of children where the disease involved the cervical lymph nodes (scrofula), the intestinal tract, or the meninges. It is now increasingly being recognised that infection in childhood is the precursor of reactivated adult disease and that many infected children may remain asymptomatic, undiagnosed, and untreated [24, 25]. Thus zoonotic TB is of particular concern for developing countries, but where bTB controls are effective, human *M. bovis* or *M. caprae* isolates are uncommon and rare in countries where bTB has been eradicated [2, 16, 18, 22, 26, 27]. *M. bovis* may affect humans of any age, and while the majority opinion is that human-to-human spread of *M. bovis* must be a very rare event, it does occur particularly amongst immunocompromised individuals [16, 25, 28–30]. O'Reilly and Daborn also referred to a small outbreak of tuberculosis in The Netherlands in 1994 caused by *M. bovis* which likely involved transmission from human to human [7]. The control and eradication of zoonotic TB requires the early recognition of preclinical infection in animals and the prompt removal of any infected animals in order to eliminate a future source of infection for other animals and for humans [31].

4. Transmission of Infection

O'Reilly and Daborn cite Sigurdsson who pre 1945, conducted experimental studies in laboratory animals which indicated that the size of the particles carrying the mycobacteria is of critical importance in determining infectivity [7]. This work also refers to the findings of research workers who, as early as the first decade of the 20th century, demonstrated that at least 10 mg of bovine tubercle bacilli are necessary to cause alimentary infection in calves whereas 0.01 mg, a 1000

times smaller dose, produces an inhalation infection. Dean et al. demonstrated that <10 viable bacilli are sufficient to cause established tuberculosis pathology reflecting that seen in naturally infected field reactor cattle but they did not observe a dose-related effect in the pathology score up to 1,000 CFUs [32].

The respiratory route is accepted as the primary method of infection spread in all species. However, it is clear that there are other less common methods of spread such as oral, occupational, congenital and via wounds [17, 19, 28, 33–37]. The postmortem evidence regarding the frequency of tuberculosis of the mammary glands in tuberculous cows appears to depend on the extent and duration of infection in the cow and is thus somewhat conflicting. Francis quotes incidences of 0.5–19.5% in tuberculous cows and 5–31% in cows with generalized tuberculosis [35]. Analysis of milk in countries with no bTB eradication programme continues to show similar levels of *M. bovis* detection [38, 39]. Even in countries with a bTB eradication programme where unpasteurised milk is routinely fed to calves on farm, a high prevalence of infection within those calves will indicate the probable presence of one or more cows with *M. bovis* in milk and require appropriate follow-up epidemiological investigation [19].

The transmission of *M. bovis* between cattle is dependent on a number of factors, including frequency of excretion, route of infection, the infective dose, the period of communicability, and host susceptibility. It is also possible that a range of highly specific conditions must occur for fine aerosols to be produced and for transmission to take place [40]. Transmission and observational studies suggest that the required conditions are unlikely to exist when a tuberculous animal is in the early stages of infection [40]. This view is also supported by studies conducted in cattle, which have indicated that bacterial shedding is, at best, transient and involves extremely low numbers of bacilli [41]. In man and badgers also the risk of transmission increases as disease progresses, and these species usually only become highly infectious when the disease is advanced and large numbers of organisms are being excreted [37, 42, 43]. Little et al. demonstrated transmission between naturally infected badgers and calves housed with them after a lapse of 6 months [44]. Field experience also indicates that cattle in the early stages of disease or with discrete walled-off lesions do not commonly transmit *M. bovis* to in-contact animals [40, 45–47]. On balance, the current evidence suggests that while some animals in the early stages of disease do excrete low numbers of *M. bovis*, in-contact animals do not readily acquire infection [40, 41].

4.1. Environmental Transmission. Various durations of environmental survival of *M. bovis* are reported in the literature depending on the conditions under which the research has been conducted. Early work suggested that *M. bovis* is a highly resistant organism surviving in cow faeces, for at least 5 months in winter, 4 months in autumn, 2 months in summer up to 2 years in soil; 4 months in liquid manure stored underground, and 1-2 months in soil during the

summer months [48]. Despite the prevalence of clinically advanced cases of bovine tuberculosis at the time that they were conducting their studies, early 20th Century, Williams and Hoy comment on the great difficulty they experienced in finding animals with naturally infected faeces such that 76% of samples from known tuberculous cows gave negative results and the irregularity of positive results with naturally infected faeces led them to conduct their work with artificially infected faeces [48]. O'Reilly and Daborn also discuss where Maddock in 1935-1936 reported grazing paddocks, with naturally infected cows and artificially infected calves, so as to produce pastures with a heavier infection burden than would be likely to occur naturally [7]. When the infected animals were removed, tuberculosis-free calves grazed these pastures for a 3-week period following intervals of 1, 2, and 3 months. On subsequent tuberculin testing and postmortem examination, all the calves proved to be free from any evidence of tuberculosis. O'Reilly and Daborn also detail how Schellner in 1959 experimentally irrigated pasture plots with 10^2–10^{12} *M. bovis* per ml of water and after intervals of 7, 14, and 21 days allowed 56 heifers to graze the plots [7]. Only 2 of 14 animals which grazed a plot irrigated 1 week previously became infected; all the others remained healthy. Little et al. failed to isolate *M. bovis* from a large number of environmental samples taken during and after a transmission study while in the same study badger faeces were positive for *M. bovis* [44]. Duffield and Young, working in North Queensland, were able to reisolate *M. bovis* from moist soil held in shade and darkness but not from any substrate held in sunlight or from faeces after 4 weeks [49]. They were not able to reisolate *M. bovis* from any substrate under any condition at or from 8 weeks. Thus, while *M. bovis* artificially deposited on soil or sterilised faeces stored away from sunlight may survive for several months, under natural conditions *M. bovis* appears to die out more quickly as in-contact animals do not readily acquire infection [7, 40, 48, 49].

4.2. Wildlife. Tuberculosis was described as a reemerging disease at the interface of domestic animals and wildlife by Palmer who cautioned that it will not be possible to eradicate *M. bovis* from livestock until transmission between wildlife and domestic animals is halted, and he advises that this will require a collaborative effort between stakeholders [50]. Corner has presented a detailed review of the role of wildlife as reservoirs of *M. bovis* differentiating between those that act as maintenance hosts or disease reservoirs and those that are spill-over or dead end hosts in which disease is not self-sustaining and which therefore do not maintain disease in an environment [51]. Some wildlife species, principally the badger in the United Kingdom and Ireland, the Australian possum in New Zealand (but not in Australia), and previously water buffalo in Australia, have been recognised as significant reservoirs of *M. bovis* with endemic self-maintaining infection in these species constituting a major obstacle to disease control programmes [52–54]. Wildlife infection is also an issue in other countries such as Canada, where *M. bovis* reservoirs in elk and deer cause occasional problems in livestock; Spain where *M. bovis*

reservoirs in deer and in particular wild boar, pose a threat to Lynx an endangered species and South Africa where multiple species are infected in conservation areas [8, 12, 55–58]. In Australia, elimination of wild water buffalo and feral cattle from areas where infection was endemic was a major component of the eradication campaign [54, 59]. Postmortem surveillance, epidemiological risk assessment, and the implementation of strict cattle movement controls finally brought disease under control, and Australia is now bTB-free [52, 59]. New Zealand has similarly employed strict population control measures against infected possum populations, and very considerable progress has been made [54, 60]. Countries where population control measures for infected wild populations must necessarily be limited, for example, badgers in the UK and Ireland where the badger is a protected species, have succeeded in reducing high incidence disease levels in cattle and maintaining them at relatively low levels by a sustained test and cull programme such that bTB is no longer a significant threat to humans. While the indications are that badgers can excrete mycobacteria from the respiratory, digestive, and urinary tract as well as in exudates from skin lesions transmission of *M. bovis* infection among badgers appears to be mainly by the respiratory route and, although there is an overall trend for increased prevalence with age, the acquisition of infection apparently occurs most frequently in young animals due to pseudovertical transmission from mother to cub [61].

4.3. Human to Cattle Transmission. Francis described how in Denmark and Sweden, towards the end of their respective successful bovine TB eradication programmes, there was concern about the risk posed to cattle herds from infected humans [33]. O'Reilly and Daborn state that transmission of *M. bovis* infection from humans to cattle is usually direct and by the respiratory route but that indirect spread via bedding and/or hay contaminated with urine from human renal excreters was reported by Huitema in 1969 in The Netherlands and by Schliesser in 1974 in Germany [7]. They provided details from Huitema as to how *M. bovis* infected humans were the source of infection in 50 cattle herds in the Netherlands where a total of 636 tuberculin reactor cattle were identified, of which 497 were confirmed postmortem as *M. bovis* infected and where 24 of 50 *M. bovis* infected patients had urogenital tuberculosis, the others mostly pulmonary tuberculosis. The patients with urogenital tuberculosis had infected 259 (41%) of the reactor animals. Citing Schliesser they stated that, in Germany, *M. bovis* infection in cattle is rare but, when it occurs, man-to-cow transmission is a principal cause and where, in 1 study, 12 patients had infected 114 cattle in 16 different herds: 9 of the 12 had genitourinary tuberculosis and 1 such patient had infected 48 cattle in 4 different herds. In 1987 Grange and Collins stressed that man might be a continuing important source of disease in cattle in Ireland and that urinary tract disease may be a hidden source of infection, and they warned that many patients with renal tuberculosis, especially older patients, had clear radiographs and only vague symptoms [62]. Srivastava reported detection of *M. tuberculosis* in cattle

and also from milk on some farms in North India raising suspicion that infection had spread from humans [39].

5. Tuberculin Tests

Having discovered the "Tubercle bacillus" in 1882 Koch went on, in 1890, to demonstrate the properties of a tuberculin he had developed. The possibility of using this tuberculin to test cattle in order to identify those with TB was very quickly recognised, and by 1891 cattle testing was operating extensively [35]. Almost it would seem simultaneously the possibility of using tuberculin and tuberculin tests as a tool to eradicate bTB was also recognised. Bovine tuberculosis had become a problem that was exacerbated by the gradual intensification of cattle production in the postindustrial revolution era [35]. In the late 19th and early 20th Century it appeared that the generally infectious nature of the "Tubercle bacillus" and then also the zoonotic implications of bTB were not well appreciated. Thus it would appear that the motivation for control of bTB during this time was predominantly economic [35]. Even today in many countries or regions the adoption or not of a bTB eradication programme may depend on economic factors as there are often many other conflicting demands for scare resources. Hence, while many underdeveloped countries have problems with TB in cattle and at least some, also in wildlife, not all have or can afford compulsory or comprehensive bTB control programmes.

Finland was the first country, in the late 1890s, to commence a successful bTB eradication programme [35]. It was relatively quickly established that bTB could be eradicated by the use of tuberculin tests when these were used with knowledge of the strengths and limitations of the test being used. Once a test and removal programme was commenced for bovines, the incidence of clinical cases of bTB rapidly declined as infected animals were removed from the population. Thus, economic losses due to bTB declined simultaneously as the cattle population became healthier. Buxton and Glover describe how Moussu and Mantoux in 1908 elaborated the value of the intradermal tuberculin test when they described the type of response it elicited in tuberculous and nontuberculous animals [63]. By 1910 Finland was already using the, then new, intradermal test in their eradication programme. Other countries gradually also commenced eradication programmes as various tuberculin test methodologies were developed and refined. Richie, describing a number of the different methods of tuberculin testing employed, speaks of the subcutaneous test which depended on temperature records over time, a short thermal test, the ophthalmic and palpebral tests, the double intradermal test, the Stormont test, and the vulval test, all now discarded from general use [64]. Christiansen and Stubb are cited by Buxton and Glover as having, in 1910, selected the side of the neck as the site for injection of tuberculin because it gave the most consistent results regarding the presence or absence of tuberculosis infection in cattle [63]. Baisden et al. in 1951 confirmed the greater sensitivity of the neck over the caudal fold and that the neck is the most

sensitive site [65]. Paterson detailed how sensitivity is greater in sites on the neck nearer the head and diminishes in sites near the shoulder and in those adjacent to the nuchal crest, and he recommended that injection should therefore be in the middle third of the neck [66]. The relative sensitivities of the different parts of the neck were confirmed by Good et al. [67]. Paterson also detailed how the test is interpreted primarily on a herd basis, taking into consideration the history of the herd but with sometimes difficulties arising in dealing with an individual animal [66]. Tuberculin testing of cattle has in many areas succeeded in eradicating bTB, and there is no doubt that where the disease was confined only to cattle a test and cull programme would succeed.

Tuberculin tests, which avail of a cell-mediated response to Mycobacteria, have now been used for the diagnosis of tuberculosis and preclinical infection in man and animals for more than 100 years [68]. In humans asymptomatic and radiographically negative persons, with no history of BCG vaccination, who are positive to tuberculin test, are regarded as latently infected. Only approximately 5% of infected humans develop clinical symptoms within a year of infection and 5–10% of latently infected persons go on to develop chronic progressive TB owing to reactivation during their lifetime [69]. In cattle tuberculin tests are based on detection of the specific immunological response following exposure to *M. bovis* or indeed *M. caprae* at some period previously. Following exposure infection will have occurred and either progressed or become quiescent under control by the animal whose response is based on the infective dose and its own inherent immune system.

Monaghan reviewed the most common tuberculin tests in use today, namely, the caudal fold test (CFT) and the Single intradermal test (SIT) which both use only bovine tuberculin PPD and the Single intradermal comparative tuberculin test (SICTT), which uses bovine and avian tuberculin PPD in combination [68]. The use of the word single in describing these tuberculin tests distinguishes them from the now obsolete double intradermal test, which regards the first injection of tuberculin as a "sensitising" injection. There are a number of national bTB eradication programmes in the Europe Union using either the SIT or SICTT where, when one or more animals in a herd show a positive response to the test, statutory controls are applied at herd level [70, 71]. Both the SIT and SICTT methodologies including test interpretation and test intervals are described in the EU trade Directive 64/432/EEC and also by the OIE [15, 72]. The SICTT has been used extensively in the Irish bTB eradication programme and has proven to be a very safe means to test and screen the Irish cattle population [73]. The caudal fold test is widely used in the USA and New Zealand and was also used in Australia during their bovine TB eradication campaign. There are also other regions of the world where this is the routine test of choice with or without use of the SICTT before animal removal.

To assess the efficacy of a particular tuberculin test methodology various parameters such as the test sensitivity, specificity, and predictive value are evaluated for the environment, the level of disease in the population, and the conditions in which the test is performed [74]. If more

than one type of test is available, the relative values of these appraisals will dictate which test may be most useful in particular situations in order to maximise the performance of the test. In 1959 Ritchie pointed out that it is vital to use a tuberculin of potency greater than that to which the majority of infected animals will respond [64]. The balance of evidence appears to favour the use of tuberculin of sufficient potency for the detection of tuberculosis in cattle for the eradication of the disease. In several countries, bovine tuberculin is considered to be of acceptable potency if its estimated potency guarantees per bovine dose at least 2000 IU (±25%) in cattle. Field trials have confirmed the scientific basis supporting this potency level [75]. In cattle with diminished allergic sensitivity, a higher dose of bovine tuberculin is needed, and, in national eradication campaigns, doses of up to 5000 IU are recommended [15]. The use of a highly potent bovine tuberculin increases the sensitivity of the test. However, test specificity is not only influenced by the purity, potency, and dosage of the tuberculin and strictness of interpretation of the response in the animal it is also influenced by sensitization of the animal. The choice of the SICCT, being a more specific test than the Single Intradermal test (SIT) or any other tuberculin test using bovine PPD alone, for the Irish and UK eradication programmes, was influenced by the abundance of nonspecific causes of sensitization. This choice was validated by Lesslie and Hebert and O'Reilly and MacClancy, in 1975, who found that 8–12% of apparently noninfected cattle in Ireland and the UK react positively to the SIT but not to the SICTT [74, 76]. While the single most important cause of sensitization is exposure to *M. bovis,* other pathogenic mycobacteria, for example, *Mycobacterium paratuberculosis* subsp. *avium*, and nonpathogenic environmental *Mycobacteria* such as *M. hiberniae,* are abundant in the Irish environment and cause nonspecific sensitisation to bovine tuberculin PPD [77, 78]. In the majority of cases the SICTT serves to differentiate between responses from exposure to *M. bovis* and other nonspecific Mycobacteria.

5.1. Tuberculin. In 1959 Paterson described tuberculin as the most important diagnostic agent in eradication schemes for tuberculosis and it remains so today [66]. The methods of preparation of tuberculin and the ways in which it has been applied to the diagnosis of tuberculosis date from Koch's original "tuberculin" preparation in 1890 when he initially thought he had discovered a cure for tuberculosis. Monaghan remarks on how quickly the principal advantages and problems associated with the use of tuberculin as a diagnostic test were, within a year of its first use, tabulated and the conclusion drawn by a committee at the University of Pennsylvania that "tuberculin is of value in the diagnosis of tuberculosis in cattle" [68]. For the purpose of testing animals modern-day tuberculin is a purified protein derivative (tuberculin PPD, bovine or avian) prepared from the heat-treated products of growth and lysis of *M. bovis* or *M. avium* (as appropriate) capable of revealing a delayed hypersensitivity in an animal sensitised to microorganisms of the same species. Administration of Tuberculin confers

no protection to acquiring infection or from progression to clinical disease in an already infected animal. Buxton and Glover also credited Siebert et al. with developing the precipitation phase in the manufacture of PPD, in 1934, so as to ensure removal of high-molecular-weight proteins, which had previously been responsible for sensitisation of the subject following injection. PPD also eliminated many of the nonspecific features of the old tuberculin [63, 79]. Paterson, citing Seibert at al., claimed that the advantages of PPD lie in the use of a pure active principle, such that successive batches contain the same amount of protein, the process of preparation is reproducible from batch to batch, and the protein yield per batch constitutes a valuable control measure [79]. Production methods have largely been standardised and under EU Regulations Tuberculin PPD is a licensed product required to be manufactured under Good Manufacturing Practice conditions and to comply with the European Pharmacopoeia and thus also conform to OIE requirements [15, 72, 80]. The preparatory method ensures that PPD tuberculin consists of a mixture of small water-soluble protein molecules and this protein content can be helpful in the chemical standardisation of tuberculin [81]. The protein content of tuberculin, however, does not predict its biological activity and consequently Directive 64/432/EEC as amended sets out the minimum requirement for tuberculin potency and requires that potency assays must be performed in guinea pigs where the response is compared to a reference standard [72, 81]. Performance of the assay is described by the OIE [15].

However, while these routine assays are most reliable when carried out in tuberculous guinea pigs sensitised with living virulent *M. bovis* the guinea pig potency is not necessarily representative of the clinical potency in cattle [66, 82–84]. Paterson recommends that guinea pigs be used for the control at preparation with occasional check assays in cattle but that if the type of tuberculin is changed or if a change in character is suspected that appeal must be to the assay in cattle [66]. Changes in manufacturing and production procedures may also result in fluctuations in tuberculin potency and there may also be considerable variability in potency between batches of tuberculin, including those produced in the same centre [83, 85, 86]. Tuberculin potency fluctuation was seen during 1990–1992 associated with Good Laboratory Practice adaptations and in 2000 associated with changes instigated as a consequence of EU requirements in relation to Transmissible Spongiform Encephalopathies (unpublished observations—DAFF records). Therefore, periodic validation of bovine PPD potency, on routine bovine tuberculin supply, in naturally infected tuberculous cattle is recommended [81, 85]. According to WHO Technical Report Series no. 745, potency testing should be performed in the animal species and under the conditions in which the tuberculins will be used in practice [87]. This means that bovine tuberculins should be assayed in naturally infected tuberculous cattle. As this requirement is difficult to accomplish, routine potency testing is conducted in guinea pigs. However, periodic testing in tuberculous cattle remains necessary, and standard preparations always require calibration in cattle.

The frequency of testing in cattle can be reduced if it is certain that the standard preparations are representative of the routine issue tuberculins and that the production procedures guarantee consistency [15]. Notwithstanding the EU and OIE specifications there are tuberculins of lower potency available and care should be exercised in selecting tuberculin as its potency has a considerable impact on test performance [15, 67, 72, 81, 88].

5.2. Test Limitations. In common with all tests and assays the tuberculin test is not perfect. As tuberculin eradication programmes advanced in different countries around the world field experience progressively showed that not all infected animals gave a good response to tuberculin. Examples of poor responders cited by Ritchie include anergic animals or those exhibiting reactions to both avian and mammalian tuberculin, those in advanced stage of disease, animals with confined infection notably in the udder, those with localised infection often in the lymphatic glands that has become inactive (latent), and periparturient cows [64]. He goes on to say that it is essential that the tuberculin be of sufficient potency to produce a reaction in the maximum number of infected animals and to use a tuberculin of potency greater than that to which the majority of infected animals will respond. He warns, however, that the highly potent tuberculin required to detect bovine infection tends to increase the frequency of reactions associated with cross-sensitisations arising from other organisms such as the human and avian types (*M. tuberculosis* and *M. avium*, resp.) and other (nonpathogenic) mycobacteria. Cross-sensitisation also appears to have caused problems during the Danish bTB eradication programme—Ritchie quoting Plum from 1937 and 1939 [64]. In 1962 Karlson reported that nonspecific responses to tuberculin were seen in all countries where eradication measures applied and that it was a widespread problem of a serious nature [89]. Karlson also reported that the sensitivity to mammalian tuberculin by cattle exposed to *M. tuberculosis* disappears when the human source of exposure is removed [89]. Rushford also reported that nonspecific responses were an issue using the single caudal fold tuberculin test in Australia [90]. Lamont in 1947 discussed at length the phenomenon of periparturient desensitisation and that instead the calf receiving the colostrums of an infected cow became passively sensitised for 4–6 weeks [79]. Lamont also postulated reasons why the test cannot succeed under certain circumstances namely that

(1) in a case of recent infection a response has had insufficient time to develop,

(2) *postmortem* finding of encapsulated lesions in test-negative animals may be because the response has disappeared due to lack of stimulation from repeated doses of mycobacterial antigens,

(3) lack of a positive response to tuberculin during "active" infection occurs particularly in advanced cases but that this desensitisation could be produced by injecting tuberculin [79].

While tuberculin tests are imperfect, they have been shown to be effective and they have succeeded in reducing the incidence of bTB and indeed many countries have succeeded in eradicating bovine TB with their use [71, 91]. One can see from the rapid progress Ireland made in the initial 5 years of their compulsory programme how quickly a country can pass from having high disease incidence (animal incidence 17%) to having a relatively low incidence (animal incidence 0.4%) [52]. However, such problems as described above by both Ritchie and Lamont still exist and continue to be manifest in eradication programmes which employ tuberculin tests [64, 79]. Delayed hypersensitivity may not develop for a period of 3–6 weeks following infection. Thus, if a herd/animal is suspected to have been in contact very recently with infected animals, delaying testing should be considered in order to reduce the probability of false-negatives. As the sensitivity of the test is less than 100%, it is unlikely that eradication of tuberculosis from a herd will be achieved with only a single tuberculin test [15].

Tuberculin test-negative animals are found, at slaughter, with evidence of encapsulated lesions confirmed as caused by *M. bovis*. Where there is no active infection ongoing in the herd from which the animal was sourced the infection appears to relate to exposure some time, even perhaps years, previously [45]. In other cases where there is ongoing infection in the herd of origin, it may well have been as a result of recrudescence of tuberculosis in a previously infected animal and there are still other herds with ongoing problems with TB infection where perhaps desensitization owing to successive short-interval skin tests may be a contributing factor (see [45, 92], DAF records unpublished).

With regard to desensitisation produced by the injection of tuberculin Buxton and Glover in 1939 cited Cuillé and Chelle (1935) as having demonstrated a progressive loss of skin response when several injections are made [63]. Coad et al. have confirmed that repeated SICTT led to increasing desensitisation at subsequent tests [92]. Paterson quoting Swindle et al. (1950) pointed out that full sensitivity at and for 2 to 3 inches (5–7.5 cm) around the tuberculin injection site is only recovered after 6–8 weeks [66]. Ritchie also spoke of the region immediately adjacent to the original inoculation that had in some but not all instances become desensitised with reactions less marked but that full sensitivity was regained by about the 6th week [64]. Doherty et al. and more recently Coad et al. have also confirmed this now well-recognised phenomenon [92, 93]. Coad et al. caution that the period of desensitisation may be longer than previously thought and that successive short-interval skin tests will result in progressive desensitisation, which should be considered when faced with "inconclusive-reactor" skin test responses [92]. They further cautioned that the possibility of repeat testing resulting in false-negative test outcomes in infected cattle with indeterminate test responses also cannot be excluded.

As a management tool in eradication test interpretation is standard or more severe dependent on the history of the herd, the level of infection within a particular group of cattle, and the epidemiological assessment of the outbreak. As eradication progresses epidemiological investigation and

data analysis become more important. In addition the use of ancillary tests, group removal, and/or full herd depopulation may also be required to accelerate eradication. Particularly towards the end of an eradication programme the response to the detection of an infected herd may be full-herd depopulation in order to ensure that no infected animals may remain. In countries with low incidence of bTB herd depopulation may be an effective response to a serious outbreak of bTB in a herd. Full-herd depopulation on the other hand is unlikely to be a significant part of the initial stages of an eradication programme in a high incidence country. To alleviate some of the problems experienced with tuberculin tests considerable research efforts have been deployed in the effort to develop blood based assays which could be used either to augment or perhaps eventually replace tuberculin testing in cattle [94–98]. Other than the Interferon-γ assay, which is approved for use in the EU and by the OIE as an ancillary test for the purpose of identifying additional infected animals in known infected herds, the majority of such assays remain at the research and development stage [72, 94].

5.3. Development of Testing Policies. Among animal health professionals there has been ongoing discussion concerning the linkage between policy and science [99–101], for example "the Role of Science in Food Policy" discussions held in Brussels in October 2010 on the initiative of the President-in-Office of the Council of Agriculture Ministers, Sabine Laruelle, the Federal Public Service for Health, Food Chain Safety, and the Environment under the Belgian Presidency of the Council of the European Union. Adapting policy to reflect scientific knowledge as it becomes available is not new to the field of tuberculosis. Paterson refers to the wealth of information on the experimental and natural pathogenicity of mycobacteria in the reports of the Royal Commissions on tuberculosis dating from 1907, 1909, 1911, and 1913 [66]. Ritchie in the same 1959 publication referring to the eradication of tuberculosis is quite clear that the experience of other countries and publications in the scientific literature of the day was taken into account when designing the British eradication programme [64]. Thus the experience of the USA where as early as 1900 measures to prevent both entries of infected animals from Europe and disease spread within the States commenced and where infection was eliminated on a geographic basis formed the foundation for the British programme. Also considered were the programmes from Finland where tuberculosis was brought under State control in 1898, from Denmark, where an eradication plan was introduced in 1922, from The Netherlands, which commenced control in Friesland early in the 20th century, and from Canada, which introduced an accredited herd plan in 1919. The exchange of experience between countries went in both directions as evidenced when The Netherlands, in order to overcome the problem of nonspecific reactions to tuberculin occurring in tuberculosis-free areas, introduced a comparative skin test in 1950, adopting the English directives, which they later in turn modified [102]. In 2006 More and Good reviewed the scientific and policy

advances in the tuberculosis eradication programme in Ireland over the previous 20 years [52]. Other authors have also described disease epidemiology in bovines and other species including wild-life reservoirs, disease surveillance, risk evaluation, and risk management during control and eradication programmes [54, 56, 59, 60].

6. Discussion and Conclusion

In countries with bTB eradication programmes, operating on a test and cull basis, incidence rapidly declines and clinical evidence of tuberculosis in cattle is seldom encountered because the intradermal tuberculin test enables presumptive diagnosis and elimination of infected animals during the preclinical stage. Prior to the adoption of national bTB eradication campaigns, however, clinical signs associated with tuberculosis in cattle, with associated economic impact, were commonly observed [15].

Research continues into test development particularly blood-based assays [94, 96, 98]. With a view to further reducing TB levels in cattle considerable research effort is being expended, in the UK and Ireland, on the development of a vaccine to protect badgers from TB and thus to reduce transmission both between badgers and from badgers to cattle [91, 103, 104]. The UK has also been exploring the efficacy of cattle vaccination. The possibility of developing genetic lines of cattle with higher resistance to infection with *M. bovis* without impacting negatively on other desirable genetic traits is an exciting prospect [105, 106]. Scientific advances will undoubtedly continue and these will be incorporated into bTB eradication or control programmes as appropriate. Other policy adaptations will result as a consequence of country or regional specific epidemiological studies and/or data analysis. Eradication programmes should also be continuously monitored for effectiveness, with a view to identifying and evaluating the constraints to progress and implementing necessary modifications to the programme as required.

Control and eradication of bTB is a desirable objective both from an animal health perspective and also because of zoonotic implications. National bTB eradication programmes have been or are still operated in many countries throughout the world. Some of the South American countries, many of which have had voluntary programmes for a number of years, are at this time considering implementation of national compulsory programmes. In other countries, particularly in the developing world, the issue is still being debated [38, 39]. Most Member States of the EU, having commenced bTB eradication programmes with a high disease incidence, are now recognised as officially TB free under the trading directive [72]. Difficulties remain however, in some EU member states which still run eradication programmes, notably Greece, Ireland, Spain, UK and to a lesser extent Italy and Portugal. Other EU countries have intermittent or localised problems with bovine tuberculosis and thus must maintain vigilance [2, 71, 72].

Tuberculin tests remain the primary tool for eradication in the bovine, and the choice of which tuberculin test to

use for primary screening is dependent on the prevalence of mycobacteria and other cross-sensitising agents in the local environment. Tuberculin potency is critical to test performance and thus in selecting a tuberculin supply particular care should be taken to evaluate the potency assays performed during the manufacturing process. The performance of independent potency checks on tuberculin is worthy of consideration particularly in the target species. When clinical cases are removed and test and cull programmes are in operation in-contact animals do not readily acquire infection. However, for effective control of bTB the disease must be addressed in all infected maintenance species in the same ecosystem. Consequently other species sharing the environment with cattle must be risk assessed to identify potential maintenance hosts, and where other species will constitute an impediment to final eradication of tuberculosis in the bovine appropriate control strategies should be developed and/or adapted taking into consideration the experience in other countries with similar problems. Human sources of infection must be considered during epidemiological investigation of outbreaks. Data collection and data and epidemiological analysis capability must be incorporated into control and eradication programmes so that progress and the constraints to progress may be evaluated. Lessons learned elsewhere during the operation of control and eradication programmes should be considered and incorporated as appropriate. Further scientific developments in the area of vaccine production and delivery and in genomics to breed increasingly disease-resistant livestock can be expected to further the goals of bovine TB eradication in the future.

References

[1] R. Brosch, S. V. Gordon, M. Marmiesse et al., "A new evolutionary scenario for the *Mycobacterium tuberculosis* complex," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 6, pp. 3684–3689, 2002.

[2] W. M. Prodinger, A. Brandstätter, L. Naumann et al., "Characterization of *Mycobacterium caprae* isolates from Europe by mycobacterial interspersed repetitive unit genotyping," *Journal of Clinical Microbiology*, vol. 43, no. 10, pp. 4984–4992, 2005.

[3] R. G. Hewinson, H. M. Vordermeier, N. H. Smith, and S. V. Gordon, "Recent advances in our knowledge of *Mycobacterium bovis*: a feeling for the organism," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 127–139, 2006.

[4] Z. Cvetnic, V. Katalinic-Jankovic, B. Sostaric et al., "*Mycobacterium caprae* in cattle and humans in Croatia," *International Journal of Tuberculosis and Lung Disease*, vol. 11, no. 6, pp. 652–658, 2007.

[5] M. T. Javed, A. Aranaz, L. de Juan et al., "Improvement of spoligotyping with additional spacer sequences for characterization of *Mycobacterium bovis* and *M. caprae* isolates from Spain," *Tuberculosis*, vol. 87, no. 5, pp. 437–445, 2007.

[6] E. L. Duarte, M. Domingos, A. Amado, and A. Botelho, "Spoligotype diversity of *Mycobacterium bovis* and *Mycobacterium caprae* animal isolates," *Veterinary Microbiology*, vol. 130, no. 3-4, pp. 415–421, 2008.

[7] L. M. O'Reilly and C. J. Daborn, "The epidemiology of *Mycobacterium bovis* infections in animals and man: a review," *Tubercle and Lung Disease*, vol. 76, supplement 1, pp. 1–46, 1995.

[8] A. L. Michel, M. L. Coetzee, D. F. Keet et al., "Molecular epidemiology of *Mycobacterium bovis* isolates from free-ranging wildlife in South African game reserves," *Veterinary Microbiology*, vol. 133, no. 4, pp. 335–343, 2009.

[9] D. J. O'Brien, S. M. Schmitt, D. E. Berry et al., "Estimating the true prevalence of *Mycobacterium bovis* in free-ranging elk in Michigan," *Journal of Wildlife Diseases*, vol. 44, no. 4, pp. 802–810, 2008.

[10] K. C. VerCauteren, T. C. Atwood, T. J. DeLiberto et al., "Sentinel-based surveillance of coyotes to detect bovine tuberculosis, Michigan," *Emerging Infectious Diseases*, vol. 14, no. 12, pp. 1862–1869, 2008.

[11] J. A. Drewe, A. K. Foote, R. L. Sutcliffe, and G. P. Pearce, "Pathology of *Mycobacterium bovis* Infection in Wild Meerkats (*Suricata suricatta*)," *Journal of Comparative Pathology*, vol. 140, no. 1, pp. 12–24, 2009.

[12] I. W. Esple, T. M. Hlokwe, N. C. Van Gey Pittius et al., "Pulmonary infection due to *Mycobacterium bovis* in a black rhinoceros (*Diceros bicomis* minor) in South Africa," *Journal of Wildlife Diseases*, vol. 45, no. 4, pp. 1187–1193, 2009.

[13] M. G. Candela, E. Serrano, C. Martinez-Carrasco et al., "Coinfection is an important factor in epidemiological studies: the first serosurvey of the aoudad (*Ammotragus lervia*)," *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 28, no. 5, pp. 481–489, 2009.

[14] J. Pérez, J. Calzada, L. León-Vizcaíno, M. J. Cubero, J. Velarde, and E. Mozos, "Tuberculosis in an Iberian lynx (*Lynx pardina*)," *Veterinary Record*, vol. 148, no. 13, pp. 414–415, 2001.

[15] World Organisation for Animal Health (OIE), "Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2009. Chapter 2.4.7: Bovine tuberculosis adopted," May 2009, http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.07_BOVINE_TB.pdf.

[16] P. A. LoBue, D. A. Enarson, and C. O. Thoen, "Tuberculosis in humans and animals: an overview," *International Journal of Tuberculosis and Lung Disease*, vol. 14, no. 9, pp. 1075–1078, 2010.

[17] C. Thoen, P. LoBue, and I. De Kantor, "The importance of *Mycobacterium bovis* as a zoonosis," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 339–345, 2006.

[18] R. De La Rua-Domenech, "Human *Mycobacterium bovis* infection in the United Kingdom: incidence, risks, control measures and review of the zoonotic aspects of bovine tuberculosis," *Tuberculosis*, vol. 86, no. 2, pp. 77–109, 2006.

[19] P. Doran, J. Carson, E. Costello, and S. J. More, "An outbreak of tuberculosis affecting cattle and people on an Irish dairy farm, following the consumption of raw milk," *Irish Veterinary Journal*, vol. 62, no. 6, pp. 390–397, 2009.

[20] M. C. Hlavsa, P. K. Moonan, L. S. Cowan et al., "Human tuberculosis due to *Mycobacterium bovis* in the United States, 1995-2005," *Clinical Infectious Diseases*, vol. 47, no. 2, pp. 168–175, 2008.

[21] I. N. De Kantor, P. A. LoBue, and C. O. Thoen, "Human tuberculosis caused by *Mycobacterium bovis* in the United States, Latin America and the Caribbean," *International Journal of Tuberculosis and Lung Disease*, vol. 14, no. 11, pp. 1369–1373, 2010.

[22] O. Cosivi, J. M. Grange, C. J. Daborn et al., "Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries," *Emerging Infectious Diseases*, vol. 4, no. 1, pp. 59–70, 1998.

[23] R. Cicero, H. Olivera, A. Hernández-Solis, E. Ramírez-Casanova, and A. Escobar-Gutiérrez, "Frequency of *Mycobacterium bovis* as an etiologic agent in extrapulmonary tuberculosis in HIV-positive and -negative mexican patients," *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 28, no. 5, pp. 455–460, 2009.

[24] Y. K. Amdekar, "Tuberculosis—persistent threat to human health," *Indian Journal of Pediatrics*, vol. 72, no. 4, pp. 333–338, 2005.

[25] J. T. Evans, E. G. Smith, A. Banerjee et al., "Cluster of human tuberculosis caused by *Mycobacterium bovis*: evidence for person-to-person transmission in the UK," *The Lancet*, vol. 369, no. 9569, pp. 1270–1276, 2007.

[26] P. D. O. Davies, *Clinical Tuberculosis*, Chapman & Hall, Boca Raton, Fla, USA, 1st edition, 1994.

[27] P. R. Ingram, P. Bremner, T. J. Inglis, R. J. Murray, and D. V. Cousins, "Zoonotic tuberculosis: on the decline," *Communicable Diseases Intelligence*, vol. 34, no. 3, pp. 339–341, 2010.

[28] J. K. Schönfeld, "Human-to-human spread of infection by *M. bovis*," *Tubercle*, vol. 63, no. 2, p. 143, 1982.

[29] S. Bilal, M. Iqbal, P. Murphy, and J. Power, "Human bovine tuberculosis—remains in the differential," *Journal of Medical Microbiology*, vol. 59, no. 11, pp. 1379–1382, 2010.

[30] S. Godreuil, E. Jeziorski, A. L. Bañuls, T. Fraisse, P. Van De Perre, and M. L. Boschiroli, "Intrafamilial cluster of pulmonary tuberculosis due to *Mycobacterium bovis* of the African 1 clonal complex," *Journal of Clinical Microbiology*, vol. 48, no. 12, pp. 4680–4683, 2010.

[31] FSAI, *ZoonoticTuberculosis and Food Safety*, Food Safety Authority of Ireland, Dublin, Ireland, 2nd edition, 2008.

[32] G. S. Dean, S. G. Rhodes, M. Coad et al., "Minimum infective dose of *Mycobacterium bovis* in cattle," *Infection and Immunity*, vol. 73, no. 10, pp. 6467–6471, 2005.

[33] J. Francis, *Tuberculosis in Animals and Man: A Study in Comparative Pathology*, Cassell & Co. Ltd., London, UK, 1958.

[34] H. Posthaus, T. Bodmer, L. Alves et al., "Accidental infection of veterinary personnel with *Mycobacterium tuberculosis* at necropsy: a case study," *Veterinary Microbiology*, vol. 149, no. 3-4, pp. 374–380, 2011.

[35] J. Francis, *Bovine Tuberculosis: Including a Contrast with Human Tuberculosis*, Staples Press, London, UK, 1947.

[36] M. O. Ozyigit, S. Senturk, and A. Akkoc, "Suspected congenital generalised tuberculosis in a newborn calf," *Veterinary Record*, vol. 160, no. 9, pp. 307–308, 2007.

[37] A. Nolan and J. W. Wilesmith, "Tuberculosis in badgers (*Meles meles*)," *Veterinary Microbiology*, vol. 40, no. 1-2, pp. 179–191, 1994.

[38] V. C. Jha, Y. Morita, M. Dhakal et al., "Isolation of Mycobacterium spp. from milking buffaloes and cattle in Nepal," *Journal of Veterinary Medical Science*, vol. 69, no. 8, pp. 819–825, 2007.

[39] K. Srivastava, D. S. Chauhan, P. Gupta et al., "Isolation of *Mycobacterium bovis* & *M. tuberculosis* from cattle of some farms in north India—possible relevance in human health," *Indian Journal of Medical Research*, vol. 128, no. 1, pp. 26–31, 2008.

[40] J. M. Griffin and L. A. Dolan, "The role of cattle-to-cattle transmission of *Mycobacterium bovis* in the epidemiology of tuberculosis in cattle in the Republic of Ireland: a review," *Irish Veterinary Journal*, vol. 48, pp. 228–234, 1995.

[41] T. McCorry, A. O. Whelan, M. D. Welsh et al., "Shedding of *Mycobacterium bovis* in the nasal mucus of cattle infected experimentally with tuberculosis by the intranasal and intratracheal routes," *Veterinary Record*, vol. 157, no. 20, pp. 613–618, 2005.

[42] D. Menzies, "Effect of Treatment on Contagiousness of Patients with Active Pulmonary Tuberculosis," *Infection Control and Hospital Epidemiology*, vol. 18, no. 8, pp. 582–586, 1997.

[43] A. Rouillon, S. Perdrizet, and R. Parrot, "Transmission of tubercle bacilli: the effects of chemotherapy," *Tubercle*, vol. 57, no. 4, pp. 275–299, 1976.

[44] T. W. A. Little, P. F. Naylor, and J. W. Wilesmith, "Laboratory study of *Mycobacterium bovis* infection in badgers and calves," *Veterinary Record*, vol. 111, no. 24, pp. 550–557, 1982.

[45] F. J. Olea-Popelka, E. Costello, P. White et al., "Risk factors for disclosure of additional tuberculous cattle in attested-clear herds that had one animal with a confirmed lesion of tuberculosis at slaughter during 2003 in Ireland," *Preventive Veterinary Medicine*, vol. 85, no. 1-2, pp. 81–91, 2008.

[46] J. W. Wilesmith and D. R. Williams, "Tuberculosis lesions in reactor cows," *The Veterinary record*, vol. 119, no. 2, p. 51, 1986.

[47] M. A. Schoenbaum, B. H. Espe, and B. Behring, "Epidemic of bovine tuberculosis cases originating from an infected beef herd in Oklahoma, USA," *Preventive Veterinary Medicine*, vol. 13, no. 2, pp. 113–120, 1992.

[48] R. S. Williams and W. A. Hoy, "The Viability of Bovinus (Bovinus) on Pasture Land, in Stored Faeces and in Liquid Manure," *Journal of Hygiene*, vol. 30, pp. 413–419, 1930.

[49] B. J. Duffield and D. A. Young, "Survival of *Mycobacterium bovis* in defined environmental conditions," *Veterinary Microbiology*, vol. 10, no. 2, pp. 193–197, 1984.

[50] M. V. Palmer, "Tuberculosis: a reemerging disease at the interface of domestic animals and wildlife," *Current Topics in Microbiology and Immunology*, vol. 315, pp. 195–215, 2007.

[51] L. A. L. Corner, "The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 303–312, 2006.

[52] S. J. More and M. Good, "The tuberculosis eradication programme in Ireland: a review of scientific and policy advances since 1988," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 239–251, 2006.

[53] A. I. Ward, J. Judge, and R. J. Delahay, "Farm husbandry and badger behaviour: opportunities to manage badger to cattle transmission of *Mycobacterium bovis*?" *Preventive Veterinary Medicine*, vol. 93, no. 1, pp. 2–10, 2010.

[54] N. E. Tweddle and P. Livingstone, "Bovine tuberculosis control and eradication programs in Australia and New Zealand," *Veterinary Microbiology*, vol. 40, no. 1-2, pp. 23–39, 1994.

[55] G. Wobeser, "Bovine Tuberculosis in Canadian wildlife: an updated history review article compte rendu," *Canadian Veterinary Journal*, vol. 50, no. 11, pp. 1169–1176, 2009.

[56] A. Parra, A. García, N. F. Inglis et al., "An epidemiological evaluation of *Mycobacterium bovis* infections in wild game animals of the Spanish Mediterranean ecosystem," *Research in Veterinary Science*, vol. 80, no. 2, pp. 140–146, 2006.

[57] C. Gortázar, M. J. Torres, J. Vicente et al., "Bovine tuberculosis in Doñana Biosphere Reserve: the role of wild ungulates as disease reservoirs in the last Iberian lynx strongholds," *PLoS ONE*, vol. 3, no. 7, Article ID e2776, 2008.

[58] A. L. Michel, R. G. Bengis, D. F. Keet et al., "Wildlife tuberculosis in South African conservation areas: implications and challenges," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 91–100, 2006.

[59] B. Radunz, "Surveillance and risk management during the latter stages of eradication: experiences from Australia," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 283–290, 2006.

[60] T. J. Ryan, P. G. Livingstone, D. S.L. Ramsey et al., "Advances in understanding disease epidemiology and implications for control and eradication of tuberculosis in livestock: the experience from New Zealand," *Veterinary Microbiology*, vol. 112, pp. 211–219, 2006.

[61] R. M. Anderson and W. Trewhella, "Population dynamics of the badger (*Meles meles*) and the epidemiology of bovine tuberculosis (*Mycobacterium bovis*)," *Philosophical Transactions of the Royal Society of London*, vol. 310, no. 1145, pp. 327–381, 1985.

[62] J. M. Grange and C. H. Collins, "Bovine tubercle bacilli and disease in animals and man," *Epidemiology and Infection*, vol. 99, no. 2, pp. 221–234, 1987.

[63] J. B. Buxton and R. E. Glover, "Tuberculin tests in cattle," A.R.C. Report Series 4, Privy Council Agricultural Research Council, London, UK, 1939.

[64] J. N. Ritchie, "Tuberculois," in *Diseases due to Bacteria*, A. W. Stableforth and I. A. Galloway, Eds., vol. 2, pp. 713–744, Buterworths, London, UK, 1959.

[65] L. A. Baisden, A. Larsen, and T. H. Vardaman, "Relative sensitivity of different skin areas of cattle to intradermal tests," *American Journal of Veterinary Research*, vol. 12, no. 45, pp. 273–275, 1951.

[66] A. B. Paterson, "Tuberculois," in *Diseases due to Bacteria*, A. W. Stableforth and I. A. Galloway, Eds., vol. 2, pp. 671–687, Buterworths, London, UK, 1959.

[67] M. Good, T. A. Clegg, E. Costello, and S. J. More, "The comparative performance of the single intradermal test and the single intradermal comparative tuberculin test in Irish cattle, using tuberculin PPD combinations of differing potencies," *The Veterinary Journal*. In press.

[68] M. L. Monaghan, M. L. Doherty, J. D. Collins, J. F. Kazda, and P. J. Quinn, "The tuberculin test," *Veterinary Microbiology*, vol. 40, no. 1-2, pp. 111–124, 1994.

[69] R. J. North and YU. J. Jung, "Immunity to tuberculosis," *Annual Review of Immunology*, vol. 22, pp. 599–623, 2004.

[70] J. P. Caffrey, "Status of bovine tuberculosis eradication programmes in Europe," *Veterinary Microbiology*, vol. 40, no. 1-2, pp. 1–4, 1994.

[71] F. J. Reviriego Gordejo and J. P. Vermeersch, "Towards eradication of bovine tuberculosis in the European Union," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 101–109, 2006.

[72] European Commission, "Council Directive of 26 June 1964 on animal health problems affecting intra-Community trade in bovine animals and swine (64/432/EEC, with later amendments). Office for Official Publications of the European Communities, Consolidated legislation (CONSLEG)," 2009, http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri= CONSLEG:1964L0432:20091218:EN:PDF.

[73] M. Good, I. Higgins, and P. Maher, "The tuberculin test— a safe means to test a cattle population for bovine tuberculosis," *Irish Veterinary Journal*, vol. 60, no. 11, pp. 680–684, 2007.

[74] I. W. Lesslie and C. N. Hebert, "The use of dilute tuberculins for testing cattle," *The British veterinary journal*, vol. 121, no. 9, pp. 427–436, 1965.

[75] L. M. O'Reilly, "Field trials to determine a suitable injection dose of bovine PPD tuberculin for the diagnosis of bovine tuberculosis in naturally infected cattle," *Developments in Biological Standardization*, vol. 58, pp. 695–763, 1986.

[76] L. M. O'Reilly and B. N. MacClancy, "A comparison of the accuracy of a human and a bovine tuberculin PPD for testing cattle with a comparative cervical test," *Irish Veterinary Journal*, vol. 29, no. 4, pp. 63–70, 1975.

[77] L. M. O'Reilly and B. N. Mac Clancy, "Estimation of the sensitivity, specificity and predictive value of the intradermal tuberculin test," *Irish Veterinary Journal*, vol. 32, pp. 127–128, 1978.

[78] R. Cooney, J. Kazda, J. Quinn, B. Cook, K. Müller, and M. Monaghan, "Environmental mycobacteria in Ireland as a source of non-specific sensitisation to tuberculins," *Irish Veterinary Journal*, vol. 50, no. 6, pp. 370–373, 1997.

[79] H. G. Lamont, "Tuberculin testing," *The Veterinary Record*, vol. 59, no. 32, pp. 407–409, 1973.

[80] European Commission, "Directive of the European Parliament and of the Council, of 6 November 2001 on the Community code relating to veterinary medicinal products (2001/82/EC with later amendments). Office for Official Publications of the European Communities, Consolidated legislation (CONSLEG)," 2009, http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2001L0082:20090807:EN:PDF.

[81] J. Haagsma and A. Eger, "Quality testing of tuberculins and its practical consequences for the diagnosis of bovine tuberculosis," in *Proceedings of the 2nd International Conference on Animal Tuberculosis in Africa and the Middle-East*, Rabat, Morocco, October 1995.

[82] I. Davidson, "Laboratory assay methods for bovine tuberculin PPD (report of the Expert Group on Veterinary Sera and Vaccines of the European Pharmacopoeia Commission)," *Developments in Biological Standardization*, vol. 58, pp. 607–616, 1986.

[83] R. Dobbelaer, L. M. O'Reilly, A. Genicot, and J. Haagsma, "The potency of bovine PPD tuberculins in guinea-pigs and in tuberculous cattle," *Journal of Biological Standardization*, vol. 11, no. 3, pp. 213–220, 1983.

[84] M. Good, T. A. Clegg, F. Murphy, and S. J. More, "The comparative performance of the single intradermalcomparative tuberculin test in Irish cattle, using tuberculin PPD combinations from different manufacturers," *Veterinary Microbiology*. In press.

[85] J. Haagsma, L. M. O'Reilly, R. Dobbelaer, and T. M. Murphy, "A comparison of the relative potencies of various bovine PPD tuberculins in naturally infected tuberculous cattle," *Journal of Biological Standardization*, vol. 10, no. 4, pp. 273–284, 1982.

[86] J. Haagsma, "Potency testing of bovine tuberculins," *Developments in Biological Standardization*, vol. 58, pp. 689–694, 1986.

[87] World Health Organisation (WHO), "Requirements for biological substances no. 16, annex 1: requirements for tuberculins," Tech. Rep. 745, WHO, Geneva, Switzerland, 1987, http://whqlibdoc.who.int/trs/WHO_TRS_745.pdf.

[88] D. Bakker, A. Eger, J. Mcnair et al., "Comparison of commercially available PPDs: practical considerations for diagnosis and control of bovine tuberculosis," in *Proceedings*

of the 4th International Conference on Mycobacterium bovis (poster), Dublin, Ireland, August 2005.

[89] A. G. Karlson, "Non-specific or cross-sensitivity reactions to tuberculin in cattle," in *Advances in Veterinary Science*, vol. 7, pp. 148–153, Academic Press, London, UK, 1962.

[90] B. H. Rushford, "Investigations into the problem of non-specific reactors to the single caudal fold tuberculin test in Victorian dairy cattle," *Australian Veterinary Journal*, vol. 40, pp. 406–411, 1964.

[91] S. J. More, "What is needed to eradicate bovine tuberculosis successfully: an Ireland perspective," *Veterinary Journal*, vol. 180, no. 3, pp. 275–278, 2009.

[92] M. Coad, D. Clifford, S. G. Rhodes, R. G. Hewinson, H. M. Vordermeier, and A. O. Whelan, "Repeat tuberculin skin testing leads to desensitisation in naturally infected tuberculous cattle which is associated with elevated interleukin-10 and decreased interleukin-1 beta responses," *Veterinary research*, vol. 41, no. 2, p. 14, 2010.

[93] M. L. Doherty, M. L. Monaghan, H. F. Bassett, and P. J. Quinn, "Effect of a recent injection of purified protein derivative on diagnostic tests for tuberculosis in cattle infected with *Mycobacterium bovis*," *Research in Veterinary Science*, vol. 58, no. 3, pp. 217–221, 1995.

[94] E. Gormley, M. B. Doyle, T. Fitzsimons, K. McGill, and J. D. Collins, "Diagnosis of *Mycobacterium bovis* infection in cattle by use of the gamma-interferon (Bovigam) assay," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 171–179, 2006.

[95] M. Coad, S. H. Downs, P. A. Durr et al., "Blood-based assays to detect *Mycobacterium bovis*-infected cattle missed by tuberculin skin testing," *Veterinary Record*, vol. 162, no. 12, pp. 382–384, 2008.

[96] H. M. Vordermeier, A. Whelan, P. J. Cockle, L. Farrant, N. Palmer, and R. G. Hewinson, "Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle," *Clinical and Diagnostic Laboratory Immunology*, vol. 8, no. 3, pp. 571–578, 2001.

[97] J. M. Pollock, M. D. Welsh, and J. McNair, "Immune responses in bovine tuberculosis: towards new strategies for the diagnosis and control of disease," *Veterinary Immunology and Immunopathology*, vol. 108, no. 1-2, pp. 37–43, 2005.

[98] C. Whelan, E. Shuralev, G. O'Keeffe et al., "Multiplex immunoassay for serological diagnosis of *Mycobacterium bovis* infection in cattle," *Clinical and Vaccine Immunology*, vol. 15, no. 12, pp. 1834–1838, 2008.

[99] W. D. Hueston, "Science, politics and animal health policy: epidemiology in action," *Preventive Veterinary Medicine*, vol. 60, no. 1, pp. 3–12, 2003.

[100] L. King, "Impacting policy through science and education," *Preventive Veterinary Medicine*, vol. 62, no. 3, pp. 185–192, 2004.

[101] J. G. Greis and D. N. Wear, "Conducting science in the public eye," *Journal of Forestry*, vol. 100, no. 7, pp. 46–49, 2002.

[102] J. Haagsma, "Tuberculosis and tuberculin," in *"Strictly Scientific and Practical Sense". A century of the Central Veterinary Institute in the Netherlands 1904—2004* , P. Verhoef, J. M. van Leeuwen, and P. H. Bool, Eds., Erasmus Publishing, Rotterdam, The Netherlands, 2007.

[103] L. A. L. Corner, E. Costello, S. Lesellier, D. O'Meara, and E. Gormley, "Vaccination of European badgers (*Meles meles*) with BCG by the subcutaneous and mucosal routes induces protective immunity against endobronchial challenge with *Mycobacterium bovis*," *Tuberculosis*, vol. 88, no. 6, pp. 601–609, 2008.

[104] S. Lesellier, L. Corner, E. Costello et al., "Immunological responses and protective immunity in BCG vaccinated badgers following endobronchial infection with *Mycobacterium bovis*," *Vaccine*, vol. 27, no. 3, pp. 402–409, 2009.

[105] M. L. Bermingham, S. J. More, M. Good, A. R. Cromie, I. M. Higgins, and D. P. Berry, "Genetic correlations between measures of *Mycobacterium bovis* infection and economically important traits in Irish Holstein-Friesian dairy cows," *Journal of Dairy Science*, vol. 93, no. 11, pp. 5413–5422, 2010.

[106] S. Brotherstone, I. M. S. White, M. Coffey et al., "Evidence of genetic resistance of cattle to infection with *Mycobacterium bovis*," *Journal of Dairy Science*, vol. 93, no. 3, pp. 1234–1242, 2010.

Review Article

Multihost Tuberculosis: Insights from the Portuguese Control Program

Mónica V. Cunha,[1] Madalena Monteiro,[2] Paulo Carvalho,[2] Paula Mendonça,[2] Teresa Albuquerque,[1] and Ana Botelho[1]

[1] INRB, I.P./LNIV-Instituto Nacional dos Recursos Biológicos/Laboratório Nacional de Investigação Veterinária, Unidade de Sanidade Animal, Laboratório de Bacteriologia, Estrada de Benfica 701, 1549-011 Lisboa, Portugal
[2] INRB, I.P./LNIV-Instituto Nacional dos Recursos Biológicos/Laboratório Nacional de Investigação Veterinária, Unidade de Sanidade Animal, Laboratório de Patologia, Estrada de Benfica 701, 1549-011 Lisboa, Portugal

Correspondence should be addressed to Mónica V. Cunha, monica.cunha@lniv.min-agricultura.pt

This paper describes the current situation of animal tuberculosis in Portugal, reviewing the accomplishments and constraints of the 2001–2009 period. Notwithstanding the substantial progress achieved with the implementation of a comprehensive test and cull scheme, notification, postmortem inspection and surveillance at slaughterhouses, herd and animal prevalence have unexpectedly increased in 2009. In parallel, the recent awareness of tuberculosis in local free-ranging wildlife species causes concern regarding the final steps towards eradication, demanding new approaches to the existing disease control policies.

1. The National Policy on Bovine Tuberculosis

Although herd prevalence of bovine tuberculosis (bTB) in Portugal (0.20 in 2010) remains markedly below the values of other European countries, it is presently the fourth cause for rejection of cattle meat [1]. Apart from being an economically detrimental disease affecting the productivity and international trade of livestock [2], tuberculosis (TB) may potentially have a severe impact on protected and/or endangered wild animal species and, as a zoonosis, on human public health [3].

Considerable progress in bTB control has been observed in Portugal after the implementation of a comprehensive eradication scheme in 1992 approved by the European Union (Council Decision 92/299/CE) based on the single intradermal comparative tuberculin test (SICTT) of cattle aged over 6 weeks and the slaughter of reactor animals. The interferon-γ test (*Bovigam®*) is used complementarily to SICTT in nonofficially TB free herds that hold animals with doubtful reactions to the intradermal test or in herds that consecutively hold positive SICTT animals (chronic bTB herd infection).

In 2001, the program became cofinanced by the European Union (in the framework of Council Directive 64/432, as amended). The National Veterinary Authority (DGV), the National Reference Laboratory (NRL-INRB-LNIV), the Regional Veterinary Services Directorates, the regional laboratories from the Ministry of Agriculture, the municipal and private veterinarians from breeders organisations are the entities engaged in the bTB control program acting at the central, regional, or local level. The national program covers the whole territory of mainland Portugal, while a specific surveillance program is applied to the insular region of Azores, an important dairy region that has remained relatively untouched by the disease.

2. The Bovine Population in Portugal

While in the past decade, the number of existing farms in Portugal has decreased to almost half, the number of animals decreased only slightly, indicating that, on average, farms are increasing in size. In 2009, the total number of herds was 37,584 (Figure 1(a)) and the number of cattle was 1,060,831,

(a)

- —△— Herd prevalence
- —■— Animal prevalence
- ■ Reactor animals
- □ Reactor herds

(b)
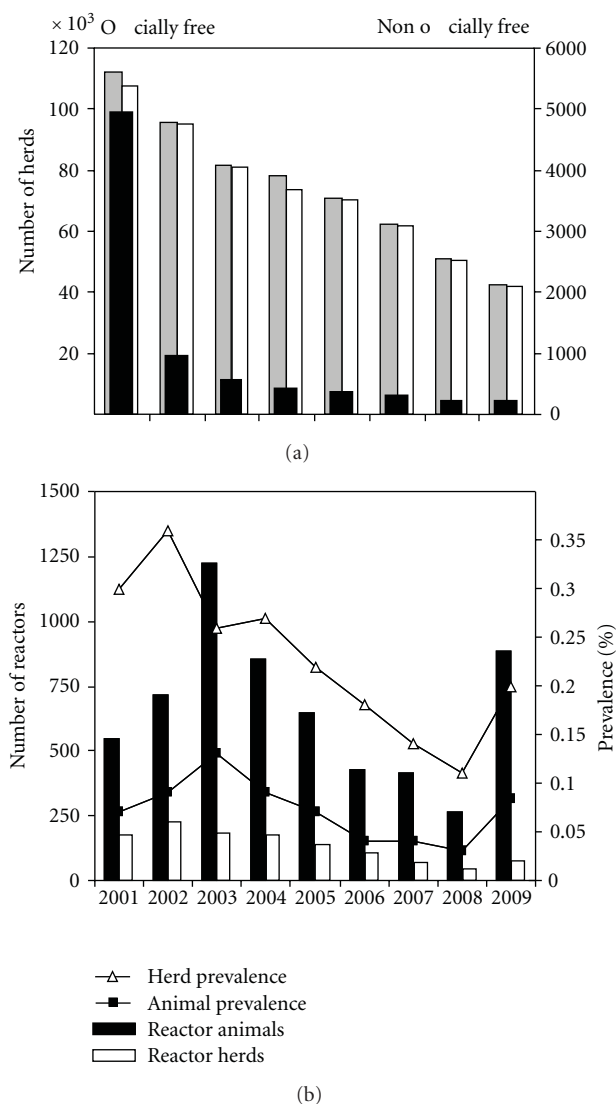
FIGURE 1: (a) Number of herds officially bTB free (white bars) and nonofficially bTB free (black bars) among the total number of herds (indicated by grey bars) in Portugal, for the period 2001–2009. Officially bTB free herds refer to herds that exclusively comprise animals exempt of disease symptoms and enclosing animals that yielded negative reactions to two consecutive official intradermal tests (applicable for cattle aged over 6 weeks). Source: [4]. (b) Number of reactor animals and reactor herds and animal/herd prevalence in Portugal for the period 2001–2009.

giving a national average of 28 animals per farm [5]. In the Northwest part of the country, the herds are usually small dairy farms, with approximately 25,000 dairy herds holding 300,000 animals. In the South-Central part, larger beef herds predominate: the Alentejo region has 4,430 beef herds comprising 523,704 animals [1].

3. Clinical and Laboratory Evaluation of Bovine Tuberculosis

An animal submitted to routine control testing under the National eradication plan is clinically considered bTB

suspect if it yields (a) a clear SICTT-positive reaction (skin reaction of >4 mm around the tuberculin injection site and/or clinical symptoms around the inoculation site such as extensive or diffuse edema, exudates, necrosis, or a pronounced inflammatory reaction of the regional lymph nodes), (b) two consecutive doubtful reactions to the intradermal test, which must be performed with an interval of, at least, 42 days, (c) a positive reaction to the interferon-γ test, and (d) a doubtful reaction to SICTT, while belonging to an herd previously confirmed as bTB infected and holding, at least, another animal with a positive SICTT reaction in the same screening test. Animals in such conditions undergo sanitary slaughter followed by official veterinary inspection and laboratory investigation of the organs and lymphatic nodes at the NRL. Finally, only the isolation of *Mycobacterium bovis* or *M. tuberculosis* during culture for mycobacteria or the observation of typical TB lesions during histopathological analysis of the organs confirms the animal as being TB infected. Animals with *postmortem* suspect lesions detected at abattoir during routine slaughter are also considered TB suspects and undergo laboratory investigation for isolation of the agent. If the suspect animal belongs to a previously confirmed TB-infected herd, it is immediately considered infected without the need of laboratory investigation.

4. Bovine Tuberculosis Surveillance Results: The Situation from 2001–2009

In Portugal, since the test and slaughter policy was put in force, 99.31% herds have been officially declared free of bTB infection (Figure 1(a)) (results from 2008), meaning that all herd animals are exempt of disease symptoms, all animals aged over 6 weeks yielded a negative reaction to two consecutive official intradermal tests, and, also, all animals introduced in the herd must arise from other officially free bTB herds. The trend in herd prevalence has been slowly improving for the past decade (Figure 1(b)) in parallel with the incidence of infected animals that has been steadily decreasing, with the lowest value verified in 2008 (0.16; 264 animals) (Figure 1(b)) [4]. However, a marked heterogeneity is observed in the prevalence rates across the six affected regions of Portugal mainland: during the 2001–2009 period, the percentage of infected herds varied from 0.05–0.1 in Beira Litoral to 0.29–1.24 in Alentejo region [4]. These geographical differences may be related to differences in herd sizes, management practices (i.e., extensive regimen) and the presence of tuberculosis-infected wildlife favouring interspecies transmission.

Unexpectedly, in 2009, a significant increase in animal prevalence was registered: 885 infected animals were detected, corresponding to 76 infected herds (59 newly infected) (Figure 1(b)). Herd prevalence and herd incidence increased twofold, while animal prevalence increased more than threefold by comparison to 2008 [1, 4, 5] (Figure 1). Moreover, persistent breakdowns occurred consistently in some regions of mainland Portugal, where the largest livestock farms are located [1, 4].

Evaluation of the reasons behind the recent increase of bTB in Portugal is not straightforward and may be a combination of genuine disease increase with higher vigilance efficacy. In fact, the increasing effort of the official authorities to raise the awareness of the various stakeholders engaged in the National bTB control program, and, consequently, to improve the *antemortem* and *postmortem* diagnosis in livestock may have biased the prevalence numbers of the last year. It is well known that the scarcity of qualified veterinary inspectors and trained personnel limits the efficiency of *antemortem* and *postmortem* examination. The effort of the authorities to train specialized personnel may have, apparently, led to an increase of the sensitivity of veterinary inspection with a greater number of specimens with visible lesions suggestive of TB being submitted to bacteriological and histopathological analysis: in 2009, 58 animals exhibiting confirmed bTB lesions were detected at abattoir, while, in the previous year, only 20 confirmed carcasses were reported [5]. In addition, during 2009, the supplier of the tuberculin used in the intradermal test was changed. Differences in the exact composition and potency of tuberculin used in the test may also have accounted for an increase of positive cases [1].

In 2009, among the 856,696 animals of the mainland submitted to the intradermal test, 716 were skin reactors, while 245 (including confirmation tests) gave positive results in the interferon-γ test (out of 2657 tested animals) [5]. Altogether, 885 animals were reported by the National Veterinary Authority as being infected with bTB [4, 5]. In 2009, only 22 animals that underwent sanitary slaughter presented typical tuberculous lesions during veterinary inspection; conversely, 58 animals that tested negative by the intradermal test presented suspect lesions at routine slaughter that were later confirmed as bTB [5], raising concern about the sensitivity of the test. Presently, all skin test positive animals are subjected to abattoir inspection and selected organs undergo histopathology and mycobacterial culture analysis. In 2009, 64.3 per cent of the samples sent to the NRL were confirmed as TB-positive through the isolation of *M. bovis* or *M. caprae* [5].

5. Wildlife Research

The difficulties existing in the final eradication steps of bTB in Portugal in livestock have raised attention to TB infection in free-ranging wildlife. Depending on the region of the world, numerous species of mammals are susceptible to *M. bovis* and even to *M. caprae* infection [3, 6]. Potential obstacles to bTB control in livestock are the existence of reservoirs in wildlife and the putative presence of the causative agent(s) in the environment [6]. Although there is no accurate census of the population of deer and wild boar in Portugal, it is recognized that the abundance and density of these species in the territory were largely increased in the last decades [7], in parallel with the proliferation of large game hunting areas/reserves and an increase of artificial management such as fencing, feeding, and watering. A few national studies have identified two regional foci where wildlife species are infected with *M. bovis* with growing evidence of being disease reservoirs and implicated

in a number of tuberculosis breakdowns in cattle [8–11]. Specifically, in Idanha-a-Nova county located in the Central East part of the country, around Tejo International River, and in Moura-Barrancos situated in the South East part of Alentejo region along Guadiana River, wild boar (*Sus scrofa*) and deer (mainly red deer-*Cervus elaphus*) are infected with *M. bovis* or *M. caprae* [8–12]. Although these two foci are geographically separated, they share common characteristics: (a) they are two of the most important hunting regions holding important densities of these two wild ungulate species that are also key game species; (b) the highest prevalence of TB in cattle is in these two regions; (c) larger beef herds and extensive management production systems predominate, favoring transmission between livestock and wildlife.

6. Bacteriology-Based Diagnosis from 2002 to October 2010

Organ or lymph node samples from either domestic or wild species sent to the NRL for the confirmation of TB infection undergo processing according to OIE standard procedures [13]. After homogenisation, decontamination and neutralisation, samples are inoculated onto egg-based solid and liquid media (Lowenstein-Jensen with pyruvate, Stonebrinks and BACTEC 9000) and incubated for a minimum of 8 weeks at 37 C. Ziehl-Nielsen stains are routinely performed and species identification of presumptive mycobacterial isolates is based on the restriction fragment length polymorphism analysis of the PCR-amplified *gyrB* gene after digestion with *Rsa*I and *Sac*II to distinguish *M. bovis* and *M. caprae* from the other members of *Mycobacterium tuberculosis* complex [14], or using the commercial reverse hybridization assays *INNO-LiPA Mycobacteria* (Innogenetics, Belgium) or *GenoType Mycobacterium* (Hain diagnostics, Germany).

M. bovis is, as expected, the main species/ecotype of the *Mycobacterium tuberculosis* complex (MTC) isolated from the majority of animal tuberculosis cases in the country, both in domesticated cattle and in wild species. Nevertheless, *M. caprae* has also been isolated from cattle under extensive management and wild boar [12, 15], despite being primarily associated with disseminated caprine TB in goats [12]. More than thirty eight per cent of 5477 samples from suspect cattle submitted to culture during the 2002–2010 period were confirmed as TB positive. Around 1% ($n = 23$) of the mycobacterial isolates obtained from cattle were identified as *M. caprae* while 92% were *M. bovis*. The remaining isolates belonged to *Mycobacterium avium* complex or to environmental nontuberculous mycobacteria. Forty per cent of the samples analyzed from farmed goat (*Capra aegagrus hircus*, $n = 124$) were confirmed as caprine TB, 85% of the cases being related to *M. caprae* infection.

During the same period, 343 samples from wild boar and 544 samples from red deer were received at the NRL for the confirmation of tuberculosis. These samples invariably originated from nonfenced, free-ranging populations of deer and boar hunted in two out of most important big game hunting counties of the country: Idanha-a-Nova and Moura-Barrancos. Although veterinary inspection is not

compulsory in hunting game, suspect samples were sent to the NRL following gross pathological evaluation performed systematically in the field by local veterinarians in hunting game activities that request official veterinarian assistance. Isolation of *M. bovis* or *M. caprae* occurred in, approximately, 51% of the tissue samples examined from red deer and 63% from wild boar. As in the case of tuberculosis in cattle, TB infection caused by *M. bovis* was definitely most frequent, although a few *M. caprae* isolates ($n = 3$, 1.4%) were also detected in boar [8, 12].

7. Insights from Molecular Epidemiology

DNA fingerprinting has been applied to a proportion of the isolates obtained from different host species to improve our understanding of the epidemiology of the disease at the local and national level. The molecular analysis of *M. bovis* and *M. caprae* isolates obtained in Portugal from skin test reactor animals, farmed domestic animals, and wild animals has been performed by the NRL. Although, initially, DNA fingerprinting was only applied to selected isolates based on geographical, host species, and herd persistent breakdowns criteria, from 2009 onwards, genotyping is being applied to all *M. bovis* and *M. caprae* isolates. The combination of spoligotyping and MIRU-VNTR using a hierarchical two step approach has contributed to the disclosure of the prevalent *M. bovis* and *M. caprae* strains involved in TB infection [8, 10, 12, 15]. Fifty spoligotyping patterns (www.mbovis.org) have been obtained from five animal species (farmed cattle, goat and sheep, wild boar, and red deer) that resulted from the characterization of 546 *M. bovis* (49 patterns) and 22 *M. caprae* isolates (one pattern) from 2002 to 2009 (Table 1). The molecular characterization of *M. bovis* and *M. caprae* isolates from red deer and boar has confirmed the apparent high genotypic diversity observed in cattle isolates with the disclosure of 27 spoligotypes, some being shared with cattle, while others appear to be specific to wild hosts [8]. These molecular epidemiology studies have positively correlated the presence of certain *M. bovis* genotypes in infected red deer with bTB occurrence in cattle from the same region by the same strain (as revealed by spoligotyping and MIRU-VNTR) but also in wild boar populations, providing evidence of disease interaction between several host species [8].

The most frequent spoligotyping profiles isolated in Portugal during the 2002–2009 period was SB0121 (23%), followed by SB0119 (12%). The relative prevalence of the predominant *M. bovis* genotypes varies according to the animal host: SB0121 and SB0119 are the most prevalent in cattle, while, in deer, SB0121 and SB0122 predominate, whilst SB1264 and SB0121 are more frequent in the wild boar isolates genotyped so far. The pattern of SB0119 differs from SB0121 by the absence of one spacer (spacer 15). Several studies of the direct repeat region in closely related strains of *M. tuberculosis*, whose polymorphism is explored in spoligotyping, have concluded that the evolutionary trend of this region is primarily by loss of single DVRs or multiple contiguous DVRs [16]. Remarkably, ten other profiles isolated until the present moment in Portugal also

exhibit a spacer deletion in relation to the profile of SB0121, suggesting the presence of a clonal complex [15]. Spoligotype SB0121 is also the most frequent pattern isolated in Spain [17], the second most predominant type in France [18] and among the top five in Italy [19]. This finding is consistent with the regular trading of animals among these Mediterranean countries. In contrast, SB0121 is infrequent in the British Isles (less than 1%) [20], where SB0140 is prevalent (35.9%) [20]. Interestingly, SB0140 strains have been isolated from all geographical regions of mainland Portugal. Regular cattle trading between Portugal and the British Isles occurred until 1996 (before the bovine spongiform encephalopathy epidemics-related embargo). The profile SB0120, commonly referred as BCG-like, corresponding to the most frequent type in France [18] and Italy [19], and representing 4% of Spanish isolates [17], is relatively infrequent in Portugal (3.2%), yet it has been isolated from three geographical regions.

MIRU-VNTR using a panel of eight validated *loci*, VNTR 3232, ETR-A, ETR-B, ETR-C, QUB11a, QUB11b, MIRU26, and MIRU4 [10], has been used complementarily to spoligotyping, being particularly useful to differentiate strains within common spoligotype groups. Genotyping of 337 isolates has already generated 127 allelic profiles [8, 15]. Polymorphisms are particularly marked among SB0121 strains, the most frequent and geographically dispersed spoligotype [10]. Furthermore, MIRU-VNTR has enabled tracking of multigenotype infected herds, reactivation cases, and, also, the assessment of intra- and interspecies transmission of *M. bovis* and/or *M. caprae* [8, 10].

8. Geographical Differences

The geographical localization of some of the *M. bovis* molecular types in the Iberian Peninsula possibly reflects localized spread of strain types and the attempt to eradicate the disease with a test and cull protocol. In addition, the historical trade of cattle and the migration of free-ranging wildlife between Portugal and Spain, particularly of deer which are nonterritorial and migratory, have also each played a part in the commonalities observed among the Iberian countries. Although the spoligotype pattern of SB0121 and related strains are not unique to Portugal and Spain, cumulative data suggests that the Iberian strains may have evolved from a single common ancestor ([15, 17], unpublished results).

Regarding the characterization of domestic farmed species isolates from the mainland, the geographical region of Entre Douro e Minho (EDM), where nearly 50% of the national herds are located, exhibits the highest strain diversity. In fact, for that region, the discriminatory power, *D*, evaluated using the Hunter and Gaston index [21, 22] and calculated with the website application http://insilico.ehu.es, is of 0.92. Unexpectedly, we also found a high discriminatory index for spoligotyping in the regions where the incidence of tuberculosis is most important: Alentejo (AL, $D = 0.88$), Trás-os-Montes (TM, $D = 0.89$), and Beira Interior (BI, $D = 0.81$). In contrast, the lowest discriminatory index is obtained for Beira Litoral (BL, $D = 0.8$), the less affected TB

TABLE 1: Patterns, animal host, and prevalence of the 21 most prevalent *M. bovis* and *M. caprae* spoligotypes isolated in mainland Portugal from 2002 to 2009 (joint results from [8, 15]). The patterns are listed in decreasing order of prevalence.

Spoligotypes	Animal species					Number of isolates	Percentage of total
	Cattle	Goat	Sheep	Deer	Boar		
SB0121	116			10	4	130	22.9
SB0119	58		1	6	4	69	12.1
SB0140	29			2		31	5.5
SB0886	28					28	4.9
SB1090	21				1	22	3.9
SB1095	21				1	22	3.9
SB0157	7	12			3	22	3.9
SB0295	19	1			1	21	3.7
SB1174	16			3	1	20	3.5
SB1264	6			8	6	20	3.5
SB0265	9	1		6	3	19	3.4
SB1172	16			2		18	3.2
SB0120	12			5	1	18	3.2
SB0124	16					16	2.8
SB0122	2			10		12	2.1
SB0134	8					8	1.4
SB1195				4	4	8	1.4
SB0856	4			3	1	8	1.4
SB1266				4	3	7	1.2
SB1273	6			1		7	1.2
SB1483	5			1		6	1.1
other	44			9	3	56	9.8

region. While most patterns are distributed throughout the country, a small number of patterns are restricted to specific regions [15]. Differences in herd sizes and management practices may account for differences among geographical regions. While in the most affected regions, such as Alentejo and Beira Interior, the predominant management practices are the extensive regimen and large grazing areas, where cattle farms can overlap wildlife habitats and interspecies transmission may occur, in the low TB prevalence regions, there is a considerably higher number of herds but with fewer animals and stricter confinement regimens.

The genetic relatedness of *M. bovis* isolates from wild species from regions near the border of Portugal and Spain has also suggested that contact between wild animals ranging among contiguous regions of both countries may account for a considerable number of new bTB cases.

9. Insights from Lesion Distribution and Host Species Differences

Pathology evaluation and the study of lesion distribution, appearance and structure of TB diseased animals may contribute to the understanding of TB disease and the associated pathogenesis, namely, as a mean to identify the routes of infection and excretion [23].

The histopathological analysis of the organs and/or lymph nodes collected from suspect animals is, at the moment, mainly (but not exclusively) performed at the NRL.

Systematic information regarding the presence of lesions in cattle in relation to the category of slaughter is reported in Table 2 for the period 2005–2009. For the same period, we present in Table 3 the number of samples exhibiting lesions confirmed as bTB by histopathology per organ and lymph node. The numbers presented are a subset of the total results of histopathology available for mainland Portugal, reporting only to the samples analyzed at the NRL in Lisbon ($n = 933$ tissue samples for 2005–2009).

Considering that for 203 animals analyzed at the Lisbon Lab, there is no information available regarding the category of slaughter, at least 297 suspect animals (31.8% out of 933) were detected during routine slaughter from 2005 to 2009 (Table 2). Among these, 170 (57.2%) were confirmed by histopathology as bTB positive. Notably, a significant number of positive tested animals did not show any visible lesions for the period under analysis ($n = 188$, 43.8%) (Table 2).

Regarding the macroscopic lesions observed in bovine, in our experience, lymph nodes are the most commonly affected tissue and, especially, those of the respiratory system ($n = 32.8\%$ out of 933 samples) and also the head (18.3%) (Table 3). A variable number of lesions were observed, and these were characterised by the presence of a yellowish caseous necrosis (Figure 2(a)) accompanied by some degree of mineralization; the size of these lesions were very heterogeneous, ranging from a few millimetres to the entire organ. At the microscopic level, all lesions

Table 2: Number of bTB suspect tissue samples received for histopathological analysis from cattle (n = 933) per year, category of slaughter, and presence or absence of bTB lesions per year as indicated by histopathology, for the period 2005–2009.

Category of slaughter		2005		2006		2007		2008		2009		Total (no lesions)
		n	%	n	%	n	%	n	%	n	%	
Routine slaughter		54	**43**	41	**34**	18	**12**	54	**33**	130	**35**	297 (127)
TB eradication program		45	**36**	44	**37**	78	**52**	62	**37**	204	**55**	433 (188)
Information not available		27	**21**	35	**29**	55	**36**	50	**30**	36	**10**	203 (99)
Total		126		120		151		166		370		933 (414)
Hystopathological results	No lesions	68	**54**	68	**57**	56	**37**	74	**45**	148	**40**	414
	TB lesions	58	**46**	52	**43**	95	**63**	92	**55**	222	**60**	519

Table 3: Number and percentage of bovine tissue samples (organs and lymph nodes) confirmed as bTB positive by histopathology. Results concerning the period 2005–2009 are presented per year and anatomical localization.

Year		2005		2006		2007		2008		2009		Total (%)
		n	%	n	%	n	%	n	%	n	%	
	head	14	**24**	6	**12**	5	**5**	23	**25**	47	**21**	95 (**18.3**)
	thoracic	16	**28**	21	**40**	19	**20**	48	**52**	66	**30**	170 (**32.8**)
	abdominal	2	**3**	1	**2**	2	**2**	1	**1**	2	**1**	8 (**1.5**)
Affected lymph nodes	unknown	21	**36**	13	**25**	62	**65**	13	**14**	80	**36**	189 (**36.4**)
	head and thoracic	2	**3**	7	**13**	3	**3**	1	**1**	10	**5**	23 (**4.4**)
	head and abdominal	0	**0**	0	**0**	1	**1**	0	**0**	2	**1**	3 (**0.6**)
	thoracic and abdominal	0	**0**	2	**4**	1	**1**	1	**1**	10	**5**	14 (**2.7**)
	head, thoracic, and abdominal	0	**0**	0	**0**	1	**1**	0	**0**	0	**0**	1 (**0.2**)
Other organs affected	lungs	10	**17**	9	**17**	35	**37**	23	**25**	72	**32**	149
	liver	0	**0**	2	**4**	2	**2**	6	**6**	11	**5**	21

observed were characteristic of bovine tuberculosis [24] (Figure 2(b)). Granulomas observed in the lungs were, sometimes, adjacent to the bronchioles, revealing erosion of the wall and allowing leakage of the infected material into the lumen (Figure 2(c)). Ziehl-Neelsen staining of the affected tissue sections indicated a very small number of acid-alcohol fast bacilli. In some of the positive tuberculin tested animals in whom no macroscopic lesions were detected, small granulomas with no necrotic core and formed only by epithelioid and multinucleated giant cells were detected (Figure 2(d)). These represent an early stage of the infection.

In red deer, the lungs and mesenteric lymph nodes were the most affected organs (Table 4) as previously described by other authors [25]. The lesions found presented a marked heterogeneity regarding their size, ranging from very small necrotic foci to large thin-walled abscesses, sometimes reaching 5 cm wide. In the centre of these, a pale yellowish, necrotic, and purulent material was seen (Figure 3(a)). The centre of these lesions were filled by necrotic debris, scarcely mineralized containing debris, and neutrophils which also showed signs of necrosis. A narrow layer of mononucleated macrophages, lymphocytes, and a variable number of multinucleated giant cells enveloped this core (Figure 3(b)). These lesions are usually enclosed by a fibrous capsule. The pulmonary granulomas observed were variable in terms of dimensions as well as in terms of limits definition. Some of them were confluent, and they all showed central necrosis involved by mononucleated macrophages,

neutrophils, lymphocytes, and multinucleated giant cells. Sometimes, the lesions were diffuse presenting an infiltration of the lung alveoli by mononucleated macrophages, neutrophils and multinucleated giant cells (Figure 3(c)).

In wild boar, the macroscopic lesions observed indicate that the most affected organs are lymph nodes although we have no information regarding the anatomical localization of a considerable part of the analyzed samples, since they arrived to the NRL without such identification (Table 4). Well-circumscribed granulomas with central necrosis and marked mineralization were observed. Microscopically, these granulomas were characterised by the presence of a partially mineralized necrotic core involved by mononucleated macrophages, lymphocytes, and rare multinucleated giant cells and surrounded by a well-defined fibrotic capsule (Figure 3(d)).

10. Challenges for the Future

The surveillance reports of the last year concerning bovine tuberculosis in Portugal suggest the possibility of a (re)emergent disease, demanding the creation of a large strategic framework combining prioritized research, improved management strategies, and the consistent engagement of stakeholders. There has been a growing effort in raising awareness of the stakeholders to the importance of bTB control and to the involvement in the programmed actions of the eradication plan. This has been accomplished

(a)

(b)

(c)

(d)
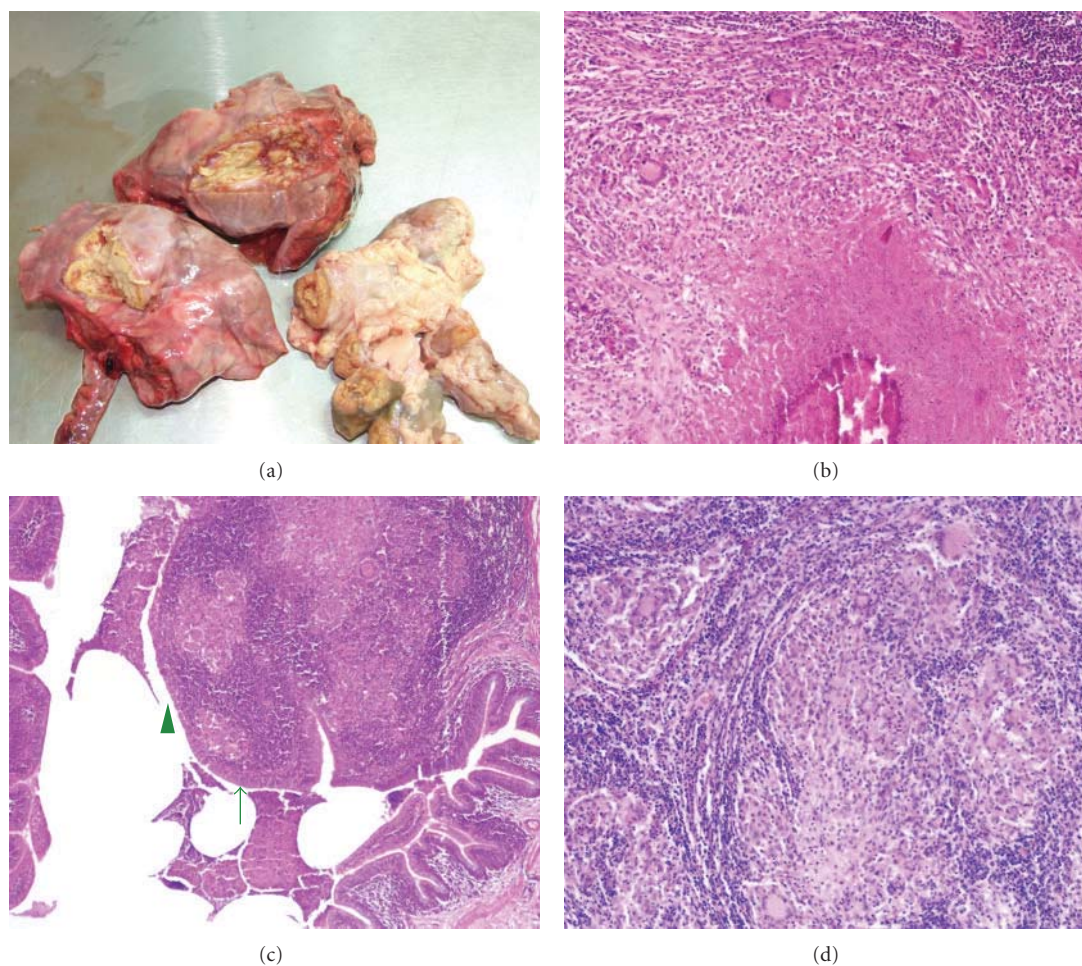
Figure 2: Typical macroscopic and microscopic lesions from bovine. Legend: (a) Lung and lymph node with extensive yellowish caseous necrosis; (b) Lymph node (100x)—a typical tuberculous granuloma with central necrosis, mineralization and multinucleated giant cells; (c) Lung (40x)—intrabronchial granuloma. Note the multinucleated giant cells. Arrow: bronchiolar epithelium; arrowhead: eroded bronchiolar epithelium; (d) Lymph node (100x)—microgranulomas with multinucleated giant cells and epithelioid cells.

Table 4: Results of the histopathological analysis of tissue samples from wild boar ($n = 153$) and red deer ($n = 356$) per year for the period 2005–2009, indicating the number of samples presenting lesions in relation to their anatomical localization.

Year	2005		2006		2007		2008		2009		Total	
	Boar	Deer	Boar	Deer	Boar	Deer	Boar	Deer	Boar	Deer	Boar	Deer
Lesions	**1**	**18**	**12**	**21**	**3**	**13**	**46**	**54**	**57**	**81**	**119**	**187**
Lung	1	8	1	10	0	4	1	26	3	29	6	77
Liver	0	0	0	1	0	1	0	0	0	0	0	2
Spleen	0	0	1	0	0	0	0	0	1	0	2	0
Lung + LMD	0	3	2	1	0	0	0	5	2	6	4	15
HLN	0	0	0	0	0	0	4	0	7	0	11	0
LMD	0	0	0	0	0	0	0	3	0	0	0	3
LMES	0	2	1	5	0	1	1	3	1	15	3	26
LMD + LME	0	0	0	1	0	0	0	0	0	0	0	1
NIL	0	5	7	3	3	7	40	17	43	31	93	63
No lesions	**0**	**2**	**1**	**4**	**11**	**27**	**9**	**132**	**13**	**4**	**34**	**169**
Total	**1**	**20**	**13**	**25**	**14**	**40**	**55**	**186**	**70**	**85**	**153**	**356**

Legend: LMD: mediastinal lymph nodes; LMES: mesenteric lymph nodes; HLN: head lymph nodes; NIL: nonidentified lymph nodes; Lung + LMD: lesions detected both in the lungs and in the mediastinal lymph nodes; LMD + LMES: lesions detected both in mediastinical and mesenteric lymph nodes.

(a)

(b)

(c)

(d)
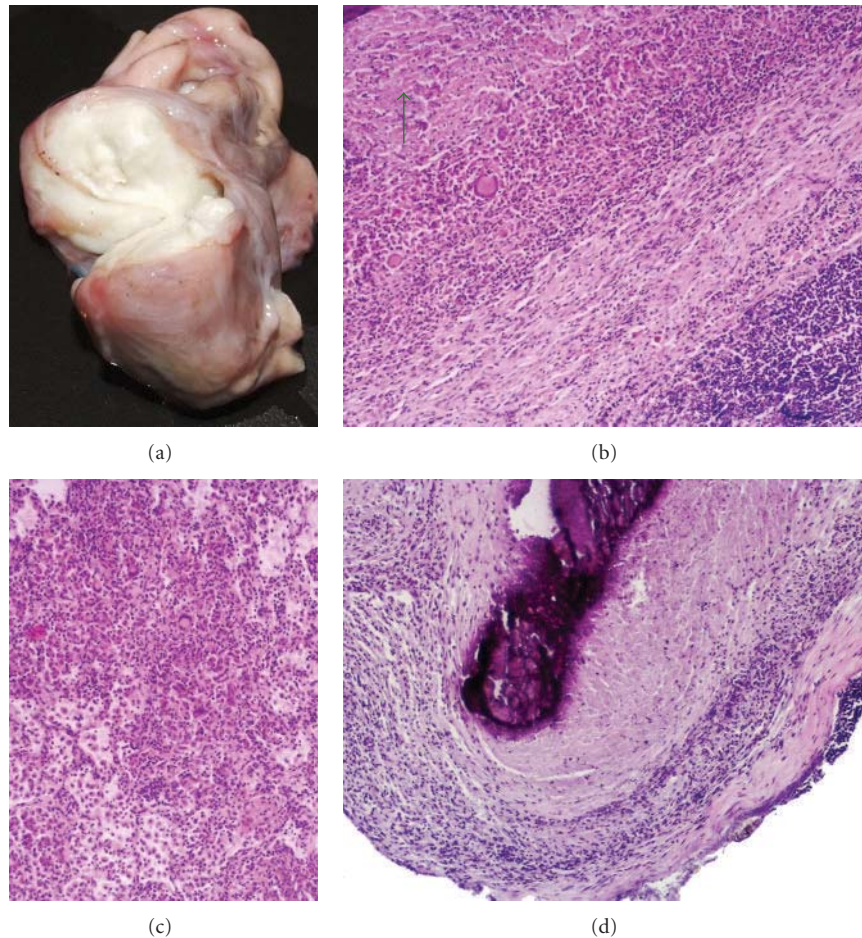
FIGURE 3: Typical lesions from deer and wild boar. Legend: macroscopic (a) and microscopic (b) images of red deer abscesses containing (a) a pale yellowish necrotic and purulent material and (b) (100x) necrotic area surrounded by epithelioid cells and multinucleated giant cells (as indicated by arrow); (c) micrograph of deer lung (100x): note the diffuse infiltration by epithelioid cells, neutrophils and multinucleated giant cells; (d) (40x) micrograph of a lymph node from wild boar: note the granuloma with extensive necrosis and mineralization.

through the education and training of veterinarians, closer followup and supervision of field veterinarians that perform the tuberculin test, training of abattoir workers and slaughterhouse inspectors, registry, control of animal movements and premovement testing, surveillance of wildlife and definition of risk areas for TB transmission, and the adoption of polyphasic laboratory practices (blood-based laboratory tests and the combination of traditional culture methods with molecular methods) to improve animal TB diagnosis and knowledge of the associated epidemiology. All breakdowns detected via testing or slaughterhouse inspection are investigated through epidemiological queries with the aim of determining if the infection was introduced via infected cattle. Movements are traced back. While spoligotyping was initially applied to a subset of strains as a particular initiative of the National Reference Laboratory, at the present time, in certain problematic areas, such as those that overlap wildlife, the determination of *M. bovis* strain type is now also requested by the National Veterinarian Authority to be compared with the local and national database.

Efforts have been undertaken to gain information on geographical distribution and prevalence rates of TB in certain wild species. The research findings of the last three years have contributed to define the risk areas for TB prevalence in wildlife and transmission from and to livestock, causing concern for the possibility of disease spread to other, apparently, unaffected areas. Problems in wildlife appear, so far, to be localized in the South-Central part of the country, with a greater emphasis on Tejo International region [8, 9, 11]. Monitoring studies in wildlife hosts in other dedicated study areas should, however, be performed.

The successful eradication of TB from countries with reservoirs in wild animals requires focus on the surveillance of wildlife reservoirs of *M. bovis* infection. Particularly, it demands progress into the development of sensitive and specific diagnosis assays available for use in field conditions in susceptible mammal host species as an important component of the strategies to improve bTB control efforts [3, 6]. Moreover, while bTB surveillance in cattle is homogeneous throughout the country, official veterinary inspection is not compulsory in big game-hunting activities, unless the

meat is introduced in the commercial route. Therefore, differences in the standards of postmortem inspection by veterinarians may account for the discrepancies in the TB incidence/prevalence rates that have been observed in the wildlife surveys conducted in Portugal. Efforts should be undertaken to improve this situation. Furthermore, TB surveillance in wildlife has been almost exclusively focused on examination of samples from hunter-killed deer and boar. This method underestimates the prevalence of TB, because animals are sampled only if they present macroscopic lesions.

The acknowledgment of the presence of TB in wildlife in the country presents a new challenge on approaches to disease control with an impact on the existing surveillance policies. Sanitary inspection in large game species should become systematic and compulsory. A surveillance network should be established all over the country to examine deer and wild boar in other unstudied areas but also to include, in known TB problematic areas, other abundant, widely distributed, animal species, such as those that scavenge on the carcasses of potentially infected animals (e.g., foxes) and/or have important social networks (badgers) and/or live as a group. In addition, efforts to prevent contacts between wildlife and livestock in risk areas should be undertaken, especially in areas of important artificial management. Fencing should be advocated and separate feed and water for different species should be warranted to prevent disease transmission. Limiting feeding of wildlife species to reduce the overall population and aggregation should also be considered in certain, highly affected areas. Management strategies should be combined at all hierarchical levels and effective communication improved through national, regional, and local networks. Management and research outputs should become clearly available to the community. Failure to adapt control programs and management strategies to the present state of the art in livestock and wildlife may perpetuate the disease, endangering the economic investment completed so far.

References

[1] "Bovine Tuberculosis Sub-Group Task Force," Tech. Rep. SANCO/11672/2010, European Commission, Health & Consumers Directorate-General, 2010.

[2] W. Amanfu, "The situation of tuberculosis and tuberculosis control in animals of economic interest," *Tuberculosis*, vol. 86, no. 3-4, pp. 330–335, 2006.

[3] A. L. Michel, B. Müller, and P. D. van Helden, "*Mycobacterium bovis* at the animal-human interface: a problem, or not?" *Veterinary Microbiology*, vol. 140, no. 3-4, pp. 371–381, 2010.

[4] *Programa de Erradicação da Tuberculose Bovina de 2010*, Direcção Geral de Veterinária, DGV, Portugal, 2010.

[5] *Tuberculose Bovina: Relatório Técnico*, Direcção Geral de Veterinária, DGV, Portugal, 2010.

[6] L. A. L. Corner, "The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 303–312, 2006.

[7] J. Vingada, C. Fonseca, J. Cancela, J. Ferreira, and C. Eira, "Ungulates and their management in Portugal," in *European Ungulates and Their Management in the 21st Century*, M.

[8] Apollonio, R. Andersen, and R. J. Putman, Eds., pp. 392–418, Cambridge University Press, Cambridge, England, 2010.

[8] M. V. Cunha, F. Matos, A. Canto et al., "Implications and challenges of tuberculosis in wildlife ungulates: a molecular epidemiology perspective," *Research in Veterinary Science*. In press.

[9] M. Vieira-Pinto, J. Alberto, J. Aranha et al., "Combined evaluation of bovine tuberculosis in wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) from Central-East Portugal," *European Journal of Wildlife Research*, pp. 1–13, 2011.

[10] E. Duarte, M. Domingos, A. Amado, M. V. Cunha, and A. Botelho, "MIRU-VNTR typing adds discriminatory value to groups of *Mycobacterium bovis* and *Mycobacterium caprae* strains defined by spoligotyping," *Veterinary Microbiology*, vol. 143, no. 2–4, pp. 299–306, 2010.

[11] N. Santos, M. Correla-Neves, S. Ghebremichael, G. Källenius, S. B. Svenson, and V. Almeida, "Epidemiology of *Mycobacterium bovis* infection in wild boar (*Sus scrofa*) from Portugal," *Journal of Wildlife Diseases*, vol. 45, no. 4, pp. 1048–1061, 2009.

[12] E. L. Duarte, M. Domingos, A. Amado, and A. Botelho, "Spoligotype diversity of *Mycobacterium bovis* and *Mycobacterium caprae* animal isolates," *Veterinary Microbiology*, vol. 130, no. 3-4, pp. 415–421, 2008.

[13] *OIE, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, vol. 2, 6th edition, 2008.

[14] S. Niemann, D. Harmsen, S. Rüsch-Gerdes, and E. Richter, "Differentiation of clinical *Mycobacterium tuberculosis* complex isolates by *gyrB* DNA sequence polymorphism analysis," *Journal of Clinical Microbiology*, vol. 38, no. 9, pp. 3231–3234, 2000.

[15] F. Matos, M. V. Cunha, A. Canto, T. Albuquerque, A. Amado, and A. Botelho, "Snapshot of *Mycobacterium bovis* and *Mycobacterium caprae* infections in livestock in a bovine tuberculosis low incidence scenario," *Journal of Clinical Microbiology*, vol. 48, pp. 4337–4339, 2010.

[16] J. D. Van Embden, T. Van Gorkom, K. Kremer, R. Jansen, B. A. Van Der Zeijst, and L. M. Schouls, "Genetic variation and evolutionary origin of the direct repeat locus of *Mycobacterium tuberculosis complex* bacteria," *Journal of Bacteriology*, vol. 182, no. 9, pp. 2393–2401, 2000.

[17] S. Rodríguez, B. Romero, J. Bezos et al., "High spoligotype diversity within a *Mycobacterium bovis* population: clues to understanding the demography of the pathogen in Europe," *Veterinary Microbiology*, vol. 141, no. 1-2, pp. 89–95, 2010.

[18] N. Haddad, A. Ostyn, C. Karoui et al., "Spoligotype diversity of *Mycobacterium bovis* strains isolated in France from 1979 to 2000," *Journal of Clinical Microbiology*, vol. 39, no. 10, pp. 3623–3632, 2001.

[19] M. B. Boniotti, M. Goria, D. Loda et al., "Molecular typing of *Mycobacterium bovis* strains isolated in Italy from 2000 to 2006 and evaluation of variable-number tandem repeats for geographically optimized genotyping," *Journal of Clinical Microbiology*, vol. 47, no. 3, pp. 636–644, 2009.

[20] R. G. Hewinson, H. M. Vordermeier, N. H. Smith, and S. V. Gordon, "Recent advances in our knowledge of *Mycobacterium bovis*: a feeling for the organism," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 127–139, 2006.

[21] P. R. Hunter and M. A. Gaston, "Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity," *Journal of Clinical Microbiology*, vol. 26, no. 11, pp. 2465–2466, 1988.

[22] P. R. Hunter, "Reproducibility and indices of discriminatory power of microbial typing methods," *Journal of Clinical Microbiology*, vol. 28, no. 9, pp. 1903–1905, 1990.

[23] J. P. Cassidy, "The pathogenesis and pathology of bovine tuberculosis with insights from studies of tuberculosis in humans and laboratory animal models," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 151–161, 2006.

[24] Jubb Kennedy and Palmer, *Pathology of Domestic Animals*, vol. 2, 5th edition, 2007.

[25] J. C. Rhyan and D. A. Saari, "A comparative study of the histopathologic features of bovine tuberculosis in cattle, fallow deer (*Dama dama*), sika deer (*Cervus nippon*), and red deer and elk (*Cervus elaphus*)," *Veterinary Pathology*, vol. 32, no. 3, pp. 215–220, 1995.

Review Article

A Review of Bovine Tuberculosis in the Kafue Basin Ecosystem

Musso Munyeme[1] and Hetron Mweemba Munang'andu[1, 2]

[1] *Department of Disease Control, School of Veterinary Medicine, University of Zambia, P.O. Box 32379, Lusaka 10101, Zambia*
[2] *Section of Aquatic Medicine and Nutrition, Department of Basic Sciences and Aquatic Medicine,*
  Norwegian School of Veterinary Science, P.O. Box 8146 Dep., 0033 Oslo, Norway

Correspondence should be addressed to Musso Munyeme, munyeme@yahoo.co.uk

The Kafue basin ecosystem is the only remaining natural habitat for the endangered Kafue lechwe antelope (*Kobus leche Kafuensis*). However, hydroelectricity power production, large-scale sugar plantations, commercial fishing and increasing livestock production are threatening its natural existence and sustainability. Further, increasing human settlements within and around the Kafue basin have resulted in decreased grazing grounds for the Kafue lechwe antelopes despite a corresponding increase in cattle population sharing the same pasture. Baseline epidemiological data have persistently reported findings of bovine tuberculosis (BTB) in both wild and domestic animals, although these have been deficient in terms of describing direct evidence in the role of either lechwe antelopes or cattle in the reported observations. Despite the current literature being deficient in establishing the casual role and transmission patterns of BTB, a bimodal route of infection at the livestock/wildlife interface has been postulated. Likewise, it is not known how much of (BTB) has the potential of causing disease in humans. This paper, seeks to underline those aspects that need further research and update available data on BTB in the Kafue basin with regards to the prevalence, distribution, risk factors, threats on wildlife conservation, livestock production, public health implications, and possible mitigatory measures.

1. Introduction

The origins of mycobacterial infections despite being age-old diseases have been a subject of much debate [1–4]. However, through the works of Brosch and coworkers, they have been able to demonstrate that the genome of *M. bovis* is smaller than that of *M. tuberculosis* and that *M. bovis* has undergone numerous deletions compared to *M. tuberculosis* implying that the origin of *M. bovis* is *M. tuberculosis* [4]. It has been demonstrated that the first six ancestral *M. tuberculosis* strains that resemble the last common ancestor before the separation of *M. tuberculosis* and *M. africanum* are all human pathogens with *M. bovis* being the final member of a separate lineage that branched from the progenitor of *M. tuberculosis* isolates [4]. Further, other molecular biological studies involving DNA typing have shown that *M. tuberculosis* has been present longer than *M. bovis* [5]. Similarly, Rothschild and coworkers successfully applied spoligotyping to a 17,000-year-old skeletal specimen of an extinct North American bison and the pattern revealed

that the respective bacteria were probably not related to *M. bovis* or *M. microti,* but best fitted *M. africanum* and *M. tuberculosis* patterns [6].

Although members of the *Mycobacterium tuberculosis* complex (MTC) are responsible for the majority of mycobacterial infections worldwide, nontuberculous mycobacteria (NTM) a group of atypical mycobacteria or mycobacteria other than tuberculosis (MOTT) are increasingly becoming more of a public health significance [1, 4]. The *Mycobacterium tuberculosis* complex includes very closely related species of mycobacteria among them: *Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium microti, Mycobacterium bovis, Mycobacterium caprae*, and *Mycobacterium pinnipedii* [4]. Nontuberculous mycobacteria (NTM) include both slow growing mycobacteria (SGM) where colony formation requires at least seven days and rapid growing mycobacteria (RGM) forming colonies in less than seven days [1].

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis* (*M. bovis*), a member of the MTC [1, 4], has been

shown to have a very wide host range with a potential to cause zoonotic tuberculosis [7–9]. In Zambia, BTB was reported in cattle as far back as 1947, when the Veterinary Department diagnosed the disease in cows at Nega Nega, Kabwe, and Mazabuka [10]. The veterinary annual report of 1956, highlighted a number of areas where the disease was diagnosed; Abercorn (now Mbala), Broken Hill (now Kabwe), Mazabuka, Monze, Namwala, and Kalomo [10]. The abattoir compilation done by the Veterinary Department in the same report indicated that 1.6% of cattle slaughtered at an abattoir in Lusaka had tuberculous lesions; 2% at an abattoir in Livingstone; 5.2% Mazabuka and 16.8% of slaughtered animals from Namwala [10].

Studies on the epidemiology of BTB in Zambia have indicated that the disease is not homogenously distributed, however, high prevalence rates have been recorded within and around the Kafue basin an area with extensive overlap in terms of grazing land from wild and domestic animals [11–13]. Additionally, the lechwe antelopes have been described as feral reservoirs of BTB in Zambia [14, 15]. The disease has a historical presence in the Kafue basin that predates the identification of the area as a protected ecosystem and Ramsar Site no.530 [16]. Despite the continued reduction in annual rainfall figures, the Kafue basin still remains as one of the few lacurstrine wetland ecosystems in Zambia supporting a surging cattle population estimated at 300,000 animals [17] at a carrying density of 50 animals per square kilometre and approximately 38,000 lechwe antelopes [16] on a 6,000 square kilometre wetland [18]. The basin has further been identified as an important livestock production zone, a recognition that is threatened by the sustained and high BTB prevalence in both wild and domestic animals [19–23]. It is to a greater extent characterised by high cattle BTB herd level prevalence of around 50%, whereas a comparatively lower herd prevalence averaging 5.6% has been determined in areas outside the basin [11, 19]. Likewise, the corresponding Kafue lechwe antelopes have been shown to have a higher prevalence rate [15, 22], raising questions on a possible interspecies transmission of the disease between cattle and Kafue lechwe antelopes, however hampered by the lack of direct evidence to conclusively ascertain this assertion. Notwithstanding the elaboration of risk factors associated with BTB in the Kafue basin [11, 19], the genetic diversity and/or relatedness of lechwe and cattle BTB needs molecular epidemiological studies to elaborate on this relationship.

2. Bovine Tuberculosis in Kafue Lechwe Antelopes (*Kobus leche kafuensis*)

It has been reported that in the last 75 years, the Kafue lechwe population has declined by 85% from 250,000 animals in 1931 to 38,000 in 2005 (Table 1) [16]. The Kafue lechwe antelope, which is endemic only to the Kafue basin of Zambia, is particularly vulnerable given the persistent reports of high tuberculosis prevalence, high poaching rates, and high human settlement pressure coupled with increasing grazing pressure on few available pasturelands

TABLE 1: Population of the Kafue lechwe (*Kobus leche kafuensis*) between 1970 and 2005.

Year	No. animals	Reference
1970	94,075	Bell et al., 1973
1971	93,215	Bell et al., 1973
1972	93,158	Bell et al., 1973
1973	109,612	Osborne et al., 1975
1975	80,774	Osborne et al., 1975
1981	45,867	Howard et al., 1983
1983	41,155	Howard et al., 1983
1987	50,715	Howard et al., 1987
1988	65,018	Howard et al., 1988
1989	47,145	Jeffrey et al., 1991
1990	44,538	Jeffrey et al., 1991
1991	68,872	Jeffrey et al., 1991
1993	64,940	Kapungwe, 1993
1994	50,000	Jeffrey, 1994
1999	45,000	Kampamba et al., 1999
2001	42,119	Kamweneshe et al., 2002
2005	38,000	Chansa and Kapamba, 2010

from resurging cattle herds among other biological and anthropological factors (Table 2). The situation is further compounded by the reduction in suitable habitat through the rapid encroachment by the invasive alien mimosa weed (*Mimosa pigra*) and the disruption of the flooding cycle by the damming of the Kafue River upstream at Itezhi-tezhi [24].

Bovine tuberculosis in the Kafue basin has persisted since 1969 during which after a cropping exercise, it was realized that 14.0% of the lechwe antelopes had BTB [25]. The disease was only confined to lechwe antelopes. These findings prompted the Zambian government at that time to embark on building an abattoir specifically for screening wild animals at Lochinvar National Park. By 1971 Gallagher and coworkers through this facility had examined 125 lechwe antelopes slaughtered under a cropping exercise and recorded a prevalence of 36% (45/125) [25]. In 1972, they examined a total of 86 animals and recorded a prevalence of 33.7% (29/86) (Table 3) [25]. Between 1973 and 1974, they recorded a prevalence rate of 49% [25].

Of the other species examined by Rottcher [26, 27], BTB was only detected in an adult eland (*Taurotragus oryx*) that had generalized lesions involving the lungs, pleural, and mediastinal lymph nodes [26, 27]. This was one of the first reports that indicated the possibility of a spillover effect to other animals. More recent studies by Pandey [13] showed a prevalence rate of 19.2% ($n = 177$) in the Lochinvar NP, while Munyeme and coworkers [15] recorded a prevalence of 24.3% ($n = 119$) for the period from 2004 to 2008 across the North and South banks of the Kafue flats, indicating the continued existence of BTB in the Kafue lechwe (Table 3).

TABLE 2: Factors influencing BTB in the livestock/wildlife interface areas of the Kafue basin.

Host Factors	Environmental factors	Pathogen
High Cattle densities	Swampy/Marshy environments	Showed maintenance in lechwe antelopes for a very long time establishing reservoir host potential
High Lechwe densities	Moist pastures for most parts of the year	Shown potential for interspecies and intraspecies spread
High Cattle/lechwe interaction heightening potential of aerosol route of infection	Moist soil conditions	
Lechwe lekking behavior	Shrinking grazing grounds	
	Alien invasive weeds (*Mimosa pigra*) spreading on already shrinking pastures	
	Overlapping grazing grounds for lechwe antelopes and cattle	

3. Bovine Tuberculosis in Cattle of the Kafue Basin

In the Kafue basin, cattle ownership is a symbol of status and wealth (Figure 1). In this very complex social system of mutual obligation, cattle are often used as a medium of exchange in place of money. As a result there is a lot of exchange of cattle between families and villages implying that cattle are neither kept in closed nor stable populations due to these movements within and between herds and kraals. Still, cattle from different villages and families can be kept together in one large herd especially during transhumance and those which become permanently resident in the interface areas away from the villages more for security reasons. In short, cattle are central to the economic and social activities among the indigenous communities of the basin [28, 29].

Three types of cattle enterprises according to grazing strategy and herd size exist in the Kafue basin. The village resident herds (VRH) are small herds kept within the confines of the villages all year round. As herd sizes increase, most cattle owners start taking their animals to the wetlands (deep into the wildlife grazing zones) in search of pasture during the drier months (May to October) where grazing land is abundant. These herds return to the villages during the rainy season (November to April). This practice of taking animals to the flood plains in dry months is known as transhumance, and the cattle herds that practice this type of grazing strategy are known as transhumant herds (TH). However, some transhumant herds become very large to be supported around villages, such herds resort to be permanently resident within the wetlands drawing back to higher grounds when there are floods, but without going back to the villages. Such herds are known as interface herds (IFH). When these different types of enterprises were studied in detail, it was found that their BTB prevalence varied among them [19]. In IFH, BTB prevalence was found to be high [19]. Epidemiological studies on risk factors of BTB in the Kafue basin indicate geographical differences based on the type of cattle enterprise [11, 19]. However, the reasons for the observed spatial variations in BTB prevalence in Zambia need further elaboration to be able to conclusively ascertain

TABLE 3: Bovine tuberculosis in the Kafue lechwe of the Kafue basin.

Year	Total animals examined	Number infected	Percent affected	Reference
1956	2	2*	100%	Leroux, 1956
1962	—	—	14.0%	Anonymous, 1962
1971	125	45	36.0%	Gallagher et al., 1972
1972	86	29	33.7%	Gallagher et al., 1972
1973–1976	300	90	30.0%	Dillman, 1976
1976-1977	141	46	32.6%	Rottcher, 1978
1976-1977	38	33	23.4%	Rottcher, 1978
1976-1977	147	5	3.4%	Rottcher, 1978
1977	7	7*	100%	Clancey, 1977
1977	63	33	52.4%	Clancey, 1977
1986	41	33*	80.5%	Krauss et al., 1986
1990	92	15	16.3%	Stafford, 1991
1998	177	34	19.2%	Pandey, 1998
2004–2008	119	29	24.3%	Munyeme et al., 2010

* Samples from cachectic/and or dead animals collected from the Kafue flats.

the deterministic factors of BTB occurrence and sustenance in the basin. In combination to geographical and biological factors, studies have also intimated to a greater extent significant anthropological determinants for the observed differences within and outside the Kafue Basin [12, 13, 28]. Other, studies in more or less similar ecosystems, have also indicated that the type of cattle management becomes a significant risk factor for BTB transmission given the type of existing risk factors in that particular ecosystem [30, 31]. This becomes more elaborate when the likelihood of cattle movement in that enterprise is high [32], an important point for consideration in the Kafue basin given the high
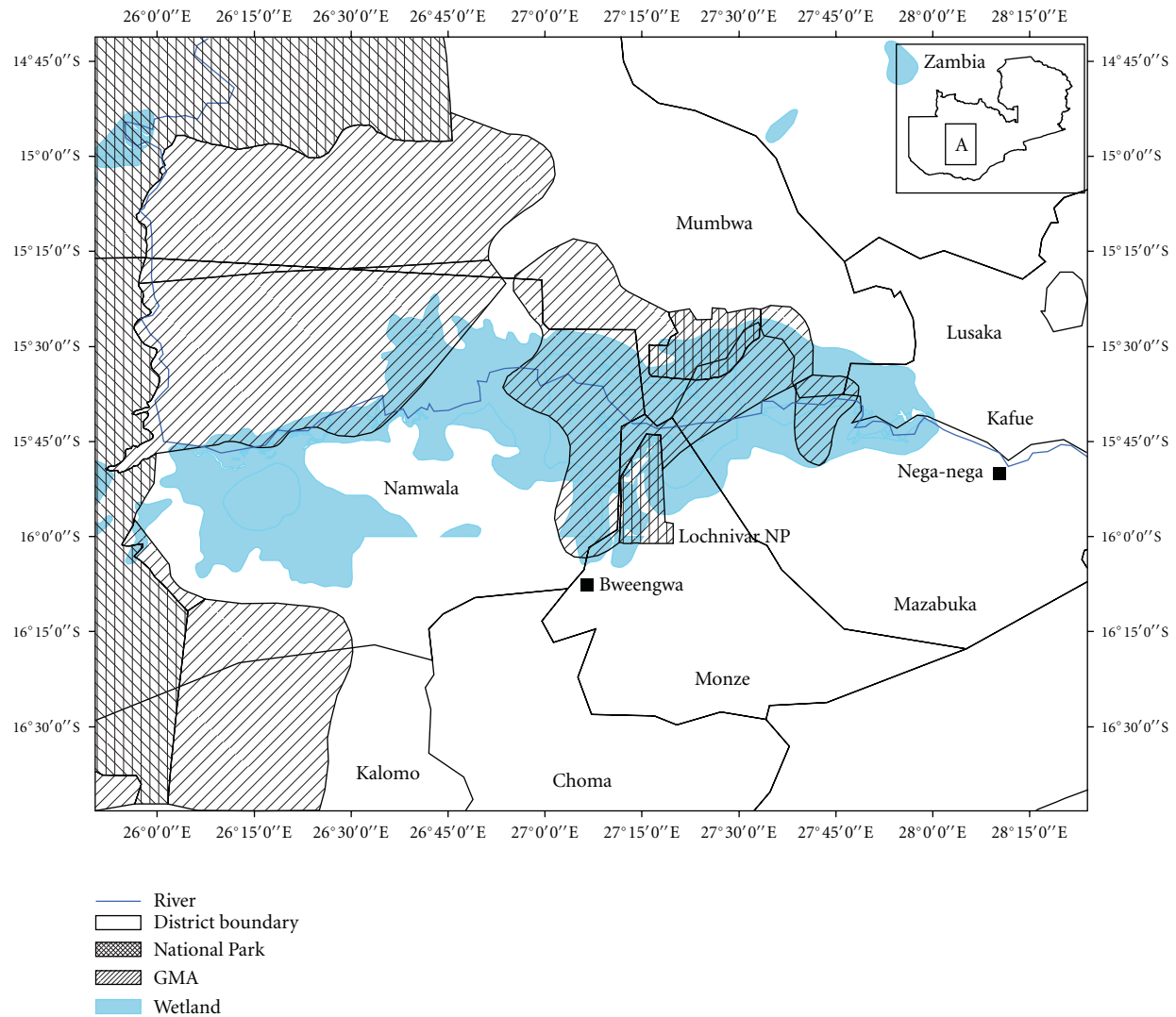
FIGURE 1: Map of the Kafue basin, insert Map of Zambia showing the location of the basin.

interaction patterns between different cattle herds and wild animal populations.

4. Conservation Implications

Due to the population decline in the Kafue lechwe (Table 1), the Zambia Wildlife Authority (ZAWA) has embarked on conservation strategies aimed at saving the remaining population from possible extinction [16]. Lack of empirical evidence on the factors contributing to the significant decline of the Kafue lechwe population has various scholars to postulate different causal factors [13, 22, 23, 33]. However, what is common amongst all the scholars is that they point out likelihood of BTB playing either a primary significant immunosuppressant role due to its chronic nature or a proxy role for other coinfections such as parasites and possible nutritional and other related stress factors [13, 22, 23, 33].

As a response to ZAWA's call to conserve the remaining lechwe antelopes, *ex-situ* conservancies have started rearing

the Kafue lechwe outside government protected areas on private-public partnerships (PPPs) where the conserved animals are kept on private game ranches but still considered government property. Thus far, approximately 700 Kafue lechwe antelopes have been translocated from the Kafue basin into game ranches. In order to promote the successful rearing of these animals on game ranches, it is imperative that a "BTB free breeding stock" is raised for translocation purposes to game ranches. The danger of translocating BTB infected animals is that they could serve as a source of new infection in new naïve areas subsequently introducing the disease to other animal species in *ex situ* conservancies which would end up reaching the human food chain. Inadvertently, BTB has since been detected from Kafue lechwe on game ranches [34]. Given that all Kafue lechwe antelopes currently reared on game ranches in Zambia originate from the Kafue basin particularly form Lochinvar NP, it is likely that the disease was introduced from lechwe that were translocated from the Kafue basin. The translocation of the Kafue lechwe

to game ranches was carried out without prescreening for BTB. Hence, it is imperative that a "BTB free herd" of Kafue lechwe is generated as breeding stock for translocating to game ranches and for further conservation purposes.

5. Economic Implications

Unlike the developed world that recognised the importance of eradicating the disease from cattle, most African countries, Zambia inclusive, argue that BTB is not a disease of national economic importance and as such, there is lack of both political will and intervention measures from respective governments. However, these arguments that BTB in cattle and wildlife in African countries does not need intervention based on economical reasoning may not be entirely justifiable. It can be argued that western-based methods of cost-benefit analysis may underestimate the value of a loss of a cow through abattoir condemnation of a carcass as a result of generalised BTB, without mentioning the threat posed to abattoir workers [7, 8]. However, the real value of an animal in a Zambian pastoral community is not only based on commercial value at the point of sale. This is so because the majority of the benefits obtained from traditional animals in rural Zambian pastoral communities are intangible and incommensurable such as social security, social status, transportation means, and credit worthiness among others. Thus to assess the real economic impact of BTB in the traditional livestock sector of Zambia, there is need for reliable and accurate epidemiological and socioeconomic information regarding the exact impact of the disease, thus its spread, maintenance, prevalence, socioperturbations, abattoir condemnations, and so forth.

Diagnosis of tuberculosis in wildlife has far reaching and serious consequences both nationally, and internationally. The current creation of Transfrontier Conservation Areas (TFCA) in Southern African countries with the translocation of wild animals across borders cannot be done with infected wildlife populations. Thus despite the perceived low returns in controlling BTB by many African countries, the accompanying benefits from eradication of the problem are incomparable. Further, a BTB free state will mean that certain sanitary mandates will have been achieved and can foster a country to enter certain highly lucrative and competitive dairy product and beef markets at a global stage, a direct economic benefit that most policy makers in African countries are oblivious of. Further benefits are through increased production efficiency to subsistence farming with majority of resource poor traditional farmers entirely dependent on livestock as the only source of livelihood. The lack of infectious communicable diseases in wildlife populations may mean increased ecotourism which is a direct source of much needed foreign revenue in Zambia and other developing countries alike. The net results of these economic benefits from disease-free livestock and wildlife may have a substantial boost and trickledown effect to the country's Gross Domestic Product (GDP).

This BTB free state can be achieved by developing rational and realistic strategies capable of controlling *M. bovis*

infection in wild and domestic animal populations. However, eradication of BTB at the livestock/wildlife interface area is costly considering the need for sustained and long-term intensive surveillance. In addition, each ecosystem has its unique challenges, just like in the Kafue basin where control strategies must take into consideration the complex nature of cattle ownership and other local practices of the cattle owners [35]. Factors relating to interaction patterns within and between herds of cattle and wildlife must be analyzed and possible transmission routes identified before any intervention strategies are proposed [35]. Control measures should be applied both for wild and domestic animal hosts simultaneously. In wildlife hosts, the identification and removal of infected animals and the creation of *ex situ* disease-free populations for future restocking into depleted National Parks is one of the most viable control options. However, with wildlife, the detection of infected animals is made difficult by the mode of restraint which is prohibitively expensive as it is based on delivering chemicals through darts (projectiles) fired from special guns. Limiting interaction between domestic and wild animals with simultaneous application of control strategies across species is recommended. Eradication campaigns in cattle such as test and slaughter schemes, despite having been shown as unsuitable for Africa [7], can still be applied together with other surveillance systems. These control measures must be backed by an animal disease control fund for the indemnification of cattle owners. Unfortunately, due to cost implications, such a scheme in most African countries still faces serious challenges.

6. Public Health Implications

In Zambia, the burden of *M. bovis* infection in humans is still unknown more so that the disease is clinically indistinguishable from that caused by *M. tuberculosis* [7, 12]. To a greater extent, such information still remains unavailable in most developing countries [7]. However, epidemiological studies conducted in high cattle rearing areas within Zambia have intimated possible BTB association between cattle and human populations although these studies have not been conclusive enough [12]. However, risks of disease especially those of zoonotic nature such as BTB remain a major threat to pastoral communities although the real extent of this threat is yet to be elucidated [12]. It is likely that despite the paucity of information in this region, coupled by the nonpasteurisation of milk, cattle is predisposed as a likely source of zoonotic TB for man [36]. Studies have indicated a high proportion of pastoral communities within the Kafue basin not pasteurizing their milk as they want to consume it in a soured form as relish or local traditional yoghurt [28, 37]. The lack of regular testing of cattle herds compounded by the lack of funds to indemnify affected farmers means the problem of BTB is likely to be widespread as it still remains without mechanisms of detection and control in place.

The relatively low incidence of development of open (infectious) pulmonary tuberculosis due to *M. bovis* in man is almost certainly due to immunological factors which

- ● Lechwe sampling sites
- ● Cattle sampling sites
- 🐾 Swamps and dambos
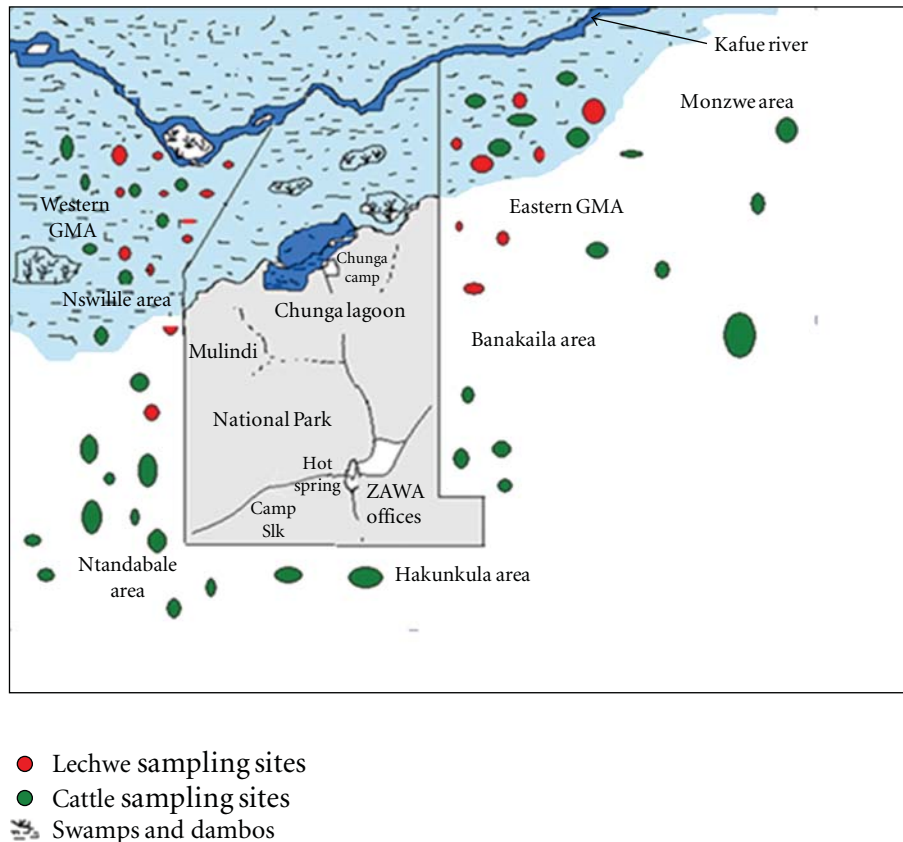
FIGURE 2: Map of Lochinvar indicating interacting sites of lechwe and cattle of the Kafue basin.

can be abrogated in HIV/AIDS. This should be cause of concern given the impact of the HIV/AIDS pandemic in resource poor countries such as Zambia with high prevalent BTB in livestock. Given the lack of diagnostic services in most rural settings where BTB prevalence is high in cattle populations, possible cases of BTB may actually go undetected [12]. With *M. bovis* being naturally resistant to a first line antituberculosis drug (pyrazinamide) and a threat of its possible circulation in humans may cause concern despite the probability being remote. In Zambia, the general lack of knowledge on zoonotic tuberculosis [37] poses another risk factor for the ease of contracting the disease [37]. Observations like these are very important in the establishment of viable workable control programs in future when public awareness campaigns and education will be sought.

7. Transmission

In the Kafue basin, *M. bovis* infection has persisted in both lechwe and cattle for a very long time without understanding the conditions the causal relationship between cattle and lechwe antelopes [15]. It has however been observed by various scholars that disease is well maintained in both cattle and lechwe antelopes and that both species have subsequently become effective disseminators [15, 20, 22]. However, up to

now, intra- and interspecies transmission routes of infection between cattle and lechwe herds are yet to be illustrated. Thus there is need for further research to understand the transmission dynamics of the disease between lechwe antelopes and cattle. However, the gregarious nature of lechwe antelopes with higher herd densities obtained in drier seasons is thought to facilitate intraspecies transmission of *M. bovis* within the lechwe antelopes themselves [22, 29, 38]. Yearly seasonal floods are also thought to play a role in the propagation and dissemination of micro-organisms in the environment (a point which needs further study and elaboration), while overcrowding of animals during lekking (mating season) with extralarge assemblages at watering points enhances the direct animal to animal transmission due to the contagious nature of the disease [39]. Available literature on gross pathological distribution of tuberculous lesions in both cattle and lechwe antelopes intimate a respiratory route of infection [13, 15, 22] with over 60% of tuberculous lesions in both cattle (Figure 3) and lechwe antelopes (Figure 4) being confined to the lungs (Figures 3 and 4). This figure may be higher considering that abattoir-based meat inspection relies heavily on visible gross lesions which may be missed if such lesions are discrete and small. Such findings indicate that environmental contamination of pasture may be a less effective method of interspecies transmission between lechwe and cattle in the Kafue basin. Biological plausibility of disease transmission of *M. bovis*

FIGURE 3: Caseous granulomatous material in the Lung and draining lymph nodes of a cow during routine abattoir surveillance in Namwala District close to the Kafue basin.
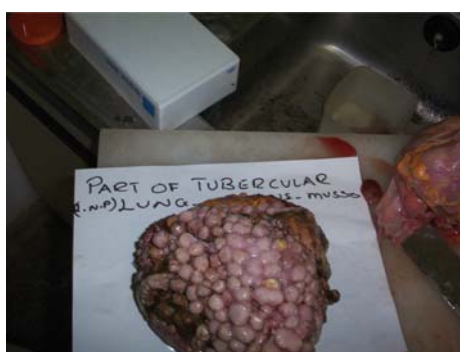
FIGURE 4: Severe Caseous granulomatous lung from a sick lechwe antelope that was seen lagging behind a large herd from the Lochinvar Game Management Area of the Kafue basin.

(a)

(b)

FIGURE 5: Lechwe antelopes and cattle grazing together in the Game Management Areas of Lochinvar National Park of the Kafue basin (left picture indicates the Eastern Game Management Area, whilst the right picture indicates the Western Game Management Area).

dictates that both environmental, host and agent attributes be optimal for transmission [34, 39], a feature which a few studies have remotely elaborated are reminiscent in the Kafue basin region [19]. Our previous work has indicated that grazing strategy apart from being a major predictor variable for BTB status in cattle, was also found to act as a proxy variable for other risk factors considered pivotal in both the maintenance and transmission of BTB between cattle and lechwe antelopes [35].

Epidemiological studies have shown that the grazing range of Kafue lechwe and cattle extensively overlap with the density of interaction increasing extensively during the dry season when transhumant herds (TH) migrate deep into the lechwe grazing grounds with further reductions of watering points coupled by few remaining good pastures [40]. Studies have shown high cattle and lechwe interaction points during the drier months (Figure 2) [40]. Lechwe antelopes and cattle are usually seen grazing together during this period (Figure 5). The absence of predators in the Kafue basin areas limits the transmission BTB to nonbovid species unlike the Kruger NP where the disease has been reported to cross into nonbovid species such as lions, cheetahs, and hyenas [41, 42]. Hence, tuberculous animals live longer and have a long period to transmit the disease to other animals.

8. Control

It has been observed that once BTB establishes itself in feral reservoirs the likelihood of eradication becomes complicated [42]. Additionally, the existence of a livestock/wildlife population further complicates the situation due to the likelihood of a bidirectional mode of transmission with possible contamination of the environment [43].

When control measures are envisaged, it is important to consider factors at play in the epidemiology of the disease. Further, the choice of workable control measures and strategies should take into account all key factors unique to each different ecosystem. A number of factors have been observed to be associated with BTB in cattle herds [44]. Oloya and coworkers observed that BTB was associated with different types of drinking water sources [45]. The same study also indicated that BTB is linked to specific geographical regions of production although they did not conclusively state the factors responsible for this observation [45]. BTB has also been shown to be associated with communal grazing, animal breed type and husbandry practices [44]. Studies have also shown that herd size has an influence on the prevalence of BTB [12, 30, 31, 46, 47]. Taken together, these factors are vital in formulating workable control strategies for cattle BTB. However, in free living wildlife populations, control measures such as test and slaughter schemes applicable to cattle are impractical.

Nevertheless, at the livestock/wildlife interface areas, more detailed studies are needed to understand factors related to the maintenance, spread, and transmission of the disease. Owing to impracticability of other control measures, the key factor at the livestock/wildlife interface area is to reduce or to completely remove interspecies contact. Selective cropping of old debilitated animals can also be used to remove would-be chronic shedders of the disease. Given the resource position of most developing countries, the use of vaccines at the moment is still impracticable.

9. Conclusion

Once policies to control bovine tuberculosis at the live-stock/wildlife interface areas has been envisaged, the determination of the role of wildlife, domestic animals, and the environment in the maintenance and spread of mycobacterial pathogens is important. This requires further research on the ecological and biological disease determinants of mycobacterial infections at the livestock/wildlife interface. In summation, it is important to base control policies on objective and empirical evidence that have taken into account critical deterministic factors of disease maintenance, dissemination, occurrence, and susceptibility.

References

[1] R. C. Huard, M. Fabre, P. de Haas et al., "Novel genetic polymorphisms that further delineate the phylogeny of the Mycobacterium tuberculosis complex," *Journal of Bacteriology*, vol. 188, no. 12, pp. 4271–4287, 2006.

[2] S. T. Cole, R. Brosch, J. Parkhill et al., "Deciphering the biology of mycobacterium tuberculosis from the complete genome sequence," *Nature*, vol. 393, no. 6685, pp. 537–544, 1998.

[3] S. T. Cole, R. Brosch, J. Parkhill et al., "Erratum: Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence," *Nature*, vol. 396, no. 6707, p. 190, 1998.

[4] R. Brosch, S. V. Gordon, M. Marmiesse et al., "A new evolutionary scenario for the Mycobacterium tuberculosis complex," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 6, pp. 3684–3689, 2002.

[5] H. D. Donoghue et al., "Molecular studies of Mycobacterium tuberculosis DNA from naturally mummified 18th century Hungarians," in *Proceedings of the 22nd Annual Congress of the European Society of Mycobacteriology*, Berlin, Germany, 2001.

[6] B. M. Rothschild, L. D. Martin, G. Lev et al., "Mycobacterium tuberculosis complex DNA from an extinct bison dated 17,000 years before the present," *Clinical Infectious Diseases*, vol. 33, no. 3, pp. 305–311, 2001.

[7] O. Cosivi, J. M. Grange, C. J. Daborn et al., "Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries," *Emerging Infectious Diseases*, vol. 4, no. 1, pp. 59–70, 1998.

[8] G. Moda, C. J. Daborn, J. M. Grange, and O. Cosivi, "The zoonotic importance of *Mycobacterium bovis*," *Tubercle and Lung Disease*, vol. 77, no. 2, pp. 103–108, 1996.

[9] C. Gortazar, J. Vicente, S. Samper et al., "Molecular characterization of Mycobacterium tuberculosis complex isolates from wild ungulates in south-central Spain," *Veterinary Research*, vol. 36, no. 1, pp. 43–52, 2005.

[10] Anonymous, *Annual Report of the Department of Veterinary and Tsetse Control Services*, R. Ministry of Agriculture and Rural Development, 1957.

[11] M. Munyeme, J. B. Muma, K. L. Samui et al., "Prevalence of bovine tuberculosis and animal level risk factors for indigenous cattle under different grazing strategies in the livestock/wildlife interface areas of Zambia," *Tropical Animal Health and Production*, vol. 41, no. 3, pp. 345–352, 2009.

[12] A. J. C. Cook, L. M. Tuchili, A. Buve, S. D. Foster, P. Godfrev-Faussett, and G. S. Pandey, "Human and bovine tuberculosis in the monze district of zambia-a cross-sectional study," *British Veterinary Journal*, vol. 152, no. 1, pp. 37–46, 1996.

[13] G. S. Pandey, *Studies of the Infectious Diseases of the Kafue lechwe (Kobus leche kafuensis) with Particular Reference to Tuberculosis in Zambia*, Azabu University, Tokyo, Japan, 1998.

[14] R. G. Bengis, F. A. Leighton, J. R. Fischer, M. Artois, T. Mörner, and C. M. Tate, "The role of wildlife in emerging and re-emerging zoonoses," *Revue Scientifique et Technique*, vol. 23, no. 2, pp. 497–511, 2004.

[15] M. Munyeme, J. B. Muma, V. M. Siamudaala, E. Skjerve, H. M. Munang'andu, and M. Tryland, "Tuberculosis in Kafue lechwe antelopes (*Kobus leche Kafuensis*) of the Kafue Basin in Zambia," *Preventive Veterinary Medicine*, vol. 95, no. 3-4, pp. 305–308, 2010.

[16] W. Chansa and G. Kampamba, "The population status of the Kafue Lechwe in the Kafue Flats, Zambia," *African Journal of Ecology*, vol. 48, no. 3, pp. 837–840, 2010.

[17] Anonymous, *Annual Report of the Department of Veterinary Services and Livestock Development*, Edited by G. Printers, Government of the Republic of Zambia, Lusaka, Zambia, 2004.

[18] B. Kamweneshe, R. Beilfesus, and C. Simukonda, *Population and Distribution of the Kafue lechwe and Other Large Mammals on the Kafue Flats, Zambia*, WWF Partners for Wetlands, Lusaka, Zambia, 2002.

[19] M. Munyeme, J. B. Muma, E. Skjerve et al., "Risk factors associated with bovine tuberculosis in traditional cattle of the livestock/wildlife interface areas in the Kafue basin of Zambia," *Preventive Veterinary Medicine*, vol. 85, no. 3-4, pp. 317–328, 2008.

[20] I. Macadam, J. Gallagher, and J. McKay, "Experimental tuberculosis in lechwe antelope in Zambia," *Tropical Animal Health and Production*, vol. 6, no. 2, pp. 107–109, 1974.

[21] H. Kraus et al., "Wildlife as a potential source of infection in domestic animals—studies on game in Zambia," *Animal Research and Development*, vol. 24, pp. 41–58, 1986.

[22] K. J. Stafford, "A review of diseases of parasites of the Kafue lechwe (*Kobus leche Kafuensis*)," *Journal of Wildlife Diseases*, vol. 27, no. 4, pp. 661–667, 1991.

[23] N. D. Kock, G. Kampamba, S. Mukaratirwa, and J. du Toit, "Disease investigation into free-ranging Kafue lechwe (*Kobus leche Kafuensis*) on the Kafue Flats in Zambia," *Veterinary Record*, vol. 151, no. 16, pp. 482–484, 2002.

[24] J. S. Dillman, *Compiled Final Report, Veterinary Wildlife Research Section*, Department of Veterinary Services, Ministry of Agriculture, Lusaka, Zambia, 1976.

[25] J. Gallagher, I. Macadam, J. Sayer, and L. P. van Lavieren, "Pulmonary tuberculosis in free-living lechwe antelope in Zambia," *Tropical Animal Health and Production*, vol. 4, no. 4, pp. 204–213, 1972.

[26] D. Rottcher, "Veterinary wildlife," Final Report, Department of Veterinary and Tsetse Control Service, Lusaka, Zambia, 1978.

[27] R. C. V. Jeffery, C. H. Malambo, and R. Nefdt, "Wild mammal surveys of the Kafue flats," A Report to the Director, National Parks and Wildlife Service, Chilanga, Zambia, 1991.

[28] A. C. Sitima, *Variability of Mycobacterium bovis in Traditionally Processed Sour Milk and the Prevalence of Bovine Tuberculosis in Namwala District of Zambia, in Disease Control*, University of Zambia, Lusaka, Zambia, 1997.

[29] V. M. Siamudaala et al., "Veterinary challenges regardding the utilisation of the Kafue lechwe (*Kobus leche Kafuensis*) in Zambia," in *Conservation and Development Interventions: At the Wildlife/Wildlife Interface: Implication for Wildlife, Livestock and Human Health*, Durban, South Africa, 2003.

[30] G. Ameni, K. Ameni, and M. Tibbo, "Bovine tuberculosis: prevalence and risk factor assessment in cattle and cattle owners in Wuchale-Jida district central Ethopia," *Journal of Applied Research in Veterinary Medicine*, vol. 1, pp. 1–4, 2003.

[31] B. Asseged et al., "Bovine tuberculosis:a crosssectional and epidemiological study in and arround Addis Ababa," *Bulletin of Animal Health Production in Africa*, vol. 48, pp. 71–80, 2000.

[32] C. N. L. Macpherson, "The effect of transhumance on the epidemiology of animal diseases," *Preventive Veterinary Medicine*, vol. 25, no. 2, pp. 213–224, 1995.

[33] E. M. Kapungwe, *Carrying Capacity and Management of the Kafue lechwe (Kobus leche kafuensis) in the Kafue Flats, Zambia*, University of Zimbabwe, Harare, Zimbabwe, 1993.

[34] U. Zieger, G. S. Pandey, N. P. J. Kriek, and A. E. Cauldwell, "Tuberculosis in Kafue lechwe (*Kobus leche Kafuensis*) and in a bushbuck (Tragelaphus scriptus) on a game ranch in Central Province, Zambia," *Journal of the South African Veterinary Association*, vol. 69, no. 3, pp. 98–101, 1998.

[35] M. Munyeme, J. B. Muma, E. Skjerve et al., "Risk factors associated with bovine tuberculosis in traditional cattle of the livestock/wildlife interface areas in the Kafue basin of Zambia," *Preventive Veterinary Medicine*, vol. 85, no. 3-4, pp. 317–328, 2008.

[36] P. N. Acha and B. Szyfres, "Zoonotic tuberculosis," in *Zoonoses and Communicable Diseases Common to Man and Animals*, Pan American Health Organization/World Health Organization, Washington, DC, USA, 1987.

[37] M. Munyeme, J. B. Muma, H. M. Munang'andu, C. Kankya, E. Skjerve, and M. Tryland, "Cattle owners' awareness of bovine tuberculosis in high and low prevalence settings of the wildlife-livestock interface areas in Zambia," *BMC Veterinary Research*, vol. 6, p. 21, 2010.

[38] J. Gallagher, I. Macadam, J. Sayer, and L. P. van Lavieren, "Pulmonary tuberculosis in free-living lechwe antelope in Zambia," *Tropical Animal Health and Production*, vol. 4, no. 4, pp. 204–213, 1972.

[39] L. A. L. Corner, "The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 303–312, 2006.

[40] H. K. Mwima, "Wildlife research and managent in Zambia with special reference to some protected areas where wild and domestic animals co-exist," in *The Effects of Enlargement of Domestic Animal Pasture on the Wildlife in Zambia*, Lusaka, Zambia, 1995.

[41] A. L. Michel, M. L. Coetzee, D. F. Keet et al., "Molecular epidemiology of *Mycobacterium bovis* isolates from free-ranging wildlife in South African game reserves," *Veterinary Microbiology*, vol. 133, no. 4, pp. 335–343, 2009.

[42] A. L. Michel, R. G. Bengis, D. F. Keet et al., "Wildlife tuberculosis in South African conservation areas: implications and challenges," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 91–100, 2006.

[43] R. G. Bengis, R. A. Kock, and J. Fischer, "Infectious animal diseases: the wildlife/livestock interface," *Revue Scientifique et Technique*, vol. 21, no. 1, pp. 53–65, 2002.

[44] M. K. Omer, E. Skjerve, Z. Woldehiwet, and G. Holstad, "A cross-sectional study of bovine tuberculosis in dairy farms in Asmara, Eritrea," *Tropical Animal Health and Production*, vol. 33, no. 4, pp. 295–303, 2001.

[45] J. Oloya, J. B. Muma, J. Opuda-Asibo, B. Djønne, R. Kazwala, and E. Skjerve, "Risk factors for herd-level bovine-tuberculosis seropositivity in transhumant cattle in Uganda," *Preventive Veterinary Medicine*, vol. 80, no. 4, pp. 318–329, 2007.

[46] R. R. Kazwala, D. M. Kambarage, C. J. Daborn, J. Nyange, S. F. H. Jiwa, and J. M. Sharp, "Risk factors associated with the occurrence of bovine tuberculosis in cattle in the Southern Highlands of Tanzania," *Veterinary Research Communications*, vol. 25, no. 8, pp. 609–614, 2001.

[47] R. R. Kazwala, L. J. M. Kusiluka, K. Sinclair, J. M. Sharp, and C. J. Daborn, "The molecular epidemiology of *Mycobacterium bovis* infections in Tanzania," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 201–210, 2006.

Review Article

Bovine Tuberculosis and the Establishment of an Eradication Program in the United States: Role of Veterinarians

Mitchell V. Palmer and W. Ray Waters

Infectious Bacterial Diseases of Livestock Research Unit, National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, 1920 Dayton Avenue, Ames, IA 50010, USA

Correspondence should be addressed to Mitchell V. Palmer, mitchell.palmer@ars.usda.gov

The significance of the identification of *Mycobacterium bovis* as a zoonotic pathogen in 1882 was not initially recognized. After years of research by veterinarians, and other scientists, the importance of *M. bovis* as a pathogen and the public health ramifications, were appreciated. Veterinarians played pivotal roles in the creation of improved meat and milk inspection, diagnosis of *M. bovis* infected cattle, and in time, a bovine tuberculosis eradication program that would impact every cattle producer in the country. After overcoming many challenges, the 93-year-long program has decreased disease prevalence from 5% to <0.001%. Today, years of hard work by practitioners, researchers and regulatory officials alike, have yielded a program with a net benefit of almost $160 million per year.

> "There is perhaps no single disease that has aroused more interest and that is of greater significance to the livestock industry of this country than bovine tuberculosis [1]."—Veranus A. Moore, State Veterinarian of New York (1911).

1. Ancient Origins of *Mycobacterium bovis* in North America

The *Mycobacterium tuberculosis* complex is composed of several related species, including *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium canettii*, *Mycobacterium pinnipedii*, *Mycobacterium caprae,* and the recently identified *Mycobacterium mungi* [2]. Genomic analysis suggests that animal-adapted strains of *M. bovis* evolved from human-adapted strains of *M. tuberculosis* or a recent ancestor common to both *M. tuberculosis* and *M. bovis*, similar to *M. africanum* [3]. DNA sequencing of fossils, containing lesions consistent with tuberculosis, suggests that *M. tuberculosis* complex was present in North America during the Pleistocene era [4]. Others propose that bovids carried *M. tuberculosis* complex over the Bering Strait during the late Pleistocene era and that tuberculosis was present when settlers arrived in North America. Still others believe tuberculosis accompanied settlers upon their arrival to North America [5].

2. Human versus Bovine Tuberculosis: Koch's Assumptions

A clear understanding of the relationship between *M. tuberculosis*, *M. bovis,* and disease in humans and animals has historically been a source of debate. In 1882, Robert Koch declared that the tubercle bacilli from humans and cattle were one and the same, and accordingly, human and bovine forms of tuberculosis were identical. In so doing, Koch

apparently overlooked the work of the French physician, Jean Antoine Villemin, who in 1868, using rabbits, described the greater virulence of the bovine tubercle bacillus compared to the human tubercle bacillus [6]. Less widely known were declarations from veterinarians such as James Law of Cornell University, who, in 1877, stated that bovine tuberculosis was a communicable disease, transmitted through inoculation or ingestion of the tubercle. He also emphasized the danger tuberculosis represented for highly prized herds as well as humans. Although a highly controversial position to take, Law believed that humans could become infected from eating undercooked meat or fresh milk from tuberculous cattle [7]. Law's opinion was bolstered in 1883 by a resolution from the Fourth International Veterinary Congress at Brussels, which recommended that tuberculous cows only be used for human consumption when disease was localized and the animal was still in good physical condition. They also advised against consumption of milk from tuberculous cows [7].

Following Koch's announcement, veterinarians, bacteriologists, and others set out to conduct comparative observations on the virulence of bacilli from cattle and humans. Tubercle bacilli from cattle were transmitted, often with fatal consequences, to horses, donkeys, swine, cats, dogs, sheep, goats, rabbits, guinea pigs, and by accidental inoculation, humans. In 1900 Ravenel, a physician from South Carolina, reported that three Pennsylvania veterinarians, in separate incidents, were infected with the bovine tubercle bacillus via accidental skin inoculation while performing postmortem examinations [8]. Similar accounts were reported, as well as numerous case reports of infection resulting from ingestion of milk from tuberculous cows [9].

In 1883, shortly after Koch's announcement, physicians, Emanuel Klein and Heneage Gibbs, noted differences between bovine and human tubercle bacilli, and conducted small animal experiments with material obtained from tuberculous cows. They repeatedly showed the guinea pig was susceptible to both human and bovine tubercle bacilli; however, the rabbit was only susceptible to bovine tubercle bacilli [6]. Veterinarian Edmond Nocard, of the Veterinary College, Alfort, Paris stated, "all mammalia, including monkeys, become tuberculous after ingestion of milk from tuberculous cows. It would be absurd to contend that man alone offers an exception to the rule" [10]. Experimental transmission studies conducted by Theobald Smith, a physician scientist working for the Veterinary Division of the Bureau of Animal Industry (BAI, precursor to the present day Agricultural Research Service in USDA), and veterinarians Austin Peters and Langdon Frothingham, used calves experimentally inoculated with sputum from tuberculous humans to demonstrate that human bacilli possessed a low virulence for cattle [11]. Smith should be credited with being one of the first to observe differences between human and bovine tubercle bacilli. In 1895, Smith visited Koch in Europe and described his findings. By 1901, Koch had concluded that there was a difference between the bovine and human tubercle bacilli, but that there was little danger of transmission of the bovine bacillus to man. Koch reported these findings at the 1901 Tuberculosis Congress in London, absent credit to Smith. Recognition for Smith's findings

would not come until the 1908 International Congress on Tuberculosis in Washington, DC [12]. Koch's assertion that the bovine tubercle bacillus posed minimal risk to humans was ardently challenged by leading veterinarians, such as Edmond Nocard, Bernard Bang, and John McFadyean who, in the interest of public health, were working towards eliminating bovine tuberculosis. Needless to say, the battle to repress bovine tuberculosis did not begin without debate. Many accepted Koch's view that tuberculosis of cattle was no danger to humans [13]. Given his prominent standing in the field of tuberculosis, this opinion was widely embraced, by all but a few scientists. An excerpt from Koch's remarks at the 1901 Tuberculosis Congress in London illustrates the obstacle faced by veterinarians and public health authorities that did not share Koch's opinion:

> "I should estimate the extent of infection by the milk and flesh of tubercular cattle, and the butter made of their milk, as hardly greater than that of hereditary transmission, and I, therefore, do not deem it advisable to take any measures against it."

Koch's statement on the minimal risk associated with consuming milk or meat from tuberculous cows, had far-reaching ramifications and led to various mistaken beliefs. Case in point, as late as 1928, prominent scientists such as Nobel prize winner Emil von Behring and Albert Calmette, coinventor of the vaccine Bacillus Calmette-Guerin (BCG) believed that bovine bacilli, after long residence in the human body, took on characteristics of the human bacilli [14].

After being faced with evidence from other scientists, by 1908 at the Sixth International Congress on Tuberculosis, Koch modified his position, stating [14]:

> "I admit that bovine infection can occasionally occur, and I desire not to be understood as disregarding the endeavors to extirpate bovine tuberculosis, as far as these endeavors are dictated by agricultural and economic reasons. But I mean that it would be wrong to give to those proposals the leading place in front of the efforts to combat human tuberculosis."

Koch clearly felt that the public health aspects of bovine tuberculosis were minimal and that eradication efforts should be motivated by animal health and economic concerns. The debate left both layperson and government policy maker confused concerning the proper amount of public health concern to provide bovine tuberculosis. Many governments engaged the opinion of expert scientists for advice. In Great Britain, a Royal Commission on Tuberculosis was formed to review the current knowledge and provide guidance to policy makers. The Pennsylvania State Livestock Sanitary Board, the New York City Health Department, the US BAI, and the American Veterinary Medical Association carried out similar investigations [14, 15]. One member of the Royal Commission was noted veterinary scientist Sir John McFadyean. In their Interim Report of 1907, it was clear that they felt Koch was in error, stating:

"Facts indicate that a very large proportion of tuberculosis contracted by ingestion is due to tubercle bacilli of bovine source.... A very considerable amount of disease and loss of life, especially among the young, must be attributed to the consumption of cows' milk containing tubercle bacilli.... Our results clearly point to the necessity of measures more stringent than those presently enforced be taken to prevent the sale or the consumption of such milk."

3. Variability in Virulence between Isolates

Despite apparent host preferences between bovine and human tubercle bacilli, numerous studies near the turn of the century suggested that cattle and humans were susceptible to both pathogens. In 1898, Ravenel of the Pennsylvania Livestock Sanitary Board fed human sputum to four calves resulting in tuberculous lesions in all four, including extensive lesions in two of the animals [16]. Ravenel also intravenously inoculated a calf with a suspension of a culture obtained from a tuberculous mesenteric gland from a child that had died of tuberculous meningitis—the calf died 17 days later with severe tuberculous nodules within the lungs and liver [16]. In 1902, addressing the American Public Health Association in New Orleans, veterinarian microbiologist, Daniel E. Salmon presented reports of inoculation of tuberculous material from humans into cattle by various routes resulting in lesions and often, severe disease [16]. The French veterinarian Jean-Baptiste Chauveau also demonstrated that administration of lung emulsions from tuberculous humans to cattle resulted in severe tuberculous lesions [9]. In contrast, performing studies for the British Royal Tuberculosis commission of 1895, professor and physician Sidney Martin demonstrated that sputum from man was less virulent for animals than was tubercular material from cattle [9]. Others were unable to infect cattle with human tuberculous material. The variability in outcome between the studies is not surprising considering early reports of wide-ranging virulence of the human tubercle bacillus [17]. Kossel, speaking on behalf of a Koch-appointed German tuberculosis commission, also reported differences in virulence between bovine isolates [9]. The German commission tested 39 different freshly prepared cultures made from tuberculous lesions obtained from humans. Inoculating cattle, 19 resulted in no disease, 9 induced very minimal foci in the prescapular glands after 4 months, 7 resulted in marked disease of the prescapular glands without dissemination, and 4 caused generalized tuberculosis in cattle recipients. Thus, it was impossible to determine the source of a culture by its effects when inoculated into cattle [9]. Descriptions of these early studies are not clear as to whether investigators knew the precise identity of inoculated strains. It is possible that some isolates from humans were not human tubercle bacilli, but were actually *M. bovis*. Generally, however, conclusions from such comparative studies revealed that bacilli from cattle were more virulent for animals than that obtained from humans [13]. As summarized by Salmon in 1904, "the bovine bacillus, being proved more virulent for all animals upon which it has been tried, is also more virulent for man" [9].

4. Public Health Concern: Zoonotic Potential and Role of Veterinarians in Early Public Health

The public health implications of bovine tuberculosis were suggested early by Chauveau, who, beginning in 1865, demonstrated the ability to transmit tuberculosis between cattle through ingestion of diseased material [18]. He reasoned that in man, as well as in animals, transmission of tuberculosis was possible through the consumption of meat or milk from diseased animals.

In 1900, tuberculosis was the leading cause of death in the United States. More than 25% of all deaths were due to airborne diseases, with tuberculosis being the most important [19]. It is estimated that approximately 10% of all human tuberculosis cases were the result of exposure to tuberculous cattle or cattle products [20]; more distressing, 25% of tuberculosis cases in children were caused by *M. bovis* [21]. Rates of bovine tuberculosis were higher in herds housed in close-confinement compared to those found on open pastures, resulting in higher disease prevalence in dairy cattle compared to beef cattle. Bovine tuberculosis was more common in herds close to major cities, since these were often the most limited in pasture and, therefore, utilized more indoor housing. With this consideration, the state veterinarian of Alabama, Charles A. Cary, implemented an "open air clause" to their state program stating: "all dairy cows shall be given each day at least six hours exercise in the open air [22]." Cary was a native of Iowa, but studied in Germany with the most renowned veterinary scientists of the day.

As early as 1885, there was substantial evidence of the infectiousness of milk from tuberculous cows. Cornell's James Law identified bovine tuberculosis as the most important zoonotic disease of the time [23]. Respected physicians such as Harold C. Ernst preached the dangers of milk from tuberculous cows, especially for children. In 1889, studies conducted by Ernst and veterinarian Austin Peters at the Harvard Medical School bacteriology laboratory, demonstrated that cows affected with tuberculosis in any part of the body could produce milk containing the bacillus, irrespective of the presence or absence of lesions in the udder [23]. An opinion also held by Danish veterinarian Bernard Bang [24]. Although veterinarians were generally in agreement with the conclusions reached by Bang and others, regrettably, Ernst's opinions did not have widespread approval among physicians. A survey in 1890 of 18,000 physicians, showed that many doctors denied the danger of milk as a source of tuberculosis for their patients [7]. Among 1013 physicians responding to the question as to whether they had ever seen a case of tuberculosis that could be traced to consumption of milk, 893 answered "no." Only 11 stated they had seen cases that could be causally linked to consumption of milk [23].

Veterinarians, public health officials, and consumers were key to the growing demand for meat and milk free from contamination by tubercle bacilli [11, 13]. It is estimated that, in 1917, bovine tuberculosis was responsible for approximately 15,000 deaths in the US; 3-times the number dying from all food-borne illnesses today [25]. Veterinarians understood early the public health concerns of bovine tuberculosis. At a meeting of the United States Veterinary Association in 1887, veterinarians called for increased inspection of dairies and slaughterhouses to reduce the amount of tuberculous meat and milk reaching consumers [26]. As noted by Dr. Cary of Alabama, "if we do not want to eat the stuff ourselves, we had better bury or burn it".

As early as 1899, many important factors in the pathogenesis and epidemiology of bovine tuberculosis were known, or at least hypothesized. Physicians and veterinarians alike established that (1) TB is a contagious disease of cattle propagated by cohabitation of tuberculous and healthy animals, (2) TB could be transmitted by feeding products from infected animals, (3) tubercle bacilli could be visualized microscopically in milk from animals with tuberculosis, but lacking lesions of the udder [11], (4) milk from cows with tuberculosis, but lacking udder lesions could transmit disease orally to guinea pigs, rabbits, pigs, and calves [11], (5) milk from the Boston milk supply could transmit disease to rabbits [11], (6) calves generally acquire infection by feeding on infected milk from tuberculous cows, (7) older cattle generally acquire infection through cohabitation with infected animals, (8) congenital tuberculosis although observed, is rare, (9) animals with widely disseminated disease often show no clinical signs, and (10) animals with either severe or mild disease can shed tubercle bacilli.

At the 1907 annual meeting of the US Association of Livestock Sanitary Boards, state veterinarian of Colorado, Dr. Charles G. Lamb emphasized the need for veterinarians, livestock sanitary boards, and public health officials to work together in the control of contagious disease, especially bovine TB. Citing transmission of TB via milk as an example, Dr. Lamb pointed out the need for a veterinarian on all public health boards. Therefore, the "One Health Concept," prevalent today, which obscures the line between human and animal medicine, has long been recognized as a beneficial strategy for zoonotic disease control.

As early as 1905, the state of Iowa implemented laws stating "that every owner, manager, or operator of a creamery shall before delivering to any person, any skim milk, cause the same to be pasteurized at a temperature of at least 185 degrees Fahrenheit." Addressing the 19th annual meeting (1915) of the US Livestock Sanitary Association, W. B. Barney, a prominent Holstein breeder from Iowa, declared that pasteurization should be made compulsory in the US and should include routine inspections of equipment and appropriate records of operation. However, there was widespread opposition throughout the country stemming from beliefs that pasteurization would increase cost to consumers and heating of milk would destroy nutritional value.

5. Meat Inspection-Differences in Approach

Opinions differed between European and American approaches to meat inspection and bovine tuberculosis. In his book, "Handbook of Meat Inspection, 4th ed," German veterinarian, Robert Von Ostertag states: "at present, the view is generally entertained that, in undoubted cases of local tuberculosis, the meat is harmless while in generalized cases it is harmful." Addressing the Royal Commission of 1896, Sir John MacFadyean stated: "I doubt whether there are ten people in this country in the year who contract tuberculosis through meat." In 1894 Europe, the inspection of meat focused on sanitation; animals with diseases not thought contagious to man were considered safe and proper for human consumption. Cattle carcasses affected by bovine pleuropneumonia, foot and mouth disease, or cows in advanced gestation were all used as food. At the same time in the US there was a widespread aversion to eating the meat from animals afflicted with any disease, or in the advanced stages of pregnancy [27]. At the beginning of the 20th century, stewardship of US meat inspection fell upon the BAI. It was the opinion of the BAI that their duty was to protect the consumer from meat which was offensive or repugnant, as well as that which was actually dangerous to public health [27]. Accordingly, inspectors of the BAI were instructed to condemn carcasses from all animals having acute diseases or high fevers, as well as diseases communicable to the consumer and carcasses of periparturient females. Such oversight by the BAI applied only to abattoirs processing meat for international or interstate sale [27]. In 1904, state laws regarding disposition of tuberculous cattle varied from state to state, resulting in confusing and sometimes illogical outcomes. Confusion can be traced to at least 2 relevant, but contradictory, laws, that being livestock sanitary laws that prohibited the use of meat from animals suffering from contagious disease and meat inspection laws that allowed the use of meat from tuberculous cattle with limited disease if diseased portions were trimmed away. In Montana and New Jersey, state regulations prohibited the use of meat from all cattle with contagious disease. Consequently, tuberculous carcasses were to be destroyed [22]. At the same time in Nebraska, meat inspection laws allowed the slaughter of tuberculous cattle if it was felt that the cattle would pass state meat inspection [22]. Complicating the matter was the issue of where the tuberculous cattle would be slaughtered and processed. Many states prohibited shipment of diseased livestock. Inspection of meat by the Federal Government began in 1891, under the direction of the BAI and its chief Salmon [27]. The federal meat inspection act of 1906 [28] by USDA held that "all carcasses affected with tuberculosis and showing emaciation shall be condemned." Although condemnation of all tuberculous carcasses was the call, reality held that carcass disposition was dependent on disease severity [29]. Carcasses with little visible disease were passed for food after removal of diseased portions. Carcasses displaying moderate disease could be rendered into lard or tallow after diseased portions were removed. Severely diseased carcasses were condemned as unfit for food or other

products [29]. From 1906 to 1916, 1.8% of cattle slaughtered were retained due to concerns about tuberculosis. In most cases, disease was localized and the affected portions trimmed away, allowing the rest of the carcass to be used for human consumption [25, 28]. However, in 288,000 carcasses disease was so widespread that the entire carcass was condemned and not used for food. Tuberculosis was not the only reason for carcass condemnation; however, it was responsible for condemnation in 68% of condemnation cases [25].

Although compliance to the 1906 USDA regulation was not complete, the potential condemnation of all carcasses regardless of extent of disease would eventually have two consequences: (1) an urgent need to establish indemnity for producer support and (2) a greater emphasis on eradication as even those animals with moderate disease were deemed unfit for consumption as unrendered products. The first state to implement systematic meat inspection was Alabama, where under the direction of the State Veterinarian, Charles A. Cary, organized inspection began in 1896, in Montgomery, AL. Cary would go on to establish meat and milk ordinances in all of Alabama's major cities. Montana assembled one of the first dairy inspection laws in the country. Under the direction of veterinarian M.E. Knowles, every city in Montana was to have a veterinarian, paid by the state, to inspect dairy cows and their products [22].

A description of the American approach can be found in Connecticut veterinarian Ingram's remarks at the 1917 US Livestock Sanitary Association meeting titled "Municipal Meat Inspection" [30]. Ingram and his fellow veterinarians called for "an inspection to determine if meat comes from an animal with a contagious disease to protect the consumer from dangers from which he cannot protect himself." Ingram went on to describe an attitude still relevant today:

> "Currently, federal meat inspection service is limited to meat for interstate or foreign shipment—thus, meat used intrastate is not subject to federal inspection. This responsibility relies on municipal or state inspectors. There is a need to inspect all facilities including smaller, out of the way, slaughterhouses, as these are often objectionable and dangerous to public health. In contrast, central abattoirs afford commercial advantages such as better equipment and options for by-products and are more conveniently inspected. As with Europe, it is preferable for the central abattoirs to be owned by the municipality. State and municipal inspectors should become aware of and follow federal inspection rules, as they are most complete. Advantages of inspectors being veterinarians are that they are aware of animal diseases and potential dangers to human health. The slaughterers should not pay inspectors. Public is suspicious of meat that does not have official stamp of inspection—thus, it fetches a lower price."

6. Tuberculin: The Foundation for an Eradication Campaign

In 1890 at a meeting of the International Congress of Medicine in Berlin, Koch announced that he had isolated a substance from tubercle bacilli that could both render guinea pigs refractory to tuberculosis and arrest the disease, even when in an advanced state. As such, Koch was describing his substance as both a preventive and a treatment. He, and others, originally characterized the substance as "paratoloid," a combination of the terms alkaloid and ptomaine (toxic substances released by bacteria) [31]. "Paratoloid" was used as a synonym for "tuberculin" as late as 1901 [32]; however, most physicians and veterinarians knew it as "Koch's lymph." It was soon discredited as both a preventive and a treatment, but Koch had observed that many tuberculous patients injected subcutaneously with tuberculin developed systemic reactions including hyperthermia. Veterinarians recognized these clinical signs as a possible means of detection. Almost immediately, veterinarians in Russia, Denmark, Great Britain, and the US began using Koch's tuberculin to diagnose tuberculosis in cattle. Six months after Koch's announcement of tuberculin, Professor Gutmann of the Veterinary Institute of Dorpat, Russia, used it as a diagnostic aid in cattle [33, 34]. Professor Eber, a veterinarian from Berlin, was one of the first to summarize the accuracy of the tuberculin test. In 1891, he collected statistics on tested cattle and reported a specificity of approximately 87% [35]. Prior to the development of the tuberculin test, veterinarians relied on physical examination to diagnose bovine tuberculosis. With limited diagnostic tools, veterinarians only identified one in ten live tuberculous cows [20].

In 1892, Pennsylvania veterinarian, Leonard Pearson, tested a herd of Jersey cattle belonging to Mr. Joseph Gillingham of Claremont Farms. Pearson was assistant professor of veterinary medicine at the University of Pennsylvania and later became Dean of the Veterinary Department of the University of Pennsylvania [36] and Pennsylvania's State Veterinarian. Mr. Gillingham was a trustee of the University [33]. Pearson obtained the tuberculin used in this first test from Europe. A total of 79 animals were tested, 51 of which showed positive reactions to the test. The entire herd was destroyed [7]. This first test in the US was by injection of 0.2–0.4 cc of Koch's tuberculin diluted 1 : 9 in 1% carbolic acid subcutaneously in the right scapular region [37]. In some infected animals, body temperature increased gradually over 24 hrs whereas in others body temperature did not begin to rise until 20 hrs after injection. Still, in other animals, body temperature rose dramatically at 10 hrs, and then declined by 12 hrs. This variability in change in temperature foreshadowed difficulties associated with this particular application of the test. This first whole-herd test drew much attention and much criticism. Many noted veterinarians of the day, Dr. Samuel Dixon of the Academy of Natural Science in Philadelphia, and Dr. W.L. Zuill, professor of veterinary surgery at the University of Pennsylvania, were decidedly opposed to tuberculin as a means of diagnosis of bovine tuberculosis, believing it toxic and harmful [33]. Dr. Zuill headed a special commission to

investigate tuberculin's "curative, preventive, and diagnostic properties" [7]. Dr. Dixon remarked to Mr. Gillingham, prior to testing of his prized Jersey herd, that he (Gillingham) was foolish to sacrifice so many valuable animals and that Dr. Pearson was nothing but an enthusiastic dreamer [33]. Pearson on the other hand confidently declared the testing a complete success and commended Gillingham for his broad-mindedness, stating that this event was a step to the passage of laws relating to tuberculosis and Gillingham's sacrifice would come to be a blessing to every cattle breeder and consumer of cattle products in the US [33]. This historic test was followed by testing of herds belonging to agricultural colleges and experiment stations in Maine, New Jersey, Wisconsin, and Vermont. Dr. Pearson went on to test some of the best herds in America from 1892 to 1895. In 1893, veterinarian E. C. Schroeder conducted the first official test for the BAI, testing a herd of 34 cattle near New Charlotte, NY. Fifteen animals showed positive reactions. Thirteen of the fifteen had typical lesions of tuberculosis upon postmortem examination [38].

Tests were voluntary, at the expense of the farmer, and no indemnity was paid for slaughtered reactors, which were buried. Pearson soon developed a plan to have reactors appraised, killed under official supervision, and the meat, if considered suitable, used for food [33]. Key elements of this plan were eventually adopted throughout the US. Education of livestock owners by veterinarians was an important factor. The efficacy of tuberculin testing was not difficult to demonstrate, since many reactors were necropsied on the farms where they were tested. These necropsies drew large, sometimes antagonistic audiences; however, veterinarians were able to demonstrate tuberculous lesions in most animals. After such displays, once skeptical onlookers were some of the first to request testing of their herds [39]. In other cases, owners of reactor animals were encouraged to follow the animals to market and, with the local veterinarian, observe the postmortem examination after slaughter [40]. Education of the public concerning bovine tuberculosis was listed as one obstacle to disease eradication [41]. Presenting information in a manner understandable to the layman and possessing a thorough knowledge of the subject, specifically science-based information, were areas identified for improvement [42]. The goal of early 20th century veterinarians to educate the public was best summarized by D. F. Luckey, State Veterinarian of Missouri and President of the US Livestock Sanitary Association in 1907, "it is said that the highest end of education is to bring the general public to the point where it can appreciate the scientific work and know whose advice to follow [43]."

In the infancy of tuberculin testing, many cattlemen objected to the tuberculin test, alleging it was inaccurate and induced disease or caused abortion [13]. Initially, tuberculin was administered subcutaneously and required monitoring of the animal for a rise in body temperature. This necessitated the veterinarian to make several preinjection temperature measurements as well as regular measurements for 24 hrs after injection [18], limiting the number of animals that could be examined by a single veterinarian in any given day. During is early use very little in tuberculin testing

practice was standardized. Several methods of tuberculin administration were practiced, including the subcutaneous, ophthalmic, intrapalpebral, and intradermal [44]. In some cases, more than one method was used on the same animal [45]. Tuberculin from Germany was commercially available; however, this tuberculin was 10-fold stronger than tuberculin distributed free of charge by the USDA's BAI. In 1910, State Veterinarian of New York, Veranus A. Moore, believed detection of cattle in the early stages of disease, through tuberculin testing, was not necessary and that veterinarians should use physical exam to determine the high shedders, which he deemed most important for removal [46]. Moore believed the US approach to tuberculosis eradication was too harsh, considering the chronic nature of the disease. Dr. Moore insisted that the control of the disease should be in the hands of the owner, not the government. Others, such as prominent supporter of the dairy industry, and former Governor of Wisconsin (1889–1891), William D. Hoard championed the use of the tuberculin test to eradicate bovine tuberculosis. Cows at his own dairy farm were routinely tested with tuberculin. He attributed the tuberculosis-free status of his herd to regular tuberculin testing, slaughter of test positive cattle, and rigorous quarantine and testing of all introductions to the herd [47]. Many dairy farmers did not share this opinion. Hoard's 45-year campaign for tuberculin testing cost his magazine, *Hoard's Dairymen,* thousands of subscriptions and significant lost revenue.

Numerous rumors and misconceptions circulated concerning the test; however, the most ardent objection was that the test was inaccurate. Others feared that such a program would decimate the cattle population and lead to shortages of milk and meat [48]. Bills to ban the tuberculin test were debated in state legislatures [49]. Cases concerning tuberculin testing of cattle reached Supreme Courts of Minnesota, Nebraska, Michigan, Ohio, Washington, Illinois, California, and Iowa [50]. The Iowa Supreme Court considered a case from Mitchell County where the plaintiffs declared that the tuberculin test was, in fact, not a test because accuracy and dependability were lacking. As proof they cited cases where healthy cows tested positive for tuberculosis and tuberculous cows tested negative. The Iowa Supreme Court; however, upheld the constitutionality and legitimacy of tuberculin test stating, "…careful reading of the evidence produced convinces us that the test is reliable, useful, and advantageous" [51].

This, among other factors, led to what is known as the "Iowa Cow War." In 1931, in Tipton, Iowa, hundreds of farmers, opposed to compulsory testing, confronted state veterinarians and sheriffs sent to test herds owned by W.C. Butterbrodt and E.C. Mitchell. Farmers blocked roads, threatened veterinarians, and splattered them with eggs, water, and feces. Less than 2 weeks later veterinarians and sheriffs arrived at the farm of Jake Lenker to be met by a force of more than 200 farmers, many of whom immediately doused the veterinarians with water, mud, and rotten eggs. Iowa State Veterinarian, Peter Malcom, was one of the last to escape the mob, but not before the gas line of his car was broken, radiator filled with mud, windows smashed, and tires punctured with pitchforks [50, 52]. Unmoved,

Malcom said he would test every cow in Cedar County. In response to the unrest, enraged Iowa Governor Daniel Turner imposed martial law, and the next day over 1800 national guardsmen arrived in Tipton, exiting the train to march through crowds of angry protesters. The guardsmen were there to protect veterinarians and ensure that testing continued. Accompanied by armed guardsmen, veterinarians returned to Lenker's farm only to find the cattle gone. Lenker had sold his cattle rather than submit to testing. Lenker was arrested and arraigned for contempt of court and released under $10,000 bond. National Guard troops remained in Tipton for 2 months [50].

Between 1892 and 1915, as veterinarians became more accustomed to the use of tuberculin, methods began to vary dramatically. As noted in 1915 by New York State Veterinarian John G. Wills, "when tuberculin first came into use, the procedure employed was somewhat more exacting and in some respects more scientific than was later observed after it had become a more familiar agent" [45]. By 1915, the number of temperature readings, before and after tuberculin administration, had been reduced [41]. In 1915, at a meeting of the Livestock Sanitary Association, Wisconsin State Veterinarian, O. E. Eliason lamented that "the value of the test depended too much on the ability, competency, and experience of the examiner" [45]. Standard procedures and official oversight of tuberculin testing were needed. In spite of these limitations, use of the subcutaneous tuberculin test, in a test and slaughter program, reduced disease prevalence in the District of Columbia from 18.87% in 1909 to 0.84% in 1918. In human medicine in 1908, Charles Mantoux, expanding on work by Clemens Freiherr von Pirquet, developed the intracutaneous (intradermal) tuberculin test. By 1921, the intradermal method of tuberculin testing became the official method approved by the BAI [38, 53]. Tuberculin testing would impact farms in every state and county. Between 1917 and 1940 veterinarians administered over 232 million tuberculin tests, resulting in the destruction of 3.8 million cattle. [20].

7. Plugging the Test

Among other idiosyncrasies, administration of the tuberculin test could render an animal unresponsive to subsequent tests for up to 8 weeks. Early on, it was recognized that animals might need to be retested due to equivocal reactions. The elapsed time between the initial test and retest was unsettled; being set at widely different periods by various authorities [37]. To provide guidance, livestock sanitary boards conducted experiments to determine the optimal time between testing. Guidelines of 1912 recommended a 4 to 7 day interval with the retest tuberculin being 3-times the strength of tuberculin used in the initial test. Theory held that the initial injection of tuberculin desensitized the animal to make them less receptive to the retest. Current recommendations do not allow retesting for 60 days, and USDA accredited veterinarians must conduct the test [54]. However, during the early 1900s, livestock owners could test their herd privately and sell reactors to unsuspecting buyers. Retesting by the new buyer would result in a negative test. For all practical purposes, healthy cattle could not be differentiated from diseased cattle. This practice was known as "plugging the test," and unscrupulous cattle buyers specialized in this practice. One of the worst offenders was likely the leading cattle dealer in the US, James Dorsey of Gilberts, IL, who was credited with creating 10,000 new foci of bovine tuberculosis across the US, Canada, and Mexico through the practice of plugging the test. [20, 25]. The actions of Dorsey, and other disreputable cattle buyers, created not only a public health predicament, exposing thousands of families to tuberculous cattle and cattle products such as raw milk, but also created hardships for fellow cattlemen when many states refused shipments of Illinois cattle, or introduced tuberculosis into their own herds through purchase of infected animals [25]. By 1914, at least 12 states refused to accept cattle from Illinois, except under certificate of federal inspection. Dorsey was finally indicted in 1915, after selling diseased cattle for over 10 years. Dorsey was sentenced to 8 years in a federal penitentiary. It is estimated that Dorsey was responsible for thousands of cases of human tuberculosis, in comparison to the 47 cases of typhoid fever ascribed to the epitome of contagion transmission, "Typhoid Mary" [25]. Dorsey had become quite wealthy after years of fraudulent dealings, and it could be argued that Dorsey's money and influence extended into the political arena. In 1920, after serving only half of an 8-year sentence, Dorsey was pardoned by President Woodrow Wilson [25].

Unfortunately, dishonest veterinarians also practiced deception. Some veterinarians invented mixtures, including antipyretics, that when given to the animal shortly before tuberculin testing, would prevent a rise in temperature [55]. Other dishonorable veterinarians, issued certificates stating the animal had not reacted to tuberculin, without ever conducting the test [56]. By 1914, in large part due to the influences of James Dorsey, railroad companies were warned not to accept health certificates from Illinois veterinarians, except those from a federal veterinarian or a certificate certified and signed by O. E. Dyson, State Veterinarian of Illinois [25]. Deception was so widespread and severe, other states barred, or discouraged, reputable veterinarians from traveling to Illinois to administer tuberculin tests.

8. Controversy over Different Models of Tuberculosis Control

From the outset, there was controversy over the system for control of bovine TB. Danish veterinarian Bernard Bang proposed a control model without slaughter, that was appealing to producers, as it prevented loss of critical genetic resources [22]. In Bang's model, farmers were encouraged to segregate their cattle into two herds: animals that reacted to tuberculin and those that showed no reaction to tuberculin and were thus considered healthy. Each herd was under the control of separate handlers and housed in separate stables. Calves were to be removed at birth and milk from the dam pasteurized before use. Slaughter of animals from the subherd could only be done under supervision of a competent meat inspector. With this system, the healthy

herd progressively increased in size whereas the tuberculous herd slowly diminished. The healthy herd was continually monitored through tuberculin testing to detect latent or recently infected animals. Such practices would minimize disease spread within a herd through contaminated feed, water, bedding, sputum, feces, and through aerosols of such materials. In many herds, however, tuberculosis was introduced by attempts to improve herd genetics through the importation of superior breeding stock. While Bang's model was widely used in Europe, it was less popular within the US. Initially attractive to owners of expensive breeding animals, the cost of maintaining a subherd was prohibitive for many. Extra time and expense involved in caring for reactors, as well as poor public acceptance of pasteurized milk were major deterrents. As stated by Illinois veterinarian, Dr. Charles P. Lovejoy,

> "In four different instances, I advised the owner of the herd to isolate his reacting cattle and breed them out. In each and every case, I was asked to go and kill them. Bad news spreads very quickly. These men said they might as well kill their whole herd as to have isolated cattle, after it had become known that the disease existed" [22].

This sentiment was common; thus, most state/territory veterinarians favored a "test and remove" approach and rarely applied Bang's model.

The test and remove approach was not without difficulties and controversy. Primary problems were the issues of indemnity, product use, and quarantine [22]. According to a 1906 regulation, carcasses of animals suffering from any contagious disease were to be destroyed or buried. In practice, however, diseased portions were trimmed away and the remainder of the carcass used for food. In cases of disseminated disease, the entire carcass was destroyed and not used for human consumption. Some cattle suspected of failing inspection would be sold to buyers that would route them to uninspected plants. If suspicious meat was sold, the reputation of the owner, butcher, and veterinarian (including regulatory veterinarians) were at stake. To circumvent this consequence, reactor cattle were shipped to distant public abattoirs such as Chicago, decreasing the possibility that local citizens would become aware of the presence of tuberculosis in a given herd [22].

In 1904, the President of the US Livestock Sanitary Association and Arizona territorial veterinarian, J. C. Norton stated, "a rule requiring a certificate of tuberculin test to accompany interstate shipment would greatly assist state veterinarians in their work to encourage further testing and would prevent the creation of new centers of infection." By 1909, 24 states had enacted tuberculin test barriers for entry of cattle into their states with varying stringency and timing related to date of shipment. However, application of the test varied tremendously from state to state. S. H. Ward, state veterinarian of Minnesota noted:

> "It does not require any extended argument to convince us that the question to be discussed is the necessity for some uniformity" ... "let regulations be drawn up by the Bureau of Animal Industry."

Dr. Alonzo D. Melvin, BAI chief, responded in favor, noting the immense undertaking necessary and the current lack of funds and personnel to implement such a plan. Regardless, there was a sentiment that oversight and control of tuberculin testing for interstate shipment of cattle be transferred from the states to the BAI.

In 1883, a veterinary division was created within USDA. The division was assigned a 7-acre plot of land outside Washington, DC, on which they were to conduct research [38]. Progress was made in 1884 when the BAI was formed by congressional mandate to "prevent the exportation of diseased cattle, and to provide means for the suppression and extirpation of bovine pleuropneumonia and other contagious diseases [5]." The BAI was placed under the management of it's first chief, Daniel E. Salmon [22]. Power was given to the Secretary of Agriculture to condemn animals capable of spreading disease across state lines [20]. Salmon surrounded himself with capable, forward thinking scientists such as physician, pathologist Theobald Smith (1884) and veterinarians, Fred L. Kilbourne (1885), Cooper Curtice (1886), Veranus Moore (1886), and E. C. Schroeder [38]. Fueled by success in eradicating bovine pleuropneumonia, officials would find tuberculosis to be a more formidable and widespread challenge. By 1900, the BAI was requiring tuberculin testing of all cattle imported into the US from foreign countries. Beginning in 1906, the Pathological Division and Experiment Station of the BAI, now under the direction of veterinarian Alonzo D. Melvin, conducted research on establishing tuberculosis free herds through the process of test and removal. Herds in Maryland, Virginia, and the District of Columbia were annually tested over a period of 12 years. Over 17,000 cattle were tested; the prevalence of positive reactors decreased from 18.87% to 0.17%. Epidemiological investigations, by the BAI, revealed that most reactors after 1917 were the result of cattle movement, animals introduced into the study herds from outside tuberculous herds. Not until 1919 did Congress appropriate the first federal funds specifically targeted to investigate the cause, mode of spread, treatment, and prevention of bovine tuberculosis [36].

Under the leadership of veterinarians such as Daniel. E. Salmon (1884–1905), Alonzo D. Melvin (1905–1917), John R. Mohler (1917–1943), Arthur W. Miller (1843–1945), and Bennett T. Sims (1945–1954), veterinarians, and other scientists, employed by the BAI conducted basic and applied research in the years before and after the establishment of a national program in 1917, Interestingly, Dr. Melvin died, while still BAI chief, in 1917 of tuberculosis that had been diagnosed by Dr. Mohler some 16 years earlier [38]. Noteworthy research included differentiation between bovine and human tubercle bacilli, differential virulence of the bovine and human tubercle bacilli in cattle [9, 57], morphological and biochemical differences in cultures of human and bovine tubercle bacilli [9, 57], transmissibility of bovine tubercle bacilli from cattle to swine [58], immunization of cattle to prevent tuberculosis [59], tuberculin potency testing,

alternative routes of tuberculin administration [53, 59], and use of test and remove methods to remove tuberculosis from a herd.

9. State's Role in Eradication Program

Pennsylvania was one of the first states to establish a coordinated, organized effort to repress bovine tuberculosis [13]. At the turn of the century, Pennsylvania was home to 6 million people and 2 million cattle. The statewide estimated prevalence of bovine tuberculosis was 2-3%; however, there were herds with 30 to 100% of animals infected. In 1895, the Pennsylvania State Livestock Sanitary Board was created, composed of the Governor, Secretary of Agriculture, Dairy and Food Commissioner, and State Veterinarian. The board was free to create and enforce its own rules and regulations. Initial funding of $40,000/yr was used to address tuberculosis, anthrax, glanders, and rabies. Money was also available for the state to produce its own tuberculin and anthrax vaccines. The program began with herd owners voluntarily making application for their herd to be tested. Owners were asked to provide reasons why they desired their herd to be tested. Obviously, owners most interested in creating and maintaining a herd free of tuberculosis were first to enter the program. Test-positive animals were either euthanized and indemnity paid based on appraisal, or reacting animals could be quarantined as a sub herd and cared for apart from the rest of the herd (Bangs model of tuberculosis control). The owner agreed to disinfect the premises, and correct any other conditions in order to keep his herd free of tuberculosis. An interesting feature of the early Pennsylvania program was a concerted effort in public education. In regions where reactors were identified, circulars and articles in agricultural reports were distributed. Efforts were made to euthanize and examine reactors in the same region where they were identified; thereby, interested parties could see lesions of tuberculosis. By 1899, over 33,000 cattle had been tested with 13.7% testing positive for tuberculosis. Indemnity payments totaled $102,909, an average of $22.56/head. In 1898, a requirement that all cattle entering Pennsylvania be tested was added to the program. The program was well received by Pennsylvania producers and many more applications for testing were received than could be handled with available funding. In addition to strong public support, support from organized producer groups and the involvement of the state's many practicing veterinarians were identified as factors for success.

Massachusetts was also an early participant in tuberculosis eradication [56]. However, different laws were passed in 1892, 1894, 1895, and 1896 resulting in confusion over which program was in effect in a given situation [7]. The potential for success in Massachusetts was severely impeded when the State Legislature passed a law restricting the use of tuberculin to confirmation of a diagnosis made by physical exam [7]. Such a law prevented the systematic and widespread testing of all herds crucial to the success in other states. Recognizing the inadequacy of such a program, neighboring Maine refused to accept cattle from Massachusetts that were deemed to be tuberculosis-free by physical exam only [56].

10. Conception of a National Eradication Program

At an 1884 meeting of livestock sanitary officials, a committee with representatives from 8 states and the BAI recommended the formation of the US Livestock Sanitary Association, later known as the US Animal Health Association (USAHA). The first formal meeting was held in Fort Worth, Texas, in 1897. Attendees included representatives from state and territorial sanitary boards, state and territorial veterinarians, and five delegates named by the US Secretary of Agriculture. By the thrid annual meeting of the US Livestock Sanitary Association, held in 1899, bovine tuberculosis was widely discussed—surpassing "Texas Fever" as the major topic. Attendees observed postmortems on cattle that reacted to the still controversial tuberculin test. A resolution was passed recognizing that bovine TB is a contagious disease that was spreading, use of tuberculin was the best means for recognizing the disease in live animals, and states should authorize methods to control it. Bureau Chief Salmon pledged that the USDA would follow the recommendations set forth by the US Livestock Sanitary Association; thereby, laying the ground work for a close working relationship between sanitary boards, state veterinarians, and federal veterinarians and establishing a role for the US Livestock Sanitary Association (USAHA) in guiding federal government on establishing and implementing livestock disease regulatory programs. At the eighth annual meeting of the US Livestock Sanitary Association in 1904, a special committee was formed to (1) deal with TB vigorously, (2) determine the reliability of the tuberculin test, (3) determine methods of disposing of infected animals, and (4) and define rules governing interstate shipment of dairy cattle and infected cattle for slaughter. This special committee was the basis for the current USAHA committee on bovine tuberculosis, again demonstrating the role for state and local veterinarians in conception and implementation of a federal eradication program still in use today. By 1921, the committee on tuberculosis divided is efforts into 7 categories, which are still applicable today: education, state-federal cooperation, regulations, administration, prevention, public health, and finances [60].

A milestone in the eradication program was the notion of accredited herds [61], a concept that remains relevant today. A special committee composed of five state and federal veterinarians, and 5 representatives from livestock breeder associations developed the original plan. A proposal outlining requirements of accredited herds was unanimously adopted at the 1917 meeting of the US Livestock Sanitary Association [62]. Accredited herds would receive a certificate of accreditation from the relevant state authority, and the BAI. The certificate was valid for 1 year. The certificate declared that tuberculosis had not been present within the herd for 2 years. Under certification cattle could be shipped interstate from accredited herds with no further tuberculin testing. In return, producers agreed to regular tuberculin testing of the entire herd as well as accurate and complete animal identification practices [61, 62]. In 1917, there were no accredited herds in the US, but by 1927 there were over

96,000 accredited herds comprising 1.5 million cattle with an additional 1.3 million herds having been tested once in the process of accreditation [63].

Having observed success in various states, the BAI, under the direction of Melvin, obtained funds ($75,000, equivalent to ~$1.27 million in 2010) from congress in 1917 to create the Tuberculosis Eradication Division headed by Tennessee veterinarian John A. Kiernan. In late 1917, at a meeting of the US Livestock Sanitary Association, the first Uniform Methods and Rules (UM & R) for tuberculosis-free herds was approved [36]. Sixty days after the adoption of the UM & R, the first tuberculosis-free herd was accredited under the new guidelines. The herd was owned by the US Soldiers Home in Washington, DC. By 1921, eradication field offices were established in 46 states [36]. Such offices were created and operated with cooperation from state livestock sanitary boards. Motivated by a desire not to place state and federal veterinarians in direct competition with local veterinarians, as well as a critical need for more veterinarians able to conduct tuberculin testing, the accredited veterinarian program was established in 1918 [64]. This allowed private practitioners, subject to a practical exam administered by the BAI, to conduct tuberculin testing. The idea stemmed from a similar program administered by the BAI to test horses for various diseases prior to export to Canada. That program consisted of 63 practitioners in 12 different states. By the close of 1920, there were over 5500 veterinarians accredited by the BAI to assist with cattle testing for tuberculosis. With assistance from practitioners, the testing phase of the program exploded. In 1901, approximately 200,000 animals were tested, and the prevalence of positive reactors ranged from 3.9% to 100% depending on region; bovine tuberculosis was more common in northeastern and north central states where dairy cattle were more common than in western states where beef cattle predominated [36]. On average, in 1917, 5% of US cattle were tuberculous, including 10% of dairy cattle and 1-2% of beef cattle [25]. By 1935, 25 million cattle were tested with a disease prevalence of 1.5% [21]. From 1917 to 1941, almost 25,000,000 cattle were tested for bovine tuberculosis. The percent reactors removed from herds declined from 4.9% in 1918 to 0.3% in 1941. This led Secretary of Agriculture, Claude R. Wickard, to declare, "the United States is now practically free of bovine tuberculosis" [65] as every county in the country had reached the modified accredited status (<0.5 per cent of animals tested that react positively). The 23-year cost to taxpayers was ~$200,000,000 plus the cost to farmers [65]. Secretary Wickard's statement in 1941 was in stark contrast to the 1917 statement, by Henry Wallace (Secretary of Agriculture 1933–1940), that eradication of tuberculosis seemed an "impossible undertaking" [20].

Progress in tuberculosis eradication in the US was relatively rapid compared to that seen in parts of Europe. Bovine tuberculosis has never been as prevalent in the US as it has been in parts of Europe, and although it is tempting to compare the programs and rate of progress between countries, there are significant differences that make such comparisons problematic. In the early 1900s, the prevalence of disease in the US was significant, but relatively low at approximately 5%. By 1941, every county in the US had a prevalence <0.5% and was considered accredited free of bovine tuberculosis [20]. Reducing the prevalence to levels below 0.5% was achievable at a relatively low cost [20]. The prevalence rate in Europe during this same period was much higher (25–80%) thus decreasing by 4.5% would have made little difference in the overall prevalence and decreasing to a level below 0.5% would have required the slaughter of enough cattle to create food shortages [20]. Fear of negative public perception and food shortages contributed to the less aggressive approaches adopted by most European countries compared to that of the US and BAI. Even Bernard Bang advocated a less aggressive approach, fearing food shortages more than contaminated meat and milk. In view of these sentiments, it was not until 1945 that most European countries imposed mandatory test and slaughter programs as well as compulsory milk pasteurization [20]. Regardless, the effective and relatively rapid diminution in disease prevalence in the US is generally attributed to the stringent application of the test and slaughter method of control. Between 1917 and 1945, there were 3,891,950 tuberculin reactors slaughtered at a cost of $250,000,000 to the federal, state, and local governments involved [66].

11. Conclusions

Veterinarians, and others, that completed the "impossible undertaking" of establishing the US bovine tuberculosis eradication program should be proud of their efforts. Those efforts range from the early pioneers in infectious disease research to the many accredited private practitioners that administer thousands of caudal fold tests every day. At the inception of the eradication program, policy decisions often arose from argumentative conversations in open forums between livestock producers, state and local veterinarians, federal and public health officials, as well as many others. On the surface, it appears as an undirected and unorganized affair; however, it led to a formal process for input and debate, still in use today as exemplified by the USAHA annual meetings. The result has been to decrease disease prevalence from 5% in 1917 to approximately 0.001% today [67]. Although eradication has not yet been achieved, the program has seen a steady decline in prevalence, decreasing by 90% every 20 years [68]. Early efforts focused on eliminating tuberculosis from breeding stock and providing uninfected animals for replacements. Federal funding for bovine TB efforts increased from $75,000 in 1917 to $500,000 in 1918 and to $1.5 million in 1919 [20]. State spending on eradication also increased from $2 million in 1918 to $13 million in 1927. In the first 2 decades of the program, state funding outpaced federal funding 2:1. As the federal government made available emergency funding in the mid-1930s, state funding decreased to half of federal funding [20].

The USDA estimated the cost of the program to livestock producers, at the onset was approximately $40 million annually [20]. It is estimated that over the period 1917 to 1962, the cost of the program was $258 million in

1918 dollars and $3 billion in 2003 dollars [20]. Annual benefits during this same time period are estimated at $98.7 million per year, equivalent to 12-times the annual costs [20]. Costs and benefits of the eradication program can also be viewed from the perspective of public health and disease prevention. The effort to eradicate bovine tuberculosis resulted in a significant decrease in human tuberculosis due to *Mycobacterium bovis*. It is estimated that the eradication program, combined with pasteurization of milk, prevented over 25,000 deaths annually [20]. Other analyses, focused on the livestock sector suggest that during the first years of the program costs may have outweighed benefits; however, benefits have consistently outweighed costs for the majority of the campaign [68]. As a whole, net annual benefits of the program are approximated at $159 million; the program returning over $13 billion to the economy since its conception [68].

References

[1] V. A. Moore, "Bovine tuberculosis, its problems and control," in *Proceedings of the 15th Annual Meeting of the United States Livestock Sanitary Association*, pp. 51–56, Chicago, Ill, USA, 1911.

[2] K. A. Alexander, P. N. Laver, A. L. Michel et al., "Novel *Mycobacterium tuberculosis* complex pathogen, M. mungi," *Emerging Infectious Diseases*, vol. 16, pp. 1296–1299, 2010.

[3] N. H. Smith, R. G. Hewinson, K. Kremer, R. Brosch, and S. V. Gordon, "Myths and misconceptions: the origin and evolution of *Mycobacterium tuberculosis*," *Nature Reviews Microbiology*, vol. 7, no. 7, pp. 537–544, 2009.

[4] B. M. Rothschild, L. D. Martin, G. Lev et al., "*Mycobacterium tuberculosis* complex DNA from an extinct bison dated 17,000 years before the present," *Clinical Infectious Diseases*, vol. 33, no. 3, pp. 305–311, 2001.

[5] G. Diamant, "Regulatory veterinary medicine: and they blew a horn in Judea," *Journal of the American Veterinary Medical Association*, vol. 172, no. 1, pp. 45–54, 1978.

[6] L. Cobbett, "The relation between animal and human tuberculosis," in *The Causes of Tuberculosis*, Cambridge Public Health, pp. 185–195, Cambridge University Press, 1917.

[7] E. B. Miller, "Tuberculous cattle problem in the United States," *Historia Medicinae Veterinariae*, vol. 14, no. 1-2, pp. 1–64, 1989.

[8] M. P. Ravenel, "Three cases of tuberculosis of the skin due to inoculation with bovine tubercle bacillus," *Philadelphia Medical Journal*, vol. 6, pp. 125–126, 1900.

[9] D. E. Salmon, *Bovine Tuberculosis and Public Health*, United States Department of Agriculture, 1904.

[10] L. Shrady and G. F. Stedman, "Human and bovine tuberculosis," *Medical Record and Weekly Journal of Medicine and Surgery*, 1902.

[11] H. C. Ernst, *Infectiousness of Milk: Result of Investigations Made for the Trustees of the Massachusetts Society for Promoting Agriculture*, The Riverside Press, Cambridge, Mass, USA, 1895.

[12] C. E. Dolman, "Theobald Smith, 1859–1934: a fiftieth anniversary tribute," *ASM News*, vol. 50, pp. 577–580, 1984.

[13] L. Pearson, "The Pennsylvania plan for controlling tuberculosis of cattle," in *Proceedings of the American Veterinary Medical Association*, W. L. Williams, Ed., pp. 161–182, American Veterinary Medical Association, Ithaca, NY, USA, 1899.

[14] D. C. Lochead, "Transmissibility of bovine tuberculosis to the human," in *Proceedings of the 32nd Annual Meeting of the United States Livestock Sanitary Association*, pp. 526–547, Chicago, Ill, USA, 1928.

[15] A. D. Melvin, "Tuberculosis-summary of methods and results to date," in *Proceedings of the 15th Annual Meeting of the United States Livestock Sanitary Association*, pp. 76–79, Chicago, Ill, USA, 1911.

[16] D. E. Salmon, *Report of the Committee on Animal Diseases and Animal Food*, American Public Health Association, New Orleans, La, USA, 1902.

[17] A. J. Lartigau, "A study of the variation in virulence of the bacillus tuberculosis in man," *Journal of Medical Research*, vol. 62, pp. 152–162, 1901.

[18] L. Pearson and M. P. Pearson, Eds., *Tuberculosis of Cattle and the Pennsylvania Plan for Its Repression*, Commonwealth of Pennsylvania Department of Agriculture, Harrisburg, Pa, USA, 1901.

[19] E. M. Crimmins and G. A. Condran, "Mortality variation in U.S. cities in 1900: a two-level explanation by cause of death and underlying factors," *Social Science History*, vol. 7, no. 1, pp. 31–60, 1983.

[20] A. L. Olmstead and P. W. Rhode, "An impossible undertaking: the eradication of bovine tuberculosis in the United States," *Journal of Economic History*, vol. 64, no. 3, pp. 734–772, 2004.

[21] J. D. Roswurm and A. F. Ranney, "Sharpening the attack on bovine tuberculosis," *American Journal of Public Health*, vol. 63, no. 10, pp. 884–886, 1973.

[22] J. C. Norton, "President's annual address," in *Proceedings of the 7th Annual Meeting of the Interstate Association of Livestock Sanitary Boards*, pp. 7–35, St. Louis, Mo, USA, 1904.

[23] B. G. Rosenkrantz, "The trouble with bovine tuberculosis," *Bulletin of the History of Medicine*, vol. 59, no. 2, pp. 155–175, 1985.

[24] "The British Congress on Tuberculosis," *Journal of American Medical Association*, vol. 37, no. 6, pp. 387–388, 1901.

[25] A. L. Olmstead and P. W. Rhode, "The Tuberculous cattle trust: disease contagion in an era of regulatory uncertainty," *Journal of Economic History*, vol. 64, no. 4, pp. 929–963, 2004.

[26] R. A. Packer, "Veterinarians challenge Dr. Robert Koch regarding bovine tuberculosis and public health," *Journal of the American Veterinary Medical Association*, vol. 196, no. 4, pp. 574–575, 1990.

[27] D. E. Salmon, "The federal meat inspection," in *Yearbook of Agriculture*, pp. 67–80, Government Printing Office, Washington, DC, USA, 1894.

[28] G. Ditewig, "Meat inspection service of the United States Department of Agriculture," in *Yearbook of Agriculture*, pp. 71–97, Government Printing Office, Washington, DC, USA, 1916.

[29] A. D. Melvin, "The economic importance of tuberculosis of food-producing animals," in *The 25th Annual Report of the Bureau of Animal Industry for the Year 1908*, pp. 97–107, Bureau of Animal Industry, Washington, DC, USA, 1910.

[30] F. A. Ingram, "Municipal meat inspection," in *Proceedings of the 20th Annual Meeting of the United States Livestock Sanitary Association*, pp. 191–195, Chicago, Ill, USA, 1916.

[31] H. C. Ernst, *Koch's Treatment of Tuberculosis*, Damrell and Upham Publishers, Boston, Mass, USA, 1891.

[32] G. M. Gould, *The Practitioner's Medical Dictionary*, P. Blakiston's Son and Co., Philadelphia, Pa, USA, 2nd edition, 1910.

[33] C. J. Marshall, "Progress in controlling bovine tuberculosis," *Journal of the American Medical Association*, vol. 80, pp. 625–633, 1932.

[34] C. J. Marshall, "Tuberculin test and retest," *Journal of the American Medical Association*, vol. 5, pp. 308–337, 1917.

[35] R. Carswell, "Value of the negative subcutaneous tuberculin test," *The British Medical Journal*, vol. 1, pp. 990–992, 1936.

[36] U. G. Houck, *The Bureau of Animal Industry of the United States Department of Agriculture: Its Establishment, Achievements and Current Activities*, Hayworth Printing Company, Washington, DC, USA, 1924.

[37] C. H. Higgins, "Channels of infection and localization in tuberculosis," *Journal of the American Medical Association*, vol. 52, pp. 299–308, 1917.

[38] O. H. V. Stalheim and W. M. Moulton,, "Part II: veterinary medicine in the United States department of agriculture," in *100 Years of Animal Health 1884–1984*, V. Wiser, L. Mark, and H. G. Purchase, Eds., pp. 19–62, Associates of the National Agricultural Library, Beltsville, Md, USA, 1986.

[39] A. J. Glover, "Methods of popular education in an anti-tuberculosis campaign," in *Proceedings of the 15th Annual Meeting of the United States Livestock Sanitary Association*, pp. 42–47, Chicago, Ill, USA, 1911.

[40] E. Z. Russell, "Bovine tuberculosis," in *Proceedings of the 21st Annual Meeting of the United States Livestock Sanitary Association*, pp. 20–26, Chicago, Ill, USA, 1917.

[41] D. F. Luckey, "Obstacles in the way of controlling tuberculosis of cattle," in *Proceedings of the 10th Annual Meeting of the Interstate Association of Livestock Sanitary Boards*, pp. 31–47, Springfield, Ill, USA, 1906.

[42] T. Butler, "The value of farmer's institutes in educating the public on contagious diseases of livestock," in *Proceedings of the 10th Annual Meeting of the Interstate Association of Livestock Sanitary Boards*, pp. 56–59, Springfield, Ill, USA, 1906.

[43] D. F. Luckey, "President's annual address," in *Proceedings of the 11th Annual Meeting of the Interstate Association of Livestock Sanitary Boards*, pp. 7–11, Richmond, Va, USA, 1907.

[44] K. F. Meyer, "Newer methods of tuberculin testing," in *Proceedings of the 14th Annual Meeting of the United States Sanitary Livestock Association*, pp. 56–62, Chicago, Ill, USA, 1910.

[45] J. G. Wills, "How should a tuberculin test be applied to insure accuracy in results?" in *Proceedings of the 20th Annual Meeting of the United States Livestock Sanitary Association*, pp. 132–137, Chicago, Ill, USA, 1916.

[46] W. A. Evans, "Sanitary milk supply for cities and towns from a health commissioners standpoint," in *Proceedings of the 14th Annual Meeting of the United States Livestock Sanitary Association*, pp. 1115–1118, Chicago, Ill, USA, 1910.

[47] W. D. Hoard, "Tuberculosis control from a laymen's standpoint," in *Proceedings of the 14th Annual Meeting of the United States Livestock Sanitary Association*, pp. 69–71, Chicago, Ill, USA, 1910.

[48] E. T. Faulder, H. M. O'Rear, C. E. Cotton, and C. C. Hisel, "Report of the special committee on tuberculosis," *Journal of the American Veterinary Medical Association*, vol. 83, pp. 518–520, 1933.

[49] J. M. Hackney, "Tuberculosis and the dairy industry," in *Proceedings of the 23rd Annual Meeting of the United States Livestock Sanitary Association*, pp. 38–44, Chicago, Ill, USA, 1919.

[50] A. L. Olmstead and P. W. Rhode, "Not on my farm! Resistance to bovine tuberculosis eradication in the United States," *Journal of Economic History*, vol. 67, no. 3, pp. 768–809, 2007.

[51] P. Malcolm, "Problems of tuberculosis control and its relation to sanitary science and food hygiene," *Journal of the American Medical Association*, vol. 80, pp. 96–99, 1932.

[52] "Husbandry: at lenker's place," *Time*, 1931.

[53] S. E. Bruner, "Result of a project to determine the comparative value of the subcutaneous-intradermal tuberculin tests," in *Proceedings of the 25th Annual Meeting of the United States Livestock Sanitary Association*, pp. 107–113, Chicago, Ill, USA, 1921.

[54] U.S. Department of Agriculture and Animal and Plant Health Inspection Service, *Bovine Tuberculosis Eradication Uniform Methods and Rules*, US Government Printing Office, Washington, DC, USA, 2005.

[55] M. P. Ravenel, "The tuberculosis problem," in *Proceedings of the 15th Annual Meeting of the United States Livestock Sanitary Association*, pp. 62–76, Chicago, Ill, USA, 1911.

[56] A. Peters, "Obstacles to enforcing regulations requiring the tuberculin test in interstate cattle traffic," in *Proceedings of the 37th Annual Meeting of the American Veterinary Medical Association*, pp. 202–215, Saint Paul, Minn, USA, 1900.

[57] T. Smith, "A comparative study of bovine tubercle bacilli and of human bacilli from sputum," *Journal of Experimental Medicine*, vol. 3, pp. 451–511, 1898.

[58] A. D. Melvin, *Hearings before the committee on Agriculture. Agricultural Appopriations Bill, Congress*, Government Printing Office, Washington, DC, USA, 1909.

[59] A. D. Melvin, *The 28th Annual Report of the Bureau of Animal Industry*, Bureau of Animal Industry, Washington, DC, USA, 1913.

[60] J. A. Kiernan, "Report of the committee on tuberculosis of the United States Livestock sanitary associatio," in *Proceedings of the 25th Annual Meeting of the United States Livestock Sanitary Association*, pp. 92–106, Chicago, Ill, USA, 1921.

[61] J. A. Kiernan, "The accredited-herd plan in tuberculosis eradication," in *Yearbook of Agriculture*, vol. 1918, pp. 215–220, Government Printing Office, Washington, DC, USA, 1918.

[62] J. A. Kiernan, "Proposed regulations for accrediting herds of cattle," in *Proceedings of the 21st Annual Meeting of the United States Livestock Sanitary Association*, pp. 128–129, Chicago, Ill, USA, 1917.

[63] J. A. Kiernan, "Bovine tuberculosis being suppressed," in *Yearbook of Agriculture*, vol. 1926, pp. 180–183, Government Printing Office, Washington, DC, USA, 1926.

[64] J. A. Kiernan, "Tuberculosis eradication," in *Proceedings of the 22nd Annual Meeting of the United States Livestock Sanitary Association*, pp. 29–52, Chicago, Ill, USA, 1918.

[65] E. Hastings, "The eradication of bovine tuberculosis," *American Journal of Veterinary Research*, vol. 3, pp. 3–9, 1942.

[66] W. H. Feldman, "Animal tuberculosis and its relationship to the disease in man," *Annals of the New York Academy of Sciences*, vol. 48, pp. 469–505, 1947.

[67] D. L. Whipple and M. V. Palmer, "Reemergence of tuberculosis in animals in the United States," in *Emerging Diseases of Animals*, C. Brown and C. A. Bolin, Eds., pp. 281–299, ASM Press, Washington, DC, USA, 2000.

[68] M. J. Gilsdorf, E. D. Ebel, and T. W. Disney, "Benefit and cost assessment of the U.S. bovine tuberculosis eradication program," in *Mycobacterium bovis Infection in Animals and Humans*, C. O. Thoen, J. H. Steele, and M. J. Gilsdorf, Eds., pp. 89–99, Wiley-Blackwell Publishing Professional, Ames, Iowa, USA, 2nd edition, June, 2006.

Review Article

Comparative Gamma Delta T Cell Immunology: A Focus on Mycobacterial Disease in Cattle

Brandon L. Plattner[1] and Jesse M. Hostetter[2]

[1] Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada N1G 2W1
[2] Department of Veterinary Pathology, 2720 Veterinary Medicine Complex, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA

Correspondence should be addressed to Jesse M. Hostetter, jesseh@iastate.edu

A theme among many pathogenic mycobacterial species affecting both humans and animals is a prolonged asymptomatic or latent period that can last years to decades. The mechanisms that favor progression to active disease are not well understood. Pathogen containment is often associated with an effective cell-mediated or T-helper 1 immune profile. With certain pathogenic mycobacteria, such as *Mycobacterium avium* subspecies *paratuberculosis*, a shift to active clinical disease is associated with loss of T-helper 1 immunity and development of an ineffective humoral or T-helper 2 immune response. Recently $\gamma\delta$ T cells have been shown to play a role early in mycobacterial infections and have been hypothesized to influence disease outcome. The purpose of this paper is to compare recent advancements in our understanding of $\gamma\delta$ T cells in humans, cattle, and mice and to discuss roles of $\gamma\delta$ T cells in host response to mycobacterial infection.

1. Introduction

The host immune response to mycobacterial infection is complex, and significant differences exist among a diverse group of mycobacterial pathogens and host species infected. A common theme among many pathogenic mycobacterial species in both humans and animals is a prolonged asymptomatic or latent period that can last years to decades. An effective cell-mediated or T helper-1 (Th-1) immune response during latency correlates to control of pathogen proliferation and disease progression. It is interesting that during this latent period bacterial numbers are usually very low and their detection difficult. In ruminants infected with *Mycobacterium avium* subspecies *paratuberculosis* (*Map*), progression from the asymptomatic phase to clinical disease is associated with a loss of Th-1 responses and development of an ineffective humoral or T helper-2 (Th-2) response [1]. Clinical disease in these animals is characterized by extensive poorly organized macrophage infiltrates into the intestine, which harbor tremendous numbers of *Map* bacilli. The mechanisms that induce this shift in immune responses remain unknown. A growing area of interest in the pathogenesis of mycobacterial infection is the role of a subset of T lymphocytes, the gamma delta ($\gamma\delta$) T cells. $\gamma\delta$ T cells are interesting in that they appear to have a diverse set of immunological functions that span innate to adaptive responses. Recently, $\gamma\delta$ T cells have been shown to play a role early in mycobacterial infection and have been hypothesized to influence the outcome of long-term infection. The purpose of this paper is to compare the recent advances in our understanding of $\gamma\delta$ T cells in humans, cattle, and mice and to discuss the $\gamma\delta$ T cell responses that occur during mycobacterial infection in these species.

2. $\gamma\delta$ T Cells

First described in humans in 1986 [2] and in cattle in 1989 [3], the $\gamma\delta$ T cell receptor (TCR) has not been well characterized compared to the more widely studied $\alpha\beta$ TCR. Since their discovery, the immunobiology of $\gamma\delta$ T cells has been most studied in humans and mice, and the data have indicated that these cells have a variety of functions

during both innate and adaptive immunity. Key differences in $\gamma\delta$ T cell biology exist between species, yet recent data have indicated several common themes that apparently span the species barrier including anatomic distribution and functional capacity: these topics are further discussed in the following sections.

3. Specialized Anatomic Distribution and Phenotype

Within lymphoid tissues, $\gamma\delta$ T cells are considered to be a minor T lymphocyte population, yet $\gamma\delta$ T cells are enriched in many organs including skin and mucosal surfaces. This distribution suggests a role for these cells during immune surveillance and antigen sampling at surfaces constantly confronted with invading pathogens [4, 5]. $\gamma\delta$ T cells are also well represented in peripheral blood mononuclear cells. $\gamma\delta$ T cells typically represent 1–10% of circulating T lymphocytes in adult humans and mice and approximately 10–25% in adult cattle, though this number can be as high as 40% in young calves [6].

Similar to α and β TCR genes, γ and δ TCR genes have variable (V), joining (J), and constant (C) regions. The δ and β genes also contain diversity (D) gene segments [7]. In humans, $\gamma\delta$ T cell subsets are defined by their γ and δ gene segment usage. In humans, $\gamma\delta$ T cells from different anatomic sites show preferential V segment usage suggesting that human $\gamma\delta$ T cell subsets have distinct functional roles [8]. For example, the two major $\gamma\delta$ T cell subsets in humans are Vδ1+ and Vδ2+ cells. Vδ2+ cells predominate in peripheral blood and have been shown to significantly expand during a variety of infectious diseases including mycobacterial diseases [9]. The Vδ1+ subset is less frequent in the blood, but is the majority subset in tissues [10, 11]. Expression of additional molecules (CD2, CD4, CD5, CD6, and CD8) on $\gamma\delta$ T cells has also been described in humans, mice, and cattle. There is considerable interspecies variability with respect to these markers suggesting that these molecules are less useful for defining functionally distinct subsets, and there is currently no species-wide $\gamma\delta$ T cell-specific marker [12, 13].

The $\gamma\delta$ TCR of cattle has been cloned and characterized, but little is known about how preferential gene segment usage correlates with tissue distribution or functionality [14, 15]. Surface expression of the cysteine-rich scavenger receptor molecule workshop cluster 1 (WC1) is most commonly used to distinguish $\gamma\delta$ T cell subsets of cattle, and expression of WC1 appears to be limited to ruminant $\gamma\delta$ T cells though WC1-like genes have been found in sheep, goats, horses, mice, pigs, and humans [16]. Further subdivision of WC1 expressing cells in cattle has been described (WC1.1, WC1.2, and WC1.3) [17, 18]. As in humans, it is thought that different phenotypes represent functionally distinct $\gamma\delta$ T cell subsets that preferentially home to different tissue localizations [13]. Based on WC1 molecule expression, bovine $\gamma\delta$ T cells are most frequently divided into two categories: the larger of these subsets has the phenotype WC1−CD2+CD3+ and is found primarily within splenic red pulp and the intestinal tract, while the second subset has the phenotype WC1+CD2−CD3+ and is

found predominantly in peripheral blood. Two additional features that fundamentally distinguish $\alpha\beta$ from $\gamma\delta$ T cells appear to be shared by humans and cattle. First, $\gamma\delta$ T cells are not clearly defined by surface expression of the CD4 or CD8 accessory molecules, and thus there is no MHC class I or MHC class II restriction. Second, $\gamma\delta$ T cells recognize unconventional antigens such as phosphorylated microbial metabolites or lipid antigens [19].

4. Ligands

Specificity of $\gamma\delta$ T cells to mycobacterial antigens in humans has been described [20]. Protein antigens such as mycobacterial heat shock protein [21, 22] and nonprotein [23, 24] antigens including phosphoantigens have been shown to induce strong $\gamma\delta$ T cell responses. In humans, the majority of studies have examined reactive patterns of the Vδ2+ subset of $\gamma\delta$ T cells. Vδ2+ cells recognize low molecular weight nonpeptide phosphate-containing metabolites produced by a variety of bacterial pathogens including mycobacteria [25]. Variations or other important ligands for Vδ2+ cells include microbial byproducts such as negatively charged alkyl phosphate antigens [26] and positively charged alkylamine antigens [27]. Many of the putative microbial ligands described for human $\gamma\delta$ T cells have autologous counterparts or endogenous metabolites of the mevalonate pathway, which are upregulated during periods of cellular stress suggesting that $\gamma\delta$ T cells also function during noninfectious processes [28]. Specific ligands for human Vδ1+ cells are less well described. Spada et al. reported that Vδ1+ cells directly recognized CD1c molecules [11], which may be a mechanism of antigen presentation to Vδ1+ cells during *M. leprae* infection [11, 29].

$\gamma\delta$ T cell ligands in cattle are not clearly defined. The majority of studies have examined the reactive patterns of WC1+ $\gamma\delta$ T cells likely because of their ease of isolation from peripheral blood. An early study by Rhodes et al. demonstrated responsiveness of bovine peripheral blood $\gamma\delta$ T cells from *M. bovis* infected calves to various mycobacterial protein antigens [30]. Work by Welsh et al. confirmed that WC1+ cells respond to both protein and nonprotein *M. bovis* antigens, and that response to mycobacterial proteins was dominant [31]. Vesosky et al. showed that WC1+ cells from healthy calves could respond to stimulation with live mycobacteria, mycobacterial cell wall, and mycobacterial culture filtrate proteins [32]. In this study, the phosphoantigen identified as a human $\gamma\delta$ T cell ligand (isopentenyl pyrophosphate, IPP) was not recognized by naïve bovine $\gamma\delta$ T cells [32]. In both humans and cattle, the interactions surrounding $\gamma\delta$ T cell activation have largely been considered to be MHC-independent [24] and TCR-dependent, although TCR-independent activation has also been shown [33]. Recent work has also demonstrated that purified human and bovine $\gamma\delta$ T cells can be directly activated by pathogen-associated molecular patterns (PAMPs) in the absence of antigen presenting cells [34], which may have significant implications for the innate role of $\gamma\delta$ T cells. Though their restriction elements during ligand recognition by bovine $\gamma\delta$ T cells remain to be fully characterized, it is

clear that $\gamma\delta$ T cells from both naïve and infected individuals have the capacity to respond to mycobacterial antigens.

5. Importance of IL-2

In $\alpha\beta$ T cells the initial encounter with specific antigen along with the appropriate costimulatory signals (CD28 of T cell binding B7 of APC) induces the synthesis of IL-2 and increased expression of the α chain of the IL-2 receptor (CD25). Subsequent binding of IL-2 to its high-affinity receptor then triggers progression through the cell cycle, proliferation, and differentiation of naïve T cells [7]. Distinct from their $\alpha\beta$ T cell counterparts, $\gamma\delta$ T cells produce minimal amounts of IL-2 upon activation, and the proliferative response of human $\gamma\delta$ T cells after antigenic stimulation is dependent on CD4+ T cell secretion of IL-2 [35]. Welsh et al. and Smyth et al. in separate studies demonstrated marked upregulation of CD25 on the surface of bovine $\gamma\delta$ T cells after encountering *M. bovis* protein antigens, but there was minimal proliferation without addition of IL-2 [31, 36]. Based on these findings, IL-2 is very likely a required secondary signal for activation of $\gamma\delta$ T cells, which ultimately drives them to proliferation after recognition of mycobacterial antigens.

6. Effects on Granuloma Formation, Maintenance

There has been recent interest in the role of $\gamma\delta$ T cells in generation and maintenance of granulomas that develop at mycobacterial infection sites. In a murine model of *Map* infection, the frequency of granuloma formation was significantly decreased in $\gamma\delta$ TCR depleted mice indicating a potential role of $\gamma\delta$ T cells in the generation of granulomas during mycobacterial infection [37]. In cattle, $\gamma\delta$ T cells have also been evaluated for a potential role in granuloma formation. Palmer et al. demonstrated that in *M. bovis* infected calves, CD4+ T cell numbers in lymph node granulomas remained constant over time. The number of CD8+ T cells and WC1+ cells was high during early-stage granulomas, but diminished as granulomas matured. The authors suggested that loss of these T cell subsets during late stages correlates with failure of the immune system to control infection [38]. In contrast, Wangoo et al. showed that late-stage lymph node granulomas from *M. bovis*-infected calves had significantly greater numbers of WC1+ T cells compared to early stage lesions, and that the WC1+ cells were spatially distributed at the peripheral zone near the fibrotic capsule [39]. However, in a separate study evaluating spatial distribution of T cell subsets, this group was unable to confirm distinct spatial relationships of the $\gamma\delta$ T cells within the granulomatous lesions [40]. Simutis et al. in 2005 identified $\gamma\delta$ T cells within poorly organized granulomatous lesions induced by subcutaneous *Map* injection in a calf model [41]. In 2009, Plattner et al. went on to show in this model that in well-organized (Th-1 polarized granulomas) there was stratification of $\gamma\delta$ T cells with respect to WC1+ and WC1− phenotypes. This stratification was lacking in poorly organized granulomas associated with *Map* infection. The conclusion from this study was that the $\gamma\delta$ T cells subsets had unique roles in directing bovine granuloma formation and function during *Map* infection [42].

7. Immediate Effector Function: Cytotoxicity

Numerous effector functions have been reported for subsets of $\gamma\delta$ T cells. Activated human Vδ2+ $\gamma\delta$ T cells have broad cytotoxic activity. Oliaro et al. demonstrated that Vδ2+ cells were able to directly lyse Brucella-infected macrophages and reduce intracellular bacterial numbers by the Fas/Fas ligand pathway [43]. Dieli et al. showed that generation of perforin and granzyme by Vδ2+ cells reduced the viability of both extracellular and intracellular *M. tuberculosis* [44, 45]. Fisch et al. specifically examined both major subsets of human $\gamma\delta$ T cells and demonstrated broad in vitro cytotoxicity by Vδ2+ cells, but importantly observed that Vδ1+ cells also exhibited this capacity [46]. In cattle, it is known that cytotoxicity mediated by bovine natural killer (NK) cells reduces intracellular viability of *M. bovis* [47]; however, the evidence for $\gamma\delta$ T cell-mediated cytotoxicity is less clear. Bovine peripheral blood-derived and antigen-stimulated $\gamma\delta$ T cells (WC1 phenotype not reported, but most likely WC1+ subset) were unable to mediate nitric oxide production and bacterial killing of *Map*-infected macrophages [48]. Other data have also suggested that cytotoxicity is a feature of bovine $\gamma\delta$ T cells during *Map* infection [49, 50].

8. Immediate Effector Function: Cytokine Secretion

It has been known for several years that a key mechanism by which T lymphocytes respond to infectious agents and mediate immune functions is secretion of specific cytokines. Upon recognition of their ligands, $\gamma\delta$ T cells are able to generate a range of proinflammatory cytokines and antimicrobial peptides [51] and provide an initial barrier until antigen-specific $\alpha\beta$ T cells have been expanded. Cytokine production by $\gamma\delta$ T cell subsets has been analyzed at the gene and protein levels in humans and cattle. Microarray analysis of stimulated human Vδ2+ cells has shown upregulation of proinflammatory genes such as tumor necrosis factor alpha (TNF-α), IFN-γ, macrophage-colony stimulating factor, IL-17, and IL-21 [52, 53]. However, secretion of some of these proteins by stimulated Vδ2+ cells has not been confirmed. Initial studies in humans showed that peripheral blood-derived $\gamma\delta$ T cells rapidly expand and produce IFN-γ in response to nonpeptide phosphate antigens [54]. Wang et al. demonstrated that human Vδ2+ cells generate IFN-γ and TNF-α as early as 2 hours following exposure to the live bacterial product *iso*-butylamine. An interesting observation in this study was that production of cytokines was cyclic and limited to periods of direct contact with live bacteria, suggesting that $\gamma\delta$ T cell activity is focused at the infection site [55]. Vδ2+ production of IFN-γ and TNF-α was also confirmed by Wesch et al. [56]. Vδ2+ cells from human

peripheral blood can be driven towards IL-4 production under specific culture conditions [56]. Depending on the physiologic or pathologic context, subsets of murine $\gamma\delta$ T cells have also been shown to produce Th2 cytokines [57]. The production of keratinocyte growth factor or connective tissue growth factor by $\gamma\delta$ T cells suggests more specialized tissue repair functions [19]. Microarray data for human-stimulated Vδ1+ cells initially demonstrated upregulation of cytokine genes that are considered important during regulatory functions such as IL-10 and IL-11 [52, 53], and recent work has confirmed the ability of Vδ1+ cells to produce IL-10 as well as transforming growth factor-beta (TGF-β) [58].

In cattle, evidence for cytokine secretion by $\gamma\delta$ T cell subsets is less clear. Buza et al. correlated IFN-γ production with changes in circulating $\gamma\delta$ T cell populations rather than CD4+ or CD8+ T cells of BCG-vaccinated calves [59]. No effects on disease pathology were observed following depletion of WC1+ $\gamma\delta$ T cells from *M. bovis*-infected calves, though increased antigen-specific IL-4, reduced innate IFN-γ, and reduced IgG2 antibody were observed [60]. WC1+ cells from *M. bovis*-infected calves proliferated strongly when stimulated with *M. bovis* extracts but produced significantly less IFN-γ compared to autologous CD4+ T cells [36]. Vesosky et al. demonstrated that while proliferation of bovine $\gamma\delta$ T cells from healthy cattle could be induced by a variety of mycobacterial antigens, the requirements for IFN-γ production were more stringent. Specifically, purified WC1+ cells produced significant amounts of IFN-γ in response to a nonprotein component of mycobacterial cell wall antigen only when antigen-presenting cells and exogenous IL-2 were added to the cultures [32]. Rogers et al. have further demonstrated that the function of bovine $\gamma\delta$ T cells varies with the expressed form of WC1. In a series of experiments, they showed that WC1.1+ and WC1.2+ cells had different proliferation potentials to various bacterial stimuli and that the WC1.1+ cells were the major producers of IFN-γ [17, 18, 61]. Further, WC1.1+ cells are preferentially recruited to the respiratory tract following intranasal BCG vaccination in calves [62]. In a fetal bovine-severe combined immunodeficient (SCID-bo) xenochimeric mouse model, WC1+ cells did not produce significant IFN-γ, but were shown to be involved in recruitment of other cells to mycobacterial infection sites [63]. These results support the hypothesis that WC1+ cells have a role in directing the Th1 bias of the immune response during mycobacterial infections. In contrast to human $\gamma\delta$ T cells, no published studies document production of IL-4 or other Th2-like cytokines from bovine $\gamma\delta$ T cells. As is the case for human Vδ1+ cells, little is known regarding cytokine secretion by WC1− $\gamma\delta$ T cells of cattle, though recent work has shown that this subset can be experimentally induced to generate significant amounts of IFN-γ [64].

Recently, $\gamma\delta$ T cells have been shown to play a role during the Th17 response. Th17 responses are defined by the production of IL-17, and are thought to play a critical role in inflammatory responses, particularly at mucosal surfaces [65]. In mice, production of IL-17 has been demonstrated from naïve $\gamma\delta$ T cells [66]. It has been proposed that $\gamma\delta$ T cells initiate Th17 responses by upregulation of IL-6 and IL-8, which in turn enhances neutrophil chemotaxis during early bacterial infections [67]. Sutton et al. recently demonstrated that murine $\gamma\delta$ T cells express the IL-23 receptor and the transcription factor RORγT and produce IL-17, IL-21, and IL-22 in response to IL-1β and IL-23 (all features of Th17 response) and that their cytokine production is independent of $\gamma\delta$ TCR ligation [68]. Okamoto Yoshida et al. have recently reported that IL-17 is essential for granuloma formation in mice during mycobacterial infection [69]. IL-17 production by $\gamma\delta$ T cells has also recently been confirmed in humans [70], but has yet to be identified in cattle.

9. Specific Effects on Other Cell Types

The ability of $\gamma\delta$ T cells to innately produce IFN-γ during mycobacterial infection is particularly interesting in the context of mycobacterial diseases. It has been proposed that early IFN-γ production at the site of infection by $\gamma\delta$ T cells could stimulate initial killing of bacteria by macrophages [71]. This could enhance antigen presentation by stimulation of infection site dendritic cells (DCs) to mature and migrate to draining lymph nodes thus initiating adaptive T cell immunity [72]. Moreover, direct influence of $\gamma\delta$ T cells on DC function has been recently explored. As the primary antigen presenting cells of the innate immune system, DCs are considered to be primary determinants of the efficacy of the T cell-mediated immune response. It is known that compared to antigen alone, the addition of exogenous IFN-γ enhances the maturation of human DCs in vitro and the potency of the ensuing Th1 immune response [73]. $\gamma\delta$ T cell-mediated enhancement of DC maturation has been documented in vitro following activation of Vδ2+ phosphoantigen-specific [74] and Vδ1+ CD1c-restricted [75] human $\gamma\delta$ T cell subsets. In these studies, there was increased expression of CD86 on cocultured DCs and enhanced IL-12 production by the DCs, which ultimately resulted in improved priming of downstream T cell responses. Leslie et al. also have demonstrated that DCs lacking $\gamma\delta$ T cell interaction (DCs matured by microbial stimuli alone) resulted in "exhausted" DC populations unable to induce efficient Th1 polarization [75]. This response was found to be partially mediated by the cytokines TNF-α and IFN-γ in a nonantigen-specific manner [75]. Vδ1+ cells are thus uniquely positioned to induce DC maturation at infection sites due to their tissue (mucosal surfaces) distribution. A reciprocal interaction where fully mature DCs stimulate $\gamma\delta$ T cells for sustained innate immune responses has been demonstrated at infection sites and in secondary lymphoid organs [76–78]. DC-$\gamma\delta$ T cell interaction has also been shown to be important in the control of mycobacterial infections in mouse models [79].

In contrast to studies with humans, studies of the cellular interactions between bovine $\gamma\delta$ T cells and DCs are few. In 1996, Collins et al. demonstrated that neither WC1− or WC1+ cells from cattle were stimulated to proliferate in response to allogeneic DCs [80]. These results contrast to CD4+ and CD8+ T cells from these calves, which were strongly induced to proliferate by DCs. Price and Hope

recently examined interactions between monocyte-derived DCs and WC1+ cells from *M. bovis*-infected calves in vitro [81]. This study demonstrated that WC1+ cells upregulated surface expression of MHC class II and CD25 (IL-2 receptor) and generated significantly greater amounts of IFN-γ when cocultured with DC. Also, the DCs produced significantly greater amounts of IL-12 when cocultured with WC1+ cells. These results further support the hypothesis that in *M. bovis*-infected cattle, $\gamma\delta$ T cells are able to provide the initial IFN-γ burst that is required for full maturation of DCs and that $\gamma\delta$ T cell-DC interaction can enhance the activation of MHC class II-restricted $\alpha\beta$ T cells.

Additional roles for $\gamma\delta$ T cells have been described in a variety of experimental systems. The ability of $\gamma\delta$ T cells to directly present antigen to $\alpha\beta$ T cells was first demonstrated in cattle [82] and pigs [83]. Collins demonstrated in cattle that B7 molecules were widely expressed on the surface of $\gamma\delta$ T cells and that antigen-primed WC1+ cells directly induced significant CD4+ T cell proliferation [82]. Similar results have been demonstrated in human Vδ2+ [84, 85] and murine $\gamma\delta$ T cells [86].

10. Regulatory Function

Immunomodulatory activity has recently been described for $\gamma\delta$ T cells of several species including mice [87] and humans [58, 88]. In the study by Kühl et al., the Vδ1+ subset was shown to have strong regulatory functions apparently mediated by their production of IL-10 and TGF-β, yet these $\gamma\delta$ T cells lack expression of the classic regulatory T cell marker and transcription factor forkhead box P3 (FoxP3) [58]. Kang et al. were successfully able to induce immunosuppressive function and FoxP3 expression in murine splenic-origin but not human blood-origin $\gamma\delta$ T cells [89]. An immunomodulatory role for bovine $\gamma\delta$ T cells was first described in cattle infected with *Map* where depletion of $\gamma\delta$ T cells was shown to enhance the proliferation of *Map* antigen-stimulated CD4+ T cells [49]. Rhodes et al. confirmed regulatory activity by bovine peripheral blood $\gamma\delta$ T cells during *M. bovis* infection when they demonstrated suppression of antigen-specific $\alpha\beta$ T cell proliferation and enhanced production of both IFN-γ and TGF-β [30]. Jutila and colleagues have also described an immunoregulatory phenotype within subsets of circulating bovine $\gamma\delta$ T cells using serial gene expression analysis [90, 91]. Recently, Hoek et al. identified and characterized regulatory function of sorted bovine $\gamma\delta$ T cells [92]. In contrast to humans and mice, bovine CD4+CD25^{high}FoxP3+ cells lacked ex vivo regulatory activity, and these authors described T regulatory cell activity by WC1.1+ and WC1.2+ cells with upregulated transcription of IL-10 but not FoxP3 or TGF-β genes [92].

11. Memory Function

In 2002, Shen et al. demonstrated the capability of primate $\gamma\delta$ T cells to mount a memory response after microbial infection [93]. Using a macaque tuberculosis model, these authors demonstrated characteristic features of memory $\gamma\delta$ T cells, which included prolonged recall response upon reinfection.

Interestingly in this study, the expansion of Vδ2+ cells in the peripheral blood was associated with clearance of detectable bacteremia [93]. In 2003 Dieli et al. demonstrated effector memory subsets of CD45RA-CD27-human Vδ2+ T cells present in the circulation and within tissues [94].

12. Conclusions

With continued exploration of $\gamma\delta$ T cell functions, it is becoming clear that $\gamma\delta$ T cells have many roles and can be regulatory or stimulatory during host defense against mycobacterial pathogens [95]. This paper has highlighted key recent findings relevant to the pathogenesis of mycobacterial infections: nonclassical mycobacterial antigens are recognized by $\gamma\delta$ T cells; $\gamma\delta$ T cells play key roles in infection site immunopathology, which is potentially mediated by $\gamma\delta$ T cell production of IL-17; $\gamma\delta$ T cells lyse infected cells and act as strong producers of IFN-γ at infection sites; interactions between $\gamma\delta$ T cells and DCs leading to mutual activation have potential to influence mycobacterial infection.

Limitations in the study of $\gamma\delta$ T cell biology such as difficulty obtaining appropriate tissues exist for many species. The study of human and bovine $\gamma\delta$ T cell biology has been largely focused on in vitro evaluation of peripheral blood-derived $\gamma\delta$ T cells. Evaluation of the less easily accessed minor $\gamma\delta$ T cell subsets is important in the future as these subsets have powerful local and downstream effector functions. There is a need for animal modeling systems that allow not only evaluation of multiple tissue-specific $\gamma\delta$ T cell subsets, but also the ability to examine these cells in the context of infection site-specific immunopathology. Cattle display some similarity with humans regarding immunopathology of mycobacterial diseases [96], and this coupled with readily-available $\gamma\delta$ T cells makes young calves a strong model choice to study the pathogenesis of mycobacterial disease.

References

[1] J. R. Stabel, "Transitions in immune responses to *Mycobacterium paratuberculosis*," *Veterinary Microbiology*, vol. 77, no. 3-4, pp. 465–473, 2000.

[2] M. B. Brenner, J. McLean, D. P. Dialynas et al., "Identification of a putative second T-cell receptor," *Nature*, vol. 322, no. 6075, pp. 145–149, 1986.

[3] C. R. Mackay and W. R. Hein, "A large proportion of bovine T cells express the $\gamma\delta$ T cell receptor and show a distinct tissue distribution and surface phenotype," *International Immunology*, vol. 1, no. 5, pp. 540–545, 1989.

[4] M. Girardi, "Immunosurveillance and immunoregulation by $\gamma\delta$ T cells," *Journal of Investigative Dermatology*, vol. 126, no. 1, pp. 25–31, 2006.

[5] M. Nanno, T. Shiohara, H. Yamamoto, K. Kawakami, and H. Ishikawa, "$\gamma\delta$ T cells: firefighters or fire boosters in the front lines of inflammatory responses," *Immunological Reviews*, vol. 215, no. 1, pp. 103–113, 2007.

[6] R. A. Wilson, A. Zolnai, P. Rudas, and L. V. Frenyo, "T-cell subsets in blood and lymphoid tissues obtained from fetal calves, maturing calve, and adult bovine," *Veterinary Immunology & Immunopathology*, vol. 53, no. 1-2, pp. 49–60, 1996.

[7] C. Janeway, *Immunobiology: The Immune System in Health and Disease*, Garland Science, New York, NY, USA, 6th edition, 2005.

[8] Y. H. Chien and M. Bonneville, "Gamma delta T cell receptors," *Cellular and Molecular Life Sciences*, vol. 63, no. 18, pp. 2089–2094, 2006.

[9] H. Das, M. Sugita, and M. B. Brenner, "Mechanisms of Vδ1 $\gamma\delta$ T cell activation by microbial components," *Journal of Immunology*, vol. 172, no. 11, pp. 6578–6586, 2004.

[10] B. Falini, L. Flenghi, S. Pileri et al., "Distribution of T cells bearing different forms of the T cell receptor γ/δ in normal and pathological human tissues," *Journal of Immunology*, vol. 143, no. 8, pp. 2480–2488, 1989.

[11] F. M. Spada, E. P. Grant, P. J. Peters et al., "Self-recognition of CD1 by γ/δ T cells: implications for innate immunity," *Journal of Experimental Medicine*, vol. 191, no. 6, pp. 937–948, 2000.

[12] A. C. Hayday, "$\gamma\delta$ cells: a right time and a right place for a conserved third way of protection," *Annual Review of Immunology*, vol. 18, pp. 975–1026, 2000.

[13] N. D. Machugh, J. K. Mburu, M. J. Carol, C. R. Wyatt, J. A. Orden, and W. C. Davis, "Identification of two distinct subsets of bovine $\gamma\delta$ T cells with unique cell surface phenotype and tissue distribution," *Immunology*, vol. 92, no. 3, pp. 340–345, 1997.

[14] N. Takeuchi, N. Ishiguro, and M. Shinagawa, "Molecular cloning and sequence analysis of bovine T-cell γ and δ chain genes," *Immunogenetics*, vol. 35, no. 2, pp. 89–96, 1992.

[15] S. L. Blumerman, C. T. A. Herzig, A. N. Rogers, J. C. Telfer, and C. L. Baldwin, "Differential TCR gene usage between WC1$^-$ and WC1$^+$ ruminant $\gamma\delta$ T cell subpopulations including those responding to bacterial antigen," *Immunogenetics*, vol. 58, no. 8, pp. 680–692, 2006.

[16] P. L. J. Wijngaard, M. J. Metzelaar, N. D. MacHugh, W. I. Morrison, and H. C. Clevers, "Molecular characterization of the WC1 antigen expressed specifically on bovine CD4$^-$CD8$^-$ $\gamma\delta$ T lymphocytes," *Journal of Immunology*, vol. 149, no. 10, pp. 3273–3277, 1992.

[17] A. N. Rogers, D. G. Vanburen, E. E. Hedblom, M. E. Tilahun, J. C. Telfer, and C. L. Baldwin, "$\gamma\delta$ T cell function varies with the expressed WC1 coreceptor," *Journal of Immunology*, vol. 174, no. 6, pp. 3386–3393, 2005.

[18] A. N. Rogers, D. G. VanBuren, B. Zou et al., "Characterization of WC1 co-receptors on functionally distinct subpopulations of ruminant $\gamma\delta$ T cells," *Cellular Immunology*, vol. 239, no. 2, pp. 151–161, 2006.

[19] S. Beetz, D. Wesch, L. Marischen, S. Welte, H.-H. Oberg, and D. Kabelitz, "Innate immune functions of human $\gamma\delta$ T cells," *Immunobiology*, vol. 213, no. 3-4, pp. 173–182, 2008.

[20] R. L. Modlin, C. Pirmez, F. M. Hofman et al., "Lymphocytes bearing antigen-specific $\gamma\delta$ T-cell receptors accumulate in human infectious disease lesions," *Nature*, vol. 339, no. 6225, pp. 544–548, 1989.

[21] A. Haregewoin, G. Soman, R. C. Hom, and R. W. Finberg, "Human $\gamma\delta^+$ T cells respond to mycobacterial heat-shock protein," *Nature*, vol. 340, no. 6231, pp. 309–312, 1989.

[22] W. Born, L. Hall, A. Dallas et al., "Recognition of a peptide antigen by heat shock-reactive $\gamma\delta$ T lymphocytes," *Science*, vol. 249, no. 4964, pp. 67–69, 1990.

[23] J. J. Fournié and M. Bonneville, "Stimulation of $\gamma\delta$ T cells by phosphoantigens," *Research in Immunology*, vol. 147, no. 5, pp. 338–347, 1996.

[24] C. T. Morita, E. M. Beckman, J. F. Bukowski et al., "Direct presentation of nonpeptide prenyl pyrophosphate antigens to human $\gamma\delta$ T cells," *Immunity*, vol. 3, no. 4, pp. 495–507, 1995.

[25] N. Shrestha, J. A. Ida, A. S. Lubinski, M. Pallin, G. Kaplan, and P. A. Haslett, "Regulation of acquired immunity by gamma delta T-cell/dendritic-cell interactions," *Annals of the New York Academy of Sciences*, vol. 1062, pp. 79–94, 2005.

[26] Y. Tanaka, C. T. Morita, Y. Tanaka, E. Nieves, M. B. Brenner, and B. R. Bloom, "Natural and synthetic non-peptide antigens recognized by human $\gamma\delta$ T cells," *Nature*, vol. 375, no. 6527, pp. 155–158, 1995.

[27] J. F. Bukowski, C. T. Morita, and M. B. Brenner, "Human $\gamma\delta$ T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity," *Immunity*, vol. 11, no. 1, pp. 57–65, 1999.

[28] N. Caccamo, S. Meraviglia, V. Ferlazzo et al., "Differential requirements for antigen or homeostatic cytokines for proliferation and differentiation of human Vγ9Vδ2 naive, memory and effector T cell subsets," *European Journal of Immunology*, vol. 35, no. 6, pp. 1764–1772, 2005.

[29] P. A. Sieling, D. Jullien, M. Dahlem et al., "CD1 expression by dendritic cells in human leprosy lesions: correlation with effective host immunity," *Journal of Immunology*, vol. 162, no. 3, pp. 1851–1858, 1999.

[30] S. G. Rhodes, G. R. Hewinson, and M. H. Vordermeier, "Antigen recognition and immunomodulation by $\gamma\delta$ T cells in bovine tuberculosis," *Journal of Immunology*, vol. 166, no. 9, pp. 5604–5610, 2001.

[31] M. D. Welsh, H. E. Kennedy, A. J. Smyth, R. M. Girvin, P. Andersen, and J. M. Pollock, "Responses of bovine WC1$^+\gamma\delta$ T cells to protein and nonprotein antigens of *Mycobacterium bovis*," *Infection & Immunity*, vol. 70, no. 11, pp. 6114–6120, 2002.

[32] B. Vesosky, O. C. Turner, J. Turner, and I. M. Orme, "Gamma interferon production by bovine $\gamma\delta$ T cells following stimulation with mycobacterial mycolylarabinogalactan peptidoglycan," *Infection & Immunity*, vol. 72, no. 8, pp. 4612–4618, 2004.

[33] G. Leclercq and J. Plum, "Stimulation of TCR Vγ3 cells by Gram-negative bacteria," *Journal of Immunology*, vol. 154, no. 10, pp. 5313–5319, 1995.

[34] J. F. Hedges, K. J. Lubick, and M. A. Jutila, "$\gamma\delta$ T cells respond directly to pathogen-associated molecular patterns," *Journal of Immunology*, vol. 174, no. 10, pp. 6045–6053, 2005.

[35] M. M. Elloso, H. C. van der Heyde, A. Troutt, D. D. Manning, and W. P. Weidanz, "Human $\gamma\delta$ T cell subset-proliferative response to malarial antigen in vitro depends on CD4$^+$ T cells or cytokines that signal through components of the IL-2R," *Journal of Immunology*, vol. 157, no. 5, pp. 2096–2102, 1996.

[36] A. J. Smyth, M. D. Welsh, R. M. Girvin, and J. M. Pollock, "In vitro responsiveness of $\gamma\delta$ T cells from *Mycobacterium bovis*-infected cattle to mycobacterial antigens: predominant involvement of WC1$^+$ cells," *Infection & Immunity*, vol. 69, no. 1, pp. 89–96, 2001.

[37] S. Tanaka, S. Itohara, M. Sato, T. Taniguchi, and Y. Yokomizo, "Reduced formation of granulomata in $\gamma\delta$ T cell knockout BALB/c mice inoculated with *Mycobacterium avium* subsp. *paratuberculosis*," *Veterinary Pathology*, vol. 37, no. 5, pp. 415–421, 2000.

[38] M. V. Palmer, W. R. Waters, and T. C. Thacker, "Lesion development and immunohistochemical changes in granulomas from cattle experimentally infected with *Mycobacterium bovis*," *Veterinary Pathology*, vol. 44, no. 6, pp. 863–874, 2007.

[39] A. Wangoo, L. Johnson, J. Gough et al., "Advanced granulomatous lesions in *Mycobacterium bovis*-infected cattle are associated with increased expression of type I procollagen, $\gamma\delta$ (WC1$^+$) T cells and CD 68$^+$ cells," *Journal of Comparative Pathology*, vol. 133, no. 4, pp. 223–234, 2005.

[40] E. Liebana, S. Marsh, J. Gough et al., "Distribution and activation of T-lymphocyte subsets in tuberculous bovine lymph-node granulomas," *Veterinary Pathology*, vol. 44, no. 3, pp. 366–372, 2007.

[41] F. J. Simutis, N. F. Cheville, and D. E. Jones, "Investigation of antigen-specific T-cell responses and subcutaneous granuloma development during experimental sensitization of calves with *Mycobacterium avium* subsp *paratuberculosis*," *American Journal of Veterinary Research*, vol. 66, no. 3, pp. 474–482, 2005.

[42] B. L. Plattner, R. T. Doyle, and J. M. Hostetter, "Gammadelta T cell subsets are differentially associated with granuloma development and organization in a bovine model of mycobacterial disease," *International Journal of Experimental Pathology*, vol. 90, no. 6, pp. 587–597, 2009.

[43] J. Oliaro, S. Dudal, J. Liautard, J. B. Andrault, J. P. Liautard, and V. Lafont, "Vγ9Vδ2 T cells use a combination of mechanisms to limit the spread of the pathogenic bacteria Brucella," *Journal of Leukocyte Biology*, vol. 77, no. 5, pp. 652–660, 2005.

[44] F. Dieli, M. Troye-Blomberg, J. Ivanyi et al., "Vγ9/Vδ2 T lymphocytes reduce the viability of intracellular Mycobacterium tuberculosis," *European Journal of Immunology*, vol. 30, no. 5, pp. 1512–1519, 2000.

[45] F. Dieli, M. Troye-Blomberg, J. Ivanyi et al., "Granulysin-dependent killing of intracellular and extracellular Mycobacterium tuberculosis by V-γ9/Vδ2 T lymphocytes," *Journal of Infectious Diseases*, vol. 184, no. 8, pp. 1082–1085, 2001.

[46] P. Fisch, K. Oettel, N. Fudim, J. E. Surfus, M. Malkovsky, and P. M. Sondel, "MHC-unrestricted cytotoxic and proliferative responses of two distinct human γ/δ T cell subsets to Daudi cells," *Journal of Immunology*, vol. 148, no. 8, pp. 2315–2323, 1992.

[47] J. J. Endsley, M. A. Endsley, and D. Mark Estes, "Bovine natural killer cells acquire cytotoxic/effector activity following activation with IL-12/15 and reduce *Mycobacterium bovis* BCG in infected macrophages," *Journal of Leukocyte Biology*, vol. 79, no. 1, pp. 71–79, 2006.

[48] F. J. Simutis, D. E. Jones, and J. M. Hostetter, "Failure of antigen-stimulated $\gamma\delta$ T cells and CD4$^+$ T cells from sensitized cattle to upregulate nitric oxide and mycobactericidal activity of autologous *Mycobacterium avium* subsp. *paratuberculosis*-infected macrophages," *Veterinary Immunology & Immunopathology*, vol. 116, no. 1-2, pp. 1–12, 2007.

[49] R. J. Chiodini and W. C. Davis, "The cellular immunology of bovine paratuberculosis: the predominant response is mediated by cytotoxic gamma/delta T lymphocytes which prevent CD4$^+$ activity," *Microbial Pathogenesis*, vol. 13, no. 6, pp. 447–463, 1992.

[50] R. J. Chiodini and W. C. Davis, "The cellular immunology of bovine paratuberculosis: immunity may be regulated by CD4$^+$ helper and CD8$^+$ immunoregulatory T lymphocytes which down-regulate gamma/delta T-cell cytotoxicity," *Microbial Pathogenesis*, vol. 14, no. 5, pp. 355–367, 1993.

[51] B. Agerberth, J. Charo, J. Werr et al., "The human antimicrobial and chemotactic peptides LL-37 and α-defensins are expressed by specific lymphocyte and monocyte populations," *Blood*, vol. 96, no. 9, pp. 3086–3093, 2000.

[52] E. Kress, J. F. Hedges, and M. A. Jutila, "Distinct gene expression in human Vδ1 and Vδ2 $\gamma\delta$ T cells following non-TCR agonist stimulation," *Molecular Immunology*, vol. 43, no. 12, pp. 2002–2011, 2006.

[53] R. L. O'Brien, C. L. Roark, N. Jin et al., "$\gamma\delta$ T-cell receptors: functional correlations," *Immunological Reviews*, vol. 215, no. 1, pp. 77–88, 2007.

[54] V. E. García, P. A. Sieling, J. Gong et al., "Single-cell cytokine analysis of $\gamma\delta$ T cell responses to nonpeptide mycobacterial antigens," *Journal of Immunology*, vol. 159, no. 3, pp. 1328–1335, 1997.

[55] L. Wang, H. Das, A. Kamath, and J. F. Bukowski, "Human Vγ2Vδ2 T cells produce IFN-γ and TNF-α with an on/off/on cycling pattern in response to live bacterial products," *Journal of Immunology*, vol. 167, no. 11, pp. 6195–6201, 2001.

[56] D. Wesch, A. Glatzel, and D. Kabelitz, "Differentiation of resting human peripheral blood $\gamma\delta$ T cells toward Th1- or Th2-phenotype," *Cellular Immunology*, vol. 212, no. 2, pp. 110–117, 2001.

[57] Y.-S. Hahn, C. Taube, N. Jin et al., "Different potentials of $\gamma\delta$ T cell subsets in regulating airway responsiveness: Vγ1$^+$ cells, but not Vγ4$^+$ cells, promote airway hyperreactivity, Th2 cytokines, and airway inflammation," *Journal of Immunology*, vol. 172, no. 5, pp. 2894–2902, 2004.

[58] A. A. Kühl, N. N. Pawlowski, K. Grollich et al., "Human peripheral $\gamma\delta$ T cells possess regulatory potential," *Immunology*, vol. 128, no. 4, pp. 580–588, 2009.

[59] J. Buza, T. Kiros, A. Zerihun, I. Abraham, and G. Ameni, "Vaccination of calves with Mycobacteria bovis Bacilli Calmete Guerin (BCG) induced rapid increase in the proportion of peripheral blood $\gamma\delta$ T cells," *Veterinary Immunology & Immunopathology*, vol. 130, no. 3-4, pp. 251–255, 2009.

[60] H. E. Kennedy, M. D. Welsh, D. G. Bryson et al., "Modulation of immune responses to *Mycobacterium bovis* in cattle depleted of WC1$^+\gamma\delta$ T cells," *Infection & Immunity*, vol. 70, no. 3, pp. 1488–1500, 2002.

[61] A. N. Rogers, D. G. Vanburen, E. Hedblom, M. E. Tilahun, J. C. Telfer, and C. L. Baldwin, "Function of ruminant $\gamma\delta$ T cells is defined by WC1.1 or WC1.2 isoform expression," *Veterinary Immunology & Immunopathology*, vol. 108, no. 1-2, pp. 211–217, 2005.

[62] S. Price, M. Davies, B. Villarreal-Ramos, and J. Hope, "Differential distribution of WC1$^+\gamma\delta$ TCR$^+$ T lymphocyte subsets within lymphoid tissues of the head and respiratory tract and effects of intranasal M. bovis BCG vaccination," *Veterinary Immunology & Immunopathology*, vol. 136, no. 1-2, pp. 133–137, 2010.

[63] R. A. Smith, J. M. Kreeger, A. J. Alvarez et al., "Role of CD8$^+$ and WC-1$^+\gamma/\delta$ T cells in resistance to *Mycobacterium bovis* infection in the SCID-bo mouse," *Journal of Leukocyte Biology*, vol. 65, no. 1, pp. 28–34, 1999.

[64] W. C. Johnson, R. G. Bastos, W. C. Davis, and W. L. Goff, "Bovine WC1$^-\gamma\delta$T cells incubated with IL-15 express the natural cytotoxicity receptor CD335 (NKp46) and produce IFN-γ in response to exogenous IL-12 and IL-18," *Developmental & Comparative Immunology*, vol. 32, no. 8, pp. 1002–1010, 2008.

[65] P. Miossec, T. Korn, and V. K. Kuchroo, "Interleukin-17 and type 17 helper T cells," *The New England Journal of Medicine*, vol. 361, no. 9, pp. 848–898, 2009.

[66] E. Lockhart, A. M. Green, and J. L. Flynn, "IL-17 production is dominated by $\gamma\delta$ T cells rather than CD4 T cells during Mycobacterium tuberculosis infection," *Journal of Immunology*, vol. 177, no. 7, pp. 4662–4669, 2006.

[67] D. J. Cua and C. M. Tato, "Innate IL-17-producing cells: the sentinels of the immune system," *Nature Reviews Immunology*, vol. 10, no. 7, pp. 479–489, 2010.

[68] C. E. Sutton, S. J. Lalor, C. M. Sweeney, C. F. Brereton, ED. C. Lavelle, and K. H. G. Mills, "Interleukin-1 and IL-23 induce innate IL-17 production from $\gamma\delta$ T cells, amplifying Th17 responses and autoimmunity," *Immunity*, vol. 31, no. 2, pp. 331–341, 2009.

[69] Y. Okamoto Yoshida, M. Umemura, A. Yahagi et al., "Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung," *Journal of Immunology*, vol. 184, no. 8, pp. 4414–4422, 2010.

[70] K. J. Ness-Schwickerath, C. Jin, and C. T. Morita, "Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vgamma2Vdelta2 T cells," *Journal of Immunology*, vol. 184, no. 12, pp. 7268–7280, 2010.

[71] K. Kubota, "Innate IFN-γ production by subsets of natural killer cells, natural killer T cells and $\gamma\delta$ T cells in response to dying bacterial-infected macrophages," *Scandinavian Journal of Immunology*, vol. 71, no. 3, pp. 199–209, 2010.

[72] C. Münz, R. M. Steinman, and S. I. Fujii, "Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity," *Journal of Experimental Medicine*, vol. 202, no. 2, pp. 203–207, 2005.

[73] G. Shankar, L. A. Pestano, and M. L. Bosch, "Interferon-γ added during Bacillus Calmette-Guerin induced dendritic cell maturation stimulates potent T1 immune responses," *Journal of Translational Medicine*, vol. 1, article 7, 2003.

[74] L. Conti, R. Casetti, M. Cardone et al., "Reciprocal activating interaction between dendritic cells and pamidronate-stimulated $\gamma\delta$ T cells: role of CD86 and inflammatory cytokines," *Journal of Immunology*, vol. 174, no. 1, pp. 252–260, 2005.

[75] D. S. Leslie, M. S. Vincent, F. M. Spada et al., "CD1-mediated γ/δ T cell maturation of dendritic cells," *Journal of Experimental Medicine*, vol. 196, no. 12, pp. 1575–1584, 2002.

[76] J. Ismaili, V. Olislagers, R. Poupot, J. J. Fournié, and M. Goldman, "Human $\gamma\delta$ T cells induce dendritic cell maturation," *Clinical Immunology*, vol. 103, no. 3 I, pp. 296–302, 2002.

[77] A. Martino, R. Casetti, A. D'Alessandri, A. Sacchi, and F. Poccia, "Complementary function of $\gamma\delta$ T-lymphocytes and dendritic cells in the response to isopentenyl-pyrophosphate and lipopolysaccharide antigens," *Journal of Clinical Immunology*, vol. 25, no. 3, pp. 230–237, 2005.

[78] M.-C. Devilder, S. Maillet, I. Bouyge-Moreau, E. Bonnadieu, M. Bonneville, and E. Scotet, "Potentiation of antigen-stimulated Vγ9Vδ2 T cell cytokine production by immature dendritic cells (DC) and reciprocal effect on DC maturation," *Journal of Immunology*, vol. 176, no. 3, pp. 1386–1393, 2006.

[79] F. Dieli, N. Caccamo, S. Meraviglia et al., "Reciprocal stimulation of $\gamma\delta$ T cells and dendritic cells during the anti-mycobacterial immune response," *European Journal of Immunology*, vol. 34, no. 11, pp. 3227–3235, 2004.

[80] R. A. Collins, P. Sopp, K. I. Gelder, W. I. Morrison, and C. J. Howard, "Bovine γ/δ TcR$^+$ T lymphocytes are stimulated to proliferate by autologous Theileria annulata-infected cells in the presence of interleukin-2," *Scandinavian Journal of Immunology*, vol. 44, no. 5, pp. 444–452, 1996.

[81] S. J. Price and J. C. Hope, "Enhanced secretion of interferon-γ by bovine $\gamma\delta$ T cells induced by coculture with *Mycobacterium bovis*-infected dendritic cells: evidence for reciprocal activating signals," *Immunology*, vol. 126, no. 2, pp. 201–208, 2009.

[82] R. A. Collins, D. Werling, S. E. Duggan, A. P. Bland, K. R. Parsons, and C. J. Howard, "$\gamma\delta$ T cells present antigen to CD4$^+\alpha\beta$ T cells," *Journal of Leukocyte Biology*, vol. 63, no. 6, pp. 707–714, 1998.

[83] H. H. Takamatsu, M. S. Denyer, and T. E. Wileman, "A sub-population of circulating porcine $\gamma\delta$ T cells can act as professional antigen presenting cells," *Veterinary Immunology & Immunopathology*, vol. 87, no. 3-4, pp. 223–224, 2002.

[84] M. Brandes, K. Willimann, and B. Moser, "Immunology: professional antigen-presentation function by human $\gamma\delta$ cells," *Science*, vol. 309, no. 5732, pp. 264–268, 2005.

[85] M. Brandes, K. Willimann, G. Bioley et al., "Cross-presenting human $\gamma\delta$ T cells induce robust CD8$^+\alpha\beta$ T cell responses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 7, pp. 2307–2312, 2009.

[86] L. Cheng, Y. Cui, H. Shao et al., "Mouse $\gamma\delta$ T cells are capable of expressing MHC class II molecules, and of functioning as antigen-presenting cells," *Journal of Neuroimmunology*, vol. 203, no. 1, pp. 3–11, 2008.

[87] K. Uezu, K. Kawakami, K. Miyagi et al., "Accumulation of $\gamma\delta$ T cells in the lungs and their regulatory roles in Th1 response and host defense against pulmonary infection with Cryptococcus neoformans," *Journal of Immunology*, vol. 172, no. 12, pp. 7629–7634, 2004.

[88] L. R. V. Antonelli, W. O. Dutra, R. R. Oliveira et al., "Disparate immunoregulatory potentials for double-negative (CD4$^-$CD8$^-$) $\alpha\beta$ and $\gamma\delta$ T cells from human patients with cutaneous leishmaniasis," *Infection & Immunity*, vol. 74, no. 11, pp. 6317–6323, 2006.

[89] N. Kang, L. Tang, X. Li et al., "Identification and characterization of Foxp3$^+\gamma\delta$ T cells in mouse and human," *Immunology Letters*, vol. 125, no. 2, pp. 105–113, 2009.

[90] J. F. Hedges, D. Cockrell, L. Jackiw, N. Meissner, and M. A. Jutila, "Differential mRNA expression in circulating $\gamma\delta$ T lymphocyte subsets defines unique tissue-specific functions," *Journal of Leukocyte Biology*, vol. 73, no. 2, pp. 306–314, 2003.

[91] N. Meissner, J. Radke, J. F. Hedges et al., "Serial analysis of gene expression in circulating $\gamma\delta$ T cell subsets defines distinct immunoregulatory phenotypes and unexpected gene expression profiles," *Journal of Immunology*, vol. 170, no. 1, pp. 356–364, 2003.

[92] A. Hoek, V. P. M. G. Rutten, J. Kool et al., "Subpopulations of bovine WC1$^+\gamma\delta$ T cells rather than CD4$^+$CD25^{high}Foxp3$^+$ T cells act as immune regulatory cells ex vivo," *Veterinary Research*, vol. 40, no. 1, 2009.

[93] Y. Shen, D. Zhou, L. Qiu et al., "Adaptive immune response of Vγ2Vδ2$^+$ T cells during mycobacterial infections," *Science*, vol. 295, no. 5563, pp. 2255–2258, 2002.

[94] F. Dieli, F. Poccia, M. Lipp et al., "Differentiation of effector/memory Vδ2 T cells and migratory routes in lymph nodes or inflammatory sites," *Journal of Experimental Medicine*, vol. 198, no. 3, pp. 391–397, 2003.

[95] W. R. Waters, M. V. Palmer, T. C. Thacker et al., "Tuberculosis immunity: opportunities from studies with cattle," *Clinical and Developmental Immunology*, vol. 2011, Article ID 768542, 11 pages, 2011.

[96] J. J. Endsley, W. R. Waters, M. V. Palmer et al., "The calf model of immunity for development of a vaccine against tuberculosis," *Veterinary Immunology & Immunopathology*, vol. 128, no. 1–3, pp. 199–204, 2009.

Research Article

The First Report of *Mycobacterium celatum* Isolation from Domestic Pig (*Sus scrofa domestica*) and Roe Deer (*Capreolus capreolus*) and an Overview of Human Infections in Slovenia

Mateja Pate,[1] Manca Žolnir-Dovč,[2] Darja Kušar,[1] Brane Krt,[1] Silvio Špičić,[3] Željko Cvetnić,[3] and Matjaž Ocepek[1]

[1] *Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1115 Ljubljana, Slovenia*

[2] *National Reference Laboratory for Mycobacteria, University Clinic for Respiratory and Allergic Diseases Golnik, Golnik 36, 4204 Golnik, Slovenia*

[3] *Laboratory for Bacterial Zoonoses and Molecular Diagnostics of Bacterial Diseases, Croatian Veterinary Institute, Savska 143, 10000 Zagreb, Croatia*

Correspondence should be addressed to Mateja Pate, mateja.pate@vf.uni-lj.si

Mycobacterium celatum, a slowly growing potentially pathogenic mycobacterium first described in humans, is regarded as an uncommon cause of human infection, though capable of inducing invasive disease in immunocompromised hosts. According to some reports, a serious disease due to *M. celatum* may also occur in individuals with no apparent immunodeficiency. In animals, an *M. celatum*-related disease has been described in three cases only: twice in a domestic ferret (*Mustela putorius furo*) and once in a white-tailed trogon (*Trogon viridis*). In this paper, we report the first detection of *M. celatum* in a domestic pig (*Sus scrofa domestica*) and roe deer (*Capreolus capreolus*). A nation-wide overview of human *M. celatum* infections recorded in Slovenia between 2000 and 2010 is also given. Pulmonary disease due to *M. celatum* was recognized in one patient with a history of a preexisting lung disease.

1. Introduction

M. celatum, a slowly growing potentially pathogenic mycobacterium, was first described in humans in 1993 [1]. Its biochemical characteristics and colony morphology are similar to those of *M. avium* complex, *M. xenopi*, *M. malmoense* and *M. shimoidei* [1]. Three types of *M. celatum* have been identified on the basis of *16S rDNA* sequence heterogeneity [1, 2], and the possibility of having two different *16S rRNA* genes within the genome has been indicated [3]. Diagnostics of *M. celatum* can be challenging as the organism can be misidentified as *M. tuberculosis* due to false positive nucleic acid probe test results [4–6]. Unambiguous identification is, therefore, provided by mycolic acid high-performance liquid chromatography or nucleic acid sequencing [1, 2].

M. celatum is an uncommon cause of human infection, but it may induce invasive disease in immunocompromised hosts. It has been reported to cause infection mainly in persons affected by *AIDS* [7–11]. However, a growing list of reports indicates that a serious disease due to *M. celatum* may occur also in non-*HIV*-infected patients [6, 12–18].

Up to date, only three cases of *M. celatum* infection have been reported in animals: two in a domestic ferret (*Mustela putorius furo*) [19, 20] and one in a white-tailed trogon (*Trogon viridis*) [21].

In the present work, we report the first detection of *M. celatum* in a domestic pig (*Sus scrofa domestica*) and roe deer (*Capreolus capreolus*). In addition, a nation-wide overview of human *M. celatum* infections recorded in Slovenia between 2000 and 2010 is given.

2. Materials and Methods

2.1. Animal Isolates. The first isolate was obtained in 2000 from a pooled specimen of lymph nodes of pigs ($n = 30$). Granulomatous changes on the submandibular lymph nodes were visible during routine tuberculosis monitoring at slaughterhouse. The second isolate was obtained in 2010 from a roe deer in the scope of a study on tuberculosis in wild animals. No gross lesions characteristic of tuberculosis were observed on the specimens collected from roe deer (Table 1).

2.2. Human Isolates. A total of 21 *M. celatum* isolates from seven patients (two males, five females) were recorded in Slovenia in the period from 2000 to 2010. *HIV* status of the affected persons was unknown except for one patient who was confirmed to be *HIV*-negative (Table 2(b)). According to the low prevalence of *AIDS* (<0.1% in 2009) [23] in Slovenia and the age of the patients, *HIV* infection was regarded as unlikely for the majority of them. All the isolates were collected and identified at the National Reference Laboratory for Mycobacteria of the University Clinic Golnik in the scope of routine diagnostics of TB and diseases caused by nontuberculous mycobacteria in humans. With the exception of one, all the isolates were cultured from sputum specimens. An overview of the human isolates included in this study is shown in Table 2(b).

2.3. Histopathology. Specimen of pig lymph nodes was subjected to Ziehl-Neelsen (ZN) and hematoxylin-eosin staining for the histopathological examination.

2.4. Bacteriology and Identification (Animal Isolates). Specimen smears were ZN stained and examined microscopically for the presence of acid-fast bacilli (AFB). Specimens of animal tissues were processed according to the protocol described by Kent and Kubica [24] and inoculated onto the following selective media: Löwenstein-Jensen (one slant supplemented with pyruvate and one slant supplemented with glycerin), Stonebrink, Middlebrook 7H10 and BBL MGIT (Mycobacteria Growth Indicator Tube, Becton Dickinson, USA). Smears of the grown bacteria were ZN stained to confirm that they comprised AFB. Identification of the isolates was based on a combination of phenotypic characteristics and molecular methods. Growth rate, growth at different temperatures, and colony morphology were recorded. According to the phenotypic features, the isolates were subjected to PCR amplification targeting the insertion sequences characteristic of *M. avium*. DNA extracted from the selected colonies was subjected to IS*1245* and FR*300* PCR amplification employing primer sets and protocols described previously [25, 26]. In addition, a hybridization assay using GenoType Mycobacterium CM and AS culture identification kits (Hain Lifescience, Germany) was performed according to the manufacturer's instructions. The pig isolate was subjected to *16S rDNA* sequencing at the National Reference Center for Mycobacteria of the Research Center Borstel in Germany. To determine the phylogenetic affiliation of the roe deer isolate, PCR using mycobacteria-specific primer pair 285/264 targeting *16S rRNA* gene [27] was performed. Ribosomal PCR amplicons were purified from agarose gels after the electrophoretic separation using PureLink Quick Gel Extraction Kit (Invitrogen, USA) following the manufacturer's instructions and were sequenced in both directions (Macrogen, Korea). The retrieved sequence fragments were submitted to megablast classification accessed through NCBI website (http://www.ncbi.nlm.nih.gov/).

2.5. Bacteriology and Identification (Human Isolates). Specimens from humans were auramine stained and examined by fluorescent microscope for AFB. All specimens were processed according to the protocol described by Vestal [28] and inoculated onto two solid media (Löwenstein-Jensen and Stonebrink) and into BACTEC MGIT 960 liquid medium (Becton Dickinson, USA). The following phenotypic characteristics were investigated: growth rate, growth at different temperatures, microscopic examination of colony morphology on Middlebrook 7H10 agar plates, and biochemical activity. Biochemical tests performed are summarized in Table 3. Identification with the GenoType Mycobacterium CM and AS culture identification kits (Hain Lifescience, Germany) was also performed, following the manufacturer's instructions. Details on the identification tests used in human isolates are given in Table 2(b).

3. Results

3.1. Pig Isolate. Bacterial growth appeared on three of the five inoculated media (Table 1). Growth was first detected after 19 days in liquid medium; nine days later, smooth filamentous (Figure 1) yellow pigmented colonies appeared on solid medium. PCR tests for *M. avium* gave negative results. Initially, the isolate was identified as a member of the genus *Mycobacterium* as diagnostic tests employed in our laboratory at the time of investigation did not enable identification of various nontuberculous mycobacteria to the species level. In 2003, GenoType Mycobacterium culture identification kit was introduced into laboratory routine work as a tool for improved identification of mycobacteria. As a result of retrospective investigation, the pig isolate was identified as *M. celatum* and was sent to the National Reference Center for Mycobacteria of the Research Center Borstel, Germany, for DNA sequencing which confirmed the isolate's identity (data on type were not available). Histopathological examination of the pig lymph node specimen revealed ZN negative partially incalcinated incapsulated granulomas.

3.2. Roe Deer Isolate. Bacterial growth occurred after 34 days on three media inoculated with the lymph node material. PCR tests for *M. avium* were negative. Using GenoType Mycobacterium CM and AS culture identification kits and DNA sequencing, the isolate was identified as *M. celatum*. Sequencing showed a duplication of electropherogram peaks after the reported insertion of additional T in position 214 [3]. Clear sequencing patterns were obtained after manual correction that revealed the presence of two different

TABLE 1: Details on diagnostics of *M. celatum* isolates from animals.

Animal	Year	Specimen	Visible lesions	Smear microscopy[2]	Growth on media	Growth time (days)	PCR	GenoType[7]	16S rDNA sequencing
Pig	2000	Submandibular lymph nodes[1]	Yes	Neg[3]	LJ-G[4], S[5], MGIT[6]	19 (MGIT) 28 (LJ-G) 34 (S)	IS1245 Neg FR300 Neg	*M. celatum*	*M. celatum*[8]
Roe deer	2010	Lymph node	No	Neg	LJ-G, S, MGIT	34 (LJ-G, S, MGIT)	IS1245 Neg	*M. celatum*	*M. celatum* type 1 and 3
		Liver	No	Neg	Neg	—	Neg	—	—

[1]Pooled specimen from 30 animals.
[2]Ziehl-Neelsen staining.
[3]Negative.
[4]Löwenstein-Jensen medium supplemented with glycerol.
[5]Stonebrink medium.
[6]Mycobacteria Growth Indicator Tube (Becton Dickinson, USA), liquid medium.
[7]GenoType Mycobacterium CM and AS culture identification kits (Hain Lifescience, Germany).
[8]Identification provided by the National Reference Center for Mycobacteria, Research Center Borstel, Germany.

TABLE 2

(a) An overview of laboratory diagnostics of mycobacteria in humans in Slovenia between 2000 and 2010

Year	No. of specimens	*M. tuberculosis*	*M. bovis*	*M. caprae*	*M. bovis* BCG	NTM[a]	*M. celatum*
2000	17016	1022	0	0	0	180	0
2001	17576	1298	0	1	0	182	0
2002	16062	1178	0	0	2	170	2
2003	15236	1132	0	0	1	213	2
2004	12268	972	0	0	3	136	0
2005	11556	923	0	0	0	211	1
2006	11753	852	0	1	2	207	2
2007	11995	874	0	2	0	182	2
2008	11566	889	0	0	0	178	5
2009	11855	817	0	0	1	299	2
2010[b]	12118	na[c]	na[c]	na[c]	na[c]	na[c]	5
Total[b]	149001	na[c]	na[c]	na[c]	na[c]	na[c]	21

[a]Nontuberculous mycobacteria.
[b]Laboratory diagnostics of specimens received in 2010 are still ongoing; as the final reports are not yet available at the time of writing, only the number of investigated specimens is given.
[c]Not available yet.

(b) Details on diagnostics of *M. celatum* from humans

Patient	Gender	Year of birth	No. of isolates	Year of isolation[1]	Sample	Microscopy[2]	Growth on media	Identification	Treatment
A	Female	1912	1	2002	Sputum	−[3]	LJ[6]	CM[10], BC[11], GT[12]	No
B	Female	1947	1	2002	Sputum	−	MGIT[7]	CM, BC, GT	No
C*	Female	1929	2	2003	Sputum	−	MGIT	CM, BC, GT	No
D	Male	1959	1	2005	Sputum	−	MGIT	CM, BC, GT	No
C*	Female	1929	2	2006	Sputum	−	MGIT	CM, GT	No
C*	Female	1929	2	2007	Sputum	±[4]	LJ, ST[8], MGIT	CM, GT	No
C*	Female	1929	5	2008	Sputum	±[5]	LJ, ST, MGIT	CM, GT	Yes
E	Female	1932	1	2009	Bronchial aspirate	−	Banič liquid medium[9]	CM, GT	No
F	Male	1971	1	2009	Sputum	−	LJ, MGIT	CM, GT	No
C*	Female	1929	4	2010	Sputum	−	LJ, ST, MGIT	CM, GT	Yes
G	Female	1974	1	2010	Sputum	−	MGIT	CM, GT	No

*Patient with *M. celatum* pulmonary disease and a history of tuberculosis and *M. avium* and *M. intracellulare*-related pulmonary mycobacteriosis; confirmed as *HIV*-negative.
[1]No *M. celatum* isolates were identified in years 2000, 2001, and 2004.
[2]Auramin fluorescent staining.
[3]Negative.
[4]One specimen positive and one specimen negative for acid-fast bacilli.
[5]Four specimens positive and one specimen negative for acid-fast bacilli.
[6]Löwenstein-Jensen medium.
[7]Mycobacteria Growth Indicator Tube (Becton Dickinson, USA), liquid medium.
[8]Stonebrink medium.
[9]In-house liquid medium containing human serum (described in [22]).
[10]Colony morphology.
[11]Biochemical tests (for details, see Table 3).
[12]GenoType Mycobacterium CM and AS culture identification kits (Hain Lifescience, Germany).

16S rRNA gene copies (i.e., type 1 and type 3) in the genome of *M. celatum* (Table 1).

3.3. *Human Isolates.* Auramine staining generated positive results in five out of 21 specimens from which *M. celatum* was later cultivated. All smear positive specimens belonged to one patient with pulmonary disease due to *M. celatum*. The vast majority of isolates grew in liquid medium; in eight out of 21 cases, the liquid medium was the only medium in which bacterial growth was detected. Colonies that appeared

TABLE 3: Results of biochemical tests performed on human *M. celatum* isolates collected between 2002 and 2005[1].

Test	Result
Semiquantitative catalase 37°C	<45 mm of foam
Catalase 68°C	+
Tween hydrolysis (5 days/10 days)	−/−
Arylsulfatase (3 days/14 days)	+/+
Tellurite reduction	+
Iron uptake	−
Tolerance to 5% NaCl	−
Tolerance to TCH[2] (10 μg/mL)	+
Growth on MacConkey agar	−
Growth at 25°C	+
Growth at 30°C	+
Growth at 37°C	+
Growth at 45°C	−
Growth rate	Slow
Colony morphology (Middlebrook 7H10 agar)	Smooth

[1] From the beginning of 2006, identification of the isolates did not include biochemical tests any more. Five *M. celatum* isolates from the period 2002–2005, on which biochemical tests were performed, displayed uniform biochemical characteristics shown in this table.
[2] TCH: thiophene 2-carboxylic hydrazide.
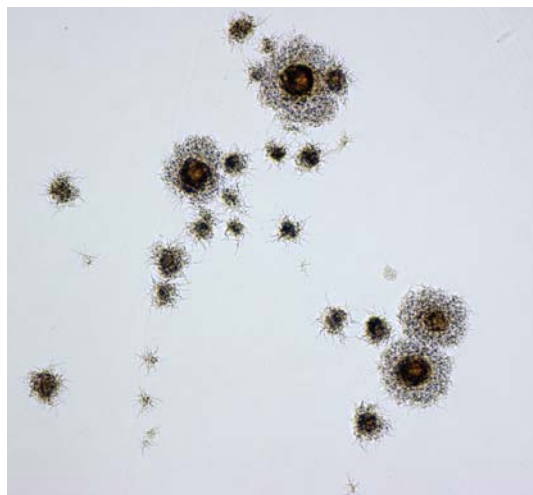
FIGURE 1: Colony morphology of pig *M. celatum* isolate grown on Middlebrook 7H10 agar plates and viewed under the microscope (×100).

on solid media were small and smooth. Until the end of 2003, the identification was based on phenotypic methods (colony morphology under stereomicroscope, biochemical tests). However, they were not discriminative enough and the isolates were identified as nonchromogenic mycobacteria. Biochemical features of the investigated isolates are shown in Table 3 and are consistent with the characteristics listed in the first description of the species [1]. GenoType Mycobacterium CM and AS culture identification kits were introduced into routine work in 2004 and have been used since for the identification of all investigated mycobacterial isolates to

the species level. The isolates of unknown mycobacterial species cultured between 2000 and 2003 were identified in retrospective. The results of bacteriological and molecular investigations of the human isolates are summarized in Table 2(b).

4. Discussion

In the first reports on *M. celatum* in animals, the organism was identified as the aetiological agent of disseminated granulomatous infections. The findings related to the infection ranged from classical tuberculous lesions with well-defined granulomas in the white-tailed trogon [21] to poorly defined granulomas described in the ferret [19]. The lesions present in the trogon were identical as those seen in classical avian mycobacteriosis caused by *M. avium* [21]. The route of infection was not determined although the lesions in the respiratory tract indicated transmission by inhalation [19, 21]. The source of infection was also unknown; however, as most mycobacteria are ubiquitous environmental saprophytes [29], exposure to soil or water seems to be the most probable source. The presence of *M. celatum* in the environment has been demonstrated by its isolation from a biofilm in an aquarium [30] and by the detection of *M. celatum*-like DNA sequence from the soil [31]. The last paper on *M. celatum* infection in animals concerns splenitis in a ferret. Common occurrence of *M. celatum* in ferrets could be explained by the supposition that they are more susceptible to mycobacterial infections as many mycobacteria, for example, *M. bovis*, *M. microti*, *M. avium*, *M. triplex*, *M. fortuitum*, *M. florentinum*, *M. interjectum*, and *M. intracellulare*, have been isolated from this animal species [32–34].

In the animal specimens investigated in this study, no AFB were detected by ZN staining of the tissue smears. Previous papers on *M. celatum* infection in animals report positive smear microscopy, but low numbers of AFB were recorded in some instances [20, 21]. The growth times of the bacteria from pig and roe deer were in between the growth times reported previously. Bacterial growth from the pig lymph node specimen was first detected after 19 days of incubation in liquid medium. Smooth filamentous and yellow pigmented colonies appeared after 28 days on one of the solid media. Bacterial growth from the roe deer lymph node specimen was detected in liquid and on two solid media after 34 days. For comparison, in a ferret with disseminated *M. celatum* infection, yellow colonies were visible after ten days [19] while small smooth and pale colonies from the ferret with splenitis appeared after eight weeks [20]. *M. celatum* types revealed in animals comprise type 1 in the trogon [21] and type 3 in the ferret with disseminated disease [19] while the type identified in the ferret with splenitis demonstrated close relatedness to the human *M. celatum* strains types 1 and 3 [20]. Our isolate from roe deer harbored two different *16S rRNA* gene copies, that is, of types 1 and 3.

The significance of *M. celatum* infection in the animals described in this paper is unclear. The presence and the location of TB-like lesions indicate that *M. celatum* is a potentially pathogenic organism for pigs which might

get infected *per os*. The granulomatous changes resembled those seen in pigs infected with *M. bovis* or *M. avium*. Therefore, *M. celatum* infection might be important from the standpoint of making correct diagnosis. In roe deer specimens, there were no gross pathological changes seen on liver and the lymph nodes; histopathological examination was not performed. The finding of *M. celatum* in the lymph node tissue could be a result of ubiquitous nature of the organism, which is likely to be widespread in the environment. In both animal species, the isolation of *M. celatum* was a rare event. Among 152 mycobacteria isolated from pigs between 2000 and 2010, the majority (92.1%) belonged to *M. avium*. A few isolates of *Mycobacterium* sp., *M. terrae* and *M. fortuitum* were also detected. *M. celatum* represented 0.67% of isolates. A more detailed overview of mycobacteria in animals in Slovenia was published recently [35]. The mycobacteria detection rate in wild animals was lower. The national study on tuberculosis in wild animals started in 2010. During this year, a total of 108 animals of various species were sampled and eight isolates of nontuberculous mycobacteria were detected. Two out of 31 roe deer were positive for mycobacteria; one isolate was identified as *M. celatum* and another as *Mycobacterium* sp.

M. celatum has been mainly recognized as the causative agent of pulmonary and disseminated infections in immunocompromised humans [16]. It may affect also immunocompetent patients [6, 12, 16]; however, some authors discussed the possibility that the host's defense failure associated with *M. celatum* infection suggests a possible hidden immunodeficiency rather than a true immunocompetence [14]. The review of the literature concerning *M. celatum* infection in non-*HIV*-infected patients revealed eight reports from seven countries in the past 16 years [6, 12–18]. Among nine cases of human *M. celatum* infection, three patients had a history of pulmonary TB [14, 17, 18], one of TB lymphadenitis and chronic obstructive pulmonary disease [15], one of diabetes mellitus [13], and one was a lung transplant recipient [16] while for three patients no underlying disease was recorded [6, 12, 16]. In all patients, *M. celatum* caused pulmonary infection with the exception of two lymphadenitis cases in children [12, 16]. Outcome of antibiotic treatment was favorable for five out of seven adult patients while in children a surgery (in one case supported by antibiotic treatment) resulted in regression of illness [17]. Smear microscopy was mostly positive for AFB. The methods used for the identification of the isolates involved phenotypic and molecular methods; definitive diagnosis was provided by *16S rDNA* sequencing in the majority of cases.

During an eleven-year period, a total of 21 *M. celatum* isolates belonging to seven patients were discovered among 149,000 specimens from humans investigated in Slovenia (Table 2(a)). *M. celatum* represented about 0.82% of isolates of nontuberculous mycobacteria identified between 2000 and 2010. With the exception of one isolate from bronchial aspirate, *M. celatum* isolates were cultivated from sputum. Smear microscopy (fluorescent auramine staining) was positive only in five sputa from one patient with pulmonary *M. celatum* disease. The remaining specimens were negative for AFB.

The occurrence of *M. celatum* in five patients, in which single sputum specimens were investigated, may be explained by the presence of this organism in the environment. The finding of these isolates could be regarded as random with no particular significance. In one patient, *M. celatum* was isolated from bronchial aspirate. Since bronchi are considered as a usually sterile site, the isolation of *M. celatum* from such a site could indicate the clinical importance of the infection. However, no further specimens of this 77-year-old patient were investigated. In order to meet the criteria for diagnosis of nontuberculous mycobacteria pulmonary disease, published by American Thoracic Society [36], several specimens should be collected within one year and evaluation of clinical and radiographic findings should be performed along with the results of bacteriology.

Therefore, among *M. celatum* isolates recovered from humans in Slovenia up to date, *M. celatum* infection was of clinical significance only in one elderly patient (born in 1929) with a history of tuberculosis (1952–1954, treated by thoracoplasty). In 2002–2003 and 2005–2006, this patient was treated also for *M. avium*-related and *M. intracellulare*-related pulmonary disease, respectively. The repeated isolation of *M. celatum* from 15 sputum specimens (among them, five smear positive) in five years confirms the clinical importance of the infection in this patient. The patient was first subjected to antibiotic therapy at the end of 2008. The outcome of the therapy is unknown as no specimens were received after the beginning of treatment course. However, the four *M. celatum* isolates obtained in 2010 and repeated therapy suggest recurrence of pulmonary infection. This case demonstrates that a preexisting lung disease represents an important risk factor for *M. celatum* pulmonary infection. This can be concluded also from the cases reported previously as the description of underlying diseases in *M. celatum*-infected patients often involved lung diseases, most frequently TB [17].

In conclusion, the finding of *M. celatum* in animals and humans suggests its presence in the environment. Its ability to induce TB-like lesions in pig indicates that the organism is potentially pathogenic for this animal species. In addition, *M. celatum* was recognized as the etiological agent of pulmonary infection in a patient with a history of a preexisting lung disease.

Acknowledgment

References

[1] W. R. Butler, S. P. O'Connor, M. A. Yakrus et al., "*Mycobacterium celatum* sp. nov.," *International Journal of Systematic Bacteriology*, vol. 43, pp. 539–548, 1993, Erratum in: *International Journal of Systematic Bacteriology*, vol. 43, pp. 868, 1993.

[2] T. J. Bull, D. C. Shanson, L. C. Archard, M. D. Yates, M. E. Hamid, and D. E. Minnikin, "A new group (type 3) of *Mycobacterium celatum* isolated from AIDS patients in the

London area," *International Journal of Systematic Bacteriology*, vol. 45, no. 4, pp. 861–862, 1995.

[3] U. Reischl, K. Feldmann, L. Naumann et al., "16s rRNA sequence diversity in *Mycobacterium celatum* strains caused by presence of two different copies of 16S rRNA gene," *Journal of Clinical Microbiology*, vol. 36, no. 6, pp. 1761–1764, 1998.

[4] W. R. Butler, S. P. O'Connor, M. A. Yakrus, and W. M. Gross, "Cross-reactivity of genetic probe for detection of *Mycobacterium tuberculosis* with newly described species *Mycobacterium celatum*," *Journal of Clinical Microbiology*, vol. 32, no. 2, pp. 536–538, 1994.

[5] Á. Somoskövi, J. E. Hotaling, M. Fitzgerald et al., "False-positive results for *Mycobacterium celatum* with the AccuProbe *Mycobacterium tuberculosis* complex assay," *Journal of Clinical Microbiology*, vol. 38, no. 7, pp. 2743–2745, 2000.

[6] J. H. T. Tjhie, A. F. Van Belle, M. Dessens-Kroon, and D. Van Soolingen, "Misidentification and diagnostic delay caused by a false-positive amplified *Mycobacterium tuberculosis* direct test in an immunocompetent patient with a *Mycobacterium celatum* infection," *Journal of Clinical Microbiology*, vol. 39, no. 6, pp. 2311–2312, 2001.

[7] E. Tortoli, C. Piersimoni, D. Bacosi et al., "Isolation of the newly described species *Mycobacterium celatum* from AIDS patients," *Journal of Clinical Microbiology*, vol. 33, no. 1, pp. 137–140, 1995.

[8] C. Piersimoni, E. Tortoli, F. De Lalla et al., "Isolation of *Mycobacterium celatum* from patients infected with human immunodeficiency virus," *Clinical Infectious Diseases*, vol. 24, no. 2, pp. 144–147, 1997.

[9] F. Garcia-Garrote, M. J. Ruiz-Serrano, J. Cosin, L. Alcala, A. Ortega, and E. Bouza, "*Mycrobacterium celatum* as a cause of disseminated infection in an AIDS patient," *Clinical Microbiology and Infection*, vol. 3, no. 5, pp. 582–584, 1997.

[10] R. A. Bonomo, J. M. Briggs, W. Gross et al., "*Mycobacterium celatum* infection in a patient with AIDS," *Clinical Infectious Diseases*, vol. 26, no. 1, pp. 243–245, 1998.

[11] S. Shahdad, M. Kaur, S. Singh, and S. Khattar, "A case of pulmonary *Mycobacterium celatum* in an Indian AIDS patient," *International Journal of Infectious Diseases*, vol. 9, no. 1, pp. 62–63, 2005.

[12] G. Haase, H. Skopnik, S. Batge, and E. C. Bottger, "Cervical lymphadenitis caused by *Mycobacterium celatum*," *Lancet*, vol. 344, no. 8928, pp. 1020–1021, 1994.

[13] I. Bux-Gewehr, H. P. Hagen, S. Rusch-Gerdes, and G. E. Feurle, "Fatal pulmonary infection with *Mycobacterium celatum* in an apparently immunocompetent patient," *Journal of Clinical Microbiology*, vol. 36, no. 2, pp. 587–588, 1998.

[14] C. Piersimoni, P. G. Zitti, D. Nista, and S. Bornigia, "*Mycobacterium celatum* pulmonary infection in the immunocompetent: case report and review," *Emerging Infectious Diseases*, vol. 9, no. 3, pp. 399–402, 2003.

[15] R. McMullan, J. Xu, T. Stanley et al., "*Mycobacterium celatum* pulmonary infection," *Ulster Medical Journal*, vol. 72, no. 2, pp. 114–116, 2003.

[16] D. C. Christiansen, G. D. Roberts, and R. Patel, "*Mycobacterium celatum*, an emerging pathogen and cause of false positive amplified *Mycobacterium tuberculosis* direct test," *Diagnostic Microbiology and Infectious Disease*, vol. 49, no. 1, pp. 19–24, 2004.

[17] C. K. Tan, C. C. Lai, C. H. Chou, and P. R. Hsueh, "*Mycobacterium celatum* pulmonary infection mimicking pulmonary tuberculosis in a patient with ankylosing spondylitis," *International Journal of Infectious Diseases*, vol. 13, no. 6, pp. e459–e462, 2009.

[18] H.-J. Jun, N. Y. Lee, J. Kim, and W.-J. Koh, "Successful treatment of *Mycobacterium celatum* pulmonary disease in an immunocompetent patient using antimicobacterial chemotherapy and combined pulmonary resection," *Yonsei Medical Journal*, vol. 51, no. 6, pp. 980–983, 2010.

[19] M. Valheim, B. Djønne, R. Heiene, and D. A. Caugant, "Disseminated *Mycobacterium celatum* (type 3) infection in a domestic ferret (*Mustela putorius furo*)," *Veterinary Pathology*, vol. 38, no. 4, pp. 460–463, 2001.

[20] E. Piseddu, M. Trotta, E. Tortoli, M. Avanzi, S. Tasca, and L. Solano-Gallego, "Detection and molecular characterization of *Mycobacterium celatum* as a cause of splenitis in a domestic ferret (*Mustela putorius furo*)," *Journal of Comparative Pathology*, vol. 144, no. 2-3, pp. 214–218, 2011.

[21] M. Bertelsen, C. Grøndahl, and S. Giese, "Disseminated *Mycobacterium celatum* infection in a white-tailed trogon (*Trogon viridis*)," *Avian Pathology*, vol. 35, no. 4, pp. 316–319, 2006.

[22] S. Banic, "A new liquid medium for the primary cultivation of tubercle bacilli," *Pathologia et Microbiologia*, vol. 29, no. 2, pp. 243–251, 1966.

[23] UNAIDS, "Global report: UNAIDS report on the global AIDS epidemic," 2010, http://www.unaids.org/documents/20101123_GlobalReport_em.pdf.

[24] G. P. Kent and G. P. Kubica, *Public Health Mycobacteriology. A Guide for the Level III Laboratory*, US Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta, Ga, USA, 1985.

[25] Z. M. Kunze, F. Portaels, and J. J. McFadden, "Biologically distinct subtypes of Mycobacterium avium differ in possession of insertion sequence IS901," *Journal of Clinical Microbiology*, vol. 30, no. 9, pp. 2366–2372, 1992.

[26] C. Guerrero, C. Bernasconi, D. Burki, T. Bodmer, and A. Telenti, "A novel insertion element from Mycobacterium avium, IS1245, is a specific target for analysis of strain relatedness," *Journal of Clinical Microbiology*, vol. 33, no. 2, pp. 304–307, 1995.

[27] P. Kirschner, B. Springer, U. Vogel et al., "Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory," *Journal of Clinical Microbiology*, vol. 31, no. 11, pp. 2882–2889, 1993.

[28] A. L. Vestal, *Procedures for the Isolation and Identification of Mycobacteria*, Centers for Disease Control, Atlanta, Ga, USA, 1977.

[29] E. Tortoli, "Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s," *Clinical Microbiology Reviews*, vol. 16, no. 2, pp. 319–354, 2003.

[30] V. Beran, L. Matlova, L. Dvorska, P. Svastova, and I. Pavlik, "Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment," *Journal of Fish Diseases*, vol. 29, no. 7, pp. 383–393, 2006.

[31] B. Z. Chilima, I. M. Clark, S. Floyd, P. E. M. Fine, and P. R. Hirsch, "Distribution of environmental mycobacteria in Karonga District, Northern Malawi," *Applied and Environmental Microbiology*, vol. 72, no. 4, pp. 2343–2350, 2006.

[32] J. R. Ragg, H. Moller, and K. A. Waldrup, "The prevalance of bovine tuberculosis (*Mycobacterium bovis*) infections in feral populations of cats (*Felis catus*), ferrets (*Mustela furo*) and stoats (*Mustela erminea*) in Otago and Southland, New Zealand," *New Zealand Veterinary Journal*, vol. 43, pp. 333–337, 1995.

[33] F. X. Emmanuel, A. L. Seagar, C. Doig, A. Rayner, P. Claxton, and I. Laurenson, "Human and animal infections with *Mycobacterium microti*, Scotland," *Emerging Infectious Diseases*, vol. 13, no. 12, pp. 1924–1927, 2007.

[34] G. W. de Lisle, R. P. Kawakami, G. F. Yates, and D. M. Collins, "Isolation of Mycobacterium bovis and other mycobacterial species from ferrets and stoats," *Veterinary Microbiology*, vol. 132, no. 3-4, pp. 402–407, 2008.

[35] M. Pate, D. Kusar, M. Zolnir-Dovc, and M. Ocepek, "Mycobacteria in animals in Slovenia: an overview of the last decade," in *Proceedings of the 30th Annual Congress of the European Society for Mycobacteriology*, p. 128, Porto, Portugal, 2009.

[36] American Thoracic Society, "Diagnosis and treatment of disease caused by nontuberculous mycobacteria," *American Journal of Respiratory and Critical Care Medicine*, vol. 156, pp. S1–S25, 1997.

Research Article

Bovine Tuberculosis in a Nebraska Herd of Farmed Elk and Fallow Deer: A Failure of the Tuberculin Skin Test and Opportunities for Serodiagnosis

W. Ray Waters,[1] Gary E. Stevens,[2] Mark A. Schoenbaum,[3] Kathy A. Orloski,[3] Suelee Robbe-Austerman,[4] N. Beth Harris,[4] S. Mark Hall,[4] Bruce V. Thomsen,[4] Arach J. Wilson,[4] Roger E. Brannian,[4] Jeffrey T. Nelson,[4] Shawn Schafer,[5] Javan Esfandiari,[6] Meghan Dutton,[6] Rena Greenwald,[6] and Konstantin P. Lyashchenko[6]

[1] *National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture (USDA), Ames, IA 50010, USA*

[2] *National Veterinary Services Laboratories (NVSL), Animal and Plant Health Inspection Service (APHIS), USDA, Lincoln, NE 68516, USA*

[3] *NVSL, APHIS, USDA, Fort Collins, CO 80526-8117, USA*

[4] *NVSL, APHIS, USDA, Ames, IA 50010, USA*

[5] *North American Deer Farmers Association, Lake City, MN 55041, USA*

[6] *Chembio Diagnostic Systems, Inc., Medford, NY 11763, USA*

Correspondence should be addressed to W. Ray Waters, ray.waters@ars.usda.gov

In 2009, *Mycobacterium bovis* infection was detected in a herd of 60 elk (*Cervus elaphus*) and 50 fallow deer (*Dama dama*) in Nebraska, USA. Upon depopulation of the herd, the prevalence of bovine tuberculosis (TB) was estimated at ∼71–75%, based upon histopathology and culture results. Particularly with elk, gross lesions were often severe and extensive. One year ago, the majority of the elk had been tested for TB by single cervical test (SCT), and all were negative. After initial detection of a tuberculous elk in this herd, 42 of the 59 elk were tested by SCT. Of the 42 SCT-tested elk, 28 were TB-infected with only 3/28 reacting upon SCT. After SCT, serum samples were collected from the infected elk and fallow deer from this herd at necropsy and tested by three antibody detection methods including multiantigen print immunoassay, cervidTB STAT-PAK, and dual path platform VetTB (DPP). Serologic test sensitivity ranged from 79 to 97% depending on the test format and host species. Together, these findings demonstrate the opportunities for use of serodiagnosis in the rapid detection of TB in elk and fallow deer.

1. Introduction

Farmed deer represent a significant alternative livestock industry with numbers exceeding 2 million in New Zealand, 1 million in China, 500,000 in the US, 400,000 in Russia, and 100,000 in Canada [1]. Farmed deer are exposed to various other livestock and to free-ranging wildlife and are moved between herds and across borders. Thus, there is an increased risk of the spread of infectious disease among and between farmed deer, traditional livestock, and free-ranging wildlife. In addition, many deer are kept in parks, preserves, and private estates for hunting, zoological, and aesthetic purposes. Intensive management promotes the spread of infectious diseases in these populations.

Mycobacterium bovis, a member of the *M. tuberculosis* complex, has a wide host range as compared to other species in this disease complex, is infectious to humans, and is the species most often isolated from tuberculous cattle. Free-ranging and captive deer are implicated in the spread of *M. bovis* to cattle [2–4] and to humans [5–8]. Spread of bovine TB in captive herds within the US is strongly linked to interherd and cross-country movement

of infected deer [3]. From 1991 to 2003, *M. bovis* was detected in 43 captive cervid herds [9]. The 1991 outbreak of bovine TB in captive elk (*Cervus elaphus*) encouraged the US government and cervid industry leaders to draft uniform methods and rules (UMR) for eradication of bovine TB in captive cervids, initially published in 1994 [3] and subsequently revised in 1999. More recently (2004–2010), *M. bovis* has been detected in 3 captive cervid farms in Indiana (elk and various other deer species), 4 captive white-tailed deer herds in Michigan, 1 captive herd in New York (red deer *Cervus elaphus*) and fallow deer (*Dama dama*), and 1 captive herd in Nebraska (present study) [10–12]. Additionally, molecular strain typing of *M. bovis* isolated from cattle in South Dakota, Nebraska, Indiana, and Kentucky matched the predominant strain of *M. bovis* in US captive cervids, suggesting transmission of *M. bovis* from captive cervids to cattle in these cases. Thus, TB in captive cervids continues to pose a significant health and regulatory concern, both for the captive cervid industry as well as for cattle producers.

In the US, farmed deer are primarily monitored by skin test and rarely by slaughter surveillance. The US program requires a negative skin test for interstate transport and includes a voluntary herd accreditation program; however, few farmed deer owners have participated in the latter. Potential reasons for low participation include inadequate handling facilities, injury and mortality resulting from handling events, poor perception by owners of the specificity of skin testing in cervids, and decreased interstate movement due to chronic wasting disease-associated restrictions in the US. For cervid producers, a blood-based TB test for initial surveillance may increase participation. Recent studies have demonstrated the potential of emerging antibody-based detection assays for use in cervids [13–18].

The present report describes the diagnosis of *M. bovis* infection in a captive elk and fallow deer farm with a high prevalence of disease. In particular, details on the case history, standard diagnostic techniques (i.e., slaughter surveillance, skin test, necropsy, histology, and mycobacterial culture), and emerging serologic methods are provided.

2. Materials and Methods

2.1. Herd History. The herd consisted of 50 fallow deer and 60 elk (includes 59 elk involved in the investigation and the TB index case detected in January, 2009). The elk herd was regularly tested by SCT as required by the TB herd accreditation program [19]. Fallow deer were not tested by SCT primarily due to the owner's concern of handling-associated injuries. Once the index case had been indentified, as many animals as possible (48 fallow deer and 52 elk) were examined postmortem for TB. Samples were collected for histopathology and culture only from those animals that had gross lesions suggestive of TB.

2.2. Single Cervical Skin Test (SCT). The SCT was applied according to the USDA bovine TB eradication, UMR [3, 19].

2.3. Isolation and Identification of Mycobacteria Spp. Tissues were processed for the isolation of *M. bovis* as described

previously [20] using a combination of the BACTEC 460 radiometric system, BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system (Becton Dickinson and Company, Sparks, MD, USA), and 4 tubes of solid media. Solid media included 2 tubes of 7H11 supplemented with OADC, pyruvate, calf serum, and lysed sheep blood (National Veterinary Services Laboratories, Ames, IA, USA) and 1 tube each of 7H10 supplemented with OADC, pyruvate (National Veterinary Services Laboratories, Ames, IA, USA), and Mycobactesel LJ (Becton Dickinson and Company, Sparks, MD, USA). Isolates of *M. bovis* were identified by a combination of Ziehl-Neelsen acid-fast staining, nucleic acid probes (AccuProbe, Gen-Probe, San Diego, CA, USA), and spoligotyping (Ocimum Biosolutions Ltd., Hyderabad, India). Identification of atypical *Mycobacteria spp.* was by 16S ribosomal DNA sequencing [21] and biochemical profiles. Sequences were then identified through the use of a mycobacterial species sequence database [22].

2.4. Histopathology. Formalin-fixed tissues were processed and stained with hematoxylin and eosin. Any granulomatous lesions were then stained with a modified Ziehl-Neelson procedure [23]. On the initial animals in the herd that were suspected of having TB based on histopathology, PCR for *IS 6110,* which identifies *M. tuberculosis* complex bacteria, was performed on formalin-fixed, paraffin embedded tissues. Test protocols followed previously described methods [24].

2.5. Multiantigen Print Immunoassay (MAPIA). MAPIA was performed as previously described by Lyashchenko et al. [16, 25]. The panel of *M. bovis* antigens included ESAT-6, CFP10, MPB59, MPB64, MPB70, MPB83, the 16-kDa protein (HspX), the 38-kDa protein (PhoS1/pstS), and Mtb8 (SecE2); three fusion proteins comprising ESAT-6/CFP10, the 16-kDa protein/MPB83, and F10 (F10 consists of CFP10, secE2, and PhoS1/pstS); two native antigens, *M. bovis* PPD (B-PPD) and culture filtrate (MBCF).

2.6. CervidTB STAT-PAK Test. The CervidTB STAT-PAK kit (Chembio Diagnostic Systems Inc., Medford, NY, USA) is a lateral-flow test used for rapid detection of antibodies specific to *M. tuberculosis* complex antigens, ESAT-6, CFP10, and MPB83 [14, 16]. The device consists of a plastic cassette containing a strip of nitrocellulose membrane impregnated with antigen. The assay employs blue latex microparticles coated with ESAT-6, CFP10, and MPB83. Twenty μL of serum and 3 drops of sample diluent are added sequentially to the sample pad. Results are read visually at 20 min. The presence of a test band of any intensity is considered a positive result whereas no band in the test area is considered a negative result.

2.7. Dual Path Platform (DPP) VetTB Test. The innovative DPP technology utilizes two nitrocellulose strips connected in a "T" shape inside the cassette device [26]. This allows independent delivery of the test sample and antibody-detecting reagent to the assay reaction area, in contrast to the single-strip format used in the CervidTB STAT-PAK test.

TABLE 1: History of herd and timeline of the outbreak investigation.

Action	Date	Number of animals	
		Elk	Fallow deer
Original herd size	2008	60	50
SCT performed	March 2008	50 (all negative)	Not tested
Index case detected	January 30, 2009	1	0
SCT performed[a]	February/March 2009	42/59 tested (3 true positive, 25/39 false negative*)	Not tested
Postmortem examinations[b]	June 2009	52	48
Serum collected	June 2009	34/52 examined	32/48 examined
Apparent prevalence[c]	June 2009	75% (40/53 TB positive)	71% (34/48 TB positive)

[a]Only 42/59 elk and none of the fallow deer were tested by SCT primarily due to animal handling and management issues. All of the elk that were tested by SCT were examined postmortem.

[b]Herd depopulation completed; however, not all of the carcasses were examined.

[c]The apparent prevalence includes the index case and is based upon the number of animals with mycobacteriosis compatible gross and microscopic lesions and/or *M. bovis* isolated from tissues.

*Necropsies were performed 3 months after SCT; thus, values may underestimate the true false negative rate.

The two recombinant antigens, MPB83 and CFP10/ESAT-6 fusion protein, are immobilized on the test strip as separate bands, allowing independent detection of antibody reactivity to each antigen. Five μL of sera is used in the assay, and results are read visually at 20 min. The presence of any of the two test bands was recorded as a positive result while no test band was considered a negative result.

2.8. Test Performance. The two rapid test formats (CervidTB STAT-PAK and DPP VetTB tests) were evaluated for test performance. Data are presented as % sensitivity ((TP/TP + FN) × 100), % specificity ((TN/TN + FP) × 100), and % accuracy ((TP + TN)/(TP + FP + TN + FN) × 100), where TP: true positive, TN: true negative, FP: false positive, and FN: false negative.

3. Results and Discussion

3.1. Case History, Skin Test, and Necropsy Findings. A timeline providing investigative actions and associated animal numbers is provided in Table 1. The index case was identified during routine slaughter surveillance inspection (January 30, 2009) when multiple granulomas were identified within the lung and thoracic lymph nodes of an elk carcass originating from a captive cervid farm in Knox County, Nebraska. As part of the USDA's National Bovine Tuberculosis Eradication Program, tissues were submitted to the National Veterinary Services Laboratories for histopathology and culture. Histopathology showed granulomatous pneumonia and lymphadenitis with acid-fast bacilli. PCR of the fixed tissue identified *M. tuberculosis* complex DNA on February 12, 2009, and *M. bovis* was subsequently isolated by culture. Approximately 10 months prior, 50/60 elk in this herd had been tested by SCT, and all were negative including the index case. After detection of the *M. bovis*-infected elk, 42 of the 59 remaining elk were tested by SCT in February/March 2009; 3/42 were SCT-positive, and the 3 SCT positive elk were euthanized and examined postmortem (March 31, 2009). Only 42 were tested by SCT due to animal handling and management issues. All 3 elk had gross and microscopic lesions consistent with TB in the lungs and pleura; PCR confirmed *M. tuberculosis* complex (April 3, 2009); *M. bovis* was subsequently isolated by culture in all 3 SCT-positive elk (May 15, 2009). Fallow deer ($n = 50$) in this mixed herd were not tested by SCT primarily due to handling concerns; however, as they had been comingled with *M. bovis*-infected elk, they were considered TB-exposed. The findings initiated quarantine of the herd and eventual depopulation of all elk and fallow deer on the premises.

Approximately 2 months after *M. bovis* was detected in the 3 SCT-positive elk, the remaining animals within the affected herd were euthanized either on the farm or at a local slaughterhouse. Postmortem examination was completed on 52 of the elk, 39 of which had gross lesions suggestive of TB that was further confirmed by culture and/or histopathology. The majority of elk had tuberculous lesions in multiple tissues, with lung lesions ($n = 23$, Figure 1(b)) being the most common, followed by lesions in the thoracic lymph nodes ($n = 19$), pleural lesions ($n = 11$), abdominal lymph node lesions ($n = 10$), and lesions within the head lymph nodes ($n = 8$). On gross inspection, the TB lesions appeared as 1 mm to 10 cm inflammatory masses with a yellow liquid necrotic center (Figure 1(a)) or as masses that were solid on cut surface and frequently mineralized. Within the 48 fallow deer in which postmortem examination was completed, 34 fallow deer had gross lesions suggestive of TB that were confirmed by culture and/or histopathology. The majority of fallow deer had lesions in multiple tissues, with lesions in the thoracic lymph nodes being most common ($n = 21$), followed by lesions in the abdominal lymph nodes ($n = 18$), lesions in the head lymph nodes ($n = 10$), and lung lesions ($n = 6$). The lesions were highly variable in size, and the majority had the gross appearance of an abscess. In general, gross lesions were more severe in elk as compared to fallow deer. Based on histopathology and/or culture results, the prevalence of TB was estimated at ~71–75% (fallow deer: 34/48; elk: 40/53, including the index case from January 30, 2009).

(a)   (b)

FIGURE 1: Gross lesions in elk. (b) demonstrates a tuberculous thoracic lesion that has eroded through the body wall. (a) demonstrates coalescing, multinodular, pulmonary granulomas. Orientation of both photographs: cranial to the left and dorsal to the top.
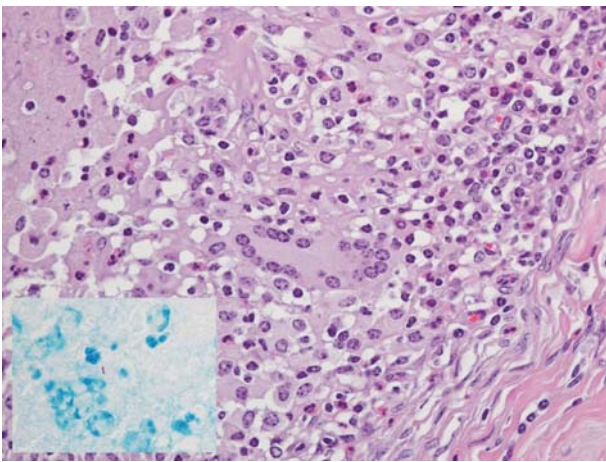

FIGURE 2: Microscopic lesions in elk. The image is a photograph of a representative pulmonary granuloma in an elk (hematoxylin and eosin stain). Note the large number of neutrophils and the single multinucleate giant cell in the center of the image. The inset image demonstrates an acid-fast bacillus (Ziehl-Neelson stain) in a pool of proteinaceous fluid, necrotic cell debris, and degenerate neutrophils.

Microscopically, the TB lesions consisted of either granulomas or pyogranulomas with rare to large numbers of acid-fast bacilli (Figure 2). The histological lesions in infected animals were quite variable between animals as previously described for TB in cervid species [27–30]. Briefly, there were three general subtypes of lesions in both the elk and the fallow deer. The first type of lesion consisted predominantly of variably sized foci containing predominantly macrophages, and epithelioid macrophages, along with low numbers of multinucleated (Langhans) giant cells. Lymphocytes and plasma cells were intermixed with small amounts of fibrous connective tissue and as small lymphocytic aggregates. These lesions were often multifocal to coalescing, but lacked a central area of necrotic cell debris. A second type of lesion was similar to the classical tubercles commonly observed in cattle infected with *M. bovis*. These lesions contained a central core of caseous necrosis with variable amounts of mineralized necrotic debris. The margins of the lesion were composed of a mixture of macrophages, epithelioid macrophages, lymphocytes, plasma cells, and occasional multinucleated (Langhans) giant cells. There was often a thin fibrous band at the outermost margins. The third type of lesion also had a large central area of necrosis; however, the central core contained large numbers of neutrophils and degenerative neutrophils. The neutrophils extended into a surrounding rim of macrophages, epithelioid macrophages, lymphocytes, plasma cells, and fibrous connective tissue. When pyogranulomas were identified in lung tissue, the adjacent bronchioles and alveoli were frequently filled with proteinaceous fluid, neutrophils, and degenerate neutrophils.

M. bovis was isolated by culture of lesions in 34/59 elk and 32/50 fallow deer. Based upon culture alone, the prevalence of TB was estimated at 58–64% for elk and fallow deer, respectively. Multiple mycobacterial isolates were recovered from one elk and two fallow deer; *M. bovis* was recovered from all three, *M. thermoresistibile* was also recovered from the elk, and *M. avium* complex from the two fallow deer. *M. avium* was also recovered from one fallow deer in which *M. bovis* was not recovered. All *M. bovis* isolates from this outbreak had the same spoligotype, octal code 666773677777600. This spoligotype was initially encountered in an isolate recovered from an elk residing in New York state in 1991 and has since sporadically resurfaced in cervid herds throughout the USA (Robbe-Austerman and Harris, unpublished observations).

Twenty-five of 39 elk which tested negative on SCT approximately 3 months prior to necropsy had microscopic lesions consistent with TB and containing acid-fast bacilli and/or *M. bovis* isolated by culture of lesions. Considering

(a)

(b)
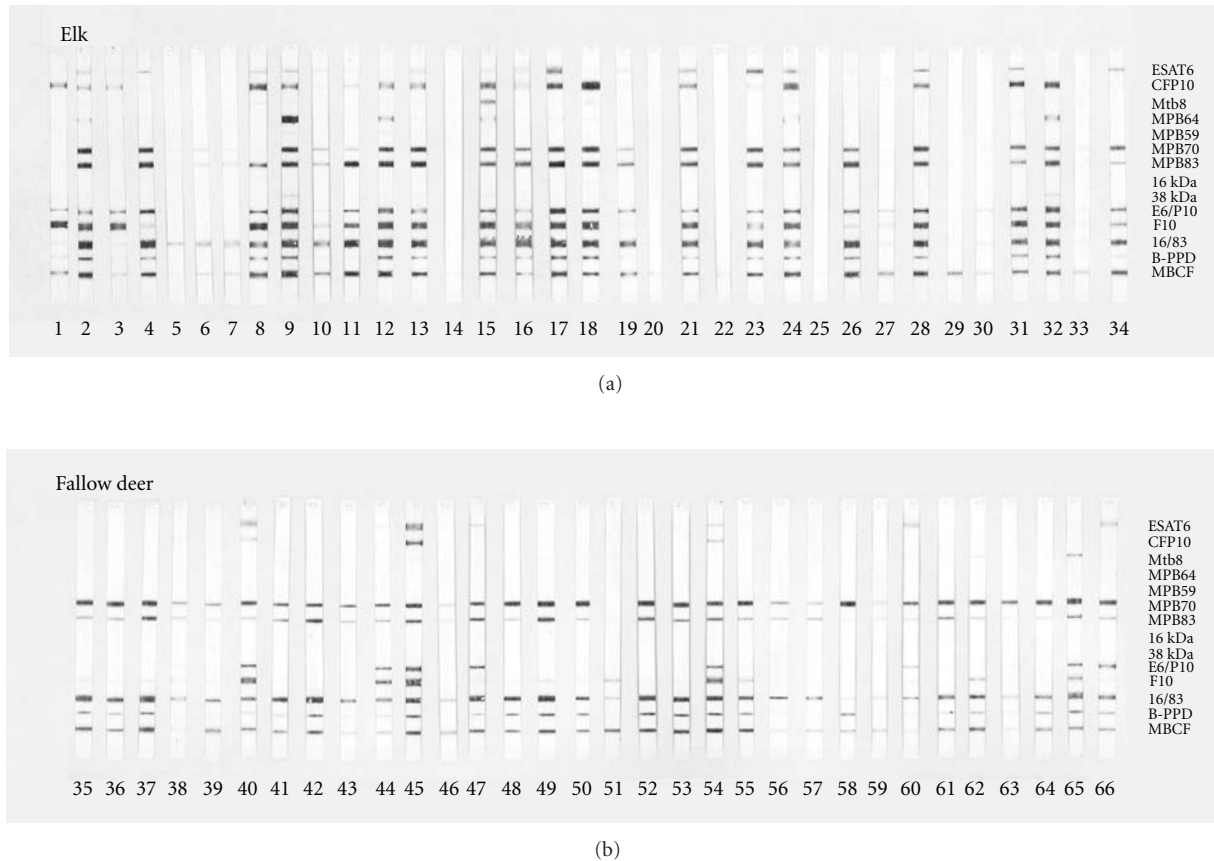
FIGURE 3: Antibody responses to recombinant and native antigens detected in elk (a) and fallow deer (b) using a multiantigen print immunoassay. Numbers in lower margins indicate unique animal identification designations. Antigens printed on nitrocellulose are indicated in the right margin. E6/P10 refers to a fusion protein of ESAT-6 and CFP10. 16/83 refers to a fusion protein of the 16kDa protein and MPB83. F10 is a fusion protein consisting of CFP10, secE2, and PhoS1/pstS. B-PPD refers to *M. bovis*-derived purified protein derivative. MBCF refers to *M. bovis*-derived culture filtrate antigen.

the initial 3 that were identified as SCT responsive, the sensitivity of SCT in elk within this herd was unexpectedly low (3/28). As all elk were considered exposed, the specificity of SCT was not possible to determine.

3.2. MAPIA. Serum was collected from 34 elk (all SCT negative ~3 months prior) and 32 fallow deer (not tested by SCT) from the Nebraska herd—all 66 animals were considered tuberculous based on histopathology and culture results. Serum was not collected from the 3 SCT-positive elk. Extensive reactivity with single *M. bovis* antigens (i.e., ESAT-6, CFP10, MPB70, and MPB83), fusion proteins (i.e., F10, ESAT-6/CFP10, and the 16-kDa protein/MPB83 fusions), and complex antigens (i.e., B-PPD and MBCF) was detected with sera from elk (Figure 3). Lesser frequency, but detectable reactivity was seen with Mtb8 and MPB64 antigens. Minimal to no reactivity was observed with MPB59, 16-kDa protein, and 38-kDa protein.

Antibody responses were generally less robust in fallow deer as compared to those in elk (Figure 3). Predominant antigen recognition patterns were similar between elk and fallow deer, although less frequent reactivity to ESAT-6 and CFP10 antigens was detected with sera from fallow deer. MAPIA was positive in 28/34 (82%) tuberculous elk and in 31/32 (97%) tuberculous fallow deer. The rates of antibody reactivity as well as antigen recognition patterns found by MAPIA in the present study appear to be in agreement with the previous report on elk experimentally infected with *M. bovis* [14].

3.3. CervidTB STAT-PAK and DPP VetTB Tests. Two rapid assays were evaluated for test sensitivity with sera from this herd ($n = 34$ elk, $n = 32$ fallow deer; all 66 were TB-positive). Additionally, sera from noninfected elk ($n = 141$) and fallow deer ($n = 107$) originating from known TB-free herds were obtained from the APHIS, NVSL bovine TB serum bank for evaluation of test specificity. Results are presented in Table 2 (elk) and Table 3 (fallow deer). Findings demonstrate high sensitivity and specificity of both test formats with elk (se : 79 to 82%, sp : 93 to 98%) and fallow deer (se : 91%, sp : 91 to 99%). The DPP VetTB assay was more accurate than CervidTB STAT-PAK test (94–97% versus 91%, resp.) in

TABLE 2: Diagnostic performance of serological tests in elk.

Assay	Sensitivity[a]	Specificity[b]	Accuracy
CervidTB STAT-PAK	82% (28/34)	93% (131/141)	91% (159/175)
DPP VetTB	79% (27/34)	98% (138/141)	94% (165/175)

[a] Elk were considered positive for bovine TB if either *M. bovis* was isolated upon culture of lesions or mycobacteriosis compatible lesions were detected upon microscopic examination of gross lesions.
[b] Sera from noninfected and SCT-negative elk were obtained from the APHIS, NVSL bovine TB serum bank for evaluation of test specificity.

TABLE 3: Diagnostic performance of serological tests in fallow deer.

Assay	Sensitivity[a]	Specificity[b]	Accuracy
CervidTB STAT-PAK	91% (29/32)	91% (97/107)	91% (126/139)
DPP VetTB	91% (29/32)	99% (106/107)	97% (135/139)

[a] Fallow deer were considered positive for bovine TB if either *M. bovis* was isolated upon culture of lesions or mycobacteriosis compatible lesions were detected upon microscopic examination of gross lesions.
[b] Sera from noninfected and SCT-negative fallow deer were obtained from the APHIS, NVSL bovine TB serum bank for evaluation of test specificity.

both cervid species. These results support the serodiagnostic potential for CervidTB STAT-PAK and DPP VetTB tests previously shown for several cervid species [13–16].

3.4. Seroreactivity of Individual Animals with Each Test. In general, the seroreactivity of individual animals between test formats was similar. With TB-infected elk sera, all 3 tests (i.e., CervidTB STAT-PAK, DPP VetTB, and MAPIA) were positive with 27/34 samples. All 3 tests were negative for TB-infected elk numbers 5, 14, 20, 22, 25, and 29. The DPP VetTB test was also negative with TB-infected elk number 33. With TB-infected fallow deer sera, all 3 tests were positive with 29/32 samples. CervidTB STAT-PAK and DPP VetTB tests were negative with TB-infected fallow deer numbers 58 and 59 and all three tests were negative with fallow deer number 51. These findings demonstrate high concordance between the serological tests used in the present study. MAPIA band intensities (indicative of levels of antibody reactivity with antigens) were generally greater in elk than those observed in fallow deer (Figure 3), presumably due to more advanced disease or prior injection of PPD for SCT in elk. These results suggest that the high-prevalence of *M. bovis* infection found in this mixed cervid herd in early 2009 had originally occurred in elk. The elk may have failed to produce an SCT response in March 2008 (as also observed in February/March 2009), presumably transmitting the infection to fallow deer upon co-mingling with elk. A lesser severity of disease and lower levels of *M. bovis*-specific antibodies support the hypothesis that the infection initiated in the elk herd prior to the fallow deer. However, disease progression and development of *M. bovis*-specific responses likely differ, potentially remarkably, between these two host species; thus, it is not clear as to which species was first infected.

Unique findings from this case report include unusually high prevalence of TB in this herd and the unexpectedly low sensitivity of SCT in elk. While purely speculative, the high prevalence of disease may be due to an extended course of infection as indicated by the severity of lesions. Reasons for

the SCT failure could be explained by anergy of cell-mediated immune responses associated with advanced stages of disease or specific host factors. Anecdotal evidence supports the potential for a predominance of antibody responses by elk to various intracellular pathogens (Waters, unpublished observations and Steven Olsen, personal communication). Another possibility, although less likely, is that disease progression within the elk occurred very rapidly, developing some time after SCT. However, prior studies with experimental TB infection in cervids would not support such a rapid progression of disease [14, 24, 30]. Further research is warranted to characterize immune responses by elk and fallow deer to various intracellular pathogens, especially *M. bovis*.

Limitations of the present study include that not all animals were evaluated by SCT, necropsy, culture, and serology; samples for histology and culture were only collected from animals with gross lesions; SCT was performed 3 months prior to necropsy to determine disease status; serologic analysis for determination of sensitivity was only performed on animals with gross lesions.

4. Conclusions

Present findings demonstrate (1) an example of an unusually high prevalence (∼71–75%) and severity of infection in a captive elk and fallow deer herd in Nebraska, USA, (2) unexpectedly poor sensitivity of SCT for detection tuberculous elk in this herd, and (3) opportunities for use of serology for the rapid detection of *M. bovis* infection in captive elk and fallow deer.

Acknowledgments

(NVSL, APHIS, USDA, Nebraska) for the postmortem examinations at the slaughter plant and on the farm, respectively; (3) numerous others from NVSL, APHIS, USDA, Nebraska Department of Agriculture, and the Nebraska Game and Parks Commission for organization and assistance with the investigation; (4) Claudia Quinn for excellent technical assistance with serological testing. USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

References

[1] J. F. T. Griffin and C. G. Mackintosh, "Tuberculosis in deer: perceptions, problems and progress," *Veterinary Journal*, vol. 160, no. 3, pp. 202–219, 2000.

[2] G. W. De Lisle, C. G. Mackintosh, and R. G. Bengis, "*Mycobacterium bovis* in free-living and captive wildlife, including farmed deer," *OIE Revue Scientifique et Technique*, vol. 20, no. 1, pp. 86–111, 2001.

[3] M. A. Essey and M. A. Koller, "Status of bovine tuberculosis in North America," *Veterinary Microbiology*, vol. 40, no. 1-2, pp. 15–22, 1994.

[4] D. J. O'Brien, S. M. Schmitt, S. D. Fitzgerald, D. E. Berry, and G. J. Hickling, "Managing the wildlife reservoir of *Mycobacterium bovis*: the Michigan, USA, experience," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 313–323, 2006.

[5] T. L. Whiting and S. V. Tessaro, "An abattoir study of tuberculosis in a herd of farmed elk," *Canadian Veterinary Journal*, vol. 35, no. 8, pp. 497–501, 1994.

[6] A. Fanning and S. Edwards, "*Mycobacterium bovis* infection in human beings in contact with elk (*Cervus elaphus*) in Alberta, Canada," *Lancet*, vol. 338, no. 8777, pp. 1253–1255, 1991.

[7] T. J. Bucholz, Bovine TB Strain Confirmed In Michigan Hunter: Hunters Reminded To Wear Gloves When Cleaning Game, *Michigan Department of Community Health*, http://www.michigan.gov/mdch/0,1607,7-132-8347-107460–M,00.html.

[8] M. J. Wilkins, J. Meyerson, P. C. Bartlett et al., "Human *Mycobacterium bovis* infection and bovine tuberculosis outbreak, Michigan, 1994–2007," *Emerging Infectious Diseases*, vol. 14, no. 4, pp. 657–660, 2008.

[9] C. E. Massengill, "Report of the committee on tuberculosis," in *Proceedings of the 109th United States Animal Health Association Annual Meeting*, vol. 109, pp. 679–718, Hershey, Pa, USA, 2005.

[10] C. E. Massengill, "Report of the committee on tuberculosis," in *Proceedings of the 108th United States Animal Health Association Annual Meeting*, vol. 108, pp. 581–616, Greensboro, NC, USA, 2004.

[11] W. C. Hench, "Current status of the US bovine tuberculosis eradication program fiscal year," in *Proceedings of the 111th United States Animal Health Association Annual Meeting*, vol. 111, pp. 747–752, Reno, Nev, USA, 2007.

[12] K. M. Connell, "Bovine tuberculosis surveillance in U.S. livestock fiscal year 2009," in *Proceedings of the 113th United States Animal Health Association Annual Meeting*, vol. 113, pp. 650–653, San Diego, Calif, USA, 2009.

[13] B. M. Buddle, T. Wilson, M. Denis et al., "Sensitivity, specificity, and confounding factors of novel serological tests used for the rapid diagnosis of bovine tuberculosis in farmed red deer (*Cervus elaphus*)," *Clinical and Vaccine Immunology*, vol. 17, no. 4, pp. 626–630, 2010.

[14] N. P. Harrington, O. P. Surujballi, J. F. Prescott et al., "Antibody responses of cervids (*Cervus elaphus*) following experimental *Mycobacterium bovis* infection and the implications for immunodiagnosis," *Clinical and Vaccine Immunology*, vol. 15, no. 11, pp. 1650–1658, 2008.

[15] R. Jaroso, J. Vicente, M. P. Martín-Hernando et al., "Antemortem testing wild fallow deer for bovine tuberculosis," *Veterinary Microbiology*, vol. 146, no. 3-4, pp. 285–289, 2010.

[16] K. P. Lyashchenko, R. Greenwald, J. Esfandiari et al., "Animal-side serologic assay for rapid detection of *Mycobacterium bovis* infection in multiple species of free-ranging wildlife," *Veterinary Microbiology*, vol. 132, no. 3-4, pp. 283–292, 2008.

[17] W. R. Waters, M. V. Palmer, J. P. Bannantine et al., "Antigen recognition by serum antibodies in white-tailed deer (*Odocoileus virginianus*) experimentally infected with *Mycobacterium bovis*," *Clinical and Diagnostic Laboratory Immunology*, vol. 11, no. 5, pp. 849–855, 2004.

[18] W. R. Waters, M. V. Palmer, J. P. Bannantine et al., "Antibody responses in reindeer (*Rangifer tarandus*) infected with *Mycobacterium bovis*," *Clinical and Diagnostic Laboratory Immunology*, vol. 12, no. 6, pp. 727–735, 2005.

[19] United States Department of Agriculture: Animal and Plant Health Inspection Service, Veterinary Services. Bovine tuberculosis eradication uniform methods and rules. U.S. Government Printing Office, Washington, DC, USA, pp.19–34, 1999.

[20] N. Hines, J. B. Payeur, and L. J. Hoffman, "Comparison of the recovery of *Mycobacterium bovis* isolates using the BACTEC MGIT 960 system, BACTEC 460 system, and Middlebrook 7H10 and 7H11 solid media," *Journal of Veterinary Diagnostic Investigation*, vol. 18, no. 3, pp. 243–250, 2006.

[21] P. Kierschner and E. C. Bottger, "Species identification of Mycobacteria using rDNA sequencing," in *Methods in Molecular Biology, Mycobacteria Protocols*, T. Parish and N. G. Stoker, Eds., vol. 101, Humana Press Inc., Totowa, NJ, USA, 1998.

[22] D. Harmsen, S. Dostal, A. Roth et al., "RIDOM: comprehensive and public sequence database for identification of Mycobacterium species," *BMC Infectious Diseases*, vol. 3, article 26, 2003.

[23] A. Penrod, "Modified Ziehl-Neelsen staining procedure," in *Standard Operating Procedure SOP-PL-38.05*, National Veterinary Services Laboratories Ames (IA), 2010.

[24] M. V. Palmer, W. R. Waters, T. C. Thacker, W. C. Stoffregen, and B. V. Thomsen, "Experimentally induced infection of reindeer (*Rangifer tarandus*) with *Mycobacterium bovis*," *Journal of Veterinary Diagnostic Investigation*, vol. 18, no. 1, pp. 52–60, 2006.

[25] K. P. Lyashchenko, M. Singh, R. Colangeli, and M. L. Gennaro, "A multi-antigen print immunoassay for the development of serological diagnosis of infectious diseases," *Journal of Immunological Methods*, vol. 242, no. 1-2, pp. 91–100, 2000.

[26] R. Greenwald, O. Lyashchenko, J. Esfandiari et al., "Highly accurate antibody assays for early and rapid detection of tuberculosis in african and asian elephants," *Clinical and Vaccine Immunology*, vol. 16, no. 5, pp. 605–612, 2009.

[27] E. B. Rohonczy, A. V. Balachandran, T. W. Dukes et al., "A comparison of gross pathology, histopathology, and mycobacterial culture for the diagnosis of tuberculosis in elk (*Cervus elaphus*)," *Canadian Journal of Veterinary Research*, vol. 60, no. 2, pp. 108–114, 1996.

[28] J. C. Rhyan and D. A. Saari, "A comparative study of the histopathologic features of bovine tuberculosis in cattle, fallow

deer (*Dama dama*), sika deer (*Cervus nippon*), and red deer and elk (*Cervus elaphus*)," *Veterinary Pathology*, vol. 32, no. 3, pp. 215–220, 1995.

[29] J. C. Rhyan, D. A. Saari, E. S. Williams, M. W. Miller, A. J. Davis, and A. J. Wilson, "Gross and microscopic lesions of naturally occurring tuberculosis in a captive herd of wapiti (Cervus elaphus *nelsoni*) in Colorado," *Journal of Veterinary Diagnostic Investigation*, vol. 4, no. 4, pp. 428–433, 1992.

[30] M. V. Palmer, W. R. Waters, and D. L. Whipple, "Lesion development in white-tailed deer (*Odocoileus virginianus*) experimentally infected with *Mycobacterium bovis*," *Veterinary Pathology*, vol. 39, no. 3, pp. 334–340, 2002.

Research Article

Preventing the Establishment of a Wildlife Disease Reservoir: A Case Study of Bovine Tuberculosis in Wild Deer in Minnesota, USA

Michelle Carstensen[1] and Michael W. DonCarlos[2]

[1] *Minnesota Department of Natural Resources, Wildlife Health Program, 5463-C West Broadway, Forest Lake, MN 55025, USA*
[2] *Minnesota Department of Natural Resources, Wildlife Research and Policy, 500 Lafayette Road, St. Paul, MN 55155, USA*

Correspondence should be addressed to Michelle Carstensen, michelle.carstensen@state.mn.us

Bovine tuberculosis (bTB) has been found in 12 cattle operations and 27 free-ranging white-tailed deer (*Odocoileus virginianus*) in northwestern Minnesota, following the state's most recent outbreak of the disease in 2005 in the northwest part of the state. Both deer and cattle have the same strain of bTB. The Minnesota Board of Animal Health has been leading efforts to eradicate the disease in Minnesota's cattle, which have included the depopulation of all infected herds, a cattle buy-out program, and mandatory fencing of stored feeds. The Minnesota Department of Natural Resources began surveillance efforts in free-ranging white-tailed deer in fall 2005. All bTB-infected deer have been found within a $16\,\text{km}^2$ area in direct association with infected cattle farms. Aggressive efforts to reduce deer densities through liberalized hunting and sharpshooting have resulted in a 55% decline in deer densities. Also, recreational feeding of wild deer has been banned. Disease prevalence in deer has decreased from 1.2% in 2005 to an undetectable level in 2010.

1. Introduction

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis*, is a chronic infectious disease that affects a wide range of mammals, including domestic cattle and humans [1]. Because of the serious economic implications and zoonotic concerns associated with bTB, efforts to eradicate this disease internationally have been extensive. Wildlife species can play an important role in the epidemiology of bTB, but it is important to distinguish between spillover and maintenance wildlife hosts. A spillover host needs continuing exposure to the bacterium from other species to maintain infection; whereas maintenance hosts can maintain infection without cross-transmission from other species of domestic or wild animals [1]. While both spillover and maintenance hosts may act as a disease vector, a true bTB wildlife reservoir with epidemiological implications for disease control requires that the maintenance host have the potential to transmit the pathogen to other species [2, 3]. Examples of wildlife reservoirs for *M. bovis* exist in various regions of the world, including African buffalo (*Syncerus caffer*, South Africa), wood bison (*B. bison athabascae*, Canada), European badger (*Meles meles*, United Kingdom), brushtail possum (*Trichosurus vulpecula*, New Zealand), European wild boar (*Sus scrofa*, Spain), elk (*Cervus elaphus*, Canada), and white-tailed deer (*Odocoileus virginianus*, United States) [3–8]. These wildlife reservoirs share some common characteristics, such as high population densities, artificial feeding, and continuous interactions at the wildlife-livestock interface, which perpetuate the disease and make bTB control efforts especially challenging. However, it may be possible to prevent the establishment of a wildlife reservoir if a spillover event can be contained, thus allowing domestic animal disease control efforts to take effect.

Bovine tuberculosis had been eradicated in Minnesota's cattle industry in 1971; however, the disease reemerged in the

state in 2005. A beef cow with thoracic lesions was discovered through routine slaughter surveillance at a slaughter facility in Wisconsin in July 2005. This cow was traced to a beef herd in northwest Minnesota; subsequent testing revealed 1.2% bTB prevalence in the herd [9]. Epidemiological investigation of trace-in and trace-out cattle movements and area herd-testing led to the detection of 4 additional bTB-positive cattle herds in the region by October 2005. All bTB-infected cattle herds were appraised, indemnified, and depopulated by the United States Department of Agriculture (USDA). Strain-typing concluded the *M. bovis* had southwestern U.S. or Mexican origin and was distinctly different than strains of *M. bovis* in Michigan and Manitoba [10]. In November 2005, the Minnesota Department of Natural Resource (MNDNR) sampled 474 hunter-harvested deer for bTB within 25 km of the infected cattle farms. One deer, harvested within 1 mile of the index cattle herd, was found infected with bTB (apparent prevalence 0.2%); strain-typing concluded that it was the same bTB strain as the cattle [11]. Deer shooting permits were issued to landowners of bTB-infected farms in January 2006, resulting in 90 additional deer being harvested, including a second bTB-positive deer. Because both bTB-infected deer were harvested in direct association with infected farms, deer shared the same strain of the disease as cattle, and no positive deer were detected in the larger area of sampling, we postulated that the wildlife infections were a result of direct spillover from bTB-positive cattle.

In 2006, surveillance efforts were intensified for both cattle and deer in northwest Minnesota, and a one-time statewide sampling effort that tested 1,554 cattle herds and 4,000 deer was conducted at the request of USDA. No bTB infection was detected outside the 4-county region of northwest Minnesota in deer or cattle. However, new cases of bTB were discovered in 2 cattle herds and 5 deer in the northwest, prompting the establishment of a Bovine Tuberculosis Management Zone as well as an inner target area, called the Bovine TB Core Area. The establishment of these control zones enabled more focused disease management efforts in deer, and the same boundaries were later adopted by the Minnesota Board of Animal Health (BAH) for disease management efforts in cattle that included increased testing requirements and movement restrictions. By the end of 2006, Minnesota had lost its Bovine TB-Free accreditation, and the entire state was demoted to Modified Accredited Advanced status [12]. This initial loss of bTB-Free accreditation prompted political and public support for aggressive disease control management, as increased cattle testing requirements and trade restrictions had major economic consequences for the entire state's cattle industry.

The MNDNR began its aggressive disease management campaign to eradicate bTB in deer by instituting a ban on recreational feeding of wild cervids in a 10,060-km^2 area of northwest Minnesota in November 2006; baiting had already been outlawed statewide since 1991. As direct or indirect transmission of *M. bovis* between wild hosts or livestock can occur at shared feed sources, restricting supplemental feeding of wild cervids can limit disease propagation [13]. Efforts to dramatically reduce deer densities in the region followed, with emphasis on the Bovine TB Core Area. During winter

2007, agency-sponsored sharpshooting of deer within the Bovine TB Core area began as an effort to further minimize disease spread outside the area by reducing deer densities and removing potentially bTB-infected individuals. Over 4 consecutive winters (2007–2010), approximately 2,600 deer were removed by ground and aerial sharpshooting in the Bovine TB Core area, including 14 bTB-positive deer. Liberalized hunting opportunities were initiated by fall 2007. This included the creation of a special deer management unit that encompassed the Bovine TB Management Area, early and late season hunts, and reduced-cost bonus permits. Landowners living within the Bovine TB Management Area were issued shooting permits to harvest deer on private lands outside the bounds of traditional deer harvest seasons.

On the cattle side of the bTB issue, the detection of 4 new bTB-infected herds in the northwest in 2007 resulted in a further loss of the state's bTB Modified Accredited Advanced status to Modified Accredited. This change fueled a political campaign that facilitated the Minnesota State Legislature to establish and fund a voluntary cattle buy-out program. By Jan 2009, 46 farms accepted the buy-out program and 6,200 cattle were removed from the Bovine TB Management Zone. Remaining farms were required to fence stored feed and winter feeding areas; the state providing funding for fencing at a 90:10 cost share with producers up to $75,000. One additional bTB-infected cattle herd was discovered during the buy-out program, bringing the total number of bTB-infected farms detected since 2005 to 12. Minnesota was granted a split-state status by USDA in 2008, which upgraded its status to Modified Accredited Advanced through most of the state, with only a 6,915-km^2 area in the northwest remaining Modified Accredited. With the last bTB-infected cattle herd being discovered in January 2009, Minnesota regained TB-Free accreditation in October 2010 in the majority of the state, with only the split-state region of the northwest remaining Modified Accredited Advanced.

Since the bTB surveillance began in deer, 27 infected deer have been discovered. All of these bTB-infected deer were harvested within 16 km of the first bTB-infected cattle herd. The last bTB-infected deer was killed in November 2009. Sharpshooting during winter 2010 in the Bovine TB Core area failed to find additional infected deer, which marked the first time that intensive culling had occurred in this area without finding bTB-positive deer. Recent hunter-harvested surveillance in fall 2010 also failed to detect any obvious cases of the disease, although final test results are pending.

Declining trends in bTB prevalence in deer and the limited geographic extent of infected individuals lends optimism that this disease may have been eradicated in the local deer herd or remains present at an undetectable level. Further, nearly all (96%) of the bTB-infected deer were older animals, born during or before 2005. To call attention to this skewed age distribution of bTB-positive deer, we coined the phrase "Alive in '05;" meaning all infected deer were born on or prior to 2005, when the disease was first discovered in the region. The apparent absence of bTB infection in younger age classes further suggested that this disease was not maintaining itself within the local deer population, but continued monitoring is required to confirm

the absence of bTB in the region. Consequently, the state will continue hunter-harvested surveillance of deer for bTB at least through 2014.

Minnesota's primary goal has been the eradication of bTB from both deer and cattle. The aim of this paper is to describe the primary management strategies implemented by MNDNR to prevent the establishment of a wildlife disease reservoir in free-ranging white-tailed deer. These strategies included, (1) rapid response to initial disease detection, (2) follow-through on monitoring the outbreak with adequate surveillance, (3) recognizing when monitoring must switch to management, (4) aggressively reducing transmission potential by reducing deer densities, limiting recreational feeding and mitigating risks at the cattle-wildlife interface, and (5) evaluation of efforts and adjusting as needed.

2. Materials and Methods

2.1. Bovine Tuberculosis Surveillance Areas. There were 3 primary surveillance areas used to monitor the bTB outbreak and focus disease management efforts. These included a (1) Split-state Zone, which was established in 2008 by USDA for disease control efforts for cattle, (2) Bovine TB Management Zone, which was established by MNDNR in 2006 and lies within the Split-state Zone, and (3) Bovine TB Core Area, also established by MNDNR in 2006 as a focal area within the Bovine TB Management Zone (Figure 1).

Minnesota's 3,884 km²-Split-state Zone is located between 48° 11′ N and 49°0′ N latitude and 94°56′ W and 95°58′ W longitude in northwest Minnesota. There were an estimated 300 cattle, 2 bison, 7 goat, and 1 captive cervid (white-tailed deer) herds in the zone [14]. Of the cattle herds, 19 were dairy operations with <200 animals per farm. The remaining cattle farms were beef cow/calf operations that averaged 80 animals per herd. Approximately 53% of the zone was publically owned by state, federal, county and tribal authorities. Private land accounted for about 3,280 km².

The 1,567 km²-Bovine TB Management Zone is located between 48°18′ N and 48°41′ N latitude and 95°15′ W and 95°49′ W longitude in northwest Minnesota. Its boundaries were defined by delineating a 16-km buffer around the 7 bTB-infected deer discovered by fall 2006. The landscape was comprised of 25% flat, mixed forest, 35% lowland marsh, and 40% open agriculture. Land ownership was approximately 57% public (state-owned), 40% private, and 3% tribal. Sixty-six cattle farms existed in the zone prior to the 2008 buy-out program, which removed 6,200 cattle from 46 herds. Twenty herds remained with approximately 1,500 cattle. Three small captive cervid operations also existed in the zone. Prefawning deer densities ranged from 2.3 to 3.1 deer/km², with reduced densities in the bTB Core Area as described below. A small, reintroduced elk (*Cervus elaphus*) population numbering 40–60 animals ranged into the southern half of the zone. From 2004–2009, 77 wild elk were tested for bTB in northwest Minnesota and all have been negative for the disease [15]. Natural predators included gray wolves (*Canis lupus*), black bears (*Ursus americanus*), coyotes (*Canis latrans*), red foxes (*Vulpes vulpes*), and bobcats (*Felis rufus*).

The 425-km²-Bovine TB Core Area is located between 48°23′ N and 48°35′ N latitude and 95°24′ W and 95°41′ W longitude and lies within the Bovine TB Management Zone. Its boundaries were defined by a 3.2-km buffer around all the bTB-infected deer found by fall 2006. Land ownership was approximately 56% public (state-owned), 41% private, and 3% tribal. Deer densities were estimated at 1.8–2.5 deer/km² in January 2007, and following 3 consecutive winters of aggressive deer removal efforts were reduced to 0.7–1.3 deer/km² in January 2010. Eight of the 12 bTB-infected cattle herds were found within the bTB Core Area.

2.2. Bovine Tuberculosis Surveillance Methods. Hunter-harvested surveillance was used to estimate apparent prevalence of bTB in the local deer population. Initial sampling goals were designed to have 95% confidence of detecting the disease at a prevalence ≥1.0% (2005–2007). Surveillance was conducted within 25-km-radius circles around known bTB-infected cattle farms. However, when Minnesota was granted Split-state Status in 2008, sampling goals were dictated by USDA, and we expanded the surveillance area to the entire Split-state Zone. Sampling requirements were set at 1,500 deer from within the Zone and 300 deer outside the Zone (2008 and 2009). In 2010, the MNDNR successfully renegotiated a reduction in sampling requirements with USDA and focused sampling intensity (with 99% confidence of detecting the disease if prevalence was ≥0.5%) within the Split-state Zone only.

In Minnesota, it is mandatory for all licensed hunters (big game only) to register their game. Hunters that registered their deer within the bTB Surveillance Area were asked to voluntarily submit cranial lymph node samples for bTB testing at designated registration stations. Only deer >1.0 year old were included in sampling efforts. Hunter information was recorded, including the hunter's name, address, telephone number, MNDNR number, and location of kill. Maps were provided to assist the hunters in identifying the location (Township, Range, Section, and Quarter-section) of the kill. Cooperating hunters were offered incentives, including a cooperator's patch and raffles for firearms donated by local and statewide sporting groups.

Additionally, MNDNR attempted to further reduce deer numbers in the posthunting season in the Bovine TB Core Area through the use of sharpshooters. The goal was to harvest as many deer as possible in hopes of removing additional bTB-infected deer from this critical area, thus, reducing the potential for deer-deer or deer-cattle transmission. Disease prevalence data generated from this removal effort were biased by targeting deer from known bTB-infected areas. During winters 2007–2010, sharpshooting from the ground was conducted by USDA-Wildlife Services (USDA-WS) professionals; supplemental sharpshooting was conducted by aerial operations during winters 2007 (Wildlife Services, Inc.) and 2008 (Tery Jon Aviation). Sharpshooter-harvested deer were transported intact to a central processing facility at Thief Lake Wildlife Management Area in northwest Minnesota. All deer were sampled, including fawns (<1.0 year old). Sample collection and handling is described below.
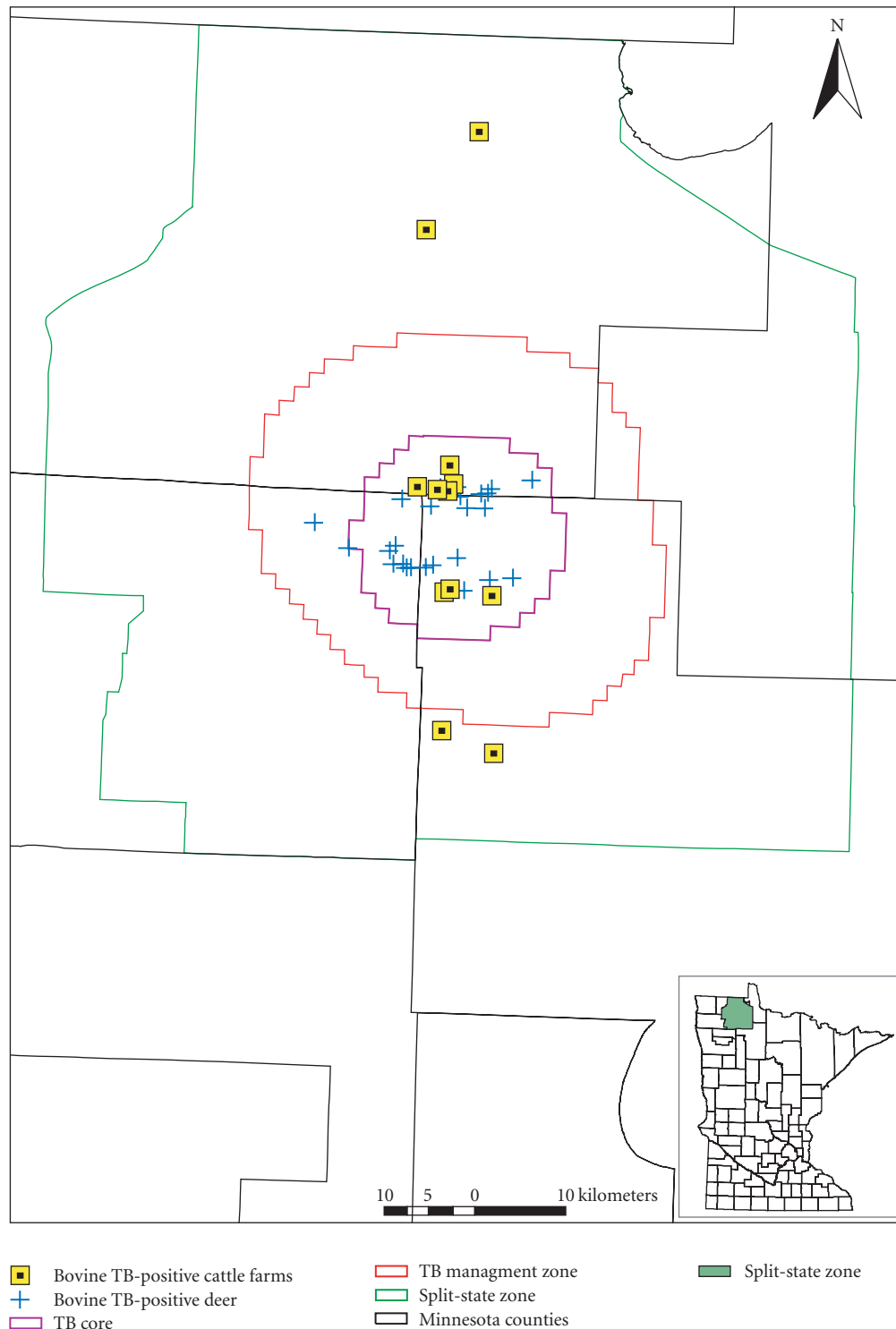
FIGURE 1: Spatial distribution of free-ranging white-tailed deer ($n = 27$) and cattle farms ($n = 12$) infected with bovine tuberculosis, 2005–2010, northwest Minnesota.

Carcasses that were free of any visible lesions were salvaged for venison and made available to the public.

2.3. Bovine Tuberculosis Sample Collection and Testing. Six cranial lymph nodes (parotid, submandibular, and medial retropharyngeal) extracted from all deer by trained personnel were submitted for testing, regardless of surveillance method. Once extracted, all lymph nodes were cross-sectioned for presence of gross lesions. Tissue collection procedures included a visual inspection of the chest cavity of

the hunter-killed deer (lungs were typically not available as carcasses were field-dressed). Any suspect carcasses (e.g., obvious lesions in chest cavity or lymph nodes) were confiscated at the registration stations, and the hunter was issued a replacement deer license at no charge. A first incisor was collected for aging by cementum annuli [16]. Suspect carcasses were transported in their entirety to the Veterinary Diagnostic Laboratory (VDL) at the University of Minnesota for further testing. Samples collected from sharpshooter-killed deer, vehicle-killed deer within the bTB Management Zone, or deer harvested by landowner shooting permit were handled as described above.

All lymph node samples were first submitted to the VDL for histological examination and acid-fast staining, and then forwarded to the National Veterinary Services Laboratories (NVSL) in Ames, IA for bacterial culture. Real-time polymerase chain reaction (RT PCR) was used to detect the presence of *M. tuberculosis complex* DNA when obvious lesions were present, which was then followed by bacterial culture. Samples that did not have obvious lesions were pooled in groups of 5 deer and also cultured. Bacterial growth identified as *M. bovis* indicates the animal was infected with bTB. These cultures were submitted for further laboratory testing to compare similarities or differences with other cultures of *M. bovis* identified within Minnesota and strains identified in the reference collection at NVSL.

2.4. Estimating Deer Densities in Bovine Tuberculosis Core Area. Deer surveys were conducted in late January—early February 2007–2010 to estimate deer densities and monitor changes in the abundance and distribution of deer within the bTB Core Area relative to ongoing management actions. The target population was free-ranging white-tailed deer within the 425-km^2 bTB Core Area. We used an equal-probability, generalized random-tessellation, stratified sampling design [17], which generated a 2D spatially balanced sample. The sampling frame included 164 Public Land Survey sections with a sample size of 72 plots (sampling rate = 0.439). Surveys were conducted by helicopter (Bell OH-58, Jet Ranger [2007-2008, 2010]; Enstrom 480B [2009]) with a pilot and 2 observers. Population estimates reflected the *minimum* numbers of deer present during the sampling interval, as estimates were not adjusted for detectability. Deer movements between sample plots were assumed to be minimal. Confidence intervals (95% CIs) were based on sampling variance only and did not include uncertainty associated with detectability or animal movements.

3. Results

3.1. Bovine Tuberculosis Prevalence and Geographic Distribution. From 2005 to 2010, 6,955 hunter-harvested deer were tested for bTB in northwestern MN, which included 12 infected deer (Table 1). Age class of hunter-harvested deer were 65% adults (≥2.0 years old) and 35% yearlings (1.0 to <2.0 years old). Annual estimates in apparent prevalence demonstrated a declining trend, from 0.53% in 2006 to 0.07% in 2009 (Table 1, Figure 2). Final test results are

(a)

(b)
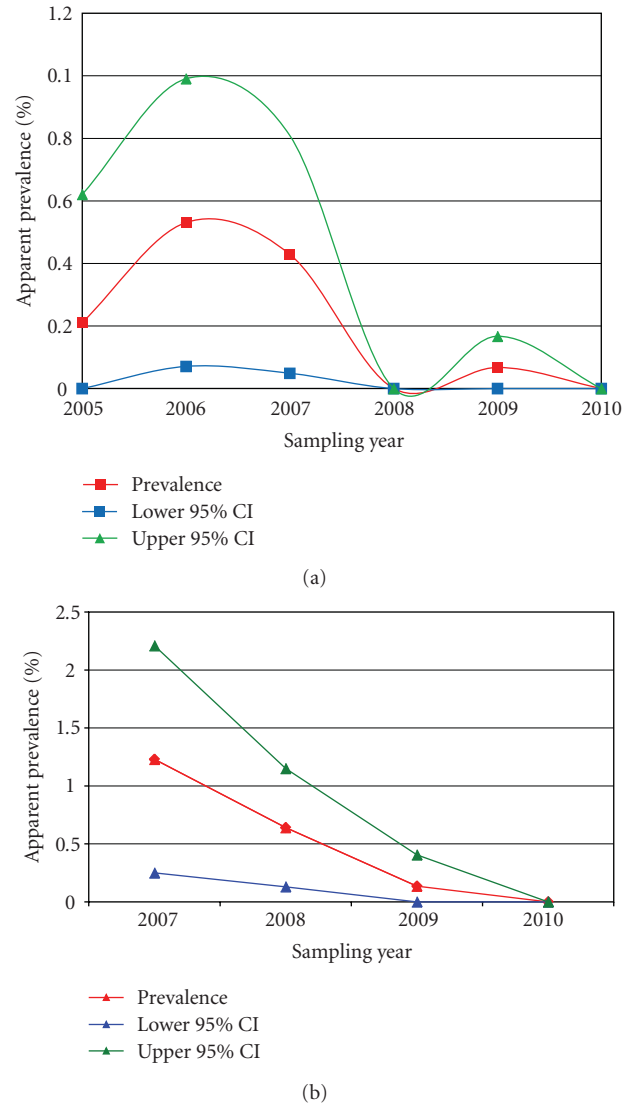
FIGURE 2: (a) Prevalence of bovine tuberculosis (bTB) in hunter-harvested deer from 2005–2010 in the Bovine TB Surveillance Zone. (b) Prevalence of bTB from sharpshooter-removed deer from 2007–2010 in the Bovine TB Core Area, northwest Minnesota.

pending for fall 2010, but no obvious cases of bTB were identified through gross examinations. Also, 4,038 hunter-harvested deer were tested outside of the northwest region during a one-time, statewide sampling effort in 2006; all deer were negative for bTB.

Sharpshooting removed 2,613 deer (22% aerial gunning and 78% ground sharpshooting) from the bTB Core Area, including 14 infected deer (Table 1). Sharpshooter-harvested deer were 63% adults, 10% yearlings, and 27% fawns (<1.0 year old). Annual estimates indicated apparent prevalence declined from 1.23% in 2007 to 0% in 2010 (Figure 2).

A total of 9,783 deer from all surveillance methods (2005–2010) were tested in northwest Minnesota for bTB and yielded 27 infected individuals (15 males, 12 females). All 27 bTB-infected deer were >1.0 year of age at harvest. Two deer harvested by sharpshooters in winter 2007 were

Table 1: Number of deer sampled for bovine tuberculosis (bTB) and testing results listed by sampling strategy, 2005–2010, northwest Minnesota.

Sampling strategy	Surveillance year						
	2005	2006	2007	2008	2009	2010	Totals
Hunter-harvested (Oct–Jan)	474	942	1,166	1,246	1,488	1,639	6,955
No. bTB-positive	1	5	5	0	1	0	
Apparent prevalence (%)	0.21	0.53	0.43	0.00	0.07	0.00[1]	
Sharpshooting (Feb–April)			488	937	738	450	2,613
No. bTB-positive			6	6	2	0	
Apparent prevalence (%)			1.23	0.64	0.27	0.00	
Landowner shooting permit		90		125			215
No. bTB-positive		1		0			
Total no. deer tested	474	1,032	1,654	2,308	2,226	2,089	9,783
Total no. bTB-positive	1	6	11	6	3	0	27

[1] Final culture results from fall 2010 hunter-harvested sampling are pending.

yearlings (1.5 years old). The remaining 25 infected deer were >2.5 years old. Twenty-six (96%) of the infected deer were born on or before 2005. The 27th infected deer was 3.5 years old at harvest in fall 2009 (born in 2006). Twenty-one (76%) of the bTB-infected deer presented with gross lesions inside the chest cavity (ribcage, lungs, and diaphragm) or cranial lymph nodes; these carcasses were confiscated. Three (10%) of the bTB-infected deer presented with gross lesions in lymph nodes only (chest cavities were clear), and 4 (14%) deer had microscopic lesions in the lymph nodes that were detected histologically.

The geographic distribution of bTB-infected deer was limited to a 16 km radius around the "index cattle herd," discovered in 2005 (Figure 1). Twenty-six of the bTB-infected deer were harvested within the boundaries of the bTB Core Area. The 27th infected deer (adult male harvested in fall 2009) was located 3.5 km outside of the western boundary of this area (Figure 1). Although this was the first case of a bTB-infected deer found outside the bTB Core, the distance was small enough that its homerange likely extended into the bTB Core Area. To be certain that this case did not represent geographic spread of the disease, winter sharpshooting efforts in 2010 were expanded to include 60 km^2 to the west of the original bTB Core Area boundary. Approximately 200 deer were removed within a 3.5 km radius around this 27th case and no additional bTB-infected deer were discovered.

3.2. Reducing Deer Densities in Critical Areas. Annual aerial surveys of the bTB Core Area estimated the deer population at 935 ($\pm$76, SE), 807($\pm$75), 664 ($\pm$44), and 422 ($\pm$64) in winters 2007–2010, respectively. The deer population in the bTB Core declined 55% from 2007 to 2010, following 3 consecutive years of winter sharpshooting and liberalized hunting opportunities (Figure 3).

Fall deer harvest in the special disease management unit, which encompassed the bTB Management Zone, registered 1,484, 840, 781, and 765 deer during falls 2007–2010, respectively. Adults and yearlings comprised 75% of deer registrations with males ranging from 47% to 56%; fawns accounted for the remaining 25% of the harvest.
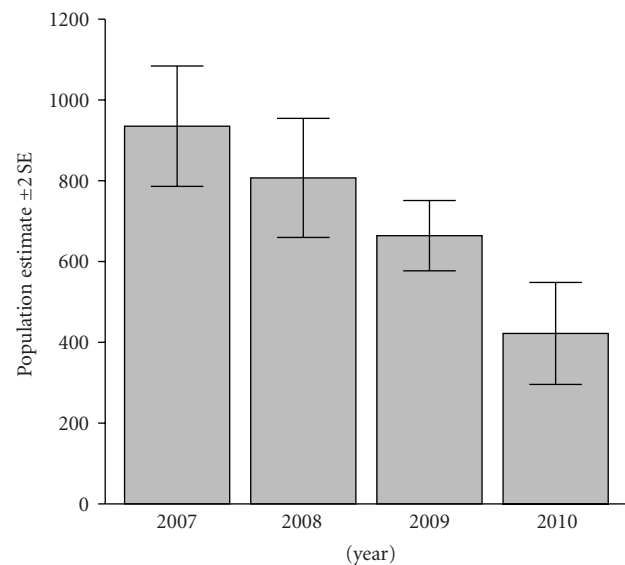
Figure 3: Population estimate of free-ranging white-tailed deer within the Bovine Tuberculosis Core Area, winters 2007–2010, northwest Minnesota.

3.3. Recreational Feeding of Deer. Recreational feeding of wild cervids was prohibited in a 10,060 km^2 area of northwest Minnesota, which encompassed and was 6.5 times larger than the bTB Management Zone, in late November 2006. Enforcement flights identified 34 suspected illegal sites during winter 2007 within the bTB Management Zone. Officers visited the sites and offered verbal warnings ($n =$ 19), written warnings, ($n = 3$), or found no illegal activity ($n = 12$). In winter 2008, enforcement efforts intensified for both illegal baiting and recreation feeding activities within the bTB Management Zone and 36 illegal sites were identified (19 baiting, 17 feeding). In these cases, officers issued verbal warnings ($n = 2$), written warnings ($n = 3$), citations ($n = 12$), or found no illegal activity ($n = 19$). Sixteen (7 baiting, 9 feeding) illegal sites were identified in winter 2009, leading to verbal warnings ($n = 1$), written warnings ($n = 1$), citations ($n = 11$), or no action ($n = 3$). In winter 2010,

only 3 complaints (1 baiting and 2 feeding) were investigated, resulting in 2 citations and 1 unfounded report.

3.4. Risk Mitigation at the Wildlife-Cattle Interface.
Wildlife risk assessments were conducted on 309 farms within the Split-State Zone in 2009, including 67 within the bTB Management Zone, as described in Knust et al. [18]. Locations of unprotected stored feed and cattle yards relative to available deer habitat were the primary factors elevating risk of disease transmission between cattle and deer. Within the bTB Management Zone, 48% of the farm risk assessments indicated moderate to high risk; 75% of this risk was attributed to feeding practices (including unprotected stored feed). Also, 83% of these farms had adequate daytime cover for deer, 43% reported deer on their farms during winter months, and 95% of the owners hunt deer on their land.

Following the cattle buy-out program in 2008, 34 fences were erected to protect stored feed and winter feeding sites throughout the bTB Management Zone. This amounted to 22,500 m of fencing. Compliance officers employed by BAH inspect these fences twice annually.

4. Discussion

When Minnesota first detected bTB in free-ranging deer, state wildlife and agricultural officials immediately contacted experts in Michigan, who have been battling a bTB outbreak in deer and cattle since 1995 [8, 19]. Many lessons could be learned from Michigan's efforts to control the disease, and their experience helped define Minnesota's approach to eradicating bTB in deer and cattle.

4.1. Rapid Response to Initial Detection.
Through epidemiological investigations, Minnesota BAH and USDA concluded that bTB was likely introduced into cattle in the northwest 3–5 years prior to the initial detection by routine slaughter surveillance [9, 14]. One major piece of evidence to support this conclusion was that herd prevalence was low among bTB-infected farms; it was <2% and 5-6% in 8 and 3 herds, respectively. This meant only one cow was found infected in most of these herds. Only one herd, the 12th bTB-infected farm (Jan 2009), had a high herd prevalence (15%) that included 3 infected cows and 9 calves (10 months old). Surprisingly, this 12th herd had tested negative during 2 previous whole herd bTB tests. Also, all bTB-infected herds were depopulated within a few months of disease detection; the removal of infected animals likely helped reduce *M. bovis* exposure risks to both cattle and deer.

MNDNR responded immediately to the initial detection of bTB in cattle by initiating hunter-harvested surveillance in free-ranging deer in fall 2005. Just prior to the bTB outbreak, MNDNR had been conducting hunter-harvested surveillance for chronic wasting disease (2002–2004) and so had a protocol in place for obtaining biological samples from deer at registration stations. More importantly, the agency also had dedicated funding available through license dollars to support disease surveillance work. Being poised financially

and logistically to respond to the bTB threat facilitated MNDNR's rapid response.

4.2. Follow-Through on Monitoring the Outbreak with Adequate Surveillance.
When MNDNR discovered the first bTB-infected deer, its significance was questionable. It was harvested within 2 km of an infected cattle farm and was the same strain of bTB. On-farm shooting permits offered in early winter 2006 produced a second bTB-infected deer that, was again, directly and similarly linked to an infected cattle farm. Although this appeared to be a simple spillover event with limited infection in free-ranging wildlife, the Michigan example of bTB establishing a wildlife reservoir in free-ranging deer concerned state and federal officials enough to warrant a geographically expanded surveillance effort in fall 2006.

Further, MNDNR decided to focus surveillance efforts on deer and not spend valuable resource dollars on evaluating infection in other wildlife species, such as furbearers or rodents. While furbearers are important wildlife reservoirs for bTB elsewhere in the world [6, 7], research conducted in Michigan on carnivores and scavengers concluded these species were only spill-over hosts that lacked the ability to effectively transmit the disease [20, 21]. Atwood et al. [22] suggested coyotes may act as useful sentinels for bTB infection in cervids; however, Sangster et al. [23] concluded testing coyotes lacked sufficient sensitivity to be effective. Given the apparent lack of epidemiological importance of furbearers in bTB transmission between deer and cattle, MNDNR chose to focus surveillance efforts solely on cervids.

The importance of adequate and timely surveillance was also learned from Michigan's bTB outbreak. Michigan's first case of bTB in a hunter-killed deer was reported in 1975; yet no surveillance in wildlife was conducted until a second grossly lesioned deer surfaced in 1994. Surveillance was initiated in 1995 within a 16 km radius of this case, and a 5% apparent prevalence of bTB was discovered [19]. This high initial prevalence suggested that bTB was maintaining itself in this deer herd for at least 2 decades before being discovered. By contrast, Minnesota's initial prevalence was only 0.2%.

4.3. Recognize When Monitoring Must Switch to Management.
The discovery of 5 additional bTB-infected deer, combined with more cattle infection and the loss of Minnesota's bTB-free accreditation, triggered a switch in MNDNR's approach to the disease outbreak. No longer were officials debating over the significance of the 2 initial deer detected in direct association with infected cattle farms. Although spillover from cattle was the likely origin of infection, the fact remained that bTB was present in wild deer, and the possibility of establishing a disease reservoir in deer needed to be addressed. MNDNR decided to develop additional methods to reduce deer numbers and infected animals, to prevent or slow the establishment of a bTB reservoir in deer.

Public support, including the cattle industry and sporting groups, was needed to effectively launch an aggressive disease control program. The loss of bTB-Free accreditation

and cattle industry pressure drove BAH to attempt to swiftly contain the outbreak in cattle and provide assurances to trading partners that disease control efforts were effective. However, the MNDNR was not operating under any regulatory framework nor was any financial support (state or federal) offered to manage the outbreak in deer. With the Michigan example in mind, the MNDNR recognized that the wildlife component of this outbreak would play a vital role in the state's efforts to regain its bTB accreditation and committed to the shared goal with BAH to eradicate the disease as soon as possible.

Establishing a dual-agency approach was challenging, as key stakeholder groups had differing priorities and ideas on how bTB should be managed in the state. Yet, a collaborative approach was launched, with shared messages of the importance of this disease to both the cattle industry and deer hunting community. Joint outreach efforts were initiated, including local media articles, public meetings, and websites, to inform the local community in northwest Minnesota of imminent changes in both deer management and cattle regulations. Local ad hoc groups formed, including a bTB Task Force, to help communicate local issues concerning bTB management to state and federal agencies. Political champions were also identified by industry groups to help fund management efforts and provide support for aggressive actions that would follow.

4.4. Aggressively Reduce Transmission Potential. Results of 5 years of bTB surveillance in deer have demonstrated that disease prevalence has been reduced to an undetectable level and the geographic extent of infected individuals is limited. Although apparent prevalence in 2005 was less than 2006 (0.21% versus 0.53%), it is unlikely that an increase in infection truly existed in the deer population between years. It is probable that the sample collected in fall 2005 was not sufficiently large and underestimated apparent prevalence. We are confident that our surveillance has been effective in identifying bTB-infected deer, as 85% of the positive cases were detected through gross examination of tissues alone. Yet every deer sampled was submitted for bacterial culture; thus our apparent prevalence estimates may accurately reflect true disease prevalence of bTB in the local deer population [24]. Michigan's surveillance program only submits grossly lesioned individuals for bacterial culture to reduce testing costs, and O'Brien et al. [24] reported this sampling method underestimated true prevalence by about 25%.

Age structure of the bTB-positive deer demonstrated a lack of infection in younger age classes. This is important because yearling prevalence has been used as a crude index of the rate of new infections, as well as evidence that deer-to-deer transmission is occurring [25]. The absence of bTB infection in younger deer strongly suggests that this disease was not maintaining itself in Minnesota's deer population. Our "Alive in '05" theory accurately predicted the birth year of 26 of the 27 bTB-infected deer as on or before 2005, when the disease was first detected and most prevalent in cattle. Assuming the disease spilled over from bTB-infected cattle just prior to 2005, and that deer-deer transmission

was absent or minimal, we would expect only this older age cohort of deer to be infected. The only outlier to this theory was the 27th bTB-infected deer born in 2006; however, this deer could have been exposed to the remaining infected cattle herds that were discovered in 2006–2009 or was an isolated occurrence of deer-deer transmission. Also, epidemiological modeling in Michigan has demonstrated a sex-based bias in infected deer, with adult males more likely to have bTB than adult females [25]. We did not observe a sex-based difference in infection rates, as both sexes were equally represented in bTB-infected individuals.

Our aerial survey data indicated a 55% reduction in deer densities within the bTB Core Area. During winters 2008–2010, the number of deer harvested by sharpshooting exceeded the early winter population estimates. While it is, of course, not possible that deer removal efforts killed >100% of the deer in the bTB Core Area, it is likely that deer were continually moving in and out of this area. There was no physical barrier to prevent movement in or out of the bTB Core Area, but immigration and emigration rates are unknown. Further, deer surveys estimated the deer population at one point in time (typically late Jan or early Feb), while the sharpshooting efforts began in late February and continued through April. It is likely that deer migration from winter range to spring-summer-fall ranges accounted for the increased movement activity noted in April, although migration rates are also poorly understood for this portion of the state.

While fall deer harvest likely contributed to the reduction of deer within the bTB Core Area, total registration within the special disease management unit dropped nearly 50% from 2007 to 2008, and remained at that reduced level through 2010. This is likely due to fewer deer being available to hunters because of winter sharpshooting. Hunters also perceived a negative effect of winter sharpshooting on deer available to harvest, thus hunting pressure was reduced [26]. Increased hunting opportunities, including special seasons and low cost bonus tags, did not effectively increase deer harvest. This was similar to experiences reported in other states, including Michigan, Wisconsin, and Illinois, where hunting opportunities were liberalized to encourage increased deer harvest for disease management [8, 27].

Efforts to restrict recreational feeding have been very successful. Since the ban were instituted in 2006, reports of illegal feeding activities have dropped markedly. Concurrent efforts to increase enforcement of baiting regulations likely increased compliance with feeding restrictions as well. In Michigan, regulatory efforts to restrict feeding and baiting have met with mixed success [28]. Much of the large-scale feeding activities there have subsided, but some smaller, covert feeding activities persist [8].

On the cattle side, the main action that dramatically reduced bTB transmission potential was the cattle buy-out program. A monumental effort to obtain adequate funding and producer buy-in to accept this voluntary program and remove 6,200 cattle from the bTB Management Zone. This $4.6 million dollar program offered the farmer $500 in addition to the slaughter value of each cow, and a $75/head annual stipend to remain out of the cattle business until the

state regains its bTB-free accreditation. Further, a 10 : 90 cost share was provided to farmers to erect deer exclusion fencing around stored feed or cattle feeding areas up to a maximum of $75,000. In 2009, 34 fences equating to 22,500 m of fencing material were installed to mitigate risk of disease between cattle and wildlife, at a cost of $815,000 to the state. Wildlife risk assessments were also conducted on all farms within the Split-state Zone [29]. Although these risk assessment were useful tools to educate farmers on practices that can mitigate risk of disease transmission between cattle and wildlife, it provided recommendations, but did not impose requirements. The only required risk mitigation practices apply to cattle farmers that receive state-sponsored fencing, where compliance is enforced and fences are inspected twice annually.

4.5. Evaluation of Efforts and Adjusting As Needed. Thus far, efforts to eradicate bTB in Minnesota have been costly. The USDA has spent nearly $70 million, BAH $12.5 million, and MNDNR $3.5 million on bTB-related expenditures [29]. The average costs for hunter-harvested deer surveillance were $350,000/year and sharpshooting ranged from $250,000 to $600,000/year, depending on whether aerial gunning was used to supplement ground removal efforts.

When MNDNR began sharpshooting deer, the public accepted the action as necessary to eradicate the disease in deer, but it was not popular. However, as the sharpshooting program persisted through 4 consecutive winters, and fewer bTB-infected deer were found each year, public tolerance declined markedly. This was of particular concern, because our primary method for disease surveillance was through hunter-harvested sampling, a voluntary process that required the cooperation of hunters. A loss of public support for MNDNR's management of bTB in deer could have made disease monitoring notably more challenging. In 2010, MNDNR was able to successfully negotiate with USDA for a reduced sampling requirement, which meant surveillance goals could be achieved through hunter-harvested surveillance alone, without the need for sharpshooting to augment sample size. Public distaste for sharpshooting motivated hunters to increase their participation in fall bTB surveillance. In fall 2010, hunters provided over 1.5x the required sample needed to satisfy the bTB sampling requirements. With sampling requirements met and no obvious cases of bTB evident in the hunter-harvested deer, no sharpshooting occurred in winter 2011.

Given all the aggressive management efforts that have occurred, did we effectively reduce bTB transmission potential? Substantial reductions in both deer densities and cattle numbers occurred from 2005 to 2010. Recreational feeding and baiting activities have declined and separation of cattle and deer on farms has improved through enhanced risk mitigation practices. Further, ongoing bTB surveillance of deer has demonstrated a decline in disease prevalence and no geographic spread of infected individuals. Bovine TB has not been found in a cattle herd since January 2009, with over a year of continued testing of herds. These results are encouraging, yet we must be cautious about prematurely pronouncing bTB eradicated in Minnesota. Continued surveillance is needed to confirm the absence of this disease in Minnesota's deer.

5. Conclusions

Minnesota's approach to bTB control employed the same basic intervention strategies as Michigan, namely, reducing transmission potential by reducing deer densities and restricting feed/baiting activities. Yet results have been very different. When active disease control was initiated in Michigan, deer were likely already a bTB reservoir, but in Minnesota, deer were likely spillover hosts. Minnesota had other key advantages from the onset of the disease outbreak. Deer densities were 6–8 times less than in Michigan, and Minnesota's bTB Core was 3 times smaller in size. Further, 60% of Minnesota's bTB Core was publically owned which facilitated aggressive deer removal efforts; Michigan's core area is only 10% public. Baiting and feeding of deer in Michigan persist, despite regulations; whereas these activities are under control in Minnesota. The MNDNR also had adequate dedicated funding in hand for initial disease management needs. Finally, public tolerance can exert a significant effect on potential disease management strategies, and thus the overall success of control efforts [30]. Minnesota was fortunate to have support from the public, industry, sporting groups, and key political champions to aggressively manage the bTB outbreak.

References

[1] R. S. Morris, D. U. Pfeiffer, and R. Jackson, "The epidemiology of *Mycobacterium bovis* infections," *Veterinary Microbiology*, vol. 40, no. 1-2, pp. 153–177, 1994.

[2] L. A. L. Corner, "The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 303–312, 2006.

[3] V. Naranjo, C. Gortazar, J. Vicente, and J. de la Fuente, "Evidence of the role of European wild boar as a reservoir of *Mycobacterium tuberculosis* complex," *Veterinary Microbiology*, vol. 127, no. 1-2, pp. 1–9, 2008.

[4] V. De Vos, R. G. Bengis, N. P. J. Kriek et al., "The epidemiology of tuberculosis in free-ranging African buffalo (*Syncerus caffer*) in the Kruger national park, South Africa," *Onderstepoort Journal of Veterinary Research*, vol. 68, no. 2, pp. 119–130, 2001.

[5] J. S. Nishi, T. Shury, and B. T. Elkin, "Wildlife reservoirs for bovine tuberculosis (*Mycobacterium bovis*) in Canada: strategies for management and research," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 325–338, 2006.

[6] R. J. Delahay, A. N. S. De Leeuw, A. M. Barlow, R. S. Clifton-Hadley, and C. L. Cheeseman, "The status of *Mycobacterium bovis* infection in UK wild mammals: a review," *The Veterinary Journal*, vol. 164, no. 2, pp. 90–105, 2002.

[7] J. D. Coleman and M. M. Cooke, "*Mycobacterium bovis* infection in wildlife in New Zealand," *Tuberculosis*, vol. 81, no. 3, pp. 191–202, 2001.

[8] D. J. O'Brien, S. M. Schmitt, S. D. Fitzgerald, D. E. Berry, and G. J. Hickling, "Managing the wildlife reservoir of *Mycobacterium bovis*: the Michigan, USA, experience," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 313–323, 2006.

[9] L. Glaser, *Minnesota's Strategy for Eliminating Bovine Tuberculosis*, Minnesota Board of Animal Health, St. Paul, Minn, USA, 2008.

[10] B. Harris, *Mycobacterium Bovis Genotyping Report*, National Veterinary Services Laboratory, Ames, Iowa, USA, 2009.

[11] M. Carstensen, D. Pauly, M. W. DonCarlos, and L. Cornicelli, "Managing bovine tuberculosis in free-ranging white-tailed deer in northwestern Minnesota: a 2007 progress report," in *Summaries of Wildlife Research Findings 2007*, pp. 1–9, Minnesota Department of Natural Resources, Wildlife Population and Research Unit, St. Paul, Minn, USA, 2008.

[12] United States Department of Agriculture, Bovine Tuberculosis Eradication: Uniform Methods and Rules, Effective, January 2005, http://www.aphis.usda.gov/animal_health/animal_diseases/tuberculosis/downloads/tb-umr.pdf.

[13] M. V. Palmer and D. L. Whipple, "Survival of *Mycobacterium bovis* on feedstuffs commonly used as supplemental feed for white-tailed deer (*Odocoileus virginianus*)," *Journal of Wildlife Diseases*, vol. 42, no. 4, pp. 853–858, 2006.

[14] L. Glaser, *Minnesota Bovine Tuberculosis Eradication Program: Application for Split-State Status*, Minnesota Board of Animal Health, St. Paul, Minn, USA, 2008.

[15] E. H. Hildebrand, M. Carstensen, E. Butler, and L. Cornicelli, "Preliminary results from herd health assessment for northwestern free-ranging elk from 2004–2009," in *Summaries of Wildlife Research Findings 2009*, pp. 135–149, Minnesota Department of Natural Resources, Wildlife Population and Research Unit, St. Paul, Minn, USA, 2010.

[16] F. F. Gilbert, "Aging white-tailed deer by annuli in the cementum of the first incisor," *Journal of Wildlife Management*, vol. 30, no. 1, pp. 200–202, 30.

[17] D. L. Stevens and A. R. Olsen, "Spatially balanced sampling of natural resources," *Journal of the American Statistical Association*, vol. 99, no. 465, pp. 262–278, 2004.

[18] B. Knust, P. C. Wolf, and S. Wells, "Use of an on-farm assessment to characterize risk," *American Journal of Veterinary Research*. In press.

[19] S. M. Schmitt, S. D. Fitzgerald, T. M. Cooley et al., "Bovine tuberculosis in free-ranging white-tailed deer from Michigan," *Journal of Wildlife Diseases*, vol. 33, no. 4, pp. 749–758, 1997.

[20] C. S. Bruning-Fann, S. M. Schmitt, S. D. Fitzgerald et al., "Bovine tuberculosis in free-ranging carnivores from Michigan," *Journal of Wildlife Diseases*, vol. 37, no. 1, pp. 58–64, 2001.

[21] T. C. Atwood, T. J. Deliberto, H. J. Smith, J. S. Stevenson, and K. C. Vercauteren, "Spatial ecology of raccoons related to cattle and bovine tuberculosis in Northeastern Michigan," *Journal of Wildlife Management*, vol. 73, no. 5, pp. 647–654, 2009.

[22] T. C. Atwood, K. C. Vercauteren, T. J. Deliberto, H. J. Smith, and J. S. Stevenson, "Coyotes as sentinels for monitoring bovine tuberculosis prevalence in white-tailed deer," *Journal of Wildlife Management*, vol. 71, no. 5, pp. 1545–1554, 2007.

[23] C. Sangster, D. Bergeson, C. Lutze-Wallace, V. Crichton, and G. Wobeser, "Feasibility of using coyotes (*Canis latrans*) as sentinels for bovine mycobacteriosis (*Mycobacterium bovis*) infection in wild cervids in and around riding Mountain National Park, Manitoba, Canada," *Journal of Wildlife Diseases*, vol. 43, no. 3, pp. 432–438, 2007.

[24] D. J. O'Brien, S. M. Schmitt, D. E. Berry et al., "Estimating the true prevalence of *Mycobacterium bovis* in hunter-harvested white-tailed deer in Michigan," *Journal of Wildlife Diseases*, vol. 40, no. 1, pp. 42–52, 2004.

[25] D. J. O'Brien, S. M. Schmitt, J. S. Fierke et al., "Epidemiology of *Mycobacterium bovis* in free-ranging white-tailed deer, Michigan, USA, 1995–2000," *Preventive Veterinary Medicine*, vol. 54, no. 1, pp. 47–63, 2002.

[26] M. Schermann and S. Wells, *Bovine Tuberculosis Transmission and Control Initiative: Final Report*, Center for Animal Health and Food Safety, University of Minnesota, St. Paul, Minn, USA, 2010.

[27] T. R. Van Deelen and D. R. Etter, "Effort and the functional response of deer hunters," *Human Dimensions of Wildlife*, vol. 8, pp. 97–108, 2003.

[28] B. A. Rudolph, S. J. Riley, G. J. Hickling, B. J. Frawley, M. S. Garner, and S. R. Winterstein, "Regulating hunter baiting for white-tailed deer in Michigan: biological and social considerations," *Wildlife Society Bulletin*, vol. 34, no. 2, pp. 314–321, 2006.

[29] B. Thompson, *Minnesota's Bovine Tuberculosis Management Plan*, Minnesota Board of Animal Health, St. Paul, Minn, USA, 2010.

[30] M. Carstensen, D. J. O'Brien, and S. M. Schmitt, "Public acceptance as a determinant of management strategies for bovine tuberculosis in free-ranging U.S. wildlife," *Veterinary Microbiology*. In press.

Research Article

Mycobacterium bovis in Swine: Spoligotyping of Isolates from Argentina

Soledad Barandiaran,[1] Marcela Martínez Vivot,[1] Eduardo Vicente Moras,[1] Angel Adrián Cataldi,[2] and Martín José Zumárraga[2]

[1] *School of Veterinary of Buenos Aires University, Chorroarín 280, C1427CWO, Buenos Aires, Argentina*
[2] *Biotechnology Institute, National Institute of Agricultural Technology (INTA), N. Repetto y De Los Reseros cc25, B1712WAA, Castelar, Buenos Aires, Argentina*

Correspondence should be addressed to Martín José Zumárraga, mzumarraga@cnia.inta.gov.ar

A total of 143 *Mycobacterium bovis* isolates of pigs, from the most productive swine area in Argentina, were typed by spoligotyping. Twenty-two different spoligotypes were identified, and 133 (93%) isolates were grouped into 12 clusters. One of them, designed SB0140, was the most frequent because it held 83 (58%) isolates. This spoligotype also grouped 362 (43%) out of 841 isolates from previously typed cattle and, thus, constitutes the most frequent in our country. In addition, 135 (94%) isolates revealed spoligotypes identical to those of cattle, showing an epidemiological link. On the other hand, there were seven novel spoligotypes, six of which were also unique since they had only one isolate each. This study aimed to identify the spoligotypes of *M. bovis* isolated from pigs to contribute to a better understanding of the distribution of bovine tuberculosis in the main productive area of Argentina.

1. Introduction

Bovine tuberculosis (BTB) is a major livestock disease in Latin America. About 70% of the cattle bred are held in areas with high disease prevalence and nearly 17% in areas virtually free from tuberculosis [1].

In Argentina, between 1969 and 2004, an average of 10 million bovine carcasses was annually submitted to official veterinary inspection. During that period, the percentage of animals condemned for tuberculosis decreased from 6.7% to 1.2% [1]. Regarding human tuberculosis, the disease caused by *Mycobacterium bovis* and that caused by *M. tuberculosis* are clinically indistinguishable. A recent work has described an incidence ranging from 0.7% to 6.2% in a main milk region of Argentina where most patients were closely related to rural activities, and a much lower national prevalence [2]. Between 1980 and 2003, the incidence of tuberculosis in Santa Fe, a province with 21% of the national dairy herd and 34% of the nation's milk production, declined from 48.1 to 20.7 per 100,000 [1]. However, the number of cases due to *M. bovis* remained stable, thus suggesting the relatively constant risk of infection [1]. In the Muñiz Hospital of Buenos Aires city, the percentage of *M. bovis* cases not associated with HIV infection significantly decreased from 0.95% between 1981 and 1991 to 0.22% between 2000–2006, whereas in the HIV/AIDS group the decrease was less pronounced (0.83% to 0.58%) [3]. The first case of person-to-person transmission of *M. bovis* in Argentina has been recently described [4].

In the United States, BTB was the most prevalent infectious disease in bovines and pigs between the XVIII century and the beginning of the XIX century, and the production loss was higher than that caused by all other diseases grouped [5].

Pigs are susceptible to *M. tuberculosis*, *M. bovis*, and the *M. avium* complex. In Argentina, the information about incidence and prevalence of tuberculosis in pigs is scarce. However, *M. bovis* is considered the primary cause of their

infection. The prevalence of lesions found in slaughterhouses from the Pampa Húmeda region during three samplings performed in 1986, 1995, and 1997, were 6.4%, 1.2%, and 0.1%, respectively, and *M. bovis* was the bacterial agent most frequently isolated in the three cases [6]. In countries where BTB has not been eradicated, such as in Argentina, pigs often become infected from cattle by oral route through ingestion of milk or other dairy products [6, 7]. Therefore, in Argentina, swine tuberculosis underwent a decline parallel to that observed in local cattle [1, 6, 8] because the eradication of BTB from cattle is the main action to avoid the transmission to swine and other hosts. Thus, the percentage of pigs condemned for tuberculosis decreased from 8.4 to 0.7% between 1969 and 2005 [9]. This panorama of the disease is also attributed to the modernization and intensification of breeding systems, which has improved the swine health conditions [9, 10]. Furthermore, in February 2009, pigs were incorporated to the National Control and Eradication Program of Bovine Tuberculosis, implemented for bovines since 1999 [11, 12]. This new resolution considers that those herds with negative skin test results with PPD once a year and the absence of tuberculosis lesions during carcass inspections in slaughterhouses are free of infection [11]. Although the skin test with PPD has a limited usefulness for individual animal diagnosis, it is a good tool to detect infected herds [13].

Approximately 80% of the swine production in Argentina is concentrated in the central region (Buenos Aires, Santa Fe, and Córdoba provinces), and it is closely related to maize production, because pigs are fed mainly on corn (65%) and soy flour (25%).

Molecular epidemiology is a helpful tool that contributes to the understanding of the dynamics of the distribution and spreading of BTB between the different hosts. One of the techniques that has improved and simplified the typing of the *M. tuberculosis* complex is spoligotyping [14]. Spoligotyping is a PCR-based method complemented with reverse line blot hybridization, in which the polymorphism consequence of rearrangements of the direct repeat (DR) region, which is composed of perfect direct 36-bp repeats and variable spacers [14]. The combination of the presence and absence of each 43 representative spacer sequences, shown with spots, represents a spoligotype.

The spoligotyping database of the Biotechnology Institute (BI) of INTA, Argentina, contains the spoligotypes of 1188 *M. bovis* isolates from different hosts from Argentina typed since 1996 and constitutes a valuable source of molecular epidemiology information that may help to contribute to the eradication program of bovine tuberculosis.

The aim of this work was to determine the *M. bovis* spoligotypes circulating among pigs from the main porcine productive region of Argentina.

2. Materials and Methods

A total of 143 isolates of *M. bovis* were obtained from culture of lymph nodes and viscera samples with tuberculosis compatible lesions from pigs from Buenos Aires ($n = 66$),

FIGURE 1: Map of Argentina. The provinces sampled are denoted in a gray scale according to the number of isolates.

Córdoba ($n = 44$), Entre Ríos ($n = 6$), La Pampa ($n = 3$), Mendoza ($n = 1$), and Santa Fe ($n = 23$) provinces (Figure 1), following a convenient sampling. Samples were obtained from the veterinary inspection of 35,000 pigs, between July 2007 and November 2008. The slaughterhouses involved in this sampling were located in Merlo, Moreno, and Tandil cities from Buenos Aires province. The samples were decontaminated using the Petroff's method and cultured in Lowenstein-Jensen and Stonebrink media at 37°C for 60 days [15]. The bacteriological typing of the isolates was performed based on the culture media, incubation temperature, growing time, colony morphology, and Ziehl-Neelsen stain.

Each *M. bovis* strain was isolated from independent animals without apparent epidemiological linkage. A loopful of colonies was transferred into a microcentrifuge tube containing $250\,\mu$L of distilled water and heated at 96°C for 45 min. Colonies were then centrifuged at 12,000 rpm for 10 min, and $5\,\mu$L of the supernatant was used for PCR to amplify the DR region. Spoligotyping [14] was carried out by using the spoligotyping kit (Isogen Biosolutions B.V., Ocimun Biosolutions Company, Ijsselstein, the Netherlands). *M. tuberculosis* H37Rv (ATCC 27294) and *M. bovis* Bacillus Calmette-Guerin (BCG) (ATCC 27289) were included as reference strains in each spoligotyping
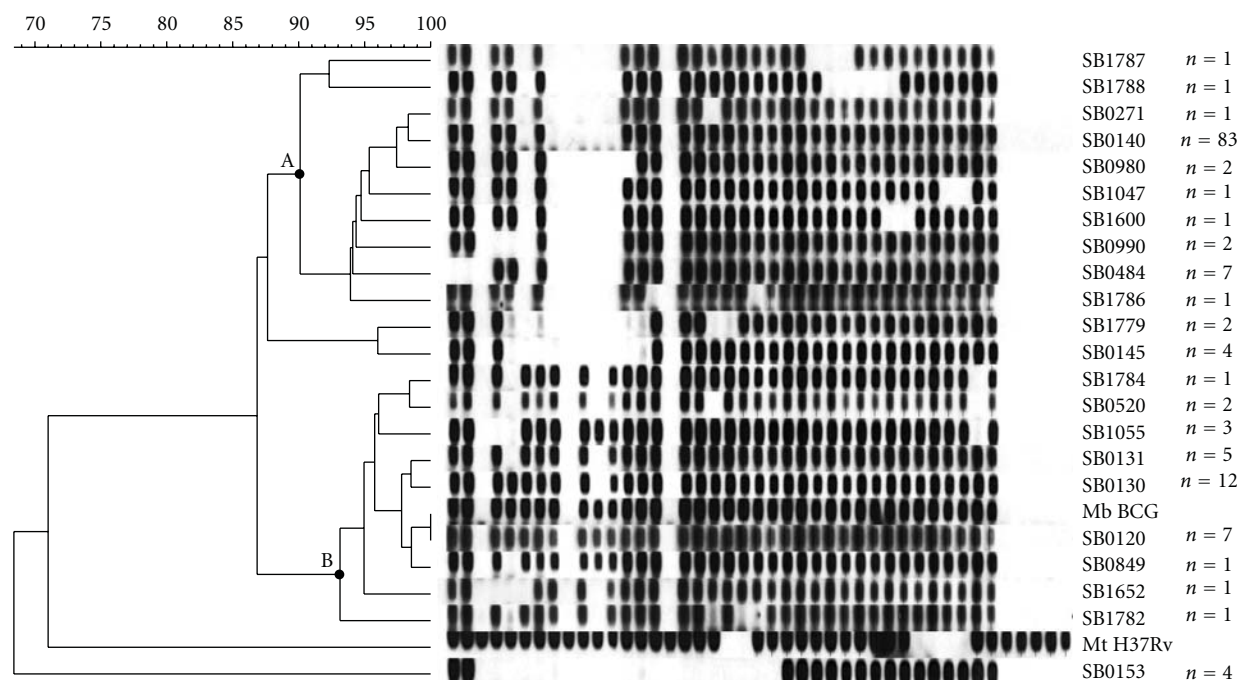
FIGURE 2: Dendrogram showing the relationship between 22 different spoligotypes identified among 143 *M. bovis* isolates of pigs from Argentina. The spoligotypes are detailed on the right of each pattern. The two families of spoligotypes were designated with letters A and B. *n*: number of isolates.

experiment. A cluster analysis of the spoligotype patterns was performed with the BioNumerics software (Windows NT, version 2.5; Applied Maths, Kortrijk, Belgium). The categorical coefficient was used to calculate the similarity of spoligotype patterns, and the UPGMA (unweighted pair-group method with arithmetic averages) method was applied to calculate a dendrogram. Clusters of isolates were defined as two or more *M. bovis* strains with identical spoligotypes. The different spoligotypes were compared against those included in the BI database, and each was assigned a number (SB code) according to the international database of the Veterinary Laboratories Agency (VLA), the United Kingdom (hosted on University of Sussex servers, http://www.mbovis.org/). When a spoligotype was not found in these databases, it was considered a novel spoligotype. Spoligotypes with only one isolate were regarded as unique spoligotypes. The discriminatory index (D) described by Hunter and Gaston and expressed by the formula of Simpson [16] was calculated to determine the discriminatory power of the spoligotyping in each province (http://insilico.ehu.es/) [17].

3. Results

All the isolates analyzed ($n = 143$) lacked spacers 3, 9, 16, and 39 to 43, characteristic of *M. bovis* strains. Twenty-two different spoligotypes were found among the 143 *M. bovis* isolates studied (Figure 2 and Table 1). One hundred thirty-three (93%) strains were grouped in 12 clusters; one of them (SB1779) was a novel spoligotype because it had not been previously reported among isolates of other hosts

from Argentina or in the VLA database. The main cluster involved 83 (58%) isolates and showed spoligotype SB0140 followed by spoligotype SB0130 with 12 (8.4%) isolates. The remaining spoligotypes grouped seven or fewer isolates. Ten (7%) isolates were unique, and seven of them were also novel (SB0849, SB1600, SB1652, SB1784, SB1786, SB1782, and SB1779) (Figure 2 and Table 1). Additionally, the cluster with the novel spoligotype (SB1779) held two isolates from the cities of Río Cuarto and General Viamonte, both in Córdoba province, which are approximately 150 km from each other. The global discriminatory power of the spoligotyping in this study was 0.65. This index was also individually calculated for each province (Table 1), being 0.62, 0.80, 0.60, and 0.40, for Buenos Aires, Córdoba, Entre Ríos and Santa Fe, respectively. These provinces provided most isolates. These results are related to the size of the clusters and the number of spoligotypes.

The dendrogram grouped the spoligotypes into two main families designed A and B, related to a similarity higher than 90% and 93%, respectively (Figure 2). The inclusion of the two most frequent spoligotypes from Argentina (SB0140 and SB0130) in each family is the main feature of these families. Moreover, 70% and 23% of the isolates were grouped in families A and B, respectively.

4. Discussion

Five of the six provinces studied are located in the most productive swine area in Argentina. When we compared the spoligotypes in the BI database, we found that 135 (94%) isolates revealed spoligotypes identical to bovine isolates,

Table 1: Number of isolates of each spoligotype by origin. The novel spoligotypes are in italic. D: discriminatory index. S: number of spoligotypes.

Province number of isolates (%)	Number of isolates by spoligotype																						D	S
	0120	0130	0131	0140	0145	0153	0271	0484	0520	*0849*	*0980*	*0990*	*1047*	*1055*	*1600*	*1652*	*1779*	*1782*	*1784*	*1786*	*1787*	*1788*		
Buenos Aires 66 (46.2%)	5	7	1	40 (60.6%)	1	2		2	1			1	1	1	1	1		1		1			0.62	15
Córdoba 44 (30.7%)	1	5	3	19 (43.2%)	1	2	1	3			2			2			2		1		1	1	0.80	14
Entre Ríos 6 (4.2%)				4 (66.7%)				1	1														0.60	3
La Pampa 3 (2.1%)				2 (66.7%)				1															0.66	2
Mendoza 1 (0.7%)					1																		1	1
Santa Fe 23 (16.1%)	1		1	18 (78.3%)	1					1		1											0.40	6
Total 143	**7**	**12**	**5**	**83**	**4**	**4**	**1**	**7**	**2**	**1**	**2**	**2**	**1**	**3**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**0.65**	**22**

showing an epidemiological link. Furthermore, seven of twenty two (32%) of the spoligotypes detected in pig isolates were detected in *M. bovis* human isolates. Conversely, seven (31.8%) spoligotypes had not been previously detected in cattle or other hosts from Argentina. Moreover, these spoligotypes were not reported in the VLA database. This finding could be due to the partial screening of the bovines with BTB in Argentina or to the existence of *M. bovis* clones circulating exclusively among pigs. Parra et al. [18] also described swine spoligotypes not detected previously in other hosts.

The main cluster detected (SB0140) grouped 43% of the 841 bovine *M. bovis* isolates from Argentina typed between 1996 and 2009 [19, 20]. This spoligotype is also frequent in Australia and New Zealand, which, like Argentina, introduced several cattle breeds in the 19th century from the United Kingdom, where this spoligotype is also prevalent [21]. Furthermore, spoligotype SB0140 was the most frequent in Buenos Aires (60.6%), Córdoba (43.2%), Entre Ríos (66.7%), and Santa Fe (78.3%) provinces, where most *M. bovis* isolates from pigs were obtained. Additionally, this spoligotype grouped 32.5, 38.9, 62.5, and 52.5% of bovine isolates of these provinces, respectively. These provinces concentrate most of the dairy farms from Argentina. Clustering of isolates has been described as an indication of active transmission of BTB [19]. Spoligotype SB0153, which was 68% related to the other types, was not integrated in families A and B and had been previously detected in only 2.7% of total *M. bovis* isolates from Argentina. Curiously, 34% of all the *M. bovis* isolates with spoligotype SB0153 belong to humans.

Taking into account the localization of most of the lesions along the digestive tract of sampled pigs (data not shown), we suggest that the infection via was the digestive route. This could be due to the fact that in Argentina pigs diets are usually supplemented with milk or other dairy derivates without thermal treatment.

Other authors have also found *M. bovis* spoligotypes of cattle in domestic pigs and other hosts (red deer and wild boar), which suggests transmission between species [18, 22, 23].

Spoligotyping is the best option for large-scale screening studies on the distribution of *M. tuberculosis* complex strains [14] and is used worldwide as the first-option typing method for *M. bovis*. Moreover, this technique is useful to identify new types from different host species [24, 25] and to detect preliminary transmission of TB between species. In order to trace transmission chains, it is necessary to perform complementary studies using more discriminatory typing methods such as the recently described VNTRs [26–29].

Future studies must be directed to evaluate the virulence and the fitness of these strains isolated from pigs, since transmission between different host species could be a selective force to increase the virulence of microorganisms. In a previous work carried out in a murine model of tuberculosis, we demonstrated that a particular *M. bovis* strain isolated from a wild boar was the most virulent compared to the *M. bovis* AN5 reference strain and other isolated from cattle and humans [30].

Considering the incidence of *M. bovis* in humans and the prevalence of the disease in cattle, especially in the provinces that hold most dairy herds, pigs could be an additional actor in the transmission chain of bovine tuberculosis to humans and cattle. This potential spillover could be controlled through the recent incorporation of swine to the National Control and Eradication Program of Bovine Tuberculosis of Argentina.

5. Conclusions

Most of the spoligotypes (68%) found in pigs had also been previously detected in cattle. There were seven novel spoligotype detected only in pigs. The most frequent spoligotype among *M. bovis* isolates studied from pigs (SB0140) was also the most prevalent in bovines from Argentina.

This work represents the first large-scale molecular typing study of *M. bovis* isolates from pigs carried out in Argentina and contributes to a better understanding of the features of tuberculosis in pigs in our country.

Acknowledgments

References

[1] I. N. De Kantor and V. Ritacco, "An update on bovine tuberculosis programmes in Latin American and Caribbean countries," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 111–118, 2006.

[2] A. Cataldi and M. I. Romano, "Tuberculosis caused by ohter members of the *M. tuberculosis* complex," in *Tuberculosis 2007 From Basic Science to Patient Care*, J. C. Palomino, S. C. Leao, and V. Ritacco, Eds., chapter 8, 1st edition, 2007.

[3] I. N. de Kantor, M. Ambroggi, S. Poggi et al., "Human *Mycobacterium bovis* infection in ten Latin American countries," *Tuberculosis*, vol. 88, no. 4, pp. 358–365, 2008.

[4] I. Etchechoury, G. E. Valencia, N. Morcillo et al., "Molecular typing of *Mycobacterium bovis* isolates in Argentina: first description of a person-to-person transmission case," *Zoonoses and Public Health*, vol. 57, no. 6, pp. 375–381, 2010.

[5] R. J. Montali, S. K. Mikota, and L. I. Cheng, "*Mycobacterium tuberculosis* in zoo and wildlife species," *OIE Revue Scientifique et Technique*, vol. 20, no. 1, pp. 291–303, 2001.

[6] A. Perez, R. Debenedetti, M. Martínez Vivot, A. Bernardelli, P. Torres, and V. Ritacco, "Tendencia de la tuberculosis porcina y validez de la inspección bromatológica para su detección en áreas de producción intensiva de Argentina," *Revue de Médecine Vétérinaire*, vol. 85, pp. 61–64, 2004.

[7] M. Martínez Vivot, D. Marticorena, M. Zumárraga et al., "Tuberculosis porcina, caracterización bacteriológica y molecular de micobacterias aisladas de cerdos faenados en La Pampa Húmeda," *InVet*, vol. 7, pp. 246–247, 2005.

[8] A. Perez and V. Ritacco, "Diagnóstico de la tuberculosis porcina en frigorífico," in *Jornadas de Divulgación Técnico Científicas*, Facultad de Ciencias Veterinarias, U.N.R., Casilda, Santa Fe, Argentina, 2001.

[9] P. Torres, "Situación de la tuberculosis bovina en la República Argentina," in *Programa Nacional de Control y Erradicación de la tuberculosis, SENASA (Servicio Nacional de Sanidad Animal)*, Secretaría de Agricultura, Buenos Aires, Argentina, 2006.

[10] L. A. Ottavianoni, A. Bernardelli, and R. J. Dubarry, "Aspectos sobre tuberculosis porcina en la provincia de La Pampa," *Veterinaria Argentina*, vol. 3, pp. 457–462, 1986.

[11] SENASA, 2009, Resolución 145/2009, http://www.senasa.gov.ar/contenido.php?to=n&in=1403&io=9279.

[12] Secretaría de Agricultura, Dirección de Sanidad Animal, Argentina, "Plan Nacional de Control y Erradicación de la Tuberculosis Bovina," Resolución no. 115/99 SENASA/SAGPyA, 1999.

[13] G. G. Magnano, M. O. Schneider, C. E. Urbani, A. Ambrogi, L. Zapata, and M. C. Jorge, "Comparación de técnicas diagnósticas de tuberculosis porcina en dos establecimientos de cría confinada en Argentina," *InVet*, vol. 12, no. 1, pp. 25–31, 2010.

[14] J. Kamerbeek, L. Schouls, A. Kolk et al., "Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology," *Journal of Clinical Microbiology*, vol. 35, no. 4, pp. 907–914, 1997.

[15] I. N. de Kantor, "Bacteriología de la tuberculosis N°11," CEPANZO OPS/OMS, Buenos Aires, pp. 26–35, 1988.

[16] P. R. Hunter and M. A. Gaston, "Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity," *Journal of Clinical Microbiology*, vol. 26, no. 11, pp. 2465–2466, 1988.

[17] S. Rodríguez, B. Romero, J. Bezos et al., "High spoligotype diversity within a *Mycobacterium bovis* population: clues to understanding the demography of the pathogen in Europe," *Veterinary Microbiology*, vol. 141, no. 1-2, pp. 89–95, 2010.

[18] A. Parra, J. Larrasa, A. García, J. M. Alonso, and J. Hermoso De Mendoza, "Molecular epidemiology of bovine tuberculosis in wild animals in Spain: a first approach to risk factor analysis," *Veterinary Microbiology*, vol. 110, no. 3-4, pp. 293–300, 2005.

[19] M. J. Zumárraga, C. Martin, S. Samper et al., "Usefulness of spoligotyping in molecular epidemiology of *Mycobacterium bovis*-related infections in South America," *Journal of Clinical Microbiology*, vol. 37, no. 2, pp. 296–303, 1999.

[20] M. J. Zumárraga, *Epidemiología molecular de la tuberculosis bovina*, Ph.D. thesis, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina, 2007.

[21] A. A. Cataldi, A. Gioffré, M. P. Santtangelo et al., "El genotipo de *Mycobacterium bovis* mayoritario en la Argentina lo es también en las Islas Británicas: la tuberculosis bovina provino de Gran Bretaña?" *Revista Argentina de Microbiologia*, vol. 34, no. 1, pp. 1–6, 2002.

[22] F. Milian-Suazo, B. Harris, C. A. Díaz et al., "Molecular epidemiology of *Mycobacterium bovis*: usefulness in international trade," *Preventive Veterinary Medicine*, vol. 87, no. 3-4, pp. 261–271, 2008.

[23] E. Costello, D. O'Grady, O. Flynn et al., "Study of restriction fragment length polymorphism analysis and spoligotyping for epidemiological investigation of *Mycobacterium bovis* infection," *Journal of Clinical Microbiology*, vol. 37, no. 10, pp. 3217–3222, 1999.

[24] M. J. Zumárraga, A. Bernardelli, R. Bastida et al., "Molecular characterization of mycobacteria isolated from seals," *Microbiology*, vol. 145, no. 9, pp. 2519–2526, 1999.

[25] A. Aranaz, E. Liébana, A. Mateos et al., "Spacer oligonucleotide typing of *Mycobacterium bovis* strains from cattle and other animals: a tool for studying epidemiology of tuberculosis," *Journal of Clinical Microbiology*, vol. 34, no. 11, pp. 2734–2740, 1996.

[26] S. Roring, A. Scott, D. Brittain et al., "Development of variable-number tandem repeat typing of *Mycobacterium bovis*: comparison of results with those obtained by using existing exact tandem repeats and spoligotyping," *Journal of Clinical Microbiology*, vol. 40, no. 6, pp. 2126–2133, 2002.

[27] R. A. Skuce, T. P. McCorry, J. F. McCarroll et al., "Discrimination of *Mycobacterium tuberculosis* complex bacteria using novel VNTR-PCR targets," *Microbiology*, vol. 148, no. 2, pp. 519–528, 2002.

[28] P. Supply, E. Mazars, S. Lesjean, V. Vincent, B. Gicquel, and C. Locht, "Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome," *Molecular Microbiology*, vol. 36, no. 3, pp. 762–771, 2000.

[29] R. Frothingham and W. A. Meeker-O'Connell, "Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats," *Microbiology*, vol. 144, no. 5, pp. 1189–1196, 1998.

[30] D. Aguilar León, M. J. Zumárraga, R. Jiménez Oropeza et al., "*Mycobacterium bovis* with different genotypes and from different hosts induce dissimilar immunopathological lesions in a mouse model of tuberculosis," *Clinical and Experimental Immunology*, vol. 157, no. 1, pp. 139–147, 2009.

Research Article

Descriptive Epidemiology of Bovine Tuberculosis in Michigan (1975–2010): Lessons Learned

Chika C. Okafor,[1, 2] Daniel L. Grooms,[1] Colleen S. Bruning-Fann,[3] James J. Averill,[4] and John B. Kaneene[1, 2]

[1] *Department of Large Animal Clinical Sciences, Michigan State University, East Lansing, MI 48824, USA*
[2] *Center for Comparative Epidemiology, Michigan State University, East Lansing, MI 48824, USA*
[3] *Veterinary Services, Animal and Plant Health Inspection Service, United States Department of Agriculture, East Lansing, MI 48823, USA*
[4] *Animal Industry Division, Michigan Department of Agriculture and Rural Development, Lansing, MI 48909, USA*

Correspondence should be addressed to Chika C. Okafor, okaforch@cvm.msu.edu

Despite ongoing eradication efforts, bovine tuberculosis (BTB) remains a challenge in Michigan livestock and wildlife. The objectives of this study were to (1) review the epidemiology of BTB in Michigan cattle, privately owned cervids, and wildlife between 1975 and 2010 and (2) identify important lessons learned from the review and eradication strategies. BTB information was accessed from the Michigan BTB Eradication Project agencies. Cattle herds (49), privately owned deer herds (4), and wild white-tailed deer (668) were found infected with BTB during the review period. BTB has occurred primarily in counties located at the northern portion of the state's Lower Peninsula. Currently used BTB eradication strategies have successfully controlled BTB spread. However additional changes in BTB surveillance, prevention, and eradication strategies could improve eradication efforts.

1. Introduction

After fifteen years of implementing the Michigan Bovine Tuberculosis Eradication Project, bovine tuberculosis (BTB) remains a challenge in Michigan livestock and wildlife. At least one BTB-infected cattle herd has been identified in Michigan annually since 1998. Because of this ongoing BTB challenge, regulatory requirements for cattle movements have affected cattle trade in Michigan. In addition, the state has spent approximately US$200 million on BTB eradication between 1994 and 2010 [1]. Annually, over US$7 million is spent on BTB surveillance in cattle alone. Additional resources are spent on indemnity payment, cleaning and disinfection of the premises of BTB infected cattle herds, wildlife surveillance, and implementation of other eradication strategies. Reviewing the epidemiology of the current BTB issues in Michigan could help advance BTB eradication strategies in Michigan, other regions of the country, or beyond.

BTB is a chronic bacterial disease caused by *Mycobacterium bovis*. *M. bovis* is primarily a pathogen of cattle but can also infect other mammals including humans. Among domestic animals, cattle are the primary reservoir. However, other animal species including deer, monkeys, European badgers, brush tailed opossums, and elephants have been shown to become endemically infected, thus serving as additional reservoirs for pathogen transmission [2].

BTB is mainly a respiratory disease and is transmitted primarily through aerosols [2, 3]. However, indirect transmission through ingestion of contaminated food items has been demonstrated in deer and cattle and is believed to be a major source of transmission in the current BTB outbreak in Michigan [4–7]. Although wildlife and domestic cattle commonly do not come in close physical contact with each other, transmission of *M. bovis* between domestic animals and wildlife has occurred over the years [8]. Domestic animals as well as wildlife are significant reservoir hosts for human tuberculosis, caused by *M. bovis*. The most common

means humans acquire BTB is through the consumption of unpasteurized or insufficiently cooked animal products from BTB-infected animal [9].

At the end of the twentieth century, tuberculosis was the leading killer of humans in the United States (US). During this time period, *M. bovis* was found to be distinct from *M. tuberculosis* and there was evidence that *M. bovis* could be passed between animals and humans, and that in humans, *M. bovis* produces symptoms that were clinically indistinguishable from *M. tuberculosis* [10]. Due to the public health and economic relevance of BTB, the US BTB Eradication Program began in 1917. The program included comprehensive testing of imported and US bred cattle, improved animal tracking, destroying skin test positive animals (reactors), strengthening meat inspection for tuberculosis lesions, and commercialization of milk pasteurization. This program proved highly effective in controlling the disease, and by the 1960s, the number of BTB-reactor cattle detected in the US had markedly declined [10].

In 1974, the last known BTB-infected cattle herd in Michigan was depopulated; however a BTB positive wild white-tailed deer was harvested by a hunter in the following year [11]. It was widely believed that the deer acquired the *M. bovis* infection as a spill-over from livestock. With no further identified cases of BTB in cattle, the state acquired BTB "accredited-free" status in 1979. At that time the extent of BTB in Michigan wildlife was unknown. A second BTB positive wild white-tailed deer was identified in 1994, nine miles from the location of the index case [11]. With this occurrence, the Michigan Bovine TB Eradication Project began in 1995. The project was charged with increasing BTB surveillance in wildlife as well as in cattle and privately owned cervid (captive or farmed deer) herds surrounding any identified BTB wild white-tailed deer. The project involves a multiagency team of experts from the Michigan Department of Agriculture (MDA), the US Department of Agriculture Animal and Plant Health Inspection Services (USDA APHIS), Michigan Department of Natural Resources (MDNR), Michigan State University (MSU), and Michigan Department of Community Health (MDCH). Wildlife surveys conducted in the spring and fall of 1995 detected further cases among wild white-tailed deer. In 1998, cases of BTB infection began to reemerge among cattle herds in the state. As a consequence, Michigan lost its BTB "accredited-free" status in June 2000 and dropped to a "modified accredited" status [12]. Despite active eradication efforts, cases of BTB are still found in Michigan cattle, privately owned cervids, and wild white-tailed deer.

The objective of this study was to conduct a comprehensive descriptive epidemiological review of BTB in cattle, privately owned cervids, and wild white-tailed deer from 1975 to July 2010. Although previous epidemiological studies on BTB in Michigan have evaluated BTB challenge in cattle [13], privately owned cervid herds[14], wildlife [15–21], or both livestock and wildlife [22], none has provided extensive descriptive account of BTB challenge in both cattle and wildlife alongside with the lessons learned since over 15 years of eradication efforts. By reviewing and understanding the epidemiology of the current disease problem combined with

a review of the strategies that have been implemented to eradicate the disease, important "lessons learned" can be identified, and new control strategies may emerge.

2. Materials and Methods

2.1. Sources of Data. Descriptive data on BTB in Michigan between 1995 and July 2010 were obtained from the partners of the Michigan BTB Eradication Project. Data on BTB infected cattle and privately owned cervid herds were obtained from MDA and USDA APHIS Veterinary Services (USDA APHIS VS). Data on BTB-infected wild white-tailed deer and other wildlife were obtained from Michigan Department of Natural Resources (MDNR) and USDA APHIS Wildlife Services (USDA APHIS WS).

2.2. Type of Data. The collected data comprised of host characteristics, geographical, and temporal distributions of the BTB-infected animals/herds. The host characteristics included type of herd operations (cattle), type of deer, origin of the infected animals into the herds, and herd size. The geographical distribution was limited to the county level. Annual records of BTB infection in animals/herds were used for temporal distribution. Additional information collected was the type of surveillance used to identify each BTB infected animal/herd, the type of BTB eradication used in each infected herd, the results of the epidemiological evaluation of the infected herds, and the various BTB eradication strategies/policies utilized by the Michigan BTB Eradication Project partners.

2.3. Data Analyses. The occurrence of BTB in cattle herds, privately owned cervids, and wild white-tailed deer was expressed either as incidence count, percentage proportion, incidence rate, herd prevalence, sample prevalence, or prevalence odds. Incidence count represented the total number of BTB-infected herds within the review period. Incidence rate was calculated as incidence count divided by the total population per year. In cattle herds, 12.5 years was used for the review time period (1998–July 2010). Captive cervid herd BTB prevalence was calculated as the number of BTB positive deer divided by the total number of deer in the herd. A BTB positive deer was classified as any deer bearing gross lesions consistent with BTB that tested positive for *M. bovis* on culture. Sample prevalence was calculated as the number of BTB positive wild white-tailed deer divided by the total number wild white-tailed deer tested. Prevalence odds of BTB infected wild white-tailed deer in an area were calculated as the probability that tested wild deer in the area were BTB positive (sample prevalence (p)) divided by the probability that the tested wild deer were not BTB positive (1-p).

3. Results

3.1. Area Description. The state of Michigan is located in the Upper Midwest region of the US. The state is made up of 83 counties and comprises two peninsulas: the Upper Peninsula (UP) and the Lower Peninsula (LP) (Figure 1).

Michigan covers approximately 37 million acres. There are approximately 43,000 miles of rivers and streams, 11,000 inland lakes, and over 4,500 miles of shoreline along the Great Lakes. A variety of forest, wetland, and grasslands provide habitat to over 15,000 native species of insects, 1,815 native species of vascular plants, and 691 native species of animals. Among animal species, 68 different native wild mammals have been identified including white-tailed deer (*Odocoileus Virginianus*), elk (*Cervus elaphus nelson*), black bear (*Ursus americanus*), coyotes (*Canis latrans*), opossum (*Didelphis virginiana*), bobcats (*Lynx rufus*), and red fox (*Vulpes vulpes fulva*) [23].

Livestock production is a significant part of the state's economy. Cattle are the most common livestock in the state and include dairy, cow-calf (beef), and feedlot operations. The 2007 agricultural census reported approximately 14,500 cattle herds in Michigan [24]. The cattle herd types (dairy, beef, mixed, and feedlot) and the total number of herds in BTB-affected counties are reported in Table 1. Other common domesticated livestock species include small ruminants such as sheep and goats, horses, swine, and poultry [24].

3.2. Cattle

3.2.1. BTB Surveillance in Cattle. Surveillance for BTB is primarily done through live animal skin testing and through tuberculosis lesion detection at slaughter facilities [25]. Common reasons for live animal testing include herd accreditation/reaccreditation, compliance with pasteurized milk ordinance (PMO) laws, herd surveillance in endemic areas of the state as required by Memorandums of Understanding with USDA ASPHIS VS, and tracing animals with lesions found on routine slaughter surveillance.

During herd surveillance, the caudal fold tuberculin (CFT) test is done on individual animals as a primary screening test [25]. All respondents (suspects) to the CFT and herd of origin are quarantined pending final classification as to whether any suspect animal is BTB infected or not. The suspects are subjected to a supplemental test, either the comparative cervical tuberculin (CCT) test or gamma interferon (γ-IFN) assay. An animal classified as CCT or γ-IFN positive responder either is designated as a "reactor" or remains a "suspect". A "suspect" animal is retested with the γ-IFN assay within 30 days or the CCT after 60 days, and if the animal is not "negative", it is automatically designated a "reactor". Reactors are purchased for diagnostic purposes, humanely euthanized, and necropsied by a veterinary pathologist [25].

At necropsy, the animal is visually inspected for possible BTB lesions. Samples are taken from lymph nodes of the head, thorax, and abdomen, as well as from all visible BTB-like lesions and submitted for further testing. Histopathological screening, acid-fast staining of tissues, polymerase chain reaction (PCR), and bacterial culture are tests that are routinely conducted on these samples. Either PCR or bacterial culture is the confirmatory test in BTB screening. If BTB is confirmed in an animal, its herd of origin is declared BTB-infected and will remain under quarantine; otherwise the herd is released from quarantine [25].

3.2.2. BTB Eradication in Cattle. In BTB-infected herds, there are two disease eradication options for the herd. One is complete herd depopulation. The other is to develop a whole herd testing and removal plan with the cooperation of the owner and governmental agencies while the herd remains under quarantine (test and remove program). This plan includes the serial performance of BTB ante mortem screening tests over time and the subsequent removal of all test positive animals as outlined in the Bovine Tuberculosis Eradication: Uniform Methods and Rules [26, 27]. The herd is released from quarantine when testing reveals a BTB negative herd after a minimum of 8 whole herd tests over approximately a 4-year period as outlined in the 1999 Uniform Methods and Rules [26]. However, USDA APHIS VS typically did not utilize a test and remove program as outlined in the 1999 Uniform Methods and Rules until Michigan found an infected dairy herd in 2000. Most infected dairy herds prefer this program to depopulation as it allows for continuation of operations and cash flow. However, depopulation remains the disease eradication of choice in most infected beef herds.

3.2.3. BTB-Infected Cattle Herds. BTB in cattle herds reemerged in Michigan in 1998 after the last known case of BTB in the state was depopulated in 1974. Comprehensive details of this BTB incident and key policy changes are detailed in Table 2. Between 1998 and July 2010, the USDA APHIS VS recorded a total of 49 BTB-infected cattle herds in Michigan. At least one BTB cattle herd has been identified yearly since 1998 with higher numbers of herds found during the period from 2000 through 2003, and the highest peak in 2001 with 8 BTB-infected herds discovered (Figure 2). Since 2004, the number of infected herds has fluctuated between 1 and 4 herds/year. The average number of cattle per BTB-infected beef and dairy herd was 84 and 147, respectively. The average size of BTB-infected herds was larger than the average Michigan herd size of 14 and 130 cattle per beef and dairy herd respectively [24]. The number of cattle in BTB infected herds ranged from 6 to 495 (Figure 3). Included in these 49 infected herds are six premises which were removed from quarantine and then discovered to be reinfected at a later date. These reinfected herds either had completed the test and remove program successfully ($n = 1$) or were depopulated ($n = 5$).

Geographically, BTB in cattle has only been found in 7 counties located in the Michigan's LP. The number of BTB-infected herds within the review period is represented in Figure 1. Within each county, the number of BTB-infected herds based on management operations is presented in Figure 4. The 6 reinfected herds comprised 1 dairy herd and 5 beef herds. The reinfected dairy herd was located in Montmorency County, while 3 beef herds were located in Alpena County, and 2 beef herds in Alcona County. Eleven herds had a previously BTB-infected herd located within a 3-mile radius: 6 were in Alcona county, 4 in Alpena, and 1 in Oscoda County. The BTB incidence rate per 1000 cattle herd-yrs ranged from 1.1 (Presque Isle) to 8.7 (Alcona) (Figure 5).
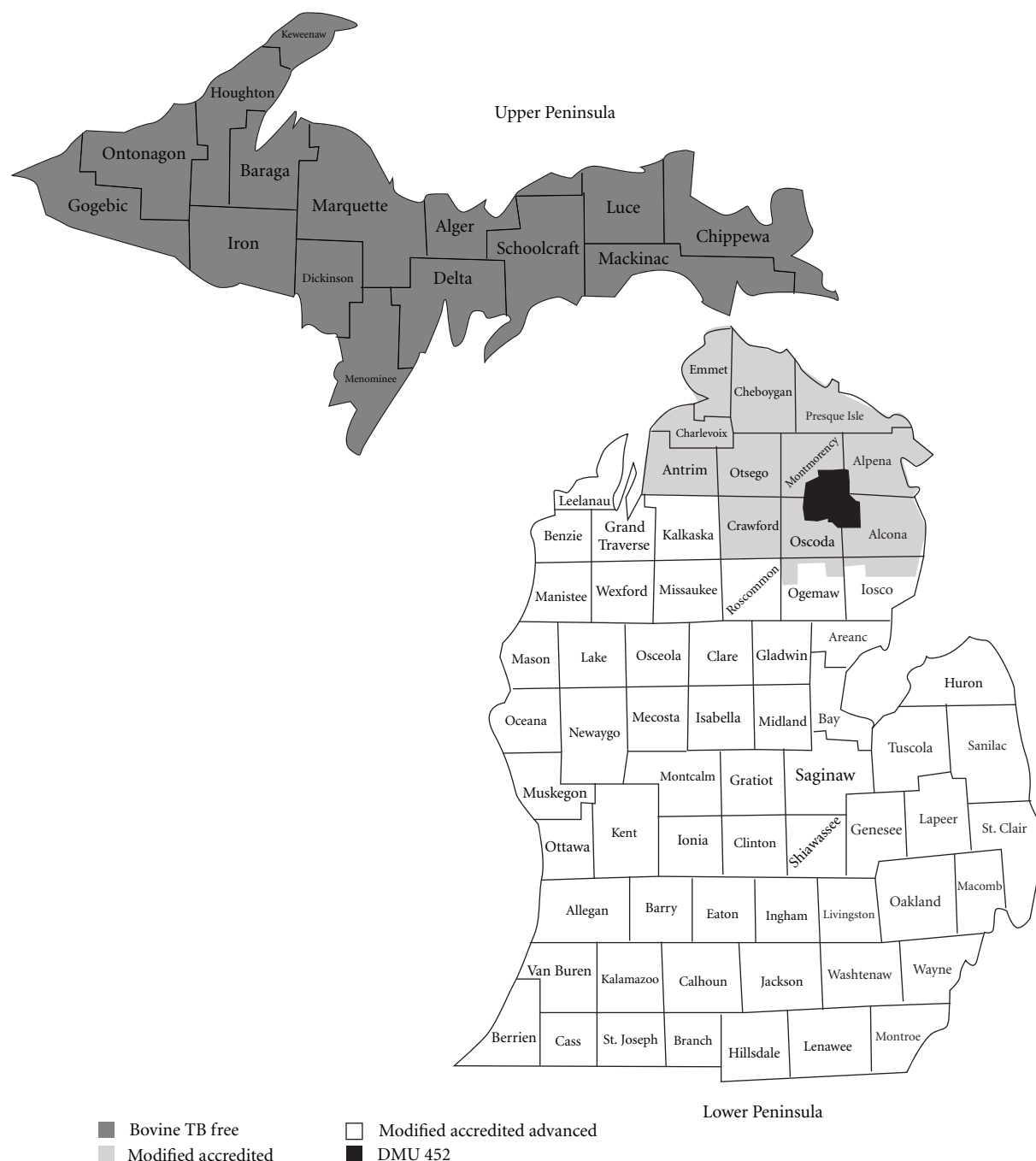
FIGURE 1: State of Michigan map showing the Upper and Lower Peninsula, counties, and BTB zones as of December 2009.

The overall number of cattle herds in each county and those found infected with BTB is presented in Table 1.

Between 1995 and July 2010, the USDA and MDA have conducted approximately 35,000 whole herd tests implemented as a part of the Michigan Bovine TB Eradication Project. During these whole herd tests, 46 of the 49 BTB infected herds (94%) were identified. One herd was identified through each of the following: movement testing, slaughter surveillance, and trace testing (investigation of herds which moved cattle to and from BTB infected herds).

Of the 49 BTB infected herds, 47 herds had a history of purchasing or moving cattle into their herd prior to being found BTB infected (open herds). The other two herds had no history of introducing new animals into the herd prior to being found BTB infected (closed herds). In regards to BTB infected animals, 147 cattle presented at necropsy with gross lesions compatible with BTB (mode = 1, median = 1, and range = 0–32), 149 cattle had histopathological lesions compatible with *M. bovis* infections (mode = 1, median = 1, and range = 0–30), 104 cattle were confirmed BTB positive

TABLE 1: Descriptive epidemiology of BTB in MI cattle, privately owned and wild white-tailed deer (1975–July 2010).

County	BTB (+) cattle herds[a]	Total cattle herds[b]	BTB Incidence rate/1000 cattle herd-yrs[a]	BTB (+) privately owned cervid herds[a]	BTB (+) wild white-tailed deer[c]	Total wild white-tailed deer tested[c]	Prevalence odds of BTB (+) wild white-tailed deer[c]
Alcona	13	119	8.7	0	240	18,451	0.0132
Alpena	21	231	7.3	0	186	18,776	0.0100
Montmorency	4	87	3.7	3	130	12,027	0.0109
Oscoda	3	80	3.0	0	74	9,624	0.0077
Presque Isle	2	140	1.1	1	13	9,404	0.0014
Antrim	3	98	2.4	0	1	5,133	0.0002
Emmet	3	135	1.8	0	2	3,413	0.0006
Others[d]	0	13,564	0	0	22	107,441	0.0002

[a] Data 1998–July 2010. [b] Data from the 2007 Michigan Agricultural census [24]. [c] Data 1975–July 2010. [d] Other counties in Michigan.
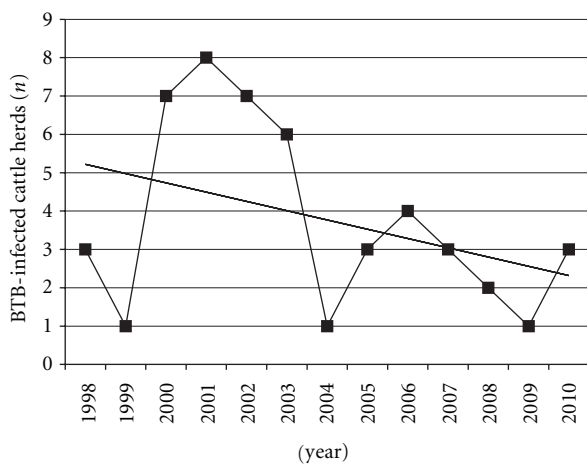
FIGURE 2: Annual incidence count of BTB cattle herds in Michigan (with linear trend line).
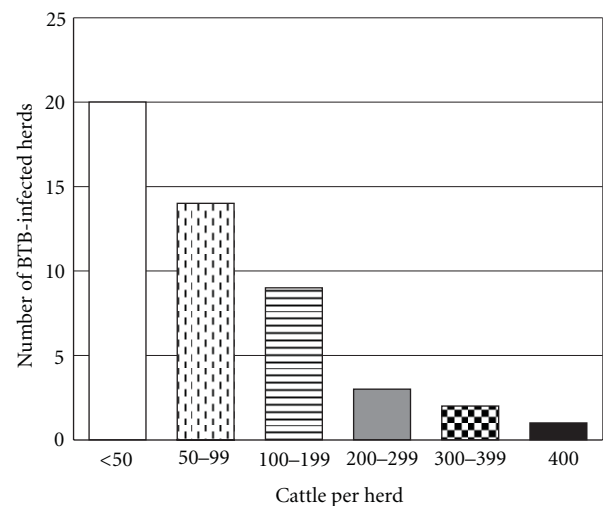
FIGURE 3: Herd sizes of BTB-infected cattle herds in Michigan, 1998–July 2010.

using PCR (mode = 1, median = 1, and range = 0–24), and 137 cattle were culture positive for *M. bovis* (mode = 1, median = 1, and range = 0–28). Out of 137 cattle confirmed through bacterial culture, 84% were raised on the farm, 7% were from unknown origin, and 9% were moved onto the farms. From the moved animals, 2 were purchased from other states, one from Ohio and the other from Texas; all others were moved through either intrastate purchases or leasing/borrowing of bulls.

The 5 most heavily BTB infected herds (greater numbers of *M. bovis* culture positive cattle) were located in the following counties: Alpena (28 cattle), Emmet (27 cattle), Alpena (9 cattle), Montmorency (9 cattle), and Presque Isle (6 cattle). These herds were each located in different counties and epidemiological investigations indicated that wildlife exposure was the most likely source of BTB transmission into the herd. The likely explanation for the higher disease prevalence in these herds is differences in individual herd management. Management practices that lead to closer confinement of cattle or sharing of focused feed and water sources are more likely to lead to intraherd transmission.

Cattle movement was believed to be the source BTB infection in the herd identified via trace testing. The source of BTB in the six infected herds located in Emmet ($n = 3$) and Antrim ($n = 3$) counties remains problematic. One herd brought no animals into the herd. Two herds brought in cattle from counties with a low prevalence of BTB in wild deer. The remaining three farms brought in cattle from counties with both high and low BTB prevalence in wild deer; however, on 1 farm the only BTB-affected animal originated from a BTB-free area. All cattle moving out of high prevalence areas were tested prior to movement for BTB with negative results. Regardless of location, all herds that were a source of livestock to BTB-affected farms had whole herd tests with no additional BTB found. In addition, all cattle farms adjoining BTB-affected farms were tested with no additional BTB-affected herds found. Finally, there is a low sample prevalence of BTB in wild white-tailed deer in these two counties (Figure 5). In one area with a high BTB prevalence in wild deer (Alcona County), five BTB infected herds had multiple possible routes of exposure. Each of the five herds shared fence-line contact

TABLE 2: Timeline of BTB in Michigan (1975–July 20010).

Time	Event
1974	(i) Last known BTB-infected cattle herd in Michigan depopulated
1975	(i) BTB-infected wild white-tailed deer harvested by a hunter in Alcona County
1979	(i) State of Michigan designated as BTB-accredited free
1994	(i) BTB-infected wild white-tailed deer harvested by a hunter in Alpena County
1995	(i) BTB surveillance of hunter killed wild white-tailed deer, cattle, and privately owned cervid herds in 16 km radius around location of 1994 BTB infected wild white-tailed deer was initiated (ii) MDNR conducted BTB surveillance in wild white-tailed deer within portions of Alcona, Alpena, Montmorency, and Oscoda counties (Deer Management Unit (DMU) 452) (iii) 18 of 403 (4.47%) wild white-tailed deer found infected with BTB (iv) Testing of all cattle and privately owned cervid herds located within 5 miles of any BTB positive wild white-tailed deer initiated
1996	(i) Statewide BTB surveillance in wild white-tailed deer and other wildlife began (ii) MDNR expanded BTB surveillance in wild white-tailed deer beyond DMU 452 to include all of Alcona, Alpena, Montmorency, and Oscoda counties (iii) Disease Control Permits issued (iv) 56 of 4,966 (1.13%) wild white-tailed deer found infected with BTB (v) 1 coyote found infected with BTB
1997	(i) The 1st privately owned white-tailed deer herd found infected with BTB (ii) 73 of 3,720 (1.96%) wild white-tailed deer found infected with BTB (iii) 2 coyotes found infected with BTB
1998	(i) 3 beef cattle herds found infected with BTB (ii) State of Michigan's BTB-free status suspended (iii) BTB testing of all cattle and cervid herds in 5-county area initiated (iv) Deer feeding banned, baiting restricted, and doe harvest increased in an Enforced Restricted Area (ERA) bordered by interstate road (I-75), state road (M-55), and shoreline of Lake Huron (v) DMU 452 expanded to encompass 5-county area (Alcona, Alpena, Montmorency, Oscoda, Presque Isle counties) (vi) Antlerless hunting permits issued liberally (1 per day) in the DMU 452 (vii) 78 of 9,057 (0.86%) wild white-tailed deer found infected with BTB (viii) 1 bear, 2 raccoons, and 2 coyotes found infected with BTB
1999	(i) Baiting regulation initiated in the northeastern Lower Peninsula of the state (ii) Unlimited antlerless hunting permits made available in the DMU 452 (iii) BTB testing for movement from any cattle herds East of I-75 and North of M-55 initiated (iv) 1 beef cattle herd found infected with BTB (v) 58 of 19,496 (0.3%) wild white-tailed deer found infected with BTB
2000	(i) Baiting/feeding of Deer and Elk banned in counties with BTB positive wild white-tailed deer (ii) Statewide official ear tag identification of cattle initiated (iii) Statewide BTB testing of all cattle herds by the end of 2003 initiated (iv) 2 dairy and 5 beef cattle herds found infected with BTB (v) State status dropped to Modified Accredited (vi) 53 of 25,858 (0.2%) wild white-tailed deer found infected with BTB
2001	(i) DMU 452 redefined to what it was in 1996 but the area shifted slightly east from the original 1996 DMU 452 (ii) USDA APHIS began fencing project on BTB high-risked cattle farms (iii) 8 beef cattle herds found infected with BTB (iv) 61 of 24,278 (0.25%) wild white-tailed deer found infected with BTB
2002	(i) BTB program changed: (a) Alcona, Alpena, Montmorency, Presque Isle counties—annual herd test of all cattle herds except feedlots was initiated. A negative BTB test required for movement of sexually active cattle if >6 months from whole herd test (WHT) (b) Cheboygan, Crawford, Iosco, Ogemaw, Oscoda, Otsego counties—Biennial WHT of all cattle herds except feedlots was initiated. A negative BTB test required for movement of sexually active cattle if >6 months from WHT

TABLE 2: Continued.

Time	Event
	(c) Antrim, Arenac, Charlevoix, Emmet, Gladwin, Kalkaska, Roscommon counties—2 WHT to be completed between 2000 and 2003. A negative BTB test required for movement of sexually active cattle if >6 months from WHT
	(i) Emmet County began annual WHT of all cattle herds except feedlots
	(ii) Antlerless hunting permits were increased for the northeast of the state
	(iii) 1 mixed, 2 dairy, and 4 beef cattle herds found infected with BTB
	(iv) 51 of 18,100 (0.28%) wild white-tailed deer found infected with BTB
2003	(i) Statewide WHT completed
	(ii) 2 dairy and 4 beef cattle herds found infected with BTB
	(iii) 32 of 17,302 (0.18%) wild white-tailed deer found infected with of BTB
2004	(i) State of Michigan acquired split state status: Modified Accredited Zone (MAZ) and Modified Accredited Advanced Zone (MAAZ)
	(ii) The gamma interferon assay approved for follow-up testing of caudal fold test suspects
	(iii) BTB program changed.
	(a) Annual WHT of all cattle herds in MAZ except feedlots initiated. Negative TB test for movement of sexually intact cattle if >60 days from WHT
	(b) Rest of Michigan-stratified random surveillance of 1500 herds every two years was initiated
	(iii) 2 dairy cattle herds found infected with BTB
	(iv) 28 of 15,131 (0.19%) wild white-tailed deer found infected with BTB
2005	(i) Upper Peninsula part of the state elevated to BTB-Free status
	(ii) 3 beef cattle herds found infected with BTB
	(iii) 16 of 7,364 (0.22%) wild white-tailed deer found infected with BTB
2006	(i) The 2nd privately owned deer herd found infected with BTB
	(ii) 2 dairy, 1 mixed, and 1 beef cattle herd found infected with BTB
	(iii) 41 of 7,914 (0.52%) wild white-tailed deer found infected with BTB
2007	(i) Implementation of official electronic identification ear tags mandatory for all cattle within the state.
	(ii) Annual WHT of feedlots within the MAZ implemented
	(iii) 1 dairy and 2 beef cattle herds found infected with BTB
	(iv) 27 of 8,316 (0.32%) wild white-tailed deer found infected with BTB
2008	(i) One time WHT of all cattle herds located in Arenac, Clare, Gladwin, Grand Traverse, Iosco, Kalkaska, Missaukee, Ogemaw, Osceola, Roscommon, and Wexford counties within 3 years initiated
	(ii) A beef and a mixed cattle herd infected with BTB
	(iii) The 3rd privately owned deer herd found infected with BTB
	(iv) 37 of 16,309 (0.23%) wild white-tailed deer found infected with BTB
2009	(i) 1 beef cattle herd found infected with BTB
	(ii) The 4th privately owned deer herd found infected with BTB
	(ii) 31 of 5,722 (0.54%) wild white-tailed deer found infected with BTB
July 2010	(i) 3 beef cattle herds found infected with BTB
	(ii) 6 of 306 (1.96%) wild white-tailed deer found infected with BTB

with another BTB infected herd and moved animals between these herds, in addition to their location within an area with a high prevalence of BTB in wild white-tailed deer. The most probable source of BTB infection in each of these herds was not clear. Epidemiological reports on all other BTB-affected herds ($n = 37$) suspected white-tailed deer to be the source of infection as all source farms and adjoining farms were tested for BTB with negative results. Wild white-tailed deer had access to all farms with BTB infected herds. All but one BTB infected cattle farm had attributes attractive to deer. These attributes included apple trees, accessible stored feed, water sources, and woodlands providing cover.

As a control measure, 43 of the BTB cattle herds (88%) were depopulated, from which 5 premises became reinfected with BTB. All animals repopulating these farms had a negative test for BTB prior to entry onto the premises. Six (12%) herds entered a test-and-remove program, of which one dairy cattle herd became reinfected at two separate times. In this herd, the first reinfection occurred after the herd was removed from quarantine. This herd was again found infected on the final whole herd test of the test and remove
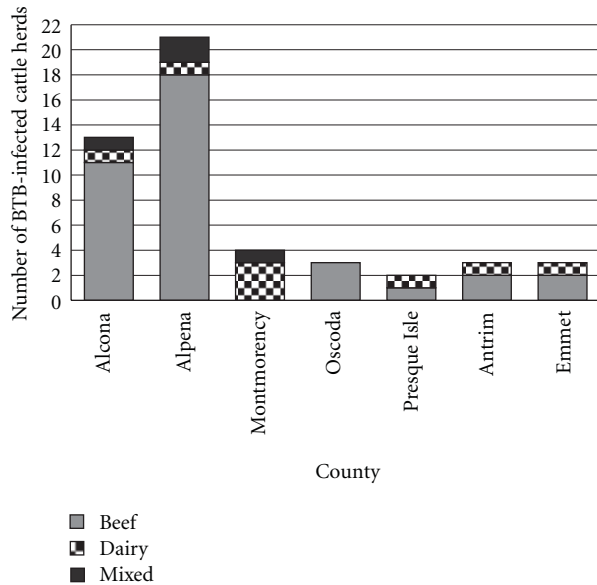
FIGURE 4: BTB incidence count in Michigan cattle herds by type, 1998–July 2010.
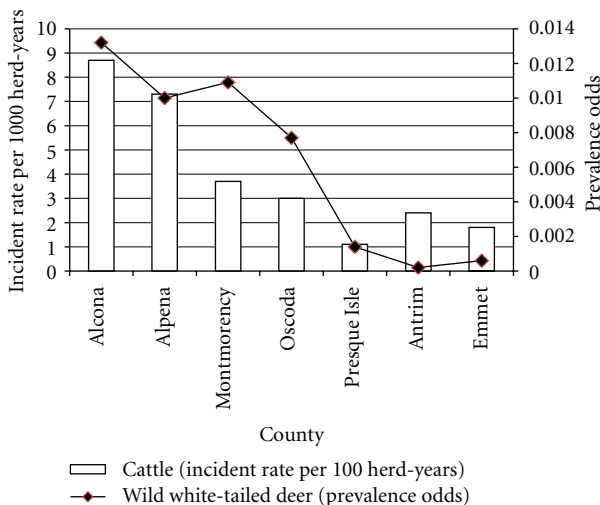

FIGURE 5: Prevalence odds and incident rate of BTB in Michigan wild white-tailed deer and cattle herds, respectively, 1975–July 2010.

program. The latest reinfection was not considered a separate infection because the herd was still quarantine at the time. Again, only BTB test negative animals were allowed onto these farms.

3.3. Privately Owned Cervids (Captive or Farmed Deer). The USDA AHPIS VS and the MDA identified 4 privately owned white-tailed deer herds infected with BTB between 1975 and July 2010. The first incidence was reported in Presque Isle County in 1997; all others were in Montmorency County in 2006, 2008, and 2009. The first [28] and the second [29] infected herds were depopulated but due to inadequacy of indemnity funds, the disease control options were changed. Hence, the third and the fourth BTB infected privately owned white-tailed deer herds became hunting only operations, placed under long-term quarantine with no live animal movement off the operation.

The first infected herd had a herd prevalence of 5.3%. Out of the 262 deer in the herd, 9 had both gross and histopathological lesions compatible with BTB at depopulation while 14 deer were confirmed using bacterial culture. In the second infected herd, the herd prevalence was 1.2%. Out of the 330 deer in this herd, 9 and 5 deer had gross and histopatholgical BTB compatible lesions, respectively, 1 tested positive by PCR and 4 tested positive by bacterial culture. There were 140 deer in the third infected herd at the time it was found infected. Beyond the one deer that made the herd positive, no other infected deer has been reported in this hunt-only herd. In the fourth (last) infected deer herd, out of the original 280 deer in the herd, 2 deer were found with gross and histopathological BTB compatible lesions and later tested BTB positive by both PCR and bacterial culture. Subsequently, no further infected deer have been reported.

3.4. Wildlife

3.4.1. BTB Surveillance in Wild White-Tailed Deer. Starting in 1995, hunter-harvested, road-killed, and other dead wild white-tailed deer were tested for BTB infection. White-tailed deer have since been tested annually for BTB [25]. Most BTB examinations occur during the fall deer hunting season. Hunters are requested to voluntarily turn in the heads of harvested wild white-tailed deer for BTB examination; in addition, carcasses bearing lesions considered suspicious by either hunters or the MDNR are collected [3]. Hunter-harvested deer accounted for 91% of all deer tested between 1975 and 2006 [3] and remain a significant source of BTB surveillance in wild white-tailed deer. The principal tissues examined are the parotid, mandibular, and medial and lateral retropharyngeal lymph nodes found in the head [11]. Unlike in cattle, only lesioned tissue is subjected to mycobacterial culture.

3.4.2. BTB Eradication in Wild White-Tailed Deer. Reduction of both deer concentration and population has been the applied BTB eradication strategies in Michigan wild white-tailed deer. Restriction/ban of baiting and supplemental feeding in wild white-tailed deer was used to reduce deer concentration, while increased deer harvest was the approach aimed at reducing deer density (Table 2). These strategies were most intensively implemented in the area with the highest sample prevalence of BTB in the wild deer (Deer Management Unit (DMU) 452). This area contains portions of Alcona, Alpena, Montmorency, and Oscoda counties and has been the "core area" of BTB challenge in Michigan. Since 1995, there has been a 57% decline in BTB transmission rate among wild white-tailed deer located with DMU 452 [30]. The total number of statewide harvested wild white-tailed deer has increased annually from approximately 100,000 in 1975 to over 400,000 in 2009 [31]. Consequently, since 1995 deer population has decreased over the years; in DMU 452, deer population dropped by 60,000 (40%) in 2009 [30].
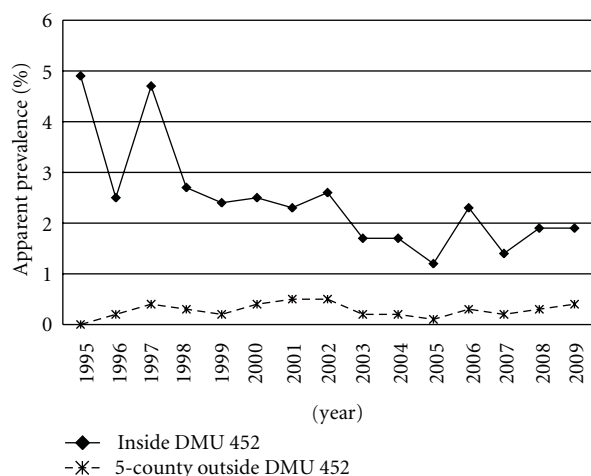
FIGURE 6: Sample prevalence of BTB in Michigan wild white-tailed deer.

3.4.3. BTB-Infected Wild White-Tailed Deer. BTB in wild white-tailed deer was first reported in Michigan in 1975 with a second case in 1994 [11]. Since then, more BTB cases have been found in white-tailed deer as well as other wildlife including elk, black bear, bobcat, coyote, opossum, raccoon, and red fox [11, 12, 15, 16, 18, 20, 22]. The increased identification of BTB in the wildlife, especially white-tailed deer, has led to numerous policy changes by the MDNR aimed at BTB eradication. Details of Michigan DNR policy changes have been reported [12, 32] as they have extensive treatment of policy implications [20, 33, 34]. The key policy changes are presented in Table 2.

The total population of white-tailed deer tested between 1975 and the end of July 2010 and those infected with BTB is presented in Table 1. Out of 184,269 white-tailed deer tested, 668 were found infected with BTB. Among the BTB infected deer, 36% were from Alcona, 28% from Alpena, 20% from Montmorency, 2% from Presque Isle, 11% from Oscoda, and 3% came from all other counties. The county with the highest prevalence odds of finding BTB white-tailed deer was Alcona (0.0133); the prevalence odds in other counties were Alpena (0.01), Montmorency (0.0109), Oscoda (0.0077), Presque Isle (0.0014), Antrim (0.0002), Emmet (0.0006), and others (0.0002) (Figure 5). Schmitt [30] reported a higher annual sample prevalence of BTB in the DMU 452 than in the surrounding counties (Figure 6).

4. Lessons Learned and Recommendations

4.1. BTB Remains an Ongoing Challenge. Despite ongoing control efforts, the continued identification of BTB in cattle and wildlife is strong evidence that BTB remains an ongoing challenge in Michigan. This is somewhat disheartening as significant resources have been expended to return Michigan to a BTB-free status. However, with the strategies that have been implemented during the past 15 years, the disease appears to have been confined to a geographical region of the state as was observed by other studies [12, 20]. The initiation of statewide BTB testing of all cattle herds in 2000 could be responsible for the spike in the BTB incidence between 2000 and 2003, as this was the first time most of these herds were ever BTB tested. Since then, there has been a declining trend in incidence and sample prevalence of BTB in cattle and wildlife, respectively, although recent evidence suggests that the downward trend has leveled off [30]. Earlier studies also observed a decline in sample BTB prevalence in Michigan's wild white-tailed deer [18, 20, 22]. These results suggest that progress has been made in the BTB eradication. However, it may be necessary to explore new and more aggressive control strategies in both cattle and wildlife that transcends political as well as social barriers, if complete eradication is to be accomplished.

4.2. Collaboration. Management of diseases that are transmissible between wildlife and domestic livestock can be a challenge and requires cooperation among their respective advocates in developing a reasonable and effective strategy that allows both to be maintained and prosper. Sharing of expertise is crucial in the eradication of a disease with many susceptible hosts as in BTB. All BTB cases in cattle and privately owned white-tailed deer herds as well as the majority of BTB infections in wildlife have been found in the northern portion of the Michigan's Lower Peninsula. The current containment of BTB to a geographical portion of Michigan is evidence of the successful collaborative efforts undertaken to eradicate BTB from Michigan. However, additional efforts and cooperation are needed to complete the eradication of BTB in Michigan. Increased cooperation between regulatory agencies, other stakeholders (e.g., hunters or local business owners), and livestock industry partners is needed. The development of a plan that is compatible with the long-term sustainability of both the livestock and wild life industries in Michigan should be targeted.

4.3. Surveillance. Whole herd testing of cattle farms has been crucial to identifying BTB infection, but this surveillance method is very expensive. Most BTB-affected cattle herds were found through annual whole herd surveillance. The state annually spends millions of dollars towards BTB control and eradication. Between 1994 and 2010, the State of Michigan has spent approximately US$200 million on BTB eradication [1]. Resources spent on whole herd testing of the livestock population contribute a significant part of the total expenses. Hidden costs rarely mentioned in the current BTB surveillance include such things such as injuries among the livestock owners, veterinarians, and technical staff that conduct BTB testing [35] and loss of production (e.g., temporary drop in milk production) that often occurs following restraint of cattle to administer and/or read ante mortem tests. Finally, there is an industry perception that the number of tests being done and the cost of surveillance in relation to the number of BTB herds found are excessive. This may lead to a decrease in long-term support of the current strategies from the cattle industry. Given these facts, there is a need for the exploration and subsequent adoption of less expensive, but just as reliable as surveillance methods

such as targeted strategies using risk-based criteria. Examples where use of targeted risk-based criteria was successful for disease eradication/control include Bovine Spongiform Encephalopathy (BSE) program, Pseudorabies in Pigs, and Brucellosis in cattle and swine. A reliable assay that could be used for BTB surveillance at points of cattle concentration, such as slaughter houses or livestock markets, would also be beneficial and could be used in conjunction with risk-based targeted strategies. Development of sophisticated targeted screening strategies would most likely produce a significant reduction in resource expenditure while at the same time maintaining the necessary rapid identification of BTB infected herds to eradicate the disease.

4.4. BTB Transmission. The prevalence odds of BTB in wild white-tailed deer are highly correlated with the incidence rate of BTB in cattle herds ($r = 0.8$ and P value $= .02$). These prevalence odds estimates, calculated from sample prevalence, do not accurately represent the true odds of BTB infection in wild white-tailed deer. The impracticality of testing all wild deer and the imperfection of available screening tests makes the true prevalence of BTB in wild deer unknown in absolute numbers [18] but a good approximation of the extent to which the sample prevalence underestimates the true prevalence has been documented [19]. Since prevalence odds remain a measure of risk, this highly positive correlation result supports the theory of interspecies transmission. Furthermore, the majority of cattle herds infected with BTB shared common environmental and management features that are conducive to wild white-tailed deer-cattle interaction. These observations further support the claim that wildlife and specifically wild white-tailed deer are a reservoir of BTB infection for livestock in Michigan [22, 36, 37]. Therefore, successfully mitigating such wildlife-cattle space interaction would be a great stride towards BTB eradication in Michigan.

With the advent of mandatory radio frequency identification (RFID) in Michigan and movement permits in the Northeast LP, it has become much easier to track cattle movement and rule in/out the possibility of cattle movement as a source of BTB transmission. This is a clear example of the utility of unique individual animal identification system in a disease control program. Using this available information in epidemiological investigations has helped in the understanding that cattle movement is not the most likely source of transmission into most of the infected herds. However, it should be noted that interherd spread has been linked to cattle movement in at least one herd and fence-line contact between infected herds has been identified as a potential mode of transmission in some of the infected Michigan herds. Therefore, these transmission modes should not be ignored and efforts to mitigate the risk of BTB spread through these transmission modes should be continued.

4.5. Wild White-Tailed Deer-Cattle Space Interactions. Wild white-tailed deer-cattle space interactions include wild white-tailed deer's access to cattle's feed and water sources, where *M. bovis* could be transmitted to cattle via ingestion

of contaminated food and water [7]. This example of space interaction would explain the lower number of BTB infected dairy herds, where the animals are primarily kept inside and usually have limited close contact with wild deer, or where livestock producers report no contact of their cattle with wild-white tailed deer. Livestock owners should sustain practices that reduce wild white-tailed deer-cattle space interactions such as storage of feed in enclosures that protect it from deer access, limiting cattle access to stagnant water sources and areas of cover that are also attractive to wild white-tailed deer, and removing feedstuffs from cattle areas that are attractive to deer (e.g., wild apple trees).

4.6. Deer Concentration and Density Reduction. Following the implementation of policies that reduced the population density and restricted practices which artificially increased wild white-tailed deer concentration, there has been a significant decreasing trend in the sample prevalence of BTB in wild white-tailed deer [20]. Since 2006, the sample prevalence has leveled-off. The cause of the slight increase in the sample prevalence of BTB in the wild deer in 2006 is not clearly known and could be associated with the epidemiology of BTB in the wild deer, which remains to be fully understood.

With policies that have led to an increased harvest [31], wild white-tailed deer population has decreased [30]. Similarly, changes in deer management practices, including restriction/ban of baiting and supplemental feeding in wild white-tailed deer, have likely helped reduce the transmission rate of BTB [30]. An increased harvest rate and a reduced transmission rate would cause a reduction in disease prevalence. Practices that have encouraged the reduction in deer concentration and deer density have likely contributed to the current containment of BTB. These practices will remain crucial for BTB eradication and should be supported.

4.7. Handling of BTB-Infected Herds. Among cattle herds infected with BTB in Michigan, depopulation has been the major strategy aimed at eradication. Depopulation is a BTB eradication strategy that is effective in areas with limited reservoirs of BTB infection and the disease challenge is not ongoing. Given the herd sizes of BTB infected cattle herds as it relates to the indemnity paid in the depopulation, this strategy is expensive and can be disrupting to the herd owner. It is even more frustrating when the depopulation strategy fails to achieve its purpose of eradicating the disease. Of the 6 herds with BTB reinfection, 5 were previously depopulated. There were no observable differences in the epidemiological data that would explain any vulnerability in those 6 cattle herds with BTB reinfection. However, detailed study of these herds and their management practices could provide insight into their vulnerability to BTB infection. Depopulation appears to not be a guaranteed BTB eradication strategy in an area with a wildlife reservoir of BTB infection and where some wildlife-livestock space interaction occurs, an observation that may have influenced a 2010 policy change in UDSA APHIS VS plans for herd-specific BTB eradication. In determining how best to handle a BTB infected cattle herd

in an area with a wildlife reservoir of BTB, measures must be taken to understand the exposure/transmission risks for BTB and then strive to mitigate those risks. In previous studies, restricting deer's access to cattle feed and water was found to be associated with reduced odds of BTB infection in cattle herds while sharing of pastures, bulls, or fence-line contacts among cattle herds, especially those in close proximity to already infected herd, was associated with increased risk of acquiring BTB into the herd [13]. Therefore adopting herd-specific wildlife risk mitigation and other biosecurity practices needs to be implemented and strictly enforced as part of the BTB eradication project.

Long-term quarantine of BTB infected privately owned white-tailed deer herds with no live animal movement off the operation is a strategy that has been implemented due to inadequacy of indemnity funds. The result of this confinement remains undetermined. There are reports of deer escaping from privately owned white-tailed deer facilities in Michigan as a result of damaged fences [38]. Also, all of the BTB-affected deer farms have fencing that could allow nose-to-nose contact between wild and captive deer. Although the true prevalence of BTB in these quarantined BTB infected privately owned white-tailed deer herds is unknown, given that the herd prevalence of BTB in the depopulated privately owned white-tailed deer herds is as high as the sample prevalence of BTB in the wild white-tailed deer in the area, any escape deer from the quarantined herds could pose a BTB risk. Therefore, depopulation of infected privately owned white-tailed deer herds would be a recommended choice if BTB eradication is to be achieved sooner rather than later.

4.8. Research. The BTB outbreak in Michigan has highlighted many knowledge gaps in our understanding about BTB. For successful eradication to occur in Michigan and in other regions of the world, significant research aimed at enhancing current eradication strategies as well as developing new eradication tools needs to be carried out. Areas of research which should be supported include the following.

(i) *Vaccine Development.* Extensive research on BTB vaccines for white-tailed deer is underway and the available results are promising [39–45]. The development of vaccines that could be successfully deployed in either livestock or wildlife and as part of a disease eradication program would be extremely beneficial globally. Vaccine may be even more important in other regions of the world without the infrastructure to implement a BTB control program using currently available strategies. Successful vaccine deployment in wildlife could transcend any social and political challenges of the current strategies which target more deer harvest. Therefore, current and future research efforts should be supported.

(ii) *New Diagnostic Assays and Strategies.* Currently available BTB diagnostic assays lack the desired sensitivity and specificity needed for effective BTB eradication in a timely manner. Development of inexpensive, accurate, and rapid diagnostic assays would be very valuable for the efficient identification of BTB

infected animals/herds. Development and evaluation of new strategies to deploy diagnostic testing, such as at points of concentration, would be of further value. Newer *ante mortem* serological [46–48] and cell-mediated immune response [49] assays show potential improvements from the currently available assays and should be encouraged through research funding.

(iii) *Disease Transmission Risk Factors.* Studies that evaluate BTB transmission risk factors have been conducted [5–7, 13]. However, inadequate research has been done on quantifying how much each known risk factor contributes to disease transmission. Understanding and quantifying risk factors important for BTB transmission within and between species would be very valuable for the successful deployment of targeted surveillance strategies and for implementing herd control programs.

(iv) *Ecology and Epidemiology of BTB in Noncattle Species.* With the emergence of BTB reservoirs in wildlife, numerous studies that strive to understand BTB epidemiology have been conducted [17, 18, 34]. The results of these studies have advanced BTB eradication efforts. However, BTB epidemiology, especially in noncattle species, is still not well understood. Better understanding of how the disease is maintained and transmitted in these "new" hosts is necessary for successful control and eradication.

(v) *Sociological Aspects of Disease Eradication Programs.* Effective disease control programs need commitment from all parties affected, whether directly or peripherally. As can be seen from a previous study [50], stakeholders attitudes have influenced the progress of BTB eradication in Michigan. Understanding societal concerns and developing strategies to mitigate these concerns is extremely important for successful deployment of a disease control program.

5. Conclusion

Despite ongoing eradication efforts, BTB remains a major challenge for Michigan. Policies and strategies implemented since 1994 have appeared to contain cases of BTB in cattle and privately owned deer herds as well as approximately 99.6% of the BTB infected wild white-tailed deer to the northern portions of the Lower Peninsula of Michigan, particularly counties in the North East portion. Active collaboration among the BTB Eradication Project partners, funding agencies, and the various stakeholders has contributed to the current progress and should be encouraged further for onward BTB eradication. Wild white-tailed deer remains the significant source of transmission of BTB to the livestock, most likely through indirect transmission. Mitigation strategies that decrease interactions and indirect transmission as well as supporting actions aimed at reducing the disease prevalence in wildlife should continue and be enhanced. These actions include decreasing wild white-tailed deer population density, decreasing opportunities

for close congregation of wild deer, and developing novel strategies for increasing resistance to BTB such as vaccines. BTB surveillance strategies associated with the current eradication program have been effective but expensive. Therefore, development of effective but less expensive disease surveillance system would be beneficial. Finally, additional research is needed to improve our understanding about BTB epidemiology as well as disease eradication techniques that would transcend social and political issues. Supporting, conducting, and implementing the results of such research would greatly improve BTB eradication efforts.

Acknowledgments

References

[1] B. Patrick, "Personal communications 2010," Animal Industry Division, Michigan Department of Agriculture, 2010.

[2] L. M. O'Reilly and C. J. Daborn, "The epidemiology of *Mycobacterium bovis* infections in animals and man: a review," *Tubercle and Lung Disease*, vol. 76, no. 1, pp. 1–46, 1995.

[3] D. J. O'Brien, S. D. Fitzgerald, T. J. Lyon et al., "Tuberculous lesions in free-ranging white-tailed deer in Michigan," *Journal of Wildlife Diseases*, vol. 37, no. 3, pp. 608–613, 2001.

[4] M. V. Palmer, D. L. Whipple, and W. R. Waters, "Experimental deer-to-deer transmission of *Mycobacterium bovis*," *American Journal of Veterinary Research*, vol. 62, no. 5, pp. 692–696, 2001.

[5] M. V. Palmer, W. R. Waters, and D. L. Whipple, "Milk containing *Mycobacterium bovis* as a source of infection for white-tailed deer fawns (*Odocoileus virginianus*)," *Tuberculosis*, vol. 82, no. 4-5, pp. 161–165, 2002.

[6] M. V. Palmer, W. R. Waters, and D. L. Whipple, "Investigation of the transmission of *Mycobacterium bovis* from deer to cattle through indirect contact," *American Journal of Veterinary Research*, vol. 65, no. 11, pp. 1483–1489, 2004.

[7] M. V. Palmer and D. L. Whipple, "Survival of *Mycobacterium bovis* on feedstuffs commonly used as supplemental feed for white-tailed deer (*Odocoileus virginianus*)," *Journal of Wildlife Diseases*, vol. 42, no. 4, pp. 853–858, 2006.

[8] M. V. Palmer, "Tuberculosis: a reemerging disease at the interface of domestic animals and wildlife," *Current Topics in Microbiology and Immunology*, vol. 315, pp. 195–215, 2007.

[9] J. M. Grange, "*Mycobacterium bovis* infection in human beings," *Tuberculosis*, vol. 81, no. 1-2, pp. 71–77, 2001.

[10] A. L. Olmstead and P. W. Rhode, "An impossible undertaking: the eradication of bovine tuberculosis in the United States," *Journal of Economic History*, vol. 64, no. 3, pp. 734–772, 2004.

[11] S. M. Schmitt, S. D. Fitzgerald, T. M. Cooley et al., "Bovine tuberculosis in free-ranging white-tailed deer from Michigan," *Journal of Wildlife Diseases*, vol. 33, no. 4, pp. 749–758, 1997.

[12] G. J. Hickling, "Dynamics of bovine tuberculosis in wild white-tailed deer in Michigan," MDNR Wildlife Division Report 3363, 2002, http://ww2.dnr.state.mi.us/Publications/Pdfs/HuntingWildlifeHabitat/Reports/WLD-Library/3301-3399/3363.Pdf.

[13] J. B. Kaneene, C. S. Bruning-Fann, L. M. Granger, R. Miller, and B. A. Porter-Spalding, "Environmental and farm management factors associated with tuberculosis on cattle farms in northeastern Michigan," *Journal of the American Veterinary Medical Association*, vol. 221, no. 6, pp. 837–842, 2002.

[14] J. B. Kaneene, M. VanderKlok, C. S. Bruning-Fann et al., "Prevalence of *Mycobacterium bovis* infection in cervids on privately owned ranches," *Journal of the American Veterinary Medical Association*, vol. 220, no. 5, pp. 656–659, 2002.

[15] C. S. Bruning-Fann, S. M. Schmitt, S. D. Fitzgerald et al., "*Mycobacterium bovis* in coyotes from Michigan," *Journal of Wildlife Diseases*, vol. 34, no. 3, pp. 632–636, 1998.

[16] C. S. Bruning-Fann, S. M. Schmitt, S. D. Fitzgerald et al., "Bovine tuberculosis in free-ranging carnivores from Michigan," *Journal of Wildlife Diseases*, vol. 37, no. 1, pp. 58–64, 2001.

[17] J. B. Kaneene, C. S. Bruning-Fann, J. Dunn et al., "Epidemiologic investigation of *Mycobacterium bovis* in a population of cats," *American Journal of Veterinary Research*, vol. 63, no. 11, pp. 1507–1511, 2002.

[18] D. J. O'Brien, S. M. Schmitt, J. S. Fierke et al., "Epidemiology of *Mycobacterium bovis* in free-ranging white-tailed deer, Michigan, USA, 1995–2000," *Preventive Veterinary Medicine*, vol. 54, no. 1, pp. 47–63, 2002.

[19] D. J. O'Brien, S. M. Schmitt, D. E. Berry et al., "Estimating the true prevalence of *Mycobacterium bovis* in hunter-harvested white-tailed deer in Michigan," *Journal of Wildlife Diseases*, vol. 40, no. 1, pp. 42–52, 2004.

[20] D. J. O'Brien, S. M. Schmitt, S. D. Fitzgerald, D. E. Berry, and G. J. Hickling, "Managing the wildlife reservoir of *Mycobacterium bovis*: the Michigan, USA, experience," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 313–323, 2006.

[21] D. J. O'Brien, S. M. Schmitt, D. E. Berry et al., "Estimating the true prevalence of *Mycobacterium bovis* in free-ranging elk in Michigan," *Journal of Wildlife Diseases*, vol. 44, no. 4, pp. 802–810, 2008.

[22] S. M. Schmitt, D. J. O'Brien, C. S. Bruning-Fann, and S. D. Fitzgerald, "Bovine tuberculosis in Michigan wildlife and livestock," *Annals of the New York Academy of Sciences*, vol. 969, pp. 262–268, 2002.

[23] J. J. Paskus, A. L. Derosier, E. H. Schools et al., "Biodiversity Assessment of Michigan Techinical Report," Tech. Rep., 2008, http://web4.msue.msu.edu/Mnfi/Reports/2007-11_Biodiversity_Assessment_of_Michigan_Technical_Report.Pdf.

[24] USDA Census of Agriculture, 2007, http://www.agcensus.usda.gov/Publications/2007/Full_Report/Volume_1,_Chapter_2_US_State_Level/st99_2_011_011.pdf.

[25] MDNR, "Bovine Tuberculosis," 2010, http://www.michigan.gov/emergingdiseases.

[26] USDA APHIS, Bovine Tuberculosis Eradication: Uniform Methods and Rules, Effective January, 1999.

[27] USDA APHIS, Bovine Tuberculosis Eradication: Uniform Methods and Rules, Effective January, 2005.

[28] M. V. Palmer, D. L. Whipple, J. B. Payeur et al., "Naturally occurring tuberculosis in white-tailed deer," *Journal of the American Veterinary Medical Association*, vol. 216, no. 12, pp. 1921–1924, 2000.

[29] D. J. O'Brien, S. M. Schmitt, K. P. Lyashchenko et al., "Evaluation of blood assays for detection of *Mycobacterium bovis* in white-tailed deer (*Odocoileus virginianus*) in Michigan," *Journal of Wildlife Diseases*, vol. 45, no. 1, pp. 153–164, 2009.

[30] S. M. Schmitt, "Bovine tuberculosis in wildlife," MDA-DNRE TB Informational Meetings, 2010, http://www.michigan.gov/documents/emergingdiseases/DNRESchmittPublicMeeting-062210_325662_7.pdf.

[31] B. J. Frawley, "Michigan Deer Harvest Survey Report 2009 Seasons," MDNR Wildlife Report no. 3513, 2010, http://www.michigan.gov/documents/dnr/report3513_327318_7.pdf.

[32] MDNR, "History of Legislation and Regulations for Bovine Tuberculosis in Michigan Wildlife," 2005, http://www.michigan.gov/documents/Bait_Feed_History_138339_7.pdf.

[33] M. M. Conner, M. R. Ebinger, J. A. Blanchong, and P. C. Cross, "Infectious disease in cervids of North America: data, models, and management challenges," *Annals of the New York Academy of Sciences*, vol. 1134, pp. 146–172, 2008.

[34] G. W. De Lisle, R. G. Bengis, S. M. Schmitt, and D. J. O'Brien, "Tuberculosis in free-ranging wildlife: detection, diagnosis and management," *OIE Revue Scientifique et Technique*, vol. 21, no. 2, pp. 317–334, 2002.

[35] M. J. Wilkins, P. C. Bartlett, L. J. Judge, R. J. Erskine, M. L. Boulton, and J. B. Kaneene, "Veterinarian injuries associated with bovine TB testing livestock in Michigan, 2001," *Preventive Veterinary Medicine*, vol. 89, no. 3-4, pp. 185–190, 2009.

[36] R. Miller, J. B. Kaneene, S. D. Fitzgerald, and S. M. Schmitt, "Evaluation of the influence of supplemental feeding of white-tailed deer (*Odocoileus virginianus*) on the prevalence of bovine tuberculosis in the Michigan wild deer population," *Journal of Wildlife Diseases*, vol. 39, no. 1, pp. 84–95, 2003.

[37] M. J. Wilkins, J. Meyerson, P. C. Bartlett et al., "Human *Mycobacterium bovis* infection and bovine tuberculosis outbreak, Michigan, 1994–2007," *Emerging Infectious Diseases*, vol. 14, no. 4, pp. 657–660, 2008.

[38] D. J. O'Brien, P. Bernardi, S. Dubay, S. Mayhew, W. E. Moritz, and D. Purol, "A Risk-based Audit of the Captive/Privately owned Cervid Industry in Michigan," 2005, Final_118651_7.pdf Final_118651_7.pdf .

[39] M. V. Palmer, T. C. Thacker, and W. R. Waters, "Vaccination with *Mycobacterium bovis* BCG strains Danish and Pasteur in white-tailed deer (*Odocoileus virginianus*) experimentally challenged with *Mycobacterium bovis*," *Zoonoses and Public Health*, vol. 56, no. 5, pp. 243–251, 2009.

[40] P. Nol, K. P. Lyashchenko, R. Greenwald et al., "Humoral immune responses of white-tailed deer (Odocoileus virginianus) to *Mycobacterium bovis* BCG vaccination and experimental challenge with M. bovis," *Clinical and Vaccine Immunology*, vol. 16, no. 3, pp. 323–329, 2009.

[41] M. V. Palmer, T. C. Thacker, and W. R. Waters, "Vaccination of white-tailed deer (*Odocoileus virginianus*) with *Mycobacterium bovis* bacillus Calmette Guerín," *Vaccine*, vol. 25, no. 36, pp. 6589–6597, 2007.

[42] M. V. Palmer, T. C. Thacker, W. R. Waters, S. Robbe-Austerman, S. M. Lebepe-Mazur, and N. B. Harris, "Persistence of *Mycobacterium bovis* bacillus calmette-guérin in white-tailed deer (*Odocoileus virginianus*) after oral or parenteral vaccination," *Zoonoses and Public Health*, vol. 57, no. 7-8, pp. e206–e212, 2010.

[43] T. C. Thacker, M. V. Palmer, and W. R. Waters, "T-cell mRNA expression in response to *Mycobacterium bovis* BCG vaccination and *Mycobacterium bovis* infection of white-tailed deer," *Clinical and Vaccine Immunology*, vol. 16, no. 8, pp. 1139–1145, 2009.

[44] P. Nol, M. V. Palmer, W. R. Waters et al., "Efficacy of oral and parenteral routes of *Mycobacterium bovis* bacille Calmette-Guerin vaccination against experimental bovine tuberculosis in white-tailed deer (*Odocoileus virginianus*): a feasibility study," *Journal of Wildlife Diseases*, vol. 44, no. 2, pp. 247–259, 2008.

[45] W. R. Waters, M. V. Palmer, D. L. Whipple, R. E. Slaughter, and S. L. Jones, "Immune responses of white-tailed deer (*Odocoileus virginianus*) to *Mycobacterium bovis* BCG vaccination," *Journal of Wildlife Diseases*, vol. 40, no. 1, pp. 66–78, 2004.

[46] W. R. Waters, M. V. Palmer, T. C. Thacker et al., "Early antibody responses to experimental *Mycobacterium bovis* infection of cattle," *Clinical and Vaccine Immunology*, vol. 13, no. 6, pp. 648–654, 2006.

[47] K. A. Fenton, S. D. Fitzgerald, J. B. Kaneene, J. M. Kruger, R. Greenwald, and K. P. Lyashchenko, "Comparison of three immunodiagnostic assays for antemortem detection of *Mycobacterium bovis* stimulation in domestic cats," *Journal of Veterinary Diagnostic Investigation*, vol. 22, no. 5, pp. 724–729, 2010.

[48] K. P. Lyashchenko, R. Greenwald, J. Esfandiari et al., "Animal-side serologic assay for rapid detection of *Mycobacterium bovis* infection in multiple species of free-ranging wildlife," *Veterinary Microbiology*, vol. 132, no. 3-4, pp. 283–292, 2008.

[49] C. C. Okafor, D. L. Grooms, S. R. Bolin, and J. B. Kaneene, "Validity of the bovine TB gamma interferon assay on blood collected during exsanguination at slaughter," in *Proceedings of the Conference of Research Workers in Animal Diseases*, Chicago, Ill, USA, 2010.

[50] M. L. Dorn and A. G. Mertig, "Bovine tuberculosis in Michigan: stakeholder attitudes and implications for eradication efforts," *Wildlife Society Bulletin*, vol. 33, no. 2, pp. 539–552, 2005.

Research Article

Lesion Distribution and Epidemiology of *Mycobacterium bovis* in Elk and White-Tailed Deer in South-Western Manitoba, Canada

Todd K. Shury[1] and Doug Bergeson[2]

[1] *Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, SK, Canada S7K 5B4*
[2] *Riding Mountain National Park, Parks Canada Agency, Wasagaming, MB, Canada R0J 2H0*

Correspondence should be addressed to Todd K. Shury, todd.shury@pc.gc.ca

Received 15 January 2011; Revised 3 March 2011; Accepted 21 March 2011

Academic Editor: Mitchell V. Palmer

Surveillance for *Mycobacterium bovis* in free-ranging elk (*Cervus elaphus*) and white-tailed deer (*Odocoileus virginianus*) from south-western Manitoba was carried out from 1997 to 2010 to describe the lesions, epidemiology, and geographic distribution of disease. Tissues were cultured from animals killed by hunters, culled for management, blood-tested, or found opportunistically. Period prevalence in elk was approximately six times higher than deer, suggesting a significant reservoir role for elk, but that infected deer may also be involved. Prevalence was consistently higher in elk compared to deer in a small core area and prevalence declines since 2003 are likely due to a combination of management factors instituted during that time. Older age classes and animals sampled from the core area were at significantly higher risk of being culture positive. Positive elk and deer were more likely to be found through blood testing, opportunistic surveillance, and culling compared to hunting. No non-lesioned, culture-positive elk were detected in this study compared to previous studies in red deer.

1. Introduction

Riding Mountain National Park (RMNP) is a 2974-hectare protected area that is part of a large elevated escarpment and is part of a UNESCO (United Nations Educational, Scientific and Cultural Organization) Biosphere Reserve. This area, which includes the Duck Mountain Provincial Park and Forest (DMPPF) is an important core habitat for a large population of elk (*Cervus elaphus)*, moose (*Alces alces*), white-tailed deer (*Odocoileus virginianus*), wolves (*Canis lupus*), and black bears (*Ursus americanus*) and is considered a southern extension of the boreal forest in Canada. Both protected areas are essentially surrounded on all sides by agricultural landscapes which include forage crop production, grain farming, and livestock production. Cattle were grazed sympatrically with wildlife within RMNP and the DMPPF until 1970 when cattle grazing was discontinued in both areas [1]. Fourteen cattle herds have been found to be infected with bovine tuberculosis (bTB) since 1991 in the area around RMNP, and several of these have been closely linked to cases of infected deer and elk [2, 3].

Local cattle producers have been involved with intensive live cattle testing and movement restrictions, resulting in negative economic consequences for these producers. The two Manitoba Game Hunting Areas that surround RMNP were designated a special management zone called the Riding Mountain Eradication Area (RMEA) in 2003. Following extensive live cattle testing for three years, cattle herds within this zone were subsequently deemed to be TB-free according to Canadian livestock standards in August of 2006. One additional herd breakdown in cattle was found within the RMEA in May of 2008 [1], but no infected cattle herds have been discovered after extensive follow-up testing since that time. All *M. bovis* isolates to date from cattle, deer, and elk share two closely related spoligotypes designated MB-1 and MB-2 including two infected wolves found within RMNP in 1978 [4, 5]. It is likely that wildlife species were initially infected as a result of contact with infected cattle, but the infection has likely spilled back to cattle since that time.

Emerging wildlife reservoirs of *M. bovis* infection have created serious negative socioeconomic consequences in the past 15 years in Europe, North America, and New Zealand,

particularly when the wildlife reservoir has significant conservation or societal value [6]. Determination of disease burden and of species acting as reservoirs is particularly challenging with infected wildlife populations. Reservoir hosts for *M. bovis* are those species that can maintain infection independently through intraspecific transmission without reinfection from another species, while spillover hosts require reinfection from another species to maintain the infection and typically do not maintain the infection in wild populations [6, 7]. Some species may act as both reservoir and spillover hosts depending on demographic and population-specific factors such as population density, presence of artificial feeding, and host immunity [8–10] and species may form reservoirs in combination [9]. In North America, white-tailed deer have been demonstrated to be a competent reservoir species in Michigan, USA while elk are considered a spillover host [8, 11]. A separate, unrelated outbreak of *M. bovis* is currently occurring in white-tailed deer in the state of Minnesota, but the disease does not appear to be spreading rapidly and deer-to-deer transmission may not be occurring in this state [12]. The epidemiology of bovine TB has been described for wild red deer in New Zealand [13, 14] and Spain [15–17], but very few references describe the epidemiology or prevalence in wild elk from North America [2, 8]. The enzootic described in this paper is even more challenging from a disease control perspective as the wildlife that make up the likely reservoir species are found within two environmentally sensitive protected areas (RMNP and DMPPF). Hunting or direct culling have typically been used as a management tools to control wildlife host density and provide samples for disease surveillance, but hunting is not currently permitted within RMNP, making disease management at a landscape scale extremely challenging [1, 17, 18]. This area is one of the last known reservoirs of *M. bovis* in Canada [18], and little is known about the status of this infection in elk and deer in this area.

This study reports on preliminary pathologic findings, lesion distribution, and descriptive epidemiology from the area around RMNP and DMPPF for both white-tailed deer and elk and provides a brief analysis of *M. bovis* confirmed cases found since 1997 in this area. Prevalence and distribution data will be presented allowing a comprehensive assessment of this long-term wildlife reservoir and a discussion of implications for future management and eradication of the disease in wildlife.

2. Methods and Materials

2.1. Sample Collection. *Mycobacterium bovis* infection was initially discovered in wild ungulates from the RMNP area in a hunter-killed bull elk in 1992, but formal surveys were not initiated until 1997 when hunter harvested elk were collected on the borders of RMNP [1, 2]. Data for this study includes deer and elk collected in the RMNP and DMPPF areas through four primary sources: (1) hunter-killed elk and deer collected as part of *M. bovis* surveillance efforts between November 1997 to January 2010 (hunter sample), (2) elk and deer collected as part of a blood testing

program within RMNP from February 2002 to May 2010 (blood test sample), (3) ground-based culls which were conducted to reduce elk and deer density and determine *M. bovis* prevalence in March 2004 (white-tailed deer only) and a February/March 2009 cull involving both elk and deer (cull sample), and (4) targeted surveillance samples which were collected opportunistically (roadkills, predation, and winter kills) and those animals destroyed because they were exhibiting clinical signs of illness (opportunistic sample). Hunter submissions typically consisted of both head and lung samples from harvested animals, but samples occasionally consisted of only the head or lungs. Blood testing was carried out through live animal capture and testing to detect antibodies and cell-mediated immunity to *M. bovis* (details provided below). A cull involving local landowners and Manitoba Conservation staff involving white-tailed deer was carried out in March of 2004 through ground-based shooting of deer in areas bordering RMNP. In 2009, culls for population reduction and surveillance were carried out within RMNP and involved helicopter net gun capture followed by euthanasia with captive bolt gun. All culled animals were transported intact to a laboratory where a full necropsy was conducted on each carcass. Head and lung samples from hunter killed animals were examined at the same laboratory (detail provided below). Targeted surveillance samples were collected opportunistically as a result of public reports and followup of predator kills for other research projects. White-tailed deer and elk were considered *M. bovis* positive if they were determined to have a positive culture on any tissue cultured for postmortem analysis.

Elk and deer captured for blood testing were primarily captured within two protected areas in south western Manitoba, Canada: RMNP and the DMPPF. Animal capture was carried out using helicopter net gunning between February 2002 and May of 2010 during winter and early spring (December to early June) (Figure 1). Elk were selected haphazardly by the helicopter crew in selected regions within RMNP and DMPPF, but virtually all elk and deer capture for blood testing occurred within these two protected areas. All captured elk were blindfolded and hobbled for short duration (10–15 minutes) and were released immediately after sampling and application of a VHF or GPS collar to allow subsequent relocation and recapture. A cotton spacer made of fire hose was attached to the collar belting to cause them to fall off within 3–6 months after capture. Sixty millilitres of whole blood was collected by jugular venipuncture and placed in either 10 mL sterile glass vials containing no additive, lithium heparin (Vacutainer), or silicone coating (Vacutainer SST). Samples without anti-coagulant were allowed to clot at room temperature and centrifuged at 3,000 rpm for 15 minutes. For the period 2004 to 2010, three blood-based assays were used to detect potentially infected cervids; a lymphocyte stimulation test (LST), a fluorescence polarization assay (FPA) [19], and a chromatographic immunoassay (Cervid Stat-Pak) [20]. An experimental polymerase chain reaction (PCR) test was also utilized on buffy coat samples in 2002 to 2004 in addition to these three tests, but it was discontinued in 2005. Serum
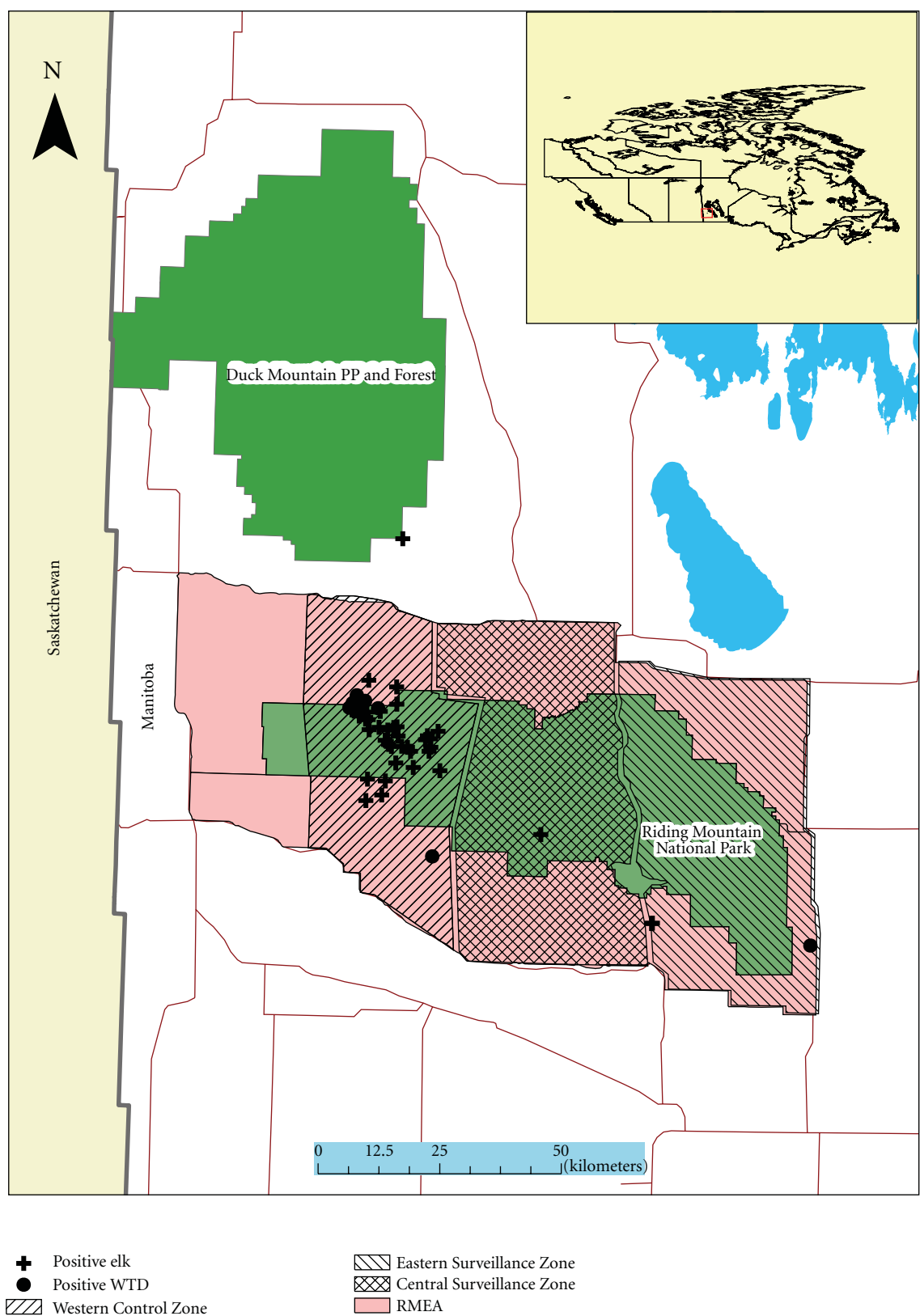
Figure 1: Locations of sampling zones and *M. bovis* culture positive elk and deer cases in south-western Manitoba from 1997 to 2010.

for the Cervid Stat-Pak evaluation was harvested and frozen at −20°C or tested immediately in some cases. Fresh whole blood with and without anticoagulant were stored at room temperature and shipped immediately upon collection to the Canadian Food Inspection Agency, Mycobacterial Diseases Centre of Expertise (MDCE), Ottawa, Ontario for evaluation using the LST and FPA, respectively. Elk testing positive (parallel interpretation) on any one of these diagnostic tests (FPA, LST, and Stat-Pak) were subsequently recaptured up to two months later using the methodology described above, euthanized with a captive bolt gun and slung by helicopter to a central laboratory for immediate necropsy. Elk testing negative to three of the four tests (LST, FPA, and RT) were not recaptured, but were monitored by aerial telemetry until their radio collars fell off within 3–12 months after capture. A subset of animals that were culled and were tested retrospectively were used to validate the sensitivity (Se = 100%, 95% confidence intervals 56.5%–100%) of the parallel testing protocol, so very few of these animals were likely truly TB positive (unpublished data). Parallel testing involving multiple tests increases the sensitivity while sacrificing specificity, resulting in numerous false positives, but few false negatives [21].

Hunter sampled elk and white-tailed deer heads and lungs were collected annually between September 1997 and January 2010 from voluntary submissions by local hunters through regular and extended hunting seasons. Submissions have been mandatory since 1999 in the RMEA and since 2000 in the Duck Mountain Provincial Park and Forest. All submitted heads and lungs were examined grossly, with specific lymphoid tissues being sent for mycobacterial culture and histopathology prior to the fall/winter of 2001/2002. Since 2002, only tissues from animals exhibiting suspect gross lesions of tuberculosis in lymphoid tissues or palatine tonsils were submitted for histopathology and mycobacterial culture. Hunting is not allowed within RMNP, but elk and white-tailed deer hunting are allowed within the DMPPF and surrounding area (Figure 1). A set of four lymph nodes were routinely evaluated in the head (medial retropharyngeal, parotid, submandibular, and lateral retropharyngeal) as well as the palatine tonsils. Lymphoid tissues were sliced thinly at 3–5 mm thickness to look for lesions typical of *M. bovis* and formalin-fixed tissue and fresh tissues were sent to the Mycobacterial Diseases Centre of Excellence (MDCE) laboratory in Ottawa, Ontario. Lung tissues were examined similarly with tracheo-bronchial and mediastinal lymph nodes being specifically targeted while lungs were palpated for abnormalities and sliced at 5 cm intervals to check for grossly visible lesions.

Elk and deer sampled opportunistically included predator killed animals, roadkilled animals, poaching investigations, winter killed animals, or animals observed with unusual clinical signs that were euthanized for necropsy. These animals were either necropsied in a laboratory or in the field depending on location.

2.2. Postmortem and Laboratory Procedures. For animals that tested positive on one or more blood tests and for the culled elk and deer, multiple tissues were collected at necropsy as part of a detailed postmortem collection procedure similar to that collected for other studies involving European badgers (*Meles meles*) [22] and subjected to mycobacterial culture, acid fast staining, and histopathological examination. Peripheral lymphoid tissues examined and collected were submandibular, medial, and lateral retropharyngeal, parotid, palatine tonsil (tonsillar crypt), prescapular, popliteal, prefemoral, supramammary/testicular, internal iliac, hepatic, portal, mesenteric, tracheo-bronchial, and mediastinal lymph nodes. Pools of tissue from body, head, abdominal, and thoracic lymph nodes were submitted for mycobacterial culture regardless of whether gross lesions were seen at necropsy or not. All other organ systems were systematically examined for gross lesions indicative of mycobacteriosis and any suspect tissue was also sent for mycobacterial culture, histopathological evaluation, and PCR testing to confirm identity of cultured mycobacteria. Harvested tissues were either frozen at −20°C or refrigerated and were shipped to the MDCE within 24 to 48 hours of collection. Formalin-fixed tissues were embedded in paraffin, cut into sections 5 mm thick, and stained with hematoxylin and eosin as well as by the Ziehl-Neelsen technique for detection of acid fast bacilli. Slides of the tissue sections were examined by a pathologist experienced in the diagnosis of TB. The tissues were cultured for mycobacteria using the method described by Rohonczy et al. [23]. Inoculated media were incubated at 37°C for 12 wk and examined every 2 weeks for evidence of bacterial growth. Elk and deer were considered TB positive if they had a positive culture for *M. bovis* on any tissue submitted for culture [19]. Spoligotyping to type cultured TB complex organisms was conducted as described previously [5]. Ages of hunter killed elk and deer at necropsy were determined by estimation of tooth wear into one of five age categories; less than one year of age, one to two years of age, three to five years of age, six to eight years of age, or greater than 8 years. Elk and deer that were culled, blood tested, or found opportunistically were aged by examination of tooth sections and counting cementum annuli [24].

2.3. Statistical Analysis. Sampled elk and deer were grouped based on sampling location into one of four risk zones created to monitor the prevalence and distribution of *M. bovis* in wildlife (Figure 1). Prevalence was estimated using the methods described in Thrusfield [21] and 95% confidence intervals were estimated using WINPEPI software version 10.1 using Wilson's score method [25]. Trend analysis on prevalence data was conducted using WINPEPI software using a two-way Cochrane-Armitage test for trend with Fishers exact 95% confidence intervals. Analyses of the proportion of culture positive animals with gross visible lesions in different tissues were compared using Upton's modified $(N-1)$ Chi-square [26].

3. Results

The overall prevalence of *M. bovis* infection in elk and white-tailed deer has been consistently very low in the area

TABLE 1: Zone specific prevalence of *M. bovis* in elk and deer from south-western Manitoba from 1997 to 2010.

Species	Sampling Year[a]	Outside[b]			Eastern Surveillance Zone			Central Surveillance Zone			Western Control Zone		
		Prevalence (%)	95% CI	No. Tested	Prevalence (%)	95% CI	No. Tested	Prevalence (%)	95% CI	No. Tested	Prevalence (%)	95% CI	No. Tested
Elk	1997/1998	0	0–65.76	2	0	0–4.87	75	0	0–11.0	31	0	0–11.35	30
	1998/1999	0	0–13.32	25	0.81	0.14–4.43	124	1.72	0.31–9.1	58	0	0–5.13	71
	1999/2000	0	0–6.02	60	1.37	0.24–7.36	73	0	0–6.02	60	1.85	0.33–9.77	54
	2000/2001	0	0–3.26	114	0	0–2.12	177	0	0–3.47	107	2.99	1.29–6.82	167
	2001/2002	0	0–4.42	83	0	0–4.01	92	0	0–11.03	31	0.00	0–5.58	65
	2002/2003	0	0–2.96	126	0	0–2.26	166	0	0–6.02	60	6.85	3.76–12.15	146
	2003/2004	0	0–3.89	95	0	0–1.83	206	0	0–5.35	68	2.46	1.06–5.64	203
	2004/2005	0.55	0.1–3.05	182	0	0–4.28	86	0	0–15.5	21	3.01	1.18–7.48	133
	2005/2006	0	0–1.62	233	0	0–5.92	61	0	0–5.5	66	1.64	0.29–8.72	61
	2006/2007	0	0–2.87	130	0	0–4.87	75	0	0–11.35	30	3.16	1.08–8.88	95
	2007/2008	0	0–3.43	108	0	0–6.42	56	0	0–27.75	10	4.39	1.89–9.86	114
	2008/2009	0	0–4.53	81	0	0–25.9	11	0	0–27.75	10	1.09	0.3–3.88	184
	2009/2010	0	0–3.56	104	0	0–29.9	9	0	0–4.58	80	1.35	0.24–7.27	74
WTD[c]	1997/1998	0	–0	0	0	0–56.1	3	0	0–56.1	3	0	0–0	0
	1998/1999	0	0–5.5	66	0	0–7.41	48	0	0–12.1	28	0	0–29.9	9
	1999/2000	0	0–10.7	32	0	0–7.71	46	0	0–8.38	42	0	0–29.9	9
	2000/2001	0	0–7	51	0	0–6.21	58	0	0–8.97	39	0	0–11.7	29
	2001/2002	0	0–0.77	494	0	0–6.11	59	0	0–11.03	31	2.86	0.51–14.53	35
	2002/2003	0	0–2.01	187	0	0–6.21	58	0	0–6.53	55	0	0–8.2	43
	2003/2004	0	0–3.5	106	0	0–1.84	205	0	0–3.66	101	1.69	0.57–4.84	178
	2004/2005	0	0–0.47	828	0	0–1.41	268	0	0–2.45	153	1.33	0.45–3.85	225
	2005/2006	0	0–0.61	623	0	0–1.79	211	0	0–3.05	122	0	0–2.28	165
	2006/2007	0	0–0.85	448	0	0–2.36	159	0	0–4.69	78	0	0–4.32	85
	2007/2008	0	0–0.97	393	0	0–3.21	116	0	0–13.8	24	0.84	0.15–4.61	119
	2008/2009	0	0–1	380	0	0–2.63	142	0	0–8.97	39	1.31	0.36–4.64	153
	2009/2010	0	0–0.78	488	1.79	0.32–9.5	56	0	0–12.1	28	0	0–5.92	61

[a] Sampling year refers to period from July to June split over two calendar years.
[b] Outside (Outside of Riding Mountain Eradication Area [RMEA]).
[c] White-tailed deer.

TABLE 2: Summary of gross pathological and culture results for infected deer and elk by number of tissues examined from south-western Manitoba.

Species	Tissues Examined	No. Examined (%)	No. Cultured	*M. bovis*		*M. avium*		*M. kansasii*		*M. terrae*		Other Mycobacteria[a]	
				No.	%	No.	%	No.	%	No.	%	No.	%
Elk	Whole carcass	446 (12.3%)	445	31	6.97	5	1.12	0	0.00	4	0.90	2	0.45
	Head & Lungs[b]	2589 (71.5%)	2567	9	0.35	5	0.19	0	0.00	6	0.23	0	0.00
	Head Only	571 (15.8%)	569	1	0.18	1	0.18	0	0.00	0	0.00	0	0.00
	Lungs Only[b]	14 (0.4%)	9	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
	Total	3620	3590	41	1.17	11	0.31	0	0.00	10	0.28	2	0.06
WTD	Whole carcass	452 (6.5%)	447	5	1.12	1	0.22	0	0.00	6	1.34	0	0.00
	Head & Lungs[b]	5198 (75.2%)	5176	5	0.10	2	0.04	1	0.02	12	0.23	0	0.00
	Head Only	1208 (17.5%)	1192	1	0.08	1	0.08	0	0.00	0	0.00	0	0.00
	Lungs Only[b]	51 (0.7%)	47	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
	Total	6909	6815	11	0.16	4	0.06	1	0.01	18	0.26	0	0.00

[a] One isolate was *M. chelonae* and one was *M. fortuitum*.
[b] Lung tissue including tracheobronchial and mediastinal lymphoid tissues.

TABLE 3: Site of gross visible lesions (GVL) in *M. bovis* positive elk and white-tailed deer from south-western Manitoba.

		Medial retropharyngeal lymph node	Parotid lymph node	Mandibular lymph node	Palatine tonsil	Lateral retropharyngeal lymph node	Lungs[a]	Body lymph nodes[b]	Abdominal lymph nodes[c]	No visible lesions
Elk	GVL	12	9	2	19	2	20	10	7	0
	Total tested	41	41	41	41	41	40	36	36	41
	Proportion	29.3	22.0	4.9	46.3	4.9	50.0	27.8	19.4	0.0
WTD	GVL	8	1	0	0	0	2	1	2	0
	Total tested	11	11	11	11	11	10	5	5	11
	Proportion	72.7	9.1	0.0	0.0	0.0	20.0	20.0	40.0	0.0

[a] Includes tracheobronchial and mediastinal lymphoid tissues.
[b] Includes prescapular, prefemoral, supramammary/testicular, and popliteal lymph nodes.
[c] Includes mesenteric, hepatic, portal, and internal iliac lymph nodes.

in and around RMNP during the period of this survey (Figure 2). Mean period prevalence over the twelve-year surveillance period was 0.89% (0.66%–1.21%) for elk and 0.15% (0.08%–0.27%) for white-tailed deer. A total of 41 culture positive elk and 11 culture positive white-tailed deer were detected through all forms of surveillance. Elk prevalence has varied quite dramatically from year to year with the highest prevalence being detected in the winter of 2002/2003 (2.01%, Figure 2) when 10 culture positive animals were found through blood testing within RMNP. Prevalence in white-tailed deer has been similarly low and consistently below 1% throughout this period. Virtually all infected elk and white-tailed deer have come from a small geographic area around the north-western border of RMNP (Table 1, Figure 1). This 1800 km² area designated the Western Control Zone where most management activities have been focussed, encompasses 37 of the 41 (90.2%) culture positive elk and 10 of 11 (90.9%) culture positive white-tailed deer found through all forms of surveillance since 1997. Annual prevalence of *M. bovis* within the Western Control Zone has been consistently higher than other

surveillance areas ranging from zero to 6.85% (Table 1). Elk from the WCZ were approximately 21.1 times more likely ($\chi^2 = 67.7$, $P < .001$) to be culture positive than elk from outside this area and white-tailed deer were approximately 49.1 times more likely ($\chi^2 = 56.4$, $P < .001$) to be culture positive compared to deer from outside this zone (based on pooled data from the other three zones for comparison). There was no evidence of a linear trend in overall prevalence for elk ($P = .827$), deer ($P = .80$) or both species combined ($P = .363$) when all data from 1997 to 2010 was examined. But if only the data from 2003 to 2010 was examined neither elk ($P = .120$) nor deer ($P = .768$) exhibit a linear trend, but both species combined exhibit a significant downward trend ($P = .019$) in this most recent time period, as can be observed in Figure 2. This time period also corresponds to a significant decline in number of elk and deer examined (Figure 2), although prevalence and sample numbers were not correlated ($\rho = -0.093$).

Mycobacterium bovis was the most common mycobacterial isolate cultured from elk, but *M. terrae* was the most frequent isolate from white-tailed deer (Table 2). *M. avium*

TABLE 4: Proportion of culture positive elk with gross visible lesions (GVL) in different tissues and body sections stratified by sex.

	Lung[a] GVL	Medial Retropharyngeal GVL	Parotid GVL	Tonsil GVL	Abdominal GVL[b]	Body GVL[c]
Male	13/20 (65.0)	6/22 (27.3)	7/22 (31.8)	11/22 (50.0)	4/15 (26.7)	5/15 (33.3)
Female	6/19 (31.6)	6/19 (31.6)	2/19 (10.5)	8/19 (42.1)	4/15 (26.7)	6/15 (40.0)
χ^2	4.24 (0.039)	0.089 (0.765)	2.63 (0.105)	0.249 (0.618)	0.0 (1.0)	0.139 (0.710)
Odds Ratio	4.02 (0.89–18.9)	0.81 (0.35–2.16)	3.97 (0.60–43.5)	1.38 (0.34–5.65)	1.0 (0.31–3.28)	0.75 (0.33–2.04)

[a] Includes tracheobronchial and mediastinal lymphoid tissues.
[b] Includes mesenteric, hepatic, portal, and internal iliac lymph nodes.
[c] Includes prescapular, prefemoral, supramammary/testicular, and popliteal lymph nodes.

TABLE 5: Prevalence of *M. bovis* in elk and white-tailed deer (WTD) stratified by sex, age category, and surveillance method from southwestern Manitoba.

Species	Age (Years)	Culture −	Culture +	Prevalence[a](%)	Odds Ratio	χ^2	P
	<1[b]	449	1	0.22	1		
	1 to 2	814	6	0.73	3.31 (0.40–152.6)	1.375	.241
	3–5	1821	11	0.60	2.71 (0.39–117.0)	0.987	.320
	6–8	508	12	2.31	10.61 (1.56–454)	7.93	.005
	>8	417	11	2.57	11.84 (1.7–510.9)	8.96	.003
Elk	Sex						
	Female[b]	2683	19	0.70	1		
	Male	1859	22	1.17	1.67 (0.86–3.27)	2.72	.099
	Surveillance Method						
	Hunted[b]	3345	9	0.27	1		
	Opportunistic	179	3	1.65	6.23 (1.07–25.2)	9.72	.002
	Culled	73	2	2.67	10.2 (1.05–50.3)	13.2	<.001
	Blood Test	945	27	2.78	10.6 (4.82–25.7)	57.5	<.001
	Age (Years)						
	<1	457	0	0.00	ND	ND	ND
	1–2[b]	2017	2	0.10	1		
	3–5	4476	3	0.07	0.68 (0.08–8.10)	0.186	.666
	6–8	220	6	2.65	27.5 (4.9–279.3)	37.4	<0.001
	>8	25	0	0.00	ND	ND	ND
	Sex						
WTD	Female	1976	1	0.05	1		
	Male	5392	10	0.19	3.66 (0.52–159.1)	1.76	.185
	Surveillance Method						
	Hunted	6735	6	0.09	1		
	Opportunistic	195	0	0.00	0 (0–29.5)	0.17	.677
	Culled	273	3	1.09	12.34 (1.98–58.1)	20.61	<.001
	Blood Test	165	2	1.20	13.61 (1.33–76.7)	17.31	.001

[a] Stratum specific prevalence (number positive/total number tested per category).
[b] Category used as the reference category for odds ratio and chi-square calculations.
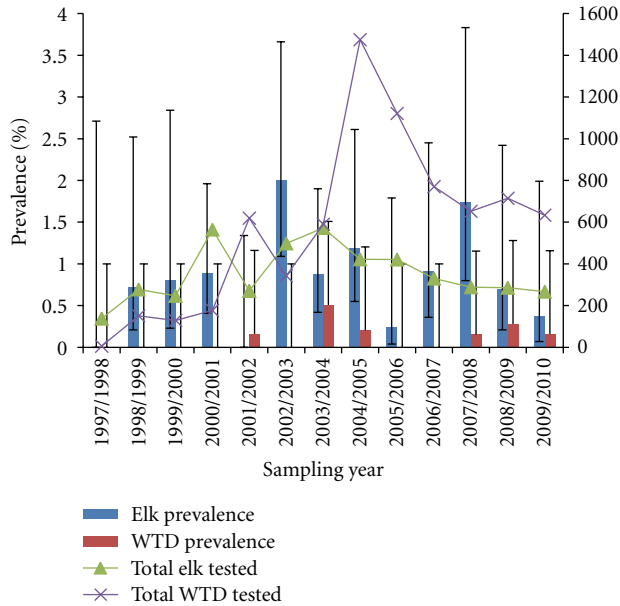
FIGURE 2: Overall estimated annual prevalence of *M. bovis* and total number of elk and white-tailed deer sampled from south-western Manitoba from 1997 to 2010.

was only cultured from elk, while *M. kansasii* was only cultured from deer. Other mycobacteria isolated included *M. fortuitum* and *M. chelonae*. All mycobacteria including *M. bovis* were most frequently isolated when the entire carcass was available for examination compared to other tissues such as the head or lungs. Thirty-one culture positive elk and 5 culture positive deer were diagnosed from examination and full necropsy of the entire carcass. Of these, 19 of 31 (61.3%) elk had gross visible lesions in the head, 15 of 31 (48.4%) had gross visible lesions in the lungs, and 25 of 31 (80.6%) had gross visible lesions in either the head lymph nodes or lungs. Three of 5 (60%) culture positive deer which had full necropsies had gross visible lesions in the head, 0 of 5 had gross visible lesions in the lungs, and 3 of 5 (60%) had gross visible lesions in either the head lymph nodes or lungs.

The most common sites of gross lesions in culture positive elk were the lungs, palatine tonsils, and retropharyngeal lymph nodes, while in white-tailed deer it was the retropharyngeal lymph node, abdomen (mesenteric lymph node), and body lymph nodes (popliteal) (Table 3). All (100%) culture positive white-tailed deer and elk exhibited at least one gross lesion compatible with *M. bovis* infection at necropsy. Gross lesions typically consisted of caseopurulent or granulomatous lesions which were either multifocal or singular and were commonly associated with some degree of mineralization. Histologically, lesions were typically well encapsulated when in lymphoid tissues and were often disseminated when in the lungs. Male elk were approximately four times more likely to have gross visible lesions in the lungs compared to female elk when stratified by sex (Table 4). Gross lesions did not vary significantly by sex for other tissues examined.

Neither sex was more likely to be *M. bovis* culture positive for both elk and deer based on proportions sampled in this study (Table 5). The prevalence of infection increased with age class in elk, but the oldest age class of deer (>8 years) had very few samples and no *M. bovis* positive animals. The majority of culture positive elk and deer were detected through blood testing, followed by opportunistic sampling and culling (Table 5), while fewer culture positive animals were detected with hunter-killed animals. At necropsy, blood-tested elk had odds of testing culture positive of 10.6 compared to hunter-killed elk, while blood-tested deer had odds of 13.6 compared to hunter-killed deer (Table 5). The seven culture positive elk in the younger age classes (less than or equal to 2 years of age) were all found prior to 2004, and no elk younger than five years of age has been found since then.

4. Discussion

Bovine tuberculosis has been consistently present in elk in the RMNP ecosystem since at least 1992 and in white-tailed deer sporadically since 2001. Culture positive elk have been found every year in this area with the exception of two years (1997/1998 and 2001/2002), while infected white-tailed deer have been detected only in certain years and not consistently from year to year despite testing large numbers of animals. For this reason, it has been suggested that elk are the primary reservoir species of *M. bovis* within this ecosystem [2]. The factors which result in this differential temporal occurrence could be related to differences in social structure, susceptibility to *M. bovis*, individual contact rate, herd immunity, and method of testing. Studies in Michigan and New Zealand suggest that elk or red deer do not act as reservoir species, but are spillover hosts instead [13, 27, 28] while data from red deer in France suggest they may act as a reservoir host in association with wild boar [27, 29]. Data presented in this study suggest that elk indeed may be a primary reservoir species, but that infected white-tailed deer may also be necessary to maintain ongoing infection in a multispecies reservoir system [9]. Infected cattle herds may also be a necessary part of this multispecies reservoir, as infected cattle herds have not been found consistently in this area despite rigorous and intensive testing [1, 3], but the role of cattle as a reservoir species is currently undetermined. It is unlikely that there are other undetected reservoir species in this ecosystem as multiple species have been assessed with negative findings to date [1, 3, 30]. This study provides some evidence that overall prevalence of *M. bovis* in both deer and elk is declining since 2003 as the number of infected cattle herds has also declined. Another piece of evidence which supports this is the lack of younger age classes of elk found positive since 2004. Since 2004, all *M. bovis* positive elk found through surveillance activities have been 5 years of age or older, but prior to 2004, five elk that were 2 years of age, 1 yearling and one calf were found to be infected. This trend is not apparent for white-tailed deer as the two most recent infected white-tailed deer were 2 years of age. Since *M. bovis* infection in cervids results in chronic disease and elk are a relatively long-lived ungulate species, especially

in a protected area, it is likely that positive cases of *M. bovis* will be continued to be detected in both elk and deer in this area for several years to come. The net force of infection is the instantaneous per capita rate that individual cervids become infected [31]. This can be estimated in wild populations infected with *M. bovis* using the proportion of young age classes found infected on cross-sectional surveys [13], as these represent relatively new infections based on short exposure times. Based on the findings of this study, the net force of infection has decreased in elk since 2004. Similar to previous studies of both red deer and white-tailed deer, age-specific prevalence of *M. bovis* increases dramatically in older age classes of both elk and deer [14, 16, 32]. Elk older than 6 years were 10 times more likely to be culture positive compared to younger age classes. Small numbers of positive deer made this association much less apparent with white-tailed deer, but the trend was similar.

The prevalence of *M. bovis* in wild elk is significantly lower in this ecosystem compared to comparable populations of red deer found in other parts of the world including New Zealand, Spain, and France where prevalence often exceeds 30%. Spatial aggregation at waterholes has been shown to be an important risk factor for infection in Spanish red deer [16], while association with other infected wildlife reservoirs such as brush-tailed possum and wild boar have been shown to be important risk factors in New Zealand and France, respectively [27, 29, 33]. The role of host density in maintenance of cervid reservoirs of *M. bovis* is somewhat equivocal with some studies finding density-dependent effects, while others have refuted this hypothesis [1, 15, 34]. Attempts to model *M. bovis* infection in wild ungulates have relied upon density-dependent transmission [35] and some studies have found positive correlations between density and prevalence [15]. Supplemental feeding and spatial aggregation around waterholes have been positively associated with spatial occurrence of *M. bovis* [10, 16], suggesting that contact structure and localized congregations may be important factors allowing maintenance and transmission of the disease in wildlife reservoirs. Elk densities were historically much higher in the RMNP area [36] and deer densities have likely been increasing since the early part of the twentieth century when white-tailed deer began colonizing this area. One of the management strategies instituted in 2003 to control *M. bovis* in this area was an attempt to keep the regional elk population at historically low levels in an attempt to reduce transmission [18]. Other strategies introduced at roughly the same time were lengthened hunting seasons, a moratorium on regional wolf trapping, and fencing of hay storage yards around RMNP [1, 37]. It appears that this combination of management factors has likely played a role in reducing the prevalence of *M. bovis* in ungulates in the RMNP area since 2003 as well as a decreasing the number of spillover events to surrounding cattle herds. Strategies to eventually eliminate bovine tuberculosis in this ecosystem are being actively considered by government agencies and local stakeholders.

The pathology of *M. bovis* infection found in elk is similar to that described in both captive and farmed elk as well as wild red deer populations in other parts of the world, with the exception that all culture positive elk had grossly visible lesions, meaning there were no culture positive elk without visible lesions (NVL) in this study. Other studies of wild red deer in Spain and New Zealand have found proportions of culture-positive elk that are NVL as high as 30% [7, 14], while studies in Canadian captive elk had proportions of approximately 7% [23]. The reason may be that a significant proportion of elk in this study were examined by a full necropsy using a detailed necropsy procedure that was designed to find *M. bovis* lesions, whereas other studies have typically used field necropsies or just examined portions of carcasses. Thus, many subtle lesions that may have been missed on a field necropsy were discovered during this study.

Other mycobacteria isolated from lesions in both elk and deer likely decrease the specificity of diagnostic tests for mycobacteria. *M. terrae* was the most common mycobacterial isolate in white-tailed deer, but previous studies have not reported isolation of *M. terrae* commonly [38]. *M. avium* was the next most common mycobacterial isolate in elk. Prior exposure to environmental mycobacteria such as *M. terrae* and other mycobacteria may play a role in sensitizing the host immune response to *M. bovis* [39, 40] and may be one factor causing individual heterogeneity in rates of infection and resistance in wild populations.

Both male elk and white-tailed deer were more likely to be culture positive for *M. bovis*, but the difference was not significant due to low sample sizes when stratified by species (Table 5). Males have generally had higher odds of testing positive to *M. bovis* in studies of both red deer and white-tailed deer [2, 32]. In the RMNP ecosystem, 10 of 11 culture-positive white-tailed deer have been male since 2001, but the low numbers of positives and higher proportion of male deer in the sample dilutes this effect. Sampling zone and surveillance method were significantly associated with *M. bovis* status in this study with animals being sampled in the Western Control zone being at a significantly higher risk of being positive for *M. bovis* than elk or deer sampled in other areas. Both elk and deer sampled through blood testing and culling were much more likely to be culture positive than animals sampled through hunting or other surveillance methods. One reason for this is that once *M. bovis* positive elk were found in the Western Control zone through blood sampling, surveillance efforts tended to focus on this area to a certain degree, increasing the likelihood of finding culture positive animals. Hunter samples tended to be more randomly distributed but are limited spatially in that none came from within RMNP. The true extent of *M. bovis* infection in this ecosystem was not fully realized until a costly and rigorous sampling program was carried out using blood tests within RMNP. Using multiple surveillance methods rather than relying on a single method was a key determinant in determining the extent of infection in wildlife in this ecosystem. Detection of *M. bovis* in wildlife species at fine spatial scales within protected areas is much more difficult [17], and this is one of the first studies to rely on blood sampling rather than traditional skin testing and hunter surveillance to determine *M. bovis* distribution in a cervid reservoir.

Similar to Michigan, *M. bovis* appears to be highly clustered in cervids in the RMNP area, but unlike Michigan,

elk are more commonly infected than white-tailed deer [8]. Reasons for this discrepancy are unknown, but are likely related to different population densities, social behaviour, and presence of baiting and feeding for hunting [10]. White-tailed deer densities in Michigan are much higher compared to south-western Manitoba [1] and the role of supplemental feeding to bait deer in Michigan [11] may act to further aggregate deer at local spatial scales. Supplemental feeding and baiting for purposes of hunting have been prohibited through legislation and enforced in the RMEA since 2002. Baiting and feeding is difficult to control in some jurisdictions, but has been relatively well accepted by local stakeholders in Manitoba. Conversely, elk population size and density are likely greater within RMNP than is found in Michigan, where elk densities are somewhat lower and not directly within the core area where *M. bovis* is found. Other factors such as habitat quality and quantity, intraspecific and interspecific contact rates, and herd immunity may also play a role in the maintenance of *M. bovis* infection in these wildlife reservoirs. Studies currently ongoing in the RMNP area hope to clarify the role of some of these important factors.

5. Conclusions

M. bovis infection has been consistently present in a relatively small geographic area located in and around the north-western part of RMNP since at least 1978, but significant annual variation in prevalence has occurred since 1997 in both elk and deer. Period prevalence in elk is approximately six times higher than deer, suggesting they may be a significant reservoir host of *M. bovis* in this ecosystem, but that infected white-tailed deer may also be required to maintain a true reservoir in this system. Pathological lesions associated with *M. bovis* infection and distribution of those lesions in wild elk and deer are very similar to those described in other parts of the world, but fewer NVL elk were found compared to red deer. The lack of culture positive animals in younger age classes of elk since 2003 indicate that the net force of infection as well as overall prevalence are declining in elk in this area, but further surveillance and monitoring will be necessary to determine if this is consistent over time. This study demonstrates that it is vitally important to sample all geographical sites occupied by *M. bovis* host species using a variety of surveillance methods if possible, or focal aggregations of disease may be overlooked for long periods of time. Both the management and surveillance of infected wildlife reservoirs is challenging and difficult, but blood-based assays were a crucial part of estimating the apparent prevalence and spatial distribution of *M. bovis* infection in this system.

Acknowledgments

References

[1] G. Wobeser, "Bovine Tuberculosis in Canadian wildlife: an updated history," *Canadian Veterinary Journal*, vol. 50, no. 11, pp. 1169–1176, 2009.

[2] V. W. Lees, S. Copeland, and P. Rousseau, "Bovine tuberculosis in elk (*Cervus elaphus manitobensis*) near Riding Mountain National Park, Manitoba, from 1992 to 2002," *Canadian Veterinary Journal*, vol. 44, no. 10, pp. 830–831, 2003.

[3] M. Koller-Jones et al., "Effect of bovine tuberculosis in wildlife on a national eradication program—Canada," in *Mycobacterium bovis Infection in Animals and Humans*, C. Thoen, J. Steele, and M. Gilsdorf, Eds., pp. 226–237, Blackwell Publishing, Ames, Iowa, USA, 2006.

[4] C. Lutze-Wallace, G. Berlie-Surujballi, Y. Barbeau, and D. Bergeson, "Strain typing of *Mycobacterium bovis* from a 1978 case of tuberculosis in a wolf (*Canis lupis*) from Manitoba," *Canadian Veterinary Journal*, vol. 46, no. 6, p. 502, 2005.

[5] C. Lutze-Wallace, C. Turcotte, M. Sabourin et al., "Spoligo-typing of *Mycobacterium bovis* isolates found in Manitoba," *Canadian Journal of Veterinary Research*, vol. 69, no. 2, pp. 143–145, 2005.

[6] G. W. de Lisle, R. G. Bengis, S. M. Schmitt, and D. J. O'Brien, "Tuberculosis in free-ranging wildlife: detection, diagnosis and management," *OIE Revue Scientifique et Technique*, vol. 21, no. 2, pp. 317–334, 2002.

[7] D. Gavier-Widén, M. M. Cooke, J. Gallagher, M. A. Chambers, and C. Gortázar, "A review of infection of wildlife hosts with *Mycobacterium bovis* and the diagnostic difficulties of the 'no visible lesion' presentation," *New Zealand Veterinary Journal*, vol. 57, no. 3, pp. 122–131, 2009.

[8] D. J. O'Brien, S. M. Schmitt, D. E. Berry et al., "Estimating the true prevalence of *Mycobacterium bovis* in free-ranging elk in Michigan," *Journal of Wildlife Diseases*, vol. 44, no. 4, pp. 802–810, 2008.

[9] D. T. Haydon, S. Cleaveland, L. H. Taylor, and M. K. Laurenson, "Identifying reservoirs of infection: a conceptual and practical challenge," *Emerging Infectious Diseases*, vol. 8, no. 12, pp. 1468–1473, 2002.

[10] G. Hickling, "Dynamics of bovine tuberculosis in wild white-tailed deer in Michigan," Tech. Rep. 3363, Michigan Department of Natural Resources, 2002, pp. 34.

[11] D. J. O'Brien, S. M. Schmitt, S. D. Fitzgerald, D. E. Berry, and G. J. Hickling, "Managing the wildlife reservoir of *Mycobacterium bovis*: the Michigan, USA, experience," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 313–323, 2006.

[12] B. Hartmann, "Comments on prevalence of bovine tuberculosis in Minnesota," *Journal of the American Veterinary Medical Association*, vol. 236, no. 3, pp. 282–283, 2010.

[13] G. Nugent, *The role of wild deer in the epidemiology and management of bovine tuberculosis in New Zealand*, Ph.D. thesis, Lincoln University, Lincoln, NZ, 2005.

[14] I. W. Lugton, P. R. Wilson, R. S. Morris, and G. Nugent, "Epidemiology and pathogenesis of *Mycobacterium bovis* infection of red deer (*Cervus elaphus*) in New Zealand," *New Zealand Veterinary Journal*, vol. 46, no. 4, pp. 147–156, 1998.

[15] J. Vicente, U. Höfle, I. G. Fernández-De-Mera, and C. Gortazar, "The importance of parasite life history and host density in predicting the impact of infections in red deer," *Oecologia*, vol. 152, no. 4, pp. 655–664, 2007.

[16] J. Vicente, U. Höfle, J. M. Garrido et al., "Risk factors associated with the prevalence of tuberculosis-like lesions in fenced wild boar and red deer in south central Spain," *Veterinary Research*, vol. 38, no. 3, pp. 451–464, 2007.

[17] C. Gortázar, M. J. Torres, J. Vicente et al., "Bovine tuberculosis in Doñana Biosphere Reserve: the role of wild ungulates as disease reservoirs in the last Iberian lynx strongholds," *PLoS ONE*, vol. 3, no. 7, Article ID e2776, 2008.

[18] J. S. Nishi, T. Shury, and B. T. Elkin, "Wildlife reservoirs for bovine tuberculosis (*Mycobacterium bovis*) in Canada: strategies for management and research," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 325–338, 2006.

[19] O. M. Surujballi, C. Lutze-Wallace, C. Turcotte et al., "Sensitive diagnosis of bovine tuberculosis in a farmed cervid herd with use of an MPB70 protein fluorescence polarization assay," *Canadian Journal of Veterinary Research*, vol. 73, no. 3, pp. 161–166, 2009.

[20] K. P. Lyashchenko, R. Greenwald, J. Esfandiari et al., "Animal-side serologic assay for rapid detection of *Mycobacterium bovis* infection in multiple species of free-ranging wildlife," *Veterinary Microbiology*, vol. 132, no. 3-4, pp. 283–292, 2008.

[21] M. V. Thrusfield, *Veterinary Epidemiology*, Blackwell Publishing, Ames, Iowa, USA, 3rd edition, 2005.

[22] M. A. Chambers, T. Crawshaw, S. Waterhouse, R. Delahay, R. G. Hewinson, and K. P. Lyashchenko, "Validation of the BrockTB Stat-Pak assay for detection of tuberculosis in Eurasian badgers (*Meles meles*) and influence of disease severity on diagnostic accuracy," *Journal of Clinical Microbiology*, vol. 46, no. 4, pp. 1498–1500, 2008.

[23] E. B. Rohonczy, A. V. Balachandran, T. W. Dukes et al., "A comparison of gross pathology, histopathology, and mycobacterial culture for the diagnosis of tuberculosis in elk (*Cervus elaphus*)," *Canadian Journal of Veterinary Research*, vol. 60, no. 2, pp. 108–114, 1996.

[24] R. E. Keiss, "Comparisons of eruption wear patterns and cementum annuli as age criteria in elk," *Journal of Wildlife Management*, vol. 33, p. 5, 1969.

[25] E. B. Wilson, "Probable inference, the law of succession, and statistical inference," *Journal of the Amercian Statistical Association*, vol. 22, p. 4, 1927.

[26] I. Campbell, "Chi-squared and Fisher-Irwin tests of two-by-two tables with small sample recommendations," *Statistics in Medicine*, vol. 26, no. 19, pp. 3661–3675, 2007.

[27] G. Zanella, A. Duvauchelle, J. Hars, F. Moutou, M. L. Boschiroli, and B. Durand, "Patterns of lesions of bovine tuberculosis in wild red deer and wild boar," *Veterinary Record*, vol. 163, no. 2, pp. 43–47, 2008.

[28] D. J. O'Brien, S. M. Schmitt, K. P. Lyashchenko et al., "Evaluation of blood assays for detection of *Mycobacterium bovis* in white-tailed deer (*Odocoileus virginianus*) in Michigan," *Journal of Wildlife Diseases*, vol. 45, no. 1, pp. 153–164, 2009.

[29] G. Zanella, B. Durand, J. Hars et al., "*Mycobacterium bovis* in wildlife in France," *Journal of Wildlife Diseases*, vol. 44, no. 1, pp. 99–108, 2008.

[30] C. Sangster, D. Bergeson, C. Lutze-Wallace, V. Crichton, and G. Wobeser, "Feasibility of using coyotes (*Canis latrans*) as sentinels for bovine mycobacteriosis (*Mycobacterium bovis*) infection in wild cervids in and around riding Mountain National Park, Manitoba, Canada," *Journal of Wildlife Diseases*, vol. 43, no. 3, pp. 432–438, 2007.

[31] P. Caley, G. Marion, and M. R. Hutchings, "Assessment of transmission rates and routes, and the implications of management," in *Management of Disease in Wild Mammals*, R. J. Delahay, G. C. Smith, and M. R. Hutchings, Eds., pp. 31–51, Springer, Tokyo, Japan, 2005.

[32] D. J. O'Brien, S. M. Schmitt, J. S. Fierke et al., "Epidemiology of *Mycobacterium bovis* in free-ranging white-tailed deer, Michigan, USA, 1995–2000," *Preventive Veterinary Medicine*, vol. 54, no. 1, pp. 47–63, 2002.

[33] T. J. Ryan, P. G. Livingstone, D. S. L. Ramsey et al., "Advances in understanding disease epidemiology and implications for control and eradication of tuberculosis in livestock: the experience from New Zealand," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 211–219, 2006.

[34] M. M. Conner, M. R. Ebinger, J. A. Blanchong, and P. C. Cross, "Infectious disease in cervids of North America: data, models, and management challenges," *Annals of the New York Academy of Sciences*, vol. 1134, pp. 146–172, 2008.

[35] C. W. McCarty and M. W. Miller, "A versatile model of disease transmission applied to forecasting bovine tuberculosis dynamics in white-tailed deer populations," *Journal of Wildlife Diseases*, vol. 34, no. 4, pp. 722–730, 1998.

[36] F. Zhao, *Environement, Earth and Resources*, M.S. thesis, Historical Overview of Bovine Tuberculosis in the Riding Mountain National Park Ecosystem, University of Manitoba, Manitoba, Canada, 2006.

[37] R. K. Brook, "Incorporating farmer observations in efforts to manage bovine tuberculosis using barrier fencing at the wildlife-livestock interface," *Preventive Veterinary Medicine*, vol. 94, no. 3-4, pp. 301–305, 2010.

[38] S. D. Fitzgerald, J. B. Kaneene, K. L. Butler et al., "Comparison of postmortem techniques for the detection of *Mycobacterium bovis* in white-tailed deer (*Odocoileus virginianus*)," *Journal of Veterinary Diagnostic Investigation*, vol. 12, no. 4, pp. 322–327, 2000.

[39] P. Nol, K. P. Lyashchenko, R. Greenwald et al., "Humoral immune responses of white-tailed deer (*Odocoileus virginianus*) to *Mycobacterium bovis* BCG vaccination and experimental challenge with *M. bovis*," *Clinical and Vaccine Immunology*, vol. 16, no. 3, pp. 323–329, 2009.

[40] B. M. Buddle, B. J. Wards, F. E. Aldwell, D. M. Collins, and G. W. De Lisle, "Influence of sensitisation to environmental mycobacteria on subsequent vaccination against bovine tuberculosis," *Vaccine*, vol. 20, no. 7-8, pp. 1126–1133, 2002.

Research Article

Ovine Paratuberculosis: A Seroprevalence Study in Dairy Flocks Reared in the Marche Region, Italy

Attili Anna Rita,[1] Ngu Ngwa Victor,[1] Preziuso Silvia,[1] Pacifici Luciana,[2] Domesi Anastasia,[2] and Cuteri Vincenzo[1]

[1] *School of Veterinary Medical Sciences, University of Camerino, via Circonvallazione 93-95, 62024 Matelica, Italy*
[2] *Regione Marche, Azienda Sanitaria Unica Regionale 10, Servizio Veterinario Sanità Animale, via Muralto, 62032 Camerino, Italy*

Correspondence should be addressed to Attili Anna Rita, annarita.attili@unicam.it

In order to fulfil the seroprevalence gap on *Mycobacterium avium* subsp. *paratuberculosis* infection in ovine dairy farms of Marche region (central Italy), a stratified study was carried out on 2086 adult female sheep randomly chosen from 38 herds selected in Ancona and Macerata provinces. 73.7% flocks resulted infected by a commercial ELISA test (Pourquier, France), with a mean seroprevalence of 6.29% of sampled sheep in both provinces. A higher number of *MAP* seropositive ewes was recorded in the large herds' consistence than in the small and medium herds' consistence ($P = 0.0269$), and a greater percentage of infected sheep was obtained among female at early/late than in peak lactation stage ($P = 0.0237$). *MAP* infection was confirmed in 12.6% of infected farms by faecal culture. The true sheep-level seroprevalence was 15.1% $\pm$ 7.3%.

1. Introduction

Paratuberculosis, known as Johne's disease (JD), is a prevalent and economically important chronic, nontreatable inflammatory bowel disease of domestic and wild ruminants as well as other mammals worldwide, including nonhuman primates [1–4]. It is on the list of "multiple species diseases" notifiable to the World Organization for Animal Health as a ruminant disease of concern [5]. The ovine paratuberculosis is an object of worry since its insidious evolution and the severity of the illness are always accompanied to a too late clinical diagnosis. It represents a sanitary and zootechnical problem of remarkable proportions because of its incidence, the lack of a valid therapeutic and preventive strategy, and the economic losses due to clinical and subclinical disease [2, 6, 7].

Mycobacterium avium subspecies *paratuberculosis* (*MAP*) has also been suggested as an aetiological agent of Crohn's disease, a chronic, granulomatous infection of the human intestine [8–10]. The bacterium has been isolated from a high percentage of Crohn's patients [11–13] although the zoonotic potential of the organism remains controversial

[8, 14]. More recently, its involvement was hypothesized for the onset of human Type I diabetes [15, 16]. People are exposed to *MAP* by direct contact with infected material and in retail milk supplies [17]. Ruminant milk has been described as a potential source through which human beings could be infected [18, 19]. Viable *MAP* has been isolated from milk and colostrum of clinically and subclinically infected cows [20], and infection of the mammary gland has been documented in small ruminants [21]. *MAP* has been demonstrated by PCR in goats' milk from bulk tanks in farms in the UK [22], the bacterium can survive pasteurisation, and cheese production processes have been shown to have little effect on the viability of *MAP* [18, 23]. Furthermore, viable bacteria have been demonstrated in hard and semi-hard cheese 120 days after production [24].

Diagnosis of subclinical infection with *MAP* in ruminant species remains one of the greatest challenges to JD control, both at the individual animal [4, 25] and herd level [26].

High shedders of *MAP* and animal with clinical signs of paratuberculosis are responsible for the greater part of the contamination of their environment, the economic damage in infected herds, and the presence of bacteria in milk [27].

To monitor the progression of a control programme, the herds need to be tested. Serology is the most practical method used for this purpose. The enzyme-linked immunosorbent assay (ELISA) is a suitable diagnostic tool to detect serum antibodies against *MAP* on a large scale, because it is possible to test large numbers of samples with a high reproducibility [25, 28]. In general, commercially available ELISA-kits for paratuberculosis have a low sensitivity [29–31]. Nevertheless, the assays have a reasonable good sensitivity to identify heavy shedders for culling in a preclinical stage [32, 33], reduce the pressure of infection in infected herds, and thus enhance the efficacy of the preventive measures [34, 35]. Control programs for Johne's disease have been established in a number of countries. In Italy, mandatory plans have not been performed in ovine population unlike other nations. Knowledge of the current herd- and sheep-level prevalence is of value today, but it will be more important as a baseline upon which evaluation of control programs can take place.

The highland provinces of Macerata and Ancona in the Marche region (central of Italy) have a sizeable number of dairy ovine populations that produces significant quantity of milk for cheese production and lambs for meat per annum. The farmers in this region have shown increased anxiety to know about the *MAP* prevalence of their herds especially those that ones involved in cheese production using unpasteurised ovine milk and the farmers that observe an unexpected decrease in milk production in their flocks. Very little is known on the epidemiology of ovine paratuberculosis in the central Italy, and *MAP* seroprevalence in the Marche region is unknown.

The objectives of the present study were as follows: (1) to estimate the individual- and herd-level seroprevalence of *MAP* among ovine dairy flocks of Ancona and Macerata provinces of Marche region, Italy and (2) to observe epidemiological factors and to examine their association with *MAP* seroprevalence among adult ovine herds in the two provinces of central Italy.

2. Materials and Methods

2.1. Study Design. A stratified study was designed in the Marche region of central Italy. The epidemiological unit of concern was the herd.

2.2. Target Population and Sample Size. At the beginning of the study, July 2008, the ovine herd consistence in Ancona and Macerata provinces registered in the Italian Anagrafe Nazionale Zootecnica archive [36] (http://statistiche .izs.it/Zootecniche/) is reported in Table 1.

The following formula was used to calculate the sample herd size: $n_{\text{inf}} = (P)(1 - P)Z^2/d^2$ [37], where n_{inf} = sample size for infinite population; P = estimated prevalence of infection [as a decimal: 0.04 (4%)]; Z = degree of confidence ($Z = 1.96$ for 95% of confidence); d = maximum difference between observed and the true prevalence that we are willing to accept [as a decimal: 0.10 (10%)]. Then, to estimate the required sample size (n_{fin}) for a finite population (N), the following conversion was done: $n_{\text{fin}} = n_{\text{inf}}/1 + (n_{\text{inf}} - 1)/N$.

Table 1: Number of herd consistence recorded by Italian Anagrafe Nazionale Zootecnica archive (National Livestock Population) (http://statistiche.izs.it/Zootecniche) on 31st July 2008.

	Number of herds	
	Ancona province	Macerata province
Milk sheep	29	35
Mixed sheep	213	240
Total	**242**	**275**

Random sampling of ovine herds was performed using the random number generator and considering the herd progressive number list.

2.3. Study Population. The data used in this study came from 2086 dairy and mixed, milk and meat, address production sheep randomly selected and reared in 38 farms representative of the herd population in the two provinces of Marche region: eighteen herds in Ancona province and twenty herds in Macerata province.

A stratified random sampling was carried out. The population was divided into subgroups according to geographical area (province), herd consistence, breed, sheep purpose, lactation stage, and clinical signs. A random sample was taken from each of these strata.

The ovine breeds reared were: "Comisana," "Fabrianese," "Massese," "Sarda," "Sopravvissana," and crosses. Most of the herds were for milk production, whereas mixed-purpose sheep were predominant in other herds.

The farms consisted of small (≤500 sheep), medium (500< sheep ≥1000), and large (≥1000 sheep) size consistence and were all semi-intensive herds. A systematic sampling was used to take serum samples for this study. The sampling represented the 10% of herds' consistence. The serum samples were collected from individually identified female sheep aged more than two years old.

2.4. Survey Design. Farmers were recruited to the study between September 2008 and July 2010. To enlist 38 dairy sheep farms, forty eight owners were contacted by telephone but ten of them did not agree to participate to an interview questionnaire and farm visit for blood sample collection. The epidemiological form was based on three main topics: animal/herd management and facilities, health status, and the sanitary measures adopted during the introduction of new animals in the flock and the formalities of faecal elimination. Each farm had one visit during the study. When seropositive ewes were found, they were submitted to a faecal sampling in a second visit.

3. Laboratory Work

3.1. Sampling. During the Annual Official Brucellosis Eradication Campaign, between September 2008 and July 2010, blood samples were collected from 2086 randomly chosen breeding ewes in lactation, aged over two years and reared in 38 farms with or without history of paratuberculosis. At

farm visit, the 10% of animals were subjected to a jugular puncture using sterile anticoagulant-free vacutainer tubes. All samples were taken to the Infectious Diseases Laboratory of the School of Veterinary Medical Sciences of the University of Camerino where, after centrifugation, the sera were harvested and stored at $-20°C$ until laboratory analysis was performed. Faecal samples were collected from seropositive sheep in all positive herds for bacteriological culturing and Ziehl Neelsen staining. Diarrhoea and wasting were observed in some sheep during sampling.

3.2. Serological Investigations. All blood samples were analysed by a commercial indirect ELISA following the instructions of the manufacturer (ELISA Paratuberculosis kit; Institut Pourquier, France). The specificity of this test was increased by preabsorption with *Mycobacterium phlei* antigen [38]. Wash steps were completed with an automated washer. Double-positive and single-negative control samples were included in all the series of ELISA; in addition to the negative and positive control samples provided by the manufacturer, two internal control serum samples were run on each plate. The cut-off as defined by the manufacturer was Sample to Positive ratio greater than or equals to 0.350 and the ratio between the mean positive control OD and the negative control OD greater than or equal to 3. Briefly, the controls and serum samples were diluted at 1 : 20 and preincubated with *M. phlei* extract which assists in binding unspecific antibodies. After washing, a diluted antiruminant horseradish peroxidase was dispensed per well in order to detect the presence of bounded antibodies. Then the Tetramethylbenzidine (TMB) enzyme substrate was used. The reaction was stopped by 1M HCl solution, and the optical density was measured at 450 nm ($OD_{450\,nm}$) using a Multiskan Ascent ELISA reader (Labsystem, Finland). All samples were tested in single and the optical density readings at 450 nm were used for the analyses. The manufacturer recommended 70% as the cut-off for positive samples. The sera within the range of 60–70% were classified as doubtful, while samples below 60% were considered as negative. The generated positive/negative binary outcome was used in all statistical analyses in this study. Doubtful samples were classified as negative and were not included in the statistical analysis of results.

In order to evaluate the true prevalence, the Rogan-Gladen [39] estimator $\{P = [AP + (SP - 1)]/[SE + (SP - 1)]\}$ was used to convert apparent prevalence values, where P = True prevalence, AP = Apparent prevalence, SP = Test specificity, and SE = Test sensitivity. An estimation had previously been made about the Pourquier's ELISA performances in ovine serum and a sensitivity of 34.9% and a specificity of 98.8% were considered [40].

3.3. Bacteriology (Microscopy and Culture). Ziehl-Neelsen (ZN) stained smears of faeces were examined microscopically and a presumptive diagnosis of paratuberculosis were made when clumps of small strongly acid-fast bacilli were found.

Culture was carried out on individual faecal sample, and a standardized basic method was used as described in the OIE terrestrial manual [5] with some modification.

Briefly, 2-g aliquot was mixed with 35 mL of sterile distilled water and shaken for 30 min. Large debris was allowed to settle for 45 min at room temperature. The aqueous layer was removed and centrifuged at 2500 g for 20 min. The resulting pellet was processed through a decontamination procedure [41]. First, the pellet was resuspended in 0.9% hexadecylpyridinium chloride (HPC; Sigma-Aldrich, Italy) in half strength ($0.5\times$) brain heart infusion broth (BHI; Becton Dickinson), incubated overnight at 37°C, and then centrifuged at 2500 rpm for 20 min to form a pellet. The pellet was resuspended in 1.2 mL 0.5 × BHI containing nalidixic acid (100 μg/mL), vancomycin (100 μg/mL), and amphotericin B (50 μg/mL) (Sigma-Aldrich, Italy) and incubated overnight at 37°C. Herrold's egg yolk medium (HEYM) agar slant containing antibiotics was inoculated with 0.2 mL of the decontaminated inoculum. All cultures were incubated at 37°C for up 20 weeks, with periodic visual assessment. Identification of *MAP* colonies was based on cultural, microscopic, and dependence to mycobactin J for growth. Typical bacterial morphology was confirmed by ZN-staining and by PCR (IS*900*) [42].

4. Outcome Variables and Statistical Analysis

A herd was defined as seropositive when one or more sheep in the herd were tested seropositive by serum ELISA test. The herd seroprevalence of *MAP* was calculated from the number of seropositive sheep divided by the total number of sheep tested at visit. Herds were dichotomized above and below the mean herd seroprevalence. Data from questionnaires, serological analysis, ZN staining, faecal culture, and molecular investigations were stored in a database and analysis was performed using a statistical software (STATA version 5; STATA Corporation, College Station, Texas, USA). The serological results were compared by province, herd size, breed and kind of production, lactation stage, and clinical signs.

Descriptive statistics were calculated to determine the apparent *MAP* prevalence at animal and herd levels. Confidence intervals at 95% of the means of clustered samples were calculated as outlined by Thrusfield [43]. Statistical differences from the ELISA results were evaluated by Chi square test. *P* Values less than 0.05 were considered significant.

5. Results

The serological investigation was performed on 2.24% of the total sheep reared in the two provinces of the Marche region. Thirty-eight randomly selected farms out of 517 dairy and mixed sheep farms representing 7.35% of the total ovine farms in the areas of study: 7.14% and 7.27% for Ancona and Macerata provinces, respectively, were evaluated. A total of 2086 animals over 2 years of age from the 38 ovine flocks were sampled for serological screening analysis. Mean and median flock sizes were 549 and 400 animals, respectively, and ranged from 110 to 1500 animals. Descriptive statistics of the study population in the two provinces are stated in Table 2.

Table 2: The sheep population in the evaluated herds in Ancona and Macerata provinces.

Study population	Variables	Number of herds		
		Macerata	Ancona	N. Total/Percentage
	Mean	550 (min: 110; max: 1500)	548 (min: 130; max: 1500)	549 (min: 110; max: 1500)
	Median	380	490	400
Herd type	Dairy	17	16	33 (86.8%)
	Mixed production	3	2	5 (13.2%)
Breed	Comisana	8	10	18 (47.4%)
	Comisana-Sarda	6	5	11 (28.9%)
	Fabrianese	1	2	3 (7.9%)
	Massese	1	1	2 (5.3%)
	Sopravvisana	1	—	1 (2.6%)
	Comisana-Massese	1	—	1 (2.6%)
	Comisana-Fabrianese	1	—	1 (2.6%)
	Comisana-Barbaresca	1	—	1 (2.6%)
Season of sampling	Spring	6	7	12 (31.6%)
	Summer	5	5	10 (26.3%)
	Autumn	5	4	9 (23.7%)
	Winter	5	2	7 (18.4%)
Lactation stage	Early/late	11	10	21 (55.3%)
	Peak	9	8	17 (44.7%)
Herd consistence	Small ($\leq$500)	12	12	24 (63.1%)
	Medium (>500 $\leq$1000)	5	3	8 (21.1%)
	Large ($\geq$1000)	3	3	6 (15.8%)
Clinical signs	Present (diarrhoea and/or wasting)	16	11	27 (71.0%)
	Absent	4	7	11 (28.9%)

The descriptive statistics on herd and sheep level and *MAP* seroprevalence for the dairy and mixed herds are presented in Table 3.

MAP antibodies were detected in 28 farms equal to 73.7% (CI_{95} 59.0–88.3, $n = 38$) of the total herds evaluated. In particular, 11 herds (28.9%) had a single seropositive sheep each and the remaining 17 herds (44.7%) had 2 or more seropositive animals (Figure 1).

In all, a total of 129 seropositive sheep were detected in the two provinces with an apparent prevalence value of 6.29% (CI_{95} 5.23–7.34; $n = 2086$).

When analysing *MAP* seroprevalence at the herd-level by province, consistence, herd type, and clinical signs, significant differences were observed between: dairy (88.2%; CI_{95} 71.2–105.3, $n = 17$) and mixed production herds (33.3%; CI_{95} 110.1–176.5, $n = 3$: $\chi^2 = 4.80$, $P = 0.0284$) in Macerata province (Figure 2), early/late (90.0%; CI_{95} 67.4–112.6) and peak lactation herds (37.5%; CI_{95} 5.8–80.8: $\chi^2 = 5.51$, $P = 0.0189$) in Ancona province and between the overt disease
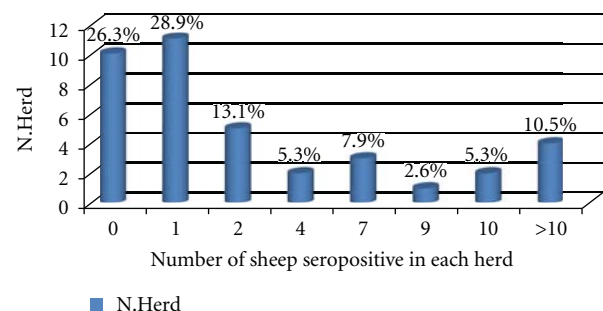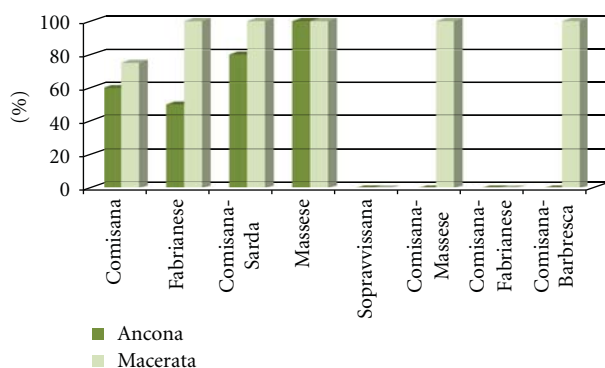

Figure 1: Number and percentage distribution of seropositive sheep over herds.

seropositive and seronegative herds ($P < 0.05$). The herd seroprevalence in relation to breeds is shown in Figure 3.

9 out of 12 herds with small consistence were found positive for *MAP* (75.0%; CI_{95} 46.3–103.7) in both provinces, while different seroprevalence values were recorded for

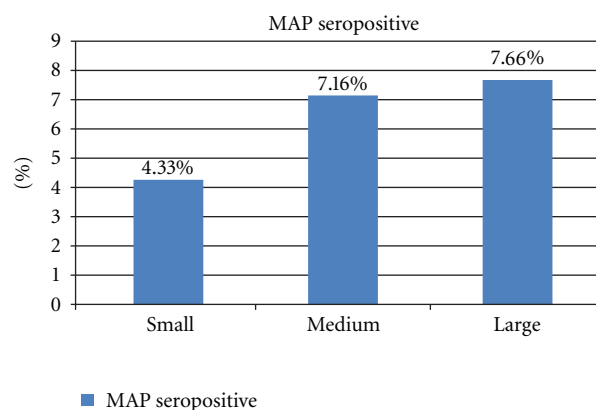TABLE 3: Descriptive statistics for dairy and mixed sheep herds.

	Total herds	Dairy	Mixed
Number of herds	38	33	5
Mean number sheep sampled/herd	55	57	38
Number of seropositive sheep/herd	3.6	4.1	0.8
Number of seropositive sheep/positive herds	5.1	4.1	1.5
% Herds ≥1 seropositive sheep	73.7	78.8	40.0
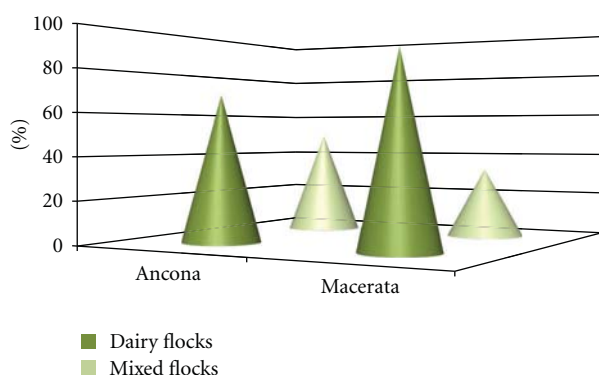
FIGURE 2: Herd seroprevalence of MAP in dairy and mixed flocks of Ancona and Macerata provinces.

FIGURE 4: Distribution of MAP seropositive sheep in the herd consistence of the studied areas.

FIGURE 3: Herd seroprevalence of MAP in relation to ovine breeds in Ancona and Macerata provinces.

medium herds: 80% (CI$_{95}$ 24.5–135.5, $n = 5$) in Macerata versus 0% (CI$_{95}$ 1.0-1.0, $n = 3$; $\chi^2 = 4.80$, $P = 0.0285$) in Ancona territory. The frequencies of antibody response against MAP infection in the different ovine farms consistence, breeds, and provinces are summarised in Table 4.

A highly significant difference was revealed comparing the seropositivity of MAP infection in the large herds and in the small herds ($\chi^2 = 4.20$, $P = 0.04$) (Figure 4).

The herd seroprevalence in Macerata province was higher in peak (88.9%; CI$_{95}$ 63.3–114.5, $n = 9$) than at early and/or at the end of lactation (72.7%; CI$_{95}$ 41.3–104.1, $n = 11$; $\chi^2 = 0.81$, $P = 0.3687$). Similar values were observed in Ancona's ovine farms where 9 out of 10 (90.0%; CI$_{95}$ 67.4–112.6) resulted positive at early/late of lactation time, while 3 out

8 (37.5%; CI$_{95}$ −5.8–80.8) at peak lactation. The difference was significant ($\chi^2 = 5.51$, $P = 0.0189$).

When clinical signs were considered, a significant difference was found in herd seroprevalence. In Macerata province the clinical signs were observed only in seropositive farms ($n = 16$), while 11 out of 12 seropositive herds of Ancona territory (91.7%; CI$_{95}$ 73.3–110.0) reared at least one sheep showed diarrhoea and/or weight loss ($\chi^2 = 10.66$, $P = 0.0011$).

In relation to the period of sampling, an increase of the positive herds is noticed in spring and in autumn but the difference was not significant ($P = 0.071$) (Figure 5). Moreover, by considering two periods in the year, the 68.2% of farms sampling in spring-summer resulted seropositive versus the 81.2% of seropositive herds tested in autumn and winter ($\chi^2 = 0.82$, $P = 0.3664$).

A homogeneous seasonal sampling distribution was revealed in relation to the province and the herd size (Table 5). The Comisana breed is the most represented in the four seasonal samplings.

Considering the totally examined animals ($n = 2086$), 129 seropositive sheep were found, equal to an overall sheep-level seroprevalence of 6.29% (CI$_{95}$ 5.23–7.34) for the investigated provinces. At the sheep level, significant differences were recorded between MAP seropositives in Macerata (7.51%; CI$_{95}$ 5.93–9.08, $n = 1079$) and Ancona province, (4.93%; CI$_{95}$ 3.57–6.30, $n = 973$; $\chi = 5.75$, $P = 0.0165$), mixed (1.82%; CI$_{95}$ 0.04–3.60; $n = 220$) and dairy sheep (6.82%; CI$_{95}$ 5.67–7.98, $n = 1832$; $\chi = 8.35$, $P = 0.0039$); sheep at early/late lactation (5.16%; CI$_{95}$ 3.86–6.47;

TABLE 4: Herd seroprevalence in relation to the breed and herd consistence in Macerata and Ancona provinces.

Breed	% Herd seroprevalence (CI$_{95}$)							
	Total herds		Small ($\leq$500 sheep)		Medium ($500 < n \geq 1000$)		Large ($\geq$1000 sheep)	
					Herd consistence			
	MC	AN	MC	AN	MC	AN	MC	AN
Comisana	75.0 (36.3–116.7) $n = 8$	60.0 (23.1–96.9) $n = 10$	60.0 $n = 5$	66.7 $n = 6$	100 $n = 1$	0 $n = 2$	100 $n = 2$	100 $n = 2$
Fabrianese	100 (1-1) $n = 1$	50.0 (−585.3–685.3) $n = 2$	100 $n = 1$	50.0 $n = 2$	—	—	—	—
Comisana-Sarda	100 (1-1) $n = 6$	80.0 (24.5–135.5) $n = 5$	100 $n = 4$	100 $n = 4$	100 $n = 2$	0 $n = 1$	—	—
Massese	100 (1-1) $n = 1$	100 (1-1) $n = 1$	—	—	—	—	100 $n = 1$	100 $n = 1$
Sopravissana	0 $n = 1$	—	—	—	0 $n = 1$	—	—	—
Comisana-Massese	100 (1-1) $n = 1$	—	100 $n = 1$	—	—	—	—	—
Comisana-Fabrianese	0 $n = 1$	—	0 ($n = 1$)	—	—	—	—	—
Comisana-Barbaresca	100 (1-1) $n = 1$	—	—	—	100 $n = 1$	—	—	—

MC = Macerata province; AN = Ancona province.
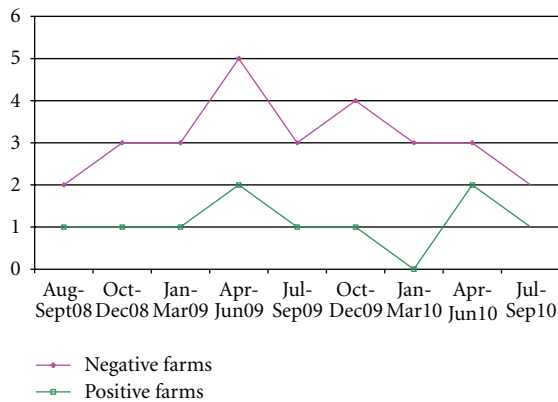
FIGURE 5: Seasonal distribution of *MAP* seropositive and seronegative farms in the two provinces.

- Negative farms
- Positive farms

TABLE 5: Herd size seasonal sampling.

| Season | N. Herd size | | |
	Small	Medium	Large
Spring	8	2	2
Summer	7	2	1
Autumn	5	2	2
Winter	4	2	1

$n = 1104$,) and those at peak lactation (7.59%; CI$_{95}$ 5.90–9.28, $n = 948$; $\chi = 5.12$, $P = 0.0237$), small (4.33%; CI$_{95}$ 2.88–5.77; $n = 763$) and medium or large farms consistence ($\chi = 7.47$, $P = 0.0063$).

MAP infection was confirmed in eleven (12.6%, CI$_{95}$ 3.9–21.7; $n = 87$) and in thirteen (14.9%, CI$_{95}$ 4.6–25.2; $n = 87$) faecal samples by bacteriological culture and Ziehl Neelsen staining, respectively. Twenty-three sheep were not detected in the second visit because of selling or death and the remaining nineteen bacteriological cultures resulted contaminated. *MAP* IS900 genome was confirmed by PCR performed on colonies obtained from each of the faecal bacteriological positive sample.

The true prevalence of *MAP* infection at sheep and herd level in the two provinces was estimated to be 15.1% ± 7.3%. (at 95% confidential limit); 18.7% in Macerata, and 11.1% in Ancona province [39, 40].

6. Discussion

Serological investigations have been described as effective tools in the establishment of the prevalence of *MAP* infection in a herd, and also to screen and confirm the diagnosis of paratuberculosis in animals that present compatible clinical symptoms [44]. A comprehensive understanding of *MAP* prevalence, incidence, and epidemiological patterns in ovine flock is of tangible value to facilitate the design of prevention,

and control programmes aim at reducing or more preferably eliminate *MAP* from farms. In this light, test selection is of critical importance in the design of such control programmes. The test selected for this current study is quick and relatively easy to perform in contrast to the culturing method which is laborious and requires an incubation period of about 8–16 weeks or more. Moreover, serological analysis would furnish both diagnosis and prognosis of the disease. In this study, an overall sheep-level and herd-level seroprevalence of 6.29% and 73.6% were obtained for the investigated territories.

The apparent prevalence observed in ovine dairy flocks sampled in this study (6.29%) supports the reports of previous researches carried out in cattle [6, 45] whereby the seroprevalence of *MAP* was observed to increase with age of animals and in relation to the herd size. Environmental factors and density-dependent effects can help in *MAP* rampant dispersal.

In this study, herd size was strongly associated with a seropositive herd status. In the large herds a higher percentage of sheep were over 2 years of age than those ones reared in the smaller herds. Thus are more likely to sample and test positive ewes due to a higher adult antibody production or because of herd size or management effects. The within herds variation of *MAP* seroprevalence, observed in the different herds consistence in this study, may be attributed to host variability in antibody production and protein enteropathy in response to *MAP* infection.

The possible within herd transmission dynamic of *MAP* could occur by continuous new *MAP* infection in lambs and high seroprevalence with eventual contamination of the environment or by transmission from dam to lambs and lamb to lamb. The high seroprevalence flocks can serve as permanent reservoirs of *MAP* that may infect other flocks via sheep movements and extensive grazing.

The seroprevalence observed in this study is dissimilar to that reported in sheep population of Umbria region and in Trapani province of Italy where the true and apparent seroprevalences were 4.8% [46] and 3.4% [47], respectively. In Australia, the prevalence was estimated to be in the range of 2.4%–4.4% [48], while in northern Greece, the herd prevalence of *MAP* infection in sheep flocks was estimated to be 21.1% [49]. Seroprevalence study of *MAP* infection in goat dairy flocks in France revealed an apparent and estimated true prevalence of 55.2% and 62.9% at herd level, while at individual animal level they were 2.9% and 6.6%, respectively [50]. However, what obtained in our study is much lower than the high seroprevalence observed in the predominantly sheep and goat flocks in the Madrid region in Spain where an apparent prevalence of 11.7% and estimated true prevalence of 44% were observed [51].

The seroprevalence of *MAP* infection was noted to be higher in Macerata than in Ancona province at both sheep and herd levels, and the difference was quite significant. The herd management practices and the animal introduction checking could be the most likely reasons for the observed difference. Good animal husbandry practice, cleanliness of the farm, manure handling, newborn-lamb care, and restriction of contact between lambs and mature animals were

observed in a greater number of farms in Ancona province than in Macerata province during farms' visit [52–54]. Furthermore, a higher percentage of sheep sampled in this study in Ancona province was at early or late stage of lactation while the majority of sheep sampled in Macerata province were at peak stage of lactation. Thus, taken into consideration the postulated hypothesis of a decreased IgG concentration in serum and an increased concentration of IgG in milk at early or late lactation compared with that of peak lactation [55], the stage of lactation could be another possible reason for the difference in *MAP* seroprevalence observed in the two provinces. An in-house comparative study carried out on milk and serum samples postulated a substantial agreement between serum and milk ELISA results (kappa value = 0.67), higher at early and late ($k = 0.78$) than in peak ($k = 0.54$) lactation, in agreement with the Immunoglobulin G (IgG) rising in the milk in the beginning and at the end of lactation (unpublished data).

We advocate strict biosecurity measures within and without the farms for proper containment of the infection. Also, sufficient housing space should be created in the large farm consistence to prevent the animal-to-animal closeness and reduce density-dependent effects which commonly lead to transmission of contagious infections. We also strongly recommended the implementation of a combination of both husbandry changes and test-and-cull methods in *MAP* subclinically infected ovine flocks without overt disease as control strategy for eliminating the pathogen in the ovine farms and preventing their spread to other farms. In *MAP*-infected farms with overt disease, vaccination is advocated for it would prevent more clinical cases, ameliorate the health status of animals exhibiting clinical symptoms, greatly reduce bacterial shedding and thus control contamination risks, and finally may lead to increase production at a highly profitable benefit-to-cost ratio.

7. Conclusions

This is the first large-scale study of *MAP* seroprevalence in the ovine population of Marche region in central Italy although until now only two provinces were studied. Serological investigations have been described as effective tools in the establishment of the prevalence of *MAP* infection in a herd, as a useful technique from which animal herd owners could make management decisions [32], and also for confirming the diagnosis of paratuberculosis in animals that present compatible clinical symptoms [56, 57]. The present study can be useful as a confirmatory finding to ascertain the endemicity of JD in the ovine dairy herds in Marche region. The provinces investigated have a rural history with zootechnical farms distributed in the sampled territory homogeneously. In almost all herds, raw milk is used to produce cheese for consumers in own laboratories close to the farm.

The results are remarkable especially considering the debate on the possibility of *MAP* playing a role in the aetiology of Crohn's disease [8, 14] and human Type I diabetes [15, 16]. The ingestion of contaminated ovine milk and cheese manufactured from raw ovine milk might lead to transmission of *MAP* to humans. Moreover, the Italian

normative (Intesa Stato Regioni of 25/1/2007), that defines the criterions of acceptability of raw milk sold directly to consumers, individualizes analytical parameters for the most important bacterial agents of food-borne illnesses but these do not include pathogens like *MAP*. Thus a precautionary approach from public health authorities should be warranted and further investigations are needed to estimate the potential risk for consumers' health.

A comprehensive understanding of *MAP* prevalence, incidence, and epidemiological patterns in ovine flock is of tangible value to facilitate the design of prevention and control programmes aimed at reducing or more preferably eliminate *MAP* from farms in Marche region.

Knowledge of the current herd- and sheep-level prevalence is of value today, and it is expected that this information will help to prioritize and direct future research and control programs in the region and, further, will be integrated into any national control campaigns.

The clustered distribution of ovine paratuberculosis in Ancona and Macerata provinces of central Italy can be managed and eliminated from sheep flocks with stringent management combined with frequent testing and culling, or by vaccination combined with management of faecal-oral transmission. The frequency of testing and level of management intervention should be determined by each farm's abilities, priorities, and finances. Furthermore, efforts should be made through the appropriate government institutions and through sheep breeder associations to recognize flocks with no history of infection or at low risk of being infected using standardized national certification guidelines. The test negative farms will serve as important source to obtain sheep for the establishment of low risk flocks.

In addition, the importance of biosecurity measures should be emphasized to avoid a flock becoming infected through the purchase of a sheep. Pilot voluntary control programs have been developed in dairy cattle farm aiming to gradually decrease the prevalence of disease in participating herds. In ovine population, the program could start with a risk assessment evaluation, and then with an implementation of management strategies to prevent transmission of infection, by testing animals before parturition. Based on likelihood ratio approach, the farm should be classified in different categories. Lamb feeding with colostrum and milk should be allowed only from negative ewes: the doubtful and weak negative sheep retested after parturition by molecular techniques. The serum is best suited as the most reliable, fastest, and easiest means of screening ovine flock for the detection of *MAP* infection, but further studies are necessary to determine if the ELISA for individual and bulk tank ovine milk samples can create opportunities for a cheaper and more feasible testing scheme in ovine Johne's disease diagnosis.

References

[1] J. M. Kreeger, "Ruminant paratuberculosis—a century of progress and frustration," *Journal of Veterinary Diagnostic Investigation*, vol. 3, no. 4, pp. 373–382, 1991.

[2] C. Cocito, P. Gilot, M. Coene, M. De Kesel, P. Poupart, and P. Vannuffel, "Paratuberculosis," *Clinical Microbiology Reviews*, vol. 7, no. 3, pp. 328–345, 1994.

[3] N. B. Harris and R. G. Barletta, "*Mycobacterium avium* subsp. *paratuberculosis* in Veterinary Medicine," *Clinical Microbiology Reviews*, vol. 14, no. 3, pp. 489–512, 2001.

[4] E. J. B. Manning and M. T. Collins, "*Mycobacterium avium* subsp. *paratuberculosis*: pathogen, pathogenesis and diagnosis," *OIE Revue Scientifique et Technique*, vol. 20, no. 1, pp. 133–150, 2001.

[5] "Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees)," chapter 2.1.11, pp. 276–291, Office International des Epizooties, Paris, France, 2008.

[6] S. L. Ott, S. J. Wells, and B. A. Wagner, "Herd-level economic losses associated with Johne's disease on US dairy operations," *Preventive Veterinary Medicine*, vol. 40, no. 3-4, pp. 179–192, 1999.

[7] I. Pavlik, J. O. Falkinham III, and J. Kazda, "Environments providing favourable conditions for the multiplication and transmission of mycobacteria," in *The Ecology of Mycobacteria: Impact on Animal's and Human's Health*, J. Kazda, I. Pavlik, J. O. Falkinham III, and K. Hruska, Eds., p. 522, Springer, Berlin, Germany, 1st edition, 2009.

[8] R. J. Chiodini and C. A. Rossiter, "Paratuberculosis: a potential zoonosis?" *The Veterinary clinics of North America*, vol. 12, no. 2, pp. 457–467, 1996.

[9] J. Hermon-Taylor, T. J. Bull, J. M. Sheridan, J. Cheng, M. L. Stellakis, and N. Sumar, "Causation of Crohn's disease by *Mycobacterium avium* subspecies *paratuberculosis*," *Canadian Journal of Gastroenterology*, vol. 14, no. 6, pp. 521–539, 2000.

[10] S. A. Naser, G. Ghobrial, C. Romero, and J. F. Valentine, "Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease," *The Lancet*, vol. 364, no. 9439, pp. 1039–1044, 2004.

[11] W. Chamberlin, D. Y. Graham, K. Hulten et al., "Review article: *Mycobacterium avium* subsp. *paratuberculosis* as one cause of Crohn's disease," *Alimentary Pharmacology and Therapeutics*, vol. 15, no. 3, pp. 337–346, 2001.

[12] I. R. Grant, "Zoonotic potential of *Mycobacterium avium* ssp. *paratuberculosis*: the current position," *Journal of Applied Microbiology*, vol. 98, no. 6, pp. 1282–1293, 2005.

[13] M. Feller, K. Huwiler, R. Stephan et al., "*Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease: a systematic review and meta-analysis," *The Lancet Infectious Diseases*, vol. 7, no. 9, pp. 607–613, 2007.

[14] L. A. Sechi, A. M. Scanu, P. Molicotti et al., "Detection and isolation of *Mycobacterium avium* subspecies *paratuberculosis* from intestinal mucosal biopsies of patients with and without Crohn's disease in Sardinia," *American Journal of Gastroenterology*, vol. 100, no. 7, pp. 1529–1536, 2005.

[15] L. A. Sechi, D. Paccagnini, S. Salza, A. Pacifico, N. Ahmed, and S. Zanetti, "*Mycobacterium avium* subspecies *paratuberculosis* bacteremia in type 1 diabetes mellitus: an infectious trigger?" *Clinical Infectious Diseases*, vol. 46, no. 1, pp. 148–149, 2008.

[16] P. S. Rani, L. A. Sechi, and N. Ahmed, "*Mycobacterium avium* subspecies *paratuberculosis* is a trigger of type-1 diabetes: destination Sardinia, or beyond?" *Gut Pathogens*, vol. 2, no. 1, pp. 1–6, 2010.

[17] J. L. E. Ellingson, J. L. Anderson, J. J. Koziczkowski et al., "Detection of viable *Mycobacterium avium* subsp. *paratuberculosis* in retail pasteurized whole milk by two culture methods and PCR," *Journal of Food Protection*, vol. 68, no. 5, pp. 966–972, 2005.

[18] R. J. Chiodini and J. Hermon-Taylor, "The thermal resistance of *Mycobacterium paratuberculosis* in raw milk under conditions simulating pasteurization," *Journal of Veterinary Diagnostic Investigation*, vol. 5, no. 4, pp. 629–631, 1993.

[19] L. C. Favila-Humara, G. G. Chávez-Gris, E. M. Carrillo-Casas, and R. Hernández-Castro, "*Mycobacterium avium* subsp. *paratuberculosis* detection in individual and bulk tank milk samples from bovine herds and caprine flocks," *Foodborne Pathogens and Disease*, vol. 7, no. 4, pp. 351–355, 2010.

[20] R. W. Sweeney, R. H. Whitlock, and A. E. Rosenberger, "*Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows," *Journal of Clinical Microbiology*, vol. 30, no. 1, pp. 166–171, 1992.

[21] S. M. Stehman, "Paratuberculosis in small ruminants, deer, and South American camelids," *The Veterinary Clinics of North America*, vol. 12, no. 2, pp. 441–455, 1996.

[22] I. R. Grant, L. M. O'Riordan, H. J. Ball, and M. T. Rowe, "Incidence of *Mycobacterium paratuberculosis* in raw sheep and goats' milk in England, Wales and Northern Ireland," *Veterinary Microbiology*, vol. 79, no. 2, pp. 123–131, 2001.

[23] N. Sung and M. T. Collins, "Effect of three factors in cheese production (pH, salt, and heat) on *Mycobacterium avium* subsp, paratuberculosis viability," *Applied and Environmental Microbiology*, vol. 66, no. 4, pp. 1334–1339, 2000.

[24] U. Spahr and K. Schafroth, "Fate of *Mycobacterium avium* subsp. *paratuberculosis* in Swiss hard and semihard cheese manufactured from raw milk," *Applied and Environmental Microbiology*, vol. 67, no. 9, pp. 4199–4205, 2001.

[25] M. T. Collins, "Diagnosis of paratuberculosis," *The Veterinary Clinics of North America*, vol. 12, no. 2, pp. 357–371, 1996.

[26] D. Jordan, "Aggregate testing for the evaluation of Johne's disease herd status," *Australian Veterinary Journal*, vol. 73, no. 1, pp. 16–19, 1996.

[27] I. R. Grant, "*Mycobacterium paratuberculosis* and milk," *Acta Veterinaria Scandinavica*, vol. 44, no. 3-4, pp. 261–266, 2003.

[28] C. van Maanen, C. Koster, B. van Veen et al., "Validation of *Mycobacterium avium* subsp. *paratuberculosis* antibody detecting ELISA," in *Proceedings of the 7th International Colloquium on Paratuberculosis*, R. A. Juste, M. V. Geijo, and J. M. Garrido, Eds., Bilbao, Spain, 2003.

[29] R. H. Whitlock, S. J. Wells, R. W. Sweeney, and J. van Tiem, "ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method," *Veterinary Microbiology*, vol. 77, no. 3-4, pp. 387–398, 2000.

[30] J. R. Stabel, S. J. Wells, and B. A. Wagner, "Relationships between fecal culture, ELISA, and bulk tank milk test results for Johne's disease in US dairy herds," *Journal of Dairy Science*, vol. 85, no. 3, pp. 525–531, 2002.

[31] M. T. Collins, S. J. Wells, K. R. Petrini, J. E. Collins, R. D. Schultz, and R. H. Whitlock, "Evaluation of five antibody detection tests for diagnosis of bovine paratuberculosis," *Clinical and Diagnostic Laboratory Immunology*, vol. 12, no. 6, pp. 685–692, 2005.

[32] R. H. Whitlock, S. J. Wells, R. W. Sweeney, and J. Van Tiem, "ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method," *Veterinary Microbiology*, vol. 77, no. 3-4, pp. 387–398, 2000.

[33] G. van Schaik, C. R. Rossiter, S. M. Stehman, S. J. Shin, and Y. H. Schukken, "Longitudinal study to investigate variation in results of repeated ELISA and culture of fecal samples for *Mycobacterium avium* subsp *paratuberculosis* in commercial dairy herds," *American Journal of Veterinary Research*, vol. 64, no. 4, pp. 479–484, 2003.

[34] D. J. Kennedy and G. Benedictus, "Control of *Mycobacterium avium* subsp. *paratuberculosis* infection in agricultural species," *OIE Revue Scientifique et Technique*, vol. 20, no. 1, pp. 151–179, 2001.

[35] G. Benedictus and C. J. H. Kalis, "Paratuberculosis: eradication, control and diagnostic methods," *Acta Veterinaria Scandinavica*, vol. 44, no. 3-4, pp. 231–241, 2003.

[36] National Livestock Population, Centri di Referenza nazionale, Anagrafe nazionale zootecnica, http://statistiche.izs.it/Zootecniche.

[37] R. D. Smith, *Veterinary Clinical Epidemiology*, Taylor & Francis, 3rd edition, 2006.

[38] C. Couquet and P. Pourquier, "Evaluation of the sensitivity, specificity and detectability of a serological ELISA test for the diagnosis of Paratuberculosis," in *Proceedings of the 6th International Colloquium on Paratuberculosis*, pp. 494–500, 1999.

[39] W. J. Rogan and B. Gladen, "Estimating prevalence from the results of a screening test," *American Journal of Epidemiology*, vol. 107, no. 1, pp. 71–76, 1978.

[40] S. Gumber, G. Eamens, and R. J. Whittington, "Evaluation of a Pourquier ELISA kit in relation to agar gel immunodiffusion (AGID) test for assessment of the humoral immune response in sheep and goats with and without *Mycobacterium paratuberculosis* infection," *Veterinary Microbiology*, vol. 115, no. 1–3, pp. 91–101, 2006.

[41] J. R. Stabel, "An improved method for cultivation of *Mycobacterium paratuberculosis* from bovine fecal samples and comparison to three other methods," *Journal of Veterinary Diagnostic Investigation*, vol. 9, no. 4, pp. 375–380, 1997.

[42] M. Moravkova, P. Hlozek, V. Beran et al., "Strategy for the detection and differentiation of *Mycobacterium avium* species in isolates and heavily infected tissues," *Research in Veterinary Science*, vol. 85, no. 2, pp. 257–264, 2008.

[43] M. Thrusfield, *Veterinary Epidemiology*, Blackwell Science, Oxford, UK, 2nd edition, 1995.

[44] R. J. Whittington, "An overview of paratuberculosis diagnostic tests," in *Proceedings of the 7th International Colloquium on Paratuberculosis*, R. A. Juste et al., Ed., Bilbao, Spain.

[45] K. A. Woodbine, Y. H. Schukken, L. E. Green et al., "Seroprevalence and epidemiological characteristics of *Mycobacterium avium* subsp. *paratuberculosis* on 114 cattle farms in south west England," *Preventive Veterinary Medicine*, vol. 89, no. 1-2, pp. 102–109, 2009.

[46] C. Maresca, G. Giorgi, F. Feliziani et al., "Indagine epidemiologica sulla paratubercolosi ovina nel territorio di una A.S.L. della regione Umbria," *Large Animals Review*, vol. 5, no. 4, pp. 71–74, 1999.

[47] S. Villari, F. Castiglione, V. Monteverde et al., "Seroprevalence of *Mycobacterium avium* subsp. *paratuberculosis (MAP)* in ovine and caprine farms in Trapani, Sicily," in *Proceedings of the 17th International Congress of Mediterranean Federation of Health and Production of Ruminants*, pp. 117–118, Perugia, Italy, May 2009.

[48] E. S. G. Sergeant and F. C. Baldock, "The estimated prevalence of Johne's disease infected sheep flocks in Australia," *Australian Veterinary Journal*, vol. 80, no. 12, pp. 762–768, 2002.

[49] Z. Dimareli-Malli, K. Stevenson, K. Sarris, and K. Sossidou, "Study of microbiological and molecular typing aspects of paratuberculosis in sheep and goats in Northern Greece," *Transboundary and Emerging Diseases*, vol. 56, no. 6-7, pp. 285–290, 2009.

[50] P. Mercier, C. Baudry, F. Beaudeau, H. Seegers, and X. Malher, "Estimated prevalence of *Mycobacterium avium* subspecies *paratuberculosis* infection in herds of dairy goats in France," *The Veterinary record*, vol. 167, no. 11, pp. 412–415, 2010.

[51] R. C. Mainar-Jaime and J. A. Vázquez-Boland, "Factors associated with seroprevalence to *Mycobacterium paratuberculosis* in small-ruminant farms in the Madrid region (Spain)," *Preventive Veterinary Medicine*, vol. 34, no. 4, pp. 3017–3027, 1998.

[52] M. T. Collins, D. C. Sockett, W. J. Goodger, T. A. Conrad, C. B. Thomas, and D. J. Carr, "Herd prevalence and geographic distribution of, and risk factors for, bovine paratuberculosis in Wisconsin," *Journal of the American Veterinary Medical Association*, vol. 204, no. 4, pp. 636–641, 1994.

[53] W. J. Goodger, M. T. Collins, K. V. Nordlund et al., "Epidemiologic study of on-farm management practices associated with prevalence of *Mycobacterium paratuberculosis* infections in dairy cattle," *Journal of the American Veterinary Medical Association*, vol. 208, no. 11, pp. 1877–1880, 1996.

[54] R. J. Whittington and E. Sergeant, "Progress towards understanding the spread, detection and control of *Mycobacterium avium* subsp *paratuberculosis* in animal populations," *Australian Veterinary Journal*, vol. 79, no. 4, pp. 267–278, 2001.

[55] S. S. Nielsen, Y. T. Gröhn, and C. Enevoldsen, "Variation of the milk antibody response to paratuberculosis in naturally infected dairy cows," *Journal of Dairy Science*, vol. 85, no. 11, pp. 2795–2802, 2002.

[56] S. S. Nielsen, K. K. Nielsen, and A. Huda, "Diagnostic techniques for paratuberculosis," in *Bulletin of the International Dairy Federation, IDF*, vol. 362, pp. 5–17, 2001.

[57] P. K. Singh, S. V. Singh, A. V. Singh, and J. S. Sohal, "Screening of tissues and serum by culture, PCR and ELISA for the detection of *Mycobacterium avium* subspecies *paratuberculosis* from cases of clinical ovine Johne's disease in farmer's flocks," *Indian Journal of Animal Sciences*, vol. 78, no. 10, pp. 1052–1056, 2008.

Research Article

Apparent Prevalence of Beef Carcasses Contaminated with *Mycobacterium avium* subsp. *paratuberculosis* Sampled from Danish Slaughter Cattle

Hisako Okura,[1] Nils Toft,[1] Nicola Pozzato,[2] Annalucia Tondo,[2] and Søren Saxmose Nielsen[1]

[1] *Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Grønnegårdsvej 8, 1870 Frederiksberg C, Denmark*

[2] *Istituto Zooprofilattico Sperimentale delle Venezie, SCT1, Via San Giacomo 5, 37135 Verona, Italy*

Correspondence should be addressed to Hisako Okura, hisako@life.ku.dk

Presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in beef has been reported as a public health concern because asymptomatically infected cattle may contain MAP in tissues that are used for human consumption. Associations between MAP carcasses contamination and animal characteristics such as age, breed, production type, and carcass classification were assessed. Cheek muscles from 501 carcasses were sampled cross-sectionally at a Danish abattoir and tested for presence of viable MAP and MAP DNA by bacterial culture and IS*900* realtime PCR, respectively. Cheek muscle tissues from carcasses of two dairy cows were positive by culture whereas 4% of the animals were estimated with ≥ 10 CFU/gram muscle based on realtime PCR. Age was found to be associated with carcass contamination with MAP. The observed viable MAP prevalence in beef carcasses was low. However, detection of MAP and MAP DNA in muscle tissues suggested that bacteremia occurred in slaughtered cattle.

1. Introduction

Paratuberculosis is a chronic infection caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in cattle and other ruminants. MAP infection in cattle is often given attention due to its possible connection to Crohn's disease (CD) in humans. CD is a chronic granulomatous ileocolitis, and patients with CD experience chronic weight loss, diarrhea, and chronic pain throughout their lives [1]. There is contradictory evidence that either support or dispute the association between MAP and CD [2, 3]. Human exposure to MAP from livestock could be via milk and meat products. For the latter, abattoirs are critical points to reduce MAP in meat and the risk of exposure to humans [4].

Infections in cattle occur primarily in calfhood, and may gradually progress to clinical disease after the incubation period, which ranges from a few months to the lifetime of an animal [5]. Although the exact time course is unclear, infected cattle may follow several stages corresponding to the changes that occur in histological and immune response such as establishment in intestines, cell-mediated immune responses, humoral immune reactions, and tissue destruction and bacteremia [6]. MAP in infected animals is primarily confined to the intestines and associated lymph nodes, but when the infection progresses, MAP is spread within the animal, although little is known about the timing and trigger mechanisms [7].

The infection prevalence of MAP at the slaughterhouse has been established, with previous studies reporting 16% and 1% in Danish dairy and nondairy cattle, respectively [8], 16% in culled dairy cattle in North America [9], and 34% in cull cows in the US [10]. However, the infection prevalence differs from carcass contamination prevalence, because not all infections have progressed to bacteremia.

The relationship between MAP infection and presence of MAP in different tissues of infected animals, in different stages of infection has been reported [11–15]. These studies show that MAP can be isolated from tissues other than

the primal infection site, including animals without clinical signs. For example, a study in the US reported disseminated infections in 57% of cows (12/21) with no clinical signs of disease [13]. Furthermore, MAP has been isolated from blood and diaphragm muscle of four slaughtered animals with only two animals exhibiting clinical signs of MAP infection [14].

Previous studies [10, 16], assessed the prevalence of MAP-contaminated carcasses were done without considering fecal contamination. Wells et al. [10] estimated that 80% of cull cows had MAP-positive hides whereas only 34% had MAP-positive ileocecal lymph nodes. This finding suggests that fecal contamination at the abattoirs is frequent. However, the prevalence of MAP contamination in tissues used for human consumption has not been established. It might be optimal if presence of MAP in carcasses can be linked with factors that are readily available at the farm or at abattoirs so that possible human exposure to MAP through beef could be minimized.

The objective of this study was to estimate the prevalence of MAP contamination in Danish beef carcasses and to characterize the contamination based on information collected at the farm or abattoirs, such as age, herd of origin, and fat code. Here, carcass contamination was defined as muscle tissue that tested positive by either culturing or PCR.

2. Materials and Methods

2.1. Collection of Samples. A cross-sectional sample from carcasses was obtained at a cattle abattoir in Denmark on two consecutive days in October 2009. All information was recorded with the animal identification number on the ear tag. On the slaughter line, samples were collected at the evisceration point, where postmortem examination was conducted. At the postmortem examination, an incision was made in the masseter muscle by a postmortem technician, and a piece of cheek muscle (approximately 20 grams each) from both sides were collected and placed in a separate container. Knives were washed in hot water (82°C) between animals. Swab samples were collected at the incision site of the cheek using a cotton swab (Transport Swabs, product number TSS, OXOID Ltd. Greve, Denmark) in order to examine if there was fecal contamination at the site. Data on birth date, herd of origin, breed, sex and fat code were obtained from the Danish Cattle Database. Fat codes indicate the amount of fat on the outside of the carcasses and in the thoracic cavity ranging from 1 (very thin) to 5 (very fat) [17].

2.2. Swab Preparation and Analyses. Swab samples were kept at 4°C until plating on MacConkey (for gram-negative bacteria) and Slanetz agar plate (for enterococci). Samples were incubated for 20 hours at 37°C followed by visual examination of the plates. Samples resulting in more than one colony on either agar plate were considered to have fecal contamination.

2.3. Muscle Samples Preparation and MAP Culture. Cheek muscle samples were stored at −18°C and transported to

Istituto Zooprofilattico Sperimentale delle Venezie, Verona, Italy. Each sample was processed in parallel for MAP culture and realtime PCR, modifying the methods already validated for bovine fecal samples [18]. In brief, 3 grams of muscle were trimmed and added to 3 mL of sterile saline solution in a stomacher bag with a filter and homogenized for 2 min at maximum speed (BagMixer, Interscience, St. Nom, France). Then, 500 μL of the homogenate was placed into a 2 mL screw-cap tube for DNA extraction. The remaining sample was homogenized for a further 1 min with 8 mL of 0.75% hexadecylpyridinium chloride in half-strength brain heart infusion for decontamination. The liquid supernatant of the homogenate was transferred into 15 mL tubes, incubated at 37°C for 2 hours, and centrifuged at 900 g for 30 min. Pellets were resuspended in 600 μL of sterile water and 200 μL inoculated into each of 2 tubes containing 5 mL of 7H9+ liquid medium, specific for MAP culture. The 7H9+ liquid medium was prepared in house and contained 0.37% Middlebrook 7H9 powder (Becton-Dickinson, Franklin Lakes, NJ, USA) supplemented with 8% OADC enrichment (Becton-Dickinson, Franklin Lakes, NJ, USA), 0.08% Casitone (Becton-Dickinson, Franklin Lakes, NJ, USA), 16% egg yolk, 0.4% Glycerol (Sigma-Aldrich, Germany), 1 μg/mL Mycobactin J (ID Vet, Montpellier, France), and 0.1 mL/tube PANTA PLUS (Becton-Dickinson, Franklin Lakes, NJ, USA). The tubes were incubated at 37°C and tested by realtime PCR at 4, 8, and 12 weeks of culture.

2.4. DNA Extraction and Realtime PCR Detection. DNA extraction was carried out starting from 500 μL of muscle homogenates or 300 μL of 7H9+ inoculated medium diluted with 200 μL of sterile water. Samples were added to screw-cap tubes containing 300 mg of glass beads (120–150 μm Sigma-Aldrich, Germany) and subjected to a bead beating step in the Fast Prep FP120 (Qbiogene, Irvine, CA, USA) instrument set twice at 6.5 m/s for 45 second. The DNA was then extracted with MagMAX 96 Viral Isolation Kit according to the manufacturer's instructions, using "MICROLAB STARLET" automated extraction platform (Hamilton Robotics, Bonaduz, Switzerland). For MAP detection, 900 nM primers (Map668F 5′-GGCTGATCGGACCCG-3′, Map791R-5′-TGGTAGCCAGTAAGCAGGATCA-3′) and 200 nM probe (Map718 5′-FAM-ATACTTTCGGCGCTGGAACGCGC-TAMRA) were used [18]. For the amplification of muscle samples, an internal control targeting endogenous bovine GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used by adding 10 nM of each primer (gapDHF 5′-GCATCGTGGAGGGACTTATGA-3′ and gapDHR 5′-GGGCCATCCACAGTCTTCTG-3′) and 50 nM of probe (5′-FAM-CACTGTCCACGCCATCACTGCCA-TAMRA). Amplification mix was completed with 1X TaqMan Universal PCR Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and 2 μL of extracted DNA in a final volume of 10 μL. The program for realtime PCR was 2 min at 50°C followed by 10 min at 95°C, 40 cycles at 95°C for 15 s, and 60°C for 1 min. Realtime amplification was performed with Rotor-Gene 6000 (Corbett Life Science, Concorde, NSW, Australia).

2.5. Analytical Sensitivity of Realtime PCR and Quantification of MAP Cell Number in Artificially Contaminated Bovine Muscle Samples. Analytical sensitivity of the realtime PCR was evaluated using bovine meat from an uninfected cow spiked with tenfold dilutions of MAP ATCC 19698 (10^0 to 10^5 CFU), prepared according to Hugues et al. [19]. Colony counts of the inocula were performed in HEYM slants. Each sample was processed in triplicate, and results were calculated as CFU/mL of homogenate (ratio muscle/saline solution was 1 : 1). A standard curve for MAP numbers (CFU/mL) in beef samples was produced as a result of the threshold cycle (Ct) values.

2.6. Case Definitions. Eight definitions of MAP DNA occurrence in carcasses were explored regarding muscle PCR and swab test results. Ct values of 35, 36, 37, or 38 in the realtime PCR were considered to define a muscle tissue sample as positive. If one of 2 muscle tissue samples was positive in PCR, the carcass was defined as being contaminated with MAP DNA. Furthermore, the data were evaluated both including and excluding samples with fecal contamination. All eight definitions were explored for the assessment of possible risk factors whereas results of the assessment of the analytical sensitivity were used to estimate the MAP contamination prevalence.

2.7. Risk Factors. Four factors, namely, age, breed group, fat code, and herd type were examined in the statistical analyses. Age was dichotomised as 2 years old and older or less than 2 years old, because disease progression leading to bacteremia depends on time since infection. Sampled animals comprised 10 different breeds, and these were grouped into three: dairy (Danish Red, Holstein, and Jersey), beef (Angus, Charolais, Hereford, Highland, Limousine, and Simmental), and crossbred. Five levels of the fat code were grouped into three: 1 (very thin), 2 (thin), and 3 or higher (normal to fat), because infected animals with progressed disease would be expected to have reduced body fat. Three herd types (dairy, beef, and veal) were considered, because dairy herds have a higher MAP prevalence than beef herds [8], and veal herds are different from the other two in terms of age and management practices. "Dairy" was used for the animals born in and slaughtered from a milk-producing herd. "Beef" was for the animals born and slaughtered from a non-milk-producing herd. "Veal" was for the animals born in a dairy herd but moved to a nondairy herd.

2.8. Statistical Analyses. Prevalence of MAP DNA in tissue samples was calculated and further stratified by risk factors. Descriptive statistics were carried out by summarizing the PCR result in relation to the risk factors in combination with the different case definitions described above. A multivariable logistic regression including PCR result as response variable and the abovementioned four risk factors was carried out to determine factors affecting MAP DNA occurrence. All data analyses were performed using the free software R [20].
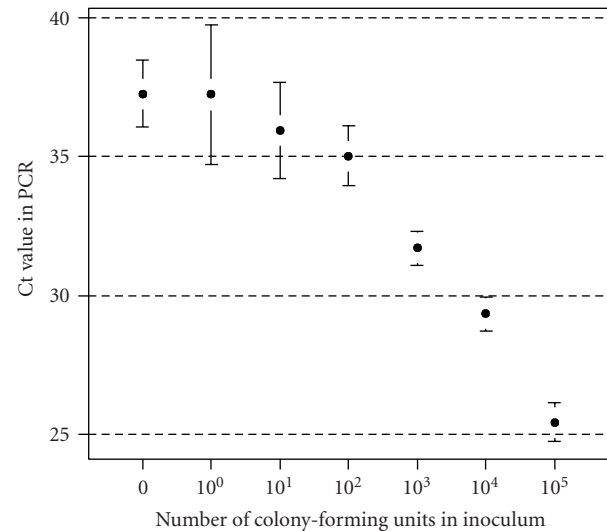
Figure 1: Quantification of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) cell number by IS*900* realtime PCR inoculating tenfold dilution of MAP ATCC 19698 artificially contaminated bovine muscle samples.

3. Results

In total, 1030 muscles tissue samples were collected from 515 animals. After excluding cattle for which identification number did not match information in the Danish Cattle Database, two samples from each of 501 beef carcasses were available. Of the 501 animals, 15 swab samples were not tested. For the 501 animals, ages ranged from 0.2 years to 14.3 years (median 3.2 years) with 304 older than 2 years of age. There were 362, 76, and 63 dairy, beef, and cross-bred breeds, respectively. Thirty-eight percent (192/501) of the animals were classified with fat code 3 or higher, and the median was 3 with first and third quartiles of 2 and 3, respectively. Three hundred and thirteen animals were from dairy herds, 73 were from veal herds, and 115 were from beef herds (Table 1).

Cheek muscles from two dairy cows (3.7 and 5.6 years of age) were positive by culture with no indication of fecal contamination. These samples were confirmed to be MAP using PCR, but the samples were not positive using the direct PCR. Association between culture positivity and risk factors (age, fat code, breed type, and herd type) was not observed due to small number of culture positives.

Concentration of MAP in beef samples was estimated as number of CFU per gram of beef from the standard curve obtained as a result of analyses of artificially contaminated bovine muscle samples (Figure 1). Exact quantification of MAP in beef at low concentrations ($<10^2$ CFU in inoculums) was impossible, but considering the Ct values in low concentrations, the detection limits would be around 10^1 CFU in inoculums of which corresponding Ct-values would be around 36. This cutoff was therefore selected for the prevalence estimation. From the standard curve, the concentrations of MAP in carcasses were very low (<10 CFU/gram; Figure 1).

TABLE 1: Number- and group-specific percentage (in brackets) of muscle tissue samples from slaughtered cattle from which *Mycobacterium avium* subsp. *paratuberculosis* DNA was detected by IS*900* PCR at threshold cycle value of 36, 37, and 38 by age, type, of herd, breed type and fat code. Positive results were defined in relation to possible fecal contamination to the tissue samples.

Variable	Level	Including samples with fecal contamination				Excluding samples with fecal contamination			
		Total no. in group	PCR threshold cycle value			Total no. in group	PCR threshold cycle value		
			36	37	38		36	37	38
		501	20 (4)	28 (6)	42 (8)	385	16 (4)	22 (6)	34 (9)
Age	<2 years	197	16 (8)	21 (11)	31 (16)	159	3 (1)	4 (2)	8 (4)
	≥2 years	304	4 (1)	7 (2)	11 (4)	226	13 (8)	18 (11)	26 (16)
Herd type	Dairy	313	17 (5)	22 (7)	30 (10)	235	13 (6)	17 (7)	23 (10)
	Beef	115	2 (2)	3 (3)	7 (6)	88	2 (2)	3 (3)	7 (8)
	Veal	73	1 (0)	3 (4)	5 (7)	62	1 (2)	2 (3)	4 (6)
Breed group	Dairy	362	18 (5)	26 (7)	35 (10)	278	14 (5)	20 (7)	27 (10)
	Beef	76	1 (1)	1 (1)	4 (5)	62	1 (2)	1 (2)	4 (6)
	Cross	63	1 (2)	1 (2)	3 (5)	45	1 (2)	1 (2)	3 (7)
Fat code	1	66	1 (2)	3 (5)	8 (12)	56	1 (2)	3 (5)	7 (13)
	2	162	9 (6)	14 (9)	15 (9)	125	7 (6)	10 (8)	11 (9)
	≥3	272	10 (4)	11 (4)	19 (7)	203	8 (4)	9 (4)	16 (8)

A total of 54 muscle tissue samples gave detectable fluorescence before a Ct-value of 40 was reached. The Ct-values of these samples were in the range from 31.9 to 39.9 with a median of 36.8. Distributions of Ct-values in the different categories are shown in Table 2. At 35 Ct-value, MAP DNA was detected in seven tissue samples from six dairy cows and one beef cattle with age between 1.0 and 6.3 years old, and these were positive without fecal contamination (1.4%). Overall, apparent prevalences of MAP-contaminated carcasses taking fecal contamination into account were 4%, 6% and 9% at Ct-values 36, 37, and 38, respectively (Table 1). At the selected cutoff of 36 Ct-values, 4% of the carcasses were deemed to be contaminated with ≥10 CFU/gram MAP muscle tissue.

Odds ratios and P values resulting from the univariable analyses suggested that age was consistently associated with PCR positivity regardless of choice of PCR cutoff as well as indication of fecal contamination: the odds of having MAP DNA were 2- to 10-times higher for cattle of 2 years old and older compared to young animals (Table 3). Therefore, in the multivariable analyses, it was decided to include age as a main effect in combination with other risk factors. The odds ratio suggested that breed was associated with PCR positivity at Ct-value of 37. However, production type and breed group were not included in the model at the same time because these two factors were highly correlated. The multivariable model of risk factors associated with the presence of MAP DNA in carcasses did not alter the univariable model with age as an explanatory variable (data not shown).

4. Discussion

The apparent prevalence of MAP culture positive was very low (2/501), and 4% (16/385) of carcasses were contaminated with ≥10 CFU/gram muscle among a cross-sectional sample of animals as deemed by PCR. The ideal cutoff for deeming an animal with MAP DNA contamination could not be determined, but the odds ratios (OR) from the univariable analyses (Table 3) suggested that it is likely between 36 and 38 Ct-values. The culture and the PCR indicated that concentrations in general were low.

A previous study describing isolation of MAP in muscle tissues reported that the prevalence of MAP in diaphragm muscle was 13% (6/47) among nonrandomly selected cattle at slaughter [14]. Other studies reported high prevalences of on-carcass contamination with MAP by testing swab samples before and after intervention and suggest that carcass contamination was a result of cross-contamination [10, 16]. Different prevalence of infection of cows could be an explanation, but also different sample site and way of sample collection could explain this difference because cheek muscle might be less likely to be exposed to contaminated environment than brisket or anal region.

True prevalences of MAP infection in adult Danish cattle have been estimated to be 16% among dairy cattle [8]. The observed apparent MAP prevalence of 4% of animals with ≥10 CFU/gram muscle in beef carcasses was lower than the infection prevalences of dairy cattle. However, this might reflect an actual low prevalence, because a recent study reported that MAP was isolated from muscle tissue and peripheral lymph nodes from 11% and 55%, respectively, of cattle with clinical paratuberculosis, which indicate a proportion of clinically diseased animals might have bacteremia [21]. The apparent prevalence was lows but the result indicates frequent occurrence of bacteremia in cattle to be slaughtered for human consumption.

Age was found to be associated with carcass contamination, which is not surprising because disease progression is

Table 2: Summary of threshold cycle value of IS*900* PCR to detect *Mycobacterium avium* subsp. *paratuberculosis* DNA in beef carcasses by age, herd type, breed group, and fat code of the cattle.

Variable	Level	PCR threshold cycle value					
		N	Mean	SD	Q_1	Median	Q_3
Overall		54	36.73	1.78	35.67	36.80	37.87
Age	<2 years	15	36.71	2.13	35.94	37.02	37.91
	≥2 years	39	36.73	1.65	35.75	36.56	37.86
Herd type	Dairy	36	36.36	1.80	35.47	36.23	37.54
	Veal	5	36.74	0.81	36.53	36.62	37.38
	Beef	13	37.74	1.64	37.20	37.88	38.93
Breed group	Dairy	42	36.48	1.78	35.57	36.30	37.66
	Beef	8	37.91	1.15	37.18	38.04	38.44
	Cross	4	36.90	2.13	36.27	37.20	37.83
Fat code	1	10	37.25	1.53	36.68	37.34	37.79
	2	19	36.06	2.03	35.02	36.28	37.00
	≥3	25	37.02	1.55	35.93	37.20	37.89

Table 3: Odds ratios (OR) and 95% confidence interval in parenthesis resulting from univariable logistic regression of risk factors for occurrence of *Mycobacterium avium* subsp. *paratuberculosis* DNA in beef carcasses.

Variable	Including samples with fecal contamination PCR threshold cycle value			Excluding samples with fecal contamination PCR threshold cycle value		
	36	37	38	36	37	38
Old						
<2 years	1	1	1	1**	1**	1**
≥2 years	2.7 (1.0–9.5)	2.0 (0.9–5.2)	1.9 (1.0–4.1)	10.1 (1.0–196.3)	3.4 (1.2–11.8)	2.5 (1.1–6.0)
Herd type						
Beef	1	1	1	1	1	1
Dairy	3.2 (0.9–20.7)	2.8 (1.0–12.1)	1.6 (0.7–4.2)	2.5 (0.7–16.3)	2.2 (0.7–9.6)	1.3 (0.5–3.3)
Veal	0.8 (0.0–8.3)	1.6 (0.3–8.9)	1.1 (0.3–3.7)	0.7 (0.0–7.5)	0.9 (0.1–5.9)	0.8 (0.0–2.8)
Breed group						
Beef	1	1	1	1	1**	1
Dairy	3.9 (0.8–71.1)	5.8 (1.2–104.4)	1.9 (0.7–6.6)	3.2 (0.6–59.2)	4.7 (1.00–85.7)	1.5 (0.6–5.1)
Cross	1.2 (0.1–31.0)	1.2 (0.1–31.0)	0.9 (0.2–4.2)	1.4 (0.1–35.7)	1.4 (0.1–35.7)	1.2 (0.1–31.0)
Fat code						
≥3	1	1	1	1	1	1
2	1.5 (0.6–3.9)	2.2 (1.0–5.2)	1.4 (0.7–2.7)	1.4 (0.5–4.1)	1.9 (0.7–4.5)	1.1 (0.5–2.5)
1	0.4 (0.0–2.2)	1.1 (0.3–3.7)	1.8 (0.7–4.3)	0.4 (0.0–2.5)	1.2 (0.3–4.2)	1.7 (0.6–4.2)

** Significant at $P \leq .05$.

known to be associated with age [7]. We also hypothesized that breed, production type, and fat code were associated with carcass contamination; therefore, we investigated the multivariable model including these factors. The odds ratios suggest that this may be true, but the association was not significant. Cattle testing positive for MAP DNA were generally 2 years and older, and these cattle were more likely to be from dairy herds and vice versa for beef cattle. Therefore, confounding between age and production type was present, and it could not be determined if age

or production type was the actual explanatory variable. However, given that MAP infections are chronic, it appears more likely that age was the relevant predictor of MAP occurrence. No association between carcass contamination with MAP DNA and fat code of the carcasses was detected, which is consistent with findings of McKenna et al. [9].

One limitation of the study was that our muscle tissue sample was limited to cheek muscles. The two culture positive samples were confirmed to be MAP using PCR, but the samples were not positive using the direct PCR. A

reason for not being able to culture MAP from PCR-positive sample could be low concentrations or freezing treatment that is known to reduce MAP viability [22]. MAP load in two culture positive samples were also low. Previous studies that detected MAP in muscle from cattle with clinical signs also reported low concentrations [14, 21]. We collected muscle samples from both cheeks to increase sensitivity, because if bacteremia does occur, MAP should be distributed evenly in the body, but with respect to the difficulties in detection of MAP with low concentration, it might have been better with more samples or samples from other sites. The prevalence presented in the study was apparent prevalence. Test accuracy estimates (i.e., sensitivity and specificity) were not available. Therefore, the apparent prevalences could underestimate the true prevalences [21].

5. Conclusion

Two out of 501 (0.4%) animals tested positive for viable MAP, and 20/501 (4%) animals were found to have a concentration of ≥ 10 CFU/gram muscle tissue positive for MAP DNA, which indicates that the probability of carcass contamination with MAP and concentration of MAP is low. Age was the only factor that was found to be significantly associated with the occurrence of MAP DNA in muscle tissues; however, there were indications of other biologically important risk factors. The study provided quantitative data for presence of MAP in slaughtered cattle, but only age could be identified as an animal characteristic useful for risk characterization.

Acknowledgments

References

[1] R. J. Chiodini, "Crohn's disease and the mycobacterioses: a review and comparison of two disease entities," *Clinical Microbiology Reviews*, vol. 2, no. 1, pp. 90–117, 1989.

[2] M. A. Behr and V. Kapur, "The evidence for *Mycobacterium paratuberculosis* in Crohn's disease," *Current Opinion in Gastroenterology*, vol. 24, no. 1, pp. 17–21, 2008.

[3] I. Abubakar, D. Myhill, S. H. Aliyu, and P. R. Hunter, "Detection of *Mycobacterium avium* subspecies *paratubercubsis* from patients with Crohn's disease using nucleic acid-based techniques: a systematic review and meta-analysis," *Inflammatory Bowel Diseases*, vol. 14, no. 3, pp. 401–410, 2008.

[4] M. M. Eltholth, V. R. Marsh, S. Van Winden, and F. J. Guitian, "Contamination of food products with *Mycobacterium avium paratuberculosis*: a systematic review," *Journal of Applied Microbiology*, vol. 107, no. 4, pp. 1061–1071, 2009.

[5] S. S. Nielsen, *Paratuberculosis in Dairy Cattle—epidemiological studies used for design of a control programme in Denmark*, Doctor medicinae veterinaria thesis, 2009.

[6] S. S. Nielsen and N. Toft, "Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-γ assay and faecal culture techniques," *Veterinary Microbiology*, vol. 129, no. 3-4, pp. 217–235, 2008.

[7] P. M. Coussens, "*Mycobacterium paratuberculosis* and the bovine immune system," *Animal Health Research Reviews*, vol. 2, no. 2, pp. 141–161, 2001.

[8] H. Okura, S. S. Nielsen, and N. Toft, "Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection in adult Danish non-dairy cattle sampled at slaughter," *Preventive Veterinary Medicine*, vol. 94, no. 3-4, pp. 185–190, 2010.

[9] S. L. B. McKenna, G. P. Keefe, H. W. Barkema et al., "Cow-level prevalence of paratuberculosis in culled dairy cows in Atlantic Canada and Maine," *Journal of Dairy Science*, vol. 87, no. 11, pp. 3770–3777, 2004.

[10] J. E. Wells, J. M. Bosilevac, N. Kalchayanand et al., "Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in ileocecal lymph nodes and on hides and carcasses from cull cows and fed cattle at commercial beef processing plants in the United States," *Journal of Food Protection*, vol. 72, no. 7, pp. 1457–1462, 2009.

[11] W. Y. Ayele, M. Bartos, P. Svastova, and I. Pavlik, "Distribution of *Mycobacterium avium* subsp. paratuberculosis in organs of naturally infected bull-calves and breeding bulls," *Veterinary Microbiology*, vol. 103, no. 3-4, pp. 209–217, 2004.

[12] M. M. Dennis, M. C. Antognoli, F. B. Garry et al., "Association of severity of enteric granulomatous inflammation with disseminated *Mycobacterium avium* subspecies paratuberculosis infection and antemortem test results for paratuberculosis in dairy cows," *Veterinary Microbiology*, vol. 131, no. 1-2, pp. 154–163, 2008.

[13] M. C. Antognoli, F. B. Garry, H. L. Hirst et al., "Characterization of *Mycobacterium avium* subspecies *paratuberculosis* disseminated infection in dairy cattle and its association with antemortem test results," *Veterinary Microbiology*, vol. 127, no. 3-4, pp. 300–308, 2008.

[14] M. Alonso-Hearn, E. Molina, M. Geijo et al., "Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from muscle tissue of naturally infected cattle," *Foodborne Pathogens and Disease*, vol. 6, no. 4, pp. 513–518, 2009.

[15] K. L. Bower, D. J. Begg, and R. J. Whittington, "Culture of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from blood and extra-intestinal tissues in experimentally infected sheep," *Veterinary Microbiology*, vol. 147, no. 1-2, pp. 127–132, 2011.

[16] W. J. Meadus, C. O. Gill, P. Duff, M. Badoni, and L. Saucier, "Prevalence on beef carcasses of *Mycobacterium avium* subsp. *paratuberculosis* DNA," *International Journal of Food Microbiology*, vol. 124, no. 3, pp. 291–294, 2008.

[17] Anonymous, "Council Regulation (EC) No 1234/2007 of 22 October 2007 establishing a common organisation of agricultural markets and on specific provisions for certain agricultural products (Single CMO Regulation) (EC 1234/2007)," *Official Journal of the European Union*, vol. L299, 2010.

[18] N. Pozzato, J. Gwozdz, M. Gastaldelli, K. Capello, C. D. Ben, and E. Stefani, "Evaluation of a rapid and inexpensive liquid culture system for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine faeces," *Journal of Microbiological Methods*, vol. 84, no. 3, pp. 413–417, 2011.

[19] V. M. Hughes, K. Stevenson, and J. M. Sharp, "Improved preparation of high molecular weight DNA for pulsed-field gel electrophoresis from mycobacteria," *Journal of Microbiological Methods*, vol. 44, no. 3, pp. 209–215, 2001.

[20] R Development Core Team, "R: A Language and Environment for Statistical Computing," 2010.

[21] L. A. Reddacliff, I. B. Marsh, S. A. Fell, S. L. Austin, and R. J. Whittington, "Isolation of *Mycobacterium avium* subspecies paratuberculosis from muscle and peripheral lymph nodes using acid-pepsin digest prior to BACTEC culture," *Veterinary Microbiology*, vol. 145, pp. 122–128, 2010.

[22] S. Khare, L. G. Adams, J. Osterstock, A. Roussel, and L. David, "Effects of shipping and storage conditions of fecal samples on viability of Mycobacterium paratuberculosis," *Journal of Clinical Microbiology*, vol. 46, no. 4, pp. 1561–1562, 2008.

Research Article

Mycobacteria in Terrestrial Small Mammals on Cattle Farms in Tanzania

Lies Durnez,[1, 2, 3] Abdul Katakweba,[4] Harrison Sadiki,[5] Charles R. Katholi,[6] Rudovick R. Kazwala,[5] Robert R. Machang'u,[4] Françoise Portaels,[2] and Herwig Leirs[1, 7]

[1] Evolutionary Ecology Group, Department of Biology, University of Antwerp, 2020 Antwerp, Belgium
[2] Mycobacteriology Unit, Department of Microbiology, Institute of Tropical Medicine, 2000 Antwerp, Belgium
[3] Entomology Unit, Department of Parasitology, Institute of Tropical Medicine, 2000 Antwerp, Belgium
[4] Pest Management Centre, Sokoine University of Agriculture, Morogoro, Tanzania
[5] Department of Veterinary Medicine and Public Health, Sokoine University of Agriculture, Morogoro, Tanzania
[6] Department of Biostatistics, School of Public Health, University of Alabama at Birmingham, Birmingham, AL 35209, USA
[7] Danish Pest Infestation Laboratory, University of Aarhus, 2800 Kongens Lyngby, Denmark

Correspondence should be addressed to Lies Durnez, ldurnez@itg.be

The control of bovine tuberculosis and atypical mycobacterioses in cattle in developing countries is important but difficult because of the existence of wildlife reservoirs. In cattle farms in Tanzania, mycobacteria were detected in 7.3% of 645 small mammals and in cow's milk. The cattle farms were divided into "reacting" and "nonreacting" farms, based on tuberculin tests, and more mycobacteria were present in insectivores collected in reacting farms as compared to nonreacting farms. More mycobacteria were also present in insectivores as compared to rodents. All mycobacteria detected by culture and PCR in the small mammals were atypical mycobacteria. Analysis of the presence of mycobacteria in relation to the reactor status of the cattle farms does not exclude transmission between small mammals and cattle but indicates that transmission to cattle from another source of infection is more likely. However, because of the high prevalence of mycobacteria in some small mammal species, these infected animals can pose a risk to humans, especially in areas with a high HIV-prevalence as is the case in Tanzania.

1. Introduction

The genus *Mycobacterium* comprises more than 140 named species recognized currently [1], of which several are pathogenic; most of them are environmental mycobacteria that may cause opportunistic infections. The pathogenic species are responsible for some important diseases in humans and animals in the developed world as well as in developing countries, namely, tuberculosis (TB), leprosy, and Buruli ulcer [2]. Susceptibility to mycobacterial infections can be higher in patients with underlying conditions such as human immunodeficiency virus-acquired immunodeficiency syndrome (HIV-AIDS), sarcoidosis, silicosis, or emphysema. With the rising number of HIV-AIDS patients in Africa, TB and in some extent other mycobacterial diseases, caused by, for example, *M. avium*

complex, are an important cause of morbidity and mortality [3]. Mycobacterial diseases in cattle such as bovine tuberculosis (BTB), caused by *Mycobacterium bovis*, and atypical mycobacterioses (e.g., paratuberculosis caused by *M. avium* subsp. *paratuberculosis*) can also have serious implications on public health and on economy [4–6]. Therefore, the control of BTB and atypical mycobacterioses is important. In countries with a wildlife reservoir of *M. bovis*, BTB in cattle is more difficult to control. In the UK, New Zealand, the United States, and Africa, a number of animals have been found to be infected with and act as a reservoir for *M. bovis*, namely, the European badger (*Meles meles*), brushtail possums (*Trichosurus vulpecula*), white-tailed deer (*Odocoileus virginianus*) and bison (*Bison bison*), and the African buffalo (*Syncerus caffer*), respectively [7].

Not much research has been conducted on the wildlife reservoir of *M. bovis* in sub-Saharan Africa and research has focused mainly on the presence of *M. bovis* in South Africa [8–11]. In Tanzania in 2005, *M. bovis* was demonstrated in free-ranging wildlife, namely, in wildebeest, topi, the lesser kudu, and lions in the Serengeti national park, the Tarangire National Park, and the Ngorongoro crater in Tanzania [12]. More information is present on the prevalence of *M. bovis*-infection in cattle in Tanzania. This prevalence ranges between 0.2% and 14% and that of atypical mycobacterioses in cattle between 0% and 13.0% [6, 13–17].

Also, other pathogenic mycobacterial species including *M. microti*, *M. avium*, and *M. marinum* have been found in wild animals [18–21]. Although these mycobacteria are less virulent for humans, they do cause infections or diseases in humans, especially when humans are immunocompromized [3, 22, 23]. As such, animals could transmit mycobacteria to humans [24].

Until present, little research is performed on the reservoir status of rodents and insectivores for mycobacteria [19, 21, 25–27] although they are hosts for pathogens causing diseases in humans and livestock [28, 29] such as leptospirosis, plague, and toxoplasmosis [30]. Cattle farms can be prone to rodent infestations because of the abundant amounts of shelter, water, and food [31], thus augmenting the rate of direct or indirect contact between rodents and cattle and the risk of disease transmission. Moreover, rodents are sensitive to experimental infection with mycobacteria that can cause disease in cattle, for example, *M. bovis* and *M. avium* subsp. *paratuberculosis* [32, 33] and recent studies have shown that African rodents and insectivores can carry mycobacteria of the *M. avium*-complex [21, 26]. In developed areas (UK and New-Zealand), rodents and insectivores were found to carry *M. bovis* [29, 34, 35] but with a low estimated transmission risk [20, 36]. However, in Africa, other rodent and insectivore species are present and the risks of rodent-borne diseases (both for humans and cattle) are higher because of a higher contact rate between humans, cattle, and rodents.

Therefore, the aim of this study was to evaluate the possibility of a rodent/insectivore reservoir for *M. bovis* and other mycobacteria in Tanzania, from which cattle (and humans) could be infected. We have collected small mammals in cattle farms with a known tuberculin status. The single comparative intradermal tuberculin test (SCITT) can detect cattle exposed to *M. bovis* as well as atypical mycobacteria [37, 38]. A positive SCITT test can thus serve as an indication for exposure to mycobacteria. By targeting the small mammal collection in cattle farms housing animals with known reactor status, we can get an indication of the transmission direction or the involvement of other source(s) of infection as summarized in Table 1.

2. Materials and Methods

2.1. Trapping Sites. A total of 26 cattle farms were chosen in and around Morogoro, a medium-sized city 200 km west of Dar es Salaam (37.26–37.49°E; 6.18–6.52°S). These farms can be divided into two reactor types based on the single comparative intradermal tuberculin test (SCITT) conducted in the cattle residing on the farms in 2005 and 2006 [14]. For all animals, the "current reactor status" at the moment the trapping took place was known. For the trapping period of 2005, the "future reactor status" of the farms was known (i.e., the SCITT-results of 2006). For the trapping period of 2006, the "past reactor status" of the farms was known (i.e., the SCITT-results of 2005). Other trapping sites included a grass field around the slaughterhouse in Morogoro, and a quarter in Morogoro where a high prevalence of mycobacteria in rodents and insectivores was observed in a previous study, namely, Mwembesongo [21].

The trapping took place in the wet and dry season of both 2005 and 2006.

2.2. Sample Collection. Three types of live traps were used: Sherman LFA Live Traps, Box traps, and big wire cage traps [21]. Peanut butter with maize bran and fresh maize cobs were used as bait [21].

The animals were processed in the laboratory following a standard protocol as described by Durnez et al. [21]. In brief, the animals were euthanized with chloroform, and external characteristics and measurements such as weight and head-body length were recorded. During necropsy, pieces of liver, spleen, lung, mesenteric lymph nodes, and external lesions if present were taken for detection of mycobacteria. The carcasses were kept in formalin and sent to the University of Antwerp for further identification to species level: primary identification was confirmed, and skulls were removed and cleaned to identify the animals to species level.

2.3. Pooling of Samples and Detection and Identification of Mycobacteria. The samples were pooled in a stratified way: the same organs were pooled per one to six individuals of the same species trapped at the same trapping site. A flow chart of the pooling procedure is given in Figure 1. The number of animals in a pool depended on the trapping number per species at a trapping site (resulting in 1 to 6 animals per pool). In this way, 645 individual animals were pooled into 307 groups of individuals. For each group, the four different organ homogenates collected from each animal were pooled separately, resulting in 1228 pools to be tested. A subset of samples was used to test whether pool screening and individual screening gave comparable prevalence estimations. In this subset of samples, the pooled results and the individual results were available.

The pools were analyzed for the presence of mycobacteria by culture and PCR as described before [21]. In short, the organs were homogenized and decontaminated to reduce overgrowth of nonmycobacterial organisms [39], before inoculating them on culture media (Löwenstein-Jensen, Stonebrink, and Löwenstein-paratuberculosis medium [40]) and performing DNA extraction (described in [41]) and PCR (described in [14]) with inhibition check.

Cultivation took place at 37 degrees C [39] for ten to twelve months.

The mycobacteria isolated on culture were checked for acid fastness using Ziehl-Neelsen staining (ZN) and

TABLE 1: Interpretation of possible differences in prevalences of mycobacteria in small mammals in relation to the reactor status of the farm on which the small mammals were collected.

Analysis in relation to	Possible difference in prevalence of mycobacteria in small mammals collected in reacting and nonreacting farms	Indication on transmission direction and the involvement of other source(s) of infection*
(1) Current reactor status	(a) No difference	Transmission between small mammals and cattle might occur, but cattle and small mammals probably have a different source of infection
	(b) Higher prevalence in currently reacting as compared to non reacting farms	Transmission between small mammals and cattle might occur, but common source of infection more probable.
	(c) Higher prevalence in currently nonreacting as compared to reacting farms	Transmission between small mammals and cattle might occur, but cattle and small mammals probably have a different source of infection
(2) Future reactor status	(a) No difference	Transmission from small mammals to cattle might occur, but cattle also has another source of infection
	(b) Higher prevalence in future reacting as compared to non reacting farms	Tranmission from small mammals to cattle may occur, either directly or indirectly
	(c) Higher prevalence in future nonreacting as compared to reacting farms	Transmission from small mammals to cattle might occur, but cattle has another, probably more important, source of infection
(3) Past reactor status	(a) No difference	Transmission from cattle to small mammals might occur, but small mammals also have another source of infection
	(b) Higher prevalence in past reacting as compared to non reacting farms	Transmission from cattle to small mammals may occur, either directly or indirectly
	(c) Higher prevalence in past nonreacting as compared to reacting farms	Transmission from cattle to small mammals might occur, but small mammals have another, probably more important, source of infection

*Another source of infection can be other wild or domestic animals, the environment, or humans.

identified to species-level by biochemical methods and by sequencing the 16 S rRNA gene [42].

2.4. Collection and Analysis of Milk Samples. Every trapping period, from every milking cow on the cattle farms where small mammals had been trapped, a milk sample (1 to 10 mL per cow) was collected. The milk samples were kept at $-20°$C and analyzed in Belgium by culture and PCR as described by Durnez et al. [14].

2.5. Data Analysis. For the results of the pooled samples, the data analysis was based on the use of likelihood ratio tests (LRTs) in the usual parametric model for pool testing. The pool screening sampling model is basically that of a Bernoulli trial with success probability $\theta = [1 - (1 - p)^n]$, where n is the pool size and p is the infection rate in the population of interest. The random variable which is denoted by X is the result of the testing of the pool and has value 1 if the pool is positive and 0 if the pool is negative.

Thus, the probability model describing the sampling is given by the probability mass function

$$f_x(x \mid n, p) = \left[1 - (1 - p)^n \right]^x \left[(1 - p)^n \right]^{1-x}, \quad x \in \{0, 1\}. \tag{1}$$

This model has a long history in the statistics literature [43–47] and is described in detail in all of these papers. The investigator collects pools of various sizes, n_i, and after testing the pool knows the value of the result, denoted by x_i. Thus, for any pool, given the pair (n_i, x_i), the only unknown quantity in the model is the value of p. The standard method for estimating p is the method of maximum likelihood [48]. This depends on the likelihood function which in this case is

$$L(p) = \prod_{j=1}^{m} \left[1 - (1 - p)^{n_j} \right]^{x_j} \left[(1 - p)^{n_j} \right]^{1-x_j}, \tag{2}$$

where m is the number of pools tested. The maximum likelihood estimate (MLE) is found by maximizing $L(p)$ as a function of p. We note that no special adjustments are required for the inequality of the pool sizes, because this is a feature which is part of the model. This sampling model is the basis for constructing any standard likelihood ratio type test. Tests for a one way or two way design are constructed by replacing p by a linear model in the factors of interest. In this case, a coding scheme analogous to cell mean coding in standard analysis of variance is convenient. Likelihood ratio test methods are a standard technique in statistics. Details of the actual implementation of such tests in
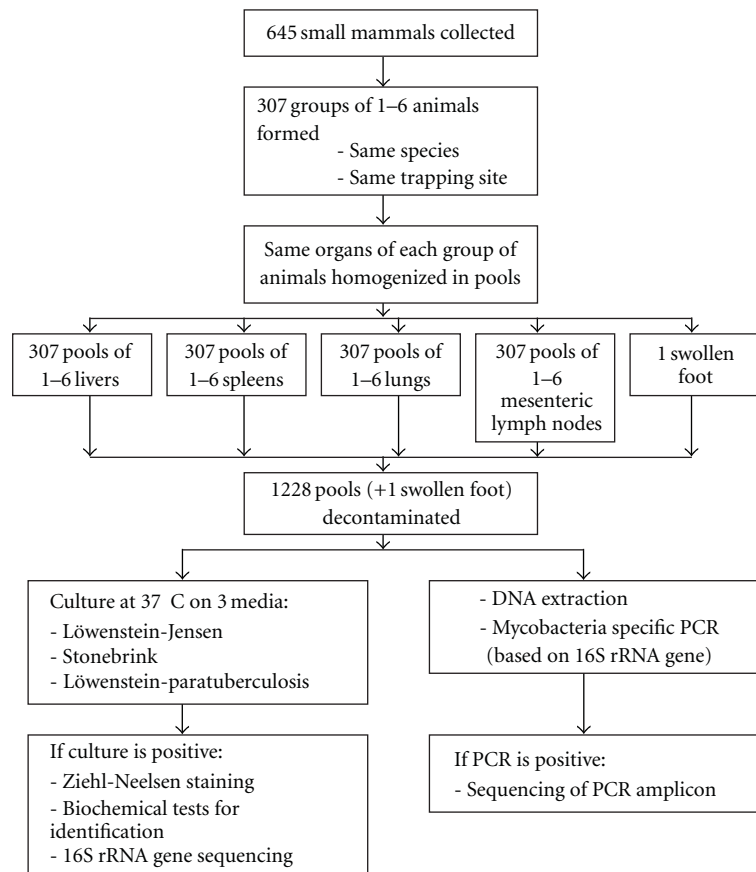
Figure 1: Flow chart of pooling procedure.

this case are computationally complex and requires extensive mathematics but would add nothing to the subject matter of this paper. The calculations were done using a custom FORTRAN program running under MS Windows developed by one of the authors (C. R. Katholi). The core of the estimation is the optimization software used, in this case the program NLPQLP [49–51].

A kappa-test (using SPSS 16.0) was used to compare the results of the individual versus pooled samples analysis.

3. Results

3.1. Trapping Results. The number of animals trapped in this study are listed per species in Table 2.

3.2. Mycobacterial Results

3.2.1. Comparison of Pool Prevalence Estimation versus Individual Prevalence Estimation. For culture and PCR, the respective Kappa-values, comparing the results of the pooled samples and the individual samples, were 0.691 and 0.876, showing a high concordance. For culture, the estimated prevalence with pool prevalence estimation was 6.8% (95% CI: 2.8%–13.3%), while with individual tests the prevalence was estimated to be 7.6% (95% CI: 3.5%–14.0%). For PCR,

these estimated prevalences were respectively 3.3% (95% CI: 0.8%–8.2%) and 3.8% (95% CI: 1.2%–8.8%).

3.2.2. Mycobacteria Detected in Rodents and Insectivores. The number of positive groups for mycobacteria and the estimated prevalence are listed in Table 2, the identification of the mycobacteria in Table 3. A total of 44 groups out of 307 tested positive for mycobacteria in culture or PCR, which makes a total estimated prevalence of 7.3%. The estimated prevalence of mycobacteria in *C. gambianus* was higher than in *M. natalensis* ($P = .011$) and in *R. rattus* ($P < .001$), while no significant difference was observed with *C. hirta* ($P = .123$). *C. hirta* also carried more mycobacteria than *R. rattus* ($P < .001$), while no difference was observed with *M. natalensis* ($P = .164$). *M. natalensis* carried significantly more mycobacteria than *R. rattus* ($P = .028$).

When testing for a difference between organs, the liver was found to be the least infested with mycobacteria, while the lung was most prone to contain mycobacteria (Page's test statistic $L = 115.5$; $\alpha < 0.01$). The positivity of mycobacteria in the different organs per animals species is given in Table 4.

A total of 33 groups out of 233 groups of animals trapped on cattle farms tested positive for mycobacteria, with an estimated prevalence of 7.0%. Since we were interested in the relation between the SCITT reactor status of the farm (current, past, or future) and the mycobacterial presence in

TABLE 2: Rodents and insectivores trapped in and around Morogoro and the prevalence of mycobacteria in the different animal species.

Animal species	Total number of animals trapped[a]	Number of groups analyzed for mycobacteria	Number of groups positive for mycobacteria	Estimated mycobacterial prevalence (95% confidence interval)	95% confidence intervals for zeroestimates of *M. bovis* and *M. avium* subsp. *Paratuberculosis* prevalence
Rodents					
Rattus rattus (Linnaeus, 1758)	268 (216/1/51)	94	7	2.8% (1.0–5.7%)	0–0.71%
Mastomys natalensis (Smith, 1834)	165 (142/23/0)	91	12	7.5% (3.7–13.1%)	0–1.15%
Cricetomys gambianus Waterhouse, 1840	36 (12/2/22)	32	8	23.9% (10.3–42.7%)	0–5.19%
Mus spp.	29 (2/1/26)	22	0	0% (0–6.4%)	0–6.4%
Grammomys surdaster Mathey, 1971	3 (3/0/0)	3	0	0% (0–47.3%)	0–47.3%
Gerbilliscus vicina (Matschie 1911)	2 (0/2/0)	2	0	0% (0-61.7%)	0-61.7%
Squirrel (not identified)	1 (1/0/0)	1	0	0% (0–85.3%)	0–85.3%
Insectivores					
Crocidura hirta Peters, 1852	137 (127/9/1)	58	15	12.5% (6.8–20.4%)	0–3.3%
Atelerix albiventris Wagner, 1841	4 (1/3/0)	4	2	50% (7.7–92.3%)	0–38.1%

[a] (on cattle farms/around slaughterhouse/in Mwembesongo).

rodents and insectivores, the analyses were performed for the current, past, and future reactor status of the farms, when enough data were available. Results are summarized in Table 5.

Additionally, two-way ANOVA analyses revealed no effect of season (dry or wet) on the prevalence of mycobacteria in rodents and insectivores in RR and NR farms (data not shown). One-way ANOVA analyses showed that there was no difference in the prevalence of mycobacteria in farms that changed reactor status (NR to RR or RR to NR) during the study as compared to farms of which the reactor status remained the same (NR or RR) (data not shown).

The prevalence of mycobacteria in rodents and insectivores trapped around the slaughterhouse and in Mwembesongo is listed in Table 5. No significant difference was found in the prevalence between rodents and insectivores trapped on these sites. When comparing the prevalence of mycobacteria in the animals trapped around the slaughterhouse and trapped in the NR and RR farms (current reactor status), a significant difference was observed ($P = .001$) with a significant interaction in the two-way ANOVA ($P = .04$). For insectivores, a significantly higher prevalence was observed in slaughterhouse as compared to NR farms ($P = .007$), while no difference was observed with RR farms ($P = .280$). No difference was observed for the past or future reactor status of the farms. Also, no difference was observed between the prevalence of mycobacteria in rodents trapped in Mwembesongo and in the NR or RR farms.

Out of 226 milk samples collected on the same farms where animals had been trapped, 6 (2.7%) were positive for mycobacteria by culture and 12 (5.3%) by PCR. The identifications of the mycobacteria are listed in Table 6.

4. Discussion

This study is the first to investigate mycobacteria present in rodents and insectivores collected on the farms in relation to the tuberculin reactor status of the cattle residing on these farms. The rationale for this study was that pathogens that infect more than one host species, as is the case for many pathogenic mycobacteria, are likely to be encountered in several host populations, some of which may constitute infection reservoirs [53]. However, this means that the presence of infection in a wild animal population does not prove that the animal species is a reservoir of the infectious agent [4]. To get more information on the possible reservoir status of a certain host, the data of the presence of infection in that host should, therefore, be analysed in relation to data of infections in the target population. In this respect, it is important to acknowledge the existence of different host types; that is, a maintenance host, in which infection can persist by intraspecies transmission alone, and a spillover host, in which infection will not persist indefinitely unless there is reinfection from another species. Both maintenance and spillover hosts may transmit infection to other species, but this difference is important when control of a host species is considered [4].

In general, rodents and insectivores can come into contact with mycobacteria through the environment by feeding, contact with soil, and contact with the feces of wild and domestic animals or humans. These mycobacteria can pass through the stomach of these animals without being digested, since they are resistant to acid. Pathogenic and opportunistic mycobacteria can pass through the stomach and can survive in tissues and organs. In this way, they can

TABLE 3: Mycobacteria detected in rodent and insectivores in and around Morogoro, Tanzania.

Mycobacteria[a]	Small mammal species	Detected by PCR or culture
Human risk group 1[c]		
M. duvalii***	*C. gambianus*	Culture
M. gordonae	*A. albiventris*	PCR
M. gordonae-like	*C. gambianus*	PCR
M. gordonae-like**	*C. hirta*	Culture
M. gordonae-like	*C. hirta*	PCR
M. gordonae-like	*M. natalensis*	PCR
M. moriokaense*	*R. rattus*	Culture
M. mucogenicum	*M. natalensis*	PCR
M. nonchromogenicum	*C. hirta*	Culture
M. nonchromogenicum*[b]	*R. rattus*	Culture
M. nonchromogenicum-like	*M. natalensis*	Culture
M. nonchromogenicum-like	*R. rattus*	PCR
M. sphagni-like	*R. rattus*	PCR
M. terrae	*C. hirta*	Culture
M. terrae***	*C. gambianus*	Culture
M. terrae	*R. rattus*	Culture
Human risk group 2[c]		
M. chelonae var. *niacinogenes*	*M. natalensis*	PCR and culture
M. genavense-like	*C. hirta*	PCR
M. intracellulare***	*C. gambianus*	Culture
M. intracellulare	*C. hirta*	Culture
M. intracellulare	*C. hirta*	PCR and culture
M. intracellulare	*C. hirta*	Culture
M. intracellulare	*M. natalensis*	PCR and culture
M. intracellulare-like	*C. gambianus*	Culture
M. intracellulare-like	*C. gambianus*	Culture
M. intracellulare-like	*C. hirta*	Culture
M. scrofulaceum-like	*C. gambianus*	Culture
M. szulgai	*M. natalensis*	PCR
MAIS	*C. gambianus*	PCR and culture
MAIS	*C. gambianus*	Culture
MAIS	*C. hirta*	PCR and culture
Recently described species, not yet classified[c]		
M. alsiensis	*M. natalensis*	PCR and culture
M. chimaera	*C. hirta*	Culture
M. chimaera-like	*C. hirta*	Culture
M. colombiense	*C. hirta*	PCR and culture
M. frederiksbergense-like	*M. natalensis*	PCR
M. goodii*[b]	*R. rattus*	Culture
M. immunogenum	*R. rattus*	PCR
M. septicum	*A. albiventris*	PCR
M. septicum	*M. natalensis*	PCR
M. septicum	*M. natalensis*	PCR

[a] *, ** and *** point out mycobacteria detected in the same group of animals but in different organs.
[b] These mycobacteria were first detected in 2005 in *R. rattus* trapped on a farm and were later detected in 2006 in the milk of cattle residing on the same farm (see Table 6).
[c] The classification in human risk groups is based on the clinical point of view in which human risk group 1 contain species that never or with extreme rarity cause disease. Human risk group 2 are species that normally live freely in the environment but also cause opportunistic infections in humans. Human risk group 3 are the obligate pathogens (*M. tuberculosis* complex and *M. leprae*) [52].

TABLE 4: Positivity of mycobacteria (in %) in different organs for all animals and for the main animal species collected.

	Liver	Spleen	Lung	Mesenteric lymph nodes
All animals	1.6 (0.8–2.7)	2.1 (1.1–3.4)	3.2 (2.0–4.8)	1.9 (1.0–3.2)
C. gambianus	5.9 (1.0–17.4)	5.9 (9.0–17.4)	8.9 (2.2–21.8)	8.3 (2.0–20.4)
C. hirta	2.2 (0.6–5.7)	5.5 (2.4–10.5)	5.3 (2.3–10.1)	3.9 (1.4–8.3)
M. natalensis	1.2 (0.2–3.7)	1.2 (1.2–3.7)	3.7 (1.5–7.4)	3.7 (1.5–7.4)
R. rattus	0.38 (0.020–1.7)	0.38 (0.020–1.7)	1.6 (0.48–3.6)	0.76 (0.13–2.3)

Page's test for order tests the following hypothesis:
H_0: liver = spleen = lymph = lung; H_a: liver < spleen < lymph < lung.
Test statistic L = 115,5; $\alpha < 0.01$.

TABLE 5: Prevalence of mycobacteria in rodents and insectivores trapped on cattle farms, around the slaughterhouse and in Mwembesongo. RR: positive tuberculin reactor status; NR: negative tuberculin reactor status. The P values given are significance values for the difference between RR and NR farms.

	Cattle farms									SH	MS
	Current reactor status			Past reactor status			Future reactor status				
	RR	NR	P	RR	NR	p	RR	NR	P		
Total	8.8%	2.9%	.014*	4.1%	8.9%	.495	8.3%	11.4%	.459	13.1%	6.1%
Rodents	6.2%	3.1%	.216	2.6%	0.0%	.438	9.1%	6.4%	.538	6.9%	6.2%
C. gambianus	12.5%	0.0%	.448	na	na	na	na	na	na	na	na
M. natalensis	9.7%	0.0%	.062	4.0%	0.0%	.673	10.4%	12.6%	.828	na	na
R. rattus	3.0%	4.4%	.644	1.1%	0.0%	.497	7.3%	5.6%	.703	na	na
Insectivores	18.2%	2.4%	.009*	9.8%	61.0%	.019*	9.4%	15.2%	.508	36.0%	na
C. hirta	18.2%	2.5%	.010*	10.0%	61.0%	.019*	9.4%	15.0%	.507	na	na

* The difference is statistically significant at $P < .05$.
na: not applicable because of insufficient or no data.
SH: Slaughterhouse.
MS: Mwembesongo.

be spread over long distances with the migration of these animals [27] and even if they are not part of the maintenance reservoir, they can play a role as transport host.

4.1. No Evidence for Rodents and Insectivores as Reservoir Hosts for M. bovis or M. avium Subsp. paratuberculosis. In previous studies in the UK and New Zealand, the prevalences of *M. bovis* in rodents and insectivores ranged from 0.4 to 2.8% and from 1.2 to 5%, respectively [20, 29, 34, 35, 54]. *M. avium* subsp. *paratuberculosis* was also found previously in rodents and insectivores in the Czech Republic and Greece at prevalences ranging from 1.3%, to 5.9% and from 1.7% to 2.6%, respectively [55, 56].

In the present study, rodents and insectivores were collected on cattle farms, some of which housed cattle infected with *M. bovis* and/or atypical mycobacterioses [14]. Although we have not detected *M. bovis* or *M. avium* subsp. *paratuberculosis* in the small mammals trapped on the cattle farms in Morogoro, African rodents or insectivores could still be a reservoir for these mycobacteria. As it has been the case in previous studies [20], for some species, not enough animals were trapped to definitely conclude that they do not carry *M. bovis* or *M. avium* subsp. *paratuberculosis*, as shown by the wide confidence intervals of the zero estimates for some species in Table 2. Moreover, although we have used different types and sizes of traps, some species of rodents and

insectivores will not be caught in these traps because of their size. For example, most of the shrews species are too small to trigger the traps used in this study. Therefore, we could have missed some crucial species.

For the two species trapped in significant numbers, namely, *R. rattus* and *M. natalensis*, the confidence intervals show that they do not play a significant role as carriers of *M. bovis* or *M. avium* subsp. *paratuberculosis* in the studied area. A closely related species of *R. rattus*, *R. norvegicus*, is experimentally not sensitive to infection with *M. bovis* [32] although it has been found to carry *M. bovis* in the UK [34, 35] but at low prevalences (1.2–2.2%).

4.2. Rodents and Insectivores as Hosts for Other Mycobacteria. Tuberculin tests in cattle have revealed a high prevalence of atypical mycobacterioses in Tanzanian cattle [14]. Atypical mycobacteria, such as *M. avium* subsp. *paratuberculosis*, can also have an effect on the cattle farm production [5], providing an economic incentive to prevent them in cattle. Therefore, all rodent and insectivore samples collected in the present study were also analyzed for the presence of atypical mycobacteria.

Analysis of the prevalences of mycobacteria in rodents and insectivores in relation to the reactor status of the farm on which the small mammals were collected gave us an indication on whether transmission between small mammals

Table 6: Mycobacteria detected in cow milk on the cattle farms.

Mycobacteria	Detected by PCR or culture
Human risk group 1[b]	
M. neoaurum	PCR
M. nonchromogenicum	Culture
M. nonchromogenicum[a]	Culture
M. gordonae	PCR
Human risk group 2[b]	
M. asiaticum	Culture
M. szulgai-like	Culture
Recently described species, not yet classified [b]	
M. engbaekii	Culture
M. goodii[a]	PCR
M. lacticola	PCR
M. septicum	PCR

[a]These mycobacteria were first detected in 2005 in *R. rattus* trapped on a farm and were later detected in 2006 in the milk of cattle residing on the same farm (see Table 3).
[b]The classification in human risk groups is based on the clinical point of view in which human risk group 1 contain species that never or with extreme rarity cause disease. Human risk group 2 are species that normally live freely in the environment but also cause opportunistic infections in humans. Human risk group 3 are the obligate pathogens (*M. tuberculosis* complex and *M. leprae*) [52].

and cattle would be possible, or if another source of infection would have to be involved.

4.2.1. Rodents. For *M. natalensis* there was no difference in the prevalence of mycobacteria between the different farm types for the present, future, and past reactor status. Another source of infection should thus be available for cattle and *M. natalensis* to become infected with mycobacteria. This other source could be another wild or domestic animal, the environment, or humans.

Some of the isolated mycobacteria, namely, *M. chelonae*, *M. intracellulare,* and *M. szulgai,* are known pathogens to humans, causing pulmonary disease, soft skin, or disseminated infections in immunocompetent (*M. intracellulare*), immunocompromized, or predisposed patients (*M. chelonae* and *M. szulgai*) [52]. Their impact on the health of cattle or on their milk production is not clear, however, although they have been isolated from cattle in several studies [14, 57, 58]. Most of the mycobacteria detected in *M. natalensis* were detected in the lung or mesenteric lymph nodes, so they may potentially be excreted by the animals through feces or respiratory secretions. In the present study, no fecal specimens were examined, but we have shown in Benin that 15.5% of small mammal's feces contain mycobacteria [26].

For *R. rattus*, the detection rate of mycobacteria is low (2.8%), with no mycobacteria belonging to human risk group 2 (Table 3) and, similarly to *M. natalensis*, no difference between farm types. However, interestingly, *M. nonchromogenicum* and *M. goodii* were first detected

in 2005 in *R. rattus* trapped on a farm and were later detected in 2006 in the milk of cattle residing on the same farm (Tables 3 and 6). This could mean that *R. rattus* excretes these mycobacteria, for example, as a transport host, that these mycobacteria are conserved very well in the environment or that these mycobacteria are maintained in another domestic or wildlife host, from which cattle and *R. rattus* can pick up the mycobacteria as spill-over hosts. For *M. nonchromogenicum*, the source of infection is probably the environment [52]. The status and natural reservoir of *M. goodii,* however, is not yet clear, but it causes infections in both humans [59] and wildlife [60].

In accordance with a previous study [21], the highest prevalence of mycobacteria was found in *C. gambianus* (23.9%) as compared to the other rodents, with *M. intracellulare* and related mycobacteria as main mycobacteria found in this species. The difference in prevalence of mycobacteria in this animal species between reacting and nonreacting farms was not significant, possibly because of the low number of *C. gambianus* trapped in these cattle farms ($n = 12$). The elevated prevalence may indicate a potential risk to humans, since most of the infected animals were trapped in or near human dwellings in Mwembesongo.

The large difference in prevalences between the different rodent species might be due to different behavior, habitat, and food preference. Table 7 summarizes the habitat and food preference of the main animal species trapped in this study. Although their habitat ranges differ, they were all collected in the same environment around cattle farms and human dwellings. *M. natalensis, R. rattus,* and *C. gambianus* are all omnivorous and will eat whatever they will find in a human-created environment. The main difference is that *C. gambianus* use their cheek pouches to carry food and bedding material and that they regularly perform coprophagy [61]; in that way, they can have more frequent encounters with mycobacteria.

4.2.2. Insectivores. A difference in prevalence of mycobacteria between rodents and insectivores was also observed similar to a previous study [21]. This difference was only observed in reacting farms and not in nonreacting farms. Insectivores probably pick up mycobacteria from the environment through their scavenging behaviour [27]. Their coprophagy and feeding on freshly killed animals (Table 7) are possible explanations for the elevated prevalence. A difference was also found in the mycobacterial prevalences in these animals between reacting and nonreacting farms: For the current reactor status, a higher prevalence was observed in the reacting as compared to the nonreacting farms, which is an argument for a common source of infection for insectivores and cattle. For the past reactor status, a higher prevalence was observed in the nonreacting as compared to the reacting farms, also indicating that cattle are probably not the source of infection for insectivores.

Most of the mycobacteria found in the insectivore *C. hirta* were potentially pathogenic for humans (Table 3), most of which were part of the *M. avium*-complex (*M. intracellulare*, *M. chimaera*, and *M. colombiense*) [52].

TABLE 7: Habitat and food preference of the main animals species collected according to Nowak [61].

Animal species	Habitat	Food preference
C. gambianus	Forests, and villages	Vegetables, insects, crabs, snails, palm fruits, and palm kernels Regular coprophagy They use their cheek pouches to carry food and store large amounts of food in their shelters
C. hirta	Damp and dry forests, grasslands Cultivated areas Occasionally human settlements and buildings	Invertebrates Bodies of freshly killed animals (including frogs, toads and lizards) Regular coprophagy
M. natalensis	Savannah Cultivated and abandoned fields Buildings and villages	Mainly grass and other seeds, insects when available Everything that people eat when available
R. rattus	Cities, villages Cultivated fields some natural habitats	Variety of plants and animal matter: seeds, grains, nuts, vegetables, fruits Everything that people eat

The other insectivores collected in this study, the hedgehogs, are also known carriers of pathogenic mycobacteria [29, 62, 63] although only European hedgehogs have been studied in this context. In the present study, 2 out of 4 hedgehogs (*A. albiventris*) carried mycobacteria, but those mycobacteria are probably not pathogenic.

4.2.3. Organs. In general, the lung was the most prone to contain mycobacteria, followed by the mesenteric lymph nodes, the spleen, and the liver. This is consistent with transmission of mycobacteria through aerosols and through digestion. In two cases, mycobacteria were cultured from lesions: *M. terrae* from a swollen foot of *R. rattus* and a mycobacterium related to *M. intracellulare* from a swollen lymph node of *C. gambianus*. In the other animals presenting mycobacteria, no macroscopic pathomorphological lesions were observed and for most of the isolates only one colony was observed in culture, suggesting colonization rather than infection.

As mycobacteria were found in all four organs, and considering what is currently known about transmission of diseases by rodents and of diseases in general [64], several ways of transmission of mycobacteria are possible: through direct contact with rodent excreta, through ingestion of food or water contaminated with rodent excreta, through ingestion of the animal itself, through inhaling aerosolized rodent excreta, through rodent bites, or through ectoparasites.

4.3. Pool Screening Approach. The study used the pooled screening approach to save time and resources when analyzing the specimens. We have shown that although there is a slight underestimation of the prevalence when pool screening is used for mycobacterial detection, the 95% confidence intervals are as wide as with individual screening. This has been previously reported by Vansteelandt et al. [65] for viral detection. As the pooling was done in a

stratified way, per habitat, per organ and per species, there was no loss of information since several hypotheses could still be tested. If pooling would have been done in a different way, for example, pooling all organs from the same animal as has been done by some researchers [20, 66], we would have lost information, about the site of infection or colonization. Therefore, we strongly recommend the stratified pool screening method in mycobacterial reservoir research.

4.4. PCR versus Culture for Detection of Mycobacteria. For only seven pools, PCR and culture results were consistent. This probably is due to a difference in sensitivity of the methods [67]: for detection of *M. tuberculosis* in clinical samples, PCR is less sensitive than culture [68]. This is also true for mycobacteria in general. Although a specific 16S rDNA PCR is very useful to detect mycobacteria in different samples, it is not as sensitive as culture, because of the fact that its target only occurs once or twice in the mycobacterial chromosome [69]. Indeed, five of the eight pools from which more than one colony grew in culture, were also positive for PCR (see Supplementary Table S1 that could be found at doi: 10.4061/2011/495074).

A second reason for the inconsistency is the fact that the methods target other mycobacteria; for example, not all mycobacteria grow at the temperature in which the inoculated media are kept [52], while PCR targets also the DNA of dead mycobacteria (killed either by the immune system of the animal, during the transport or storage, or during the decontamination) [70]. This inconsistency has been shown and discussed in previous studies as well [21, 26].

4.5. Risk of Transmission of Mycobacteria from Small Mammals to Humans. Little is known about the prevalence of atypical mycobacterioses in the human population in Tanzania. However, Kazwala et al. [71] reported that 16%

of the mycobacterial isolates from extrapulmonary human samples in Tanzania were *M. bovis* and 13.6% were atypical mycobacteria, whereas Mfinanga et al. [72] demonstrated that 10.8% of mycobacterial isolates from extrapulmonary human samples were *M. bovis* and 47.7% were atypical mycobacteria. This shows that although few data are available, human cases of both BTB and atypical mycobacterial disease are present in Tanzania. Recently, three additional cases of invasive atypical mycobacterial disease in HIV-positive patients in Tanzania were described caused by *M. sherrisii* and *M. avium*-complex [73]. At ITM, we have records of *M. intracellulare*, *M. terrae*, *M. arupense*, *M. colombiense*, and *M. kumamotonense* isolated from clinical samples in Tanzania although the clinical significance of these mycobacterial isolates is not known. Of the mycobacteria isolated in humans, *M. intracellulare*, *M. colombiense*, and *M. terrae* were detected in small mammals in the present study. In a previous study in the same region in Tanzania, we detected also *M. intracellulare* and *M. arupense* [21]. Although the HIV prevalence has been decreasing slowly in Tanzania, it still reached 6.2% and 6% in 2005 and 2006, respectively [74]. In 2009, the HIV prevalence has decreased to 5.6%, but this still means that a substantial proportion of the Tanzanian population is more sensitive to infections with these atypical mycobacteria. All rodents and insectivores in the present study were collected in close proximity to human dwellings, which means that infected animals could pose a risk to humans with a lowered immune system.

5. Conclusion

The present study is the first to investigate the presence of mycobacteria in rodents and insectivores in relation to the reactor status of the cattle farms on which they were collected. Analysis of the presence of mycobacteria in relation to the reactor status of the cattle farms does not exclude transmission between small mammals and cattle but indicates that transmission to cattle from another source of infection is more likely. However, because of the high prevalence of potentially pathogenic mycobacteria in some small mammal species, namely, in *C. gambianus* and *C. hirta*, the infected animals can pose a risk to humans, especially in areas with a high HIV-prevalence as is the case in Tanzania.

Acknowledgments

References

[1] J. P. Euzéby, "List of Prokaryotic names with standing in nomenclature— Genus *Mycobacterium*," 2009.

[2] E. Tortoli, "The new mycobacteria: an update," *FEMS Immunology and Medical Microbiology*, vol. 48, no. 2, pp. 159–178, 2006.

[3] F. M. Collins, "Mycobacterial disease, immunosuppression, and acquired immunodeficiency syndrome," *Clinical Microbiology Reviews*, vol. 2, no. 4, pp. 360–377, 1989.

[4] L. A. Corner, "The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 303–312, 2006.

[5] S. H. Hendrick, D. F. Kelton, K. E. Leslie, K. D. Lissemore, M. Archambault, and T. F. Duffield, "Effect of paratuberculosis on culling, milk production, and milk quality in dairy herds," *Journal of the American Veterinary Medical Association*, vol. 227, no. 8, pp. 1302–1308, 2005.

[6] R. R. Kazwala, *Molecular epidemiology of bovine tuberculosis in Tanzania*, Ph.D. thesis, University of Edinburgh, 1996.

[7] G. W. de Lisle, C. G. Mackintosh, and R. G. Bengis, "*Mycobacterium bovis* in free-living and captive wildlife, including farmed deer," *Revue Scientifique et Technique*, vol. 20, no. 1, pp. 86–111, 2001.

[8] R. G. Bengis, N. P. Kriek, D. F. Keet, J. P. Raath, V. de Vos, and H. F. Huchzermeyer, "An outbreak of bovine tuberculosis in a free-living African buffalo (*Syncerus caffer-Sparrman*) population in the Kruger National Park: a preliminary report," *Onderstepoort Journal of Veterinary Research*, vol. 63, no. 1, pp. 15–18, 1996.

[9] J. A. Drewe, A. K. Foote, R. L. Sutcliffe, and G. P. Pearce, "Pathology of *Mycobacterium bovis* infection in wild meerkats (*Suricata suricatta*)," *Journal of Comparative Pathology*, vol. 140, no. 1, pp. 12–24, 2009.

[10] D. F. Keet, N. P. Kriek, M. L. Penrith, A. Michel, and H. Huchzermeyer, "Tuberculosis in buffaloes (*Synecrus caffer*) in the Kruger National Park: spread of the diseases to other species," *Onderstepoort Journal of Veterinary Research*, vol. 63, no. 3, pp. 239–244, 1996.

[11] R. M. Kirberger, D. F. Keet, and W. M. Wagner, "Radiologic abnormalities of the appendicular skeleton of the lion (*Panthera leo*): incidental findings and *Mycobacterium bovis*-induced changes," *Veterinary Radiology and Ultrasound*, vol. 47, no. 2, pp. 145–152, 2006.

[12] S. Cleaveland, T. Mlengeya, R. R. Kazwala et al., "Tuberculosis in Tanzanian wildlife," *Journal of Wildlife Diseases*, vol. 41, no. 2, pp. 446–453, 2005.

[13] S. Cleaveland, D. J. Shaw, S. G. Mfinanga et al., "*Mycobacterium bovis* in rural Tanzania: risk factors for infection in human and cattle populations," *Tuberculosis*, vol. 87, no. 1, pp. 30–43, 2007.

[14] L. Durnez, H. Sadiki, A. Katakweba et al., "The prevalence of *Mycobacterium bovis*-infection and atypical mycobacterioses in cattle in and around Morogoro, Tanzania," *Tropical Animal Health and Production*, vol. 41, no. 8, pp. 1653–1659, 2009.

[15] S. F. Jiwa, R. R. Kazwala, A. A. Aboud, and W. J. Kalaye, "Bovine tuberculosis in the Lake Victoria zone of Tanzania and its possible consequences for human health in the HIV/AIDS era," *Veterinary Research Communications*, vol. 21, no. 8, pp. 533–539, 1997.

[16] R. H. Mdegela, L. J. Kusiluka, A. M. Kapaga et al., "Prevalence and determinants of mastitis and milk-borne zoonoses in smallholder dairy farming sector in Kibaha and Morogoro districts in Eastern Tanzania," *Journal of Veterinary Medicine Series B*, vol. 51, no. 3, pp. 123–128, 2004.

[17] G. M. Shirima, R. R. Kazwala, and D. M. Kambarage, "Prevalence of bovine tuberculosis in cattle in different farming

systems in the eastern zone of Tanzania," *Preventive Veterinary Medicine*, vol. 57, no. 3, pp. 167–172, 2003.

[18] H. Bercovier and V. Vincent, "Mycobacterial infections in domestic and wild animals due to *Mycobacterium marinum, M. fortuitum, M. chelonae, M. porcinum, M. farcinogenes, M. smegmatis, M. scrofulaceum, M. xenopi, M. kansasii, M. simiae and M. genavense*," *Revue Scientifique et Technique*, vol. 20, no. 1, pp. 265–290, 2001.

[19] R. Cavanagh, M. Begon, M. Bennett et al., "*Mycobacterium microti* infection (vole tuberculosis) in wild rodent populations," *Journal of Clinical Microbiology*, vol. 40, no. 9, pp. 3281–3285, 2002.

[20] R. J. Delahay, G. C. Smith, A. M. Barlow et al., "Bovine tuberculosis infection in wild mammals in the South-West region of England: a survey of prevalence and a semi-quantitative assessment of the relative risks to cattle," *Veterinary Journal*, vol. 173, no. 2, pp. 287–301, 2007.

[21] L. Durnez, M. Eddyani, G. F. Mgode et al., "First detection of mycobacteria in African rodents and insectivores, using stratified pool screening," *Applied and Environmental Microbiology*, vol. 74, no. 3, pp. 768–773, 2008.

[22] W. Y. Ayele, S. D. Neill, J. Zinsstag, M. G. Weiss, and I. Pavlik, "Bovine tuberculosis: an old disease but a new threat to Africa," *International Journal of Tuberculosis and Lung Disease*, vol. 8, no. 8, pp. 924–937, 2004.

[23] N. A. Foudraine, D. van Soolingen, G. T. Noordhoek, and P. Reiss, "Pulmonary tuberculosis due to Mycobacterium microti in a human immunodeficiency virus-infected patient," *Clinical Infectious Diseases*, vol. 27, no. 6, pp. 1543–1544, 1998.

[24] F. Biet, M. L. Boschiroli, M. F. Thorel, and L. A. Guilloteau, "Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium-intracellulare* complex (MAC)," *Veterinary Research*, vol. 36, no. 3, pp. 411–436, 2005.

[25] R. D. Cavanagh, X. Lambin, T. Ergon et al., "Disease dynamics in cyclic populations of field voles (*Microtus agrestis*): cowpox virus and vole tuberculosis (*Mycobacterium microti*)," *Proceedings of the Royal Society—Biological Sciences*, vol. 271, no. 1541, pp. 859–867, 2004.

[26] L. Durnez, P. Suykerbuyk, V. Nicolas et al., "Terrestrial small mammals as reservoirs of *Mycobacterium ulcerans* in benin," *Applied and Environmental Microbiology*, vol. 76, no. 13, pp. 4574–4577, 2010.

[27] O. Fischer, L. Mátlová, J. Bartl, L. Dvorská, I. Melichárek, and I. Pavlík, "Findings of mycobacteria in insectivores and small rodents," *Folia Microbiologica*, vol. 45, no. 2, pp. 147–152, 2000.

[28] N. G. Gratz, "Rodents and human disease: a global appreciation," in *Rodent Pest Management*, I. Prakash, Ed., pp. 101–169, CRC Press, Boca Raton, Fla, USA, 1988.

[29] I. W. Lugton, A. C. Johnstone, and R. S. Morris, "*Mycobacterium bovis* infection in New Zealand hedgehogs (*Erinaceus europaeus*)," *New Zealand Veterinary Journal*, vol. 43, pp. 342–345, 1995.

[30] N. G. Gratz, "The burden of rodent-borne diseases in Africa south of the Sahara," *Belgian Journal of Zoology*, vol. 127, pp. 71–84, 1997.

[31] B. G. Meerburg, *Zoonotic risks of rodents in livestock production*, Ph.D. thesis, University of Amsterdam, 2006.

[32] K. A. Clarke, S. D. Fitzgerald, L. S. Zwick et al., "Experimental inoculation of meadow voles (*Microtus pennsylvanicus*), house mice (*Mus musculus*), and Norway rats (*Rattus norvegicus*) with *Mycobacterium bovis*," *Journal of Wildlife Diseases*, vol. 43, no. 3, pp. 353–365, 2007.

[33] P. Collins, P. R. Matthews, A. McDiarmid, and A. Brown, "The pathogenicity of *Mycobacterium avium* and related mycobacteria for experimental animals," *Journal of Medical Microbiology*, vol. 16, no. 1, pp. 27–35, 1983.

[34] T. J. Bosworth, "Further observations on the wild rat as a carrier of *Brucella abortus*," *Journal of Comparative Pathology and Therapeutics*, vol. 53, pp. 42–49, 1940.

[35] T. W. Little, C. Swan, H. V. Thompson, and J. W. Wilesmith, "Bovine tuberculosis in domestic and wild mammals in an area of Dorset. III. The prevalence of tuberculosis in mammals other than badgers and cattle," *Journal of Hygiene*, vol. 89, no. 2, pp. 225–234, 1982.

[36] R. J. Delahay, A. N. De Leeuw, A. M. Barlow, R. S. Clifton-Hadley, and C. L. Cheeseman, "The status of *Mycobacterium bovis* infection in UK wild mammals: a review," *Veterinary Journal*, vol. 164, no. 2, pp. 90–105, 2002.

[37] J. C. Hope, M. L. Thom, B. Villarreal-Ramos, H. M. Vordermeier, R. G. Hewinson, and C. J. Howard, "Exposure to *Mycobacterium avium* induces low-level protection from *Mycobacterium bovis* infection but compromises diagnosis of disease in cattle," *Clinical and Experimental Immunology*, vol. 141, no. 3, pp. 432–439, 2005.

[38] C. O. Thoen and E. D. Ebel, "Diagnostic tests for bovine tuberculosis," in Mycobacterium Bovis *Infection in Animals and Humans*, C. O. Thoen, J. H. Steele, and M. J. Gilsdorf, Eds., pp. 49–53, Blackwell, Ames, Iowa, USA, 2006.

[39] H. L. Rieder, T. M. Chonde, H. Myking et al., *The Public Health Service National Tuberculosis Reference Laboratory and the National Laboratory Network*, International Union Against Tuberculosis and Lung Disease, Paris, France, 1998.

[40] F. Portaels, A. De Muynck, and M. P. Sylla, "Selective isolation of mycobacteria from soil: a statistical analysis approach," *Journal of General Microbiology*, vol. 134, no. 3, pp. 849–855, 1988.

[41] G. Mangiapan, M. Vokurka, L. Schouls et al., "Sequence capture-PCR improves detection of mycobacterial DNA in clinical specimens," *Journal of Clinical Microbiology*, vol. 34, no. 5, pp. 1209–1215, 1996.

[42] V. V. Levy-Frebault and F. Portaels, "Proposed minimal standards for the genus *Mycobacterium* and for description of new slowly growing *Mycobacterium* species," *International Journal of Systematic Bacteriology*, vol. 42, no. 2, pp. 315–323, 1992.

[43] J. T. Barker, *Statistical estimators of infection potential based on PCR pool screening with unequal pool sizes*, Ph.D. thesis, University of Alabama, Birmingham, UK, 2000.

[44] C. L. Chiang and W. C. Reeves, "Statistical estimation of virus infection rates in mosquito vector populations," *American Journal of Hygiene*, vol. 75, pp. 377–391, 1962.

[45] R. Dorfman, "The detection of defective members in large populations," *Annals of Mathematical Statistics*, pp. 436–440, 1943.

[46] C. R. Katholi, L. Toe, A. Merriweather, and T. R. Unnasch, "Determining the prevalence of *Onchocerca volvulus* infection in vector populations by polymerase chain reaction screening of pools of black flies," *Journal of Infectious Diseases*, vol. 172, no. 5, pp. 1414–1417, 1995.

[47] C. R. Katholi and T. R. Unnasch, "Important experimental parameters for determining infection rates in arthropod vectors using pool screening approaches," *American Journal of Tropical Medicine and Hygiene*, vol. 74, no. 5, pp. 779–785, 2006.

[48] Casella and Berger, *Statistical Inference*, Duxbury, Pacific Grove, Calif, USA, 2001.

[49] Y. H. Dai and K. Schittkowski, "A sequential quadratic programming algorithm with non-monotone line search," *Pacific Journal of Optimization*, vol. 4, pp. 335–351, 2008.

[50] K. Schittkowski, "NLPQL: a fortran subroutine solving constrained nonlinear programming problems," *Annals of Operations Research*, vol. 5, pp. 485–500, 1986.

[51] K. Schittkowski, "NLPQLP: a fortran implementation of a sequential quadratic programming algorithm with distributed and non-monotone line search—user's guide, version 2.2," Tech. Rep., Department of Computer Science, University of Bayreuth, 2006.

[52] S. C. Leão, A. Martin, G. I. Mejia et al., Practical handbook for the phenotypic and genotypic identification of mycobacteria. With the financial support of INCO, DEV and CA, 2004.

[53] D. T. Haydon, S. Cleaveland, L. H. Taylor, and M. K. Laurenson, "Identifying reservoirs of infection: a conceptual and practical challenge," *Emerging Infectious Diseases*, vol. 8, no. 12, pp. 1468–1473, 2002.

[54] R. J. Delahay, C. L. Cheeseman, and R. S. Clifton-Hadley, "Wildlife disease reservoirs: the epidemiology of *Mycobacterium bovis* infection in the European badger (*Meles meles*) and other British mammals," *Tuberculosis*, vol. 81, pp. 43–49, 2001.

[55] M. Florou, L. Leontides, P. Kostoulas et al., "Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from non-ruminant wildlife living in the sheds and on the pastures of Greek sheep and goats," *Epidemiology and Infection*, vol. 136, no. 5, pp. 644–652, 2008.

[56] M. Kopecna, I. Trcka, J. Lamka et al., "The wildlife hosts of *Mycobacterium avium* subsp *paratuberculosis* in the Czech Republic during the years 2002–2007," *Veterinarni Medicina*, vol. 53, no. 8, pp. 420–426, 2008.

[57] L. Dvorska, L. Matlova, M. Bartos et al., "Study of *Mycobacterium avium* complex strains isolated from cattle in the Czech Republic between 1996 and 2000," *Veterinary Microbiology*, vol. 99, no. 3-4, pp. 239–250, 2004.

[58] M. F. Thorel, H. F. Huchzermeyer, and A. L. Michel, "*Mycobacterium avium* and *Mycobacterium intracellulare* infection in mammals," *Revue Scientifique et Technique*, vol. 20, no. 1, pp. 204–218, 2001.

[59] V. M. Katoch, "Infections due to non-tuberculous mycobacteria (NTM)," *Indian Journal of Medical Research*, vol. 120, no. 4, pp. 290–304, 2004.

[60] P. D. van Helden, N. C. Gey van Pittius, R. M. Warren et al., "Pulmonary infection due to *Mycobacterium goodii* in a spotted hyena (*Crocuta crocuta*) from South Africa," *Journal of Wildlife Diseases*, vol. 44, no. 1, pp. 151–154, 2008.

[61] R. M. Nowak, *Walker's Mammals of the World*, vol. 1-2, The John Hopkins University Press, Baltimore, Md, USA, 6th edition, 1999.

[62] J. P. Tappe, I. Weitzman, S. Liu, E. P. Dolensek, and D. Karp, "Systemic *Mycobacterium marinum* infection in a European hedgehog," *Journal of the American Veterinary Medical Association*, vol. 183, no. 11, pp. 1280–1281, 1983.

[63] R. S. Windsor, D. S. Durrant, and K. J. Burn, "Avian tuberculosis in pigs: *Mycobacterium intracellulare* infection in a breeding herd," *Veterinary Record*, vol. 114, no. 20, pp. 497–500, 1984.

[64] M. Shakespeare, *Zoonoses*, Pharmaceutical Press, London, UK, 2001.

[65] S. Vansteelandt, E. Goetghebeur, and T. Verstraeten, "Regression models for disease prevalence with diagnostic tests on pools of serum samples," *Biometrics*, vol. 56, no. 4, pp. 1126–1133, 2000.

[66] G. W. de Lisle, G. F. Yates, P. Caley, and R. J. Corboy, "Surveillance of wildlife for *Mycobacterium bovis* infection using culture of pooled tissue samples from ferrets (*Mustela furo*)," *New Zealand Veterinary Journal*, vol. 53, no. 1, pp. 14–18, 2005.

[67] M. A. Pfaller, "Application of new technology to the detection, identification, and antimicrobial susceptibility testing of mycobacteria," *American Journal of Clinical Pathology*, vol. 101, no. 3, pp. 329–337, 1994.

[68] D. I. Ling, L. L. Flores, L. W. Riley, and M. Pai, "Commercial nucleic-acid amplification tests for diagnosis of pulmonary tuberculosis in respiratory specimens: meta-analysils and meta-regression," *PLoS ONE*, vol. 3, no. 2, Article ID e1536, 2008.

[69] G. B. Fogel, C. R. Collins, J. Li, and C. F. Brunk, "Prokaryotic genome size and SSU rDNA copy number: estimation of microbial relative abundance from a mixed population," *Microbial Ecology*, vol. 38, no. 2, pp. 93–113, 1999.

[70] R. Fukushima, S. Kawakami, H. Iinuma, and K. Okinaga, "Molecular diagnosis of infectious disease," *Nippon Geka Gakkai Zasshi*, vol. 104, no. 7, pp. 518–522, 2003.

[71] R. R. Kazwala, C. J. Daborn, J. M. Sharp, D. M. Kambarage, S. F. Jiwa, and N. A. Mbembati, "Isolation of *Mycobacterium bovis* from human cases of cervical adenitis in Tanzania: a cause for concern?" *International Journal of Tuberculosis and Lung Disease*, vol. 5, no. 1, pp. 87–91, 2001.

[72] S. G.. Mfinanga, O. Morkve, R. R. Kazwala et al., "Mycobacterial adenitis: role of *Mycobacterium bovis*, non-tuberculous mycobacteria, HIV infection, and risk factors in Arusha, Tanzania," *East African Medical Journal*, vol. 81, no. 4, pp. 171–178, 2004.

[73] J. A. Crump, J. van Ingen, A. B. Morrissey et al., "Invasive disease caused by nontuberculous mycobacteria, Tanzania," *Emerging Infectious Diseases*, vol. 15, no. 1, pp. 53–55, 2009.

[74] WHO, Global Health Observatory Database, 2011.

Research Article

A Study of the Persistence of *Mycobacterium bovis* in the Environment under Natural Weather Conditions in Michigan, USA

Amanda E. Fine,[1,2] Carole A. Bolin,[3] Joseph C. Gardiner,[4] and John B. Kaneene[1]

[1] *Department of Large Animal Clinical Sciences and Center for Comparative Epidemiology, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824-1314, USA*

[2] *Wildlife Conservation Society, P.O. Box 485, Post Office 38, Ulaanbaatar 211238, Mongolia*

[3] *Diagnostic Center for Population and Animal Health, Michigan State University, 4125 Beaumont Road, Lansing, MI 48910-8104, USA*

[4] *Department of Epidemiology, College of Human Medicine, Michigan State University, B601 West Fee Hall, East Lansing, MI 48824, USA*

Correspondence should be addressed to John B. Kaneene, kaneene@cvm.msu.edu

Reisolation of *Mycobacterium bovis* from inoculated substrates was used to follow the persistence of viable *M. bovis* bacteria exposed to natural weather conditions over a 12-month period. Environmental factors were recorded continuously, and factors affecting *M. bovis* persistence (i.e., temperature, season, and substrate) were studied using survival analysis and Cox's proportional hazards regression. Persistence of *M. bovis* in the environment was significantly shorter in the spring/summer season, characterized by the highest average daily temperatures over the 12-month period. *M. bovis* persisted up to 88 days in soil, 58 days in water and hay, and 43 days on corn. These studies demonstrate that *M. bovis* bacteria persist long enough to represent a risk of exposure for cattle and/or wildlife and strengthen evidence that suggests cattle farm biosecurity and efforts to eliminate supplemental feeding of white-tailed deer will decrease the risk of bovine TB transmission among and between cattle and deer populations.

1. Introduction

An endemic focus of bovine tuberculosis (TB), caused by a single strain of *Mycobacterium bovis*, has been identified in white-tailed deer (*Odocoileus virginianus*) in northeast lower Michigan [1–3]. Spillover of *M. bovis* infection from white-tailed deer to cattle is suspected in the majority of the 52 cattle farms in the same region of the State identified as bovine TB positive since intensive surveillance for TB in Michigan livestock was reinitiated in 1998 (Michigan Department of Agriculture; USDA/APHIS/VS). The emergence of a wildlife reservoir for bovine TB in Michigan, and evidence of disease transmission between infected free-ranging white-tailed deer populations and domestic cattle, has forced a reevaluation of the understanding of the epidemiology of bovine TB in North America. Disease transmission between deer and cattle in Michigan is thought to occur in the absence of close contact between the species [4]. This has raised questions about the role of indirect transmission of *M. bovis* in the epidemiology of bovine TB and identified a need to investigate the persistence of *M. bovis* in the environment and the potential role of contaminated substrates in the transmission of *M. bovis* among and between wildlife and cattle populations.

The persistence of *M. bovis* in the environment and role of indirect transmission in the epidemiology of bovine TB in Michigan has been debated since the current TB epidemic in Michigan was first described in 1997 [2]. Bovine TB infection in white-tailed deer today is likely linked to the large number of cattle infected with *M. bovis* in Michigan during the late 1950's [5], however, the establishment and persistence of *M. bovis* in free-ranging white-tailed deer in northeast Michigan is thought to have been influenced by the long-term practice of Winter feeding of deer in the region [2].

These piles of feed, set out to attract deer and improve their productivity and Winter survival, are thought to contribute to the transmission of TB among white-tailed deer by (1) increasing local density and contact between animals and (2) providing a site for the indirect transmission of TB through contamination of the feed by infected deer shedding *M. bovis* in their saliva or nasal discharges and the subsequent infection of a naïve deer by consumption of contaminated feed [2, 6]. This is supported by evidence that suggests that specific supplemental feeding practices, generally indicative of large-scale feeding operations, are associated with an increasing risk for bovine TB in deer in Michigan [7].

The role of *M. bovis* contaminated environmental substrates in the interspecies transmission of bovine TB between cattle and deer has also been investigated [8, 9]. Although *M. bovis* was not identified from any of the environmental substrates tested, particular cattle management practices and environmental factors were identified that have been shown to be associated with tuberculosis on cattle farms in northeast Michigan in the past [10]. These practices likely facilitate the indirect transmission of bovine TB from deer to cattle via *M. bovis* contaminated substrates. The factors and practices identified included the presence of ponds or open water in cattle areas, maintaining cattle outside more than 50% of the time, feeding, and watering cattle outside and not protecting feed intended for cattle from deer.

Evidence suggests that opportunities for the indirect transmission of *M. bovis* between white-tailed deer and cattle exist in northeast Michigan under current cattle and deer management practices. Data on the persistence of *M. bovis* on various environmental substrates and the factors that influence its survival are essential to the further understanding of the complexity of bovine TB transmission and epidemiology in Michigan. In addition to contributing to our understanding of bovine TB dynamics in this system, information regarding the persistence of *M. bovis* in the environment will support efforts to improve protocols for cattle farm biosecurity and the maintenance of appropriate restrictions on feeding and baiting free-ranging white-tailed deer and other wildlife.

Experimental studies conducted in New Zealand, Australia, South Africa, Great Britain, and Ireland, have shown that *M. bovis* persists in typical environmental substrates for varying amounts of time [11–15].

This study was designed to describe the persistence of the Michigan strain of *M. bovis* in typical environmental substrates (corn, hay, soil, and water) exposed to natural weather conditions in Michigan. Factors affecting the length of persistence, or survival time, of *M. bovis* in the environment were also investigated. The objective of the study was to determine whether or not *M. bovis* can survive in environmental substrates for sufficient lengths of time to serve as a source of infection for cattle and/or wild deer.

2. Materials and Methods

2.1. Culture, Media, and Growth Conditions.
A Michigan strain of *Mycobacterium bovis* was obtained from a frozen culture of *M. bovis* originally isolated in 2002 from the retropharyngeal lymph node of a naturally infected 2-year-old Holstein cow from Michigan. The animal was classified as a reactor on a comparative cervical test and had gross and microscopic lesions consistent with bovine TB at necropsy. The frozen *M. bovis* culture was added to 10 mL of Middlebrook 7H9 Broth with Middlebrook ADC Enrichment for cultivation of mycobacteria (Becton-Dickinson, Cockeysville, Md, USA). Multiple 10 mL vials of *M. bovis* inoculated 7H9 Broth were incubated at 37°C for 21 to 30 days. The exact final concentration of colony forming units of *M. bovis* liquid culture stock (CFU/mL) was determined by monitoring turbidity and then performing colony counts on Selective 7H11 agar (Becton-Dickinson) plates inoculated with exactly 100 μL of *M. bovis* liquid stock, a 1 : 10, 1 : 100, 1 : 1000 and a 1 : 10000 dilution. Blood and CNA agar plates were inoculated with *M. bovis* liquid culture stock to monitor for contamination.

2.2. Environmental Substrates.
Substrates selected for testing included grass hay, soil, water, and shelled corn. Grass hay was collected from the feed storage area of the Michigan State University (MSU) Large Animal Veterinary Teaching Hospital in East Lansing, Mich, USA. Soil was collected from the Baker Woodlot (Rachana Rajendra Neotropical Bird Sanctuary) located in the south central section of the MSU campus. Water was collected from the large pond at the center of the Baker Woodlot and from the Red Cedar River at the Farm Lane Bridge on the MSU campus. Shelled corn was purchased in 20-pound (~9 kg) bags from a local feed store. Environmental substrates were stored at 4°C with no exposure to light.

A set of environmental substrates consisted of 4 samples each of grass hay, soil, water, and shelled corn for a total of 16. Ball Half-Pint (236 mL) Regular Can-or-Freeze Jars were filled with 5 gm of hay, 10 gm of soil, 10 gm of corn, or 10 mL of water. Half of the sample-filled jars were autoclaved for 2 hours at 121°C and 20 psi to sterilize the contents. Each sample set of 16 was identified with uniquely colored tape and a label denoting the sample type, sample set number, the autoclave status, and shade or nonshade treatment.

2.3. Facility (Laboratory and Outdoor Enclosure).
Mycobacterium bovis sample inoculation, sample processing, and *M. bovis* isolation procedures were all performed in the biosafety level III (BL3) laboratory in the Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University (MSU).

The *M. bovis* environmental persistence studies were carried out in a structure erected on a concrete slab along the north fence of the livestock containment facility south of the DCPAH at MSU. The structure consisted of an enclosed "cage" 4.88 m × 7.62 m × 2.44 m with a galvanized steel frame covered with 2.44 mm fencing (chain link-type fence with 2.44 mm holes) on all sides including the top. The bottom rail of the cage was flush with the concrete slab or buried below the ground surface. Any gaps below the bottom rail were closed with 0.61 m × 1.22 m wooden beams. A
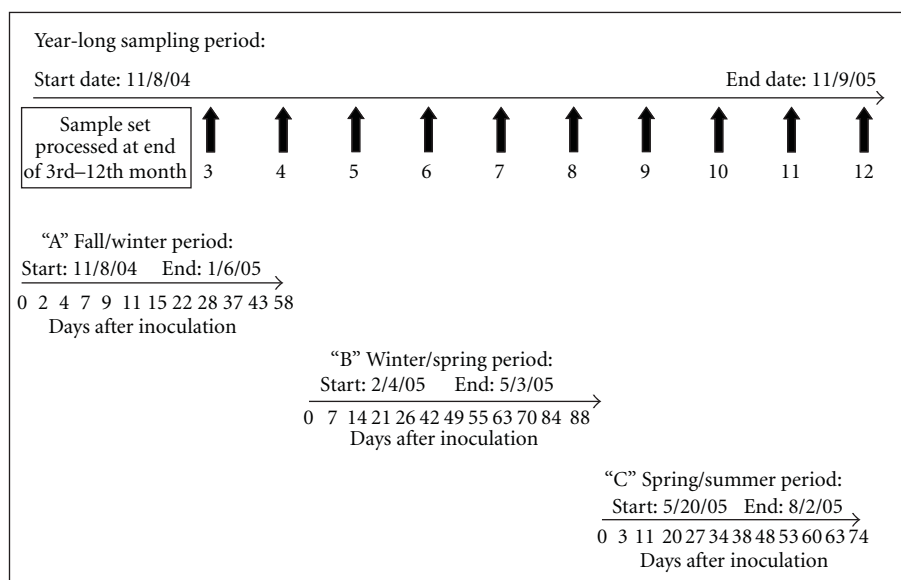
FIGURE 1: Timing of processing samples after *M. bovis* inoculation over year-long and seasonal sampling periods.

locked door was built into one side of the enclosure with a minimum clearance with the concrete slab and doorframes.

The fencing excluded all birds and small mammals. The structure was built to exclude livestock and/or deer in the unlikely event that they gain entrance to the fenced containment facility and access to the experimental enclosure was limited to authorized individuals.

Specified sets of *M. bovis* inoculated environmental substrates were placed within secondary clear plastic containers on 2 lines of steel tables set up within the enclosure. Sample containers on 1 line of steel tables were covered with black shade cloth. All secondary sample containers were lined with gravel and sand and secured with wire mesh covers.

2.4. Environmental Monitoring. A WeatherHawk weather station, Division of Campbell Scientific, Inc., was positioned at the center of the enclosure. The station was powered by a solar panel charged battery pack. Environmental data collected included rainfall (mm), wind speed (m/sec), temperature (°C), humidity level (%), and solar radiation (W/m²). Evapotranspiration, a combination of solar radiation, temperature, wind speed, and humidity, was also calculated with the WeatherHawk Virtual Weather software. The weather station was programmed to record data at 20-minute intervals, 24 hours a day. Environmental data was downloaded from the weather station to an Excel (Microsoft Corporation, Redmond, Wash, USA) spreadsheet on a desktop computer using a wireless system. Temperature and precipitation data were also collected over the same time periods from a weather station in the Michigan Automated Weather Network (MAWN) in Hawks City, Presque Isle County, Mich, USA in the bovine TB area.

2.5. Inoculation with M. bovis. Each substrate sample was inoculated with 50,000 CFUs of a strain of *Mycobacterium*

bovis originally isolated from the lymph node of a cow in Michigan that tested positive for bovine TB and presented at necropsy with pathologic lesions typical of *M. bovis* infection. Samples were inoculated in the BL3 laboratory. Sample jars were then sealed with plastic, leak-proof lids and transported to the outdoor experimental enclosure, located 500 meters from the BL3 laboratory, in sealed and labeled coolers. Each sample set was then placed in the secondary containers within the enclosure described above. The plastic lids were removed from each sample jar and the wire mesh was placed over the secondary containers and secured.

2.6. Study Design and Sampling. The persistence of *M. bovis* in environmental substrates was evaluated over 4 sampling periods as presented in **Figure 1**. For each sampling period, 12 sets of 16 samples (4 grass hay, 4 corn, 4 water, and 4 soil) were inoculated with *M. bovis* which allowed for sample processing for *M. bovis* isolation at time 0 and at 11 additional time points. The first sample period spanned 12 months. Samples were placed in the enclosure on November 8, 2004. Sample sets were processed monthly with the final set processed on November 9, 2005. Three additional sampling periods were nested within the sampling year to allow for the collection of seasonal data. The first seasonal sampling period "A" was for 58 days during the Fall/Winter (November 8, 2004–January 6, 2005). The second sampling periods "B" was for 88 days during the Winter/Spring (February 4, 2005–May 3, 2005). The third sampling periods "C" was for 74 days during the Spring/Summer (May 20, 2005–August 2, 2005), and a set of samples was processed monthly from November, 2004 to December, 2005. At the start of each seasonal sampling period twelve sets of 16 *M. bovis* inoculated samples were placed in the environmental sample enclosure (**Figure 2**). The persistence of *M. bovis* over time was determined by processing the sample sets for

FIGURE 2: Photograph of samples of *M. bovis* inoculated substrates in their primary containers placed within their secondary containers inside the experimental environmental sample enclosure. Shown here are the "nonshaded" treatments from 4 sample sets (total of 32 sample jars).

mycobacterial culture and attempting to isolate *M. bovis* at time 0 and 11 additional time points. The size of the environmental sample enclosure limited the number of sample sets that could be placed in the enclosure at any given time which limited the number of sets per sampling period to 12. The schedule for sample set processing was varied slightly from season to season in an attempt to capture both the pattern of *M. bovis* die off over time (number of colony forming units isolated from 100 μL of processed sample) and the time point of the last positive and first negative *M. bovis* culture from a processed sample recorded for a particular sample substrate. As indicated in Figure 1, sample sets from the "Fall/Winter" period were collected every other day for 2 weeks and then weekly over 58 days, sample sets from the "Winter/Spring" were collected weekly over 88 days and sample sets from the "Spring/Summer" were collected on the third day and then approximately weekly over a 74 day period.

2.7. Environmental Sample Processing. At the scheduled sampling point a set of samples was collected from the environmental sample enclosure and transported (with lids replaced and in a sealed cooler) to the BL3 laboratory for processing. Sample substrates were allowed to come to room temperature before processing if frozen at collection. All samples were processed using the TB Culture Kit with Lytic Decon II (Integrated Research Technology, LLC, Quest Diagnostics Inc., Baltimore, Md) and a protocol standardized by Fine et al. for processing environmental samples (hay, soil and water) for mycobacterial culture [16]. Collected samples were processed within their original container (Ball 1/2 pint (236 mL) regular jar). If necessary, as was the case with
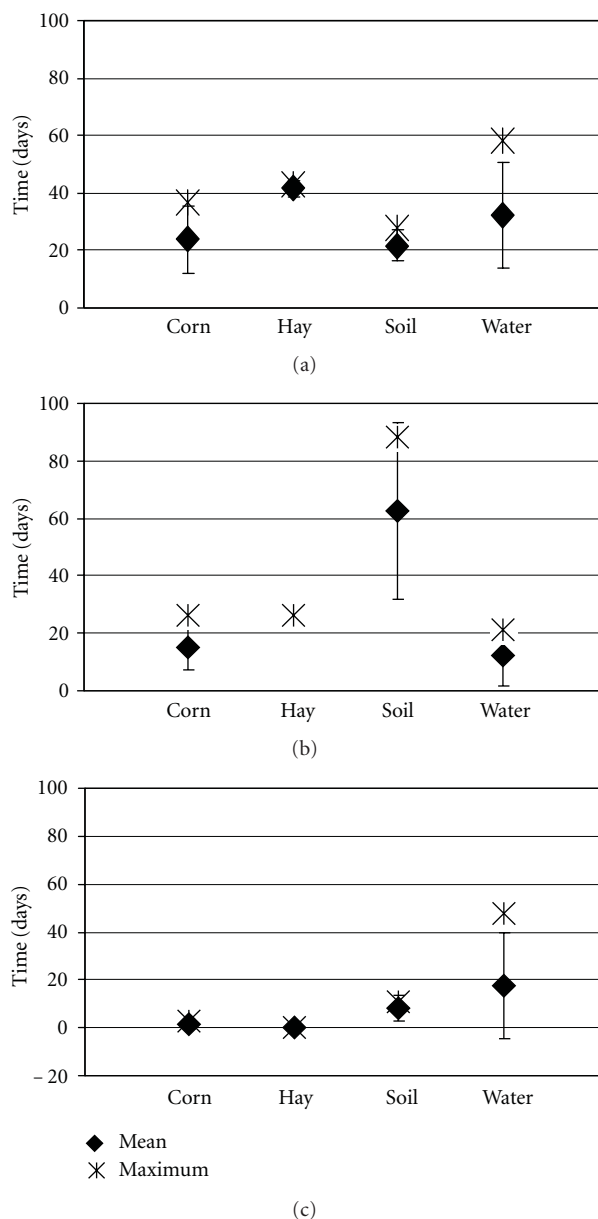
FIGURE 3: The mean and maximum survival of *M. bovis* in corn, hay, soil, and water exposed to environmental conditions in Fall/Winter (a), Winter/Spring (b), and Spring/Summer (c). Error bars represent standard deviation.

desiccated samples, sterile water (5–10 mL) was added to the solid substrates. Samples were pulverized and homogenized by securing a blade unit and gasket on the jars, inverting and blending them for 30 seconds on high with a household blender.

The samples were placed upright and allowed to settle for 30 minutes. The top 5 mL of fluid from each sample was removed and transferred to a 50 mL conical tube containing 10 mL of Decontamination Solution (20X Tris-citrate Buffer, CB-18 Stock, NALC, and water). Samples were mixed with a vortex machine and incubated at 37°C for 75 minutes. Sterile water was added to the 50 mL mark on each tube,

mixed and centrifuged at 3,000 g for 20 minutes. Pellet-containing tubes were decanted completely. A pipette was used to remove all but 1–3 mL of liquid from samples without a visible pellet. The pellet was resuspended in the supernatant backwash. One mL of sterile water was added and mixed. A 0.5 mL sample was transferred to a 2.0 mL labeled cryogenic vial and frozen at $-80°C$ and maintained for additional experiments or follow-up testing. One mL of 2X Resuspension Solution (10X-Enzyme Stock-*Trichoderma harzianum* extract, lysozyme and *Lysobacter* extract, and NALC) was added to each sample and samples were incubated for 45 minutes at $37°C$.

2.8. Mycobacterial Culture and Isolation. CB-18 processed samples were inoculated onto solid 7H11 Selective plates (Becton-Dickinson) by transferring $100 \mu L$ of processed sample onto the plates and spreading the sample equally across the media with individual L-shaped spreaders. Two culture replicates were set up for each sample and the plates were incubated at $37°C$ for 12 weeks and examined weekly for colony formation. Typical *M. bovis* colonies were counted and recorded on laboratory data sheets. Acid-fast smear analysis was performed to confirm the presence of acid-fast bacteria using standard protocols for slide preparation, staining, and examination [17]. A subset of the acid-fast positive isolates was confirmed to be *Mycobacterium tuberculosis* complex species group using a genetic probe (AccuProbes, Gen-Probe, San Diego, Calif, USA).

2.9. Data Analysis. Mycobacterial culture results, recorded on laboratory data sheets for each sampling period, were entered in an Excel spreadsheet (Excel, Microsoft Office XP Professional, Redmond, Wash, USA). Data from the weather records and mycobacterial culture results were imported into SAS software (SAS version 9.0, SAS Institute, Inc., 2003, Cary, NC, USA) and combined. Summaries of the weather records for each sampling period were created and descriptive statistics were generated for the persistence of *M. bovis* on each sample type for all sampling periods. Statistical analyses were carried out with SAS software.

Differences between mean *M. bovis* survival time (persistence) in shade/nonshade treated samples and sterilized/nonsterilized treated substrates were compared using t-tests. To test for significant differences in the mean survival time (persistence) in sample types and sampling seasons (Fall/Winter, Winter/Spring, and Spring/Summer), we used analysis of variance (ANOVA) with the Bonferroni adjustment of P-values for multiple comparisons. Assessment of associations between the bovine TB status of experimental samples (positive versus negative) and covariates (sample type, shade/nonshade, and sterilized/nonsterilized substrate) were conducted using the Cochran-Mantel-Haenszel Chi-square tests or 2-tailed Fisher's exact test where appropriate.

Survival analyses were used to compare the persistence of *M. bovis* in the environment between seasons and to study the effects of environmental factors on *M. bovis* survival across the seasons. The time from sample inoculation to the first negative bovine TB culture after the last positive bovine TB culture was used as the survival time, or *M. bovis* persistence period, for each sample. The Log rank and Wilcoxon tests were used to compare the survival distributions for *M. bovis* persistence in the environment across the 3 seasons tested. The survival function or Kaplan-Meier curves for *M. bovis* persistence in each of the three seasons and in each of the 4 substrates across the three seasons were plotted. Cox's proportional hazards regression was used to study the effects of the nonweather-related covariates (sample type, shade/nonshade, and sterilized/nonsterilized substrate) and season on *M. bovis* survival in the environment.

Model selection in Cox regression was used to identify specific weather or seasonal factors that influenced the survival of *M. bovis* in the environment. The weather data were summarized as daily means, maxima and minima for rainfall, wind speed, temperature, humidity, barometer readings, solar radiation, and evapotranspiration. Spearman correlation coefficients (r) were computed to identify potential areas of multicollinearity between the weather-related risk factors.

A multivariable Cox proportional hazards regression model was developed based on an initial evaluation of univariable models for each weather-related risk factor. The model contained all weathered-related risk factors that were significant at P-value $< .15$ on the likelihood ratio test (LRT). Highly correlated weather-related risk factors were removed due to redundancy of information and multicollinierity (e.g., solar radiation and evapotranspiration were both highly correlated with temperature). Purposeful selection of covariates and a modified stepwise method of variable evaluation with the entry P-value of .15 and the "stay" or removal P-value of .20 were used to build the final multivariable model for *M. bovis* survival in the environment.

3. Results

Detailed results from *M. bovis* inoculated substrates are presented in this section for the three sampling periods outlined in Figure 1 representing the seasons "A" Fall/Winter, "B" Winter/Spring and "C" Spring/Summer. Detailed results are not provided for the year-long sampling period outlined in Figure 1 because no *M. bovis* bacteria was isolated from samples processed at months three through twelve. The initial 2 months of the 12-month sampling period was covered by sampling period "A" Fall/Winter, with data on weather conditions presented in Table 1 and data on *M. bovis* persistence presented in Table 2.

3.1. Mycobacterial Culture. One hundred and ninety-two sample replicates from each of the three sampling periods (total 576) were processed for *M. bovis* isolation. Contamination of cultures with mold and other nonmycobacterial species was detected in 13% of the samples processed in sampling period Fall/Winter "A" and Winter/Spring "B" and 50% of the samples processed in sampling period Spring/Summer "C". Sample substrates that were sterilized before *M. bovis* inoculation had significantly lower odds of

TABLE 1: Weather conditions recorded over the three sampling periods at the site of the *Mycobacterium bovis* environmental persistence study and temperature and precipitation data from the TB area of Michigan for the same time.

|	Fall/Winter "A" 11/8/04–1/6/05 Average (Min.–Max)	Winter/Spring "B" 2/4/05–5/3/05 Average (Min.–Max)	Spring/Summer "C" 5/20/05–8/2/05 Average (Min.–Max)
Temperature (°C)	0.35 (−20.83–21.06)	2.82 (−14.44–29.72)	21.72 (4.83–36.94)
Temperature (°C) TB Area	−2.75 (−27.50–12.80)	−0.35 (−22.20–28.80)	19.55 (0.70–35.80)
Precipitation (mm)	1.71 (0.00–20.83)	0.91 (0.00–18.54)	3.13 (0.00–30.48)
Precipitation (mm) TB Area	1.49 (0.00–22.35)	0.78 (0.00–12.70)	2.02 (0.00–24.64)
Humidity (%)	85.92 (27.00–100.00)	66.07 (4.00–100.00)	62.44 (10.00–100.00)
Solar Radiation (W/m^2)	24.29 (0.00–347.00)	79.99 (0.00–1040.00)	149.95 (0.00–1170.00)
Evapotranspiration (mm)	0.03 (0.00–1.02)	0.18 (0.00–2.29)	0.38 (0.00–3.30)
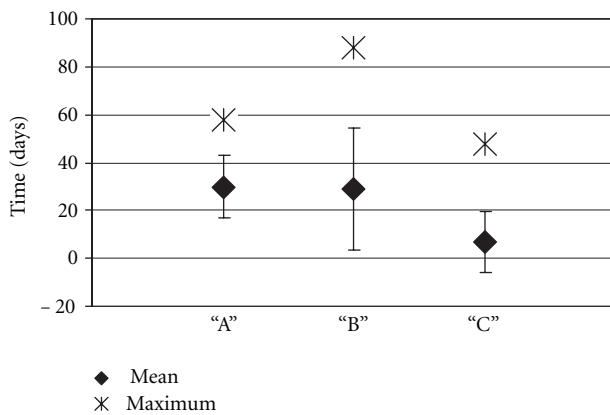
FIGURE 4: The overall mean and maximum survival time (persistence) of *M. bovis* in all substrates exposed to natural environmental conditions in Fall/Winter "A", Winter/Spring "B", and Spring/Summer "C". Error bars represent standard deviation.

contamination across all sampling periods ("A": $\chi^2 = 20.28$, $P < .01$; "B": $\chi^2 = 10.29$, $P < .01$; "C": $\chi^2 = 4.7$, $P < .05$).

3.2. Environmental Conditions. The coldest sampling period was the Fall/Winter "A", Winter/Spring "B" was intermediate, and Spring/Summer "C" was the warmest with the greatest amount of precipitation, highest solar radiation, and greatest degree of evapotranspiration (Table 1). Daily average temperatures and daily average rainfall recorded at the Michigan Automated Weather Network weather station in Hawk City, Presque Isle Country, Mich, USA in the endemic bovine TB area over the same time periods revealed similar trends in temperature patterns (highs and lows) and similar patterns of precipitation. As expected, the average daily temperature at the northern weather station in the bovine TB endemic region was approximately 3 degrees lower across all sampling periods (Table 1).

3.3. Persistence of M. bovis in the Environment. M. bovis persisted in substrates exposed to environmental conditions for an average of one month in cool Fall/Winter and Winter/Spring conditions and for an average of 7 days in warmer Spring/Summer conditions (Table 2). Both the time from inoculation to the last positive *M. bovis* sample and
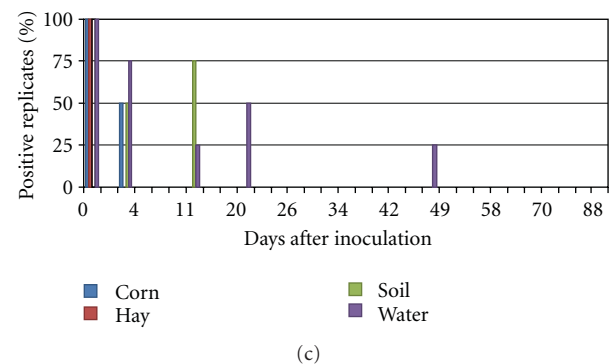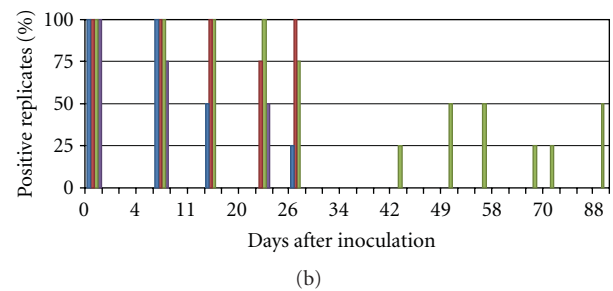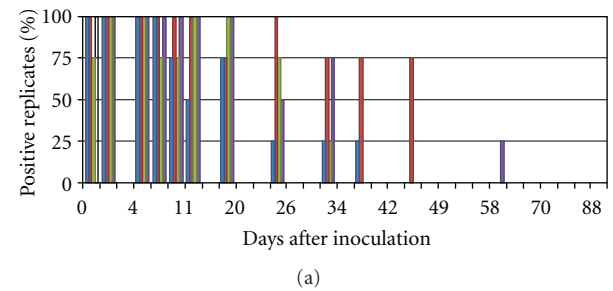
FIGURE 5: The percent ($n = 4$ of each substrate) of *M. bovis* positive replicates of corn, hay, soil, and water at each sampling point in Fall/Winter (a), Winter/Spring (b), and Spring/Summer (c).

the time from inoculation to first negative after last positive *M. bovis* sample are presented. The average and maximum survival times were lowest in the Spring/Summer sampling period "C". The maximum survival time across all substrate types and sampling seasons was recorded in a soil sample

TABLE 2: Duration of *M. bovis* persistence in the environment on corn, hay, water and soil samples in season "A", "B" and "C".

	Days to last positive		Days to 1st negative after last positive	
	Mean (standard deviation)	Maximum (days)	Mean (standard deviation)	Maximum (days)
Fall/Winter "A"				
Corn	24.00 (11.75)	37.00	29.75 (13.94)	43.00
Hay	41.50 (3.00)*	43.00	54.25 (7.5)*	58.00
Soil	21.75 (5.32)	28.00	28.75 (6.18)*	37.00
Water	32.25 (18.23)	58.00	38.50 (14.80)	58.00
All samples	29.88 (12.88)	58.00	37.81 (14.59)	58.00
Winter/Spring "B"				
Corn	15.25 (7.89)*	26.00	24.50 (12.12)*	42.00
Hay	26.00 (0.00)	26.00	42.00 (0.00)	42.00
Soil	62.75 (30.63)*	88.00	68.25 (23.41)*	88.00
Water	12.25 (10.50)*	21.00	18.25 (9.39)*	26.00
All samples	29.06 (25.56)	88.00	38.25 (23.61)	88.00
Spring/Summer "C"				
Corn	1.50 (1.73)	3.00	7.00 (4.62)	11.00
Hay	0.00 (0.00)	0.00	3.00 (0.00)	3.00
Soil	8.25 (5.50)	11.00	15.75 (8.50)	20.00
Water	17.75 (22.01)*	48.00	23.50 (22.05)*	53.00
All samples	6.88 (12.48)	48.00	12.31 (13.53)	53.00

* Significant difference in means (ANOVA) among substrate type Bonfferoni $P \leq .05$.

in the Winter/Spring "B" period. The soil sample was *M. bovis* positive at the final sampling point of 88 days. The shortest survival period was recorded in a hay sample in the Spring/Summer "C" period. The sample was positive at time 0 but negative at the 1st sampling point at 3 days. The mean and maximum survival time in each substrate type in each season is presented in Figure 3. The overall mean and maximum survival time across all substrate types in each season is presented in Figure 4.

The number of *M. bovis* positive replicate samples, and the number of *M. bovis* colonies recovered per sample, drop off quickly over the first 7 to 14 days of exposure to environmental conditions. The isolation of *M. bovis* from substrates exposed to environmental conditions was more intermittent after 14 days and positive samples were often identified based on the isolation of less than 5 *M. bovis* colonies per $100\,\mu$L of sample. The percent of *M. bovis* positive replicates recorded at each sampling point and the number of colony forming units isolated are displayed in Figures 5 and 6.

3.4. Effects of Nonseasonal Factors on the Persistence of M. bovis in the Environment.

The effect of substrate type on the persistence of *M. bovis* in the environment was variable. In the Spring/Summer period "C" survival was significantly longer in water. In the Fall/Winter "A" period survival appeared to be significantly longer in hay, and in the Winter/Spring "B" period survival appeared to be significantly longer in soil. Survival probability curves for *M. bovis* in soil, corn, hay and water across all seasons are illustrated in Figure 7. The curves appear similar and log rank statistics confirm that the survival curves for the

different substrates types are not significantly different from one another ($\chi^2 = 5.03$, $P = .17$). Among all seasons there is no significant difference in the survival of *M. bovis* in one sample type versus another.

No significant associations were found between the bovine TB status of a sample and whether or not it was sterilized before *M. bovis* inoculation. The placement of the inoculated samples in shade or direct sunlight did not have a statistically significant effect on *M. bovis* survival, but the mean survival time was longer across all samples and seasons in those placed under shade. The difference in mean survival time in the shaded and nonshaded samples approached significance in the Fall/Winter "A" and Spring/Summer "C" seasons (Table 3).

3.5. Effects of Season on M. bovis Persistence in the Environment.

Figure 8 illustrates the survival probability curves of *M. bovis* organisms exposed to environmental conditions in Fall/Winter "A", Winter/Spring "B", and Spring/Summer "C". The log rank statistics were associated with highly significant differences (chi-square = 19.88, $P < .0001$) for between season probabilities. An analysis of maximum likelihood estimates when other covariates (shade/nonshade, substrate type, sterilized/nonsterilized substrates, and interaction between shade/nonshade and season) were added to the model makes it clear that it is the season that drives the difference in the survival probability (Table 4).

3.6. Effects of Weather on M. bovis Persistence in the Environment Across Seasons.

The Cox's proportional hazard regression model, used to determine the relative influence of various weather related factors that together contribute to

TABLE 3: Mean duration in days of *M. bovis* persistence in the environment on corn, hay, water and soil samples in season "A", "B", and "C" in shaded and nonshaded conditions.

	Shade mean (Std. Dev.)	No shade mean (Std. Dev.)	*t* statistic	*P*-value
Fall/Winter "A"	12.79 (12.27)	9.58 (9.38)	−1.68	.10
Winter/Spring "B"	18.40 (20.65)	17.41 (20.48)	−0.20	.84
Spring/Summer "C"	7.18 (12.52)	2.38 (4.03)	−1.48	.15

TABLE 4: Cox's proportional hazard regression model for persistence of *M. bovis* over three different sampling periods (Fall/Winter "A", Winter/Spring "B" and Spring/Summer "C") or seasons.

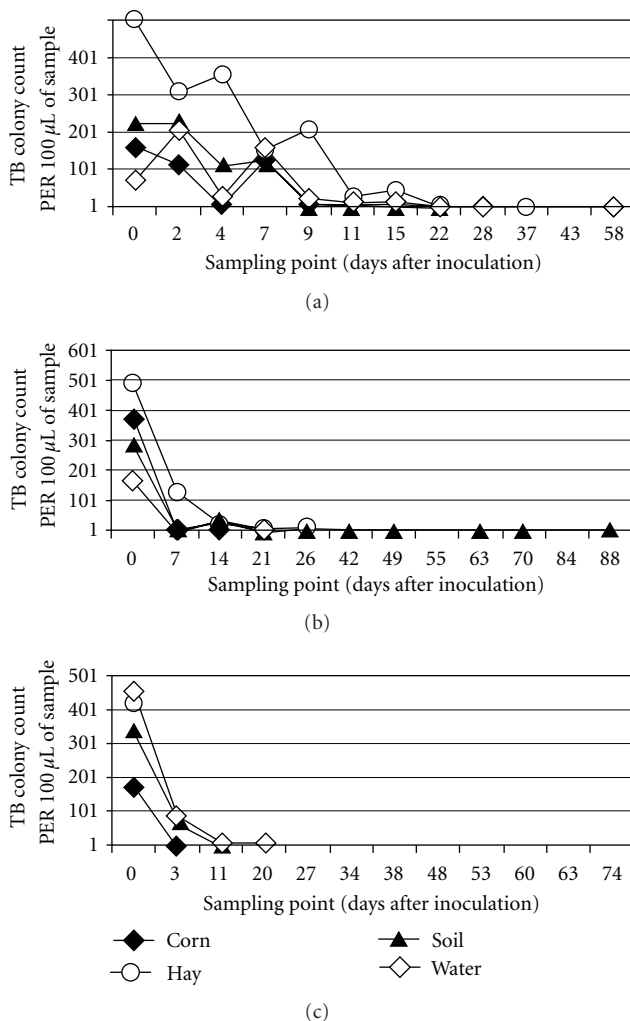| Risk factor	Parameter estimate	Standard error	Hazard ratio (95% confidence interval)
Season A	−2.13	0.49	0.12 (0.05–0.32)
Season B	−1.94	0.49	0.14 (0.5–0.38)
Season C	0	—	—
Sample Type	−0.28	0.16	0.76 (0.55–1.05)
Sterilized	−0.50	0.35	0.61 (0.31–1.20)
Shade	−0.68	0.33	0.51 (0.26–1.05)

FIGURE 6: The average number of *M. bovis* colony forming units isolated on solid media from $100\,\mu$L of processed sample (corn, hay, soil and water) over time in Fall/Winter (a), Winter/Spring (b), and Spring/Summer (c).

seasonal differences in the environmental persistence of *M. bovis*, revealed that temperature is the most influential factor in *M. bovis* survival. A number of the weather-related factors recorded throughout the sampling periods were significantly associated with the survival of *M. bovis* in the environment, however, many of these factors correlated with one another. The univariable hazard ratios and 95% confidence intervals for the weather-related factors tested are presented in Table 5. Although all of these variables were significant at the $P < .15$ level, evapotranspiration and solar radiation were removed due to redundancy. The final multivariable Cox proportional hazard regression model is presented in Table 6.

4. Discussion

Mycobacterium bovis is an obligate intracellular pathogen but it has been shown to survive in the environment, outside a host, for substantial periods of time under favorable conditions [11–15, 18–24]. Although all of these studies demonstrated the persistence of *M. bovis* in the environment, their experimental nature, the use of high bacterial loads, and the variability of results have generally led to the conclusion that the environmental persistence of *M. bovis* does not play a significant role in the epidemiology of bovine TB through the indirect transmission of the pathogen among or between susceptible species.

The outbreak of bovine TB in northeast Michigan, and the establishment of a wildlife reservoir (white-tailed deer) for *M. bovis* in the region, has renewed interest in the characterization of *M. bovis* persistence in the environment and its role in the epidemiology of bovine TB in North America. This study clearly demonstrates that the Michigan strain of *M. bovis* persists in the environment under typical Michigan weather conditions. The study mimicked, to the greatest degree possible, the natural conditions under which *M. bovis* would be deposited on substrates in the environment and the weather to which the organisms would be exposed. Since

TABLE 5: Univariable analysis of the influence of each weather related variable on the hazard (survival) of *M. bovis* in the environmental using the score test in Cox regression.

Weather factor (average)	Score Chi-square	*P*-value
Solar Radiation	7.50	.0062
Humidity	2.46	.1165
Temperature	9.85	.0017
Precipitation	5.44	.0197
Evapotranspiration	17.19	<.0001

TABLE 6: Multivariable Cox proportional hazards model of weather related factors associated with the survival of *M. bovis* in the environment.

Risk factor	Parameter estimate	Standard error	Hazard ratio (95% confidence interval)
Temperature*	0.06	0.02	1.06 (1.02–1.10)

* Results of purposeful selection of covariate and modified stepwise modeling in Cox regression. Only temperature remains in the model.
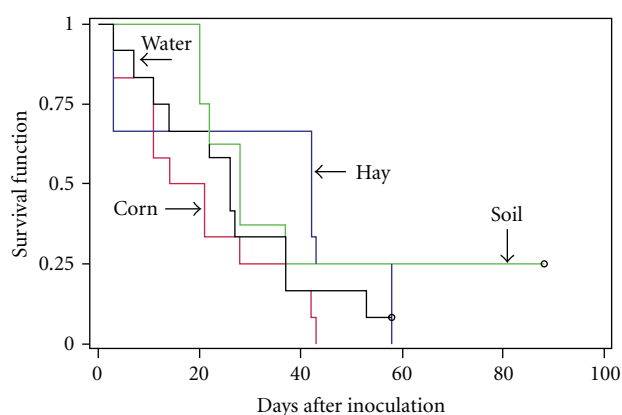
FIGURE 7: Survival probabilities of *M. bovis* in water, corn, hay, and soil across all seasons tested.
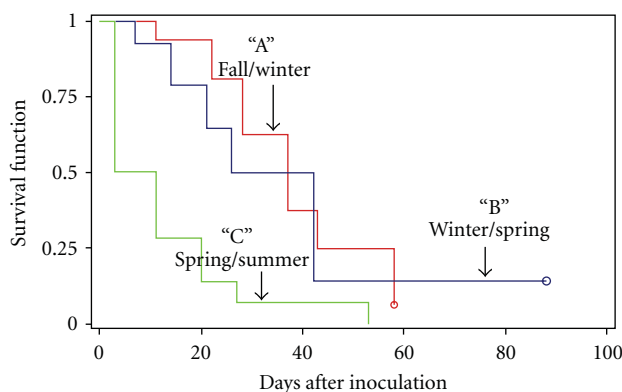
FIGURE 8: Survival probabilities of *M. bovis* exposed to natural environmental conditions in "A", "B", and "C" sampling periods.

the study site was approximately 200 miles to the south of the endemic bovine TB area in Michigan, basic indicators of environmental conditions (average daily temperature and precipitation) were compared between the two areas by using data from a weather station in the Michigan Automated Weather Network located in Hawk City, Presque Isle County. Daily temperature changes and precipitation at the study site

and the northern Michigan weather station were graphed and compared visually. The temperature cycles (periods of high and low temperatures) and patterns of precipitation recorded at the northern Michigan weather station mimicked those observed at the study site, however, the temperature in the northern TB endemic area was on average of 3°C cooler throughout the sampling periods. These slightly cooler temperatures in the north could be expected to support slightly longer persistence of *M. bovis* in the environment.

4.1. The Persistence of M. bovis in the Environment. The general pattern of *M. bovis* persistence in the environment, across all seasons and in all substrate types, was an initial decline in the numbers of recoverable bacilli over a 7-to-14-day period and then the persistence of small numbers of *M. bovis* bacilli for up to 4 to 12 weeks. The persistence of the Michigan strain of *M. bovis* on corn, hay, soil, and water recorded in this natural environmental exposure study confirms the findings of laboratory-based studies conducted earlier in which the Michigan strain of *M. bovis* was found to persist for up to 12 weeks on feed (hay, corn, sugar beets, apples, carrots, and potatoes) stored at 46°F and 0°F and for shorter periods when stored at 75°F [25]. The similarity between the findings of the laboratory-based studies on *M. bovis* persistence and this one, in which the experimentally infected substrates were exposed to natural environmental conditions, indicates that failure to detect *M. bovis* in the environment in known areas of *M. bovis* transmission is likely associated with the highly clustered spatial distribution of contaminated substrates under natural conditions and the inability to pinpoint the exact location of a *M. bovis* contaminated substrate for sampling, and not the inability of *M. bovis* to survive in the environment in a viable state.

The results of early studies performed in Europe and designed to characterize the persistence of *M. bovis* in the environment, have been scrutinized due to the large numbers of *M. bovis* bacilli used to experimentally inoculate of substrates [18–21]. In this study the initial bacterial load used to inoculate the substrates was 50,000 CFU of *M. bovis*. This amount of *M. bovis* is more than the minimum infective oral dose of *M. bovis* established through experimental studies

with the Michigan strain of *M. bovis* in both cattle (5,000 CFU) and white-tailed deer (300 CFU) [26, 27]. The 50,000 CFU inoculums used is thought to be indicative of the amount of *M. bovis* that could be deposited by a bovine TB-infected and shedding animal. The 50,000 CFU inoculum is smaller than the number of *M. bovis* bacilli recorded in the exudates from a lesion in an infected brush-tailed possum in New Zealand (5×10^8 CFU/mL) [28] but larger than number of bacilli (approximately 70 CFU) detected in the nasal mucous of an experimentally infected cow [29]. The amount of *M. bovis* used to inoculate substrates is believed to be relevant both in terms of the probability of contamination of environmental substrates through shedding of *M. bovis* from an infected animal and the likelihood of ingestion of *M. bovis* bacilli present in the environment by a susceptible host. This is particularly true in the 1st 7 days of exposure of *M. bovis* to environmental conditions when the number of CFUs recovered from the experimentally inoculated samples remained high.

4.2. Factors Influencing the Persistence of M. bovis in the Environment. This study was designed to characterize the persistence of *M. bovis* in a number of substrates exposed to natural environmental conditions over a 12-month period. A review of the literature indicated the necessity to supplement the 12-month study with a number of seasonal experiments designed to capture the effects on the persistence of *M. bovis* under different weather conditions. The persistence of *M. bovis* during the Michigan Spring/Summer season (May 20th to August 2nd) was significantly shorter than the persistence recorded in the Fall/Winter season (November 8th to January 6th) and Winter/Spring season (February 4 to May 3). The Spring/Summer season was associated with higher average daily temperatures, greater intensity of solar radiation, and higher loss of moisture through evapotranspiration. These findings are in agreement with those reported elsewhere in which an increase in temperature and a loss of moisture were found to be associated with a decrease in the persistence of *M. bovis* in the environment [12–14, 22].

Other factors, including substrate type, did not significantly affect the pattern of *M. bovis* persistence. The detection of *M. bovis* in a soil sample at 88 days (the final sampling point) in the Winter/Spring sampling period is in agreement with other studies that recorded the longest survival of *M. bovis* in cool, moist soil that presumably protects the bacilli from desiccation and provides an organic environment that supports its persistence [12, 19, 20]. Detection of *M. bovis* in a water sample at 48 days in the Spring/Summer sampling period, in which all other substrate types were negative at 20 days, indicates that even in the presence of high temperatures and intense solar radiation, viable *M. bovis* can persist under moist conditions.

The sterilization of substrates before *M. bovis* inoculation did not affect the persistence of *M. bovis,* but it did significantly decrease the occurrence of contaminated bacterial cultures. Presterilized substrates were not used exclusively in this study because decreased survival of *M. bovis* in sterile substrates has been reported [12, 15].

The location of the *M. bovis* inoculated substrates under shade had an effect on persistence. The mean survival time was longer for samples under shade than those exposed to direct sunlight. This was true in the Fall/Winter season and the Spring/Summer season but not in the Winter/Spring season. This apparent lack of a protective effect of shade during the Winter/Spring season is likely due to the fact that the cloth used to cover the "shaded" samples during this sampling period was severely damaged by wind and removed. It was replaced before the final Spring/Summer sampling period.

Survival analysis was used to evaluate the impact of weather over the three seasons on the persistence of *M. bovis* in the environment. Univariate analysis using Cox's proportional hazard regression indicated that the survival probability or persistence of *M. bovis* was decreased as temperatures increased, solar radiation intensified, and evapotranspiration (a measure of moisture loss from a system) increased. The effects of average precipitation and percent humidity lost their significance in the presence of temperature. Since solar radiation and evapotranspiration are both directly related to temperature, the final conclusion was that temperature drives the seasonal effect seen in *M. bovis* persistence in the environment.

4.3. Implications of the Persistence of M. bovis in the Environment. The evidence that viable *M. bovis* persists in the Michigan environment under near natural conditions has significant implications on the efforts to control and eventually eliminate the occurrence of bovine TB in the region. Authors have argued when examining other systems of bovine TB in other parts of the world that the conditions that contribute to *M. bovis* persistence in the environment also make the organisms inaccessible to susceptible hosts [13, 30]. This is not the case in Michigan. The types of substrates tested (soil, water, hay, and corn) are present in and around cattle farms in northeast Michigan. Additionally, white-tailed deer have access to these substrates on many cattle operations. Although many feed piles are exposed to sunlight and Summer temperatures, there are periods of the year throughout the region in which low temperatures, cloud cover, and the location of feed and water sources under the cover of a forest canopy or otherwise protected from the elements, would facilitate the long-term (4–12 weeks) survival of *M. bovis* bacilli deposited by a bovine TB-infected animal.

The elimination of feeding and baiting sites for white-tailed deer and other wildlife should remain a component of the efforts to reduce bovine TB prevalence in this population and part of the management recommendations designed to reduce deer-to-deer bovine TB transmission events. Bovine TB eradication programs designed to eliminate the occurrence of bovine TB in cattle must consider *M. bovis* contaminated feed or water as a possible route for the indirect transmission of bovine TB between infected white-tailed deer and cattle. Farm biosecurity measures focused on the elimination of the possibilities of cross contamination of feed and water sources should be added to the current protocols designed to eliminate cattle-to-cattle and the direct

transmission of bovine TB. Specifically, cattle should be fenced out of open water sources, and they should be provided an alternative source of water. Cattle should not be fed hay on the ground in the woods or in pasture adjacent to wooded areas and crops fields known to attract white-tailed deer. The programs designed to encourage the fencing of feed storage areas should continue, but emphasis should also be placed on the fencing and protection of cattle feeding areas.

5. Conclusions

The data clearly indicate that there is a real potential for the indirect transmission of *M. bovis* among and between cattle and white-tailed deer populations in Michigan. Persistence of *M. bovis* can be expected to be longer in cooler seasons. Practices that facilitate the cross contamination of substrates by infected and susceptible animals should be restricted at all times but especially during the cooler seasons of the year.

Failure to isolate *M. bovis* from environmental substrates collected from areas with known bovine TB transmission, is likely due to the inability to pinpoint the exact location of environmental contamination for sampling and less to do with the persistence of the *M. bovis* bacilli in the environment. Difficulties in isolating viable *M. bovis* from environmental substrates due to the limitations of sample processing and mycobacterial culture will also continue to hinder the accurate assessment of the quantity of viable *M. bovis* organisms in the environment. However, this study indicates that the organisms do persist over a time period that would allow a susceptible animal to become exposed and infected with *M. bovis* from an environmental source. Indirect transmission of *M. bovis* plays a role in the interspecies transmission of bovine TB and will continue to hinder programs designed to eliminate the disease if not addressed.

The recorded persistence of *M. bovis* in the Michigan environment under natural weather conditions strongly suggests the potential contribution of indirect means to the transmission of bovine TB in the region. These data supplement those produced through experimental *M. bovis* disease transmission studies that have proven the feasibility of indirect transmission of *M. bovis* among and between cattle and white-tailed deer. They also support the analyses of observational data on *M. bovis* infection in cattle and white-tailed deer in Michigan that indicate the importance of indirect transmission in the interspecies transmission of *M. bovis* in the region.

Acknowledgments

References

[1] D. J. O'Brien, S. M. Schmitt, S. D. Fitzgerald, D. E. Berry, and G. J. Hickling, "Managing the wildlife reservoir of *Mycobacterium bovis*: the Michigan, USA, experience," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 313–323, 2006.

[2] S. M. Schmitt, S. D. Fitzgerald, T. M. Cooley et al., "Bovine tuberculosis in free-ranging white-tailed deer from Michigan," *Journal of Wildlife Diseases*, vol. 33, no. 4, pp. 749–758, 1997.

[3] D. L. Whipple, J. L. Jarnagin, and J. B. Payeur, "DNA fingerprinting of *Mycobacterium bovis* isolates from animals in northeast Michigan," in *Proceedings of the IX International Symposium of the World Association of Veterinary Laboratory Diagnosticians, World Association of Veterinary Laboratory Diagnosticians*, College Station, Tex, USA, 1999.

[4] T. J. DeLiberto, K. C. Vercauteren, and G. W. Witmer, "The ecology of *Mycobacterium bovis* in Michigan," in *Activities Report and Conference Proceedings, Michigan Bovine Tuberculosis Eradication Project*, Lansing, Mich, USA, 2004.

[5] G. Frye, "Bovine tuberculosis eradication: the program in the United States," in Mycobacterium bovis *Infection in Animals and Humans*, C. O. Thoen and J. H. Steele, Eds., pp. 119–129, Iowa State University Press, Ames, Iowa, USA, 1995.

[6] G. J. Hickling, "Dynamics of bovine tuberculosis in wild white-tailed deer in Michigan," Wildlife Division Report No. 3363, Michigan Department of Natural Resources, 2002.

[7] R. Miller, J. B. Kaneene, S. D. Fitzgerald, and S. M. Schmitt, "Evaluation of the influence of supplemental feeding of white-tailed deer (*Odocoileus virginianus*) on the prevalence of *bovine tuberculosis* in the Michigan wild deer population," *Journal of Wildlife Diseases*, vol. 39, no. 1, pp. 84–95, 2003.

[8] A. E. Fine, D. J. O'Brien, S. R. Winterstein, and J. B. Kaneene, "An effort to isolate *Mycobacterium bovis* from environmental substrates during investigations of bovine tuberculosis transmission sites (cattle farms and wildlife areas) in Michigan, USA," submitted.

[9] G. Witmer, A. E. Fine, J. Glonfriddo et al., "Epizootiologic survey of *Mycobacterium bovis* in wildlife and farm environments in northern Michigan," *Journal of Wildlife Diseases*, vol. 46, no. 2, pp. 368–378, 2010.

[10] J. B. Kaneene, C. S. Bruning-Fann, L. M. Granger, R. Miller, and B. A. Porter-Spalding, "Environmental and farm management factors associated with tuberculosis on cattle farms in northeastern Michigan," *Journal of the American Veterinary Medical Association*, vol. 221, no. 6, pp. 837–842, 2002.

[11] T. W. A. Little, P. F. Naylor, and J. W. Wilesmith, "Laboratory study of *Mycobacterium bovis* infection in badgers and calves," *Veterinary Record*, vol. 111, no. 24, pp. 550–557, 1982.

[12] B. J. Duffield and D. A. Young, "Survival of *Mycobacterium bovis* in defined environmental conditions," *Veterinary Microbiology*, vol. 10, no. 2, pp. 193–197, 1984.

[13] R. Jackson, G. W. de Lisle, and R. S. Morris, "A study of the environmental survival of *Mycobacterium bovis* on a farm in New Zealand," *New Zealand Veterinary Journal*, vol. 43, pp. 346–352, 1995.

[14] M. Tanner and A. L. Michel, "Investigation of the viability of *M. bovis* under different environmental conditions in the Kruger National Park," *Onderstepoort Journal of Veterinary Research*, vol. 66, no. 3, pp. 185–190, 1999.

[15] J. S. Young, E. Gormley, and E. M. H. Wellington, "Molecular detection of *Mycobacterium bovis* and *Mycobacterium bovis* BCG (Pasteur) in soil," *Applied and Environmental Microbiology*, vol. 71, no. 4, pp. 1946–1952, 2005.

[16] A. E. Fine, *The role of indirect transmission in the epidemiology of bovine tuberculosis in cattle and white-tailed deer in Michigan*, Ph.D. dissertation, Michigan State University, East Lansing, Mich, USA, 2006, p. 157.

[17] P. T. Kent and G. P. Kubica, *Public Health Mycobacteriology: A Guide for Level III Laboratory*, U.S. Department of Health and Human Services, Centers for Disease Control, Public Health Service, Atlanta, Ga, USA, 1985.

[18] S. R. Williams and W. A. Hoy, "The viability of *B. tuberculosis* (bovinus) on pasture land, in stored faeces and in liquid manure," *Journal of Hygiene*, vol. 30, pp. 413–419, 1930.

[19] E. C. G. Maddock, "Studies on the survival time of the bovine tubercle bacillus in soil, soil and dung, in dung and on grass, with experiments on the preliminary treatment of infected organic matter and the cultivation of the organism," *Journal of Hygiene*, vol. 33, pp. 103–117, 1933.

[20] E. C. G. Maddock, "Further studies on the survival time of the bovine tubercle bacillus in soil, soil and dung, in dung and on grass, with experiments on feeding guinea-pigs and calves on grass artificially infected with bovine tubercle bacilli," *Journal of Hygiene*, vol. 34, pp. 372–379, 1934.

[21] E. C. G. Maddock, "Experiments on the infectivity for healthy calves on bovine tubercle bacilli discharged in dung upon pasture. Part 1. From tubercular calves fed with emulsion of tubercle bacilli 1934–1935. Part 2. From tubercular cows passing tubercle bacilli in their dung 1935–1936," *Journal of Hygiene*, vol. 36, pp. 594–601, 1936.

[22] C. Wray, "Survival and spread of pathogenic bacteria of veterinary importance within the environment," *Veterinary Bulletin*, vol. 45, pp. 543–550, 1975.

[23] D. J. van Donsel and E. P. Larkin, "Persistence of *Mycobacterium bovis* BCG in soil and on vegetables spray-irrigated with Sewage effluent and sludge," *Journal of Food Protection*, vol. 40, no. 3, pp. 160–163, 1977.

[24] E. Mitscherlich and E. H. Marth, "Mycobacterium bovis," in *Microbial Survival in the Environment: Bacteria and Rickettsiae Important in Human and Animal Health*, pp. 235–242, Springer, Berlin, Germany, 1984.

[25] M. V. Palmer and D. L. Whipple, "Survival of *Mycobacterium bovis* on feedstuffs commonly used as supplemental feed for white-tailed deer (*Odocoileus virginianus*)," *Journal of Wildlife Diseases*, vol. 42, no. 4, pp. 853–858, 2006.

[26] M. V. Palmer, W. R. Waters, and D. L. Whipple, "Lesion development in white-tailed deer (*Odocoileus virginianus*) experimentally infected with *Mycobacterium bovis*," *Veterinary Pathology*, vol. 39, no. 3, pp. 334–340, 2002.

[27] M. V. Palmer, W. R. Waters, and D. L. Whipple, "Investigation of the transmission of *Mycobacterium bovis* from deer to cattle through indirect contact," *American Journal of Veterinary Research*, vol. 65, no. 11, pp. 1483–1489, 2004.

[28] B. L. Smith, "Tuberculosis in the opossum," *New Zealand Veterinary Journal*, vol. 20, p. 199, 1972.

[29] T. McCorry, A. O. Whelan, M. D. Welsh et al., "Shedding of *Mycobacterium bovis* in the nasal mucus of cattle infected experimentally with tuberculosis by the intranasal and intra-tracheal routes," *Veterinary Record*, vol. 157, no. 20, pp. 613–618, 2005.

[30] R. S. Morris, D. U. Pfeiffer, and R. Jackson, "The epidemiology of *Mycobacterium bovis* infections," *Veterinary Microbiology*, vol. 40, no. 1-2, pp. 153–177, 1994.

Research Article

Leaching of *Mycobacterium avium* subsp. *paratuberculosis* in Soil under *In Vitro* Conditions

Eran A. Raizman,[1] Mussie Y. Habteselassie,[2] Ching C. Wu,[1] Tsang L. Lin,[1] M. Negron,[1] and Ronald F. Turco[3]

[1] *Department of Comparative Pathobiology School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907, USA*
[2] *Department of Crop and Soil Sciences, University of Georgia, Griffin, GA 30223, USA*
[3] *Agronomy Department, College of Agriculture, Purdue University, West Lafayette, IN 47907, USA*

Correspondence should be addressed to Eran A. Raizman, eraizman@gmail.com

Mycobacterium avium subsp *paratuberculosis* (Map), the causative agent of Johne's disease, has a robust ability to survive in the environment. However, the ability of Map to migrate through soil to drainage tiles or ground water, leave the farm, and leak into local watersheds is inadequately documented. In order to assess the ability of Map to leach through soil, two laboratory experiments were conducted. In the first study, 8 columns (30 cm long each) of a sandy loam soil were treated with pure cultures of Map. Two soil moisture levels and two Map concentrations were used. The columns were leached with 500 mL of water once a week for three weeks, the leachate was collected, and detection analysis was conducted. In the second experiment, manure from Map negative cows (control) and Map high shedder cows (treatment) were deposited on 8 similar columns and the columns were leached with 500 mL of water once a week for four weeks. Map detection and numeration in leachate samples were done with RT-PCR and culture techniques, respectively. Using RT-PCR, Map could be detected in the leachates in both experiments for several weeks but could only be recovered using culture techniques in experiment one. Combined, these experiments indicate the potential for Map to move through soil as a result of rainfall or irrigation following application.

1. Introduction

Mycobacterium avium subsp *paratuberculosis* (Map), the causative agent of Johne's disease (JD) in domestic and wild ruminants is known to be ubiquitous in the dairy farm environment [1]. Additionally, Map has long been implicated as a possible cause of Cohn's disease in humans [2]; however, this is still strongly debatable. Several reports indicate that Map will survive for long periods of time under various *in vitro* physical and environmental conditions, controlled by the amount of water, urine, manure, and temperatures [3–5]. Results suggest that long-term survival could occur near shaded animal management locations and locations that receive frequent introductions of manure [6]. Using dam water and sediment columns, one study [6] showed that Map could survive up to 48 and 36 weeks in shade or semiexposed location, respectively, and survival in sediment

was 12 to 26 weeks longer than survival in water columns. In a Map-inoculated liquid manure slurry stored under anaerobic conditions, Map was shown to survive 252 days at 5°C and 98 days at 15°C [7]. One study [8] found that Map could survive up to 175 days in Map-inoculated liquid manure storage. A large study on 108 Minnesota dairy farms [1] found that a predictable location to recover Map isolates was in alleyways and manure storage areas. Similar results were obtained in another study, which used approximately 100 dairies across the USA [9]. This is important since land-spreading of bovine manure is a common practice on many dairy operations [10, 11]. Also, the survival of Map in the soil may be influenced by the type of soil present [11] as was established for other bacteria [12, 13]. The potential of Map to leach through soil to enter a farm from the environment or enter a local watershed is unknown. A recent study investigated the processes controlling Map transport through

aquifer material and found that, compared to other bacteria, Map transport in the soil is lower [14]. Another factor that remains unknown is the ability of Map to survive during the leaching process. Only recently, Map absorption in soil particles was studied and it was found that of the organism added to the columns 83% were estimated to be retained in chromatography columns packed with clay and silica soils [15]. Cho et al. [16] showed that fecal bacteria can migrate into the subsurface and cause significant contamination of vadose systems (subsurface soil) especially when manures are applied repeatedly. For other enteric bacteria it was shown [17] that bacterial leaching to tile-drains could exceed 71,000 organisms $100\,mL^{-1}$ when driven with high rates of water infiltration. The objective of this study was to assess the potential of Map to leach through columns of soil under laboratory conditions. Our hypothesis was that Map has the ability to leach through a soil column when introduced as pure culture inoculum or via feces.

2. Material and Methods

2.1. Soil. The soil type used in the study was Tracy (Coarse-loamy, mixed, active, mesic Ultic Hapludalfs), collected from the Pinney-Purdue Agriculture Center located on the county line between Porter and LaPorte counties, Indiana, USA. We used this soil because it is the most common soil type in the US Midwest. An aggregate sample was generated by combining soil from ten separate locations across the field. The soil sample was obtained from area where there is no livestock production, sieved (4 mm) and stored in closed plastic bags at room temperature until use. Soil parameters for the Tracy soil includes pH 5.5, 1.5% organic matter, 6 ppm Na, 103 ppm NO_3^-–N, 72 ppm P (Bray I Olsen), 155 ppm K, 148 ppm Mg, 787 ppm Ca, 9 ppm SO_4^{2-}S, and 2.9 ppm Zn. Soil analysis was performed by Harris Laboratory (Lincoln, NE). Prior to packing the columns, the soil was passed through a 2 mm sieve. The soil moisture content was determined by drying subsamples of the soil at 105°C for 24 hours and determining its water loss gravimetrically.

2.2. Soil Columns Setup. The bottom of PVC tubes (40 cm length × 8 cm diameter) was covered with a wire mesh and cheese cloth (Figure 1). The lower 30 cm of the tubes was marked and enough soil added to provide a final bulk density of $1\,gm\,cm^{-3}$ based on the mass of soil put into a specific volume of the column. In order to assess the impact of the initial soil moisture content on Map movement during leaching, the soil was adjusted to two moisture levels: −0.1 or −0.03 MPa (14 and 15.2%, resp.), before it was placed into the columns. These two soil moisture suctions were chosen because they bracket the range of optimal water potential in soil for microbial processes [18]. The soil was brought to the two moisture levels by adding water to the soil slowly in a dropwise fashion while mixing to achieve as uniform distribution as possible [19, 20].

2.3. Preparation of Map Inoculum. Map inoculums were prepared from fecal sample of naturally infected cattle pre-
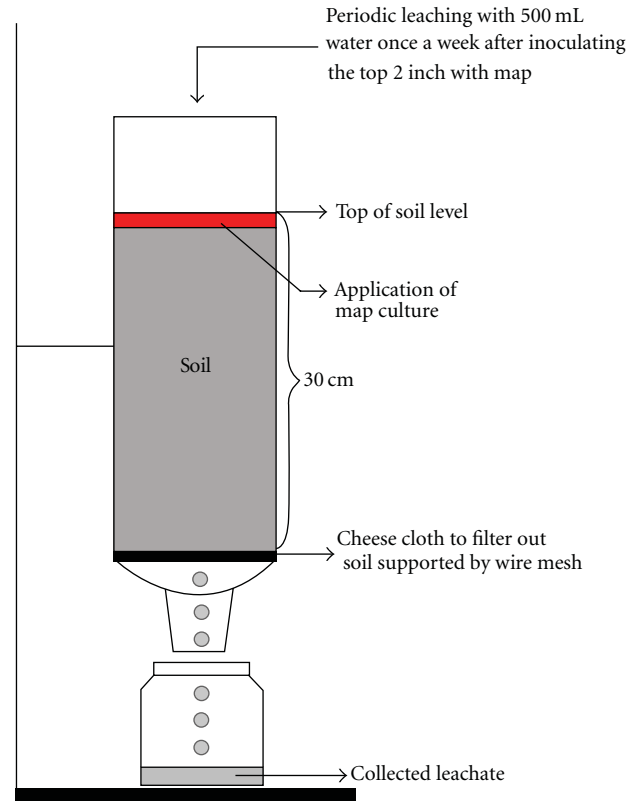
FIGURE 1: A column setup for leaching study of *Mycobacterium avium* subsp *paratuberculosis* in Tracy soil from northwest Indiana.

viously confirmed to be positive based on serum ELISA and liquid fecal cultures (procedures described by Whitlcok and Rosenberg, 1990) [21]. The liquid culture was incubated for up to 6 weeks and acid fast staining was applied to samples at 4.5 and 6 weeks. Map specific PCR testing was performed to the sample with positive acid fast result. Samples positive by Map specific PCR were reported as Map positive. Liquid culture positive samples were streaked on to Herold's Egg York agar to obtain isolated colonies and subsequently passed to liquid culture medium again to propagate to desired concentration and used as the stock inoculums.

2.4. Map Recovery from Columns. To mimic natural rainfall, each soil column received once a week 500 mL distilled and deionized water in a dropwise fashion to achieve a uniform distribution, which is equivalent approximately 150 mm of rain. Leachates from each column were collected into a 100 mL sterile bottle placed below the PVC columns. Twenty-four hours after water was added, the leachates volume was measured and centrifuged (7500 g; 10 minutes). The supernatant was poured out and the remaining sediment was resuspended in 1 mL sterile water for Map detection and enumeration.

2.5. Preculture Map Detection with PCR. The recovered pellet in 1 mL of sterile water was centrifuged at 2500 g for 10 minutes. The supernatant was removed and the pellet was used

for DNA extraction and Real Time PCR using Tetracore MAP extraction system and DNA test kit (VetAlert, Tetracore, Inc., Rockville, MD). The manufacture's procedures were followed.

2.6. Bacterial Culture. The TREK ESP liquid culture system (TREK Diagnostics Systems, Inc., Westlake, OH 44145) was used for the bacterial culture. The double incubation method as described previously [21] was used to prepare samples, with the modification that 1mL of sample replaced 2 g of feces. Samples were placed in 30 mL of sterile water, mixed and allowed to stand for 30 minutes. To reduce the number of other fast growing bacteria, five millilitres of the surface fluid were decontaminated with 25 mL of 0.9% hexadecylpyridinium chloride (Sigma-Aldrich, St Louis, MO) in half-strength brain heart infusion BHI broth and allowed to stand at 37°C for 24 hours After centrifugation at 900 g for 30 minutes, the pellet was resuspended in 1 mL of half-strength BHI broth with vancomycin (10 μg/mL; (Sigma-Aldrich, St Louis, MO), nalidixic acid (60 μg/mL; (Sigma-Aldrich, St Louis, MO), and amphotericin B (40 μg/mL; (Sigma-Aldrich, St Louis, MO) and incubated at 37°C for 24 hours [22]. Prepared soil was cultured using the ESP para-JEM culture bottles and incubated in the ESP machine (Trek Diagnostic Systems, Inc., Cleveland, OH). Samples were removed from the ESP machine at 4, 5, and 6 weeks and evaluated by acid-fast staining. This procedure was done to increase test specificity in order to ensure the presence of Map versus another acid-fast positive bacterium. Culture results were recorded as +, 2+, and 3+, which are equivalent to low, moderate, and high bacterial load (<10 colonies-per-tube, (CPT), 10–50 CPT, and >50CPT, resp.). Fecal culture sensitivity is estimated to be between 40 and 80% depending on the bacterial load. Using fecal culture, the test specificity is nearly 100%. Nevertheless, no such information is available on the test performance using soil leachates. Samples that were acid-fast positive were confirmed using IS*900* PCR to detect the IS*900* gene. DNA was extracted from ESP par a-JEM culture by the guanidine isothiocyanate-glass bead lysis method [23]. Alcohol-precipitated DNA extracts were resuspended in 40 μL of sterile water. The IS900 segment and primers used for amplification as well as the procedures performed for this PCR were reported previously [24–26]. Briefly, each 50 μL reaction contained 200 μM each of d ATP, dCTP, and dGTP; 1 μM dUTP; 3.0 mM Mg Cl$_2$; 10 mM Tris-HCl, pH 8.3; 50 mM KCL; 0.01% gelatine; 0.5 μM each of primers IS90/150C (5′-CCGCTAATTGAGAGATGC-GATTGG-3′) and IS900/921 (5′-AATCAACTCCAGCAG-CGCGGCCTCG-3′); 1U urail-*N*-glycosylase (UNG, Epicenter Technologies, Madison, WI); 2.5 *Taq* polymerase (PE Applied Biosystems, Foster City, CA); and 2 μL of DNA extract. In addition, each batch run included positive and template-negative controls, as well as UNG control (IS amplicons containing dU residues).

2.7. Experiment 1. In this experiment a total of 8 columns were used. Before the inoculation procedure soil samples from each column were cultured for Map and resulted neg-

ative. The pure culture strain of Map was introduced into the top 5 cm of eight of the columns. The cell suspension was uniformly applied to the top layer through injection by syringe. The cells were applied at two cell concentrations (10^8 and 10^7 cells mL^{-1}: 10 mL per tube). The soil columns were leached with 500 mL water as described above once a week for three weeks (Figure 1). The leachates were harvested as described above. The experiment was done in duplicates except for the control column.

2.8. Experiment 2. Feces from two cows known to be naturally infected with Map from previous testing were used for the Map-positive fecal inoculum. These samples were kept frozen (−70°C) after being collected rectally from the cows while a subset of the samples was cultured to assess bacterial load using a solid media as described elsewhere [27]. Results of this initial culture indicated that feces bacterial load was over >100 CFU/g. Once Map concentration was determined in the sample and soil columns were ready to be inoculated, the fecal samples were thawed at room temperature for 2 hours. Feces obtained from a fourth lactation cow known to be negative by repeated fecal culture and serum ELISA served as the negative fecal inoculums control specimen. These samples were frozen and thawed as described above.

2.9. Experiment Procedure. In this experiment we used eight PVC columns filled with 30 cm of Tracy soil packed to a bulk density of 1 gm cm^{-3} at a starting moisture content corresponding to −0.03 MPa. For the treatment group, 50 g of the manure was mixed well and evenly spread on the top 2 cm of soil of each of the 4 treatment columns (no. 5–8) in similar fashion to the control group. Similarly, 50 g of Map negative feces were evenly spread on top of the 4 control columns and mixed into the top 2 cm of soil to on farm mimic manure spreading. Twenty-four hours after the manure was deposit, columns were leached with 500 mL of distilled water as described above. Thereafter this procedure to harvest samples was repeated once a week for 7 weeks. Samples collected on weeks 0, 2, 4, 5, 6, and 7 were processed for Map detection. The rest of the procedure was the same as described for experiment one.

2.10. Statistical Analysis. Map data that are expressed as −, +, 2+, and 3+ in Table 1 were ranked as 1, 2, 3, and 4, respectively, for the purpose of statistical analysis. The ranks were then subject to a nonparameteric one way ANOVA analysis (Chi-Square and Wilcoxon tests) to investigate the statistical significance of the effect of antecedent soil moisture level, initial Map inoculum size, and time on the level of Map in the leachats. Leachate volume data were also subject to one-way ANOVA to test for statistical significance on the effect of the antecedent soil moisture level. All statistical analyses were done in SAS (2002-2003, SAS Institute, Inc., North Carolina) at significance level of $\alpha = 0.05$.

3. Results

3.1. Experiment 1. On average 322 mL and 345 mL leachates were collected from the soil columns with initial soil

TABLE 1: Result summary from Experiment 1 after application of a pure *Mycobacterium paratuberculosis* culture to the top of 30 cm soil column of sandy loam soil from Indiana, USA.

Moisture content	Initial noculumsize (cfu ml^{-1})	Week after bacterial inoculation					
		1		2		3	
		RT − PCR	Culture + IS900	RT − PCR	Culture + IS900	RT − PCR	Cultur + IS900
−0.1	10^8	+	−	−	−	+	−
−0.1	10^8	−	−	−	−	−	−
−0.1	10^7	−	−	−	−	−	−
−0.1	10^7	−	−	−	−	−	−
−0.03	10^8	−	−	−	−	+	−
−0.03	10^8	+	3+	−	−	+	−
−0.03	10^7	+	3+	−	−	−	−
−0.03	10^7	+	2+	+	−	+	−

Culture results were recorded as +, 2+, and 3+, which are equivalent to low, moderate, and high bacterial load on agar (<10 colonies-per-tube, (CPT), 10–50 CPT, and >50 CPT, resp.).

moisture content of −0.1 MPa and −0.03 MPa, respectively. The difference in leachat volume between the two treatments was not statistically significant ($P = .79$). The results of RT-PCR, bacterial culture, and PCR-IS900 show that Map was detected in the leachate samples collected from the treatment columns with both concentrations of Map (10^7 and 10^8 CFU (Table 1) over three weeks time. Week by week statistical analysis of the data indicated that the initial inoculum size did not have any significant effect on PCR and culture-based Map levels in the leachate (e.g., $P = 1.0$, week-1 PCR; $P = .3173$, week 2 PCR; and $P = .186$, week 3 PCR). The same was true for the antecedent soil moisture level except for week 1 in which the culture-based Map level in the leachate was significantly higher ($P = .045$) from the columns in which the antecedent soil moisture level was 0.03 MPa. Culture-based Map level decreased with time in the leachate, and this decrease was statistically significant ($P = .038$). The PCR-based Map levels, however, did not show any significant decrease over time ($P = .362$).

3.2. Experiment 2. On average 410 mL and 425 mL of leachate were collected from each of the control and the treatment soil columns, respectively, 24 hours after the columns were leached with 500 mL distilled water. This difference was not statistically significant but traceable to the fact that despite all efforts to maintain the same soil density in all columns, column no. 6 of the treatment group produced little leachate over the course of the study (on average <100 mL).

Results from RT-PCR indicate that immediately following fecal material application to the surface of the treatment columns, all of the leachates were negative for Map. At two weeks post application three of the four treatment columns were positive, but near the limits of detection which is 35 cycle threshold (Ct). The three columns produced Ct values of 34.9, 34.81, and 34.43. At four weeks post manure application, one column of the treatment group was positive (Ct = 34.31). At weeks five, six, and seven post treatment, all columns were RT-PCR negative. At all time points, when tested with the liquid media culture, no fecal Map bacteria

were detected in the leachate recovered from any of the eight columns.

4. Discussion

Contamination of food and water by microorganisms from animal manure has become a topic of concern in the last decade especially in regard to non-point-source manure contamination as a result of pastured animals or manure intentionally spread onto fields as fertilizer or waste [28]. The results of our first experiment using Map inoculum showed that it is possible to recover Map from soil columns that are leached with water. We, however, do not have an explanation why two samples from both moisture contents were negative on week two and positive on week 3. One possible explanation is that Map was retained in the soil and only after sufficient water was poured it could be washed to the bottom of the column. Map trends to clump and intermittent detection are not uncommon. Our second experiment assessed the leaching ability of Map which originated from a manure matrix that contains fecal bacteria and possibly other inhibitors such as organic and inorganic materials to migrate in the column. The results of this experiment indicated that with manure as a source of Map, only a few cells could leach through the 30 cm soil column. Nevertheless, we could not assess if these were viable cells. The authors are well aware that epidemiologically this is a crucial factor in terms of Map transmission whether to livestock or to human. Despite the fact that RT-PCR results were very close to the recommended cut-off value, we are confident that they were accurate because these results were constant over several sampling period and over 3 of the 4 treatment columns. Hexadecylpyridinium chloride (HPC), alone or in combination with the antibiotics vancomycin and natamycin, is used in the decontamination process during the sample preparation for culture [21]. Several studies reported that HPC decontamination resulted in a significant reduction in the number of culture-positive milk samples [12, 29]. It is possible that this process killed the few viable cells that were

shown on RT-PCR and hence culture results were negative. It was shown that the interaction of enteric bacteria with the soil environment can differ from that in the absence of manure components and this can affect the retention and transport through the soils [30]. Manure application, alters the organic and inorganic components of the soil, which have a great influence on the survival and mobility of bacteria in the soil [30]. Since the number of bacteria added via feces was lower than with the pure culture application, a comparison between the two methods is difficult. It is plausible, however, that the difference in Map leaching ability between the two experiments is partly due to the manure environment with its biotic and abiotic components. PCR test sensitivity and specificity have been previously compared to fecal culture, whether solid or liquid media [31–34]. In one study, however, test sensitivity was shown to be directly associated with the manure bacterial load [33]. Specifically to the PCR method used in the current study Alinovi et al. [34] assessed test sensitivity and specificity of 60% and 97%, respectively. In this initial study we have demonstrated that Map could be mobile in the environment and this finding suggests that a large-scale investigation is warranted. The possibility that Map can leach through soils to possibly enter groundwater or exit the system in tile water where it could enter the water supply used for human consumption is especially alarming in light of the considerable evidence that Map may be involved in the etiology and pathophysiology of Crohn's disease in humans [35]. It is important to consider that we only used one type of soil (Tracy), but this soil is the most common soil found near Indiana's dairy operations. Map's movement through soil may also be influenced by the physical, chemical, and biological properties of the soil as was established for other bacteria [12–15]. Further understanding of Map leaching and survival in different soil types will enable us to better explain the spatial cluster of Map seropositivity found to be associated with loamy soils with silt or sand content in Indiana [11]. Thus, the results from our study indicate that further research is needed to determine how Map may move through the environment, especially as leachate in soils.

References

[1] E. A. Raizman, S. J. Wells, S. M. Godden et al., "The distribution of Mycobacterium avium subsp. paratuberculosis in the environment surrounding Minnesota dairy farms," Journal of Dairy Science, vol. 87, no. 9, pp. 2959–2966, 2004.

[2] R. J. Chiodini and C. A. Rossiter, "Paratuberculosis: a potential zoonosis?" The Veterinary Clinics of North America. Food animal practice, vol. 12, no. 2, pp. 457–467, 1996.

[3] P. P. Vishnevskii, E. G. Manatsev, V. V. Chernyshev, and N. S. Ustoichivost, "Paratuberkuliznykh batsill Ione," Soviet Veterinary, vol. 11–12, pp. 89–93, 1940.

[4] R. Lovel, M. Levi, and J. Francis, "Studies on the survival of Johne's bacilli," Journal of Comparative Pathology, vol. 54, pp. 120–129, 1944.

[5] A. B. Larsen, S. Richard, B. S. Merkal, and T. H. Vardman, "Survival time of Mycobacterium paratuberculosis," American Journal of Veterinary Research, vol. 17, no. 64, pp. 549–551, 1956.

[6] R. J. Whittington, I. B. Marsh, and L. A. Reddacliff, "Survival of Mycobacterium avium subsp. paratuberculosis in dam water and sediment," Applied and Environmental Microbiology, vol. 71, no. 9, pp. 5304–5308, 2005.

[7] J. B. Jørgensen, "Survival of Mycobacterium paratuberculosis in slurry," Nordisk Veterinaermedicin, vol. 29, no. 6, pp. 267–270, 1977.

[8] S. K. Grewal, S. Rajeev, S. Sreevatsan, and F. C. Michel Jr., "Persistence of Mycobacterium avium subsp. paratuberculosis and other zoonotic pathogens during simulated composting, manure packing, and liquid storage of dairy manure," Applied and Environmental Microbiology, vol. 72, no. 1, pp. 565–574, 2006.

[9] J. E. Lombard, T. M. Byrem, B. A. Wagner, and B. J. McCluskey, "Comparison of milk and serum enzyme-linked immunosorbent assays for diagnosis of Mycobacterium avium subspecies paratuberculosis infection in dairy cattle," Journal of Veterinary Diagnostic Investigation, vol. 18, no. 5, pp. 448–458, 2006.

[10] D. Morse Meyer, I. Garnett, and J. C. Guthrie, "A survey of dairy manure management practices in california," Journal of Dairy Science, vol. 80, no. 8, pp. 1841–1845, 1997.

[11] M. P. Ward and A. M. Perez, "Association between soil type and paratuberculosis in cattle herds," American Journal of Veterinary Research, vol. 65, no. 1, pp. 10–14, 2004.

[12] C. P. Gerba, C. Wallis, and J. L. Melnick, "Fate of wastewater bacteria and viruses in soil," Journal of Irrigation and Drainage Engineering, vol. 101, no. 3, pp. 157–174, 1975.

[13] W. T. Frankenberger Jr., "Fate of wastewater constitutents in soil groundwater: pathogen," in Irrigation with Reclaimed Municipal Wastewater, G. S. Pettygrove and T. Asano, Eds., pp. 14-1–14-25, Lewis Publishers, Chelsea, Mich, USA, 1985, California State Water Resources Control Board Report Number 84-1.

[14] C. H. Bolster, K. L. Cook, B. Z. Haznedaroglu, and S. L. Walker, "The transport of Mycobacterium avium subsp. paratuberculosis through saturated aquifer materials," Letters in Applied Microbiology, vol. 48, no. 3, pp. 307–312, 2009.

[15] N. K. Dhand, J. A. L. M. L. Toribio, and R. J. Whittington, "Adsorption of Mycobacterium avium subsp. paratuberculosis to soil particles," Applied and Environmental Microbiology, vol. 75, no. 17, pp. 5581–5585, 2009.

[16] J. C. Cho, H. B. Cho, and S. J. Kim, "Heavy contamination of a subsurface aquifer and a stream by livestock wastewater in a stock farming area, Wonju, Korea," Environmental Pollution, vol. 109, no. 1, pp. 137–146, 2000.

[17] C. A. Scott, L. D. Geohring, and M. F. Walter, "Water quality impacts of tile drains in shallow, sloping, structured soils as affected by manure application," Applied Engineering in Agriculture, vol. 14, no. 6, pp. 599–603, 1998.

[18] R. F. Harris, "Effect of water potential in microbial growth and activity," in Water Potential in Soil Microbiology Special Publication no. 9, J. F. Parr, M. R. Gardner, and L. F. Elliott, Eds., pp. 23–95, Soil Science Society of America, Madison, Wis, USA, 1981.

[19] R. L. Mahler and A. G. Wollum, "The influence of water potential on survival of rhizobia in a Goldsboro loam sand," Soil Science Society of America Journal, vol. 44, pp. 988–992, 1980.

[20] R. L. Mahler and A. G. Wollum, "The influence of irrigation and Rhizobium japonicum strains on yield of soybean grown in a lakeland sand," Agronomy Journal, vol. 73, pp. 647–651, 1981.

[21] R. H. Whitlock and A. E. Rosenberger, "Fecal culture protocol for *Mycobacterium paratuberculosis*: a recommended procedure," *Proceedings, Annual Meeting of the United States Animal Health Association*, vol. 94, pp. 280–285, 1990.

[22] J. S. Sung, H. H. Jun, E. J. B. Manning, and M. T. Collins, "Rapid and reliable method for quantification of Mycobacterium paratuberculosis by use of the BACTEC MGIT 960 system," *Journal of Clinical Microbiology*, vol. 45, no. 6, pp. 1941–1948, 2007.

[23] P. Kirchner, M. Meier, and E. C. Bottiger, "Genotypic identification and detection of mycobacteria-facing novel and uncultured pathogens," in *Diagnostic Molecular Microbiology: Principles and Applications*, D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White, Eds., pp. 173–190, American Society for Microbiology, Washington, DC, USA, 1993.

[24] P. H. Vary, P. R. Andersen, E. Green, J. Hermon-Taylor, and J. J. McFadden, "Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease," *Journal of Clinical Microbiology*, vol. 28, no. 5, pp. 933–937, 1990.

[25] T. E. Secott, A. M. Ohme, K. S. Barton, C. C. Wu, and F. A. Rommel, "*Mycobacterium paratuberculosis* detection in bovine feces is improved by coupling agar culture enrichment to an IS900-specific polymerase chain reaction assay," *Journal of Veterinary Diagnostic Investigation*, vol. 11, no. 5, pp. 441–447, 1999.

[26] C. C. Wu, T. E. Secott, T. E. J. Stewart, and T. L. Lin, "Identification and subspeciation of *Mycobacterium avium* isolates by PCR amplification of a fibronectin-attachment protein gene element," in *Proceedings of the 8th International Colloquium on Paratuberculosis*, pp. 438–442, 2005.

[27] S. J. Wells, R. H. Whitlock, C. J. Lindeman, and T. Fyock, "Evaluation of bacteriologic culture of pooled fecal samples for detection of *Mycobacterium paratuberculosis*," *American Journal of Veterinary Research*, vol. 63, no. 8, pp. 1207–1211, 2002.

[28] J. V. Gagliardi and J. S. Karns, "Leaching of Escherichia coli O157:H7 in diverse soils under various agricultural management practices," *Applied and Environmental Microbiology*, vol. 66, no. 3, pp. 877–883, 2000.

[29] A. Gao, J. Odumeru, M. Raymond, and L. Mutharia, "Development of improved method for isolation of Mycobacterium avium subsp. paratuberculosis from bulk tank milk: effect of age of milk, centrifugation, and decontamination," *Canadian Journal of Veterinary Research*, vol. 69, no. 2, pp. 81–87, 2005.

[30] A. Unc and M. J. Goss, "Transport of bacteria from manure and protection of water resources," *Applied Soil Ecology*, vol. 25, no. 1, pp. 1–8, 2004.

[31] Y. Fang, W. H. Wu, J. L. Pepper et al., "Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of Mycobacterium avium subsp. paratuberculosis in bovine fecal samples," *Journal of Clinical Microbiology*, vol. 40, no. 1, pp. 287–291, 2002.

[32] J. R. Stabel and J. P. Bannantine, "Development of a nested PCR method targeting a unique multicopy element, ISMap02, for detection of Mycobacterium avium subsp. paratuberculosis in fecal samples," *Journal of Clinical Microbiology*, vol. 43, no. 9, pp. 4744–4750, 2005.

[33] S. J. Wells, M. T. Collins, K. S. Faaberg et al., "Evaluation of a rapid fecal PCR test for detection of Mycobacterium avium subsp. paratuberculosis in dairy cattle," *Clinical and Vaccine Immunology*, vol. 13, no. 10, pp. 1125–1130, 2006.

[34] C. A. Alinovi, M. P. Ward, T. L. Lin, G. E. Moore, and C. C. Wu, "Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of Mycobacterium avium ssp. paratuberculosis," *Veterinary Microbiology*, vol. 136, no. 1–2, pp. 177–179, 2009.

[35] R. J. Greenstein, "Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease," *The Lancet Infectious Diseases*, vol. 3, no. 8, pp. 507–514, 2003.

Research Article

Long-Term Survival of *Mycobacterium avium* subsp. *paratuberculosis* in Fecal Samples Obtained from Naturally Infected Cows and Stored at $-18°C$ and $-70°C$

Eran A. Raizman,[1] L. A. Espejo,[2] and S. J. Wells[2]

[1] *320 Fernleaf Dr West Lafayette, IN 47906, USA*
[2] *Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108, USA*

Correspondence should be addressed to Eran A. Raizman, eraizman@gmail.com

The objective was to evaluate the survival of Mycobacterium paratuberculosis (Map) in naturally infected dairy cows feces under long periods of freezing at $-18°C$ and $-70°C$. Samples were collected from cows previously tested positive with serum ELISA or fecal culture, or with clinical signs of Johne's disease. Samples were stored at $-18°C$ and/or $-70°C$ and recultured in Herrold's egg yolk media every 3–6 months. A proportional odds mixed model was used for data analysis. Sixty nine fecal samples were stored for different periods between September 2002 and January 2005. Of these, 45 (65%) were stored at $-18°C$ and 24 (35%) at $-70°C$. Average number of days between repeated culture dates was 98 and 84 for $-18°C$ and $-70°C$, respectively. Median number of repeated cultures was 6 and 4 for samples stored at $-18°C$ and $-70°C$, respectively. After adjusting for initial sample bacterial load, the effects of temperature or number of thawing and refreezing cycles on Map viability were not significant. The probability that a sample decreases from high to moderate-low bacterial load and from moderate-low to negative bacterial load was 13.5% per month. Although this study found gradual reduction of Map concentration in stored fecal samples through time, overall survival in $-18°C$ can ease fecal samples management in laboratories with low-processing capacity or lack of $-70°C$ freezer.

1. Introduction

Mycobacterium paratuberculosis (Map), the causative agent of Johne's disease (JD) in domestic and wild ruminants, is known to survive for long periods of time in different environmental conditions [1, 2]. This is especially true in the dairy farm environment near shaded animal management locations where frequent introductions of manure are occurring [3, 4]. Several reports describe long-term *in vitro* Map survival under various physical and environmental conditions including survival in water, urine, and manure and at extreme temperatures [3, 5, 6]. The organism was found to survive in pond water for up to 270 days under *in vitro* conditions and up to 246 days in bovine feces under natural conditions where environment temperature varied between $-3°C$ and $23°C$ [7]. Viable bacteria were isolated after storing the same samples for five months at $-14°C$, then

at $4°C$ for five months and finally at $38°C$ for eight months. Bacteria stored for 12 months at $-14°C$ and then at $4°C$ for five months were also viable [5].

The value of fecal culture in the diagnosis of JD in livestock and especially in cattle has long been established [8]. Fecal samples are normally collected directly from the rectum or from the environment and transported to the laboratory for further processing. Limited processing laboratory capacity may hinder the ability to test all samples immediately. One solution that has been proposed to overcome this problem is to store the specimens at $-70°C$ until facilities are available to complete the sample processing [9]. This would allow a single collection of samples, regardless of herd size or when the laboratory is ready to process the samples, assuming that storage space is available. Furthermore, sample storage can also be used for research studies performed over a long period of time. However, limited information is available on

the viability of Map under these conditions and most studies have used relatively small sample sizes of fecal specimens [9–11]. In a recent study using 11 cows, it was found that the samples storage at −20°C for one week had adverse effects on the viability of Map, whereas short-term storage of fecal samples at 4°C (48 hours to one week) and longer-term storage (3 months) at −70°C had no negative effect on Map survival [10]. Thus, while the authors evaluated Map viability under different shipping and storage conditions, storage was evaluated only up to 3 months. Other than this study, the impact on Map survival of long-term storage under −18°C has not been evaluated, as far as the authors could assess in the published literature. Long-term viability of Map under these conditions can often reduce laboratory costs significantly for JD research. This is especially true in laboratories where the availability of −70°C freezers is very limited due to their high cost. Therefore, the objective of this study was to evaluate the survival of Map in manure from naturally infected cows over long periods of freezing at −18°C and −70°C. Our hypothesis was that Map long-term viability at −18°C and −70°C would not be significantly different.

2. Material and Methods

We used a targeted sampling approach where fecal samples were collected from cows known to be infected with JD through previous serum ELISA or fecal culture testing. We also sampled cows with clinical signs consistent with JD (i.e., chronic diarrhea, progressive weight loss, and reduced milk production) regardless of previous fecal culture results. Fecal samples were collected using a disposable obstetric glove and placed in a 40 mL plastic container for transportation to the laboratory in an iced cooler. A portion of each sample was sent for initial fecal culture, and the rest was divided into two equal portions and stored at −18°C and −70°C in a plastic container appropriately labeled with the sample ID, herd name, date of collection, and date of last culture. When manure sample was too small to be divided, priority was made to store samples at −18°C over −70°C. Our sample size included a total of 51 cows from 17 herds. Of these, 35 cows were sampled once, 27 (77%) at −18°C and 8 (23%) at −70°C. Fourteen cows were sampled twice; one of the pair samples was stored at −18°C and the other 13 at −70°C. Two cows were sampled 3; times of these, one was stored only at −18°C and the other two at both −18°C and −70°C. Samples were recultured approximately every 3–8 months. All samples were processed at the Minnesota Veterinary Diagnostic Laboratory using a previously described bacterial culture method [12]. Briefly, a sedimentation culture procedure was used with 72 hours of sedimentation prior to inoculation of 4 tubes containing Herrold's egg yolk medium. Colony counts were recorded weekly for 16 weeks, and final results were reported as negative, light (mean of 0.25 to 9 colonies/tube (CPT)), moderate (mean of 10 to 49 CPT), moderate-heavy (mean of 50–100 CPT), and heavy (>100 CPT) bacterial load. Each sample was recultured until results were negative in two consecutive cultures or until the fecal material was exhausted.

3. Statistical Analysis

All statistical analysis was performed using SAS (SAS Institute version 9.1).

To test our hypothesis, a proportional odds mixed model approach (PROC GLIMMIX) was used. We assumed a multinomial distribution for the response variable with 3 levels (0 = negative fecal culture, 1 = moderate-low fecal shedding (1–50 CPT), and 2 = high fecal shedding (>50 CPT)). Independent variables were time in months (continuous) which is the total time that samples were kept in the freezer, storage temperature (dichotomous) (−18°C or −70°C), and initial culture result (ordinal categorical with 4 levels of fecal bacterial load). Each fecal sample had a different time to testing; therefore, the model assumed repeated measures (fecal culture) for the same experimental units (fecal samples) and testing time was not constant among fecal samples. As a result, time of testing was also included in the model as a categorical random variable. Because some of the cows have more than one sample tested, we used a nested structure of the data, which is sample nested within cow. In order to test the effect of the number of thawing and refreezing cycles of the samples on Map viability, we ran the same model described above once together with the main effect (months) and then as a main effect. In the final model, a P value of <0.05 was considered statistically significant.

4. Results

A total of 69 samples from 51 cows were stored for different periods between September 2002 and January 2005. Of these, 45 (65%) were stored at −18°C and 24 (35%) at −70°C. Map was cultured at the initiation of the study from all 69 samples. Of the 45 samples stored at −18°C, 35 (78%) and 10 (22%) samples started the study with high and moderate-low bacterial load, respectively. Of the 24 samples stored at −70°C, 21 (88%) and 3 (12%) were high and moderate-low bacterial load, respectively. The average number of days between repeated cultures was 98 (minimum = 40, maximum = 128) and 84 (minimum = 67, maximum = 207) for the −18°C and −70°C storage temperatures, respectively. The median number of repeated cultures was 6 (maximum = 13, minimum = 2) and 4 (maximum = 9, minimum = 2) for samples stored at −18°C and −70°C, respectively. Before adjusting for initial sample bacterial load, the median survival days was 549 (maximum 1016, minimum = 124) and 740 (maximum = 1042, minimum = 112) for samples stored at −18°C and −70°C, respectively. This difference was statistically significant (P < 0.01; Table 1). After adjusting for initial fecal sample bacterial load, the temperature effect was not statistically significant (P = 0.084). Similarly, the effect of the number of thawing and refreezing cycles on Map viability was not found to be statistically significant (P = 0.71) and hence was not included in the final model. The overall estimated Odds Ratio for bacterial load reduction for each month in the freezer was 1.13 (95% CI 1.01 and 1.20) per month in the freezer; therefore, the probability that a sample viability decreases from moderate-low to bacterial load or

TABLE 1: Parameter estimates (standard error—SE, odds ratio—OR and the OR 95% confidence interval—CI) of the proportional odds mixed model for changes on the fecal culture results across time for *Mycobacterium avium* subsp. *paratuberculosis* in dairy cows in Minnesota, USA.

Parameter	Levels	Estimate	SE	OR	CI	*P* value
Intercept*	0	−6.44	0.54			<0.01
	1	−2.43	0.34			<0.01
	2	Reference	—	—	—	—
Months, n	1	0.13	0.02	1.14	1.10, 1.18	<0.01
Temperature, C°	−18°	0.79	0.31	2.20	0.82, 5.99	0.08
	−70°	Reference	—	—	—	—
Initial bacterial load**	1	4.38	0.56	79.80	13.4, 478.02	<0.01
	2	2.47	0.53	11.82	2.21, 62.97	0.02
	3	1.65	0.46	5.18	1.19, 22.85	0.04
	4	Reference	—	—	—	—

* 0: negative, 1: mean of 0.25–50 colonies/tube (CPT)), and 2: mean of >50 CPT.

** 1: mean of 0.25–9 colonies/tube (CPT)), 2: mean of 10–49 CPT, 3: heavy (mean of 50–100 CPT), and 4: >100 CPT.

from low to negative bacterial load was 13% per month. Because the proportional odds model did not converged when the interaction temperature of storage by time was included, the analysis was stratified by storage temperature to evaluate the effect of time in months on the probability of decreasing one category the bacterial culture results. A similar proportional odds model used for the full data set was fitted separately for samples storage at −18 and −70. The results of the model indicated that the probability of decreasing one category the bacterial culture result was 13% (95% CI 8.3–19.4; OR = 1.13 95% CI 1.08–1.19) for samples storage at −18 and 14.7% (95% CI −22.4–70.4; OR = 1.15 95% CI 0.78–1.70), for samples storage at −70, after adjusting by initial bacterial culture result in both models.

5. Discussion

The current study represents the first evaluation of the long-term viability of Map in fecal samples from naturally infected cows when stored at −18°C and −70°C, using a relatively large sample size and more than two years of storage time. Previous studies used a small sample size over a shorter time period [9–11]. In our study, Map survived at −18°C on average 540 days (i.e., 18 months). This period of time is sufficient to allow low capacity laboratories as well as laboratories without −70°C freezers the ability to perform Map testing and research. Our findings suggest that overall Map viability on the long term was not significantly affected by the number of thawing and refreezing cycles compared to storage time. It is possible, however, that, with a larger sample size, a significant impact would be assessed. The methodology used in this study, however, was not designed to address the specific issue of thawing and refreezing. The authors acknowledge that to most accurately assess freeze thaw, aliquots should be made at the beginning of the study where some aliquots would be subjected to freezing and thawing cycles while others are continuously frozen until the date of culture.

Giving the long-term survival in both temperatures, this can ease sample management for long-term research using either −18°C or −70°C. It is important to consider, however, that our sampling targeted high bacterial load cow shedders and results may not apply to low-to-moderate shedders, which are more commonly found during routine herd testing. Additional research is required on a larger sample size of low-to-moderate bacterial load fecal samples to determine whether long-term storage is a viable option for these samples as well. While the freezing and thawing cycles in our study do not exactly mimic these cycles in the nature, our study results provide additional evidence of the ability of Map to survive extreme environmental conditions on farms. It is important to consider that daily cycles of thawing and refreezing are common in the northern latitudes of the north hemisphere.

While a clear tendency for decreasing Map viability over time was observed in the current study, several samples resulted in an increased bacterial load between two consecutive cultures. We do not have a definitive explanation for this lack of repeatability, but it is plausible that fecal sample homogeneity was not the same in each sample processing, perhaps due to non uniform distribution of Map within the sample. This is, however, in contrast to what was reported in previous study which showed that the distribution of Map cultivable quantities was relatively uniform in fecal samples obtained from both subclinical and clinical animals [13]. The authors, however, did not quantify fecal bacterial load as we did in the current study.

A proportional odds model (also known as ordinal logistic regression) was chosen to analyze the data because the results of the fecal culture are normally classified in categories (negative, low, and high) rather than the average of colonies per tube. This classification system provides information about not only the shedding status of the cows, but also the risk of the disease transmission. An important assumption of this type of model is the proportionality of the odds for each level of the outcome fecal culture

result. That assumption was initially evaluated using the Chi-square score test and plotting the odds against the fixed effects. Data could also be analyzed using a nominal logistic regression, where there is no assumption of proportionality of the odds; however, that type of model requires even larger amount of data because more parameters need to be estimated. In our model, we tested the effect of the initial culture result on the probability of a subsequent decrease in bacteria cultured (response 0, 1, and 2). Samples with high bacterial load on the first fecal culture after collection had a higher probability of reduction in Map concentration than samples with moderate-low initial bacterial load. In other words, the proportion of bacterial death that must occur in order to decrease the fecal culture bacterial load from moderate-low to negative is smaller than the proportion of bacterial death that must occur in order to decrease fecal culture bacterial load from high to moderate-low. Therefore, the odds of decreasing the culture are higher on moderate-low initial bacterial load cultures. While a recent study addressed different temperatures and shipping methods, different initial bacterial loads were not addressed at all [10]. Additionally, our stratified analysis indicates that the odds for reduction in Map viability overtime was statistically significant for samples stored in $-18°C$ but not significant for samples stored at $-70°C$. This is an important fact to consider when the use of either freezer is considered whether for research or diagnostics.

The current study was a natural experiment, and the weaknesses of the design were addressed on the analysis when possible. Nevertheless, the main weakness of this study is the lack of constant time interval between repeated cultures and that a different number of samples was used in the two storage temperatures. We believe, however, that the lack of constant time interval was addressed in the statistical model by using a random intercept for storage time and hence did not affect the results of this study. Also, a targeted sampling approach was used in this study and samples were not randomly selected from cows. However, we do not believe that this affected the study results since the subject of the study was the Map organism independent of the animal it was shed from. Additionally, to overcome possible biased estimates due to nonrandom allocation of the samples between the two treatments, we included the variable of the initial culture results to control/adjust for nonrandom allocation of the samples based on initial fecal culture results. Despite that in our analysis we controlled for possible known biases by adjusting for those variables that could not be controlled on the study design, it was impossible to control for unknown sources of biases such as random allocation does.

In summary, this study has shown that Map can survive over long periods (median approximately 18 months) at both $-18°C$ and $-70°C$ and the difference in survivorship between both storage temperatures was shown not to be statistically significant. Nevertheless, Map in samples storage at $-70°C$ showed a trend for twice (OR = 2.2) longer survival which can result from the relatively small sample size stored at $-70°C$. This implies that fecal samples can be stored for long periods in case a laboratory does not have

the capacity to process all the samples at one time or if a laboratory does not have a $-70°C$ freezer. Additionally, the results of this study are an additional indication that Map has the ability to survive long periods of time under extreme environmental conditions that can be found on many dairy farms and crop fields. Our study has shown that this ability, however, depends on the initial bacterial load of the manure. Future studies need to address Map survival with low initial bacterial load (0.25–10 CPT) and to perform the analysis using the real number (continuous) CPT rather than the categorical culture result, which will determine the survival curve against storage time per treatment.

References

[1] R. J. Whittington, I. B. Marsh, and L. A. Reddacliff, "Survival of *Mycobacterium avium* subsp. *paratuberculosis* in dam water and sediment," *Applied and Environmental Microbiology*, vol. 71, no. 9, pp. 5304–5308, 2005.

[2] R. J. Whittington, D. J. Marshall, P. J. Nicholls, I. B. Marsh, and L. A. Reddacliff, "Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment," *Applied and Environmental Microbiology*, vol. 70, no. 5, pp. 2989–3004, 2004.

[3] J. E. Lombard, B. A. Wagner, R. L. Smith et al., "Evaluation of environmental sampling and culture to determine *Mycobacterium avium* subspecies *paratuberculosis* distribution and herd infection status on US dairy operations," *Journal of Dairy Science*, vol. 89, no. 11, pp. 4163–4171, 2006.

[4] E. A. Raizman, S. J. Wells, S. M. Godden et al., "The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms," *Journal of Dairy Science*, vol. 87, no. 9, pp. 2959–2966, 2004.

[5] A. B. Larsen, R. S. Merkal, and T. H. Vardaman, "Survival time of *Mycobacterium paratuberculosis*," *American Journal of Veterinary Research*, vol. 17, no. 64, pp. 549–551, 1956.

[6] P. P. Vishnevskii, E. G. Manatsev, V. V. Chernyshev, and N. S. Ustoichivost, "Paratuberkuliznykh batsill Ione," *Soviet Veterinary*, vol. 11-12, pp. 89–93, 1940.

[7] R. Lovel, M. Levi, and J. Francis, "Studies on the survival of Johne's bacilli," *Journal of Comparative Pathology*, vol. 54, pp. 120–129, 1944.

[8] R. S. Merkal, A. B. Larsen, and K. E. Kopecky, "Comparison of examination and test methods for early detection of paratuberculous cattle," *American Journal of Veterinary Research*, vol. 29, no. 8, pp. 1533–1538, 1968.

[9] W. D. Richards and C. O. Thoen, "Effect of freezing on the viability of *Mycobacterium paratuberculosis* in bovine feces," *Journal of Clinical Microbiology*, vol. 6, no. 4, pp. 392–395, 1977.

[10] S. Khare, L. G. Adams, J. Osterstock, A. Roussel, and L. David, "Effects of shipping and storage conditions of fecal samples on viability of *Mycobacterium paratuberculosis*," *Journal of Clinical Microbiology*, vol. 46, no. 4, pp. 1561–1562, 2008.

[11] W. D. Richards, "Effects of physical and chemical factors on the viability of *Mycobacterium paratuberculosis*," *Journal of Clinical Microbiology*, vol. 14, no. 5, pp. 587–588, 1981.

[12] S. J. Wells, R. H. Whitlock, C. J. Lindeman, and T. Fyock, "Evaluation of bacteriologic culture of pooled fecal samples for detection of *Mycobacterium paratuberculosis*," *American Journal of Veterinary Research*, vol. 63, no. 8, pp. 1207–1211, 2002.

[13] G. J. Eamens, M. J. Turner, and R. J. Whittington, "Sampling and repeatability of radiometric faecal culture in bovine Johne's disease," *Veterinary Microbiology*, vol. 119, no. 2-4, pp. 184–193, 2007.

Research Article

The Effect of *Mycobacterium avium* Complex Infections on Routine *Mycobacterium bovis* Diagnostic Tests

Claire Barry,[1] David Corbett,[1] Douwe Bakker,[2] Peter Andersen,[3] Jim McNair,[4] and Sam Strain[4]

[1] *Veterinary Sciences Division, Agri-Food and Biosciences Institute, Stoney Road, Stormont, Belfast BT4 3SD, Northern Ireland*

[2] *Central Veterinary Institute, P.O. Box 65, Edelhertweg 15, 8200 AB Lelystad, The Netherlands*

[3] *Department of Infectious Immunology, Statens Serum Institut, Artillerivej, 2300 Copenhagen, Denmark*

[4] *Veterinary Sciences Division, Agri-food and Biosciences Institute, Stoney Road, Stormont, Belfast BT4 3SD, Northern Ireland*

Correspondence should be addressed to Sam Strain, samuel.strain@afbini.gov.uk

Bovine tuberculosis (bTB) is diagnosed in naturally infected populations exposed to a wide variety of other pathogens. This study describes the cell-mediated immune responses of cattle exposed to *Mycobacterium avium* subspecies *paratuberculosis* (Map) and *Mycobacterium avium* subspecies *avium* with particular reference to routine antemortem *Mycobacterium bovis* diagnostic tests. The IFN-γ released in response to stimulated blood was found to peak later in the Map-exposed group and was more sustained when compared to the Maa-exposed group. There was a very close correlation between the responses to the purified protein derivatives (PPD) used for stimulation (PPDa, PPDb, and PPDj) with PPDa and PPDj most closely correlated. On occasion, in the Map-infected cattle, PPDb-biased responses were seen compared to PPDa suggesting that some Map-infected cattle could be misclassified as *M. bovis* infected using this test with these reagents. This bias was not seen when PPDj was used. SICCT results were consistent with the respective infections and all calves would have been classed skin test negative.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (Map), a gram positive bacterium, is the causative agent of Johne's disease in cattle. It has a wide host range which includes cattle, sheep, goats, deer, and other nonruminants such as rabbits [1]. The recognised transmission routes are via the faecal-oral route through the consumption of contaminated milk or colostrum and *in utero* [2]. Infection can be cleared by the immune system or progress to subclinical or clinical disease. The proportion of animals that progress to disease will probably vary depending upon the host, the infective dose, and the age of exposure [3–5]. Cattle are most susceptible to infection during their first months of life although older animals can be infected [5–7]. Clinical signs do not usually appear for 3–5 years after infection [4, 6]. Clinical signs include chronic enteritis, diarrhoea, weight loss, and eventual death [8] making it an important constraint on animal health, welfare, and productivity. The direct costs of the disease are difficult to measure, however, in dairy herds it has been estimated that subclinical infection can depress milk production by 10% [9, 10]. However, if infection confounds the diagnosis of other diseases such as *M. bovis*, the actual national cost of infection may be grossly underestimated.

Early Map diagnosis is difficult because of the length of time taken for cattle to develop an antibody response or for clinical signs to develop therefore many infected animals remain on farms where they can readily shed organisms and possibly infect others. Current antibody serology tests vary widely in terms of sensitivity and specificity making comparisons difficult [11–13]. However, with the complete sequencing of the Map genome [14] new antigens are being

discovered which may improve the performance of serology tests [15]. The gold standard of disease diagnosis is tissue/faeces culture which, although accurate, can take months to complete and is likely to be insensitive in preclinically infected animals [16] so is not ideal for disease eradication programs. An alternative diagnostic tool is measuring the Map-specific early cell-mediated immune response (CMI) [17]. This is already routinely used in many countries to diagnose bTB infections in cattle in conjunction with the skin test and is recognised by the Office International des Epizooties for diagnosing *M. bovis* infections [18]. Several studies have demonstrated that the IFN-γ assay is more effective at detecting early Map infections than antibody tests [19, 20]. One of the limitations with these diagnostic tests is the possible misdiagnosis due to cross reactivity with other Mycobacteria. Álvarez et al. have shown that a dual Map and *M. bovis* infection in cattle may reduce the sensitivity of the IFN-γ test to detect bTB [21]. To eliminate this cross reactivity, it may become essential to develop more specific tests, for example, using ESAT-6/CFP10 in the IFN-γ assay to help differentiate between *M. bovis* and Map infections [22, 23].

Map infection in cattle is global. While many studies have been undertaken to determine prevalence within a country, they are not easily comparable due to the different methods used and the cattle tested in the studies not reflecting the overall population. Herd level prevalences within European countries have been estimated to be between 3 and 68% [24]. A 2009 UK government report has estimated that the UK dairy herd prevalence of Johne's disease is 34.7% (95% ci 27.6%–42.5%) [25]. Given this, it is very likely that Johne's disease is present in virtually all countries which currently have bTB. Bovine TB eradication programs are heavily dependent on diagnostic tools to accurately identify affected animals and to distinguish them from animals exposed to environmental mycobacteria. Most current diagnostic tests depend upon the purified protein derivatives (PPDs) of *M. bovis* (PPDb) and Maa (PPDa). Current bTB eradication programs compare the delayed-type hypersensitivity (DTH) reactions of these two tuberculins in the single intradermal comparative cervical tuberculin (SICCT) skin test. Given that these are crude, poorly characterised, can vary with manufacturer [26] and that the Map, Maa, and *M. bovis* mycobacteria are genetically similar [27], there is a strong likelihood that an infection with Map may give responses to either reagent that could affect both the sensitivity and specificity of current tests [21, 23].

The purpose of this paper is to assess the CMI response of cattle experimentally infected with either Maa or Map and if there is any impact on the specificity of current *M. bovis* diagnostics.

2. Materials and Methods

2.1. Animals and Experimental Design.
All calves used were male Friesian-Holsteins sourced in Northern Ireland from *M. bovis* and Map free herds and were tested for exposure to *M. bovis*, Map, and Maa using the IFN-γ release assay

beforehand. Throughout each experiment, the calves were housed in a secure, level 2 containment facility and were blood sampled weekly. In the first experiment, 12 calves aged approximately 16 weeks (ranging from 15 to18 weeks) were infected with Map and kept for 39 weeks before being euthanized and examined at postmortem [28]. In the second experiment, 6 calves were infected with Maa at approximately 20 weeks (ranging from 19 to 22 weeks) of age and then kept for 64 weeks after which they were euthanized and examined at postmortem. Both groups were kept for the maximum length of time practically possible in a level 2 containment facility. In the third experiment, 12 calves were infected with Maa at 18 weeks of age (ranging from 17–19 weeks old) and were kept for 8 weeks before postmortem examination. All experiments complied with UK Home Office approved licence conditions and were subject to regular inspections.

2.2. Inoculation of Calves.
The strain of Map used was a local, low-passage bovine strain courtesy of Dr. Grant, Queens University Belfast, and the strain of Maa used was NCTC 8559. The Map culture was grown in 7H9 Middlebrook media supplemented with OADC and mycobactin J (BD Diagnostics), and the Maa culture was grown in 7H9 Middlebrook media supplemented with OADC (BD Diagnostics). Each inocula was made using cultures harvested in the log phase of growth, and a count was performed using a wet weight method [29]. The innocula were resuspended in buffered saline and the calves infected orally. Calves were administered 10^8 CFU of Map in experiment 1 (optimised by a titration in an earlier study—data not shown) and 10^9 CFU of Maa in experiments 2 and 3. In the absence of other evidence, 10^9 CFU was chosen as the Maa dose to ensure a sufficient immune response.

2.3. IFN-γ Release Assay.
Blood samples were taken by jugular venepuncture into heparinised Vacutainer tubes (BD Diagnostics) and transported to the laboratory within 1 hour. The blood was then immediately stimulated with either PBS (nil control), avian-purified protein derivatives (PPDa) at $4\,\mu$g/mL final concentration (Veterinary Laboratories Agency (VLA)), bovine-purified protein derivative (PPDb) at $8\,\mu$g/mL final concentration (VLA), and johnin-purified protein derivative (PPDj) at $4\,\mu$g/mL final concentration (Central Veterinary Institute (CVI)), ESAT-6 at $2\,\mu$g/mL final concentration (Statens Serum Institut), and pokeweed mitogen (Sigma) (positive control). After 24 hours, the plasma was removed and tested in duplicate using a sandwich ELISA for the detection of bovine IFN-γ (Bovigam, Prionics). The IFN-γ released is expressed as a Net OD (OD of antigen minus OD of PBS). The cut-off for an animal to be classed as bTB-positive animal in Northern Ireland is if PPDb OD minus PBS OD is greater than 0.1 and PPDb OD minus PPDa OD is greater than 0.05 OD. This cutoff has been used in the following results and discussion.

2.4. SICCT Test.
A single intradermal comparative cervical tuberculin (SICCT) test was conducted. Three discreet sites were clipped on each animal and measured for skin thickness
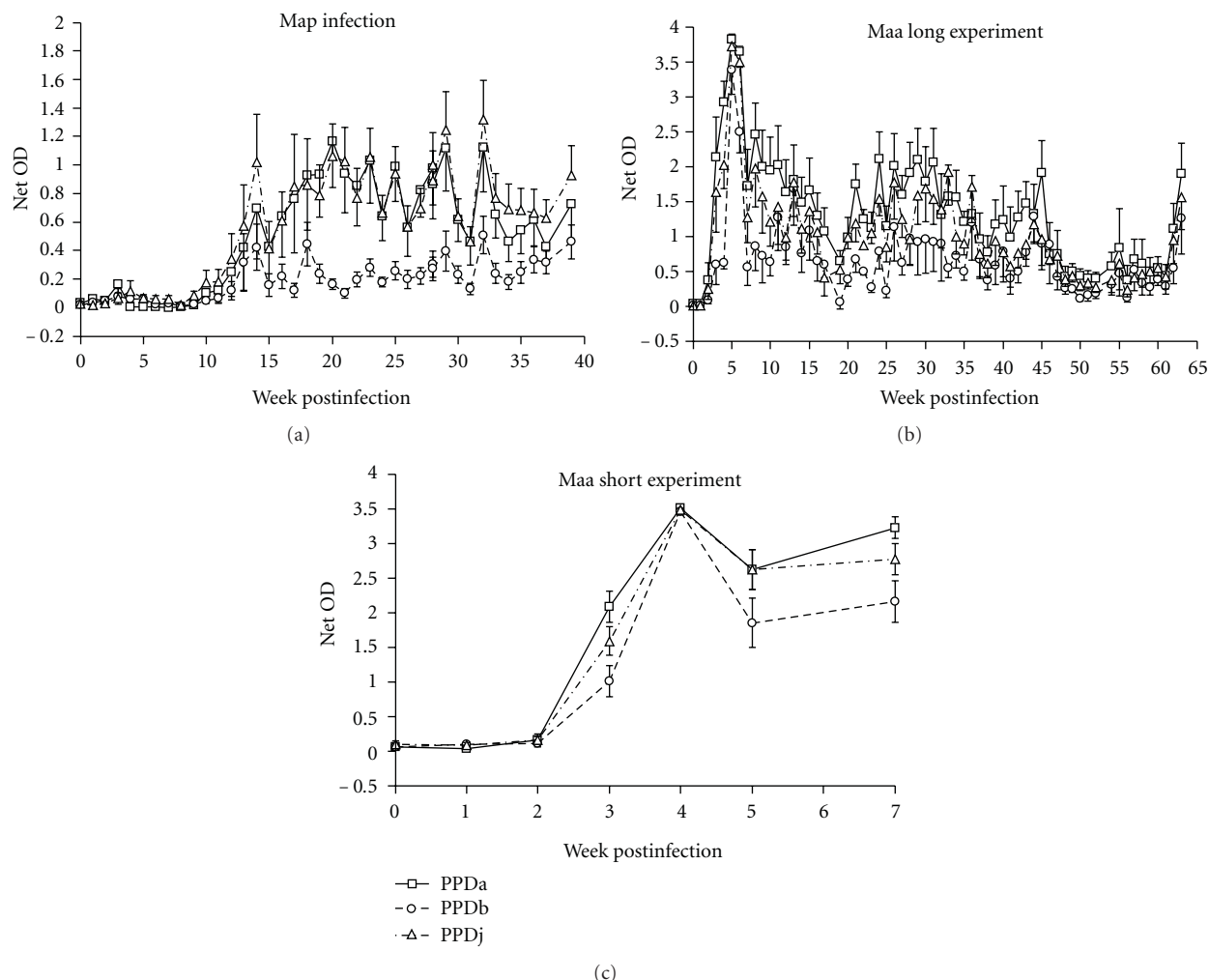
(a)

(b)

(c)

FIGURE 1: (a) Mean IFN-γ net OD values from whole blood stimulated with PPDa, PPDb, and PPDj tuberculins from 12 Map-exposed calves over 39 weeks. For clarity, only one side of the standard error of measurement bar is shown for PPDa and PPDj data points. (b) Mean IFN-γ net OD values from whole blood stimulated with PPDa, PPDb, and PPDj tuberculins from 6 Maa-exposed calves over 64 weeks. (c) Mean IFN-γ net OD values from whole blood stimulated with PPDa, PPDb, and PPDj tuberculins from 12 Maa-exposed calves over 7 weeks.

by callipers and injected intradermally with $100\,\mu$L PPDa (VLA), PPDb (VLA), and PPDj (CVI) tuberculins. Each injection site was checked to ensure intradermal delivery. The skin thickness of each site was measured 72 hours later. Using standard test criteria, an animal is classified as bTB skin test positive if there is a skin reaction to PPDb at least 5 mm greater than that to PPDa. All injections and measurements were performed by the same person to limit operator variation.

2.5. Statistics. Data analysis was carried out using GenStat 12th edition (VSN international). Skin test reactions were compared between infection groups using the Kolmogorov-Smirnov test. The comparisons were between PPDa and PPDb, PPDa and PPDj, and PPDj and PPDb. Correlation coefficients for IFN-γ responses to the three tuberculins were estimated using Kendall's Rank Correlation Coefficients. Graphs were produced using FigSys, (Biosoft).

3. Results

3.1. Experiment 1—Map Infection. The purpose of experiment 1 was to expose 12 calves to a known dose of Map and to measure the IFN-γ released *in vitro* by antigen stimulated PBMCs to study CMI responses. In summary, all calves elicited a CMI response after infection which remained over the course of the experiment. Figure 1(a) shows the mean IFN-γ released in response to blood stimulated with PPDa, b, and j tuberculins. The graph indicates there is a high degree of cross reactivity between the tuberculins with all three showing a similar pattern of response over time with the peak IFN-γ response achieved between weeks 14 and 25 postinfection. All tuberculin comparisons of IFN-γ responses were significantly correlated ($P < .001$) with the response to PPDa and PPDj most closely correlated. However, in 6 out of the 12 calves there was a PPDb bias over PPDa at some time points (Figure 2(a)) suggesting that these animals may, on occasion, be misclassified as bTB
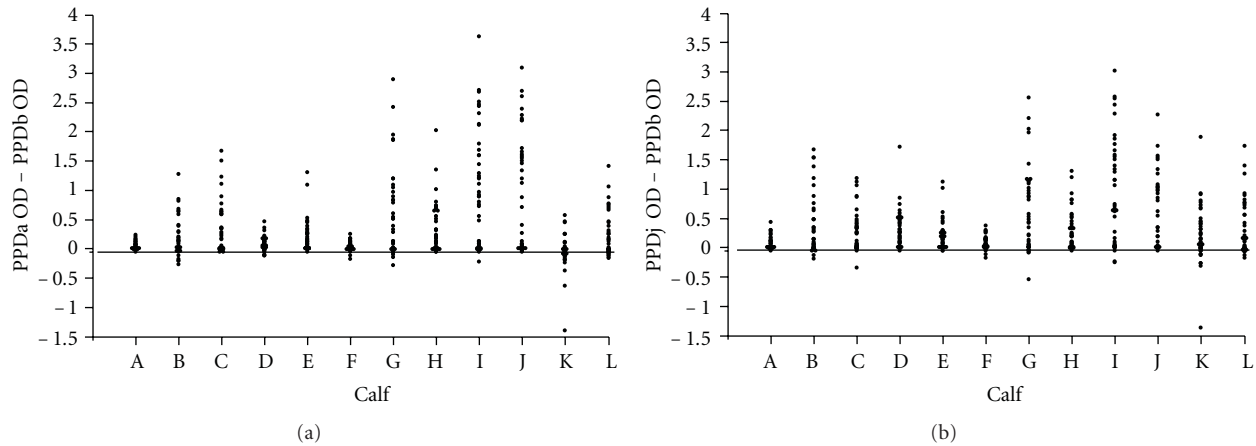
(a)

(b)

FIGURE 2: (a) Difference between IFN-γ responses to PPDa and PPDb over the course of experiment 1 at weekly intervals with Map exposed cattle. A cut-off of 0.05 OD has been applied to highlight cattle with a higher response to PPDb than PPDa. (b) Difference between IFN-γ responses to PPDa and PPDb over the course of experiment 1 at weekly intervals with Map-exposed cattle. A cut-off of -0.05 OD has been applied to highlight cattle with a higher response to PPDb than PPDj.

positive using the current cut-offs used in Northern Ireland (see Section 2.3 for further details). 8% of incidences would have been misclassified with one animal (K) accounting for most (44% of its time points); although, in preinfection bleeds the animal would have tested negative. Importantly, the overall percentage of incidences was less than 5% if the comparison was between PPDb and PPDj (Figure 2(b)). In general the greatest immune response was seen with PPDj, followed by PPDa and then PPDb. No calf was misclassified using the SICCT (Figure 3(a)), and there were no significant differences between PPDa and PPDj skin reactions but there was a significant response difference between PPDa and PPDb ($P = .05$).

3.2. Experiment 2—Maa Infection. The purpose of experiment 2 was to expose 6 calves to a known dose of Maa and to measure the IFN-γ released *in vitro* by antigen stimulated PBMCs to study CMI responses. The Maa group calves' mean IFN-γ response was different to the Map group's. Initially, there was a rapid response of released IFN-γ to PPDa, b, and j tuberculins peaking at weeks 4 and 5 (Figure 1(b)). It then decreased to a low level towards the end of the experiment. There was a high degree of correlation between the tuberculins with a consistent bias in the responses with PPDa having the greatest response and PPDb the lowest. This was also confirmed with the results of the SICCT tests (Figure 3(b)) which show that no calf would have been misclassified and that PPDa induced the greatest delayed-type hypersensitivity (DTH) reaction compared to PPDj and PPDb.

3.3. Experiment 3—Truncated Maa Infection. The purpose of repeating the Maa infection in experiment 3 was to measure the IFN-γ response immediately after exposure up to and including the IFN-γ peak (Figure 1(c)). The responses to PPDa, b, and j were muted compared to experiment 2 but this could reflect a different group of calves being

used. However, response patterns were consistent showing a rapid IFN-γ response between weeks 4 and 5. Again the mean response bias was highest with PPDa then PPDj and then PPDb. However, the SICCT responses were more pronounced compared to experiments 1 and 2. Most calves showed a stronger response to PPDa compared to PPDj or PPDb. This is consistent with the SICCT being performed at the peak IFN-γ response phase. As with the previous 2 experiments, no calf's infection status was misclassified using the SICCT test (Figure 3(c)).

3.4. Mycobacterium bovis Differentiation. ESAT-6 [30, 31], an *M. tuberculosis* complex-specific antigen, was tested at most time points in all three experiments to examine its differentiation potential and to ensure that the PPDb response seen (Figures 1(a), 1(b), and 1(c)) was not due to prior exposure to *M. bovis* (data not shown). No animal showed any consistent response in any of the three experiments indicating that the PPDb responses were due to cross reactivity from exposure to either Map or Maa organisms and not from bTB infection. This supports the use of ESAT-6 as a more specific antigen for use in bTB diagnostics.

4. Discussion

The series of experiments described above demonstrates the development of infection models to define the immune responses following exposure to two nontuberculous species of mycobacteria, Map and Maa [3, 29]. Both of the species are considered to confound specificity of bTB diagnosis in cattle [21, 23]. Using IFN-γ release assays we demonstrated the development of cell-mediated immune responses to three tuberculin reagents, PPDa, PPDb, and PPDj. The IFN-γ responses to these tuberculins had a slower onset in the group exposed to Map compared to Maa, and responses remained elevated for a longer period of time postexposure.
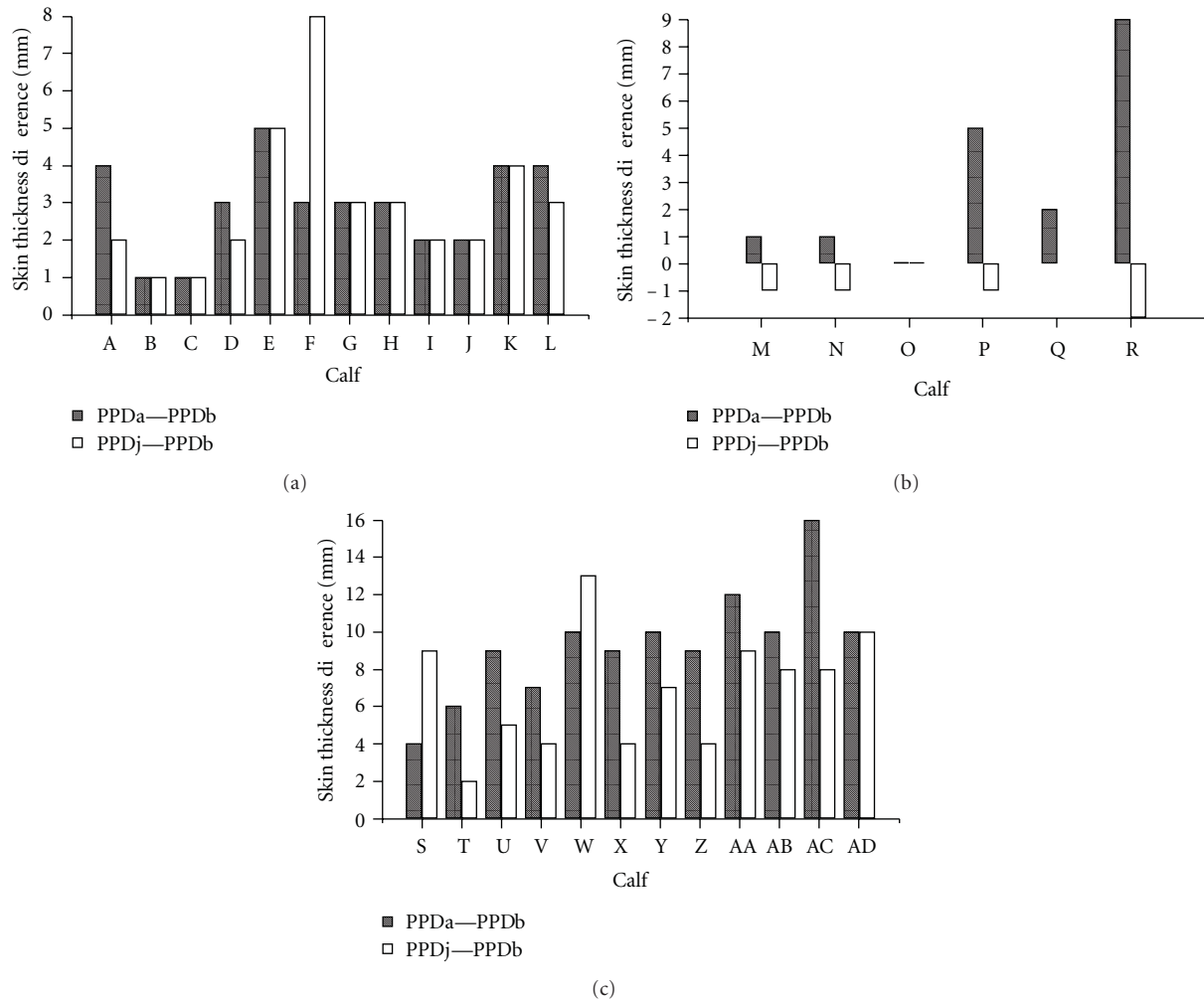
(a)

(b)

(c)

Figure 3: (a) Mean differences in skin thickness of 12 calves exposed to Map. Measurements were made at 39 weeks postinfection using PPDa, PPDb, and PPDj tuberculins. (b) Mean differences in skin thickness of 6 calves exposed to Maa. Measurements were made at 64 weeks postinfection using PPDA, PPDb, and PPDj tuberculins. (c) Mean differences in skin thickness of 12 calves exposed to Maa. Measurements were made after 7 weeks postinfection using PPDa, PPDb, and PPDj tuberculins.

The subsequent, reduced peaks of the Maa group could be explained by the organism being reexposed to the animals' immune system, thus eliciting a more effective CMI response resulting in the organism being cleared; however, this remains uncertain from these results.

It is known that in calves infected with Map there may be a slow progressive infection which takes years to develop into a clinical state [5–7]. As Map infection progresses, the bovine immune response is increasingly exposed to bacterial antigens resulting in the development of humoral and cell-mediated immune responses [32]. In comparison, infection or exposure to Maa would appear to provoke a transient immune response which in our model resulted in a peak of IFN-γ released at approximately 5 weeks postinfection and thereafter declined. In comparison, the IFN-γ response to Map peaked much later and remained high at 39 weeks postinfection.

All IFN-γ PPD responses were significantly correlated to each other ($P < .001$). However, the strongest IFN-γ correlation among individual calves in the Map infection model was between PPDj and PPDa with the weakest between PPDa and PPDb. These correlations are consistent with those seen in a naturally infected herd (data not shown). The IFN-γ responses of individual calves indicate that if tested for bTB using cut-offs currently used with the Northern Ireland IFN-γ program, the majority would have been negative; however, there are some time points which would have classed the calves as positive. Therefore, this has potentially important implications for current diagnostic policies, for example, large-scale screening of animals in a Map-infected region is likely to lead to a proportion of animals incorrectly identified as *M. bovis* infected. However, all animals, in each experiment, would have been correctly identified as bTB negative using current skin test cut-offs. Most animals displayed a skin test reaction bias towards PPDa then PPDj and PPDb with the truncated Maa experiment resulting in

the highest responses. This is probably due to the test being performed at the peak CMI response.

A recognised aspect of the IFN-γ test using tuberculins is the significant number of animals identified as *M. bovis* positive without any other evidence for infection [33]. While a significant proportion of these are likely to be truly infected or exposed, it is possible that some may be infected with Map. Therefore, a large-scale field study is warranted to identify if Johne's infected animals is one factor that leads to the apparent reduced specificity of the *M. bovis* IFN-γ test, as suggested in this study. This work suggests that the use of better defined antigens could be a useful adjunct to disentangle Map infected from *M. bovis*-infected animals, for example, through the use of ESAT-6.

5. Conclusions

This study illustrates the very high degree of cross reactivity between PPDa, b, and j tuberculins and also differences in the kinetics of the CMI responses after exposure to Maa and Map. Diagnostic comparative tests are essential where animals have been exposed to *M. avium* complex organisms to avoid misclassification of *M. bovis* infection status. This is particularly important if the animals are tested soon after exposure to Maa. The Map experimental model highlighted the potential for misidentification as *M. bovis* positive in Map infected cattle using PPDa and PPDb. Therefore, additional specific antigens may have to be incorporated into future diagnostics, for example, ESAT-6. Given that PPDa induces a skin test response in Maa-and Map-infected cattle, careful consideration should be given to the analysis and interpretation of tests in field cattle to avoid misdiagnosis of Map infection. Therefore, efforts should be made to develop comparative tests to identify Map-exposed animals from *M. bovis*-and Maa-exposed animals using mycobacterial species-specific diagnostics.

Acknowledgments

References

[1] K. Stevenson, J. Alvarez, D. Bakker et al., "Occurrence of *Mycobacterium avium* subspecies *paratuberculosis* across host species and European countries with evidence for transmission between wildlife and domestic ruminants," *BMC Microbiology*, vol. 9, article no. 212, pp. 1–13, 2009.

[2] R. J. Whittington and P. A. Windsor, "In utero infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: a critical review and meta-analysis," *Veterinary Journal*, vol. 179, no. 1, pp. 60–69, 2009.

[3] D. J. Begg and R. J. Whittington, "Experimental animal infection models for Johne's disease, an infectious enteropathy caused by *Mycobacterium avium* subsp. *paratuberculosis*," *Veterinary Journal*, vol. 176, no. 2, pp. 129–145, 2008.

[4] R. J. Chiodini, H. J. Van Kruiningen, and R. S. Merkal, "Ruminant paratuberculosis (Johne's disease): the current status and future prospects," *The Cornell Veterinarian*, vol. 74, no. 3, pp. 218–262, 1984.

[5] T. Doyle, "Susceptibility to Johne's disease in relation to age," *The Veterinary Record*, vol. 65, no. 23, pp. 363–365, 1953.

[6] J. Payne and J. Rankin, "A comparison of the pathogenesis of eperimental Johne's disease in calves and cows," *Research in Veterinary Science*, vol. 2, pp. 175–179, 1961.

[7] P. A. Windsor and R. J. Whittington, "Evidence for age susceptibility of cattle to Johne's disease," *Veterinary Journal*, vol. 184, no. 1, pp. 37–44, 2010.

[8] C. J. Clarke, "The pathology and pathogenesis of paratuberculosis in ruminants and other species," *Journal of Comparative Pathology*, vol. 116, no. 3, pp. 217–261, 1997.

[9] G. Benedictus, A. A. Dijkhuizen, and J. Stelwagen, "Economic losses due to paratuberculosis in dairy cattle," *Veterinary Record*, vol. 121, no. 7, pp. 142–146, 1987.

[10] A. W. Stott, G. M. Jones, R. W. Humphry, and G. J. Gunn, "Financial incentive to control paratuberculosis (Johne's disease) on dairy farms in the United Kingdom," *Veterinary Record*, vol. 156, no. 26, pp. 825–831, 2005.

[11] H. Köhler, B. Burkert, I. Pavlik et al., "Evaluation of five ELISA test kits for the measurement of antibodies against *Mycobacterium avium* subspecies *paratuberculosis* in bovine serum," *Berliner und Munchener Tierarztliche Wochenschrift*, vol. 121, no. 5–6, pp. 203–210, 2008.

[12] S. L. B. McKenna, G. P. Keefe, H. W. Barkema, and D. C. Sockett, "Evaluation of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* using tissue and fecal culture as comparison standards," *Veterinary Microbiology*, vol. 110, no. 1–2, pp. 105–111, 2005.

[13] S. S. Nielsen and N. Toft, "Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-γ assay and faecal culture techniques," *Veterinary Microbiology*, vol. 129, no. 3–4, pp. 217–235, 2008.

[14] L. Li, J. P. Bannantine, Q. Zhang et al., "The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 35, pp. 12344–12349, 2005.

[15] J. P. Bannantine, D. O. Bayles, W. R. Waters, M. V. Palmer, J. R. Stabel, and M. L. Paustian, "Early antibody response against *Mycobacterium avium* subspecies *paratuberculosis* antigens in subclinical cattle," *Proteome Science*, vol. 6, article no. 5, pp. 1–12, 2008.

[16] L. de Juan, J. Álvarez, B. Romero et al., "Comparison of four different culture media for isolation and growth of type II and type I/III *Mycobacterium avium* subsp. *paratuberculosis* strains isolated from cattle and goats," *Applied and Environmental Microbiology*, vol. 72, no. 9, pp. 5927–5932, 2006.

[17] J. R. Stabel, "Production of γ-interferon by peripheral blood mononuclear cells: an important diagnostic tool for detection of subclinical paratuberculosis," *Journal of Veterinary Diagnostic Investigation*, vol. 8, no. 3, pp. 345–350, 1996.

[18] OIE, World Organisation for Animal Health, *Manual of Standards for Diagnostic Tests and Vaccines*, World Organisation for Animal Health, O.I.d.E. (OIE), Office International des Epizooties, Paris, France, 4th edition, 2000.

[19] J. M. Gwozdz, K. G. Thompson, A. Murray, M. P. Reichel,

B. W. Manktelow, and D. M. West, "Comparison of three serological tests and an interferon-γ assay for the diagnosis of paratuberculosis in experimentally infected sheep," *Australian Veterinary Journal*, vol. 78, no. 11, pp. 779–783, 2000.

[20] A. Huda, G. Jungersen, and P. Lind, "Longitudinal study of interferon-gamma, serum antibody and milk antibody responses in cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*," *Veterinary Microbiology*, vol. 104, no. 1–2, pp. 43–53, 2004.

[21] J. Álvarez, L. de Juan, J. Bezos et al., "Effect of paratuberculosis on the diagnosis of bovine tuberculosis in a cattle herd with a mixed infection using interferon-gamma detection assay," *Veterinary Microbiology*, vol. 135, no. 3–4, pp. 389–393, 2009.

[22] J. M. Pollock and P. Andersen, "The potential of the ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of tuberculosis," *Journal of Infectious Diseases*, vol. 175, no. 5, pp. 1251–1254, 1997.

[23] C. Aagaard, M. Govaerts, V. Meikle et al., "Detection of bovine tuberculosis in herds with different disease prevalence and influence of paratuberculosis infection on PPDB and ESAT-6/CFP10 specificity," *Preventive Veterinary Medicine*, vol. 96, no. 3–4, pp. 161–169, 2010.

[24] S. S. Nielsen and N. Toft, "A review of prevalences of paratuberculosis in farmed animals in Europe," *Preventive Veterinary Medicine*, vol. 88, no. 1, pp. 1–14, 2009.

[25] *SB4022: An Integrated Strategy to Determine the Herd Level Prevalence of Johne's Disease in the UK Dairy Herd*, DEFRA, 2009.

[26] I. Schiller, H. M. Vordermeier, W. R. Waters et al., "Comparison of tuberculin activity using the interferon-γ assay for the diagnosis of bovine tuberculosis," *Veterinary Record*, vol. 167, no. 9, pp. 322–326, 2010.

[27] J. P. Bannantine, E. Baechler, Q. Zhang, L. Li, and V. Kapur, "Genome scale comparison of *Mycobacterium avium* subsp. *paratuberculosis* with *Mycobacterium avium* subsp. *avium* reveals potential diagnostic sequences," *Journal of Clinical Microbiology*, vol. 40, no. 4, pp. 1303–1310, 2002.

[28] R. W. Sweeney, J. Uzonna, R. H. Whitlock, P. L. Habecker, P. Chilton, and P. Scott, "Tissue predilection sites and effect of dose on *Mycobacterium avium* subs. *paratuberculosis* organism recovery in a short-term bovine experimental oral infection model," *Research in Veterinary Science*, vol. 80, no. 3, pp. 253–259, 2006.

[29] M. E. Hines, J. R. Stabel, R. W. Sweeney et al., "Experimental challenge models for Johne's disease: a review and proposed international guidelines," *Veterinary Microbiology*, vol. 122, no. 3–4, pp. 197–222, 2007.

[30] B. M. Buddle, T. J. Ryan, J. M. Pollock, P. Andersen, and G. W. de Lisle, "Use of ESAT-6 in the interferon-γ test for diagnosis of bovine tuberculosis following skin testing," *Veterinary Microbiology*, vol. 80, no. 1, pp. 37–46, 2001.

[31] L. A. H. van Pinxteren, P. Ravn, E. M. Agger, J. Pollock, and P. Andersen, "Diagnosis of tuberculosis based on the two specific antigens ESAT-6 and CFP10," *Clinical and Diagnostic Laboratory Immunology*, vol. 7, no. 2, pp. 155–160, 2000.

[32] J. R. Stabel, "Host responses to *Mycobacterium avium* subsp. *paratuberculosis*: a complex arsenal," *Animal Health Research Reviews*, vol. 7, no. 1–2, pp. 61–70, 2007.

[33] M. D. Welsh, J. McNair, S. W. J. McDowell et al., "The Northern Ireland interferon-gamma (IFN-g) testing programme," *Cattle Practice*, vol. 16, no. 2, pp. 136–139, 2008.

Research Article

A New Experimental Infection Model in Ferrets Based on Aerosolised *Mycobacterium bovis*

Lyanne McCallan,[1] David Corbett,[1] Peter L. Andersen,[2] Claus Aagaard,[2] David McMurray,[3] Claire Barry,[1] Suzan Thompson,[1] Samuel Strain,[1] and Jim McNair[1]

[1] *Veterinary Sciences Division, Agri-food and Biosciences Institute, Stoney Road, Belfast BT4 3SD, UK*

[2] *Department of Infectious Immunology, Statens Serum Institut, 5 Artillerivej, 2300 Copenhagen S, Denmark*

[3] *Department of Microbial and Molecular Pathogenesis, Texas A&M University System Health Science Center, College Station, TX 77840-7896, USA*

Correspondence should be addressed to Jim McNair, jim.mcnair@afbini.gov.uk

There is significant interest in developing vaccines to control bovine tuberculosis, especially in wildlife species where this disease continues to persist in reservoir species such as the European Badger (*Meles meles*). However, gaining access to populations of badgers (protected under UK law) is problematic and not always possible. In this study, a new infection model has been developed in ferrets (*Mustela furo*), a species which is closely related to the badger. Groups of ferrets were infected using a Madison infection chamber and were examined postmortem for the presence of tuberculous lesions and to provide tissue samples for confirmation of *Mycobacterium bovis* by culture. An infectious dose was defined, that establishes infection within the lungs and associated lymph nodes with subsequent spread to the mesentery lymph nodes. This model, which emphasises respiratory tract infection, will be used to evaluate vaccines for the control of bovine tuberculosis in wildlife species.

1. Introduction

The persistence of the bovine tuberculosis problem in cattle and other species on a worldwide basis has led to the reappraisal of control programmes to counter this disease. The current direction for research into the control of bovine TB includes evaluation of vaccines that may be deployed in wildlife populations, including badgers (*Meles meles*) [1, 2]. This work has been largely driven through an experimental model approach [3]. Despite considerable advances in our understanding of the pathogenesis and immunology of the disease and the impact of vaccination in a range of species, there remains a significant paucity of information relating to vaccine efficacy. Significant advances have been made through the use of *M. bovis* BCG; yet, some studies reported partial protection in badgers against experimental challenge [3, 4]. There remains a need to evaluate other types of vaccines (protein subunit vaccines for example) that may induce enhanced protection from infection or even boost *Mycobacterium bovis* (*M. bovis*) BCG generated immunity [5].

The use of free ranging badgers taken from wildlife populations and held in captivity, to study vaccine efficacy, is problematic, primarily since the badger is a protected species under UK legislation and removal of wild animals for experimentation is very emotive to the public. Using a surrogate species, such as the ferret (*Mustela furo*), brings a number of advantages that include the availability of animals from licensed suppliers, the capacity to control a number of significant experimental features, and the opportunity to adopt a multifaceted approach to the study (controlled infection dose, pathogenesis, immunology, and culture confirmation).

The ferret has been used in various experimental models to study a number of human pathogens to which it is susceptible [6–8]. However, this species is also susceptible to infection with *M. bovis* [9] and it is closely related to the European badger (both are members of the Mustelidae), making it an attractive surrogate species in which to develop an aerosol infection model when wild badgers are unavailable.

The main purpose of this study was to establish an experimental model of infection in the ferret using aerosolised *M. bovis* by defining the optimum challenge dose that could be used to induce disease. In doing so, we intend to use this model in future studies to measure the efficacy of protein subunit vaccines.

2. Materials and Methods

2.1. Experimental Rationale and Design. The purpose of this study was to establish an *M. bovis* infectious dose delivered by aerosol, to initiate infection and establish the disease in ferrets. This is part of a larger study to measure the efficacy of antibovine tuberculosis vaccines for wildlife species. In order to define the optimal *M. bovis* challenge dose, we infected ferret groups with either 1×10^2 CFU or 5×10^3 CFU of *M. bovis* strain AF2122/97 (a kind gift from Dr M. Vordermeier, VLA, Weybridge) and carried out postmortem examinations at 8 and 20 weeks post infection (PI).

Prior to the commencement of any procedures, approval to carry out ferret-based experiments was given by the UK Home Office and the AFBI Ethics Review Committee and all procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

2.2. Ferrets. Ferrets used in this experiment were born into litters within a two-week period and were fully weaned when they arrived into AFBI. Sixteen male ferrets were obtained from approved UK suppliers, through Harlan Laboratories, UK. Upon arrival at the biosecure containment level 3 suite, ferrets were split into two groups of 8 depending on litter and age and housed in bespoke pens with environmental enrichment. The mean age (SD) for the group of ferrets on arrival at AFBI was 6.4 (SD 0.51) weeks. At the time of infection, the mean age (SD) was 20.4 (0.51) weeks. This building was maintained under negative air pressure, with high-efficiency particulate air (HEPA) filtration. In addition to cleaning each pen and replacing food (James Wellbeloved, UK) and water on a daily basis, each ferret was handled and examined for any signs of infection (demeanour, movement, bite wounds, etc.). The individual weight of each ferret was recorded on a weekly basis throughout the duration of the experiment to provide an additional measure of health.

2.3. Preparation of M. bovis for Aerosol Experiments. M. bovis strain AF2122/97, a virulent field strain isolated from a cow found to have caseous lesions in the lung and bronchomediastinal lymph nodes, was used as the inoculum for aerosol exposure. Unicellular *M. bovis* stock cultures were prepared as described previously [10]. For aerosol exposure, aliquots of unicellular *M. bovis* stock cultures were thawed and diluted to the desired concentration in sterile 0.01 M phosphate buffered saline (PBS), pH 7.2 immediately before use.

2.4. Aerosol Exposure. All ferrets were exposed to *M. bovis* using a Madison aerosol chamber, essentially as described by Rodgers et al. [10]. Two groups of ferrets were established

and were infected with either 1×10^2 or 5×10^3 CFU *M. bovis*. Prior to infection unicellular cultures at 10^6 CFU per mL, was placed in the aerosol chamber nebulizer jar with the exposure cycle pre set to deliver the appropriate infection dose. Following the timed exposure period, the infection chamber was purged with clean, filtered air and the ferret groups were returned to their pens. During each infection cycle the air stream carrying aerosolized *M. bovis* was sampled into an all glass impinger (AGI) containing phosphate buffered saline (PBS) to calculate the actual infective dose (CFU) delivered to each group. One hundred μl of PBS from each impinger (undiluted, 1 in 10, 1 in 100 and 1 in 1000 dilutions in PBS) were inoculated in quadruplicate onto 7H11-OADC agar plates and incubated at 37°C for up to 6 weeks.

2.5. Faecal Sampling. A sample of faeces was collected from the latrine area of each pen every week for the duration of the experiment, to monitor excretion of *M. bovis* in faeces. Samples were split into two aliquots and decontaminated using either 0.075% (final volume) hexadecylpyridinium chloride (HPC) or 5% (final volume) oxalic acid followed by centrifugation at 3000 g for 15 min. Pellets were resuspended in 1 mL 0.01 M sterile phosphate buffered saline (pH 7.2) and inoculated onto the BACTEC MGIT 960 culture system. Suspect *M. bovis* culture positive samples were stained using the Ziehl-Neelsen method. Samples from faeces shown to contain typical acid-fast rods were confirmed as *M. bovis* positive using Variable Number Tandem Repeat (VNTR) typing to confirm re-isolation of AF2122/97 [11].

2.6. Postmortem Examination and Lesion Score. During the design of this experiment, we decided to use the culture of *M. bovis* from tissues as the primary indicator for infection. The reason for this was that there was likely to be very limited amounts of tissue available for laboratory confirmation techniques. Any attempt to prepare tissues for bacteriology and histopathology would compromise both techniques and experience gained from previous experiments in cattle that demonstrated a superior sensitivity with culture techniques compared to histopathology. In addition, *M. bovis* induced lesions are difficult to discern in ferrets, with bacteriology greatly enhancing detection of *M. bovis* infection [12].

At the end of each experimental period, each ferret was euthanized using isofluorothane gas followed by pentobarbitone overdose and examined for the presence of tuberculous lesions. Prior to dissection, the fur on each animal was clipped and washed in chlorhexidine surgical scrub to reduce the potential for microbial contamination. Carcasses were dissected and tissue samples carefully removed into sterile containers for further examination and for preparation prior to inoculation onto culture media. During carcass dissection, a standardised protocol was followed to ensure consistency and accuracy. Firstly, the lungs were removed and cut into four quarters representing right upper and lower and left upper and lower sections. The kidneys, liver, and spleen were then removed followed by the mesenteric lymph node. Finally, the submandibular lymph nodes were removed and, if visible, the retropharyngeal lymph nodes. Prior to

preparation for culture, all tissues were dissected in detail to identify and measure lesions, if present.

Tuberculous lesions, if present, were measured, recorded and assigned a lesion score according to their presence and extent. When lesions were absent, a score of zero was recorded. If one small focus of infection was seen a score of 1 was recorded. When several foci or an area of necrosis (measuring 5 mm or greater) was observed, a score of 2 was given and when multiple lesions and/or extensive necrosis was observed, a score of 3 was recorded. Only lesions that had been confirmed as tuberculosis following isolation of *M. bovis* were used in the analysis of postmortem scores. All tissues removed for examination and culture were assigned a postmortem score.

All postmortem examinations were carried out within the containment level three suite, under negative air pressure, where complete sterility could not be guaranteed. For this reason, all tissue samples were considered to be potentially contaminated through exposure to the environment.

2.7. Preparation of Tissue Homogenates for Culture. A total of 156 samples were submitted for both qualitative and quantitative bacteriological culture at 8 weeks and 20 weeks PI, with sampled tissues including the submandibular, mesenteric, and lung-associated lymph nodes (LNs), as well as the lungs, liver, spleen, and kidneys. Each tissue sample was trimmed to remove fat, weighed, dissected carefully to reveal lesions indicative of tuberculosis and then sliced into approximately 5 mm cubes. The cubed tissue samples were then placed into double thickness stomacher bags (Seward, Worthington, UK) with between 5 and 10 mL PBS (depending on the weight of sample) and homogenised in a stomacher (Biomaster 80, Seward) at high speed for 2 min. Tissue homogenates were decanted into sterile universal bottles for inoculation into media for qualitative and quantitative culture. Urine was recovered from the bladder in most cases but not in sufficient volumes to allow a full bacteriological analysis. Despite this, samples were swabbed onto 7H11-OADC agar plates and incubated at 37°C for up to 6 weeks.

2.8. Qualitative Culture. For qualitative culture, tissue homogenates were inoculated into the BACTEC MGIT 960 culture system and incubated for up to 56 days. Samples identified as positive by the BACTEC MGIT 960 system were stained using the Ziehl-Neelsen method. Samples containing typical acid-fast rods were presumed to be positive for *M. bovis* and analysed further by VNTR to indentify the strain [11].

2.9. Quantitative Culture. For quantitative culture, 100 μl of tissue homogenates (undiluted, 1 in 10, 1 in 100 and 1 in 1000 dilutions in PBS) were inoculated in quadruplicate onto 7H11-OADC agar plates and incubated at 37°C for up to 6 weeks. Tissue homogenates were decontaminated in 0.075% (final volume) hexadecylpyridinium chloride (HPC) prior to inoculation onto 7H11-OADC agar plates. *M. bovis* colonies were counted weekly from 3 weeks onwards and the average colony counts at 6 weeks post-inoculation used to calculate the total number of CFU per g of sample. *M. bovis* colonies were initially identified on the basis of colony morphology and a selection of colonies were prepared for VNTR typing to confirm isolation of AF2122/97 [11].

2.10. Data Analysis. Where appropriate, data was analysed using GraphPad InStat version 3.05 for Windows NT. Data was analysed by one-way analysis of Variance (ANOVA) with Tukey-Kramer multiple comparisons test.

3. Results

3.1. Clinical Observations. All ferrets were examined on a daily basis for signs of ill health and bite wounds, were observed feeding and drinking regularly, and appeared to be in good health during the entire experimental period. During this period, no adverse clinical signs were observed in any of the ferrets. A number of ferrets had bite wounds between the shoulders, but these were considered to be minor and did not warrant treatment. The weight of each ferret was recorded every week and used as an indirect measure of health status. During the experimental period, all ferrets either maintained or gained weight, with the exception of two animals where the weight loss was 4.8% and 12.8%. There was no significant difference between the mean weight of each group at time point zero (preinfection) or at the time when postmortem examinations were carried out (data not shown).

3.2. Aerosol Exposure. At the outset, the intention was to infect each group of ferrets with either 1×10^2 CFU or 5×10^3 CFU *M. bovis*. The actual dose delivered was estimated by capturing aerosolised *M. bovis* from the airstream delivered to the Madison chamber for culture onto 7H11 media. The actual dose delivered to each group was calculated to be 0.8×10^2 CFU (lower dose) and 1.1×10^3 CFU (higher dose).

3.3. Culture of M. bovis from Faeces. A total of 49 faeces samples were collected during the experiment for qualitative culture using the BACTEC MGIT 960 system. Samples collected prior to infection were culture negative for *M. bovis*. After infection and up to 20 weeks after infection (end of experiment), a total of 10 faeces samples were found to be positive for *M. bovis* (between 5 and 18 weeks post infection), of which nine originated from the group infected with 1.1×10^3 CFU. Isolates obtained from all ten positive faeces were confirmed as AF2122/97 using the VNTR typing method. Culture positive faeces samples were only detected by the BACTEC MGIT 960 system after decontamination with 5% oxalic acid. Corresponding samples decontaminated with 0.075% HPC were negative at the end of the incubation period (56 days).

3.4. Postmortem Examination and Lesion Score. Ferrets were euthanised at either 8 or 20 weeks PI and examined postmortem. The greater number of lesions confirmed as tuberculous, was found in the lungs and lung-associated lymph nodes. When present, suspect lesions had the appearance of discrete, pale, circumscribed areas which were not

TABLE 1: Isolation of *M. bovis* from a range of tissues taken from 4 ferrets infected with 0.8×10^2 CFU and examined postmortem at 8 weeks post infection.

Tag number	432			481			523			564		
Tissue	LS[1]	QL[2]	QN[3]	LS	QL	QN	LS	QL	QN	LS	QL	QN
Submandibular LN	0	−	−	0	−	−	0	−	−	0	+	24.3
Retropharyngeal LN	0	−	−	NS[4]	NS	NS	NS	NS	NS	0	+	31.7
Mesenteric LN	0	+	1.94	0	+	16.3	0	−	32.8	1	+	1080
Upper left lung	0	−	−	0	+	2.96	0	−	−	0	+	36.8
Lower left lung	0	+	−	0	+	−	0	−	74.4	0	+	14.9
Upper right lung	0	+	5.4	0	+	−	0	−	−	0	+	−
Lower right lung	0	+	−	0	+	32	0	−	81.8	0	+	−
Lung associated LN	0	+	−	0	+	11.8	2	−	1190	1	+	46.6
Kidney	0	−	−	0	−	−	0	−	−	0	0	152
Liver	0	−	88	0	+	−	0	−	−	0	+	−
Spleen	0	+	−	0	−	−	0	−	−	0	+	8.5
Kidney (2)	0	−	−	NS	NS	NS	NS	NS	NS	NS	NS	NS

[1] LS: lesion score.
[2] QL: qualitative bacteriology.
[3] QN: quantitative bacteriology ($\times 10^2$ CFU/g).
[4] NS: not sampled.

necrotic or caseous. Typically, lesions were less than 5 mm in diameter and did not coalesce. In each infection group, the tissues where lesions were most likely disclosed were the lungs and lung associated lymph nodes. Postmortem scores ranged between 1 and 3 and were consistently higher with the greater infection dose (Tables 1 to 4). After the lungs, the mesenteric lymph nodes were the site most likely to find lesions, although the postmortem scores were lower, between 1 and 2. Only three additional lesions were found with a PM score between 1 and 2, and these were in the spleen tissue of ferrets infected with 1×10^3 CFU (Tables 1–4). A range of tissues were examined and sampled for bacterial culture. These included the lungs, lung-associated lymph nodes, the submandibular and mesenteric lymph nodes, spleen, liver, and kidneys. If visible, the retropharyngeal lymph nodes were also removed. When examined at 8 weeks PI, the mean postmortem score for lesions disclosed in the group infected with 0.8×10^2 CFU was 1 (SEM 0.56) compared to 9.25 (SEM 1.1) in the group infected with 1.1×10^3 CFU. A mean postmortem score of 1.25 (SEM 1.25) was calculated for the group infected with 0.8×10^2 CFU and examined postmortem at 20 weeks, compared to 7.75 (SEM 1.31) for the group infected with 1.1×10^3 CFU examined at 20 weeks PI (Figure 1). The differences in the postmortem score between groups infected with 0.8×10^2 and 1.1×10^3 CFU and examined at 8 weeks PI were statistically significant ($P < .001$). Differences were also statistically significant at the $P < .01$ level between the group infected with 0.8×10^2, examined at 8 weeks PI and the group infected with 1.1×10^3 CFU at 20 weeks PI, and the group infected with 0.8×10^2 CFU at 20 weeks PI compared to the group infected with 1.1×10^3 CFU and examined at 8 weeks PI (Figure 1).

3.5. *Culture of M. bovis from Tissue Samples.* During postmortem examination, a number of urine samples were removed for culture; however, *M. bovis* was not isolated from any of these samples. Of the 156 tissue samples submitted, *M. bovis* was isolated from 102 samples (65%) using a combination of qualitative and quantitative culture. Isolates obtained from a representative panel of culture positive samples were confirmed as AF2122/97, by VNTR. The number of samples from which *M. bovis* was isolated (qualitative culture), and the concentration of *M. bovis* (CFU/g $\times 10^2$) in the samples (quantitative culture) is shown in Tables 1 to 4.

At 8 weeks PI, *M. bovis* was isolated from 29/43 samples (69%) from the group infected with 0.8×10^2 CFU using a combination of qualitative and/or quantitative culture compared to 30/42 samples (71%) from the group infected with 1.1×10^3 CFU. Estimates of bacterial counts varied widely within both groups examined at 8 weeks PI (Tables 1 and 2). The differences between mean group values for bacterial load were not statistically significant. In both the 0.8×10^2 and 1.1×10^3 CFU groups at 8 weeks PI, *M. bovis* was consistently isolated from the lower respiratory tract (lungs, lung-associated lymph nodes) and mesenteric lymph nodes with only occasional isolation from the extrapulmonary sites (submandibular and retropharyngeal LN, kidney, liver, and spleen).

At 20 weeks PI, *M. bovis* was isolated from 16/40 (40%) samples from the group infected with 0.8×10^2 CFU using a combination of qualitative and/or quantitative culture compared to 32/40 (80%) samples from the group infected with 1.1×10^3 CFU. Estimates of bacterial counts varied widely within both groups examined at 20 weeks PI (Tables 3 and 4). The differences between mean group values for

TABLE 2: Isolation of *M. bovis* from a range of tissues taken from 4 ferrets infected with 1.1×10^3 CFU and examined postmortem at 8 weeks post infection.

Tag number	349			358			728			884		
Tissue	LS[1]	QL[2]	QN[3]	LS	QL	QN	LS	QL	QN	LS	QL	QN
Submandibular LN	0	+	−	0	−	−	0	−	−	0	−	−
Retropharyngeal LN	NS[4]	NS	NS	0	−	−	NS	NS	NS	0	−	−
Mesenteric LN	1	+	−	2	+	−	1	+	−	1	+	−
Upper left lung	1	+	−	0	+	−	2	+	−	2	+	−
Lower left lung	2	+	34	1	+	−	2	+	−	0	−	−
Upper right lung	2	+	21.6	0	+	−	2	+	−	0	−	−
Lower right lung	2	+	21.7	2	+	−	3	+	−	2	+	−
Lung associated LN	0	+	52.3	2	+	−	2	+	7	2	+	−
Kidney	0	−	−	0	−	−	0	−	0.22	0	−	−
Liver	0	−	38.5	0	−	−	0	+	26.3	0	−	−
Spleen	2	+	51.1	1	+	−	0	+	0.19	0	−	2.31

[1] LS: lesion score.
[2] QL: qualitative bacteriology.
[3] QN: quantitative bacteriology ($\times 10^2$ CFU/g).
[4] NS: not sampled.

TABLE 3: Isolation of *M. bovis* from a range of tissues taken from 4 ferrets infected with 0.8×10^2 CFU and examined postmortem at 20 weeks post infection.

Tag number	262			456			940			78968		
Tissue	LS[1]	QL[2]	QN[3]	LS	QL	QN	LS	QL	QN	LS	QL	QN
Submandibular LN	0	−	−	0	−	2.1	0	−	−	0	−	−
Retropharyngeal LN	NS[4]	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Mesenteric LN	1	+	25.7	0	+		0	+	70	0	+	7.0
Upper left lung	2	+	−	0	−	417	0	−	−	0	−	−
Lower left lung	2	+	−	0	−	−	0	−	−	0	−	−
Upper right lung	0	−	−	0	−	−	0	−	−	0	−	−
Lower right lung	0	−	−	0	−	−	0	−	−	0	−	−
Lung associated LN	0	+	62.5	0	+	−	0	−	−	0	+	80.6
Kidney	0	−	−	0	−	−	0	−	−	0	−	−
Liver	0	+	−	0	+	−	0	−	−	0	−	−
Spleen	0	+	0.78	0	+	−	0	−	−	0	+	−

[1] LS: lesion score.
[2] QL: qualitative bacteriology.
[3] QN: quantitative bacteriology ($\times 10^2$ CFU/g).
[4] NS: not sampled.

bacterial load were not statistically significant. At 20 weeks PI, *M. bovis* was isolated from all of the mesenteric and lung-associated LN cultured from the 0.8×10^2 CFU infection group, whereas *M. bovis* was isolated on only a few occasions from lungs, and extra-pulmonary sites. In contrast, *M. bovis* was isolated consistently from the lungs and spleen as well as the lung associated LN and mesenteric LN in the 1.1×10^3 CFU group at 20 weeks, with only occasional isolation from kidney and liver tissues. *M. bovis* was isolated from only one submandibular LN in the 0.8×10^2 CFU infection group at 20 weeks PI.

3.6. Strain Confirmation by VNTR. In total, 65 putative *M. bovis* isolates from AGI, faeces, and tissue samples were analysed further by VNTR. All were confirmed to be *M. bovis* strain AF2122/97.

TABLE 4: Isolation of *M. bovis* from a range of tissues taken from 4 ferrets infected with 1.1×10^3 CFU and examined postmortem at 20 weeks post infection.

| Tag number | 015 | | | 507 | | | 780 | | | 89968 | | |
Tissue	LS[1]	QL[2]	QN[3]	LS	QL	QN	LS	QL	QN	LS	QL	QN
Submandibular LN	0	−	−	0	−	−	0	−	−	0	−	−
Retropharyngeal LN	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Mesenteric LN	1	+	239	0	+	20.6	0	+	224	2	+	11.4
Upper left lung	0	+	2.1	3	+	387	3	+	0.21	0	+	−
Lower left lung	0	+	2.98	1	+	13.6	1	+	6.7	1	+	−
Upper right lung	0	+	0.45	3	+	1.5	3	+	2.45	0	−	−
Lower right lung	2	+	0.39	3	+	2.3	3	+	4.82	2	+	−
Lung associated LN	1	+	1550	0	+	4.3	0	+	3490	0	+	9.38
Kidney	0	−	−	0	−	19.1	0	+	−	0	−	−
Liver	1	−	0.63	0	−	−	0	+	9.48	0	+	−
Spleen	0	+	0.81	0	+	−	0	+	25.5	1	+	−

[1] LS: lesion score.
[2] QL: qualitative bacteriology.
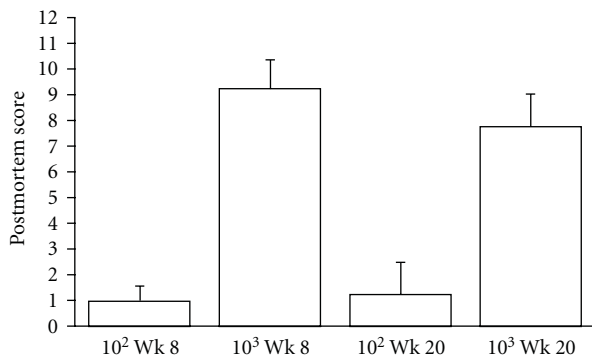[3] QN: quantitative bacteriology ($\times 10^2$ CFU/g).
[4] NS: not sampled.

FIGURE 1: The mean postmortem scores for each group of ferrets infected with either 0.8×10^2 or 1.1×10^3 CFU *M. bovis* and examined postmortem at either 8 or 20 weeks post infection. Differences between the 10^2 and 10^3 CFU infection groups at 8 weeks post infection were significant at the $P < .001$ level, while differences at week 20 post infection were significant at the $P < .01$ level.

4. Discussion

The purpose of this study was to establish an experimental model of infection in the ferret, using *M. bovis* culture as the main read out, that would induce disease and prove suitable for the future evaluation of vaccine efficacy. In addition, the lower respiratory tract was targeted in this experiment since bovine tuberculosis is principally an infectious disease of the respiratory system [13, 14]. Some tissues from the upper respiratory tract (submandibular lymph nodes) were removed for culture while others (retropharyngeal lymph nodes) proved very difficult to locate. No attempts were made to sample nasal mucus, for example, due to the need for frequent anaesthesia and its inherent risks. This rationale

follows the findings summarised by Corner et al. [15]. The immunology of vaccination and challenge in the ferret infection model will be described elsewhere.

At the outset, we intended to deliver two infection doses of *M. bovis* with a 50-fold difference in CFU, in order to measure the impact of dose on the establishment of infection. However, the actual difference between the two doses was 14-fold but we were still able to observe differences between these groups at 8 and 20 weeks post infection. At postmortem examination a number of tissues appeared to be tuberculous and only those lesions where infection was subsequently confirmed by culture, were assigned a postmortem score and used in analysis. Differences in lesion score between the two infection groups were statistically significant ($P < .001$). It would appear that despite administration of a heavier infection dose and a longer infection period, there was not a greater disease burden at 20 weeks post infection. This observation could be explained by a lack of biological significance between the two infection doses. However, since there was no significant development of disease as measured by culture confirmed lesions, this suggests that ferrets, as with badgers, may become latently infected [14, 15], rather than progress toward a more severe form of the disease.

Bacteriological culture provided a reliable and specific method for the isolation of the infection strain (*M. bovis* AF2122/97) from tissue samples obtained in this experiment. The combination of qualitative (BACTEC MGIT) and quantitative (7H11-OADC agar plates) culture resulted in isolation of *M. bovis* from 67% tissue samples submitted for culture, including decontaminated and recultured samples. In total, 69% *M. bovis* positive samples were detected by both qualitative and quantitative culture. *M. bovis* was isolated from a further 41% samples by qualitative culture alone and 11% samples by quantitative culture alone. These results illustrate that neither culture method, when used

individually, was capable of detecting all of the *M. bovis* positive samples and emphasises the importance of applying more than one culture method to maximise recovery of viable mycobacteria [16–19].

Bacterial counts varied widely with the lowest count observed in the 0.8×10^2 group examined at 8 weeks PI while the highest bacterial count was observed in the 1.1×10^3 group examined at 20 weeks. Tissues which consistently had the highest bacterial counts included the lungs, lung-associated lymph node, and mesenteric lymph nodes, with lower bacterial counts commonly observed in tissues such as the liver, spleen, and kidney. This is a feature of the aerosol infection method [10] and confirms delivery of *M. bovis* to the lower respiratory tract tissues of the lung [10, 20, 21] where infection was established. However, the finding that all mesenteric lymph nodes were infected was surprising and less easily explained. Irrespective of bacterial load, each ferret was found to have infected mesentery lymph nodes, a finding that could be peculiar to this model using this route of infection and infectious dose. Lugton et al. (1997) concluded that in naturally infected New Zealand ferrets, infection was acquired primarily by the oral route, most likely through scavenging TB-infected carrion [22]. In over 60% of naturally infected ferrets with gross lesions, the mesentery lymph nodes were found to be infected. These findings differ from those reported in this study where the route of infection and, likely, the infectious dose were very different. Gallagher et al. reported faecal excretion in 65% of naturally infected badgers with pulmonary lesions, with between 10^2 and 10^5 CFU *M. bovis* per gram of faeces [23]. Exposure initiated by inhalation of *M. bovis* can establish infection in the lungs with subsequent spread by haematogenous dissemination to distal lymph nodes, and visceral organs [24, 25]. There were no significant differences in the mean bacterial counts between the groups. However, there was considerable range in the numbers of bacteria recovered. This confirms the pattern of distribution for *M. bovis* primarily to the lung with spread to the mesenteric LN. Spread of infection to the kidneys, spleen, and liver was not confined to those ferrets with high bacterial loads in the lungs. Although this study was based on small numbers of ferrets in each infection group, this data suggests that spread of infection between various organs was influenced by a factor or factors other than bacterial load. Currently, we do not have sufficient additional data (histopathology, e.g.) to explain these findings but analysis of immunological data, when available, may provide clarification.

Faecal samples were taken from the latrine area of each pen to determine if *M. bovis* was excreted during the infection period. It was important to establish whether or not faecal excretion was a feature of this model, and, if so, would vaccination experiments (as part of the long term strategy) prevent release from infected animals? A small proportion of faecal samples were positive by BACTEC MGIT culture, with a clear bias towards groups receiving 1.1×10^3 CFU. Faecal excretion is not surprising since *M. bovis* was isolated from the mesenteric LN of all ferrets. Interestingly, *M. bovis* was isolated from faecal samples treated with oxalic acid but not from corresponding samples treated with HPC,

indicating greater toxicity of HPC for *M. bovis* cells. This is in sharp contrast to previous published findings relating to use of decontamination agents [26]; however, much of this work was carried out with tissue samples rather than faecal samples. The recovery of *M. bovis* from faeces indicates excretion of viable bacteria, which could potentially act as a source of reinfection for ferrets. It is difficult to determine whether reinfection occurred via the oral route in this experiment. In field cases of TB in ferrets gross lesions are often observed in LN draining the alimentary tract, particularly the retropharyngeal and mesenteric LN [9, 27, 28]. In addition, over 60% of ferrets displaying gross lesions at a single tissue site also had infection of the mesenteric LN [27]. Such observations have led to the assumption that TB in wild ferrets is transmitted via the oral route through scavenging of TB-infected carcasses [24], an infectious dose which is necessarily higher than doses given via the respiratory route [29].

However, Cross et al. described oral inoculation in ferrets where *M. bovis* was isolated from those lymph nodes draining the head and mesenteries, with spread to the thoracic area, possibly by bacteraemia [30]. In our model, this mechanism may also account for the spread from the thoracic cavity to the mesenteries. It is unlikely that coprophagy contributed to reinfection or enhanced spread, since typically, bacterial load was less in mesenteric lymph nodes compared to the lungs.

Despite the differences in the two intended doses, aerosol infection establishes infection primarily in the lungs and associated lymph nodes with spread to the mesentery lymph nodes and occasionally to the liver, spleen, and kidneys. The model described here resembles the natural infection seen in badgers where the infection is principally found in the respiratory tract [14]. It is also clear that isolation of *M. bovis* by culture is sensitive and specific; however, reliance on one culture method alone is not sufficient to maximise recovery of *M. bovis* from a range of tissues.

The development of an infection model in ferrets to measure vaccine efficacy is not novel. Qureshi et al. reported partial protection in ferrets vaccinated with *M. bovis* BCG and challenged with *M. bovis*, both delivered by the oral route [31]. The challenge dose for this study was 5×10^6 CFU *M. bovis*, considerably greater that the dose given to ferrets in the current study. However, proof of principle was established with demonstration of immunity to infection. In future studies, the authors aim to refine ferret vaccination and challenge to identify vaccines that are effective in the control of bovine tuberculosis.

5. Conclusions

From the findings described above, we have established an infection model in the ferret. There are a number of similarities between this model and the natural disease seen in badgers, including the establishment of respiratory disease, spread of infection to the viscera and in the potential to establish latent infection. These factors indicate that this model is pertinent to the study of bovine tuberculosis in badgers. Given the phylogenetic similarity of badgers and

ferrets, there is a distinct advantage in developing this model further to study vaccines designed to control *M. bovis* infection in wildlife species. Some questions remain unanswered; however, analysis of the immunological data from this study may clarify the role of this model and contribute toward a clearer understanding of the immune responses that follow infection and vaccination.

Acknowledgments

References

[1] R. J. Delahay, G. J. Wilson, G. C. Smith, and C. L. Cheeseman, "Vaccinating badgers (*Meles meles*) against *Mycobacterium bovis*: the ecological considerations," *The Veterinary Journal*, vol. 166, no. 1, pp. 43–51, 2003.

[2] M. A. Chambers, F. Rodgers, R. J. Delahay et al., "Bacillus Calmette-Guerin vaccination reduces the severity and progression of tuberculosis in badgers," *Proceedings of the Royal Society B*. In Press.

[3] L. A. L. Corner, E. Costello, S. Lesellier, D. O'Meara, and E. Gormley, "Vaccination of European badgers (*Meles meles*) with BCG by the subcutaneous and mucosal routes induces protective immunity against endobronchial challenge with *Mycobacterium bovis*," *Tuberculosis*, vol. 88, no. 6, pp. 601–609, 2008.

[4] S. Lesellier, L. Corner, E. Costello et al., "Antigen specific immunological responses of badgers (*Meles meles*) experimentally infected with *Mycobacterium bovis*," *Veterinary Immunology and Immunopathology*, vol. 122, no. 1-2, pp. 35–45, 2008.

[5] J. Dietrich, K. Weldingh, and P. Andersen, "Prospects for a novel vaccine against tuberculosis," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 163–169, 2006.

[6] T. Kuiken, J. van den Brand, D. van Riel, M. Pantin-Jackwood, and D. E. Swayne, "Comparative pathology of select agent influenza a virus infections," *Veterinary Pathology*, vol. 47, no. 5, pp. 893–914, 2010.

[7] S. Pillet, N. Svitek, and V. von Messling, "Ferrets as a model for morbillivirus pathogenesis, complications, and vaccines," *Current Topics in Microbiology and Immunology*, vol. 330, pp. 73–87, 2009.

[8] C. J. Sigurdson, C. K. Mathiason, M. R. Perrott et al., "Experimental chronic wasting disease (CWD) in the ferret," *Journal of Comparative Pathology*, vol. 138, no. 4, pp. 189–196, 2008.

[9] J. R. Ragg, H. Moller, and K. A. Waldrup, "The prevalence of bovine tuberculosis (*Mycobacterium bovis*) infections in feral populations of cats (*Felis catus*), ferrets (*Mustela furo*) and stoats (*Mustela erminea*) in Otago and Southland, New Zealand," *New Zealand Veterinary Journal*, vol. 43, no. 7, pp. 333–337, 1995.

[10] J. D. Rodgers, N. L. Connery, J. McNair et al., "Experimental exposure of cattle to a precise aerosolised challenge of *Mycobacterium bovis*: a novel model to study bovine tuberculosis," *Tuberculosis*, vol. 87, no. 5, pp. 405–414, 2007.

[11] R. A. Skuce, S. W. McDowell, T. R. Mallon et al., "Discrimination of isolates of *Myobacterium bovis* in Northern Ireland on the basis of variable numbers of tandem repeats (VNTRs)," *Veterinary Record*, vol. 157, no. 17, pp. 501–504, 2005.

[12] G. W. de Lisle, R. P. Kawakami, G. F. Yates, and D. M. Collins, "Isolation of *Mycobacterium bovis* and other mycobacterial species from ferrets and stoats," *Veterinary Microbiology*, vol. 132, no. 3-4, pp. 402–407, 2008.

[13] S. D. Neill, J. Cassidy, J. Hanna et al., "Detection of *Mycobacterium bovis* infection in skin test-negative cattle with an assay for bovine interferon-gamma," *Veterinary Record*, vol. 135, no. 6, pp. 134–135, 1994.

[14] D. Murphy, E. Gormley, E. Costello, D. O'Meara, and L. A. L. Corner, "The prevalence and distribution of *Mycobacterium bovis* infection in European badgers (*Meles meles*) as determined by enhanced post mortem examination and bacteriological culture," *Research in Veterinary Science*, vol. 88, no. 1, pp. 1–5, 2010.

[15] L. A. L. Corner, D. Murphy, and E. Gormley, "*Mycobacterium bovis* infection in the Eurasian badger (*Meles meles*): the disease, pathogenesis, epidemiology and control," *Journal of Comparative Pathology*, vol. 144, no. 1, pp. 1–24, 2011.

[16] L. A. Corner, "Personal communication," 2010.

[17] C. E. Stager, J. P. Libonati, S. H. Siddiqi et al., "Role of solid media when used in conjunction with the BACTEC system for mycobacterial isolation and identification," *Journal of Clinical Microbiology*, vol. 29, no. 1, pp. 154–157, 1991.

[18] L. A. Corner and C. Nicolacopoulos, "Comparison of media used for the primary isolation of *Mycobacterium bovis* by veterinary and medical diagnostic laboratories," *Australian Veterinary Journal*, vol. 65, no. 7, pp. 202–205, 1988.

[19] L. A. Corner, "Post mortem diagnosis of *Mycobacterium bovis* infection in cattle," *Veterinary Microbiology*, vol. 40, no. 1-2, pp. 53–63, 1994.

[20] C. S. Cox, *The Aerobiological Pathway of Organisms*, Wiley-Interscience Publications, Chichester, UK, 1987.

[21] D. W. Smith, D. N. McMurray, E. H. Wiegeshaus, A. A. Grover, and G. E. Harding, "Host-parasite relationships in experimental airborne tuberculosis. IV. Early events in the course of infection in vaccinated and nonvaccinated guinea pigs," *American Review of Respiratory Disease*, vol. 102, no. 6, pp. 937–949, 1970.

[22] I. W. Lugton, G. Wobeser, R. S. Morris, and P. Caley, "Epidemiology of *Mycobacterium bovis* infection in feral ferrets (*Mustela furo*) in New Zealand: II. Routes of infection and excretion," *New Zealand Veterinary Journal*, vol. 45, no. 4, pp. 151–157, 1997.

[23] J. Gallagher, R. Monies, M. Gavier-Widen, and B. Rule, "Role of infected, non-diseased badgers in the pathogenesis of tuberculosis in the badger," *Veterinary Record*, vol. 142, no. 26, pp. 710–714, 1998.

[24] J. Gallagher and P. A. Jenkins, "Mycobacterial diseases," in *Zoonoses*, pp. 155–164, Oxford University Press, Oxford, UK, 1998.

[25] R. S. Clifton-Hadley, "Badgers, bovine tuberculosis and the age of reason," *The British Veterinary Journal*, vol. 152, no. 3, pp. 243–246, 1996.

[26] L. A. Corner, A. C. Trajstman, and K. Lund, "Determination of the optimum concentration of decontaminants for the primary isolation of *Mycobacterium bovis*," *New Zealand Veterinary Journal*, vol. 43, pp. 129–133, 1995.

[27] J. R. Ragg, K. A. Waldrup, and H. Moller, "The distribution of gross lesions of tuberculosis caused by *Mycobacterium bovis* in feral ferrets (*Mustela furo*) from Otago, New Zealand," *New Zealand Veterinary Journal*, vol. 43, no. 7, pp. 338–341, 1995.

[28] I. W. Lugton, G. Wobeser, R. S. Morris, and P. Caley, "Epidemiology of *Mycobacterium bovis* infection in feral ferrets (*Mustela furo*) in New Zealand: I. Pathology and diagnosis," *New Zealand Veterinary Journal*, vol. 45, no. 4, pp. 140–150, 1997.

[29] J. M. Pollock, J. D. Rodgers, M. D. Welsh, and J. McNair, "Pathogenesis of bovine tuberculosis: the role of experimental models of infection," *Veterinary Microbiology*, vol. 112, no. 2-4, pp. 141–150, 2006.

[30] M. L. Cross, R. E. Labes, and C. G. Mackintosh, "Oral infection of ferrets with virulent *Mycobacterium bovis* or *Mycobacterium avium*: susceptibility, pathogenesis and immune response," *Journal of Comparative Pathology*, vol. 123, no. 1, pp. 15–21, 2000.

[31] T. Qureshi, R. E. Labes, M. L. Cross, J. F. T. Griffin, and C. G. Mackintosh, "Partial protection against oral challenge with *Mycobacterium bovis* in ferrets (*Mustela furo*) following oral vaccination with BCG," *International Journal of Tuberculosis and Lung Disease*, vol. 3, no. 11, pp. 1025–1033, 1999.

Research Article

Overt *Mycobacterium avium* subsp. *paratuberculosis* Infection: An Infrequent Occurrence in Archived Tissue from False TB Reactor Cattle in Michigan, USA

Scott D. Fitzgerald,[1] Steven R. Bolin,[1] Kathryn G. Boland,[2] Ailam Lim,[1] and John B. Kaneene[3]

[1] *Department of Pathobiology and Diagnostic Investigation and the Diagnostic Center for Population and Animal Health, College of Veterinary Medicine, Michigan State University, Lansing, MI 48910, USA*

[2] *Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, WA 99164, USA*

[3] *Center for Comparative Epidemiology, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824, USA*

Correspondence should be addressed to Scott D. Fitzgerald, fitzgerald@dcpah.msu.edu

The objective of this study was to retrospectively determine whether or not cattle from the state of Michigan which were classified as bovine tuberculosis reactors, based on currently approved field and laboratory testing methods, were overtly infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Included in this study were 384 adult cattle submitted to the Diagnostic Center for Population and Animal Health over a seven-year period. Cattle were tested utilizing standard methods to confirm that all cattle were lesion and culture negative for infection with *Mycobacterium bovis* at postmortem examination. Retrospective analysis of formalin-fixed, paraffin-embedded sections of ileum and ileocecal lymph node were evaluated by histopathology, acid-fast staining, and PCR assays to detect MAP. Overall, only 1.04 percent of cattle showed overt infection with MAP on visual examination of sections of ileum and/or ileo-cecal lymph node. This increased slightly to 2.1 percent of cattle likely infected with MAP after additional testing using a PCR assay. Based on these results, we found no evidence that overt infection with MAP plays a major role in the false tuberculosis reactor test results for cattle examined in this study.

1. Introduction

The identification of bovine tuberculosis (TB) in white-tailed deer in Michigan in 1994, and the subsequent identification of TB in cattle, has resulted in a long-term surveillance program for TB in cattle [1]. To date, 50 cattle herds have been found in Michigan that contained one or more *M. bovis* infected animals [2]. Thousands of cattle have been tested as suspect reactors on the caudal fold test (CFT) and comparative cervical test (CCT) or gamma interferon assay (γ-IFN), but only 138 cattle have been found infected with *M. bovis*. The large number of cattle found as false-positive reactors on field and laboratory tests, compared with the relatively small number of cattle eventually diagnosed as TB positive, is a reflection of the specificity of the currently approved antemortem diagnostic procedures when disease prevalence is low. Development of improved antemortem screening tests for detection of cattle and other species infected with *Mycobacterium bovis (M. bovis)*, the causal agent of bovine tuberculosis, has been the subject of recent research [3–10]. This activity is driven by the less than optimal sensitivity and specificity of currently approved diagnostic tests for antemortem detection of TB.

Diagnostic tests currently approved to screen for bovine tuberculosis in the USA include the CFT, the CCT, and the whole blood γ-INF assay [11]. The skin tests measure a cell-mediated immune response (delayed type hypersensitivity response) stimulated by an injection of purified protein derivative (PPD) obtained from cultured *M. bovis*. The whole blood γ-INF assay also measures a cell mediated response

(production of γ-INF by lymphocytes) after stimulation with PPD. The content of PPD is a nonstandardized and variable complex mixture of various antigenic components prepared from cultures of mycobacteria [6, 12, 13]. Many of the antigens in PPD are shared among the various species of pathogenic and nonpathogenic mycobacteria. Thus, there is concern that previous exposure of cattle, or concurrent infection of cattle, with mycobacteria other than *M. bovis* will affect the sensitivity and/or specificity of current diagnostic assays [6, 12, 14–20]. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is widespread in Michigan, and infection of cattle with that organism may affect the outcome of currently approved tests for TB [19, 21, 22].

The relatively high prevalence of cattle herds infected with MAP and cattle infected with MAP in Michigan, and the low prevalence of TB in cattle examined postmortem, prompted us to conduct a retrospective study to determine whether overt infection with MAP was an important cause of false-positive reactors in currently approved tests for TB. Because TB in cattle is a regulatory disease that has zoonotic potential, postmortem examination of cattle that are suspect for TB is focused on collection of tissues known to be targeted by *M. bovis*, and collection of additional tissues for use other than diagnosis of TB is not standard practice. To determine infection of cattle with MAP, we were limited to formalin-fixed, paraffin-embedded distal ileum and ileo-cecal lymph node. Our purpose was to examine those available tissues for microscopic lesions consistent with infection with MAP, to identify acid-fast stained organisms within the tissues, and to substantiate infection with MAP using polymerase chain reaction (PCR) assays.

2. Materials and Methods

2.1. Cattle Selection Criteria. Cattle included in this retrospective study were from 13 contiguous counties within the north-east portion of the lower peninsula of Michigan. This region of the state is under an active surveillance program for bovine tuberculosis, as small numbers of infected cattle are detected periodically in that area [23]. Cattle designated for postmortem examination were removed from the herd the day before postmortem examination and transported to an isolated and secured holding facility. All cattle in the study were examined postmortem for bovine tuberculosis at the Diagnostic Center for Population and Animal Health (DCPAH), Michigan State University, between June 7, 2001 and May 1, 2008. The cattle were adult animals (greater than two years old) and mostly female (96%). There was a distinct predominance of dairy cattle compared to beef cattle (2 : 1). Finally, only cattle purchased by the State of Michigan for purposes of tuberculosis surveillance were included. All cattle in the study were found negative for overt infection with *M. bovis* using standardized postmortem diagnostic methods [24, 25]. Lymphoid tissues from all cattle were submitted to the National Veterinary Services Laboratory (NVSL) in Ames, Iowa, for confirmation of the histopathologic lesions and for culture for *M. bovis*. The cattle were grouped according to outcome of the primary caudal fold skin test

(CFT) and the secondary comparative cervical skin test or whole blood gamma interferon assay for bovine tuberculosis. Group 1 consisted of 189 cattle that were false-positive reactors on both primary and secondary tests. Group 2 consisted of 122 cattle that were false-positive reactors on the CFT only. Group 3 consisted of 73 cattle which were negative on the CFT and were examined postmortem without a secondary test being performed; this group was considered the negative control group. The cattle included in groups 2 and 3 were from tuberculosis positive herds that were being depopulated or were cattle that had been exposed to animals that had bovine tuberculosis and were being examined postmortem for bovine tuberculosis.

2.2. Necropsy and M. bovis Diagnostics. Cattle were transported alive to the DCPAH, where they were euthanized by overdosage of intravenous barbiturates. The same diagnostic protocols were followed for all animals. Gross postmortem examinations were conducted with attention directed to examination of the animals' lungs, lymph nodes, and ileal-cecal-colic junction. Lymph nodes were harvested by anatomic region (cranial, thoracic, and abdominal), along with a section of terminal ileum. Lymph nodes were serially sectioned for gross examination. Portions of each lymph node and ileum were fixed in 10% neutral-buffered formalin for histopathology, while other portions of the same lymph nodes were shipped fresh on ice packs to the Tuberculosis Laboratory, National Veterinary Service Laboratories (NVSL), Ames, Iowa, for mycobacterial isolation and identification using previously described techniques [24, 25]. Formalin-fixed samples of lymph nodes and distal ileum were embedded in paraffin, sectioned at 5 um, and routinely processed for both hematoxylin and eosin (H&E) and Ziehl-Neelsen acid fast staining. Sections of stained tissue were examined microscopically for granulomatous inflammation and for presence of acid-fast bacilli.

2.3. Tissue Processing and DNA Extraction. Three serial sections, 20 μm thick, were cut from each block of paraffin-embedded distal ileum. The tissue sections were placed into a sterile 1.5 mL microcentrifuge tube and stored at room temperature until processed for extraction of DNA. Between blocks of paraffin-embedded tissue, the knife blade of the microtome was wiped clean with an absorbent tissue impregnated with a 10% solution of household bleach in 0.01 M phosphate buffered saline solution (pH 7.2). For DNA extraction, 1 section of paraffin-embedded ileum and ileo-cecal lymph node from each animal was placed in a microcentrifuge tube, using a sterile toothpick. The remaining sections of paraffin-embedded tissue were stored at room temperature for use as needed. Extraction of DNA and PCR were performed using previously described methods and PCR primers with slight modification [26, 27]. Briefly, a 1.5 mL microcentrifuge tube containing one section of paraffin embedded ileum was centrifuged at 16,000 × g for 1 minute at 24°C to collapse the tissue section. Approximately 200 μL of a 0.5% solution of polyoxyethylene-sorbitan monolaurate (Tween 20) in DNase and RNase free molecular

biology grade water was added to each microcentrifuge tube. The tubes were then subjected to 2 cycles of boil and snap freeze using first a 10-minute incubation at 100°C followed immediately by a 3-minute immersion into a dry ice-ethanol bath. Finally, the tubes were incubated an additional 10 minutes at 100°C and centrifuged at $3,000 \times g$ at 4°C to pellet tissue debris and float the melted paraffin to the surface. The paraffin layer was removed with a sterile toothpick, and $5 \mu L$ of the liquid phase was aspirated and inoculated into a $200 \mu L$ PCR tube containing PCR primers and $20 \mu L$ PCR reaction mixture. The DNA extraction method was tested on archived formalin-fixed, paraffin-embedded sections of ileum and ileo-cecal lymph node from 25 cattle not submitted to DCPAH for TB postmortem examination and confirmed infected with MAP by bacterial culture. The tissue blocks had been archived from 5 to 8 years at the time of DNA extraction, and the number of acid-fast organisms observed in these sections varied from numerous to none. Only sections of tissues from one culture positive animal produced negative results on PCR assay. Multiple paraffin-embedded blocks of tissues from that animal were examined microscopically, and the block that tested negative on PCR assay lacked visible acid-fast organisms.

2.4. Polymerase Chain Reaction (PCR) Assays. The PCR for MAP used primers from the IS900 sequence, 5′-CCGCTA-ATTGAGAGATGCGATTGG and 5′-AATCAACTCCAG-CAGCGCGGCCTCG, and yielded a product of 229 base pairs. This PCR was done on all 384 samples of ileum and ileo-cecal lymph node. Subsequently, 271 representative samples of ileum from all 3 groups of cattle (including all tissues that tested positive for MAP) were subjected to a PCR assay designed to detect the *M. avium* group of organisms. The PCR primers used for that assay were from the gene for 16s ribosomal RNA, 5′-AGAGTTTGATCCTGGCTCAG and 5′-ACCAGAAGACATGCGTCTTG, and yielded a product of 193 base pairs. The PCR reaction mixture included a PCR buffer, 1.5mM MgCl_2, 0.2 mM each dATP, dGTP, dCTP, and dTTP, 1.25 units HotStar Taq polymerase, $0.4 \text{pmol}/\mu L$ of each primer for IS900 or $1.0 \text{pmol}/\mu L$ of each primer for the *M. avium* group, and $5 \mu L$ of sample DNA. The PCR reaction conditions were 1 cycle of 95°C for 15 min, 50 cycles of 94°C for 1 minute, 65°C (MAP) or 61°C (*M. avium*) for 15 sec, 72°C for 2 min, followed by 1 cycle of 72°C for 10 min. PCR amplification products were analyzed by gel electrophoresis using a 1.5% agarose gel in sodium borate buffer with 0.5 mg/mL of ethidium bromide mixed into the molten gel [28].

2.5. Statistical Analysis. Associations between PCR-confirmed MAP status and *M. bovis* test status (primary and secondary test reactors, primary (CFT) reactors only, and primary test negative cattle) were measured with odds ratios for *M. bovis* test reactors in comparison with *M. bovis* test negative cattle, and Fisher's Exact Test was used to determine whether these associations were statistically significant ($P < .05$).

3. Results

3.1. Group 1 Cattle. This group of cattle were found suspect for TB on sequential primary and secondary screening tests for bovine tuberculosis. The second of the sequential screening tests, either the CCT or gamma interferon assay, is designed to reduce the number of false-positive reactors that may be attributed to previous infection of the animal with the *M. avium* group of mycobacteria. Of 189 cattle that were positive reactors on both primary and secondary tests for bovine TB, only 1 cow was positive by PCR for MAP (Tables 1 and 2). Gross or microscopic lesions consistent with Johne's disease were not observed in tissues from that cow or in tissues from any other animal in Group 1 (Table 2). Also, acid fast organisms were not found in sections of the ileum or in ileo-cecal lymph nodes from any animal in Group 1. The PCR assay designed for detection of the *M. avium* group of organisms was negative for all cattle tested in this group (Tables 1 and 2).

3.2. Group 2 Cattle. Cattle in this group were false-positive reactors on the CFT and were negative on a secondary test. Cattle that are reactors on the CFT and are negative on a second screening test for infection with *M. bovis* may have been infected with mycobacteria in the *M. avium* group or to environmental mycobacteria and not *M. bovis*. Thus, cattle in Group 2 should have been at higher risk for infection with MAP or with other members of the *M. avium* group of mycobacteria than cattle in Group 1. Three of 122 cattle in Group 2 were positive for infection with MAP by PCR (Tables 1 and 2). Two of those 3 cattle had gross lesions of Johne's disease, consisting of mild to moderate thickening of the terminal ileum wall (Table 2). These two animals also had microscopic granulomatous lesions consistent with Johne's disease including visible acid fast organisms in the ileum and granulomatous lymphadenitis in the ileo-cecal lymph nodes. Additionally, tissue sections from these animals were positive by PCR for the *M. avium* group of bacterium (Tables 1 and 2). The third animal that tested positive for MAP using PCR assay lacked gross or microscopic lesions consistent with Johne's disease and lacked visible acid fast organisms in sections of ileum or ileo-cecal lymph node. This cow was negative by PCR for the *M. avium* group of mycobacteria. None of the remaining 122 cattle in Group 2 had lesions consistent with Johne's disease, had visible acid fast organisms in sections of tissue, or were positive by PCR assay for MAP or the *M. avium* group of mycobacteria.

3.3. Group 3 Cattle. This group consisted mostly of cattle culled from TB-infected herds, but also included some cattle that moved from TB-infected herds to other herds prior to detection of TB in the herd of origin. Reasons for the animals being culled included ill thrift, lameness, mastitis, or other chronic disease conditions. The cattle in this group had been administered the CFT but were not positive reactors on the CFT. Four of the 73 cattle were found positive for MAP by PCR assay, and two of those cattle also were positive on PCR assay for the *M. avium* group of mycobacteria

Table 1: Summary of results of various screening tests for bovine tuberculosis and for results of PCR assays.

M. bovis screening test result	Number of cattle tested	MAP PCR positive	Percent MAP positive	*M. avium* PCR positive
(1) CFT and CCT positive	189	1	0.5%	0
(2) CFT positive	122	3	2.5%	2
(3) CFT negative (Control group)	73	4	5.5%	1
Totals	384	8	2.1%	3

(Tables 1 and 2). None of those 4 cows had gross lesions consistent with Johne's disease, but acid fast organisms were found in sections of ileum from one cow positive by PCR assay for both MAP and the *M. avium* group of mycobacteria (Tables 1 and 2). None of the remaining 69 cattle in this group had lesions consistent with Johne's disease, had visible acid-fast organisms in sections of tissue, or were positive by PCR assay for the *M. avium* group of mycobacteria.

3.4. Statistical Analysis. There was not a statistically significant difference between the CFT false-positive cattle in Group 2 and CFT negative cattle in Group 3. In addition, the differences between the primary and secondary test reactor group (Group 1) and the CFT negative cattle (Group 3) was close to statistically significant ($P = .0511$) (Table 3).

4. Discussion

In the current study, we attempted to determine if overt infection of cattle with MAP was a common cause of false-positive reactions in currently approved field and laboratory tests for bovine tuberculosis. This was a retrospective study that made use of samples from 384 cattle examined postmortem and diagnosed free of bovine TB. We used a series of diagnostic assays that included gross examination of the ileum for thickening, microscopic examination of hematoxylin and eosin stained sections of distal ileum and ileo-cecal lymph node for histiocytic or granulomatous infiltrates, microscopic examination of acid-fast stained sections of ileum and ileo-cecal lymph node for detection of stained organisms, and finally PCR. This diagnostic approach is similar to current recommendations for detection of Johne's disease postmortem [29].

Our results were similar with recent studies that assessed formalin-fixed, paraffin-embedded tissues for surveillance for overt Johne's disease in randomly selected cattle at slaughter [30, 31]. Very few cattle ($n = 4$, 1%) in the current study had lesions and/or presence of acid fast stained organism that would be considered consistent with overt infection with MAP. Because bovine TB is a regulatory disease and samples of fresh tissues are shipped to the NVSL for final diagnosis, bacterial culture from feces or tissue was not attempted at the DCPAH in the current study. It is likely that use of culture techniques or PCR assays on fresh tissue

would have resulted in identification of additional cattle infected with MAP in the absence of overt lesions [30, 31].

Further testing of tissue samples, using a PCR assay for the IS900, yielded positive results from an additional 4 cattle that did not show lesions or acid-fast organisms in tissues. This finding was not surprising because PCR likely is a more sensitive indicator of earlier infection with MAP than either gross or histologic lesions [32]. The PCR assay was based on IS900, a multiple copy transposable element commonly used as a target for detection of MAP [33]. This insertion sequence has been identified in mycobacteria other than MAP [34, 35]. Hence, it is possible that some of the 8 cattle that were positive on PCR may have been simultaneously infected with mycobacterium other than MAP. An attempt was made in the current study to identify cattle infected with mycobacteria other than MAP, using a group-specific PCR assay that can detect most members of the *M. avium* group, including MAP. However, that PCR assay only yielded positive results when acid-fast stained organisms were detected in tissue and when the PCR assay for MAP yielded positive results. Thus we failed to conclusively identify an animal currently infected with a mycobacterium other than MAP. A previous study also found that the PCR assay for the *M. avium* group of organisms yielded fewer positive results using formalin fixed-tissue than the PCR assay for MAP [26].

Based on antemortem laboratory tests, the prevalence of MAP in Michigan's dairy herds has been estimated recently to be about 50% with rates of infection of individual cattle estimated between 5 and 15% for most herds and infection rates higher than 15% in some herds [21, 22, 36]. The prevalence of MAP in Michigan's beef cattle herds is thought to be substantially lower than the prevalence in dairy herds. Approximately one third of the cattle in the current study were beef cattle, and none of the cattle suspected as being infected with MAP in the current study were beef cattle. The inclusion of a substantial number of beef cattle likely lowered rates of infection with MAP detected in the current study.

The cattle in Groups 1 and 2 were reactors in 1 or more field and/or laboratory tests for detection of TB in cattle. If infection with MAP influenced the results of field or laboratory tests for TB by increasing the number of false-positive reactors, cattle in Groups 1 and/or 2 likely would have higher rates of infection with MAP than cattle in Group 3, which consisted of cattle that were negative for TB on the CFT test. That outcome was not observed. Instead, infection with MAP, or other organisms possessing IS900, was identified in 5.5% cattle in Group 3, compared with 2.5% of cattle in Group 2 and 0.5% of cattle in Group 1 (Table 1). Recent reports indicate that previous or current infection with mycobacteria other than the tuberculosis group of mycobacteria adversely affects currently approved field and laboratory tests for detection of bovine TB, resulting in higher rates of false-negative tests [14, 15, 34]. Due to bovine TB being a regulatory disease, we did not attempt to detect *M. bovis* in the group of cattle that were in contact with TB-infected cattle, but had tested negative for TB on the CFT test.

Formalin fixation is known to cause DNA degradation which can compromise use of PCR assays. It is recommended to use PCR primer sets that amplify short sequences

TABLE 2: Summary of pathology and PCR results in MAP infected animals.

Study group	M. bovis test result	H & E histo. result ileum	Acid -fast result ileum	H & E histo. result lymph node[a]	Acid-fast result lymph node[a]	MAP PCR	M. avium PCR	Breed	TB status of herd
Group 1	Primary and secondary test positive.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Jersey	Neg.
Group 2	CFT Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Jersey	Pos.
Group 2	CFT Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Holstein	Pos.
Group 2	CFT Pos.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Holstein	Pos.
Group 3	CFT Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Holstein	Neg.
Group 3	CFT Neg.	Neg.	Pos.	Neg.	Neg.	Pos.	Neg.	Holstein	Pos.
Group 3	CFT Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Holstein	Pos.
Group 3	CFT Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Holstein	Pos.

[a] Lymph node was ileo-cecal lymph node.

TABLE 3: Odds ratios for M. bovis test reactors compared to M. bovis test negative cattle, by PCR-confirmed MAP status.

Study group	MAP PCR positive			Fisher's exact test P value*	Odds ratio	
	Total number	No. of Positive	Percent		Estimate	95% C I
(1) Primary and secondry test reactors	189	1	0.5	.0511	0.11	0.01–1.06
(2) CFT reactor	122	3	2.5	.4281	0.43	0.09–2.00
Groups 1 and 2 combined	311	4	1.3	.0458	0.22	0.05–0.92

* Fisher's exact test compares the difference in the odds ratios between the bovine TB test group in that row verses the bovine TB test negative group.

(less than 200 bp) when formalin-fixed tissues are assayed [37]. The PCR primer sets used in the current study amplified targets of about 200 bp in length, so our assays were at the recommended upper limit for target detection in formalin-fixed tissue. This might have affected our results and reduced the number of cattle that were found infected with MAP. However, the effect of formalin fixation of tissue would occur across all groups of cattle. The relatively low number of primary test negative cattle in this study reduced the statistical power to the point where we could not confirm statistical significance between groups of cattle. The annual number of cattle designated for TB postmortem examination that are test negative of bovine TB is small in Michigan; hence, expanding that group of cattle was not possible in the time frame of the current study.

What other factors might be contributing to the high numbers of false-positive skin test cattle? This retrospective study only evaluated one possible *Mycobacterium* sp. and its presence at a specific regional site (the terminal ileum and ileo-cecal lymph node); exposure of the animal to other environmental *Mycobacteria* spp. located in other anatomic sites is one possible factor [18]. Another cause may be nonmycobacterial infections in tested cattle, such as *Nocardia* spp. [18]. Immunization of cattle for Johne's disease or with experimental *M. bovis* BCG vaccines—neither of these vaccinations are allowed in Michigan—may also cause false skin test results [18]. Finally, this false reactor rate may simply be intrinsic to the skin tests used. When the cattle population is this far advanced along the road to

disease eradication such as is the current situation in the United States in general and Michigan in particular, using a screening test with very high sensitivity may necessitate living with a lower specificity as we attempt to detect the last few remaining infected individuals [18]. While there is no argument that it would be desirable to increase the specificity of current skin testing methods to detect *M. bovis* in cattle, cross-reaction with MAP does not appear to be a major limiting factor in the tests' utility based on this study.

In conclusion, the methods used in this study found few cattle infected with MAP and failed to find a positive association between an infection with MAP and false positive reactions in field and/or laboratory tests for bovine TB.

Acknowledgments

References

[1] S. M. Schmitt, S. D. Fitzgerald, T. M. Cooley et al., "Bovine tuberculosis in free-ranging white-tailed deer from Michigan," *Journal of Wildlife Diseases*, vol. 33, no. 4, pp. 749–758, 1997.

[2] B. Patrick, "Trace investigation finds bovine TB positive herd in Emmet County," September 2010, http://www.michigan.gov/emergingdiseases/0,1607,7-186-25804-243197-,00.html.

[3] L. R. Green, C. C. Jones, A. L. Sherwood et al., "Single-antigen serological testing for bovine tuberculosis," *Clinical and Vaccine Immunology*, vol. 16, no. 9, pp. 1309–1313, 2009.

[4] N. P. Harrington, OM. P. Surujballi, W. R. Waters, and J. F. Prescott, "Development and evaluation of a real-time reverse transcription-PCR assay for quantification of gamma interferon mRNA to diagnose tuberculosis in multiple animal species," *Clinical and Vaccine Immunology*, vol. 14, no. 12, pp. 1563–1571, 2007.

[5] M. E. Jolley, M. S. Nasir, OM. P. Surujballi et al., "Fluorescence polarization assay for the detection of antibodies to *Mycobacterium bovis* in bovine sera," *Veterinary Microbiology*, vol. 120, no. 1-2, pp. 113–121, 2007.

[6] J. M. Pollock, B. M. Buddle, and P. Andersen, "Towards more accurate diagnosis of bovine tuberculosis using defined antigens," *Tuberculosis*, vol. 81, no. 1-2, pp. 65–69, 2001.

[7] J. M. Pollock, M. D. Welsh, and J. McNair, "Immune responses in bovine tuberculosis: towards new strategies for the diagnosis and control of disease," *Veterinary Immunology and Immunopathology*, vol. 108, no. 1-2, pp. 37–43, 2005.

[8] M. Seth, E. A. Lamont, H. K. Janagama et al., "Biomarker discovery in subclinical mycobacterial infections of cattle," *PLoS One*, vol. 4, no. 5, Article ID e5478, 2009.

[9] W. R. Waters, B. J. Nonnecke, M. V. Palmer et al., "Use of recombinant ESAT-6:CFP-10 fusion protein for differentiation of infections of cattle by *Mycobacterium bovis* and by *M. avium* subsp. avium and *M. avium* subsp. *paratuberculosis*," *Clinical and Diagnostic Laboratory Immunology*, vol. 11, no. 4, pp. 729–735, 2004.

[10] W. R. Waters, M. V. Palmer, T. C. Thacker et al., "Early antibody responses to experimental *Mycobacterium bovis* infection of cattle," *Clinical and Vaccine Immunology*, vol. 13, no. 6, pp. 648–654, 2006.

[11] Anonymous, "Bovine tuberculosis eradication: uniform methods and rules, effective January, 2005," *United States Department of Agriculture, Animal and Plant Health Inspection Service*, no. 91-45-011, pp. 1–34, 2005.

[12] M. L. Monaghan, M. L. Doherty, J. D. Collins, J. F. Kazda, and P. J. Quinn, "The tuberculin test," *Veterinary Microbiology*, vol. 40, no. 1-2, pp. 111–124, 1994.

[13] I. Schiller, H. M. Vordermeier, W. R. Waters et al., "Comparison of tuberculin activity using the interferon-γ assay for the diagnosis of bovine tuberculosis," *Veterinary Record*, vol. 167, no. 9, pp. 322–326, 2010.

[14] J. Álvarez, L. de Juan, J. Bezos et al., "Interference of *paratuberculosis* with the diagnosis of tuberculosis in a goat flock with a natural mixed infection," *Veterinary Microbiology*, vol. 128, no. 1-2, pp. 72–80, 2008.

[15] J. Álvarez, L. de Juan, J. Bezos et al., "Effect of *paratuberculosis* on the diagnosis of bovine tuberculosis in a cattle herd with a mixed infection using interferon-gamma detection assay," *Veterinary Microbiology*, vol. 135, no. 3-4, pp. 389–393, 2009.

[16] L. A. Auer and S. M. Schleehauf, "Antibodies to mycobacteria in cattle not infected with *Mycobacterium bovis*," *Veterinary Microbiology*, vol. 18, no. 1, pp. 51–61, 1988.

[17] R. Cooney, J. Kazda, J. Quinn, B. Cook, K. Müller, and M. Monaghan, "Environmental mycobacteria in Ireland as a source of non-specific sensitisation to tuberculins," *Irish Veterinary Journal*, vol. 50, no. 6, pp. 370–373, 1997.

[18] R. de la Rua-Domenech, A. T. Goodchild, H. M. Vordermeier, R. G. Hewinson, K. H. Christiansen, and R. S. Clifton-Hadley, "Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques," *Research in Veterinary Science*, vol. 81, no. 2, pp. 190–210, 2006.

[19] J. R. Dunn, J. B. Kaneene, D. L. Grooms, S. R. Bolin, C. A. Bolin, and C. S. Bruning-Fann, "Effects of positive results for *Mycobacterium avium* subsp *paratuberculosis* as determined by microbial culture of feces or antibody ELISA on results of caudal fold tuberculin test and interferon-γ assay for tuberculosis in cattle," *Journal of the American Veterinary Medical Association*, vol. 226, no. 3, pp. 429–435, 2005.

[20] J. C. Hope, M. L. Thom, B. Villarreal-Ramos, H. M. Vordermeier, R. G. Hewinson, and C. J. Howard, "Exposure to *Mycobacterium avium* induces low-level protection from *Mycobacterium bovis* infection but compromises diagnosis of disease in cattle," *Clinical and Experimental Immunology*, vol. 141, no. 3, pp. 432–439, 2005.

[21] Y. Johnson-Ifearulundu and J. B. Kaneene, "Distribution and environmental risk factors for *paratuberculosis* in dairy cattle herds in Michigan," *American Journal of Veterinary Research*, vol. 60, no. 5, pp. 589–596, 1999.

[22] R. B. Pillars, D. L. Grooms, and J. B. Kaneene, "Longitudinal study of the distribution of *Mycobacterium avium* subsp. *paratuberculosis* in the environment of dairy herds in the Michigan Johne's disease control demonstration herd project," *The Canadian Veterinary Journal*, vol. 50, no. 10, pp. 1039–1046, 2009.

[23] Anonymous, "Tuberculosis in cattle and bison: state and zone designations: Michigan," *Federal Register*, vol. 69, no. 75, pp. 20805–20809, 2004.

[24] P. T. Kent and G. P. Kubica, *Public health mycobacteriology. A guide for the level III laboratory*, U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta, Ga, USA, 1985.

[25] F. S. Nolte and B. Metchock, "Mycobacterium," in *Manual of Clinical Microbiology*, P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken, Eds., pp. 400–437, American Society for Microbiology Press, Washington, DC, USA, 1995.

[26] J. M. Miller, A. L. Jenny, and J. L. Ellingson, "Polymerase chain reaction identification of *Mycobacterium avium* in formalin-fixed, paraffin-embedded animal tissues," *Journal of Veterinary Diagnostic Investigation*, vol. 11, no. 5, pp. 436–440, 1999.

[27] J. M. Miller, A. L. Jenny, and J. B. Payeur, "Polymerase chain reaction detection of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* organisms in formalin-fixed tissues from culture-negative ruminants," *Veterinary Microbiology*, vol. 87, no. 1, pp. 15–23, 2002.

[28] J. R. Brody and S. E. Kern, "Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis," *BioTechniques*, vol. 36, no. 2, pp. 214–216, 2004.

[29] M. T. Collins, I. A. Gardner, F. B. Garry, A. J. Roussel, and S. J. Wells, "Consensus recommendations on diagnostic testing for the detection of *paratuberculosis* in cattle in the United States," *Journal of the American Veterinary Medical Association*, vol. 229, no. 12, pp. 1912–1919, 2006.

[30] S. A. Martinson, P. E. Hanna, B. O. Ikede et al., "Comparison of bacterial culture, histopathology, and immunohistochemistry for the diagnosis of Johne's disease in culled dairy cows,"

Journal of Veterinary Diagnostic Investigation, vol. 20, no. 1, pp. 51–57, 2008.

[31] S. L. B. McKenna, G. P. Keefe, H. W. Barkema et al., "Cow-level prevalence of *paratuberculosis* in culled dairy cows in Atlantic Canada and Maine," *Journal of Dairy Science*, vol. 87, no. 11, pp. 3770–3777, 2004.

[32] N. B. Harris and R. G. Barletta, "*Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine," *Clinical Microbiology Reviews*, vol. 14, no. 3, pp. 489–512, 2001.

[33] T. J. Bull, J. Hermon-Taylor, I. Pavlik, F. El-Zaatari, and M. Tizard, "Characterization of IS900 loci in *Mycobacterium avium* subsp. *paratuberculosis* and development of multiplex PCR typing," *Microbiology*, vol. 146, no. 9, pp. 2185–2197, 2000.

[34] D. V. Cousins, R. Whittington, I. Marsh, A. Masters, R. J. Evans, and P. Kluver, "Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess IS900-like sequences detectable by IS900 polymerase chain reaction: implications for diagnosis," *Molecular and Cellular Probes*, vol. 13, no. 6, pp. 431–442, 1999.

[35] S. Englund, G. Bölske, and K. E. Johansson, "An IS900-like sequence found in a *Mycobacterium* sp. other than *Mycobacterium avium* subsp. *paratuberculosis*," *FEMS Microbiology Letters*, vol. 209, no. 2, pp. 267–271, 2002.

[36] C. E. Greer, S. L. Peterson, N. B. Kiviat, and M. M. Manos, "PCR amplification from paraffin-embedded tissues. Effects of fixative and fixation time," *American Journal of Clinical Pathology*, vol. 95, no. 2, pp. 117–124, 1991.

[37] R. B. Pillars, D. L. Grooms, J. A. Woltanski, and E. Blair, "Prevalence of Michigan dairy herds infected with *Mycobacterium avium* subspecies *paratuberculosis* as determined by environmental sampling," *Preventive Veterinary Medicine*, vol. 89, no. 3-4, pp. 191–196, 2009.

Research Article

Diagnosis and Molecular Characterization of *Mycobacterium avium* subsp. *paratuberculosis* from Dairy Cows in Colombia

J. A. Fernández-Silva,[1, 2] A. Abdulmawjood,[3] and M. Bülte[1]

[1] *Institute of Veterinary Food Sciences, Justus Liebig University Giessen, Frankfurter Straße 92, 35392 Giessen, Germany*

[2] *Escuela de Medicina Veterinaria (Grupo Centauro), Facultad de Ciencias Agrarias, Universidad de Antioquia, Carrera 75, No. 65-87, Medellín, Colombia*

[3] *Institute of Food Quality and Food Safety, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany*

Correspondence should be addressed to J. A. Fernández-Silva, jfernandez@agronica.udea.edu.co

The objective of this study was the serological, bacteriological and molecular diagnosis, as well as the molecular characterization of *Mycobacterium avium* subsp. *paratuberculosis* (Map) in adult cows of five Colombian dairy herds. Serum samples were tested by an indirect absorbed enzyme–linked immunosorbent assay (ELISA-C). All fecal samples were tested by pooled culture. After that, fecal samples of Map positive pools were tested individually by culture and polymerase chain reaction (PCR). In one herd, slurry and tissue samples from one animal were also taken and tested by PCR and culture. Map isolates were analyzed by the Multilocus Short Sequence Repeat (MLSSR) and the Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR) methods. ELISA produced positive results in 1.8% (6/329) of the animals and 40% (2/5) of the herds. Four fecal, two tissue, and two slurry samples from a herd were Map positive by culture and PCR. MLSSR and MIRU-VNTR revealed two different strain profiles among eight Map isolates recovered. This study reports the first molecular characterization of Map in one dairy herd in Colombia, the limitations for individual diagnosis of subclinical Map infections in cattle, and the usefulness of pooled fecal samples and environmental sampling for Map diagnosis.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (Map) is a slow growing, mycobactin-dependent acid fast bacterium that causes paratuberculosis or Johne's disease in cattle [1]. Paratuberculosis produces a considerable economic impact on the cattle industry, especially on milk production and body condition. Map has also been related to the chronic human enteritis known as Crohn's disease, but this relationship still remains controversial [2].

Enzyme-linked immunosorbent assay (ELISA), bacteriological cultivation of fecal samples, and polymerase chain reaction (PCR) are test widely used for the antemortem diagnosis of paratuberculosis in cattle herds [3–5]. On the other hand, bacteriological culture of pooled fecal samples and environmental sampling are cost-effective methods to classify herds as Map infected [6, 7]. In addition, sampling all adult cattle in every herd, environmental sampling, serial testing, and the use of two to three diagnostic tests have been recommended for herd screening and to increase the accuracy of Map diagnosis [5, 8].

Strain differentiation of Map is very useful to understand the origin of the infections and the disease transmission dynamics, to design more adequate control measures, and to improve diagnosis rates and the development of vaccines [9]. Although paratuberculosis is a notifiable disease in Colombia, the lack of an official national control program and some limitations of the diagnostic tests have contributed to the reduced local information on the disease.

The objective of this study was the serological, bacteriological, and molecular diagnosis of Map in adult cows of five Colombian dairy herds, as well as the molecular characterization of Map isolates and the comparison of results with similar studies.

TABLE 1: Information on herd management of five dairy herds examined for Map in Colombia.

Herd[a]	District	Number of cattle purchased in the last two years		Raising of own replacement heifers	Proportion of ELISA-A positive results 2007[b,d]
		2007[b]	2009		
1	Monterredondo	0	0	Yes	15% (3/20)
2	El Yuyal	10	5	Yes	20% (4/20)
3	El Yuyal	0	0	Yes	27.2% (6/22)
4	Santo Domingo	0	0	Yes	21.7% (5/23)
5	Santa Bárbara	N.A.[c]	4	Yes	NA

[a] Herd 2 and herd 4 belong to the same farmer and cattle exchange between both herds occurs frequently.
[b] According to Fernández-Silva et al. 2011 [10].
[c] NA: not applicable. This herd was not sampled in 2007.
[d] Refers to the proportion of positive animals by ELISA-A to the number of animals sampled in the herd.

2. Materials and Methods

2.1. Selection of Herds. Between November and December of 2009, five dairy herds were selected to be examined for Map (Table 1 and Figure 1). Of these five herds, four herds (herds 1, 2, 3, and 4) tested ELISA and PCR positive but culture negative for Map in a previous cross-sectional study in 2007 [10]. In this study, 13 herds without previous history of paratuberculosis and one herd (herd 1 of the present study) with previous diagnosis of Johne's disease [11] were examined. In every herd, only a sample (between 19 and 29 randomly selected adult cows, depending on the herd population) was tested. Serological testing was initially carried out to all sampled animals using a commercial lipoarabinomannan- (LAM-) based indirect ELISA test without preabsorption (Svanovir Para-TB Ab ELISA Kit, Uppsala, Sweden) (ELISA-A). For confirmation of positives samples by ELISA-A, a commercial indirect ELISA test based on detection of antibodies to protoplasmic Map antigens, including a pre-absorption step with *Mycobacterium phlei* (*M. phlei*) (ELISA paratuberculosis antibody verification, Institut Pourquier, Montpellier, France) (ELISA-B) was used. For the PCR diagnosis, two methods targeted to F57 and IS*Mav2*, and to IS*900*, respectively, were applied [10].

The four herds previously tested in 2007 and selected in the present study (herds 1, 2, 3, and 4) have never followed any structured or consistent control program for prevention or control of paratuberculosis before the first study in 2007, or in the period between both studies. However, culling of animals with nonresponsive diseases (including animals with compatible signs of Johne's disease in herd 1) or low productive or reproductive performance was done permanently. The remaining herd (herd 5) had a cow with weight loss and nonresponsive diarrhea compatible with paratuberculosis, but did not have a history of Johne's disease or a previous diagnosis of Map.

From the herds tested, only herd 2 has purchased animals before the first study in 2007 and between both studies. Herd 5 has purchased animals in the last two years before the sampling of the present study. Between herd 3 and herd 4, which belong to the same farmer, cattle exchange occurs usually. All herds raise their own replacement heifers (Table 1).

2.2. Collection of Samples. Serum and fecal samples were taken from all adult dairy cows (≥ 2 years) in every herd. In herd 2, 110 cows were sampled for feces, but only 53 of them were sampled for serum due to reluctance of farmer to sample all animals. In one herd (herd 1) that had slurry pit collecting liquid manure and wastewater from the herd's milking parlor, slurry samples were additionally taken from three different places of the pit. From one animal of the same herd (herd 1), a section of thick intestine (colon) and a mesenteric lymph node were obtained after euthanasia and necropsy due to advanced clinical symptoms compatible with Johne's disease. Information about age was collected from all animals with exception of six animals, from which farmers did not have available data at the moment of sampling.

2.3. ELISA. Serum samples ($n = 329$) were tested with an ELISA test based on detection of antibodies to Map extract (ID Screen Paratuberculosis Indirect, IDVET, Montpellier, France) (ELISA-C). This test included a pre-absorption step with *M. phlei*. ELISA-C was not carried out in duplicate due to economical reasons. A herd was considered positive if at least one animal tested positive by ELISA-C.

2.4. Bacteriological Culture. Fecal samples ($n = 386$) were examined on the basis of a strategic pooling procedure. Fecal samples were sorted on the basis of birth order of the animals, and 2 g of feces from each cow was mixed at the laboratory into pooled fecal samples of 8–12 cows per pool. After this, 3 g of the pooled fecal sample was decontaminated with 0.75% (w/v) Hexadecylpyridinium Chloride solution (0.75% HPC) for 24 h, according to standard procedures [12]. Briefly, 3 g of feces were added to a 50 mL sterile tube (Sarstedt, Nümbrecht, Germany) containing 30 mL of a 0.75% HPC. This suspension was manually mixed by shaking and vortexting and let in vertical position for 5 min at room temperature to allow the precipitation and sedimentation of big particles. Approximately 20 mL of the upper portion of the supernatant was transfer to another 50 mL sterile tube in which the whole suspension was agitated for 30 min by 200 U/min. Tubes were placed in vertical position in the dark for 24 h at room temperature. Decontaminated pooled fecal samples were centrifuged at 900 ×g during
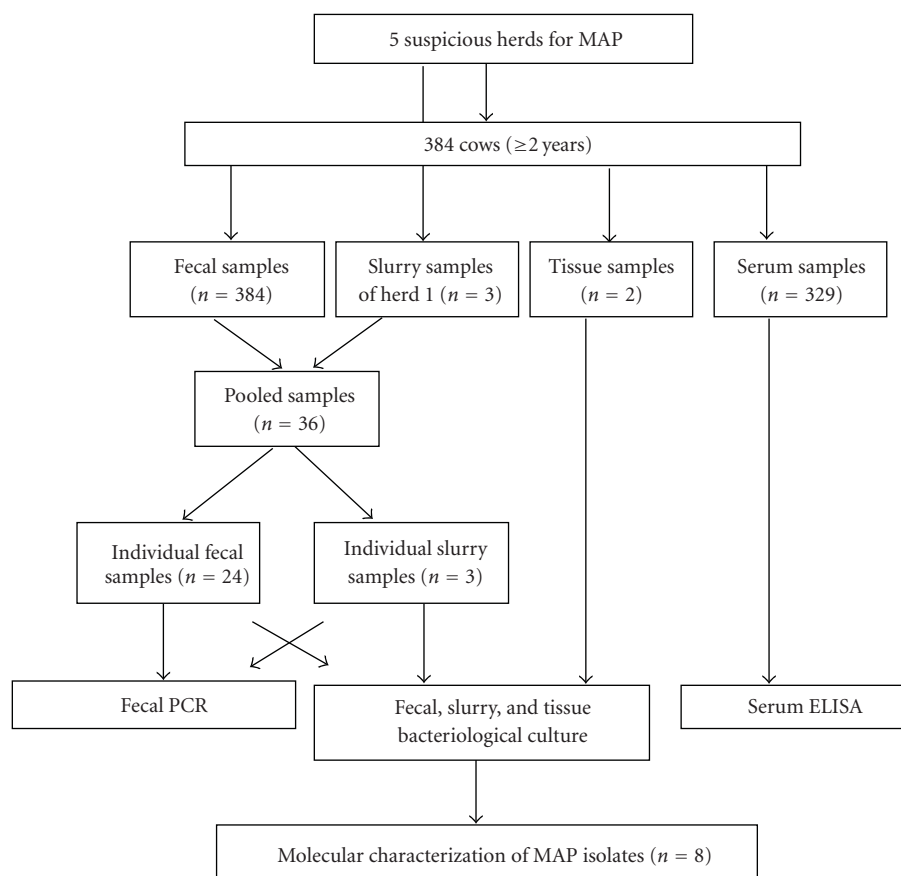
FIGURE 1: Overview of the study design.

30 min, supernatant was discarded, and two Herrold's Egg Yolk Agar medium (HEYM) slants, supplemented with mycobactin J (Prepared Culture Media, Becton Dickinson, Heidelberg, Germany) were inoculated with 300 μL of the decontaminated pellet [12]. The slants were incubated at 37°C for a maximum of 20 weeks and checked at 1-2-week intervals.

Slurry samples were also pooled, decontaminated, and inoculated as described above for fecal samples. If HEYM slants inoculated with pooled fecal or slurry samples showed mycobacterial growth, single fecal and slurry samples were cultured individually. The individual samples from a negative pool were assumed negative and not tested individually, except for the fecal samples from ELISA-C-positive animals of herd 2, which were cultured individually regardless of their culture results.

Tissue samples (colon and mesenteric lymph node) were prepared, decontaminated, and inoculated in duplicate onto HEYM slants [12]. Briefly, the colon tissue was cut open, and the mesenteric lymph node was released from adipose tissue. Both samples were cut up separately, and approximately 1 g of the respective tissue material was put in a stomacher bag with 7 mL of 0.9% (w/v) Hexadecylpyridinium Chloride solution (0.9% HPC) and was homogenized for 6 min in the stomacher. The homogenized tissue was put in a 50 mL sterile tube (Sarstedt, Nümbrecht, Germany) and shaken

at room temperature, 200 U/min for 5–10 min. After that, tubes were place in vertical position in the dark for 24 h at room temperature. After decontamination, the tubes were centrifuged at 1880 ×g, at 20°C for 20 min. The supernatant was discarded, and the sediment was resuspended in PBS-Buffer pH 7.2 and vortexed. Finally, two HEYM slants, supplemented with mycobactin J (Prepared Culture Media, Becton Dickinson, Heidelberg, Germany) were inoculated with 300 μL of the decontaminated pellet. As done with fecal samples, slants were incubated at 37°C for maximum 20 weeks and checked at 1-2-week intervals for mycobacterial growth or contamination. Contamination rate was estimated in 8.3% (3/36) for the fecal and the slurry pooled samples, and 3.7% (1/27) for the individual fecal samples (including fecal cultures from ELISA-C-positive animals of herd 2) and the tissue samples. In all contaminated samples, only one slant of the duplicate was affected. In case of mycobacterial growth, Map was confirmed by the real-time PCR method described as follows.

2.5. Polymerase Chain Reaction (PCR). PCR was carried out only on individual fecal and slurry samples that were part of positive fecal and slurry pooled samples by culture, and to fecal samples of four positive ELISA-C animals of herd 2 ($n = 27$) (Figure 1). DNA isolation from fecal and slurry samples was carried out using a commercial DNA

preparation kit (High Pure PCR Template Preparation Kit, Roche, Mannheim, Germany). Briefly, 1.5 g of bovine feces was put in a 15 mL sterile, nonpyrogenic centrifuge tube (Sarstedt, Nümbrecht, Germany). Five mL of a buffer for stabilization (Stool Transport and Recovery-S.T.A.R. buffer, Roche, Mannheim, Germany) was added to fecal sample and homogenized. This suspension was subsequently centrifuged for 1 min by 1000 ×g and 1 mL of the supernatant was put in a 2 mL conical sample tubes (Biozym Scientific, Hess. Oldendorf, Germany) containing ceramic beads, size range 1.4–1.6 mm, Genotype ZY (Zirkonoxid-Beads, Yttrium stabilized) (Sigmund Lidner, Warmensteinach, Germany). A mechanical cell disruption step was carried out in an automated biological sample lyser (Precellys 24, Bertin technologies, Montigny-le-Bretonneux, France) to achieve efficient cell lysis. The mixture was subsequently incubated at 95°C for 10 m and centrifuged 5 min by 5000 ×g. Two hundred microliters of the supernatant was added to a 1.5 mL reaction tube containing 5 μL of lysozyme (Merck, Darmstadt, Germany) solution. Further processing was done according to kit's protocol for isolation of nucleic acids from bacteria and yeast. DNA isolation was always carried out in duplicate.

DNA isolation from bacteria for Map confirmation and molecular characterization was carried out using a commercial preparation kit (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany). This preparation included overnight lysis buffer incubation at 37°C, proteinase K/AL-buffer incubation for 90 min at 56°C, and final incubation for 15 min at 95°C, as a modification of the protocol of the commercial kit. DNA from fecal samples and from bacterial culture was tested in duplicate for Map with the real-time PCR method targeted to F57 and IS*Mav2* described by Schönenbrücher et al. [13]. Samples were also tested in duplicate with the nested-PCR targeted to IS*900* described by Bull et al. [14]. Additional to the samples, a positive and a negative preparation control, as well as a blank control were included. In the PCR system, a positive Map control (DNA of a positive Map strain), a non-Map negative control (DNA of a non-Map mycobacteria), and a master-mix blank control were also included. The real-time PCR method also included an internal amplification control (IAC) to avoid the misinterpretation of false negative results [13].

2.6. Molecular Characterization. For molecular characterization of the Map strains isolated, a combination of two different genotyping methods based on PCR amplification of repetitive elements of Map genome was applied. The Multilocus Short Sequence Repeat (MLSSR) analysis was carried out by amplification of the short sequence repeats (SSRs) found in locus 1, 2, 8, and 9 according to primers and PCR conditions reported by Amonsin et al. [15]. The final PCR reaction volume (30 μL) contained GeneAmp 10x PCR Puffer (Applied Biosystems, Darmstadt, Germany), dNTP-Mix (10 μM each) (Roche, Mannheim, Germany), 0.2 μM of each primer (Eurofins MWG, Martinsried, Germany), 10% Dimethil Sulfoxide (DMSO) (Roth, Karlsruhe, Germany), 1 U of AmpliTaq Gold Polymerase 5 U/μL (Applied Biosystems, Langen, Germany), and 3 μL of DNA. A master

mixture blank (without DNA) was included as control in every PCR reaction. Seven microliters of every PCR product were mixed with 2 μL of loading buffer, and electrophoresed in a 1.5% agarose gel. All amplicons in every SSR locus were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced by an independent laboratory (Sequence Laboratories, Göttingen, Germany). MLSSR genotypes were expressed as the combination of the number of repeats found in the four loci amplified by PCR.

The Mycobacterial Interspersed Repetitive Units (MIRU) loci MIRU-1, MIRU-2, MIRU-3, and MIRU-4 were selected to analyze the Map isolates according to Bull et al. [16]. The Variable Number of Tandem Repeats (VNTRs) loci VNTR-292, VNTR-1658 (alias X3), VNTR-25, VNTR-47, VNTR-3, VNTR-7, VNTR-10, VNTR-32, and VNTR-259 were selected to analyze the Map isolates according to Overduin et al. [17], Thibault et al. [18], and Castellanos et al. [19]. For all loci, primers used were those suggested by the authors mentioned above. Except for the PCR conditions of MIRU-1, VNTR-7, and VNTR-10 carried out according to Möbius et al. [20], and of VNTR-25 and VNTR-47 according to Castellanos et al. [19]. The final reaction volume of PCR (30 μL) for MIRU-VNTR was the same as described for MLSSR. However, for the PCR amplification of VNTR-32, 5 μL of Betain (Sigma-Aldrich, Schenelldorf, Germany) was additionally added to the mix as suggested by Thibault et al. [18].

Calculation of the number of repeats per locus for MIRU-VNTR was initially performed according to the size of the amplicon determined by electrophoresis in 1.5% agarose gel. Additionally, the amplicons of representative alleles in every locus were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced by an independent laboratory (Sequence Laboratories, Göttingen, Germany). MIRU and VNTR genotypes were confirmed by the number of repeat units and the number of tandem repeats in selected sequences. MIRU-VNTR genotypes were expressed as the combination of the number of repeats found in every locus in the order MIRU-1, MIRU-2, MIRU-3, MIRU-4, VNTR-292, VNTR-1658 (alias X3), VNTR-25, VNTR-47, VNTR-3, VNTR-7, VNTR-10, VNTR-32, and VNTR-259. The INRA Nouzilly MIRU-VNTR (INMV) nomenclature as defined by Thibault et al. [18] was taken into account for ease comparison with previous studies. For this purpose only the results of loci VNTR-292, VNTR-1658, VNTR-25, VNTR-47, VNTR-3, VNTR-7, VNTR-10, and VNTR-32 were considered.

2.7. Data Analysis. The descriptive analysis of age, the estimation of standard deviation (SD), and the determination of confidence intervals 95% (95% CI) were carried out using the program packages BMPD release 8.1 (Berkeley, USA) and BIAS release 8.2 (Hochheim-Darmstadt, Germany). The estimation of the testing agreement between ELISA and culture (Cohen's kappa (κ) coefficient) was done with the program Win-Episcope 1.0 (Zaragoza, Spain). True prevalence was estimated based on the apparent prevalence obtained by ELISA-C using sensitivity (42%), and specificity (99%) values determined previously on asymptomatic infected animals [21]. The relation age versus ELISA results

Table 2: Enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and fecal culture positive results of five dairy herds examined for Map in Colombia.

Herd	District	Herd cattle population	Number of samples[a]	Serum ELISA-C	Fecal culture[c]	Fecal PCR[e]
1	Monterredondo	125	75	2	4[d]	2
2	El Yuyal	174	53[b]	4	0	0
3	El Yuyal	144	84	0	0	ND[f]
4	Santo Domingo	172	94	0	0	ND
5	Santa Bárbara	38	23	0	0	ND
Total		653	329	6	4	2

[a]Only cows over 2 years of age were sampled.
[b]In this herd, 110 animals were sampled for feces, but only 53 of them were sampled for serum.
[c]Refers to fecal samples cultured individually.
[d]Positive results of one lymph node, one colon tissue, and two slurry samples are not included.
[e]Refers to individual fecal samples part of pooled fecal samples positive by culture, and to fecal samples of positive ELISA-C animals tested individually.
[f] ND not done.

was analyzed descriptively, according to age classes defined arbitrarily.

3. Results

3.1. ELISA. ELISA-C produced positive results in 1.8% (6/329) (95% C.I.; 0.7–3.9%), negative results in 97.5% (321/329), and doubtful results in 0.6% (2/329) of the serum samples examined, as well as positive results in 40% (2/5) of the herds. Of the six positive ELISA-C samples detected, two were detected in herd 1 and four were detected in herd 2 (Tables 2 and 3). The true Map-prevalence based on ELISA-C-apparent prevalence, sensitivity (42%) and specificity (99%) was 2.2%.

The age of the animals sampled ranged between 2.2 and 14 years (mean 5.9, SD 2.8). Analysis of age of animals versus ELISA-C result (positive, negative, doubtful) revealed that the group of >11 years of age, was the group in which the highest proportion (6.3%, 1 out of 16) of ELISA-positive samples were produced (Table 4). However, it was in the group of 5.1–8 years of age in which the highest absolute number of ELISA-positive animals (n = 3) of the whole study was found. In the group of the youngest cows (>2.2–2.9 years of age), no positive result by ELISA was produced. In the group of 3–5, 5.1–8, and 8.1–10.9 years, 0.8%, 2.9%, and 1.9% of the samples produced positive results, respectively (Table 4).

3.2. Bacteriological Culture and Polymerase Chain Reaction. The strategic pooling procedure for fecal samples from the five herds resulted in 36 pools, including the slurry pool prepared from herd 1, which had a slurry pit collecting liquid manure and wastewater from the herd's milking parlor. Two pools from herd 1 out of 36 pools analyzed produced positive results by culture after 5-6 weeks of incubation with >50 Colony Forming Units (CFU)/tube. The slurry pool produced positive results by culture after 17 weeks of incubation with <10 CFU/tube. Isolates obtained from pooled samples were confirmed as Map by the real-time PCR method described above. Remaining pools of herds 2, 3, 4, and 5 did not show mycobacterial growth by culture in 20 weeks of incubation.

Table 3: Enzyme-linked immunosorbent assay (ELISA-C), polymerase chain reaction (PCR), and culture results of animals and individual slurry samples from positive pooled samples or ELISA-C-positive animals of herd 1 and herd 2.

Herd	Pool	Source	ELISA-C	PCR	Culture
1	1	C1	−	−	+
		C2	−	−	+
		C3	+	+	+
		C4	+	−	−
		C5	−	−	−
		C6	−	−	−
		C7	−	−	−
		C8	−	−	−
		C9	−	−	−
	2	C1	−	−	−
		C2	−	−	−
		C3	−	−	−
		C4	−	+	+
		C5	−	−	−
		C6	−	−	−
		C7	−	−	−
		C8	−	−	−
		C9	−	−	−
		B	−	−	−
	3	S1	N.A.	−	−
		S2	N.A.	−	−
		S3	N.A.	−	−
2	1	C1	+	−	−
	2	C2	+	−	−
	3	C3	+	−	−
	4	C4	+	−	−

C: cow, B: bull, S: slurry pit, +: positive result, −: negative result, NA not applicable.

Fecal samples that were a part of the two positive pools of herd 1 (n = 19) produced four positive results by individual culture (Table 3). All isolates showed no pigmentation and were confirmed as Map by real-time PCR. Two cows

TABLE 4: ELISA results according to group of age of 323 animals from five dairy herds.

Group of age	Positive (%)	Negative (%)	Doubtful (%)	Total (%)
		ELISA result		
2.2–2.9	0 (0.0)	32 (100)	0 (0.0)	32 (9.9)
3–5	1 (0.8)	116 (98.3)	1 (0.8)	118 (36.5)
5.1–8	3 (2.9)	101 (97.1)	0 (0.0)	104 (32.2)
8.1–10.9	1 (1.9)	51 (96.2)	1 (1.9)	53 (16.4)
>11	1 (6.3)	15 (93.8)	0 (0.0)	16 (5)

TABLE 5: Isolates of *Mycobacterium avium* subsp. *paratuberculosis* recovered in a dairy herd.

Isolate number	Source	Isolated from	MLSSR-Type[a]	MIRU-VNTR-Type[b]
			Molecular genotype	
(1)	Cow 1	Feces	A	1
(2)	Cow 2	Feces	A	1
(3)	Cow 3	Feces	A	1
(4)	Cow 3	Mesenteric lymph node	B	2
(5)	Cow 3	Colon tissue	A	1
(6)	Cow 4	Feces	A	1
(7)	Slurry pit	Slurry	A	1
(8)	Slurry pit	Slurry	B	2

[a] MLSSR-genotype A: 7g-10 g-4ggt-5tgc and MLSSR-genotype B: 7g-10g-5ggt-4tgc.
[b] MIRU-VNTR genotype 1: 3951-42332228-2 (INMV 1) and MIRU-VNTR genotype 2: 3751-32332228-2 (INMV 2).

(both ELISA-C-negative, PCR-negative, asymptomatic, 7.1 years old) were low shedders (<10 CFU/tube), one cow (ELISA-positive, PCR-positive, symptomatic, 6 years old) was a heavy shedder, and one cow (ELISA-negative, PCR-positive, asymptomatic, 9.5 years old) was a heavy shedder (>50 CFU/tube) (Table 3). The cow 3 from pool 1 (herd 1) also produced positive results by mesenteric lymph node and colon tissue culture (Tables 3 and 5). On HEYM-slants inoculated with mesenteric lymph node tissues, visible colonies grew before 16 weeks of incubation, while in those inoculated with colon tissues no visible Map colonies grew in this period of time. Four fecal samples from positive ELISA-C animals of herd 2 produced negative results by culture and PCR; these samples were all from different pooled fecal samples (Table 3). Surprisingly, although the pooled slurry sample produced positive results by culture and PCR, their individual samples ($n = 3$) were negative by PCR, and by culture after 20 weeks of incubation (Table 3).

ELISA-C results were confirmed by culture in only one symptomatic animal (cow 3) of herd 1. Thus, ELISA-C, culture, and PCR only agreed on one single animal out of four animals that delivered positive culture results. According to these results, calculated agreement between ELISA-C and culture was poor ($\kappa = 0.19$, 95% C.I. 0.09–0.29). ELISA-C did not detect Map antibodies in three serum samples from asymptomatic animals of herd 1 (Cow 1 and Cow 2 from pool 1, and Cow 4 from pool 2) that produced positive results by fecal culture. In fecal samples from two of these three animals, Map was also not detected by PCR. In one case, a cow (cow 4 of pool 1 from herd 1) produced negative results by ELISA-C, but positive results by PCR and culture (Table 3).

3.3. Comparison of Results 2007–2009. Based on results of a previous study [10], it was determined that some animals ($n = 11$) tested in 2007 were tested again by ELISA, PCR and culture in this study (2009). None of these animals have presented symptoms of paratuberculosis before 2007 or between the two studies. Results can be classified in five different categories, in which only positive findings are described, meaning that other tests produced negative results. In the category 1, one single animal produced positive results by ELISA-A and real-time PCR (molecular target F57) in 2007 and ELISA-C-positive results in 2009. In the category 2, one single animal produced positive results in ELISA A, PCR and real-time PCR (molecular target IS*Mav2*) in 2007. In the category 3, two animals produced ELISA-A and ELISA-B positive results in 2007. In the category 4, one single animal produced positive results by ELISA-A and PCR in 2007. Finally, in the category 5, six animals produced positive results by ELISA-A (Table 6).

3.4. Molecular Characterization. In total eight Map isolates were recovered. Four isolates were from fecal samples, one from mesenteric lymph node, one from colon tissue sample, and two from pooled slurry samples (Table 5). All isolates were confirmed as Map by the real-time PCR method described above. All isolates were obtained from samples from herd 1. All isolates grew within 6–16 weeks of incubation, except from samples of slurry and colon tissue, which grew after the 16th week.

The four isolates obtained from fecal samples, the isolate obtained from colon tissue, and one of the two isolates obtained from slurry sample were of the MLSSR genotype

TABLE 6: Comparison of diagnostic test results of single animals ($n = 11$) tested for Map in 2007 and in 2009

Category	2007[a]						2009		
	ELISA-A[b]	ELISA-B[c]	PCR[d]	Real-time PCR[d]	Culture	ELISA-C[e]	Culture[f]	PCR[g]	Real-time PCR[g]
1	+	−	−	+ (F)	−	+	−	−	−
2	+	−	+	+ (I)	−	−	−	N.D	N.D
3	+	+	−	−	−	−	−	N.D	N.D
4	+	−	+	−	−	−	−	N.D	N.D
5	+	−	−	−	−	−	−	N.D	N.D

[a] According to Fernández-Silva et al. 2010 [10].
[b] ELISA-A (Svanovir Para-TB Ab ELISA Kit, Svanova Biotech AB).
[c] ELISA-B (ELISA paratuberculosis antibody verification, Institute Pourquier) performed only to positive and doubtful ELISA-A samples.
[d] PCR and real-time PCR performed only to fecal samples from positive animals by ELISA-A.
[e] ELISA-C (ID Screen Paratuberculosis Indiret, IDVET) performed to herds in the present study (2009).
[f] Culture performed initially from pooled fecal samples, and then individually if pooled sample was Map positive.
[g] PCR and real-time PCR performed only to individual fecal samples from culture-positive pooled samples, and to fecal samples from ELISA-C-positive animals regardless of culture result.
+: positive, −: negative, (F): positive real-time-PCR in marker F57, (I): positive real-time-PCR in marker IS*Mav2*, ND: not done.

7g-10g-4ggt-5tgc (hereafter MLSSR-genotype A). The isolates obtained from mesenteric lymph node and the remaining isolate obtained from slurry sample were of the MLSSR genotype 7g-10g-5ggt-4tgc (hereafter MLSSR-genotype B). Similarly, the combination of MIRU-VNTR showed two different MIRU-VNTR genotypes, genotype 3951-42332228-2 (hereafter MIRU-VNTR genotype 1) and 3751-32332228-2 (hereafter MIRU-VNTR genotype 2). Interestingly, strain types A-1 and B-2 were both identified in cow 4, representing a case of double strain infection.

4. Discussion

To our knowledge, this is the first report of isolation and molecular characterization of Colombian Map-strains from dairy herds. For achievement of this goal, herds with history of Johne's disease (report of cases and/or positive diagnosis) were selected to increase the likelihood of detection and isolation of Map. Despite the importance of the cattle production in Colombia, paratuberculosis has remained relatively uninvestigated, and very limited epidemiological information and data on molecular characterization of Map were available. For about 60 years, some studies have tried to widen the clinical and epidemiological information of paratuberculosis in the country through research on diagnosis, treatment, epidemiology, and molecular biology. Now, a consistent study including a significant cattle population, using different diagnostic tests, and including the molecular characterization of the circulating causal agent is presented. As previously suggested in some studies, ELISA, PCR, and culture were used to increase sensitivity of Map detection, in order to confirm whether herds with history of Johne's disease or Map diagnosis were truly infected [22].

The lower proportion of the current ELISA-C positive results (1.8%) compared to the previous ELISA-A positive results (10.1%) in four of the five herds examined was surprising at first sight, but it is explained by the characteristics of the ELISA tests used in both studies. ELISA-C is an absorbed test using purified Map extract, IgG-conjugate and

preincubation with *M. phlei*, which are characteristics that have been considered of critical influence on the increment of specificity for the serological diagnosis of paratuberculosis [23–26]. Therefore, the use of an absorbed test (ELISA-C) has produced negative or a lower proportion of positive results in herds with previous Map diagnosis (ELISA-A and PCR), or even with previous history of clinical cases of paratuberculosis (e.g., herd 1), compared to the previous study of 2007. In this previous study, herds of a dairy region mostly without previous diagnosis of paratuberculosis were tested using an unabsorbed test that used LAM as Map antigen (ELISA-A), which produced a higher proportion of seropositives confirmed only in two animals by an absorbed ELISA (ELISA-B). Interestingly, the results of both absorbed tests, ELISA-B in 2007 and ELISA-C in 2009, produced closer results (5.1% versus 1.8%) than those obtained with the unabsorbed ELISA-A (10.1%) in 2007. This suggests that the characteristics of the tests used were determinant in the different proportions of seropositives obtained in both studies.

Furthermore, the absence of reliable preliminary epidemiological information on the disease makes also plausible that dairy herds in the region of study were of a very low prevalence or even negative for Map, or at least undetectable with the current diagnostic tests, if only cross-sectional studies instead of a longitudinal study or serial testing is carried out. In any case, these studies are the first step of the systematic epidemiological study of paratuberculosis in Colombia, and therefore further studies have to be conducted to elucidate the situation of the disease in the country.

The case of herd number 2, in which clinical paratuberculosis has never been reported, but some animals were positive by ELISA-A and PCR in 2007, and again in 2009 (four positive ELISA-C, but negative results by culture) is striking and difficult to explain. In this case, it could be possible that other mycobacteria could influence the positive results of the unabsorbed ELISA-A in 2007 and of the ELISA-C positive in 2009, making the proportion of positive animals higher than it really is [27]. In the study of 2007, atypical mycobacteria (*Mycobacterium engbaekii*) were isolated [10].

However, the lack of testing of the half of the adult cattle population of this herd for ELISA-C limits any definitive conclusion about the current negative PCR and culture results obtained from seropositive animals, taking into account the high quality of the tests employed. Additionally, the four fecal samples from the positive ELISA-C animals were cultured individually, which could have been reduced the chances of a probable concentration of low quantities of Map, if these animals were really shedders. Likewise, it is possible that the positive ELISA results, not only in herd 2, but all herds of both studies have been produced due to the interference with tuberculin from intradermal tests (caudal fold tuberculin test) [28] applied occasionally in order to be certified as free herd from bovine tuberculosis in frame of the national program for eradication of tuberculosis. In conclusion, some of these results of ELISA-A and even ELISA-C could have been simply false positives in 2007 and in 2009, respectively.

The low apparent prevalence and the true prevalence obtained (1.8 versus 2.2%) was probably related to the high specificity of test used (99%), as previously reported for studies with these characteristics [29]. Although our study was biased for prevalence determination, due to analysis of herds with previous history or diagnosis of paratuberculosis, animal level apparent prevalence calculated appeared to be lower compared to prevalences obtained in European countries [30]. However, no similar studies were found aiming at the determination of prevalence of Map infection by using the ELISA-C, which made impossible a better comparison of results.

In this study, only 0.8% of ELISA-C-positive cows were detected in the group of 3–5 years and the only symptomatic animal found was a 6- year- old cow. This results slightly disagree with a report of a higher probability of testing positive by ELISA between 2.5 and 5.5 years in infected animals [31] and with the knowledge that most clinical cases of paratuberculosis occur between 3–5 years [32]. Cattle in Colombia tend to be kept longer in production and to be culled later compared to North American or European countries. In this manner, cows can live long enough to be tested and detected by ELISA or fecal culture, or to show symptoms of paratuberculosis out of the age limits reported for other countries.

As reported before, culture of pooled fecal samples of 8–12 animals per pool permitted the examination of a high number of fecal samples by culture at low cost and with acceptable sensitivity [6, 33, 34]. The option of pooling 5 fecal samples instead of 10 was not considered due to economic reasons. Furthermore, precise information about Map within-herd prevalence was absence to take a better decision of the best pool size according to a previous modeling study [35]. This option was also discarded because of the reported insignificant difference in sensitivity between pooling 5 or pooling 10 cows in a previous study in a comparable South American cattle production system, in which it was concluded that the sensitivity of the pool is related more to the prevalence of the herd and to the infection status of the cows as with the size of the pool [6]. In the same way, a study in the United States reported acceptable sensitivity with 10 samples per pool (35%), compared to pooled samples of 5 animals (44%), leading to the conclusion that in herds with at least one high fecal shedder, pools of more than five samples might also detect Map [36]. Although some studies have concluded a better sensitivity of pooling five animals instead of 10 or more, these studies have been based on the detection of Map using radiometric fecal cultures to reliably detect low-shedders, which could be not comparable to classical bacteriological methods [37]. Other studies refer more to a theoretical calculation than to a sensitivity estimation, difficult to extrapolate to South American field conditions [35], or reported the use of five samples per pool focusing on the determination of the sensitivity of culture of pooled fecal samples compared with culture of individual fecal samples, with special attention to the number of pooled fecal samples per herd, rather than to the number of animals to be included in the pooled fecal samples [36].

The detection of a positive pooled slurry sample by culture from a positive ELISA-C herd (herd 1) agrees with the knowledge of the correlation of this finding with seropositive results, and with the higher probability of isolation from lagoon samples compared to other environmental samples [7]. The result of the single slurry samples producing negative results by individual culture and PCR has been reported and has been attributed to uneven distribution of Map in the fecal sample [6], to the lack of homogeneity in the fecal sample or to different sensitivities of individual fecal culture procedure between laboratories [34], to the presence of Map in the feces of at least one animal within the pooled fecal sample, although this animal was not detected by bacteriological culture of individual fecal samples [38], or to unclear reasons [36]. In any case, Kalis et al. properly concluded that there is an element of chance apart from the element of dilution related to the detection of Map in feces, particularly when samples contain low numbers of the organism, and the bacteria are not uniformly distributed in the fecal samples [33]. Thus, although a complete homogenization of the pooled slurry sample was achieved and the PCR systems used are very reliable, it is possible that the 3 g or 1.5 g of slurry samples taken to test by individual culture and by PCR, respectively, lacked enough Map cells to be detected by bacteriological culture in a 20-week period of incubation, and in two PCR systems (F57-Is$Mav2$-real-time and IS900-nested-PCR) carried out in duplicate. The PCR systems used, specially the real-time PCR, are strict tested for specificity, included an IAC and use multiple reaction controls, which avoid the misinterpretations of results due to disturbed contamination or very improbable false positive results [13].

Close analysis of the individual results obtained in herd 1 revealed that one single symptomatic animal producing positive results by ELISA-C and PCR, confirmed that regardless of the ELISA or PCR type used sensitivity is higher for detection of symptomatic animals and fecal high shedders [21, 39]. The results of three asymptomatic ELISA-C-negative cows that produced positive results by culture could be: two cases (Map low-shedders and PCR-negative) of the known "passing through" phenomenon previously described [40], and one case (Map high-shedder and PCR-positive) of a positive animal with undetectable antibodies.

On the other hand, the results of one ELISA-C positive-animal of herd 1, that produced negative results by culture, does not necessarily mean that the animal was not really infected, but that the shedding phase has probably not yet started (infected animal in a noninfectious phase) or was absent at the moment of fecal sampling (intermittency). Another possibility is that in this animal Map-antibodies have been detected prior to the start of bacterial shedding, which could begin later and could be then detected by PCR or culture [41]. Map is shed in feces of infected animals at all stages but at different levels and sporadically, which demands repeated testing to detect animals shedding very low number of Map, which could anyway go undetected [5].

One ELISA-C-negative animal was positive by PCR, real-time PCR, and culture. On the contrary, one ELISA-C-positive animal in the same herd showed negative results by PCR, real-time PCR, and culture. In herd 2 four ELISA-C-positive animals produced negative results by fecal PCR and fecal real-time PCR, as well as negative results by individual fecal culture. Muskens et al. found a low percentage of ELISA-positive cattle testing fecal culture-positive for all age groups included. Among their arguments, they stated a possible limited sensitivity of the fecal culture and/or false-positive ELISA test results and a nonhomogeneous distribution of Map in feces especially for low shedders [42]. In general, explanations for the poor concordance of diagnostic tests could be attributed to false-positive ELISA results, to nonhomogeneous distribution of Map in feces (especially for low shedders), to relatively low prevalence of Map infection, and very low positive predictive value of ELISAs applied. In addition, it has to be taken into account that not only the combination of different tests, but repeated sampling is necessary to achieve the identification of individual animals [5].

Although many animals sampled in a previous study (2007) were no longer in the herds at the time of the second sampling (2009), it was an interesting finding to compare the diagnostic results of animals sampled in 2007 with those results obtained from the same animals in 2009, simulating a longitudinal study or repeating testing for these animals. Changes in our diagnostic test results between 2007 and 2009 agree with studies that report fluctuations of serum ELISA, PCR, and culture results overtime [43–45]. Many test factors (sensitivity, specificity, within-herd prevalence of herd) in every diagnostic procedure influence the variability of results, when the same animals are tested more than once overtime. Particularly for ELISA, fluctuations in test results have been attributable to false-positive results on the first or on the second test, fluctuation in antibody production by the cow, application of tests to low prevalence herds, in which the positive predictive value of tests is lower, or to analytic error. Analytic error occurs when samples were not tested in duplicate as suggested by manufacturers, and repeat analysis gives negative results [43]. Nevertheless multiple testing over time increases the chance of detection of an infected animal, this would also increase the chances of a false-positive result [43]. Therefore, ELISA results have to be analyzed carefully when this test is applied for individual animal diagnosis [21, 46]. However, this is not an uniform process because,

as it has been reported, cows with negative results are less likely to change ELISA status than cows with positive results, regardless of within-herd prevalence [44].

Phenotypic characteristics of fast growth, mycobactin dependency, and no pigmentation of Colombian Map isolates coincide with the description of type II (or cattle type) strains described in previous studies [47]. The combination of MIRU-VNTR and MLSSR, as done previously [48] made possible the reliable differentiation for the first time of two Map genotypes among eight different Map isolates of one herd in Colombia. These methods were applied combined to increase the minimum discriminatory ability needed and not reached if one single method had been used, as reported before by Stevenson et al. 2009 [49]. According to MLSSR, the types isolated in our study are commonly found in cattle and other species in different countries [48, 50–52]. Interestingly, a bovine isolate from Colombia's neighbor country Venezuela has shown a different genotype (11g-10g-5ggt-5ggt), suggesting strain diversity in the northern part of the subcontinent [48].

Although comparison with other studies is very difficult because of the use of different loci for analysis, genotype 1 (INMV1) and genotype 2 (INMV 2) were previously reported as the most common genotypes found in isolates from Argentina and Venezuela [18], and in European isolates [49, 53]. Cases of double strain infection has been also reported at herd level in the United States [54], Germany [20], and the Netherlands [55], while cases of double strain infection at animal level have been reported in Germany [51].

The finding of two strain types among eight isolates recovered from herd 1, including isolates from four cows all born in the herd, but unrelated each other, and isolates of slurry samples of the slurry pit collecting liquid manure and wastewater from the herd's milking parlor, suggests the circulation of Map from and to the environment, and among different animals in the herd. In the same way, the isolation of two different types in one single animal, types that were also isolated in slurry samples, supports the idea of a highly Map contaminated environment, which leads to the infection with more than one different strain genotype in the herd. In herd 1, animal feces are used as fertilizer on the pastures and no paratuberculosis control program is carried out. It has been presenting sporadic cases of animals with symptoms of paratuberculosis confirmed by histopathology (unpublished data), and Map has been detected by PCR and serology [10, 11]. Shedding cows were relatively old cows (≥ 6 years) at the time of sampling suggesting that these animals have been contaminating the environment with Map until they are removed from the herd, contributing to the perpetuation of Map and the presentation of new infections, if no control program is established.

Regarding technical considerations of the genotyping methods, we agree that MLSSR could be less accessible and more expensive than MIRU-VNTR due to the sequencing step required [48]. This aspect could represent a limitation in some developing countries (e.g., Colombia) in which some-times sequencing has to be carried out abroad incrementing even more the costs of application of MLSSR method. However, we agree that MLSSR analysis is an excellent Map

molecular characterization method in terms of in vitro stability and discriminatory index [54], which could justify the cost of the sequencing step needed.

The results of this study confirm the presence and suggest the circulation and transmission of different Map strains types between individuals of the infected herd. In addition, the study confirmed the limitations of current tests for individual diagnosis of subclinical Map infections in cattle, and the usefulness of pooled fecal samples and environmental sampling to screen herds for Map.

Acknowledgments

References

[1] R. W. Sweeney, "Transmission of paratuberculosis," *The Veterinary clinics of North America. Food Animal Practice*, vol. 12, no. 2, pp. 305–312, 1996.

[2] "Possible links between Crohn's disease and paratuberculosis.," Report of the Scientific Comitee on Animal Health and Welfare, pp. 2–76, European Comission, Brussels, Belgium, 2000.

[3] D. L. Clark Jr., J. J. Koziczkowski, R. P. Radcliff, R. A. Carlson, and J. L. E. Ellingson, "Detection of *Mycobacterium avium* subspecies *paratuberculosis*: comparing fecal culture versus serum enzyme-linked immunosorbent assay and direct fecal polymerase chain reaction," *Journal of Dairy Science*, vol. 91, no. 7, pp. 2620–2627, 2008.

[4] S. S. Nielsen and N. Toft, "Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-γ assay and faecal culture techniques," *Veterinary Microbiology*, vol. 129, no. 3-4, pp. 217–235, 2008.

[5] K. Stevenson, "Diagnosis of Johne's disease: current limitations and perspectives," *Cattle Practice*, vol. 18, no. 2, pp. 104–109, 2010.

[6] G. van Schaik, M. Pradenas, A. Mella, and J. Kruze, "Diagnostic validity and costs of pooled fecal samples and individual blood or fecal samples to determine the cow- and herd-status for *Mycobacterium avium* subsp. *paratuberculosis*," *Preventive Veterinary Medicine*, vol. 82, no. 1-2, pp. 159–165, 2007.

[7] R. D. Berghaus, T. B. Farver, R. J. Anderson, C. C. Jaravata, and I. A. Gardner, "Environmental sampling for detection of *Mycobacterium avium* ssp. *paratuberculosis* on large California dairies," *Journal of Dairy Science*, vol. 89, no. 3, pp. 963–970, 2006.

[8] M. T. Collins, I. A. Gardner, F. B. Garry, A. J. Roussel, and S. J. Wells, "Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States," *Journal of the American Veterinary Medical Association*, vol. 229, no. 12, pp. 1912–1919, 2006.

[9] A. S. Motiwala, L. Li, V. Kapur, and S. Sreevatsan, "Current understanding of the genetic diversity of *Mycobacterium avium* subsp. *paratuberculosis*," *Microbes and Infection*, vol. 8, no. 5, pp. 1406–1418, 2006.

[10] J. A. Fernández-Silva, A. Abdulmawjood, O. Akineden, and M. Bülte, "Serological and molecular detection of *Mycobacterium avium* subsp. *paratuberculosis* in cattle of dairy herds in Colombia," *Tropical Animal Health and Production*. In press.

[11] M. M. Zapata, O. Arroyave, R. Ramírez, C. Piedrahita, J. D. Rodas, and J. G. Maldonado, "Identification of *Mycobacterium avium* subspecies *paratuberculosis* by PCR techniques and establishment of control programs for bovine *paratuberculosis* in dairy herds," *Revista Colombiana de Ciencias Pecuarias*, vol. 23, pp. 17–27, 2010.

[12] "*Mycobacterium avium* subsp. *paratuberculosis*," Working Group on diagnosis of infections in veterinary medicine (AVID) of the German Veterinary Society (DVG), 2007, http://www.dvg.net/fileadmin/Bilder/PDF_AVID_Alt/methoden/AB_AVID-Methode_Paratb.pdf.

[13] H. Schönenbrücher, A. Abdulmawjood, K. Failing, and M. Bülte, "New triplex real-time PCR assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces," *Applied and Environmental Microbiology*, vol. 74, no. 9, pp. 2751–2758, 2008.

[14] T. J. Bull, E. J. McMinn, K. Sidi-Boumedine et al., "Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease," *Journal of Clinical Microbiology*, vol. 41, no. 7, pp. 2915–2923, 2003.

[15] A. Amonsin, L. L. Li, Q. Zhang et al., "Multilocus short sequence repeat sequencing approach for differentiating among *Mycobacterium avium* subsp. *paratuberculosis* Strains," *Journal of Clinical Microbiology*, vol. 42, no. 4, pp. 1694–1702, 2004.

[16] T. J. Bull, K. Sidi-Boumedine, E. J. McMinn, K. Stevenson, R. Pickup, and J. Hermon-Taylor, "Mycobacterial interspersed repetitive units (MIRU) differentiate *Mycobacterium avium* subspecies *paratuberculosis* from other species of the *Mycobacterium avium* complex," *Molecular and Cellular Probes*, vol. 17, no. 4, pp. 157–164, 2003.

[17] P. Overduin, L. Schouls, P. Roholl et al., "Use of multilocus variable-number tandem-repeat analysis for typing *Mycobacterium avium* subsp. *paratuberculosis*," *Journal of Clinical Microbiology*, vol. 42, no. 11, pp. 5022–5028, 2004.

[18] V. C. Thibault, M. Grayon, M. L. Boschiroli et al., "New variable-number tandem-repeat markers for typing *Mycobacterium avium* subsp. *paratuberculosis* and M. avium strains: comparison with IS900 and IS1245 restriction fragment length polymorphism typing," *Journal of Clinical Microbiology*, vol. 45, no. 8, pp. 2404–2410, 2007.

[19] E. Castellanos, B. Romero, S. Rodríguez et al., "Molecular characterization of *Mycobacterium avium* subspecies *paratuberculosis* types II and III isolates by a combination of MIRU-VNTR loci," *Veterinary Microbiology*, vol. 144, no. 1-2, pp. 118–126, 2010.

[20] P. Möbius, G. Luyven, H. Hotzel, and H. Köhler, "High genetic diversity among *Mycobacterium avium* subsp. *paratuberculosis* strains from German cattle herds shown by combination of IS900 restriction fragment length polymorphism analysis and mycobacterial interspersed repetitive unit-variable-number tandem-repeat typing," *Journal of Clinical Microbiology*, vol. 46, no. 3, pp. 972–981, 2008.

[21] H. Köhler, B. Burkert, I. Pavlik et al., "Evaluation of five ELISA test kits for the measurement of antibodies against *Mycobacterium avium* subspecies *paratuberculosis* in bovine serum," *Berliner und Munchener Tierarztliche Wochenschrift*, vol. 121, no. 5-6, pp. 203–210, 2008.

[22] P. J. Pinedo, D. O. Rae, J. E. Williams, G. A. Donovan, P. Melendez, and C. D. Buergelt, "Association among results of serum ELISA, faecal culture and nested PCR on milk, blood and faeces for the detection of paratuberculosis in dairy cows," *Transboundary and Emerging Diseases*, vol. 55, no. 2, pp. 125–133, 2008.

[23] C. H. J. Kalis, H. W. Barkema, J. W. Hesselink, C. Van Maanen, and M. T. Collins, "Evaluation of two absorbed enzyme-linked immunosorbent assays and a complement fixation test as replacements for fecal culture in the detection of cows shedding *Mycobacterium avium* subspecies *paratuberculosis*," *Journal of Veterinary Diagnostic Investigation*, vol. 14, no. 3, pp. 219–224, 2002.

[24] E. A. Sugden, K. Stilwell, and A. Michaelides, "A comparison of lipoarabinomannan with other antigens used in absorbed enzyme immunoassays for the serological detection of cattle infected with *Mycobacterium paratuberculosis*," *Journal of Veterinary Diagnostic Investigation*, vol. 9, no. 4, pp. 413–417, 1997.

[25] Y. Yokomizo, H. Yugi, and R. S. Merkal, "A method for avoiding false-positive reactions in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of bovine paratuberculosis," *Nippon Juigaku Zasshi*, vol. 47, no. 1, pp. 111–119, 1985.

[26] U. Jark, I. Ringena, B. Franz, G. F. Gerlach, M. Beyerbach, and B. Franz, "Development of an ELISA technique for serodiagnosis of bovine paratuberculosis," *Veterinary Microbiology*, vol. 57, no. 2-3, pp. 189–198, 1997.

[27] J. B. Osterstock, G. T. Fosgate, B. Norby, E. J. B. Manning, M. T. Collins, and A. J. Roussel, "Contribution of environmental mycobacteria to false-positive serum ELISA results for paratuberculosis," *Journal of the American Veterinary Medical Association*, vol. 230, no. 6, pp. 896–901, 2007.

[28] R. Varges, C. D. Marassi, W. Oelemann, and W. Lilenbaum, "Interference of intradermal tuberculin tests on the serodiagnosis of paratuberculosis in cattle," *Research in Veterinary Science*, vol. 86, no. 3, pp. 371–372, 2009.

[29] S. W. Martin, "Estimating disease prevalence and the interpretation of screening test results," *Preventive Veterinary Medicine*, vol. 2, no. 1–4, pp. 463–472, 1984.

[30] S. S. Nielsen and N. Toft, "A review of prevalences of paratuberculosis in farmed animals in Europe," *Preventive Veterinary Medicine*, vol. 88, no. 1, pp. 1–14, 2009.

[31] S. S. Nielsen and A. K. Ersbøll, "Age at occurrence of *Mycobacterium avium* subspecies *paratuberculosis* in naturally infected dairy cows," *Journal of Dairy Science*, vol. 89, no. 12, pp. 4557–4566, 2006.

[32] R. J. Chiodini, H. J. Van Kruiningen, and R. S. Merkal, "Ruminant paratuberculosis (Johne's disease): the current status and future prospects," *The Cornell Veterinarian*, vol. 74, no. 3, pp. 218–262, 1984.

[33] C. H. J. Kalis, J. W. Hesselink, H. W. Barkema, and M. T. Collins, "Culture of strategically pooled bovine fecal samples as a method to screen herds for paratuberculosis," *Journal of Veterinary Diagnostic Investigation*, vol. 12, no. 6, pp. 547–551, 2000.

[34] S. Tavornpanich, I. A. Gardner, R. J. Anderson et al., "Evaluation of microbial culture of pooled fecal samples for detection of *Mycobacterium avium* subsp *paratuberculosis* in large dairy herds," *American Journal of Veterinary Research*, vol. 65, no. 8, pp. 1061–1070, 2004.

[35] G. Van Schaik, S. M. Stehman, Y. H. Schukken, C. R. Rossiter, and S. J. Shin, "Pooled fecal culture sampling for *Mycobacterium avium* subsp. *paratuberculosis* at different herd sizes and prevalence," *Journal of Veterinary Diagnostic Investigation*, vol. 15, no. 3, pp. 233–241, 2003.

[36] S. J. Wells, R. H. Whitlock, C. J. Lindeman, and T. Fyock, "Evaluation of bacteriologic culture of pooled fecal samples for detection of *Mycobacterium paratuberculosis*," *American Journal of Veterinary Research*, vol. 63, no. 8, pp. 1207–1211, 2002.

[37] G. J. Eamens, R. J. Whittington, M. J. Turner, S. L. Austin, S. A. Fell, and I. B. Marsh, "Evaluation of radiometric faecal culture and direct PCR on pooled faeces for detection of *Mycobacterium avium* subsp. *paratuberculosis* in cattle," *Veterinary Microbiology*, vol. 125, no. 1-2, pp. 22–35, 2007.

[38] S. J. Wells, S. M. Godden, C. J. Lindeman, and J. E. Collins, "Evaluation of bacteriologic culture of individual and pooled fecal samples for detection of *Mycobacterium paratuberculosis* in dairy cattle herds," *Journal of the American Veterinary Medical Association*, vol. 223, no. 7, pp. 1022–1025, 2003.

[39] K. Bögli-Stuber, C. Kohler, G. Seitert et al., "Detection of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss dairy cattle by real-time PCR and culture: a comparison of the two assays," *Journal of Applied Microbiology*, vol. 99, no. 3, pp. 587–597, 2005.

[40] R. W. Sweeney, R. H. Whitlock, A. N. Hamir, A. E. Rosenberger, and S. A. Herr, "Isolation of *Mycobacterium paratuberculosis* after oral inoculation in uninfected cattle," *American Journal of Veterinary Research*, vol. 53, no. 8, pp. 1312–1314, 1992.

[41] S. S. Nielsen, "Transitions in diagnostic tests used for detection of *Mycobacterium avium* subsp. *paratuberculosis* infections in cattle," *Veterinary Microbiology*, vol. 132, no. 3-4, pp. 274–282, 2008.

[42] J. Muskens, M. H. Mars, A. R. W. Elbers, K. Van Maanen, and D. Bakker, "The results of using faecal culture as confirmation test of paratuberculosis-seropositive dairy cattle," *Journal of Veterinary Medicine, Series B*, vol. 50, no. 5, pp. 231–234, 2003.

[43] R. W. Sweeney, R. H. Whitlock, S. McAdams, and T. Fyock, "Longitudinal study of ELISA seroreactivity to *Mycobacterium avium* subsp. *paratuberculosis* in infected cattle and culture-negative herd mates," *Journal of Veterinary Diagnostic Investigation*, vol. 18, no. 1, pp. 2–6, 2006.

[44] H. L. Hirst, F. B. Garry, and M. D. Salman, "Assessment of test results when using a commercial enzyme-linked immunosorbent assay for diagnosis of paratuberculosis in repeated samples collected from adult dairy cattle," *Journal of the American Veterinary Medical Association*, vol. 220, no. 11, pp. 1685–1689, 2002.

[45] E. Geisbauer, J. L. Khol, M. Wassertheurer et al., "Longterm investigation in an Austrian dairy herd with low prevalence of paratuberculosis detection of antibodies in blood and milk," *Veterinary Quarterly*, vol. 29, no. 4, pp. 138–148, 2007.

[46] S. L. B. McKenna, H. W. Barkema, G. P. Keefe, and D. C. Sockett, "Agreement between three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle," *Veterinary Microbiology*, vol. 114, no. 3-4, pp. 285–291, 2006.

[47] K. Stevenson, V. M. Hughes, L. De Juan, N. F. Inglis, F. Wright, and J. M. Sharp, "Molecular characterization of pigmented and nonpigmented isolates of *Mycobacterium avium* subsp. *paratuberculosis*," *Journal of Clinical Microbiology*, vol. 40, no. 5, pp. 1798–1804, 2002.

[48] V. C. Thibault, M. Grayon, M. L. Boschiroli et al., "Combined multilocus short-sequence-repeat and mycobacterial interspersed repetitive unit-variable-number tandem-repeat typing of *Mycobacterium avium* subsp. *paratuberculosis* isolates," *Journal of Clinical Microbiology*, vol. 46, no. 12, pp. 4091–4094, 2008.

[49] K. Stevenson, J. Alvarez, D. Bakker et al., "Occurrence of *Mycobacterium avium* subspecies *paratuberculosis* across host species and European countries with evidence for transmission between wildlife and domestic ruminants," *BMC Microbiology*, vol. 9, article 212, 2009.

[50] A. H. Ghadiali, M. Strother, S. A. Naser, E. J. B. Manning, and S. Sreevatsan, "*Mycobacterium avium* subsp. *paratuberculosis* strains isolated from Crohn's disease patients and animal species exhibit similar polymorphic locus patterns," *Journal of Clinical Microbiology*, vol. 42, no. 11, pp. 5345–5348, 2004.

[51] A. El-Sayed, A. A. Hassan, S. Natour et al., "Evaluation of three molecular methods of repetitive element loci for differentiation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP)," *Journal of Microbiology*, vol. 47, no. 3, pp. 253–259, 2009.

[52] J. L. Corn, E. J. B. Manning, S. Sreevatsan, and J. R. Fischer, "Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from free-ranging birds and mammals on livestock premises," *Applied and Environmental Microbiology*, vol. 71, no. 11, pp. 6963–6967, 2005.

[53] P. E. Douarre, W. Cashman, J. Buckley, A. Coffey, and J. O'Mahony, "Molecular characterization of *Mycobacterium avium* subsp. *paratuberculosis* using multi-locus short sequence repeat (MLSSR) and mycobacterial interspersed repetitive units-variable number tandem repeat (MIRU-VNTR) typing methods," *Veterinary Microbiology*, vol. 149, no. 3-4, pp. 482–487, 2011.

[54] N. B. Harris, J. B. Payeur, V. Kapur, and S. Sreevatsan, "Short-sequence-repeat analysis of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. avium isolates collected from animals throughout the United States reveals both stability of loci and extensive diversity," *Journal of Clinical Microbiology*, vol. 44, no. 8, pp. 2970–2973, 2006.

[55] K. J. E. Van Hulzen, H. C. M. Heuven, M. Nielen, J. Hoeboer, W. J. Santema, and A. P. Koets, "Different *Mycobacterium avium* subsp. *paratuberculosis* MIRU-VNTR patterns coexist within cattle herds," *Veterinary Microbiology*, vol. 148, no. 2–4, pp. 419–424, 2011.

Research Article

Detection of *Mycobacterium avium* subsp. *paratuberculosis* by a Direct In Situ PCR Method

Fernando Delgado,[1] Diana Aguilar,[2] Sergio Garbaccio,[1] Gladys Francinelli,[1] R. Hernández-Pando,[2] and María Isabel Romano[3]

[1] *Instituto de Patobiología, CICVyA, INTA, De Los Reseros y Dr. Nicolás Repetto S/N, Hurlingham, 1686 Buenos Aires, Argentina*
[2] *Experimental Pathology Section, Department of Pathology, Instituto Nacional de la Nutrición "Salvador Zubirán", Vasco de Quiroga 15, Colonia Seccion XVI, Tlalpan,14000 Mexico City, DF, Mexico*
[3] *Instituto de Biotecnología, CICVyA, INTA, De Los Reseros y Dr. Nicolás Repetto S/N, Hurlingham, 1686 Buenos Aires, Argentina*

Correspondence should be addressed to Fernando Delgado, fdelgado@cnia.inta.gov.ar

In situ detection of *Mycobacterium avium* subsp. *paratuberculosis* is useful for diagnosis and research of paratuberculosis. The aim of this paper was to detect this agent in formalin-fixed, paraffin-embedded tissue samples by a direct in situ PCR. The technique was performed on ileum or ileocaecal lymph node samples from 8 naturally infected cattle and 1 healthy calf, by using p89 and p92 primers for amplification of IS900 sequence. Moderate positive signal was detected in all positive samples and not in negative control, but tissues resulted were affected in many cases due to the enzymatic treatment and the high temperature exposition. Although the technique was useful for Map detection, the signal was lower than immunohistochemistry probably because of the fixation process. In one case, signal was higher, which might be due to the detection of spheroplasts. Thus, the described method should be recommended when others resulted negative or for spheroplasts detection.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (Map) is the causative agent of paratuberculosis (PTB), also called Johne's disease. This affects cattle, sheep, and goats and produces losses in daily and beef production. Clinical features include diarrhea and loss of weight, and the main pathologic changes are granulomatous inflammation of the intestine and mesenteric lymph nodes [1]. Additionally, PTB is suspected to be related to Crohn's disease (CD) in humans although this hypothesis is currently debated [2, 3].

Histopathology is used as a diagnostic method, but it is also a very important tool for researching PTB. Detection of Map in tissue samples increases the pathologic diagnosis and may be necessary when experimental infections are performed. Several techniques such as Ziehl Nielsen staining (ZN), immunohistochemistry (IHC), and in situ hybridization (ISH) were tested for detection of the agent [2, 4, 5], but their performances are different. ZN and IHC are easy

to perform and have high sensitivity [4–6], but false negative can arise when infection was recent or bacilli were scanty. Besides, their specificity may be considered low since ZN can not differentiate among acid fast microorganisms and antigens shared by different mycobacteria may affect IHC performance. When both are compared, ZN is cheaper, but IHC may detect antigens of Map even when the bacillus was digested in the cytoplasm of macrophages. On the other hand, ISH is specific but more expensive, hard to perform, and its interpretation may be difficult because of the lower signal obtained [5]. However, detection of Map by ISH is considered useful because it detects spheroplasts (forms of Map with deficiencies in the cell wall) which may be involved with the disease and may be not detected by ZN or IHC [2]. Besides, detection of DNA of Map may be useful when IHC and ZN are negative because bacilli and their antigens are damaged.

In situ PCR (ISP) consists of the amplification of one specific sequence of DNA in a tissue sample. It has been

described as a very sensitive and specific technique and is used for diagnosis or research in many diseases. Related to PTB, an ISP method followed by in situ hybridization was performed for detection of Map in sheep and mice tissue samples [7, 8]. Although it was useful for Map identification, a direct ISP (dISP) method which does not require the hybridization step should be easier to perform. To our knowledge, dISP was successfully used to detect the infection of *M. tuberculosis* in samples from affected and healthy human subjects [9], but not for Map detection in veterinary medicine. The aim of this paper was to detect *Mycobacterium avium* subsp. *paratuberculosis* in tissue samples of naturally infected cattle fixed in formalin paraffin embedded, by using a dISP method, and compare it with immunohistochemistry.

2. Material and Methods

2.1. Analyzed Samples. Samples of ileum or ileocaecal lymph node from nine cows were used. Eight corresponded to adult animals with clinical signs of PTB and isolation of Map from faeces. The other sample was from a calf belonging to a free herd with no changes or clinical signs, which was used as negative control. Tissues were fixed in 10% formalin solution and embedded in paraffin following the standard histological procedures. Compatible lesions and acid fast bacilli were previously confirmed by hematoxylin and eosin and ZN staining performed following routine techniques.

2.2. Direct In Situ PCR. Tissue sections ($2\,\mu$m thickness) were obtained and mounted on positive charged slides. These were deparaffinised by keeping 18 h at 60°C and immersed in xylene (30 min at 37°C), absolute ethanol at room temperature (RT), 75% ethanol (RT), 50% ethanol (RT), 25% ethanol (RT), and water (RT). Then, they were made permeable by incubation at room temperature in 0.02 mol/L HCl for 10 min, followed by 0.01% triton X-100 for 90 s. Proteins were depleted by incubation with 1 mg/L proteinase K (Gibco, Paisley, UK) for 30 min at 37°C, which was inactivated by boiling in a microwave for 15 s. Endogenous alkaline phosphatase was inactivated by immediately immersing the slides into 20% acetic acid for 15 s.

The PCR was performed by incubation of the sections with 50 mL 1X reaction buffer (Gibco, BRL), 1.5 U Taq polymerase, 2 mmol/L MgCl2, 40 mmol/L dNTP, 0.2 mmol/L dUTP labelled with digoxigenin (Boehringer Mannheim, Lewes, UK), and 60 pg each of IS900 *M. avium* subsp. *paratuberculosis* insertion sequence primers. The primers used were p89 (sequence 5′-CGTCGGGTATGGCTTTCATGTGGTTG-CTGTG-3′) and p92 (sequence 5′-CGTCGTTGGCCACC-CGCTGCGAGAGCAAT-3′), previously tested by ISH [2, 5]. The slides were sealed with the Assembly tool (Perkin Elmer, Cambridge, UK) and placed in a Touch Down thermocycler (Hybaid, Ashford, UK). PCR was undertaken with the following thermocycler conditions: 5 min at 95 8C, 35 cycles of 94 8C (1 min), 64.5 8C (1 min) and 72 8C (1 min) ending at 72 8C for 2 min. PCR products were detected with alkaline phosphatase-conjugated sheep antibodies against antidi-

goxigenin (Boehringer Mannheim) diluted 1/500. The chromogen was 5-bromo-4-chloro-3-3 indolyl phosphate toluidine salt and tetrazolium nitroblue (Boehringer Manheim) diluted 1/50. Sections were counterstained with nuclear fast red. For control of false positives, each test section was subjected to PCR without the Taq polymerase.

2.3. Immunohistochemistry. Immunohistochemistry was performed following procedures previously described [5]. Briefly, $2\,\mu$m sections were obtained, mounted on positively charged slides, and deparaffinized. Endogenous peroxidase activity was blocked with 10% hydrogen peroxide in methanol (20 min), and antigenic recovery was performed by humid heat treatment (121°C, 15 min) in citrate buffer (monohydrate citrate, 10 mM, pH 6). After cooling, slides were immersed in TBS buffer (50 mM Tris–HCl, 300 mM NaCl pH 7.6) for 20 min. A blocking step was performed (BSA (Promega) 2% in TBS, 5 min), after which 40 mL of the anti-Map antibody (rabbit polyclonal, Queen's University Belfast, Northern Ireland, UK) diluted 1/100 in TBS was added and incubated at 4°C overnight. The reaction was revealed using the LSAB2^R system (Dako Citomation System) and DAB. Slides were counterstained with Mayer Hematoxylin and coverslipped with synthetic medium.

2.4. Slides Interpretation. All preparations were observed with a conventional light microscope at 40 X, 100 X, 200 X, 400 X, and 1000 X magnification and were compared observing the same regions in all cases. The obtained results were classified as negative (−), weak (+), moderate (++), and intense (+++), according to the number of stained cells at 400 X. When abundant staining was observed at 100 X, it was classified as intense.

3. Results

3.1. In Situ PCR. All infected samples showed staining, which consisted in small blue spots inside the macrophages or Langhans giant cells. Most of them were in the cytoplasm, and few were in the nucleus of the cells (Figures 1(a) and 1(b)). The intensity of the signal was moderate in all cases (Table 1). Negative control was negative, and positive tissues which were incubated without Taq did not show any signal.

Tissue morphology was not perfectly conserved. Connective tissue was not correctly counterstained and a lot of cellular nuclei could not be clearly observed (Figure 1(d)). Areas of tissue were disrupted and removed from the slide in several cases, which made impossible the interpretation of dISP in these areas.

3.2. Immunohistochemistry. All infected tissues showed immunostaining in areas with granulomatous inflammation. Staining was inside the epithelioid and Langhans giant cells, which were distributed in the ileal mucosa and submucosa (Figure 1(c)). The signal was intense in all cases, except in sample 6, in which it was weak (Table 1). Negative control did not show immunostaining.
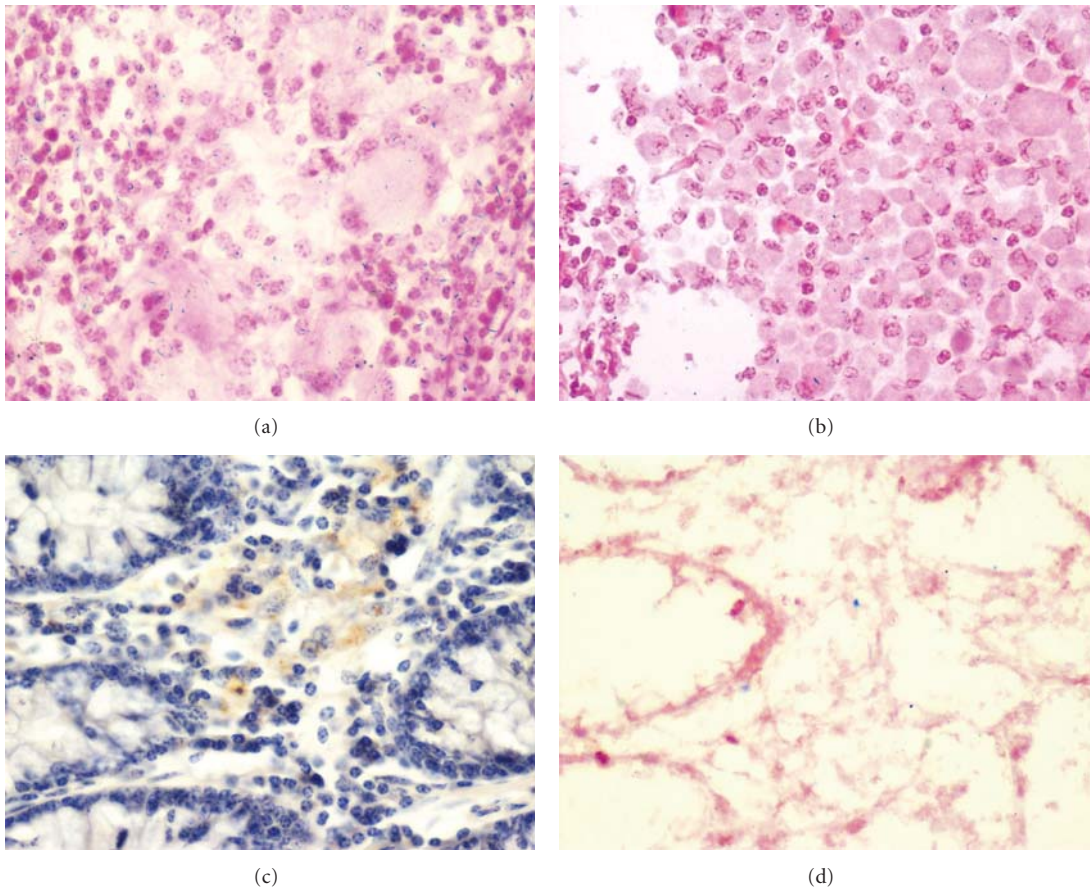
(a)

(b)

(c)

(d)

FIGURE 1: (a) Positive in situ PCR signal (small blue spots) inside the cytoplasm of epithelioid and Langhans giant cells (lamina propria, ileum). (b) Positive in situ PCR signal (small blue spots) inside the cytoplasm of macrophages (ileocaecal lymph node, cortex). (c) Positive immunostaining (brown color) inside the cytoplasm of epithelioid cells. Tissue's architecture is perfectly conserved (lamina propria, ileum). (d) Positive in situ PCR signal inside the cytoplasm of an epithelioid cell. Interpretation of this slide became very difficult due to damage on tissue's architecture (lamina propria, ileum).

TABLE 1: Analyzed samples and obtained results.

Case	Organ	Map culture	Histopathology	IHC	ISPCR
1	Ileum	Positive	Positive	+++	++
2	Ileum	Positive	Positive	+++	++
3	Ileum	Positive	Positive	+++	++
4	Ileocaecal LN	Positive	Positive	+++	++
5	Ileocaecal LN	Positive	Positive	+++	++
6	Ileum	Positive	Positive	+	++
7	Ileum	Positive	Positive	+++	++
8	Ileum	Positive	Positive	+++	++
9	Ileum	Negative	Negative	−	−

References: (IHC): immunohistochemistry, (dISP): direct in situ PCR. (+++): intense, (++): moderate, (+): weak, (−): negative.

Tissue morphology was perfectly conserved in all cases, and interpretation was easy to perform.

4. Discussion

Detection of Map in tissue samples supports pathological diagnosis and allows to determinate where the agent persists in the experimental disease [4, 8]. The obtained results indicated that dISP was able to detect Map in all formalin-fixed, paraffin-embedded tissue samples from naturally infected cattle. The blue positive signal was clearly identified in areas with pathological changes, inside the macrophages or Langhans giant cells. Negative control and positive samples incubated without Taq enzyme resulted negatively, which confirmed that obtained signal in the positive samples was due to the presence of Map.

It was described that spheroplasts may be related to the development of PTB or CD, and these forms of Map can be detected by ISH [2, 7]. In a similar way, dISP would be useful for their identification since it is based in the detection of mycobacterial DNA, and this fact may improve its sensitivity when compared to IHC. In the present paper, IHC signal was intense in all cases, except in sample 6, in which it was weak but moderate for dISP. This fact may be explained by the detection of spheroplasts in this section, which was suggested in a previous study [5].

Although the sensitivity of dISP was described as very high [9], our results indicate that its sensitivity is lower than IHC since the dISP detected signal was moderate and immunostaining was intense in most of cases. However, this difference on staining results may be related with damage on DNA probably occurred during the fixation process, because variables such as time of fixation or nature of fixative solution were not considered when sampling was done, and they can alter the DNA integrity [10]. Although IHC may also be affected by fixation [11], antigen recovery may recuperate immunogenicity Considering that the analyzed samples were not collected for dISP and fixation was not controlled, further studies will be necessary to evaluate sensitivity of dISP.

In situ hybridization was not tested in the present paper. However, the signal obtained with dISP was higher than the weak signal reported for ISH in our previous paper [5]. The cause of this difference may be related to the amplification of DNA obtained by dISP. Besides, the size of the probe, which has to penetrate the cell to hybridize the target DNA in ISH, did not affect the efficiency of dISP since primers and dNTPs are very small and constantly available.

The specificity of the dISP method is based on the amplified gene sequence. Although "IS900-like" sequences were described in other microorganisms [12, 13], IS900 is considered the gold standard in the molecular detection of Map by PCR [14]. It may be possible that these sequences affect the performance, which might decrease the specificity of the technique. The amplification of other more specific sequence of Map may reduce mismatching [7], and further studies will be necessary to determinate which sequence improves the sensitivity. However, it is critical to relate dISP staining with the histopathological changes in order to avoid false positive diagnosis.

Tissue morphology was affected when dISP was performed. This fact may be related to the enzymatic digestion and repeated exposition of the slides to high temperature in each PCR cycle since this problem was not detected with IHC. Because of this, interpretation of the latter was easier, while tissues damaged by dISP required repeating the performed test.

5. Conclusion

A dISP method against IS900 DNA sequence was successfully used for Map detection in formalin-fixed, paraffin-embedded tissue samples, which were obtained from naturally infected adult cows. Although the signal was lower than IHC, further studies should be necessary to determinate sensitivity and specificity of the technique. As it was previously described for ISH, detection of Map DNA by dISP should be useful for the detection of spheroplasts. However, the tissue's structure was affected, and its development was more difficult than IHC. At the light of these facts, this method should be performed after IHC failed to detect Map or to detect spheroplasts in tissue samples with compatible changes.

References

[1] G. W. Dunkin, "Paratuberculosis of cattle and sheep," *Proceedings of the Royal Society of Medicine*, vol. 30, no. 2, pp. 83–90, 1936.

[2] L. Sechi, M. Mura, F. Tanda et al., "Identification of *Mycobacterium avium* subsp. *paratuberculosis* in biopsy specimens from patients with Crohn's disease identified by in situ hybridization," *Journal of Clinical Microbiology*, vol. 39, no. 12, pp. 4514–4517, 2001.

[3] F. A. K. El-Zaatari, M. S. Osato, and D. Y. Graham, "Etiology of Crohn's disease: the role of Mycobacterium avium paratuberculosis," *Trends in Molecular Medicine*, vol. 7, no. 6, pp. 247–252, 2001.

[4] D. J. Brees, S. B. Reimer, N. F. Cheville, A. Florance, and C. O. Thoen, "Immunohistochemical detection of Mycobacterium paratuberculosis in formalin-fixed, paraffin-embedded bovine tissue sections," *Journal of Veterinary Diagnostic Investigation*, vol. 12, no. 1, pp. 60–63, 2000.

[5] F. Delgado, D. Etchechoury, A. Gioffré et al., "Comparison between two in situ methods for Mycobacterium avium subsp. paratuberculosis detection in tissue samples from infected cattle," *Veterinary Microbiology*, vol. 134, no. 3-4, pp. 383–387, 2009.

[6] S. A. Martinson, P. E. Hanna, B. O. Ikede et al., "Comparison of bacterial culture, histopathology, and immunohistochemistry for the diagnosis of Johne's disease in culled dairy cows," *Journal of Veterinary Diagnostic Investigation*, vol. 20, no. 1, pp. 51–57, 2008.

[7] M. Jeyanathan, D. C. Alexander, C. Y. Turenne, C. Girard, and M. A. Behr, "Evaluation of in situ methods used to detect *Mycobactenum avium subsp. paratuberculosis* in samples from patients with Crohn's disease," *Journal of Clinical Microbiology*, vol. 44, no. 8, pp. 2942–2950, 2006.

[8] E. Sanna, C. J. Woodall, N. J. Watt et al., "In situ-PCR for the detection of Mycobacterium paratuberculosis DNA in paraffin-embedded tissues," *European Journal of Histochemistry*, vol. 44, no. 2, pp. 179–184, 2000.

[9] R. Hernández-Pando, M. Jeyanathan, G. Mengistu et al., "Persistence of DNA from *Mycobacterium tuberculosis* in superficially normal lung tissue during latent infection," *Lancet*, vol. 356, no. 9248, pp. 2133–2138, 2000.

[10] C. E. Greer, S. L. Peterson, N. B. Kiviat, and M. M. Manos, "PCR amplification from paraffin-embedded tissues. Effects

of fixative and fixation time," *American Journal of Clinical Pathology*, vol. 95, no. 2, pp. 117–124, 1991.

[11] M. M. Arnold, S. Srivastava, J. Fredenburgh, C. R. Stockard, R. B. Myers, and W. E. Grizzle, "Effects of fixation and tissue processing on immunohistochemical demonstration of specific antigens," *Biotechnic and Histochemistry*, vol. 71, no. 5, pp. 224–230, 1996.

[12] D. Cousins, R. Whittington, I. Marsh, A. Masters, R. J. Evans, and P. Kluver, "Mycobacteria distinct from Mycobacterium avium subsp. paratuberculosis isolated from the faeces of ruminants possess IS900-like sequences detectable by IS900 polymerase chain reaction: implications for diagnosis," *Molecular and Cellular Probes*, vol. 13, no. 6, pp. 431–442, 1999.

[13] A. S. Motiwala, M. Strother, A. Amonsin et al., "Molecular epidemiology of Mycobacterium avium subsp. paratuberculosis: evidence for limited strain diversity, strain sharing, and identification of unique targets for diagnosis," *Journal of Clinical Microbiology*, vol. 41, no. 5, pp. 2015–2026, 2003.

[14] P. Kralik, I. Slana, A. Kralova, V. Babak, R. H. Whitlock, and I. Pavlik, "Development of a predictive model for detection of Mycobacterium avium subsp. paratuberculosis in faeces by quantitative real time PCR," *Veterinary Microbiology*, vol. 149, no. 1-2, pp. 133–138, 2011.

Research Article

Comparative Intradermal Tuberculin Testing of Free-Ranging African Buffaloes (*Syncerus caffer*) Captured for *Ex Situ* Conservation in the Kafue Basin Ecosystem in Zambia

Hetron Mweemba Munang'andu,[1] Victor Siamudaala,[2] Wigganson Matandiko,[2] Andrew Nambota,[3] John Bwalya Muma,[3] Aaron Simanyengwe Mweene,[3] and Musso Munyeme[3]

[1] Section of Aquatic Medicine and Nutrition, Department of Basic Sciences and Aquatic Medicine, Norwegian School of Veterinary Sciences, Ullevålsveien 72, P.O. Box 8146, 0033 Oslo, Norway
[2] Information and Planning Unit, Department of Research, Zambia Wildlife Authority, Private Bag 1, Chilanga, Zambia
[3] Department of Disease Control, School of Veterinary Medicine, University of Zambia, P.O. Box 32379, Lusaka 10101, Zambia

Correspondence should be addressed to Musso Munyeme, munyeme@yahoo.co.uk

Received 20 January 2011; Revised 2 April 2011; Accepted 27 April 2011

Academic Editor: Jesse M. Hostetter

Bovine tuberculosis (BTB) is endemic in African buffaloes (*Syncerus caffer*) in some National Parks in Southern Africa, whilst no studies have been conducted on BTB on buffalo populations in Zambia. The increased demand for ecotourism and conservation of the African buffalo on private owned game ranches has prompted the Zambian Wildlife Authority (ZAWA) and private sector in Zambia to generate a herd of "BTB-free buffaloes" for *ex situ* conservation. In the present study, 86 African buffaloes from four different herds comprising a total of 530 animals were investigated for the presence of BTB for the purpose of generating "BTB free" buffalo for *ex-situ* conservation. Using the comparative intradermal tuberculin test (CIDT) the BTB status at both individual animal and herd level was estimated to be 0.0% by the CIDT technique. Compared to Avian reactors only, a prevalence of 5.8% was determined whilst for Bovine-only reactors a prevalence of 0.0% was determined. These results suggest the likelihood of buffalo herds in the Kafue National Park being free of BTB.

1. Introduction

Bovine tuberculosis (BTB) has emerged to be a significant threat to wildlife conservation in sub-Saharan Africa as it affects several wildlife species especially African buffalo (*Syncerus caffer*) and Kafue lechwe (*Kobus leche Kafuensis*) [1–5]. Demographic factors such as group composition of the herd augmented with gregarious social behavioral pattern under favorable ecological disposition have been identified as major deterministic factors of disease maintenance within particular wildlife species [6–8]. Highly gregarious species that live in large herds such as the African buffalo and the Kafue lechwe (have been shown to have a high intraspecies transmission capacity [4]. Interspecies transmission between prey species and predators has been

reported in the Kruger National Park while close contact between wild ungulates and livestock is reported to be the cause of interspecies transmission at the interface areas [5]. In certain endemic wildlife populations, the disease has been associated with high mortalities that have been linked to population reductions [4, 5]. Given that the eradication of BTB in infected populations is not easy, the disease poses a significant challenge to wildlife conservation. Hence, it is imperative to reduce the risk of introducing BTB to naïve wildlife populations.

The expansion of *ex situ* conservation in Zambia has been affected by the general absence of "Africa's big five," namely, the lion (*Panthera leo*), leopard (*Panthera pardus*), elephant (*Loxodonta africana*), rhino (*Diceros bicornis*), and African buffalo (*Syncerus caffer*) on game ranches. The prohibitive

legislature leading to the absence of wild cats such as the lion, leopard and cheetah on most game ranches is for obvious reasons that in the absence of adequate protective measures wild cats are a serious threat to the general public as most game ranches are located in areas close to human habitation. Besides, most game ranches do not stock enough prey species for the survival of superpredators like lions, leopards, and cheetahs. Elephants are not kept on game ranches since most game ranches are too small to support large herds. This leaves the African buffalo as the only species of the "big five" that can be reared on game ranches. Apart from increasing revenues from ecotourism and safari hunting, rearing of buffalo on game ranches has several ecological benefits that would contribute to the conservation of other species [9]. The other main benefit of buffaloes on a game ranch is that they easily open up thickets and other inaccessible dense vegetation by antelope species through their mixed feeding as bulk grazers and browsers. Besides, rearing of buffaloes on game ranches is strongly supported as a positive *ex situ* conservation strategy that would help increase the declining buffalo populations due to poaching and uncontrolled predation by humans observed in the state owned national parks (NPs). The introduction of buffaloes captured in state owned national parks makes it necessary to screen the animals for infectious diseases like foot and mouth disease (FMD) and BTB to ensure that they are healthy. In the present study, buffaloes captured in the Kafue flats game management area (GMA) were subjected to intradermal tuberculin skin test and were only translocated to game ranches after they were declared free of BTB. We anticipate that these animals will serve as breeding stocks of "BTB-free buffalo herds" for stocking other game ranches in Zambia.

The main aim of this paper is to highlight the tuberculosis situation as it occurs in other animal species within the Kafue basin besides the lechwe antelope species. Further we want to document our current findings to what we may obtain in future given the rising interaction patterns between wild and domestic animals within the Kafue basin.

2. Materials and Methods

2.1. Study Area. The study was carried out in an area called Nanzhila which is located in the game management area of the Kafue National Park (Figure 1). The area is a wetland that supports a transhumance grazing system which allows for the coexistence of wildlife and livestock. Nanzhila is located on the western end of the basin approximately 40 km away from the Lochinvar and Blue lagoon Game Management areas which are endemic with BTB in the Kafue lechwe.

2.2. Capture of the Buffaloes. Seven buffalo herds were captured in the Kafue basin using a helicopter to drive the animals into holding facilities (Capture bomas). Herd size was estimated by taking a mean of three counts of the entire herd brought in the Capture boma. Only animals between one and two years old were captured and included in the study for purpose of generating a breeding stock to be used as a source of buffaloes for other game ranches in future. The selected animals were then driven into quarantine facilities.

There were six quarantine facilities, three on each side of the capture Boma. The facilities were adjacent to the capture Boma where captured animals were diverted for separation as well as holding them for longer periods. The quarantine facilities were fenced with hessian black cloth inside and game fence wire on the outside. They had watering points and feed was provided during the whole capture period. The animals in the quarantine facilities were anaesthetized by darting using M99 (etorphine hydrochloride/Immobilon), a super potent opioid analgesic and were reversed using a very strong opioid antagonist: M5050 (diprenorfin/Revivon,) at standard dosages as recommended by the manufacturer (Novartis, Johannesburg, South Africa). Ages were estimated by tooth development and wear [10] as well as horn development [11]. Each animal was ear-tagged with a unique number. Biological data indicating sex, age, body condition at capture, and herd size were entered in Microsoft excel sheets. The body conditions were subjectively assessed using the degree of exposure of the pelvic bony prominences around the pelvic area with those having a well-padded pelvic area scoring 1, with less or fair scoring 2 whilst those with high prominences or poor scored 3. Here in the quarantine facilities, the animals were kept for as long as three months.

2.3. Comparative Intradermal Tuberculin Skin Test. Animals were cast down using chemical restraint as explained above. Two sites of approximately 8 cm in diameter in the mid cervical area and approximately 10 to 12 cm apart, cranial-caudal, were shaved using a Gillette razor blade. The initial skin thicknesses at the two sites were then measured using vernier callipers. A comparative intradermal tuberculin test (CIDT) consisting of avian and bovine purified protein derivative (PPD) antigens (from The Central Veterinary Institute, Lelystad: The Netherlands) was used. The inoculation was carried out using the McLintock Tuberculin Testing equipment which precisely injects 0.1 ml of avian (containing 2,500 international Units) tuberculin (Lelystad) intradermal onto the cranial site and 0.1 ml of mammalian-bovine (containing 5,000 Tuberculin units) tuberculin (Lelystad) intradermal on the caudal site. After inoculation, a small nodule formed on both sites.

After seventy-two hours after inoculation, each animal was chemically restrained by darting using M99 (etorphine hydrochloride/Immobilon) and the same person who took the previous readings took a second measurement of the skin thickness. The effect of theM99 was reversed using a very strong opioid antagonist: M5050 (diprenorfin/Revivon,) at standard dosages as recommended by the manufacturer (Novartis, Johannesburg, South Africa). The skin thickness at the two sites of inoculation was measured and entered on a record sheet. Reactors were classified according to the Office International des Epizooties (OIE) standards of result interpretation [12]. Further, we arbitrarily classified avian only reactors as being negative when an increase in skin thickness of less than 2 mm PPD is recorded. Inconclusive reactors were indicated by skin thickness increases of between 2 mm and 4 mm.

A positive reaction for the intradermal comparative test was indicated by an increase in skin thickness at the bovine
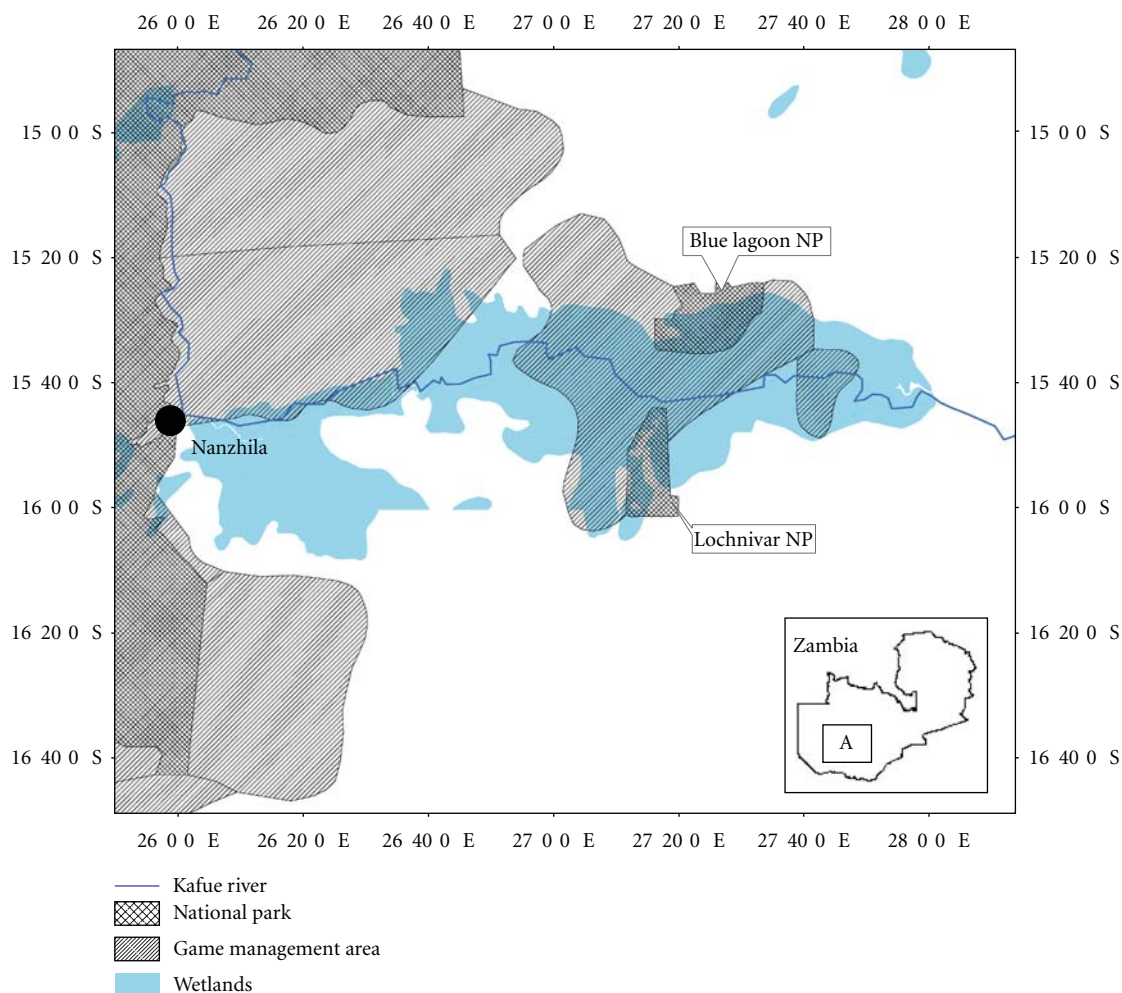
Figure 1: Map of the Kafue basin ecosystem with one big black dot (Nanzhila) showing the study site were the buffaloes were captured and quarantined for the BTB intradermal skin test. The insert shows the map of Zambia with A showing the location of the Kafue basin ecosystem.

site of injection of more than 4 mm greater than the reaction shown at the site of the avian injection. A negative reactor on CIDT was identified when there was a negative reaction to bovine tuberculin, or a positive or inconclusive reaction to bovine tuberculin that was equal to, or less than a positive or inconclusive reaction in avian test and also when negative to both [12]. Only Buffaloes that were negative CIDT were allowed to be translocated to game ranches. Inconclusive reactors were not translocated.

3. Results

A total of 86 African buffaloes were captured from 4 apparently different and independent herds (herd no. 1; (n = 21 animals), herd no 2; (n = 18 animals), herd no 3; (n = 11 animals); herd no 4; (n = 36 animals). All animals (100%) were negative for BTB on comparative intradermal tuberculin test (Table 1). With Avian reactors only, a prevalence

of 5.8% was determined (Table 1). Across all herds captured, 2.3% of the buffaloes showed an inconclusive BTB response.

4. Discussion

To our knowledge this is the first study on BTB intradermal tuberculin test in buffaloes in Zambia and precisely in the Kafue basin area (Figure 1) [4, 13]. Using the CIDT, no BTB positive reactors were found. However, the results need to be interpreted with caution given the age range of the animals. Further, the animals used in the present study were obtained from an area located on the western end of the Kafue basin unlike the Lochinvar area located further east, which has been shown to be endemic to BTB in Kafue lechwe antelopes (Figure 1). However, there has been no intradermal-tuberculin screen tests carried out on buffaloes in the Lochinvar NP as routine meat inspections have not indicated presence of the disease in this species of animals. However, it is evident that the disease is endemic in cattle in

TABLE 1: Overall results based on Avian, Bovine and Comparative tuberculin test reactor animals.

Herd No.	Total no. of buffaloes (N)	No. of buffaloes sampled (n)	Proportion positive (%) Avian reactors only	Proportion Inconclusive (%) CIDT	Proportion Positive (%) CIDT
1	213	21	1 (4.8%)	0 (0.0%)	0 (0.0%)
2	72	18	0 (0.0%)	1 (5.6%)	0 (0.0%)
3	137	11	1 (9.1%)	0 (0.0%)	0 (0.0%)
4	108	36	3 (8.3%)	1 (2.8%)	0 (0.0%)
Overall	**530**	**86**	**5 (5.8%)**	**2 (2.3%)**	**0 (0.0%)**

the area and these are likely to serve as a source of infection for other species apart from the lechwe antelopes, given that the prevalence of BTB in cattle through CIDT was found to be close to 50% [14]. Hence, it would be interesting to carry out detailed investigations on a larger scale to elucidate factors that have kept the disease in cattle and Kafue lechwe with little or no transmission to other l species. On the other hand, these findings are in line with earlier observations made by Munyeme and coworkers [4] that BTB interspecies transmission seems to be restricted in certain regions within the Kafue basin, for example, interface area where cattle and lechwe share grazing pastures. Moreover, if also animals over two years of age would have been included in the testing, more positive reactors might have been found.

Pandey had postulated that cattle on the plains could acquire BTB through contaminated pastures [15]. From this study, buffaloes do not come into direct contact with cattle as do the lechwe antelopes. All the cattle keepers and herdsmen in the Kafue basin have the same observation that cattle will never go close to Buffaloes or Buffaloes come close to cattle, yet lechwe antelopes and cattle will be seen grazing together. Another important observation by both wildlife officers and herdsmen is that lechwe antelopes do not graze near Buffalo herds.

However, the lack of evidence of BTB in Buffaloes that graze in the same cattle grazing grounds without close contact as it obtains in lechwe antelope questions the possibility of acquiring the infection through the oral route. On the other hand, Munyeme and coworkers (2010) found that the disease in both cattle and lechwe is more respiratory than intestinal, which indicates air born route of transmission and thus close contact between animals [4]. However, despite these findings, area attributable deterministic factors of disease occurrence have been found to play a pivotal role in differentiating transmission dynamics of disease patterns [7, 14]. Other studies have indicated that wildlife reservoirs of *M. bovis* are major sources of infection for grazing cattle in some countries [7]. For instance the Badger (*Meles meles*) of the United Kingdom and red deer (*Cervus elaphus*) in New Zealand are biological reservoirs for BTB in these countries [16]. However, our current findings demonstrate a unique difference to these other areas as the disease seems to be more likely to be harboured in cattle than Buffaloes.

With increasing demand for buffaloes in *ex situ* conservation, it is imperative that animals captured for the purpose of translocation from state owned NPs to game ranches should be tested for the presence of BTB and only animals free of the disease may be translocated to game ranches. This is particularly important for highly gregarious species captured from the Kafue basin being an interface area known to be endemic of the disease both in cattle and the Kafue lechwe [1, 4, 5]. Thus far, about 700 Kafue lechwe have been translocated from the Kafue basin without testing for BTB [17]. Subsequently, BTB was diagnosed from Kafue lechwe and bushbuck on a game in Chisamba [13]. Although it is not known how the disease was introduced on the game ranch, it is likely that the translocated Kafue lechwe from Lochinvar NP to the game ranch could have carried the infection thereby becoming a source of infection to the bushbucks on the game ranch. Game ranches with BTB-infected animals could become a source of transmission to other game ranchers as it is common practice for game ranchers to sell live animals to other game farmers. In the absence of screening tests, it is likely BTB can be transmitted from one game ranch to the other. The ultimate solution is to generate BTB-free herds whenever animals are captured for *ex situ* conservation from NPs and GMAs.

Detection of positive *Mycobacteria avium* reactors albeit at a relatively low prevalence intimates the likely presence of other *Mycobacteria species*. Atypical *Mycobacteria species* have been reported from Kafue lechwe in the Kafue basin [18]. MacAdams isolated *Mycobacteria avium* from Kafue lechwe around the Lochinvar NP. As pointed out by Rottcher [18], an environment with warm, humid mud fields appears beneficial for the survival of saprophytic mycobacteria and it would appear that buffaloes and lechwe pick these atypical infections from the environment. However, there is need to carry out more detailed investigation on the presence of other *Mycobacteria species* in the Kafue basin and to determine their impact on wildlife and domestic animals in the area. In order to increase the sensitivity of the screening procedure, animals can be tested using the gamma interferon assay along with the intradermal skin test although there would be need to validate the sensitivity of both assays in wildlife. As pointed out by others, intradermal tuberculin test has proved to be useful for the diagnosis of BTB in buffaloes and lions in the Kruger NP [19]. The bovine gamma interferon assay has proved to be an alternative to the tuberculin test although its application in wildlife has not been widely explored [20]. Overall, African buffaloes

and other highly gregarious species captured for *ex situ* conservation should be screened for the presence of BTB prior to translocation and only noninfected animals should be translocated in order to avoid introducing the disease into new territories.

Acknowledgments

References

[1] R. G. Bengis, N. P. Kriek, D. F. Keet, J. P. Raath, V. De Vos, and H. F. Huchzermeyer, "An outbreak of bovine tuberculosis in a free-living African buffalo (Syncerus caffer–sparrman) population in the Kruger national park: a preliminary report," *Onderstepoort Journal Of Veterinary Research*, vol. 63, no. 1, pp. 15–18, 1996.

[2] J. Gallagher, I. Macadam, J. Sayer, and L. P. van Lavieren, "Pulmonary tuberculosis in free-living lechwe antelope in Zambia," *Tropical Animal Health and Production*, vol. 4, no. 4, pp. 204–213, 1972.

[3] G. Kalema-Zikusoka, R. G. Bengis, A. L. Michel, and M. H. Woodford, "A preliminary investigation of tuberculosis and other diseases in African buffalo (Syncerus caffer) in queen Elizabeth national park, Uganda," *Onderstepoort Journal of Veterinary Research*, vol. 72, no. 2, pp. 145–151, 2005.

[4] M. Munyeme, J. B. Muma, V. M. Siamudaala, E. Skjerve, H. M. Munang'andu, and M. Tryland, "Tuberculosis in Kafue lechwe antelopes (Kobus leche Kafuensis) of the Kafue Basin in Zambia," *Preventive Veterinary Medicine*, vol. 95, no. 3-4, pp. 305–308, 2010.

[5] A. R. Renwick, P. C. L. White, and R. G. Bengis, "Bovine tuberculosis in southern African wildlife: a multi-species host-pathogen system," *Epidemiology and Infection*, vol. 135, no. 4, pp. 529–540, 2007.

[6] R. G. Bengis, R. A. Kock, and J. Fischer, "Infectious animal diseases: the wildlife/livestock interface," *Revue Scientifique et Technique de l'OIE*, vol. 21, no. 1, pp. 53–65, 2002.

[7] L. A. L. Corner, "The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: how to assess the risk," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 303–312, 2006.

[8] M. Munyeme, L. Rigouts, I. C. Shamputa et al., "Isolation and characterization of mycobacterium bovis strains from indigenous zambian cattle using spacer oligonucleotide typing technique," *BMC Microbiology*, vol. 9, Article ID 144, 2009.

[9] H. M. Munag'andu, V. M. Siamudaala, A. Nambota et al., "Disease constraints for utilization of the African buffalo (Syncerus caffer) on game ranches in Zambia," *The Japanese Journal of Veterinary Research*, vol. 54, no. 1, pp. 3–13, 2006.

[10] A. E. Jolles, "Population biology of African buffalo *(Syncerus caffer)* at Hluhluwe-Mfolozi park, South Africa," *African Journal of Ecology*, vol. 45, no. 3, pp. 398–406, 2007.

[11] J. J. R. Grimsdell, "Age determination of the African buffalo, *Syncerus caffer* sparrman," *East African Wildlife Journal*, vol. 11, no. 31, p. 54, 1973.

[12] OIE, *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, OIE Manual, 5th edition, 2004.

[13] U. Zieger, G. S. Pandey, N. P. J. Kriek, and A. E. Cauldwell, "Tuberculosis in Kafue lechwe (Kobus leche kafuensis) and in a bushbuck (Tragelaphus scriptus) on a game ranch in central province, Zambia," *Journal Of The South African Veterinary Association* , vol. 69, no. 3, pp. 98–101, 1998.

[14] M. Munyeme, J. B. Muma, E. Skjerve et al., "Risk factors associated with bovine tuberculosis in traditional cattle of the livestock/wildlife interface areas in the Kafue basin of Zambia," *Preventive Veterinary Medicine*, vol. 85, no. 3-4, pp. 317–328, 2008.

[15] G. S. Pandey, *Studies of the Infectious Diseases of the Kafue Lechwe (Kobus leche kafuensis) with Particular Reference to Tuberculosis in Zambia*, Azabu University, Tokyo, Japan , 1998.

[16] J. F. T. Griffin, J. P. Cross, D. N. Chinn, C. R. Rodgers, and G. S. Buchan, "Diagnosis of tuberculosis due to Mycobacterium-bovis in New-Zealand red deer Necrvus-Elaphus) using a composite blood-test and antibody-assays," *The New Zealand Veterinary Journal* , vol. 42, no. 5, pp. 173–179, 1994.

[17] V. M. Siamudaala, J. B. Muma, H. M. Munang'andu, and M. Mulumba, "Disease challenges concerning the utilization of the Kafue lechwe *(Kobus leche kafuensis)* in Zambia," in *Conservation and Development Interventions at the Wildlife/Livestock Interface, Implications for Wildlife, Livestock and Human Health*, O. Osofsky, S. Leaveland, W. B. Karesh et al., Eds., chapter 11, pp. 75–80, The International Union for Conservation of Nature, Gland, Switzerland and Cambridge, UK, 2005.

[18] D. Rottcher, "Wildlife diseases in Zambia—Final report," Department of Veterinary Services, Ministry of Agriculture Final report on wildlife diseases in Zambia, 93, German Agency for Technical Cooperation, Germany, 1978.

[19] A. E. Jolles, D. V. Cooper, and S. A. Levin, "Hidden effects of chronic tuberculosis in African buffalo," *Ecology*, vol. 86, no. 9, pp. 2358–2364, 2005.

[20] D. G. Grobler, A. L. Michel, L. M. de Klerk, and R. G. Bengis, "The gamma-interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (Syncerus caffer) in the Kruger National Park," *Onderstepoort Journal of Veterinary Research*, vol. 69, no. 3, pp. 221–227, 2002.

Research Article

Antibodies Induced by Lipoarabinomannan in Bovines: Characterization and Effects on the Interaction between *Mycobacterium avium* subsp. *paratuberculosis* and Macrophages *In Vitro*

Ana Jolly,[1] Silvia Beatriz Colavecchia,[1] Bárbara Fernández,[1] Eloy Fernández,[2] and Silvia Leonor Mundo[1]

[1] *Inmunología, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires (UBA), Chorroarín 280, C1427CWO Buenos Aires, Argentina*
[2] *Clínica de Rumiantes, Facultad de Ciencias Veterinarias, Universidad de Buenos Aire (UBA), Chorroarín 280, C1427CWO Buenos Aires, Argentina*

Correspondence should be addressed to Silvia Leonor Mundo, smundo@fvet.uba.ar

Lipoarabinomannan (LAM) is a major glycolipidic antigen on the mycobacterial envelope. The aim of this study was to characterize the humoral immune response induced by immunization with a LAM extract in bovines and to evaluate the role of the generated antibodies in the *in vitro* infection of macrophages with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Sera from fourteen calves immunized with LAM extract or PBS emulsified in Freund's Incomplete Adjuvant and from five paratuberculosis-infected bovines were studied. LAM-immunized calves developed specific antibodies with IgG1 as the predominant isotype. Serum immunoglobulins were isolated and their effect was examined in MAP ingestion and viability assays using a bovine macrophage cell line. Our results show that the antibodies generated by LAM immunization significantly increase MAP ingestion and reduce its intracellular viability, suggesting an active role in this model.

1. Introduction

Paratuberculosis is a chronic granulomatous enteric disease affecting ruminants. The causative agent, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), enters orally, crosses the intestinal barrier, and is phagocytized by macrophages within the lamina propria. These cells serve as the intracellular site in which MAP survives and multiplies [1, 2]. Several studies have been carried out to evaluate the MAP-macrophage interaction, due to its importance in paratuberculosis pathogenesis [3]. It has been proved that various receptors are involved in endocytosis of mycobacteria [4, 5] and that different routes of entry can alter the intracellular fate of pathogens. For example, ligation to receptors for the Fc portion of the immunoglobulins (FcR) is generally accompanied by activation of the respiratory burst [6], and

maturation of phagolysosomes [7], whereas uptake mediated by complement receptors occurs in the absence of pro-inflammatory signals [8].

Generally, the humoral immune response against mycobacterial infections has been considered nonprotective. However, evidence for an active role of B cells and antibodies in some intracellular infections has been accumulated during the last years [9–15]. As regards paratuberculosis, it is accepted that the humoral immune response appears late in the infection and probably associated with the progression of disease from a subclinical to a clinical stage [16]. However, few works have suggested that antibodies could enhance some immune mechanism against MAP. A recent report has evaluated the effect of immune serum on the MAP macrophage interaction suggesting an active role of antibodies [17]. In addition, our group has previously reported

that purified specific antibodies against MAP could enhance the MAP-macrophage interaction *in vitro* and improve the activation of the nuclear factor NF-κB in infected cells [18].

Lipoarabinomannan (LAM) is the main glycolipidic antigen on the mycobacteria envelope and has a molecular weight of approximately 40 kDa. Its structure presents similarities among pathogenic mycobacteria and differences in relation to LAM of nonpathogenic members of the genus [19, 20]. The role of LAM in mycobacterial pathogenesis has been studied by different research groups [21, 22]. Antibodies against LAM have been shown to be beneficial in passive protection experiments in murine tuberculosis models [10]. As regards paratuberculosis in bovines, the serological response against this compound has been extensively studied in order to improve diagnosis. However, little is known about the role of LAM-specific antibodies in this infection and, to our knowledge, there are no published reports on this topic.

The aim of this work was to characterize the humoral immune response induced by immunization with a LAM extract in bovines and to evaluate the role of the generated antibodies in the *in vitro* infection of macrophages with MAP.

2. Materials and Methods

2.1. LAM Extract. Mycobacterium avium subsp. *avium* (MAA) was grown to *log* phase in Dorset-Herley medium, heat-inactivated and kindly provided by Dr. A. Bernardelli (Servicio Nacional de Sanidad Animal, Argentina). The bacterial pellet was centrifuged and resuspended in PBS (NaH$_2$PO$_4$ 3 mM, Na$_2$HPO$_4$ 7.5 mM, NaCl 145 mM, pH 7.2–7.4) for further sonication. LAM was extracted from 5.2 g of total bacteria according to the method previously described elsewhere [23] and adapted to our laboratory conditions [24]. Carbohydrate concentration was determined by the phenol-sulphuric acid method [25] using glucose as standard. Protein concentration was determined by the Bradford method [26] using bovine serum albumin as standard. From these data, the percentage of protein removal achieved was estimated as total protein amount in the LAM extract $\times$ 100/initial total protein amount. The LAM extract was characterized by SDS-PAGE, stained with Bio-Rad Silver Stain (Bio-Rad Laboratories Inc., Hercules, CA, USA) modified for carbohydrate detection [27]. Electrophoresis was performed in a Mini-Protean II electrophoresis cell (Bio-Rad) on 12% polyacrylamide gels, following the manufacturer's instructions. Samples containing 5 μg carbohydrate/lane were twofold diluted in sample buffer and heat-treated (85°C, 5 min) before running (2 h at 96 mV). An ELISA was carried out using anti-LAM of *Mycobacterium tuberculosis* monoclonal antibody (mab CS-35) and purified *M. tuberculosis* LAM as pattern (both reagents were kindly provided by Dr. J. Belisle, Colorado State University, Fort Collins, CO, USA). Flat-bottomed 96-well polystyrene plates (Greiner Microlon, Greiner Bio-One North America Inc., Monroe, NC, USA) were coated with LAM extract or LAM pattern at 25 μg carbohydrate/well. An HRP-conjugated antimouse IgG (KPL, Kirkegaard & Perry Laboratories Inc., Gaithsburg, MD, USA) was used at a dilution of 1 : 500. Plates

were developed using ortho-phenylendiamine dihydrochloride (OPD, Sigma-Aldrich Corp., St. Louis, MO, USA) in citrate-phosphate buffer (Sigma-Aldrich). The reaction was stopped after 10 min by the addition of 50 μL/well of 1 M sulphuric acid, and plates were read in an OpsysMR spectrophotometer (Dynex Technologies, Chantilly, VA, USA). Results are expressed in ELISA Units (EUs), estimated as the mean optical density value obtained at 490 nm (OD) for each sample $\times$ 100/OD for the negative control. In this case, an irrelevant mab was tested as negative control.

2.2. Animals and Samples. A total of fourteen five-month-old Holstein calves from tuberculosis- and paratuberculosis-free herds from the Pampas region of Argentina were kept under field conditions during all the experimental period. Calves were randomly assigned into the LAM group ($n = 9$), which subcutaneously received 2 mg of LAM extract dissolved in 1 mL of PBS and emulsified in 1 mL of Freund's Incomplete Adjuvant (FIA, Sigma-Aldrich), or the normal control group (NC group, $n = 5$), which were mock-immunized with 1 mL of PBS emulsified in 1 mL of FIA. The first immunization was received on day 0 and the booster 35 days later. Blood samples were taken on days 0 and 65. This experiment was performed under the approval and supervision of the Institutional Committee for the care and use of experimental animals of Facultad de Ciencias Veterinarias of Universidad de Buenos Aires, Argentina.

Serum samples from five naturally infected bovines with clinical signs of paratuberculosis were included in the current study as the infected control group (IC group). The diagnosis was confirmed by fecal culture and amplification of the IS*900* fragment from isolated colonies by PCR [28].

2.3. Evaluation of Humoral Immune Response against LAM Extract

2.3.1. ELISA. Plates were coated (4°C, 2 days) with LAM extract (25 μg carbohydrate/well in PBS), washed three times with rinsing buffer (0.05% Tween 20 in PBS) and blocked with blocking buffer (0.05% Tween 20 and 10% skimmed milk in PBS). All subsequent incubations were performed at 37°C for 1 h and after each one, plates were washed three times with rinsing buffer. For comparisons of specific antibody levels, EUs of serum samples diluted 1 : 100 in blocking buffer were measured. An HRP-conjugated goat antibovine IgG (KPL) was added in a 1 : 1000 dilution. For specific isotype evaluation, HRP-conjugated sheep anti-bovine IgM, IgG1, and IgG2 antibodies (Behtyl Laboratories Inc., Montgomery, TX, USA) diluted 1 : 300 and rabbit antibovine IgG3 antibody [18] diluted 1 : 500 followed by HRP-conjugated goat antirabbit IgG (KPL) diluted 1 : 1000 were used. Plates were developed as described above. Results are expressed in EUs, using normal control sera as negative.

2.3.2. Immunoblot. To characterize the specificity of the generated antibodies, immunoblots were performed using LAM extract as antigen. Electrophoretic transfer onto nitrocellulose membranes (Trans-blot transfer medium, Bio-
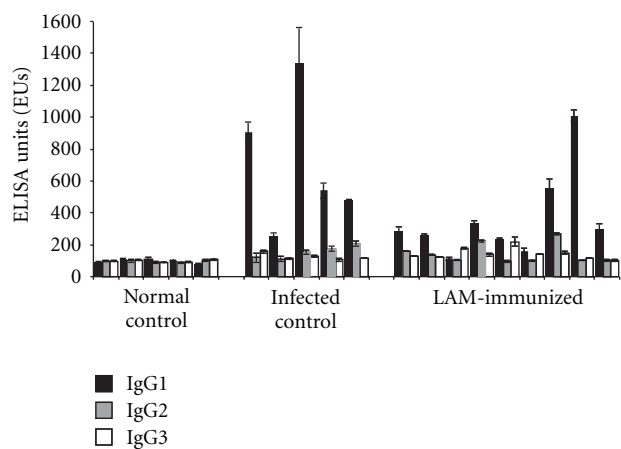
FIGURE 1: Relative levels of LAM extract-specific IgG isotypes expressed as ELISA Units (EUs) ± standard deviation of two independent measurements for each animal at 1 : 100 dilution of sera.

Rad) was carried out in a Trans-Blot SD cell (Bio-Rad) following the manufacturer's instructions. Membranes were incubated in blocking buffer and then with bovine sera diluted 1 : 250. Subsequently, HRP-conjugated goat anti-bovine IgG (KPL) was added in a 1 : 1000 dilution. The reaction was developed using 0.5 mg/mL DAB (HRP Color Development Reagent 3,3′-diaminobenzidine, Bio-Rad) and 1 μL/mL H_2O_2 100 vol. in TBS buffer (20 mM Tris, 500 mM NaCl, pH 7.5) for 10 min. All incubations were performed at 37°C for 1 h, and each step was followed by three washes in rinsing buffer. In order to confirm specificity against nonprotein molecules, a proteolytic treatment of LAM extract with 0.2 mg/mL proteinase K (Amresco Inc., Solon, OH, USA) was performed before running the SDS-PAGE, as described by Reichel et al. [29]. All sera were analyzed by immunoblot against digested and undigested LAM extract. As a digestion control, 1 mg/mL ovalbumin (OVA, Sigma-Aldrich) solution was incubated with proteinase K in the same conditions as the LAM extract. Digested and undigested OVA were evaluated with a hyperimmune bovine serum against OVA.

2.4. Precipitation and Purification of Antibodies.
Serum antibodies of two animals from LAM-immunized, infected, and normal control groups were isolated and purified for further use in functional assays. Sera were heat-inactivated at 56°C for 30 min. Ammonium sulfate precipitation (44% saturation) was performed to obtain precipitated immunoglobulins (Igs). Then, purification by protein G affinity chromatography (protein G-agarose, Exalpha Biologicals, Shirley, MA, USA) was carried out, and purified Igs were obtained. In order to check the purity of the Igs fractions, SDS-PAGE and electrophoresis were conducted. The concentration of Igs was estimated by the Bradford method, considering the percentage of gamma-globulins detected by densitometric analysis of electrophoresis. The Igs fractions were filtered by 0.22 μm and stored at −70°C until use.

2.5. Functional Evaluation of Antibodies.
These experiments were performed using the SV40-transformed bovine peritoneal macrophage cell line (Bomac) [30]. Bomac cells were cultured in RPMI-1640 medium (GIBCO, Invitrogen Corp., Carlsband, CA, USA) supplemented with 50 μg/mL gentamicin (Sigma-Aldrich) and 5% foetal calf serum (Invitrogen) at 37°C and 5% CO_2. The K-10 MAP reference strain [31], generously provided by Dr. F. Paolicchi (Instituto Nacional de Tecnología Agropecuaria, Argentina), was grown at 37°C in Middlebrook 7H9 broth (Difco, BD biosciences, Franklin Lakes, NJ, USA) containing 10% albumin-dextrose-sodium chloride, 0.05% Tween 80 (Sigma-Aldrich), and 2 μg/mL mycobactin J (Allied Monitor Inc., Fayette, MO, USA). Titration was performed by serial dilution and seeding onto 7H9 agar plates. Stock was centrifuged and frozen at −70°C in 15% glycerol medium. Before use, MAP was unfrozen and cultured overnight at 37°C, then centrifuged, disaggregated by passages through a 25-gauge needle, and resuspended in RPMI medium to a final concentration of 10^9 Colony Forming Units (CFU)/mL. Multiplicity of infection was set at 10 : 1 (bacteria : cell), and antibodies were used at a final concentration of 100 μg/mL.

2.5.1. Ingestion Assay.
Bomac cells (1×10^6 viable cells/mL) were seeded onto 20 mm × 20 mm sterile coverslips, allowed to adhere for 2 h and incubated overnight in RPMI medium. Bacteria were opsonized with precipitated Igs (100 μg/mL) from LAM-immunized, infected or normal control bovines, at 37°C for 1 h in a shaker. Immediately prior to inoculation of monolayers, the bacterial suspension was disaggregated as described above. MAP-macrophages interaction was allowed for 45 min. Cells were washed with cold PBS, fixed with 0.37% formaldehyde, and stained with Ziehl-Nielsen. A minimum of 100 cells/coverslip were counted in immersion fields (1000x) by light microscopy. The percentage of phagocytic cells (%PhC) and the mean number of internalized MAP detected in each cell (iMAP) were recorded. The phagocytic index (PI) was calculated as % PhC × iMAP [32].

2.5.2. Intracellular Viability Assay.
To evaluate the viability of ingested MAP, Bomac cells (1×10^6 viable cells/mL) were seeded onto 24-well tissue culture plates and incubated at 37°C overnight in 5% CO_2. Bacteria were opsonized and disaggregated as described above. Then, inoculated into Bomac cultures, in duplicate. After 2 h, monolayers were washed with PBS and one of each duplicate well was lysed with 0.2% sodium dodecylsulfate for initial CFU counting. The other duplicate well was incubated with RPMI medium containing 0.1 mg/mL gentamicin for 2 h in order to avoid contamination [33], then replacing it with antibiotic-free medium. Infected macrophages were cultured for 72 h and then lysed for final CFU counting. Lysates were serially plated on 7H9 agar and cultured at 37°C for 5 weeks until the CFU were counted. Results are expressed as percent change in viability (final CFU/initial CFU × 100). Additionally, an FcR-blocking assay was conducted as described by Manca et al. [34]. Bomac cells were preincubated for 1 h with 100 μg/mL of protein G-purified Igs from a normal control bovine that had been heat-aggregated.
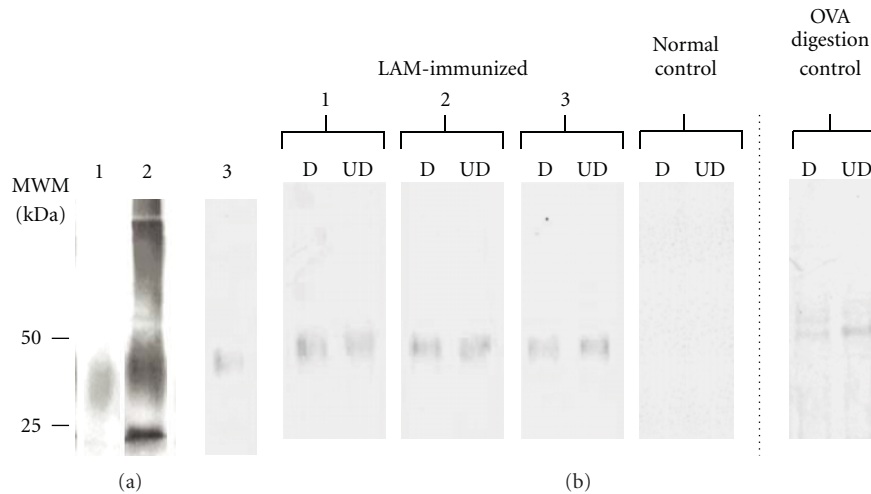
(a)

(b)

FIGURE 2: (a) Characterization of LAM extract by SDS-PAGE (lane 1: purified LAM of *M. tuberculosis*, lane 2: LAM extract, both silver-stained for carbohydrate detection) and immunoblot. (lane 3: LAM extract reactivity assessed against one representative serum from the infected control group). (b) Immunoblot of bovine sera reactivity against LAM extract. Left side of the dotted line: LAM extract was run as antigen. D and UD indicate protein-digested or undigested antigen. 1, 2 and 3 show results of three representative sera from LAM-immunized group. Normal control shows results representative for sera in that group. Right side of the dotted line: ovalbumin (OVA) was run as antigen and reactivity assessed against serum of an OVA-immunized bovine, as a positive control of protein digestion. MWM: molecular weight markers.

2.6. Statistical Analysis. Data were analyzed for statistical significance using STATISTIX 8.0 software. ANOVA followed by Tukey's test was used except for isotype analysis. In that case, Kruskal-Wallis test followed by pairwise comparisons was run. The level of significance was set at P value <.05.

3. Results

3.1. Characterization of LAM Extract. We obtained an extract containing 105 mg of total carbohydrate. Protein presence was largely reduced (46.8 mg of protein initially versus 0.4 mg of residual protein in the LAM extract), thus showing a percentage of protein removal of 99.2%. SDS-PAGE results demonstrated that the LAM extract was mostly composed of a carbohydrate mixture, as revealed by the presence of many bands when modified silver stain was performed. Indeed, the predominant component migrated similar to *M. tuberculosis* LAM (Figure 2(a), lane 1 and 2). As expected, sera from the MAP-infected control group recognized our LAM extract of MAA (Figure 2(a), lane 3). In the mab CS35-ELISA, the result obtained for our LAM extract was 493.5 ± 35.1 EU, whereas for purified tuberculosis LAM it was 657.8 ± 10.6 EU. These results show cross reactions between LAM of pathogenic mycobacteria.

3.2. Humoral Immune Response against LAM Extract. The reactivity of sera against LAM extract was assessed and the level of specific antibodies and the isotypic profile were determined. All the calves in the LAM group generated a specific humoral immune response, with antibody levels at 1 : 100 serum dilution between 180 and 790 EU. Comparable levels were detected in the infected control group (range

between 180 and 723 EU). As regards the isotype analysis, the presence of specific IgM, IgG1, IgG2, and IgG3 was evaluated in sera from LAM-immunized, infected, and normal control bovines. We could not detect specific IgM (LAM group 106.8 ± 5.5 EU, IC group 97.9 ± 15.8 EU, NC group 101.6 ± 8.1 EU). The results obtained for specific IgG1, IgG2, and IgG3 are shown in Figure 1. The humoral immune response of LAM-immunized bovines was mostly predominated by IgG1 with minor presence of IgG2 and IgG3, although statistically significant differences were found for the three of them when comparing with the normal control group ($P < .05$). In the infected control group, a similar trend was detected, with IgG1 as the only isotype with levels significantly higher than in the normal control group.

Sera were also tested against the LAM extract by immunoblot assay (Figure 2(b)). For all LAM-immunized calves, only one band of molecular weight between 25 and 50 kDa was detected. This band remained at equal strength after proteolytic digestion of the extract, demonstrating the nonprotein nature of the antigen involved (Figure 2(b), D *versus* UD lanes).

3.3. Functional Effects of Antibodies. The precipitated and purified Igs obtained (Figure 3(b), boxes) were used in functional assays.

Effect on MAP Ingestion. As shown in Figure 3(a), opsonization of MAP with specific antibodies increased the ingestion of bacteria. The antibodies from both LAM-immunized and infected control bovines showed a phagocytic index significantly higher ($P < .01$) than the one obtained when
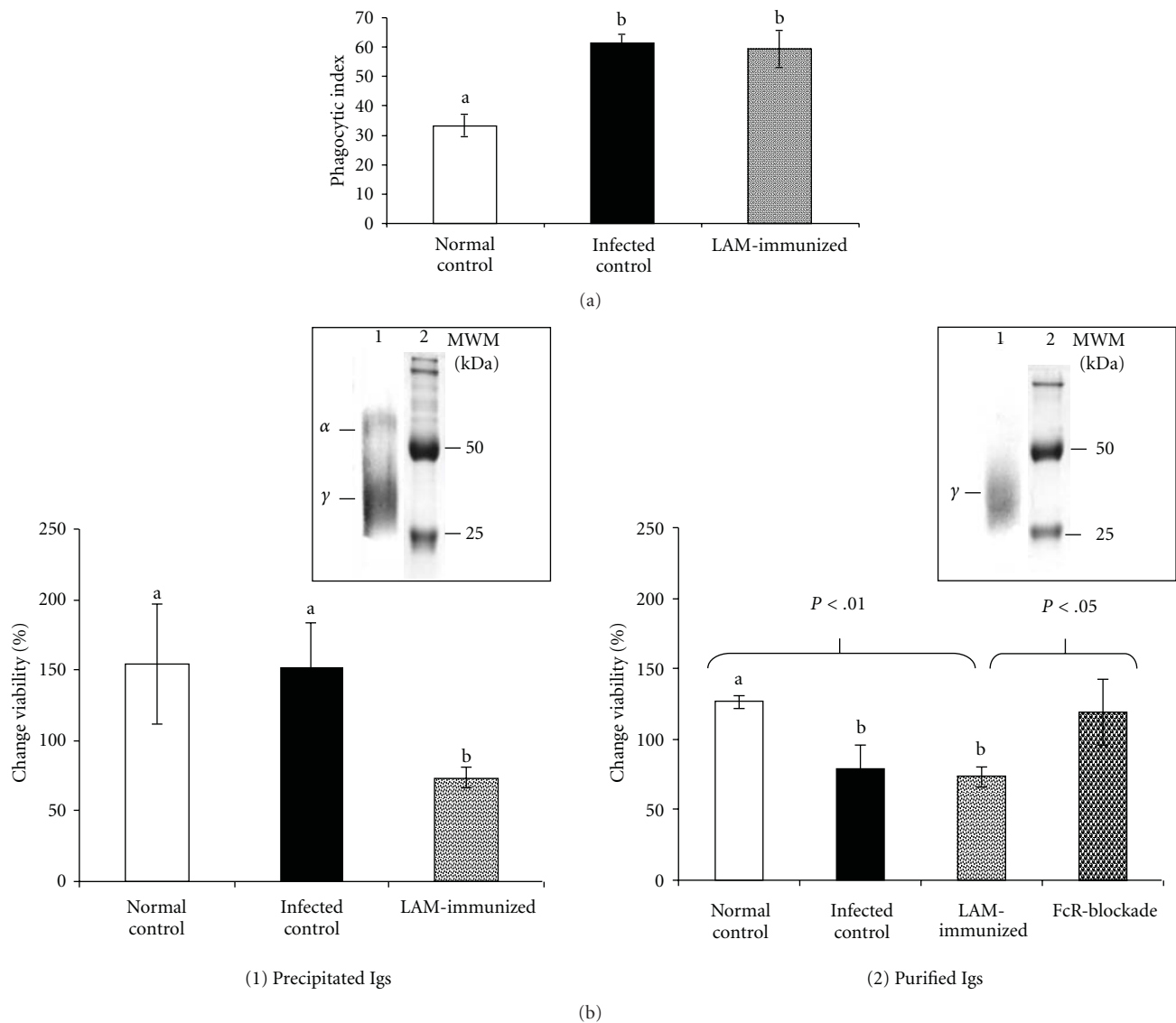
(a)

(1) Precipitated Igs

(2) Purified Igs

(b)

FIGURE 3: Functional effects of antibodies: (a) on MAP ingestion. Results are expressed as mean phagocytic index for each treatment ± standard deviation; (b) on MAP intracellular viability. Results are expressed as % change in MAP viability 72 h after the ingestion by Bomac cells. Boxes are representative of electrophoretic (lane 1) and SDS-PAGE (lane 2) pattern of precipitated and purified Igs. α and γ indicate alpha- and gamma- globulins bands. MWM: molecular weight markers. Error bars indicate standard deviation of three independent assays and different letters or numbers over bars represent significant differences between groups.

opsonizing MAP with normal control antibodies (phagocytic index of 59.4, 61.5, and 33.2, resp.).

Effect on MAP Intracellular Viability. When assays were conducted by opsonizing MAP with precipitated antibodies, LAM group Igs significantly reduced ($P < .05$) the percentage of MAP viability, as compared with that obtained for normal and infected control Igs opsonization (Figure 3(b)(1)).

When MAP was opsonized with purified Igs, comparable and more repeatable results were obtained for LAM-immunized and normal control groups ($P < .01$). For the infected control group, the result was similar to that of the treatment with LAM group Igs. Preincubation of macrophages with aggregated Igs (FcR blockade) resulted in

a significant increase of MAP viability as compared with the effect of antibodies from LAM group without previous incubation with aggregated Igs ($P < .05$) (Figure 3(b)(2)). This result suggests that the effect observed for the opsonization with LAM group Igs could be FcR-mediated.

4. Discussion

In the present work, we examined the immune response induced in cattle by immunization with a LAM extract and the effect of the generated antibodies in the MAP-macrophage interaction.

We used MAA for LAM extraction instead of MAP due to the antigenic homology between both [29] and the

faster growth in culture of the former. Besides, our ELISA and immunoblot results support cross reactions among mycobacterial glycolipids previously described [19, 20, 29] and show that the method of LAM extraction applied preserved its antigenicity. To our knowledge, this is the first report of bovine immunization using a mycobacterial LAM extract. With the immunization protocol used, we were able to induce high levels of specific antibodies with increases of all IgG isotypes and the predominance of IgG1 in most of the studied calves. IgG1 was also detected as the predominant isotype in the infected control group. Similar responsiveness against LAM was previously described in bovines with clinical paratuberculosis [35]. Taking into account that this isotype represents the most relevant immunoglobulin in mammary gland secretions of bovines [36], the presence of local specific IgG1 might be relevant for the passive protection of newborn calves.

The influence of opsonization with antibodies on MAP phagocytosis and intracellular viability has been previously evaluated [17, 18, 33, 37, 38]. However, published reports have been generally based on assays where total serum was examined as a source of antibodies. While many other serum components are known to possess opsonic activity and could affect intracellular viability of bacteria, we purified Igs for functional evaluation. The purification methodology applied was successful. However, a heavy band was observed in SDS-PAGE, probably corresponding to the presence of incompletely reduced Igs molecules, as suggested by the reactivity with an antibovine IgG antibody (data not shown).

We found that the phagocytic level of Bomac cells increased almost twofold when MAP was opsonized with antibodies from either LAM-immunized or infected bovines. MAP-phagocytosis enhancement by hyperimmune sera has been previously reported in Bomac cells [18] and bovine blood monocyte-derived macrophages (BMDMs) [17, 37]. The phagocytic levels detected herein were comparable to those published by Woo et al. for Bomac cells [38], although our indexes were lower than those described for BMDM when ingesting MAP [17, 37]. Therein, the kinetics of MAP uptake was evaluated and a significant increase in the ingestion level was detected after 60 to 120 min of incubation [17, 37]. Taking these observations into account, it is possible that the lower phagocytic index detected herein could be related to the shorter MAP-macrophages incubation time or to the use of Bomac cell line instead of BMDM.

Controversial results in viability assays opsonizing MAP with whole sera from healthy and infected bovines were published [17, 37, 38]. In our model, opsonization with precipitated Igs from normal and infected bovines shows a beneficial effect on MAP viability. However, we found different results when precipitated and purified Igs from infected bovines were used for opsonization. The presence of other opsonins in precipitated Ig fractions, such as collectins, C-Reactive protein, or fibronectin, could explain the difference found [39–41]. Noteworthy, our results show that antibodies induced by LAM immunization could significantly reduce the intracellular viability of MAP.

These results raise questions about the biological relevance of LAM antibodies in paratuberculosis. We here found

that purified antibodies present in sera from bovines immunized with LAM of MAA could reduce MAP intracellular viability as well as antibodies from MAP clinically infected cattle. However, in the natural infection, the antibodies appear late and probably associated with the progression of disease. It remains to be established whether specific antibodies present at the moment of the infection could modify the course of paratuberculosis.

Our findings provide new data about basic aspects of the role of antibodies in MAP-macrophage infection *in vitro*. More studies, using other MAP strains, especially field isolates, and macrophages derived directly from bovines, must be conducted in order to better approach to the role of antibodies in the natural MAP-macrophage interaction that takes place in the host.

References

[1] P. H. Bendixen, B. Bloch, and J. B. Jorgensen, "Lack of intracellular degradation of *Mycobacterium paratuberculosis* by bovine macrophages infected *in vitro* and *in vivo*: light microscopic and electron microscopic observations," *American Journal of Veterinary Research*, vol. 42, no. 1, pp. 109–113, 1981.

[2] C. J. Clarke, "The pathology and pathogenesis of paratuberculosis in ruminants and other species," *Journal of Comparative Pathology*, vol. 116, no. 3, pp. 217–261, 1997.

[3] D. J. Weiss and C. D. Souza, "Review paper: modulation of mononuclear phagocyte function by *Mycobacterium avium* subsp. *paratuberculosis*," *Veterinary Pathology*, vol. 45, no. 6, pp. 829–841, 2008.

[4] C. D. Souza, O. A. Evanson, S. Sreevatsan, and D. J. Weiss, "Cell membrane receptors on bovine mononuclear phagocytes involved in phagocytosis of *Mycobacterium avium* subsp. *paratuberculosis*," *American Journal of Veterinary Research*, vol. 68, no. 9, pp. 975–980, 2007.

[5] J. S. Sohal, S. V. Singh, P. Tyagi et al., "Immunology of mycobacterial infections: With special reference to *Mycobacterium avium* subspecies *paratuberculosis*," *Immunobiology*, vol. 213, no. 7, pp. 585–598, 2008.

[6] K. Yamamoto and R. B. Johnston Jr., "Dissociation of phagocytosis from stimulation of the oxidative metabolic burst in macrophages," *Journal of Experimental Medicine*, vol. 159, no. 2, pp. 405–416, 1984.

[7] V. Trivedi, S. C. Zhang, A. B. Castoreno et al., "Immunoglobulin G signaling activates lysosome/phagosome docking," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 48, pp. 18226–18232, 2006.

[8] L. S. Schlesinger, "*Mycobacterium tuberculosis* and the complement system," *Trends in Microbiology*, vol. 6, no. 2, pp. 47–49, 1998.

[9] J. U. Igietseme, F. O. Eko, Q. He, and C. M. Black, "Antibody regulation of T-cell immunity: implications for vaccine strategies against intracellular pathogens," *Expert Review of Vaccines*, vol. 3, no. 1, pp. 23–34, 2004.

[10] A. Glatman-Freedman, "The role of antibody-mediated immunity in defense against *Mycobacterium tuberculosis*: advances toward a novel vaccine strategy," *Tuberculosis (Edinb)*, vol. 86, no. 3-4, pp. 191–197, 2006.

[11] S. H. E. Kaufmann, "The contribution of immunology to the rational design of novel antibacterial vaccines," *Nature Reviews Microbiology*, vol. 5, no. 7, pp. 491–504, 2007.

[12] G. Källenius, A. Pawlowski, B. Hamasur, and S. B. Svenson, "Mycobacterial glycoconjugates as vaccine candidates against tuberculosis," *Trends in Microbiology*, vol. 16, no. 10, pp. 456–462, 2008.

[13] Y. López, D. Yero, G. Falero-Diaz et al., "Induction of a protective response with an IgA monoclonal antibody against *Mycobacterium tuberculosis* 16 kDa protein in a model of progressive pulmonary infection," *International Journal of Medical Microbiology*, vol. 299, no. 6, pp. 447–452, 2009.

[14] P. J. Maglione and J. Chan, "How B cells shape the immune response against *Mycobacterium tuberculosis*," *European Journal of Immunology*, vol. 39, no. 3, pp. 676–686, 2009.

[15] F. Abebe and G. Bjune, "The protective role of antibody responses during *Mycobacterium tuberculosis* infection," *Clinical and Experimental Immunology*, vol. 157, no. 2, pp. 235–243, 2009.

[16] J. R. Stabel, "Transitions in immune responses to *Mycobacterium paratuberculosis*," *Veterinary Microbiology*, vol. 77, no. 3-4, pp. 465–473, 2000.

[17] J. M. Hostetter, R. Kagan, and E. Steadham, "Opsonization effects on *Mycobacterium avium* subsp. *paratuberculosis*-macrophage interactions," *Clinical and Diagnostic Laboratory Immunology*, vol. 12, no. 6, pp. 793–796, 2005.

[18] S. L. Mundo, A. M. Fontanals, M. García et al., "Bovine IgG1 antibodies against *Mycobacterium avium* subsp. *paratuberculosis* protein p34-cx improve association of bacteria and macrophages," *Veterinary Research*, vol. 39, no. 1, 6 pages, 2008.

[19] J. Nigou, M. Gilleron, and G. Puzo, "Lipoarabinomannans: from structure to biosynthesis," *Biochimie*, vol. 85, no. 1-2, pp. 153–166, 2003.

[20] V. Briken, S. A. Porcelli, G. S. Besra, and L. Kremer, "Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response," *Molecular Microbiology*, vol. 53, no. 2, pp. 391–403, 2004.

[21] A. Welin, M. E. Winberg, H. Abdalla et al., "Incorporation of *Mycobacterium tuberculosis* lipoarabinomannan into macrophage membrane rafts is a prerequisite for the phagosomal maturation block," *Infection and Immunity*, vol. 76, no. 7, pp. 2882–2887, 2008.

[22] L. Sweet, P. P. Singh, A. K. Azad, M. V. Rajaram, L. S. Schlesinger, and J. S. Schorey, "Mannose receptor-dependent delay in phagosome maturation by *Mycobacterium avium* glycopeptidolipids," *Infection and Immunity*, vol. 78, no. 1, pp. 518–526, 2010.

[23] B. Hamasur, G. Källenius, and S. B. Svenson, "A new rapid and simple method for large-scale purification of mycobacterial lipoarabinomannan," *FEMS Immunology and Medical Microbiology*, vol. 24, no. 1, pp. 11–17, 1999.

[24] A. Jolly, S. Colavecchia, A. Jar, E. Fernandez, and S. Mundo, "Lipoarabinomanano (LAM) de *Mycobacterium sp.*: respuesta inmune inducida en terneros," *Investigacion Veterinaria*, vol. 8, pp. 103–109, 2006.

[25] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, "Colorimetric method for determination of sugars and related substances," *Analytical Chemistry*, vol. 28, no. 3, pp. 350–356, 1956.

[26] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[27] A. Fomsgaard, M. A. Freudenberg, and C. Galanos, "Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels," *Journal of Clinical Microbiology*, vol. 28, no. 12, pp. 2627–2631, 1990.

[28] D. M. Collins, D. M. Gabric, and G. W. De Lisle, "Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*," *FEMS Microbiology Letters*, vol. 60, no. 2, pp. 175–178, 1989.

[29] M. P. Reichel, R. Kittelberger, M. E. Penrose et al., "Comparison of serological tests and faecal culture for the detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle and analysis of the antigens involved," *Veterinary Microbiology*, vol. 66, no. 2, pp. 135–150, 1999.

[30] J. R. Stabel and T. J. Stabel, "Immortalization and characterization of bovine peritoneal macrophages transfected with SV40 plasmid DNA," *Veterinary Immunology and Immunopathology*, vol. 45, no. 3-4, pp. 211–220, 1995.

[31] E. M. Foley-Thomas, D. L. Whipple, L. E. Bermudez, and R. G. Barletta, "Phage infection, transfection and transformation of *Mycobacterium avium* complex and *Mycobacterium paratuberculosis*," *Microbiology*, vol. 141, no. 5, pp. 1173–1181, 1995.

[32] P. A. Campbell, B. P. Canono, and D. A. Drevets, "Measurement of bacterial ingestion and killing by macrophages," in *Current Protocols of Immunology*, J. E. Coligan, B. E. Bierer, D. H. Margulies, E. M. Shevach, W. Strober, and R. Coico, Eds., chapter 14, unit 14.6, John Wiley & Sons, 1999.

[33] J. Hostetter, E. Steadham, J. Haynes, T. Bailey, and N. Cheville, "Phagosomal maturation and intracellular survival of *Mycobacterium avium* subspecies *paratuberculosis* in J774 cells," *Comparative Immunology, Microbiology and Infectious Diseases*, vol. 26, no. 4, pp. 269–283, 2003.

[34] F. Manca, D. Fenoglio, G. L. Pira, A. Kunkl, and F. Celada, "Effect of antigen/antibody ratio on macrophage uptake, processing, and presentation to T cells of antigen complexed with polyclonal antibodies," *Journal of Experimental Medicine*, vol. 173, no. 1, pp. 37–48, 1991.

[35] A. D. Koets, V. Rutten, M. De Boer, D. Bakker, P. Valentin-Weigand, and W. Van Eden, "Differential changes in heat shock protein-, lipoarabinomannan-, and purified protein derivative-specific immunoglobulin G1 and G2 isotype responses during bovine *Mycobacterium avium* subsp. *paratuberculosis* infection," *Infection and Immunity*, vol. 69, no. 3, pp. 1492–1498, 2001.

[36] J. E. Butler and M. E. Kehrli Jr., "Immunoglobulins and immunocytes in the mammary glands and its secretions," in *Mucosal immunology*, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, J. R. McGee, and L. Mayer, Eds., vol. 3, chapter 103, pp. 1776–1784, Elsevier Academic Press, 3rd edition, 2005.

[37] B. G. Zurbrick and C. J. Czuprynski, "Ingestion and intracellular growth of *Mycobacterium paratuberculosis* within bovine blood monocytes and monocyte-derived macrophages," *Infection and Immunity*, vol. 55, no. 7, pp. 1588–1593, 1987.

[38] S. R. Woo, J. Sotos, A. Hart, R. G. Barletta, and C. J. Czuprynski, "Bovine monocytes and a macrophage cell line differ in their ability to phagocytose and support the intracellular survival of *Mycobacterium avium* subsp. *paratuberculosis*," *Veterinary Immunology and Immunopathology*, vol. 110, no. 1-2, pp. 109–120, 2006.

[39] L. E. Bermudez, L. S. Young, and H. Enkel, "Interaction of *Mycobacterium avium* complex with human macrophages: Roles of membrane receptors and serum proteins," *Infection and Immunity*, vol. 59, no. 5, pp. 1697–1702, 1991.

[40] N. Palaniyar, "Antibody equivalent molecules of the innate immune system: Parallels between innate and adaptive immune proteins," *Innate Immunity*, vol. 16, no. 3, pp. 131–137, 2010.

[41] M. Dec and A. Wernicki, "Conglutinin, CL-43 and CL-46—three bovine collectins," *Polish Journal of Veterinary Sciences*, vol. 9, no. 4, pp. 265–275, 2006.

Research Article

Antimicrobial Susceptibility Testing of *Mycobacterium bovis* Isolates from Michigan White-Tailed Deer during the 2009 Hunting Season

Scott D. Fitzgerald,[1] Angie M. Schooley,[2] Dale E. Berry,[2] and John B. Kaneene[3]

[1] Department of Pathobiology and Diagnostic Investigation and Diagnostic Center for Population and Animal Health,
  Michigan State University, 4125 Beaumont Road, Lansing, MI 48910, USA
[2] Tuberculosis Laboratory, Michigan Department of Community Health, Lansing, MI 48910, USA
[3] Center for Comparative Epidemiology, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824, USA

Correspondence should be addressed to Scott D. Fitzgerald, fitzgerald@dcpah.msu.edu

Michigan has had an ongoing outbreak of endemic *Mycobacterium bovis* which has been recognized within and sustained by its free-ranging white-tailed deer population since 1994. Worldwide, organisms within the *Mycobacterium tuberculosis* complex have exhibited the ability to develop resistance to antimicrobial agents, resulting in both the multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of human tuberculosis. Michigan's Bovine Tuberculosis Working Group has conducted active antimicrobial susceptibility testing on wildlife isolates of the endemic *M. bovis* organism at five-year intervals to detect any emerging drug resistance patterns. The results of 33 white-tailed deer origin isolates collected from the 2009 hunting season are reported here. There continues to be no evidence of any drug resistance except for pyrazinamide resistance. These results are likely due to the lack of antibacterial treatment applied to either wildlife or domestic animals which would provide selection pressure for the development of drug resistance.

1. Introduction

The state of Michigan has had an ongoing free-ranging white-tailed deer tuberculosis surveillance and control program since 1994 [1, 2]. It appears that deer serve as the primary reservoir host in the state of Michigan, with elk and a variety of wild carnivores and omnivores serving as spill-over hosts, and to date there have been 50 cattle herds infected by *M. bovis* [3], While some progress has been made on controlling the spread of *M. bovis* outside of the endemic 12 county area and the overall incidence rate of infection in deer has dropped from around 4.9% to approximately 1.7%, there remain ongoing programs for surveillance, and the ultimate goal is to eradicate the disease from both domestic cattle and wild deer [4]. Since *M. bovis* is also infectious to humans, a multiagency task force consisting of personnel from state departments (Michigan Department of Natural Resources

and Environment, Michigan Department of Agriculture, and Michigan Department of Community Health), from a public university (Michigan State University), and from a federal agency (United States Department of Agriculture) have formed a bovine tuberculosis task force to deal with issues concerning wildlife, domestic animals, and human health [1, 2].

Bovine tuberculosis threatens human health through direct acquisition of the disease from field dressing infected deer, from airborne transmission of the disease from infected cattle or captive deer, and from ingestion of unpasteurized milk or undercooked meat products including venison [5–7]. Over the last sixteen years, only two human cases have been identified as associated with the specific strain of *M. bovis* endemic in Michigan deer and cattle [7]. One case was an elderly individual who was raised on a farm and may have been exposed decades earlier through drinking

unpasteurized milk. A second case was a hunter who cut his hand while field-dressing a deer carcass and developed a localized cutaneous infection which has been subsequently treated successfully. Since humans do occasionally become exposed and infected by this organism, our task force has instituted regular antimicrobial susceptibility testing of deer and other wildlife isolates, so that we may recognize any developing antibacterial resistance [8].

Mycobacterial species in the *M. tuberculosis* complex undergo low-frequency spontaneous chromosomal mutations which result in genetic resistance to antituberculosis drugs. For example, *M. tuberculosis* undergoes spontaneous mutations resulting in resistance to isoniazid at a frequency of 3.5×10^{-6} and mutations resulting in rifampin resistance at a frequency of 3.1×10^{-8} [9]. However, it is the application of antituberculosis drugs which creates pressure for selection of these strains with mutations. This is generally due to improper therapeutic applications such as insufficient length of drug treatment, poor patient adherence to dosing schedules, using a single antituberculosis drug instead of the recommended multiple drug therapy, and failure to recognize pre-existing resistance in a tuberculosis case [10]. These problems then lead to the emergence of either multidrug-resistant (MDR) or extensively drug-resistant (XDR) strains of mycobacteria. MDR is defined as resistant to at least rifampicin and isoniazid, which are first-line antituberculosis drugs. XDR is defined by the World Health Organization Global Task Force on XDR-TB as resistant to rifampicin and isoniazid, as well as any member of the fluroquinolone family, and one or more of the second-line antituberculosis drugs including kanamycin, capreomycin, or amikacin [10]. Fortunately, these forms of antituberculosis drug resistance are not as prevalent in veterinary medicine as they are in human medicine because we tend to cull infected animals rather than treat them for tuberculosis [5].

The objectives for this study were to (1) take the majority of the 2009 hunter-harvested wild deer isolates of *M. bovis* from Michigan and conduct routine antimicrobial susceptibility testing by two different methodologies in order to detect any evidence of new antimicrobial resistant strains and (2) compare the 2009 data with the 1999 and the 2004 antimicrobial studies which utilized similar methods [8].

2. Materials and Methods

All culture positive deer *M. bovis* samples submitted to the Michigan Department of Community Health Tuberculosis Laboratory during the 2009 wild white-tailed deer hunting season were tested for antimicrobial susceptibility. The antimicrobial agents tested included isoniazid, streptomycin, rifampin, ethambutol, ethionamide, kanamycin, ciprofloxacin, cycloserine, capreomycin, and pyrazinamide. Isolates from 33 deer were utilized in this study.

2.1. Proportion Plate Method. Isolates were subcultured into Middlebrook 7H9 broth which was then incubated at 35°C for 2 to 6 days. Subcultures were then used to conduct 1% proportion plate susceptibility assays by previously described

methods [11, 12]. Middlebrook 7H10 agar plates were prepared with 5 mL of medium into each of four quadrants in the Petri dishes, which then had drug-impregnated discs placed into each quadrant, with one drug-free quadrant serving as a control. Powdered suspensions were utilized for the cycloserine and capreomycin preparations. Final drug concentrations in these plates were 0.2 and 1.0 μg/mL isoniazid, 2.0 and 10.0 μg/mL streptomycin, 1.0 μg/mL rifampin, 5.0 μg/mL ethambutol, 5.0 μg/mL ethionamide, 6.0 μg/mL kanamycin, 2.0 μg/mL ciprofloxacin, 30.0 μg/mL cycloserine, 10.0 μg/mL capreomycin, and 100 μg/mL pyrazinamide. Plates were inoculated to reach a colony count of between 100 and 200 colony forming units on the control quadrant containing no drug. Plates were incubated as 35°C with CO_2 for 3 weeks. Percentage resistance for each drug was calculated by dividing the total number of colonies in a quadrant by the total number of colonies in the control quadrant and multiplying the result by one hundred. A 1% standard cutoff value was used for the interpretation of resistance. Therefore, a culture with a percent resistance of less than 1% was considered susceptible to that particular drug at that concentration while a culture with a percent resistance greater than or equal to 1% was considered resistant to that particular drug.

2.2. Bactec Method. Bactec radiometric susceptibility testing was the second method used to evaluate *M. bovis* isolates for antimicrobial resistance and followed previously described methods [13]. The following antimicrobial drugs were added to Bactec 12B vials: isoniazid at 0.1 g/mL, streptomycin at 2.0 g/mL, rifampin at 2.0 g/mL, and ethambutol at 2.5 g/mL. A suspension of each isolate was diluted to a 0.5 MacFarland suspension and then inoculated into vials containing each of the drugs. A drug-free control vial was also prepared by diluting the 0.5 MacFarland suspension by 1 : 100. Vials were read each day using the Bactec 460 TB instrument until the growth index (GI) of the control vials reached 30 or greater. Susceptibility results were determined by comparing the ΔGI (difference between the current day's GI value with the GI value from the previous day's reading) of the drug vial to the ΔGI of the control vial. A resistant isolate had a ΔGI value greater than the ΔGI value of the control vial.

The Bactec method for determination of pyrazinamide was somewhat different from the other drugs tested. A 0.5 MacFarland suspension of each isolate was inoculated into a Bactec 12B vial and then grown out to a GI value of greater than 300. Next, these vials contents were inoculated into 2 pyrazinamide test medium vials. Pyrazinamide was added to one vial at a concentration of 100 μg/mL while the second vial served as a drug-free control. Vials were read each day using the Bactec 460 TB instrument until the control GI value reached greater than or equal to 200. Bactec pyrazinamide results were interpreted by dividing the GI of the drug vial by the GI of the control vial and multiplying the result by 100 to calculate the percentage resistance. Percentage of resistance less than 9% was interpreted as susceptible to pyrazinamide while percentage of resistance greater than 11% was interpreted as resistant. Percentages between 9 and

TABLE 1: Antimicrobial susceptibility results for Michigan deer from 2009.

Host Species	Bactec method	Bactec PZA[1] method	Proportion method	Proportion method PZA[1]
Deer	0/33[2]	33/33[2]	0/33[2]	33/33[2]

[1]PZA: pyrazinamide

[2]Number of resistant isolates over the total number of isolates tested.

11% were considered equivocal, and repeat of the assay was performed; this repetition of the assay was rarely needed.

3. Results

For the 2009 deer isolates of *M. bovis*, all 33 were susceptible to isoniazid, streptomycin, rifampin, ethambutol, ethionamide, kanamycin, ciprofloxacin, cycloserine, and capreomycin by the proportion plate method (Table 1). All 33 isolates were also susceptible to isoniazid, streptomycin, rifampin, and ethambutol by the Bactec method. All 33 isolates were resistant to pyrazinamide (100%) by both the proportion plate and the Bactec methods. These results indicating no drug resistance except for pyrazinamide were identical to our findings in the 1999 and 2004 surveys, in which there were 30 deer isolates tested in 1999 and 28 deer isolates tested in 2004 [8].

4. Discussion

It was expected that all deer *M. bovis* isolates would be resistant to pyrazinamide. Historically, *M. bovis* isolates are resistant to pyrazinamide because the organism does not produce the enzyme pyrazinamidase which is needed to convert pyrazinamide into pyrazinic acid, the active form of the antimicrobial agent [14]. This resistance is one of the basic features which can be used to distinguish isolates of *M. bovis* (universally resistant to pyrazinamide) from *M. tuberculosis* (commonly susceptible).

The fact that no detectable emerging antimicrobial resistant *M. bovis* strains were found is good news, especially for the rare individual who is infected with this *M. bovis* strain and must undergo therapy. It is most likely explained by the fact that wild deer and other wild spill-over hosts are commonly found to be infected with *M. bovis* only after they have been harvested by hunters or trappers. Likewise, when either domestic cattle or captive cervids in Michigan are detected as TB suspects or reactors on antemortem surveillance, they are routinely slaughtered and sent for full tuberculosis testing which includes culture and PCR assays. In either circumstance, there is no antibacterial treatment applied to infected or possibly infected individual animals, which means there is no selection pressure to favor the development of drug-resistant strains. Furthermore, the antimicrobial susceptibility surveys conducted at 50-year intervals give us a broad overview of resistance development since the disease was first recognized as endemic in Michigan deer 16 years ago.

Unlike the two previous susceptibility surveys conducted in 1999 and 2004, this survey was limited to wild deer, the primary reservoir host of *M. bovis* in Michigan. This is due to changes in the processing of tuberculosis surveillance samples. In earlier years, all wild animal samples including the spill-over hosts comprised by various wild carnivores and omnivores, used to run through the state diagnostic laboratory and the Tuberculosis Laboratory, Michigan Department of Community Health. Starting in 2005, personnel from the Wildlife Services, United Sates Department of Agriculture, took over the responsibility for nondeer wildlife surveillance, and those samples are shipped to another state for testing. Likewise, during the earlier years of the tuberculosis endemic the suspect cattle which were processed at the state diagnostic laboratory had duplicate samples collected for culture both at the Diagnostic Bacteriology Laboratory, National Veterinary Services Laboratory (NVSL), Ames, Iowa, as well as at the local Michigan Department of Community Health Laboratory. However, in recent years, samples have only been collected for NVSL, as this is a program disease for which the USDA has primary authority, and the fact that duplicate processing was considered redundant and too expensive for current fiscal budgets. Since the wild deer are considered the principal reservoir host, we feel that the current antimicrobial susceptibility survey is still valid in spite of the absence of the other wildlife and domestic cattle isolates.

While the lack of any detectable antimicrobial resistance development is good news, there remains reason for caution and continued regular surveys of this type. In July, 2009, the USDA, Animal and Plant Health Inspection Service produced a concept paper for new approaches to managing bovine tuberculosis which are currently being implemented [15]. One of their recommendations was to begin applying whole-herd depopulation judiciously and developing alternative control strategies including test and cull of individual suspect animals. This is a reasonable response to the enormous costs that USDA and state departments of agriculture must expend in order to depopulate and indemnify ever larger cattle herds, many with valuable individual breeding animals. However, we should remember that the reason why drug-resistant strains of *M. bovis* are not emerging in animals nearly as rapidly as in human populations is because of the lack of treatment-related selection pressures. No one is suggesting that individual cattle or captive cervids which are identified as tuberculosis suspects should start to receive antimicrobial therapy as we do with infected people. When whole-herd test and individual animal culling practices are employed, there is the very real opportunity for other animals in the same herd which may be harboring tuberculosis to receive limited antibacterial therapy for other conditions, such as respiratory disease, mastitis, or other localized infections. Alternatively, whole beef cattle herds may be treated with long-term low-level antibacterial agents as growth promoters. In this type of environment, asymptomatic and undetected tuberculosis carriers will be subjected to similar selection pressures that have produced the current worldwide emergence of MDR and XDR tuberculosis strains in humans. This is not meant

to find fault with newer management procedures for bovine tuberculosis but only to point out that selection pressures which promote antimicrobial resistance development may well be increasing, at least in our domestic and captive animal species. Therefore, continued surveillance for drug-resistant strains of *M. bovis* will be even more important as we move forward to ensure the safety of our milk, beef, and venison sources for human consumption.

Acknowledgments

References

[1] D. J. O'Brien, S. M. Schmitt, J. S. Fierke et al., "Epidemiology of *Mycobacterium bovis* in free-ranging white-tailed deer, Michigan, USA, 1995–2000," *Preventive Veterinary Medicine*, vol. 54, no. 1, pp. 47–63, 2002.

[2] S. M. Schmitt, D. J. O'Brien, C. S. Bruning-Fann, and S. D. Fitzgerald, "Bovine tuberculosis in Michigan wildlife and livestock," *Annals of the New York Academy of Sciences*, vol. 969, pp. 262–268, 2002.

[3] B. Patrick, "Trace investigation finds bovine TB positive herd in Emmet County," September 2010, http://www.michigan.gov/emergingdiseases/0,1607,7-186-25804-243197–,00.html.

[4] D. J. O'Brien, S. M. Schmitt, S. D. Fitzgerald, D. E. Berry, and G. J. Hickling, "Managing the wildlife reservoir of *Mycobacterium bovis*: the Michigan, USA, experience," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 313–323, 2006.

[5] C. Thoen, P. LoBue, and I. De Kantor, "The importance of *Mycobacterium bovis* as a zoonosis," *Veterinary Microbiology*, vol. 112, no. 2–4, pp. 339–345, 2006.

[6] M. J. Wilkins, P. C. Bartlett, B. Frawley, D. J. O'Brien, C. E. Miller, and M. L. Boulton, "*Mycobacterium bovis* (bovine TB) exposure as a recreational risk for hunters: results of a Michigan hunter survey, 2001," *International Journal of Tuberculosis and Lung Disease*, vol. 7, no. 10, pp. 1001–1009, 2003.

[7] M. J. Wilkins, J. Meyerson, P. C. Bartlett et al., "Human *Mycobacterium bovis* infection and bovine tuberculosis outbreak, Michigan, 1994–2007," *Emerging Infectious Diseases*, vol. 14, no. 4, pp. 657–660, 2008.

[8] M. Daly, K. L. Diegel, S. D. Fitzgerald, A. Schooley, D. E. Berry, and J. B. Kaneene, "Patterns of antimicrobial susceptibility in Michigan wildlife and bovine isolates of *Mycobacterium bovis*," *Journal of Veterinary Diagnostic Investigation*, vol. 18, no. 4, pp. 401–404, 2006.

[9] R. Johnson, E. M. Streicher, G. E. Louw, R. M. Warren, P. D. van Helden, and T. C. Victor, "Drug resistance in *Mycobacterium tuberculosis*," *Current Issues in Molecular Biology*, vol. 8, no. 2, pp. 97–112, 2006.

[10] A. Jain and P. Dixit, "Multidrug resistant to extensively drug resistant tuberculosis: what is next?" *Journal of Biosciences*, vol. 33, no. 4, pp. 605–616, 2008.

[11] P. T. Kent and G. P. Kubica, *Public Health Mycobacteriology. A Guide for the Level III Laboratory*, US Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta, Ga, USA, 1985.

[12] B. A. Forbes, N. Banaiee, K. G. Beavis et al., "Susceptibility testing of Mycobacteria, Nocardiae, and other aerobic Actinomycetes; approved standard," Document M48-A, Clinical and Laboratory Standards Institute (CLSI), Wayne, Pa, USA, 2008.

[13] S. H. Siddiqui, "Bactec 460 TB system product and procedure manual," Publication MA-0029, Becton Dickinson and Company, Sparks, Md, USA, 1996.

[14] A. S. Barouni, C. J. Augusto, M. T. P. Lopes, M. S. Zanini, and C. E. Salas, "A pncA polymorphism to differentiate between *Mycobacterium bovis* and *Mycobacterium tuberculosis*," *Molecular and Cellular Probes*, vol. 18, no. 3, pp. 167–170, 2004.

[15] Anonymous, "A new approach for managing bovine tuberculosis: Veterinary Services proposed action plan. United States Department of Agriculture, Animal and Plant Health Inspection Services, Docket No. APHIS-2009-0073," *Federal Register*, vol. 74, no. 191, p. 51116, 2009.