Veterinary Medicine International

Mycobacterial Diseases of Animals 2012

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

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Contents

Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 684720, 2 pages
doi:10.1155/2012/684720

Editorial
Mycobacterial Diseases of Animals 2012

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Received 9 September 2012; Accepted 9 September 2012

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Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium avium subsp. *avium, Mycobacterium avium* subsp. *paratuberculosis, Mycobacterium ulcerans,* and other mycobacteria are the etiologies of important diseases in humans and a wide range of animal species including cattle, sheep, goats, deer, possums, badgers, wild boar, elephants, dogs, cats, birds, amphibians, and fish. Moreover, *M. bovis* has a remarkably broad host range and is a serious zoonotic pathogen. In spite of centuries of investigation and costly eradication efforts, *M. bovis* remains an important pathogen at the interface of humans, livestock, and wildlife. *M. avium* subsp. *paratuberculosis* is one of the most widespread pathogens of dairy cattle, causing decreased production, protracted diarrhea, and emaciation.

This 2nd special issue on mycobacterial diseases of animals contains 16 papers comprising 4 reviews and 12 original research papers on various topics including immunology, epidemiology, microbiology, genomics, vaccinology, and pathology. Authors from 9 different countries provide a diverse examination of mixed topics including *M. tuberculosis*, *M. bovis,* and *M. avium* subsp. *paratuberculosis*.

The issue begins with G. M. Kassa et al. examining a cross-section of Ethiopian small ruminants and describing the isolation of *M. tuberculosis* from goats and stressing important zoonotic implications. From Spain, B. Beltrán-Beck and colleagues review the significant progress on oral vaccination of Eurasian wild boar with the vaccine *M. bovis* BCG and a new heat-killed *M. bovis* vaccine. R. A. Skuce et al. review important risk factors for bovine TB herd breakdowns in the United Kingdom and Ireland. The next 3 papers focus on immunology and diagnostics beginning with B. Fernández et al. and their analysis of an ELISA for bovine paratuberculosis. J. T. Nelson and colleagues evaluate a rapid serologic test for tuberculosis diagnosis in several deer species, while M. L. Mon et al. search for improved antigens for use in bovine paratuberculosis diagnosis.

M. V. Palmer and collaborators review in detail wildlife reservoirs of *M. bovis* and the potential for disease transmission at the livestock-wildlife-human interface. C. Mackintosh and colleagues from New Zealand describe experimental infection of red deer with *M. avium* subsp. *paratuberculosis* and examine the clinical, immunological, and pathological outcomes. Rapid diagnostic tests for both bovine tuberculosis and paratuberculosis are high priorities in the veterinary medical field, and A. Wadhwa et al. review current and future platforms for diagnostic assays. A. Lim and colleagues at Michigan State University use genomics to examine differential gene expression between true *M. bovis*-positive and -false-positive cattle.

Mycobacterium bovis can infect numerous species, but not all species have maintenance host potential. Intraspecies transmission is requisite to maintenance host potential. Fenton et al. examine potential intraspecies transmission among Virginia opossums in an experimental aerosol challenge model. Possible species differences in susceptibility to *M. bovis* infection lead G. Ameni et al. to explore potential differences in immune responses to *M. bovis* between *Bos indicus* and *Bos taurus* cattle in Ethiopia. C. Furphy and colleagues from Ireland use the molecular epidemiological tools of spoligotyping and VNTR to examine strain types of *M. bovis* in badgers and show that individual badgers can be infected with multiple strains of *M. bovis*.

One useful serological assay for tuberculosis in cattle is the multiantigen print immunoassay, which S. D. Fitzgerald et al. examine, using various interpretation criteria and suggest its use as a possible supplemental test to intradermal

tuberculin testing. K. P. Lyashchenko and colleagues describe the uncommon infection of a horse with *M. tuberculosis* and discuss possible zoonotic concerns. The highlands of Cameroon are the setting for J. Awah-Ndukum and collaborators as they use traditional tuberculin skin testing and novel serological tests to determine the prevalence of bovine tuberculosis and explore possible modifications of test interpretation to improve disease surveillance.

The editors thank the many authors for their efforts in the experimentation, labor, and time reflected in each manuscript. The lead editor thanks all editors for time spent reviewing, assigning reviews, and commenting on the many manuscripts submitted. It is the hope of the editors that this 2012 issue will prove useful to investigators, policy makers, and veterinarians involved in the study of mycobacterial diseases of animals.

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Michael D. Welsh
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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 869146, 8 pages
doi:10.1155/2012/869146

Research Article

Tuberculosis in Goats and Sheep in Afar Pastoral Region of Ethiopia and Isolation of *Mycobacterium tuberculosis* from Goat

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Received 3 February 2012; Accepted 21 May 2012

Academic Editor: Michael D. Welsh

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A cross sectional study was conducted on 2231 small ruminants in four districts of the Afar Pastoral Region of Ethiopia to investigate the epidemiology of tuberculosis in goats and sheep using comparative intradermal tuberculin skin test, postmortem examination, mycobacteriological culture and molecular typing methods. The overall animal prevalence of TB in small ruminants was 0.5% (95% CI: 0.2%–0.7%) at ≥4 mm and 3.8% (95% CI: 3%–4.7%) at cutoff ≥2 mm. The herd prevalence was 20% (95% CI: 12–28%) and 47% (95% CI: 37–56%) at ≥4 mm and ≥2 mm cut-off points, respectively. The overall animal prevalence of Mycobacterium avium complex infection was 2.8% (95% CI: 2.1–3.5%) and 6.8% (95% CI: 5.8–7.9%) at ≥4 mm and ≥2 mm cut-off points, respectively. Mycobacteriological culture and molecular characterization of isolates from tissue lesions of tuberculin reactor goats resulted in isolation of *Mycobacterium tuberculosis* (SIT149) and non-tuberculosis mycobacteria as causative agents of tuberculosis and tuberculosis-like diseases in goats, respectively. The isolation of *Mycobacterium tuberculosis* in goat suggests a potential transmission of the causative agent from human and warrants further investigation in the role of small ruminants in epidemiology of human tuberculosis in the region.

1. Background

Ethiopia has one of the largest resources of goats and sheep among African countries, with an estimated number of 21.9 million goats and 25.9 million sheep [1]. Goats and sheep contribute significantly to the economy and food security of the poor farmers in the country. About 73% of the national goat population and 25% of the sheep population are found in the lowland pastoral areas of the country [2]. In pastoralist area, goats and sheep are mainly utilized for milk and meat production and generate income to the owner. In spite of the large population and potential use of small ruminants, the production system is affected by feed shortage, poor genetic makeup of the animals, and wide spread occurrence of livestock diseases such as tuberculosis which has both economic and public health significance to the communities.

TB in goat and sheep is caused by members of *Mycobacterium tuberculosis* complex predominantly by *Mycobacterium bovis* and *Mycobacterium caprae* [3–14] and few caused by *Mycobacterium tuberculosis* [15, 16]. Epidemiological studies indicated that tuberculosis in goat and sheep has a wide global distribution and has been reported in various countries of the world including New Zealand, Sudan, Spain, Nigeria, the United Kingdom, Italy, Algeria, Ethiopia [3–17]. In Ethiopia, bovine TB has been known to be endemic in cattle; however, the status of TB in goats and sheep has not been well studied in spite of their close contact with cattle. Few studies carried out so far in central highland Ethiopia

indicated the existence of TB in small ruminants with low level of prevalence (4.2%) based on abattoir examination results [14] and 3.1% using single intradermal tuberculin test [17]. In lowland pastoral area where the large population of goats and sheep exists, the status of the disease is unknown.

Livestock in pastoralist region is major source of food and income, in addition, possession of livestock provides a measure of social status in the pastoral communities. In pastoralist communities of Ethiopia including the Afar, pastoralists' habit of consumption of raw animal product particularly milk is common and the pastoralists have close physical contact with their animals. Afar pastoralists consume both goat and sheep milk very commonly, and to protect these small ruminants from predators, the pastoralists keep these animals in very close proximity to their houses. These conditions are potential risk factors for transmission of zoonotic diseases such as TB of animal origin to human or vice versa. Goats and sheep have also common watering and grazing points with cattle that might favor the transmission of mycobacterial species among these domestic animals. Previous studies in cattle and camel of pastoral regions indicated the endemic nature of TB in the regions [18–22]. Therefore, the present study was designed to investigate the epidemiology of TB in goats and sheep and characterizes the causative agents in the Afar Pastoral Region of Ethiopia.

2. Material and Methods

2.1. Study Area. The study was conducted in four districts, namely, Amibara, Dubti, Afambo, and Chifra districts of Afar Pastoral Region. The Afar Pastoral Region is located in northeast of Ethiopia between 39°34′ and 42°28′ E longitude and 8°49′ and 14°30′ N latitude. The region shares common international boundaries with Eritrea in the northeast and Djibouti in the east, and it is characterized by an arid and semiarid climate with low and erratic rainfall [23].

In the Afar Region, there are about 4,268,000 goats and 2,464,000 sheep which are managed under pastoral and agropastoral production system [24]. Afar pastoralists own different species of domestic animals, and these animals share common watering points and grazing sites. Small ruminants usually graze/browse near their villages, while cattle and camel might travel a long distance in search of grass and browsing trees. The watering points of small ruminants are commonly shared with cattle and camel creating a close interspecies interaction among these domestic animals, and this might increases the risk of transmission of mycobacteria from cattle or camels to small ruminants or vice versa.

2.2. Study Design. A cross-sectional study was conducted in the four districts of the Afar Pastoral Region and a total of 14 subdistricts and 21 villages were included in the study based on the inclusion criteria (accessibility, security, and willingness of the pastoralists to participate in the research). All villages in each subdistrict were included after obtaining the elder clan leaders' consent to participate in the study. In this study, goat and sheep kept by an owner and his close relatives in which case if the animals share common grazing sites, watering points, and night shelter, they were considered as a herd to calculate the herd prevalence. A total of 103 flocks (herds) of small ruminants were tested by CIDT test.

2.3. Study Animals. For the CIDT test, small ruminants above the age of six months having no clinical symptom of any disease were included. Study animal-related information on each tested sheep and goat (such as sex, age, body condition score, lactation and reproductive status, and parity number) was collected and recorded at the time of the test. Each animal was dewormed with antihelmintic drug after testing. A total of 2231 small ruminants (1884 goat and 347 sheep) were tested using CIDT.

2.4. Comparative Intradermal Tuberculin Skin Test (CIDT). CIDT test was carried out by injecting both bovine purified protein derivative (PPD) and avian PPD (observe bovine and avian tuberculin, AsureQuality Company, Mt. Wellington, Auckland, New Zealand). Two sites on the skin of the midneck of the animal, 12 cm apart, were shaved, and skin thickness was measured with a caliper. One site was injected with an aliquot of 0.1 mL of 2,500-IU/mL bovine PPD into the dermis, and the other was similarly injected with 0.1 mL of 2,500-IU/mL avian PPD. After 72 h, the skin thickness at the injection site was measured and recorded. Results were interpreted according to the recommendations of the Office International des Epizooties [25] at ≥4 mm cutoff and also at ≥2 mm cutoff [26]. Thus, at cutoff ≥4 mm, if the increase in skin thickness at the injection site for bovine PPD (PPD-B) was greater than the increase in skin thickness at the injection site for avian PPD (PPD-A) and PPD-B minus PPD-A was less than 2 mm, between 2 and 4 mm, or 4 mm and above, the animal was classified as negative, doubtful, or positive reactor based on CIDT test, respectively. At cutoff ≥2 mm, if the difference between PPD-B and PPD-A was greater or equal to 2 mm, the animal was considered as positive, while if the difference is less than 2 mm, the animal was considered as negative. When the change in skin thickness was greater at PPD-A injection site, the animal was considered positive for mycobacteria species other than *Mycobacterium tuberculosis* complex. A flock (herd) was considered as positive if it had at least one tuberculin reactor animal.

2.5. Body Condition Scoring. The body condition scoring for goat and sheep was carried out using the guidelines established by Langston University and ESGIP guidelines for body condition scoring [27, 28]. Accordingly, on the basis of observation of anatomical parts such as vertebral column, ribs, and spines, the study animals were classified as lean (score 1 to 2), medium (3 to 4), or fat (greater than 5).

Age determination was carried out based on the dentition according to Vatta and his coworkers [29] and adopted ESGPIP guideline for estimation of age of sheep and goat [30].

2.6. Postmortem Examination. Tissues with suspicious lesions from five slaughtered tuberculin reactor goats were

collected aseptically from the lung lobes (left apical, left diaphragmatic, right apical, right cardiac, right diaphragmatic, and right accessory), lymph nodes of the head (retropharyngeal and mandibular), lymph nodes of lungs (mediastinal and bronchial), and mesenteric lymph nodes. Data were collected on the presence, size, and distribution of visible lesions in each carcass. Samples from tissues containing visible lesions were collected and placed into sterile universal bottles containing 5 mL of 0.9% saline solution(pH 7.2) and kept at −20°C at Semera Regional Animal Health Laboratory until they were transported to ALIPB laboratory under cold chain for isolation of the causative agents.

2.7. Isolation of Mycobacteria from Tissue Samples. Isolation of mycobacteria from tissues was done in accordance with OIE protocols [31]. Briefly, the specimens were sectioned into pieces using sterile blades and then homogenized by pestle and mortar. The homogenate was decontaminated by adding an equal volume of 4% NaOH followed by centrifugation at 1000 g for 15 minutes. The supernatant was discarded, while the sediment was neutralized by 1% (0.1N) HCl using phenol red as an indicator. Neutralization was achieved when the colour of the solution changed from purple to yellow. Thereafter, 0.1 mL of suspension from each sample was spread onto a slope of Löwenstein-Jensen (LJ) medium. Duplicates of LJ media were used; one enriched with sodium pyruvate, while the other was enriched with glycerol. Cultures were incubated at 37°C in a slant position for one week and in upright position for 11 weeks with weekly observation for mycobacterial growth. Whenever, colonies were seen, subculturing and Ziehl-Neelsen staining were performed to confirm the presence of acid fast bacilli. Positive colonies were preserved with freezing media, and some portions of the colonies were heat-killed in water bath at 80°C for 45 minutes. The frozen and heat killed isolates were stored at −20°C for future mycobacteriology and further molecular typing analysis.

2.8. Molecular Characterization of Mycobacterial Isolates. Mycobacterium genus typing was conducted as described previously [32], and spoligotyping of *Mycobacterium tuberculosis* complex isolate from goat was performed as previously described by Kamerbeek and coauthors [33]. Both methods were described in detail in previous publication [22].

2.9. Data Management and Analysis. Data were classified, filtered, coded using EpiData software and Microsoft Excel sheet, and was transferred and analyzed using STATA version 11 (Stata Corp., Collage station, TX). Pearson chi-square was used to evaluate the statistical significance of the associations of different categorical variables with skin test results. Bivariate and multivariable logistic regression analyses were performed to quantify crude and adjusted effects of prespecified risk factors on tuberculin reactivity. *P* values less than 5% were considered statistically significant. In cases of estimating the effect of different risk factors in terms of OR with corresponding 95% confidence interval, statistically significance was assumed if the confidence interval did not include one among its values.

3. Results

3.1. Animal Prevalence. On the basis of CIDT test, the animal prevalence of TB was 0.5% (10/2231) at a cutoff ≥4 mm and 3.8% (86/2231) at a cutoff ≥ 2 mm. At ≥2 mm cut-off point, there were significant differences in proportions of reactors among the four districts (χ^2 = 26.385, P = 0.000), between sheep and goat (χ^2 = 6.46, P = 0.011) and between pregnant and nonpregnant females (χ^2 = 5.342, P = 0.021) (Table 1). Multivariable logistic regression analysis at ≥2 mm cut-off point showed that older small ruminants (5 years and above) had 13 times the odds of being tuberculin reactors compared with age category less than 2 years old (adjusted OR = 13.79; CI: 2.22–85.55). Female small ruminants with parity number greater than 4 had 0.05 odds of being bovine tuberculin positivity in relative to those with less than 2 parity numbers (adjusted OR = 0.05; CI: 0.01–0.31) (Table 2). At ≥4 mm cut-off points, there was no statistical significance difference in the proportion of bovine tuberculin positivity between groups in relation to the different variables considered.

3.2. Herd Prevalence. The herd prevalence was 20% (95% CI: 12–28%) and 47% (95% CI: 37–56%) at ≥4 mm and ≥2 mm cut-off points, respectively. In multivariable logistic regression analysis, no significant association was found in herd positivity between groups in relation to district of origin, herd size category, and production system at ≥2 mm cut-off point (Table 3).

3.3. Prevalence of Mycobacterium Avium Complex Infection. According to the CIDT test result of the avian tuberculin skin reaction, the overall animal prevalence of *Mycobacterium avium* complex infection was 2.8% (95% CI: 2.1–3.5%) and 6.8% (95% CI: 5.8–7.9%) at ≥4 mm and ≥2 mm cut-off points, respectively.

3.4. Postmortem Lesions in Tuberculin Reactor Goats. Five goats which were positive to bovine TB in CIDT test at cutoff ≥ 4 mm were purchased, slaughtered, and investigated for gross tuberculous lesions. Tuberculous lesions were detected in different organs (left diaphragmatic lung, retropharyngeal lymph node, parotid lymph node, right bronchial lymph node, mesenteric lymph node, intestinal wall, and mesentery). Two of them had partially disseminated TB lesions which involved lung, intestine and the lymph nodes of thoracic and abdominal cavities. Upon incision of the lung, lesions showed a yellowish caseous material indicating a characteristic of tuberculous lesion (Figure 1). In mesentery and mesenteric lymph nodes, greenish discharge was observed in the lesions.

3.5. Molecular Typing of the Isolates from Goats. All tissue samples obtained from slaughtered tuberculin reactor goats were positive for mycobacterial growth on LJ culture medium. Further molecular characterization indicated that

Table 1: Association of different risk factors to skin test positivity at ≥2 mm cut-off point for small ruminant tuberculosis in Afar Pastoral Region of Ethiopia.

Variables	Number of animals examined	Number of positive (%)	χ^2	P value
Districts				
Chifra	396	20 (5.05)	26.385	0.000*
Dubti	237	22 (9.28)		
Afambo	117	5 (4.27)		
Amibara	1481	39 (2.63)		
Species				
Ovine	347	5 (1.44)	6.460	0.011*
Caprine	1884	81 (4.3)		
Herd size				
≤25	617	32 (5.19)	4.915	0.086
$11 < X \leq 50$	851	32 (3.76)		
>50	763	22 (2.88)		
Sex				
Male	206	11 (5.34)	1.351	0.245
Female	2025	75 (3.70)		
Age[#]				
≤2	594	18 (3.03)	2.361	0.307
$2 < X < 5$	779	36 (4.62)		
$X \geq 5$	858	32 (3.73)		
BCS				
Poor	376	13 (3.46)	1.204	0.548
Good	1116	48 (4.30)		
Fat	739	25 (3.38)		
Production system				
Pastoral	2051	80 (3.90)	0.144	0.705
Agropastoral	180	6 (3.33)		
Lactation status				
Kid/lamb	57	2 (3.51)	1.255	0.534
Lactating	760	19 (2.50)		
Nonlactating	354	13 (3.67)		
Reproductive status				
Nonpregnant	857	19 (2.22)	5.342	0.021*
Pregnant	314	15 (4.78)		
Parity number				
<2	260	10 (3.85)	4.415	0.110
$2 \leq X < 4$	304	13 (4.28)		
$X \geq 4$	324	5 (1.54)		

[#]A given age range includes its lower bound and excludes its upper bound; BCS: body condition score; *statistically significant.

one of the isolates was human type *Mycobacterium tuberculosis* (SIT149) from goat specimen (Figure 2), and the others were nontuberculosis mycobacteria species. The goat with SIT149 isolate was strong reactor to bovine tuberculin test with high skin induration difference (PPD-B minus PPD-A = 10 mm), and the postmortem examination result showed typical tuberculin lesions in lung, bronchial lymph nodes, caudal mediastinal lymph node, and also on mesenteric lymph nodes while goats from which nontuberculosis mycobacteria species were isolated have showed indurations of skin at both avium and bovine tuberculin injection site. In addition, the pathological lesions observed in postmortem examination were localized in retropharyngeal lymph nodes and mesenteric lymph nodes.

4. Discussion

Little information is available on TB in small ruminants in Ethiopia even though bovine TB is known to be endemic in cattle of Ethiopia [34]. In this study, a prevalence of 0.5% at ≥4 mm cut-off and 3.8% at ≥2 mm cut-off point was recorded in small ruminants in four districts of Afar Pastoral

Table 2: Multivariable logistic regression analysis of tuberculin reactors with various host-related risk factors at ≥2 mm cut-off point.

Variables	Number of animals examined	Number (%) of positive in CIDT	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Districts				
Chifra	396	20 (5.05)	1	1
Dubti	237	22 (9.28)	1.92 (1.03–3.61)*	—
Afambo	117	5 (4.27)	0.84 (0.31–2.29)	—
Amibara	1481	39 (2.63)	0.51 (0.29–0.88)*	0.17 (0.05–0.55)*
Species				
Ovine	347	5 (1.44)	1	1
Caprine	1884	81 (4.3)	3.07 (1.24–7.64)*	2.05 (0.42–9.94)
Herd size				
≤25	617	32 (5.19)	1	1
$11 < X \leq 50$	851	32 (3.76)	0.71 (0.43–1.18)	1.84 (0.52–6.45)
>50	763	22 (2.88)	0.54 (0.31–0.94)*	0.44 (0.14–1.34)
Sex				
Male	206	11 (5.34)	1	1
Female	2025	75 (3.70)	0.68 (0.36–1.31)	0.25 (0.04–1.74)
Age				
≤2	594	18 (3.03)	1	1
$2 < X < 5$	779	36 (4.62)	1.55 (0.87–2.76)	2.16 (0.47–9.89)
$X \geq 5$	858	32 (3.73)	1.24 (0.69–2.23)	13.79 (2.22–85.55)*
BCS				
Poor	376	13 (3.46)	1	1
Good	1116	48 (4.30)	1.25 (0.67–2.34)	1.90 (0.61–5.88)
Fat	739	25 (3.38)	0.98 (0.49–1.93)	0.75 (0.17–3.28)
Production system				
Pastoral	2051	80 (3.90)	1	1
Agropastoral	180	6 (3.33)	1.18 (0.51–2.74)	—
Lactation status				
Kid/lamb	57	2 (3.51)	1	1
Lactating	760	19 (2.50)	1.05 (0.23–4.77)	0.50(0.06–4.08)
Nonlactating	354	13 (3.67)	0.71 (0.16–3.11)	0.82(0.12–5.79)
Reproductive status				
Nonpregnant	857	19 (2.22)	1	1
Pregnant	314	15 (4.78)	2.21 (1.11–4.41)*	3.43(0.72–16.33)
Parity number				
<2	260	10 (3.85)	1	1
$2 \leq X < 4$	304	13 (4.28)	1.12 (0.48–2.59)	0.38 (0.09–1.65)
$X \geq 4$	324	5 (1.54)	0.39 (0.13–1.16)	0.05 (0.01–0.31)*

CI: confidence interval, BCS: body condition scoring, *statistically significant.

Region of northeastern Ethiopia. The result was in agreement with that of Hiko and Agga [14] who reported 4.2% in goats slaughtered at Mdjo abattoir and with a report by Tafesse and coauthors [17] who recorded a prevalence of 3.1% in goat with single intradermal tuberculin skin test. A recent study carried out on goats and sheep of central Ethiopia using CIDT also showed a low prevalence of tuberculosis (0.41% at 2 mm cut-off point) [16] which might suggests an overall low prevalence of TB in small ruminants in the country. However, our result was different from the result of a previous study done in Hamer pastoral district of southern Ethiopia, which indicated the absence of the disease in 186 goats using CIDT test [21]. This difference might be related, the difference in geographical location of the two studies in which the epidemiology of the disease might vary between these areas.

The proportion of positive reactors was significantly higher in Dubti district than the other districts which might be related to the husbandry system where small ruminants had higher interaction with cattle in Dubti districts than

Table 3: Multivariable logistic regression analysis of herd TB positivity with selected risk factors at ≥2 mm cut-off point.

Variables	Number of herds examined	Number of positive herds (%)	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Districts				
Chifra	18	10 (55.6)	1	1
Dubti	13	9 (69.2)	1.8 (0.40–8.07)	2.59 (0.49–13.73)
Afambo	6	2 (33.3)	0.40 (0.06–2.77)	2.70 (0.06–114.64)
Amibara	66	27 (40.9)	0.55 (0.19–1.58)	0.45 (0.14–1.39)
Herd size				
≤25	52	19 (36.5)	1	1
$11 < X \leq 50$	34	19 (55.9)	2.2 (0.91–5.31)	3.23 (1.21–8.60)
>50	17	10 (58.8)	2.48 (0.81–7.59)	2.48 (0.76–8.09)
Production system				
Agropastoral	8	3 (37.5)	1	1
Pastoral	95	45 (47.4)	1.5 (0.34–6.64)	6.88 (0.28–170.32)

CI: confidence interval.

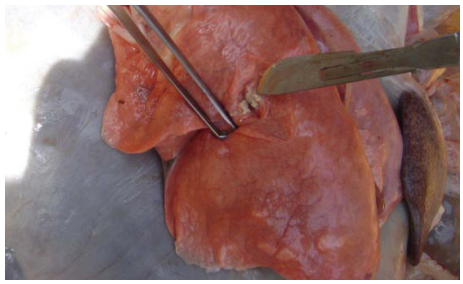

Figure 1: Tuberculous lesion from goat lung caused by *Mycobacterium tuberculosis*.

the other districts, which can favor a potential transmission of mycobacterial species between cattle and goat. Older goat and sheep showed higher proportion of positivity in tuberculin test results which might be related to the fact that older animals have longer duration and repeated chance of exposure to mycobacterial infection with their age. Similar results have been reported by other researchers in cattle [34, 35]. Female animals with more parity number showed higher proportion of positivity in tuberculin test results than in those with lower parity number. This might be related to the age of the animals as animals with high parity number were older in their age which increases their chance of exposure to mycobacterial infection in their longer life time.

Mycobacteriological culture of the tissue lesions from the five tuberculin reactors goats had resulted in the isolation of *Mycobacterium tuberculosis* and nontuberculosis mycobacteria species. In this study, *Mycobacterium tuberculosis* strain SIT149 was isolated from a goat suggesting the possibility of its transmission from human to goat. Similar strain has been isolated in camel from pastoral region in south east of Ethiopia [36]. The SIT149 strain of *Mycobacterium tuberculosis* is a dominant strain in Ethiopia [37], and it was a common isolate in human pulmonary TB patients from the same Afar Pastoral Region indicating that the isolate has been circulating in the area. Afar pastoralists have close contact with goats and sheep and often keep young goats and sheep in their house at night which might be a potential factor for transmission from human patient to animals. Previous studies in cattle of Ethiopia demonstrated that *Mycobacterium tuberculosis* was commonly isolated from tuberculous lesions of cattle in different regions of Ethiopia [38, 39].

In sheep, we observed 1.44% prevalence of TB at 2 mm cut-off point and no at 4 mm cut-off point. The result was in agreement with previous studies where sheep TB has been reported both with tuberculin skin test and postmortem examination results [3, 4, 9, 10, 12, 16].

In conclusion, this study revealed a moderately low prevalence of TB in goats and sheep of Afar Pastoral Region of Ethiopia. *Mycobacterium tuberculosis* and nontuberculosis mycobacteria were isolated as causative agents of TB in goats of the region. The isolation of the *Mycobacterium tuberculosis* in goat indicates the need for further studies to understand the interspecies transmission dynamics of *Mycobacterium tuberculosis* and the role of small ruminants in the epidemiology of human tuberculosis in pastoralist setting where potential epidemiological risk factors for infection and transmission between livestock and human exist. In addition, the identification of nontuberculosis mycobacteria from tuberculous lesions in goats indicates their importance in the epidemiology of small ruminant TB and further research is needed to identify the species and their public health significance for the pastoralist communities of the region. In general, similar to the previous studies carried out in cattle and camel of pastoral regions of Ethiopia [18, 20–22, 36] which have indicated the endemic nature of tuberculosis in these species, the result of this study also indicated the importance of tuberculosis in small ruminants of Afar Pastoral Region which further emphasizes the need to design a feasible national TB control strategy in livestock of the country.

Authors' Contribution

G. M. Kassa contributed to the design of the study, participated in collection, analysis, and interpretation of data, drafted and revised the paper. F. Abebe contributed to the design of the study, interpretation of data, and critically

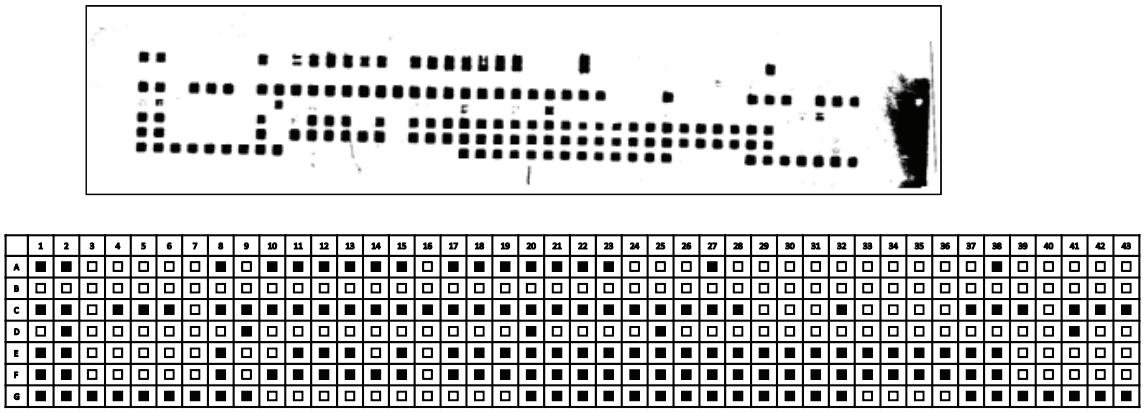

Figure 2: Scanned autorad and schematic representation showing spoligotyping pattern of isolate from the goat with tuberculous lesion caused by *M. tuberculosis*. A: *M. bovis* SB1176 (positive control); B: Qiagen H_2O (negative control); C: M. tuberculosis (positive control); D–F: sample from other animals, G: SIT149 (isolate from goat). The black rectangles represent presence of spacers, and the white rectangles indicate absence of spacers.

revised the paper. Y. Worku and M. Legesse contributed to study design, data collection, data analysis, and interpretation and critically revised the paper. G. Medhin participated in study design, data analysis, and interpretation and critically revised the paper. G. Bjune involved in study design and critically revised the paper. G. Ameni contributed to study design, data collection, data analysis, and interpretation and critically revised the paper. All authors read and approved the final paper.

Acknowledgments

The authors are grateful to Afar pastoralist communities and Afar Pastoral Regional State for willing to participate in the study and supporting the research. The authors acknowledge the Aklilu Lemma Institute of Pathobiology of Addis Ababa University, Institute of Health and Society Department of Community Medicine of University of Oslo, and Faculty of Veterinary Medicine of Addis Ababa University for the overall support of the study. They also thank TB laboratory staff at ALIPB for field and laboratory work. This study was financially supported by the Norwegian Program for Development Research and Education (NUFU PRO-2007/10198).

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 978501, 11 pages
doi:10.1155/2012/978501

Review Article

Progress in Oral Vaccination against Tuberculosis in Its Main Wildlife Reservoir in Iberia, the Eurasian Wild Boar

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Received 7 February 2012; Accepted 8 May 2012

Academic Editor: Jesse M. Hostetter

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Eurasian wild boar (*Sus scrofa*) is the main wildlife reservoir for tuberculosis (TB) in Iberia. This review summarizes the current knowledge on wild boar vaccination including aspects of bait design, delivery and field deployment success; wild boar response to vaccination with Bacillus Calmette-Guérin (BCG) and inactivated Mycobacterium bovis; and wild boar vaccination biosafety issues as well as prospects on future research. Oral vaccination with BCG in captive wild boar has shown to be safe with significant levels of protection against challenge with virulent *M. bovis*. An oral vaccination with a new heat-killed *M. bovis* vaccine conferred a protection similar to BCG. The study of host-pathogen interactions identified biomarkers of resistance/susceptibility to tuberculosis in wild boar such as complement component 3 (C3) and methylmalonyl coenzyme A mutase (MUT) that were used for vaccine development. Finally, specific delivery systems were developed for bait-containing vaccines to target different age groups. Ongoing research includes laboratory experiments combining live and heat-killed vaccines and the first field trial for TB control in wild boar.

1. Introduction

Total eradication of an infectious agent shared between wild and domestic animals is almost impossible if a native wildlife host is able to serve as a natural reservoir of the pathogen [1–3]. Tuberculosis (TB) is a chronic disease caused by infection with *Mycobacterium bovis* and closely related members of the *Mycobacterium tuberculosis* complex (MTC). TB affects not only cattle but also a range of other livestock, companion animals, and wild animals. Humans are also susceptible; hence control of the risks of zoonotic infection is a driver for disease control in animal hosts. As TB prevalence has been reduced in livestock, the relative epidemiological and socio-economic importance of wildlife reservoirs has increased and there is a corresponding need for disease management strategies to reflect this effect [4].

Disease control through vaccination of wildlife reservoirs has advantages over other approaches. When dealing with disease maintenance by native wildlife, vaccination—as opposed to culling—is a nondestructive method of controlling disease that is more acceptable to the public [5, 6]. The primary goal of a wildlife vaccine would be to reduce the prevalence of infection in the wildlife reservoir or to change the expression of the disease and limit the rate of *M. bovis* excretion [7]. Indeed, vaccination is nowadays explored as an option for TB control in all major wildlife reservoir hosts such as the brushtail possum (*Trichosurus vulpecula*) in New Zealand, Eurasian badger (*Meles meles*) the United Kingdom and the Republic of Ireland, and white-tailed deer (*Odocoileus virginianus*) in the USA, among others [8].

In Mediterranean habitats of the south-western Iberian Peninsula, the abundant and widespread native Eurasian wild boar (*Sus scofa*) is an important driver in *M. bovis* epidemiology [9], thus the need for TB control in this species. Since uninfected 2–4-month-old wild boar piglets are the preferred age class for vaccination [10], an oral

delivery system that targets piglets is needed for field application of oral tuberculosis vaccines in wild boar. After briefly introducing the role of the wild boar in tuberculosis (TB) epidemiology and the options for TB control, this review summarizes the current knowledge on wild boar vaccination including aspects of bait design, delivery, and field deployment success; wild boar response to vaccination with BCG and inactivated *Mycobacterium bovis*; wild boar vaccination biosafety issues as well as prospects on future research.

2. Tuberculosis in Eurasian Wild Boar

The Eurasian wild boar is the ancestor of the domestic pig. It is native to Eurasia and the north of Africa and has been introduced, pure or crossbred, to many other regions worldwide. TB is one of the main infections shared between wild boar and other wild and domestic animals [4]. TB has a complex epidemiology involving multiple hosts and is influenced by climate, habitat, and management factors. Consequently, the role of wild and domestic hosts in TB epidemiology varies among regions [11].

Despite the success of compulsory test and slaughter campaigns in cattle, bovine TB (bTB) is still present in the Iberian Peninsula, and the role of wildlife reservoirs is increasingly recognized [4, 11, 12]. In Mediterranean habitats of southern Portugal and south-western Spain, MTC transmission occurs among three wild ungulate species, wild boar, red deer (*Cervus elaphus)*, as well as fallow deer (*Dama dama*), cattle, and to a lesser extent other domestic and wild animals such as goats, pigs, and Eurasian badgers [4, 13, 14].

Nevertheless, there is consensus in defining the wild boar as the single most important TB reservoir host in this region [9, 13, 15–17]. Extremely high densities and high contact rates within social groups and at waterholes or focal food sources might contribute to the high TB prevalence, often over 40% prevalence [12, 13, 16, 18–20]. Wild boar experience higher levels of exposure than deer [18] and are at greater infection risk as a result of feeding on tuberculous carrion [13]. Finally, wild boar are more likely than deer to push their way under fences, facilitating contact with livestock [12]. Work on TB time trends in Iberian wild boar has shown a stable prevalence with local variability, as well as an apparent expansion of the infection to new sites [21]. Moreover, wild boar TB has already been described in at least ten European countries (Bulgaria, Croatia, France, Germany, Hungary, Italy, Portugal, Slovakia, Spain, and the UK), and evidence as wildlife reservoirs is growing beyond the peculiar high-density and intense-management systems of south-central Iberia [4].

Naturally, MTC-infected wild boar show visible lesions in over 80% of the cases and only microscopic lesions in another 9% [22]. The distribution of lesions is generalized in two thirds of the cases, meaning that they are evident in more than one anatomic region. The mandibular lymph node (mLN) is the most frequently affected organ while large generalized and lung lesions are more frequent among 1 to 2 year old subadults, which have the highest potential to excrete mycobacteria and can die due to the disease [22]. Prevalence increases with age, < 6 month-old piglets showing a mean of 10% prevalence [15].

In contrast to wild boar, the role of feral pigs as MTC reservoirs is questioned [23–25]. However, domestic pigs and free-ranging *Sus scrofa* are MTC hosts in southwestern Spain [26], the Mediterranean islands of Corsica, Sardinia and Sicily [27–29], and the Hawaiian island of Molokai [30]. In Argentina, a wild-boar-derived *M. bovis* strain proved more pathogenic than the reference cattle-derived strain in a cattle challenge model [31]. Hence, the role of suids in the maintenance of MTC deserves more attention worldwide.

3. Options for TB Control in Wild Boar

The first requisite for any disease control in wildlife is establishing a proper monitoring scheme [32]. Then, actions towards disease control can be critically assessed. TB control in wildlife reservoir hosts can eventually be achieved by different means, including (1) the improvement of biosecurity and hygiene, (2) population control through random or selective culling or through habitat management, and (3) vaccination. Ideally, tools from all three fields should be combined in an integrated control strategy.

In this context, wildlife vaccination to reduce MTC infection prevalence emerges as a valuable alternative or complementary tool in TB control [43]. Capturing wild animals to vaccinate them individually is expensive, time consuming, and difficult [44]. Therefore, the most feasible approach to deliver vaccines to wildlife is the use of oral baits.

Oral vaccination against rabies was the first successful attempt to control a disease in wildlife through vaccination [45]. Thus, oral bait vaccination has also been considered for controlling other diseases such as classical swine fever in wild boar in Europe [33, 46] or TB in several wildlife hosts worldwide [47].

4. Bait Design, Selective Delivery, and Field Deployment Success

The effective and efficient field vaccination of wildlife requires the development of baits that are stable under field conditions, safe for target and nontarget species as well as the environment, and effective in reaching the target species [48–50]. A wide variety of baits have been developed in order to deliver pharmaceuticals to wild species. Lipid-based baits have been tested to deliver BCG vaccine against TB in wild animal species that act as reservoirs hosts such as badgers in United Kingdom and Republic of Ireland [44], possums in New Zealand [51–53], and while-tailed deer in USA [54].

For free-ranging *Sus scrofa*, three different baits have been developed and used for the oral delivery of vaccines and pharmaceuticals (Table 1). All of them are made with a cereal-based matrix containing a capsule or blister to deliver the vaccine or pharmaceutical. The palatable ingredients used for the bait matrix composition stimulate chewing to open the capsules contained in the baits and releasing their content inside the oral cavity [36].

Table 1: Characteristics of commercial, registered, or patented baits designed for the delivery of vaccines or pharmaceuticals to wild boar and feral pigs. Disadvantages include particularly their suitability for vaccine delivery to piglets.

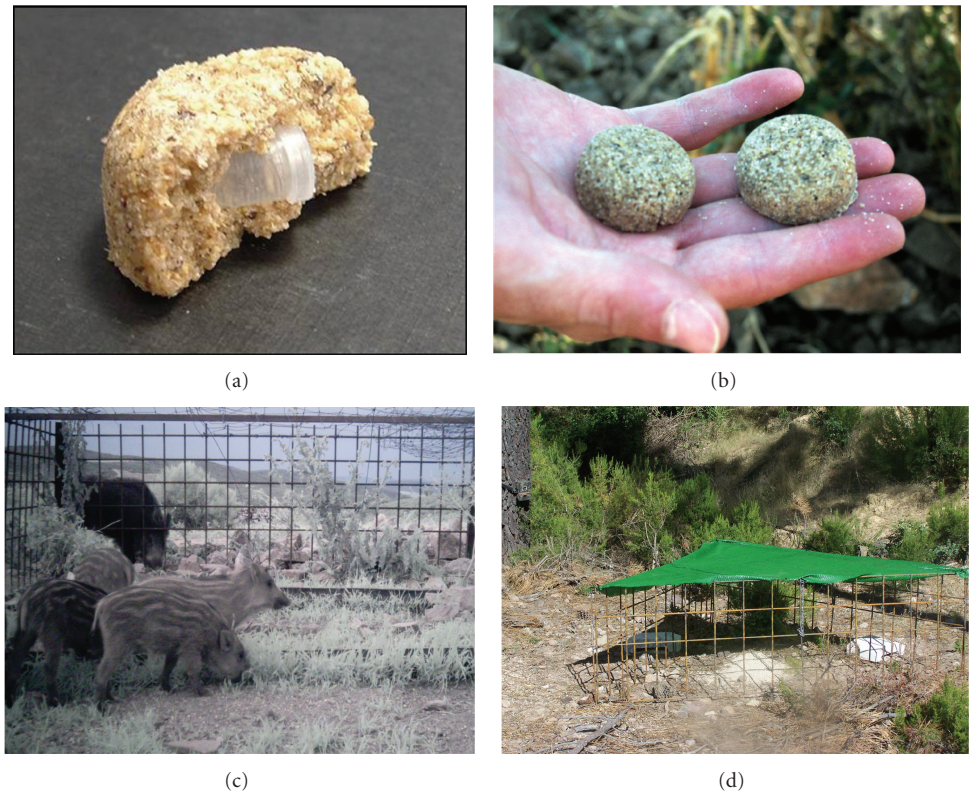
Bait	Shape and size	Use	Advantages	Disadvantages	References
Riemser	Square shape ($4 \times 4 \times 1.5$ cm)	Delivery of vaccines against classical swine fever in Europe	Resistant to water and moisture	Not resistant to warm temperatures Not completely consumed by <4.5 month-old wild boar piglets	[33–35]
IREC, Spain	Hemispherical shape (Ø3.4×1.6 cm; Figures 1(a) and 1(b))	Delivery of vaccines against *Mycobacterium bovis* in Spain	Resistant to high temperatures Well accepted by 2–4 month-old wild boar piglets	Not resistant to water and moisture	[10, 36–38]
PIGOUT	Cylindrical shape (9×5 cm)	Delivery of toxicants or pharmaceuticals to feral pigs in Australia and USA	Resistant to high temperatures	Large size would not be suitable for piglets	[39–42]

For TB vaccination, wild boar piglets (rather than already infected adults) are the main target [10]. If BCG was used, accidental bait uptake by cattle needs to be avoided [55]. Hence, purpose-designed baits and oral delivery systems selective for piglets are needed. The three baits developed for free-ranging *Sus scrofa* have been found to be highly attractive and readily ingested by animals [33, 36, 37, 39, 40]. However, both Spanish and PIGOUT baits have been found to be not target-specific enough in those areas where other wildlife species can also have access to the baits [10, 41, 42]. No data concerning target specificity of Riemser baits has been published.

Field assessment of the proportion of target and nontarget individuals that consume baits is crucial to evaluate the success of a baiting campaign. Therefore, marking agents are incorporated into baits to enable identification of consuming individuals [56]. Iophenoxic acid (α-ethyl-2-hydroxy-2,4,6-triiodebenzenepropanoic acid) and its derivatives have been used successfully to investigate baits and baiting strategies to deliver orally vaccines, contraceptives, and toxicants [41, 57–61], since they bind to protein in the blood plasma and elevate the protein-bound iodine of animals which consume them. So, these markers can be detected in the serum of animals consuming IPA-marked baits for a long time after their ingestion [62]. In the case of wild boar, Ballesteros et al. [63] found that ethyl and propyl-iophenoxic acids could be detected in animal serum up to 18 months after their consumption when doses of 5 and 15 mg/kg were delivered.

Bait consumption rate and host specificity depend directly on the delivery method employed [10, 55]. To date, three delivery systems have been designed to allow free-ranging *Sus scrofa* access to baits while preventing bait consumption by nontarget species: the Boar-Operated-System [64–66], the HogHopper [67, 68], and portable selective wild boar piglet feeders of Spanish patent [37]. The BOS consists of a metal pole onto which a round perforated base is attached. A metal cone with wide rim slides up and down the pole and fully encloses the base onto which the baits are placed. This system has been tested in United Kingdom [66] and the United States [65] showing low bait consumption by nontarget species. However, a possible disadvantage to this system is its cost [65]. The Hog-Hopper is a new box-shaped bait delivery device designed to allow feral pigs to access poison baits in the station, and to restrict other species (such as Australian native species and livestock) from taking bait. The door of the device is easily raised by feral pigs allowing them to feed on bait, but it excludes nontarget species that lack the physical attributes to lift the sliding door. Small rodents are also unable to access baits. The device has the added benefit of preventing bait from being exposed to rain, thereby preventing bait degradation [67, 68]. Selective feeders were used by Ballesteros et al. [37] in order to reduce bait consumption by nontarget species in southern Spain. These triangular-shaped feeders (side = 1 m) consist of a 1-cm-∅ metal-grid cage with an opening (15-cm wide) to allow only access of young wild boar (Figure 1(c)). A green mesh that provides shade covers the cage (Figure 1(d)). Although this system was found to be highly selective for wild boar piglets [10], occasionally small animals such as badgers can enter inside the feeders and have access to the baits [37].

The success of vaccination programs is also determined by the timing of bait delivery. For example, early summer would be the best timing for TB vaccine bait delivery to wild boar piglets in south-central Spain [10]. In addition, bait consumption by wild boar or feral pigs is better if the prebaiting period lasts longer (e.g., feeding corn weekly for three weeks) so that animals get accustomed to feed in the place where baits will be delivered [37, 69, 70]. Other factors such as baiting and/or free-ranging *Sus scrofa* densities can affect bait consumption by target species [37]. Ballesteros et al. [37] found marked-bait consumption by up to 73% wild boar piglets at a bait density of 30 baits/km^2 and using one piglet feeder per 2 km^2. These baiting densities were lower than those used in previous studies in other countries (e.g., 68 to 489 baits/km^2 [41, 57]. Therefore, in future TB vaccination experiments, it would be desirable to use higher



(a) (b)

(c) (d)

Figure 1: (a) and (b) Baits developed to deliver vaccines to wild boar in Spain. They are composed of piglet feed, paraffin, sucrose and cinnamon-truffle powder attractant. (c) Wild boar piglets consuming baits inside selective feeders. (d) Portable selective feeder used to deliver baits to young wild boar.

baiting densities to target a higher percentage of the wild boar population.

5. Tuberculosis Vaccines in Wildlife

Live vaccines are believed to confer more protection against mycobacterial infections than killed vaccines [71]. This is the case of the attenuated live strain of *M. bovis* BCG [72], which is currently the only vaccine approved for vaccination of humans against TB [73, 74]. Since 1921, BCG has been used worldwide and reports of adverse reactions arising from the use of this vaccine have been relatively uncommon [75]. Also, BCG is the most widely used vaccine for TB control in wildlife reservoirs. Experiments in controlled environments have been carried out in several host species such as badgers [76], brushtail possums [77], Cape buffalo (*Syncerus caffer*) [78], white-tailed deer [54, 79], wild boar [38], and ferrets (*Mustela furo*) [80]. In addition, recent reports on field vaccination of badgers in the UK [81] and possums in New Zealand [53] encourage the use of BCG for TB control in wildlife. Additionally, due to its long and widespread use in different species, licensing BCG for field use in wild animals is easier than licensing a newly developed vaccine [81, 82].

However, the use of BCG has some disadvantages, including (1) its variable efficacy in humans and cattle due to differences among BCG vaccine strains, trial methodology, and prior host sensitization to a variety of environmental mycobacteria [71, 83–87], (2) the possibility to cause disease or to infect nontarget individuals and cause interference with TB diagnosis [88–92], and (3) its limited half life in the environment and during vaccine preparation, shipment, or storage [93].

The use of killed vaccines would eliminate the risk of causing TB and should limit the likelihood of diagnostic interference and make field vaccination protocols cheaper. Several authors have found experimental evidence indicating that nonviable bacilli are able to produce some degree of protection to TB in guinea pigs [94–99], mice [100–103] and dogs [104]. In wildlife, only limited information exists regarding inactivated vaccines. Experiments with inactivated vaccines have been conducted in deer, brushtail possums, and recently in wild boar [105–107]. Deer were vaccinated with two doses of heat-killed BCG (5×10^7 cfu) in an oil adjuvant finding no protection against experimental challenge with virulent *M. bovis* [105]. In brushtail possums, the heat-inactivated *M. vaccae* was used to improve the effectiveness of live BCG to protect against bTB [106]. Recently, heat-inactivated *M. bovis* was found to confer protection against TB similar to BCG to wild boar [107].

6. Wild Boar Response to BCG Vaccination

The first results about wild boar response to BCG vaccination were documented in 2009, after vaccinating seven animals by the intramuscular route [108]. Later, a subsequent study by Ballesteros et al. [38] analyzed wild boar responses to oral BCG vaccination and challenge with a *M. bovis* field strain. Purpose-designed oral baits were used for the experiment [36]. The oropharyngeal route was found appropriate for wild boar experimental infection since lesions recorded resembled those of natural mycobacterial infections [109]. This research allowed defining the infection model and a lesion scoring system for wild boar TB [38], while further experiments increased the information on wild boar response to BCG vaccination [107].

In captive wild boar, BCG has shown significant levels of protection against challenge with a virulent *M. bovis* field strain. Culture scores and lesion scores of orally BCG-vaccinated wild boar were consistently lower in vaccinated than in control nonvaccinated animals [38, 107]. In addition, the reduction of the lesion and culture scores in the thoracic organs has been between 67% and 90% as compared to unvaccinated controls [107]. Vaccinated wild boar exposed to low-or-medium doses of *M. bovis* (10^2 cfu or 10^4 cfu) by the oropharyngeal route generally remain either uninfected or develop only limited lesions [38].

Antibody responses of wild boar against *M. bovis* have been detected reliably with specific serologic tests [21, 110, 111]. This enzyme-linked immunosorbent assay uses *M. bovis* purified protein derivative (bPPD ELISA test). Antibodies to bPPD increase only slightly and late after challenge and correlate with the total lesion scores of BCG vaccinated and *M. bovis* challenged wild boar [107]. Additionally, an innovative dual-path platform test (DPP test) using MPB83 and CFP10/ESAT-6 antigens has also been used to monitor antibody production in vaccination experiments in captive wild boar [107, 111]. Also, IFN-gamma production in response to bPPD has been detected in both BCG vaccinated and unvaccinated wild boar after *M. bovis* challenge [107].

7. Wild Boar Response to Vaccination with Heat-Inactivated *M. bovis*

Recently, a heat-killed *M. bovis* vaccine for oral and parenteral use was developed and tested in wild boar [107]. Each oral dose contained 6×10^6 bacteria in 2 mL of PBS, and each parenteral dose contained the same number of bacteria in 1 mL Montanide ISA 50 V (Seppic, Castres, France).

The first study incorporating this new inactivated vaccine showed that oral or parenteral vaccination with heat-inactivated *M. bovis* conferred a similar protection after challenge when compared to oral vaccination with BCG, and that the response of wild boar to both vaccines was similar. Although a high challenge dose was used (10^6 cfu), this vaccination protocol reduced the number and severity of lesions and the infection burden, particularly in the thoracic region [107].

The dynamics of antibody production, IFN-gamma response, and gene expression were similar in oral BCG- and inactivated *M. bovis*-vaccinated animals. Wild boar parenterally vaccinated with the inactivated vaccine responded to the MPB83 antigen but not to bPPD immediately after vaccination, suggesting potential use of these ELISAs to distinguish between parenterally vaccinated and exposed wild boar [107].

8. Wild Boar-Pathogen Interactions and Protection against TB

The study of host-pathogen interactions allowed identifying biomarkers of resistance/susceptibility to tuberculosis in wild boar and using these biomarkers for vaccine development [63, 107, 108, 112–115]. The expressions of some of these genes such as complement component 3 (C3) and methylmalonyl coenzyme A mutase (MUT) were shown to correlate with resistance to natural *M. bovis* infection and protection against *M. bovis* challenge in BCG-vaccinated wild boar [9, 38, 107, 108, 116]. In these experiments, C3 and/or MUT mRNA levels were higher in nontuberculous than in tuberculous adult wild boar naturally exposed to mycobacterial infection, decreased after *M. bovis* infection and increased with BCG vaccination, with higher mRNA levels in protected animals [9, 38, 107, 108, 113, 114, 116, 117]. Additionally, MUT may be genetically associated with resistance to tuberculosis in wild boar [113, 116, 117].

The mechanisms by which C3 and MUT expression contributes to resistance to mycobacterial infection remain unknown. The complement system has been shown to be involved in mycobacterial pathogenesis and *M. tuberculosis* activates the alternative pathway of complement and binds C3 protein, resulting in enhanced phagocytosis by complement receptors (CR3) on human alveolar macrophages [118, 119]. A similar mechanism may occur with *M. bovis* in which C3 opsonophagocytosis of mycobacteria by macrophages may result in the inhibition of host bactericidal responses and pathogen survival [118]. Consequently, higher levels of C3 in wild boar may allow increased binding of C3 to CR3 to promote phagocytosis and effective killing of bacteria, while interfering with CR3-mediated opsonic and nonopsonic phagocytosis of mycobacteria [114]. For MUT, a hypothesis was recently discussed to suggest that host genetically defined higher MUT expression levels result in lower serum cholesterol concentration and tissue deposits that increase the protective immune response to *M. bovis*, thus resulting in resistance to tuberculosis and better response to BCG vaccination [117].

The mechanism of protection from BCG vaccination involves a reduction of the haematogenous spread of mycobacteria from the site of primary infection. It protects against the acute manifestations of the disease, and reduces the lifelong risk of endogenous reactivation and dissemination associated with foci acquired from prior infection [120].

It is tempting to speculate that BCG protection in wild boar would involve distinct systemic and mucosal populations of effector memory T cells. Immune genes with

significant overexpression in nontuberculous than in tuberculous adult wild boar naturally exposed to mycobacterial infection include RANTES (aka Chemokine (C-C motif) ligand 5; CCL5), IFN-gamma, and IL4 [108]. The mRNA levels of these genes also increased after parenteral and oral BCG vaccination of wild boar [38, 108], thus suggesting that IFN-gamma and activated RANTES-secreting CD8 (+) and/or CD4 (+) T lymphocytes may be key players in BCG-induced protective response in wild boar. However, although it is generally recognized that humoral immunity is not important for the control of tuberculosis [121], IL4-induced antibody response against *M. bovis* may be important for tuberculosis control in wild boar. IL4 overexpression in nontuberculous and BCG-vaccinated wild boar suggests that antibodies against mycobacterial proteins may be used for disease surveillance and treatment monitoring in this species [21, 107, 110, 111] and underline the existence of host-specific responses to mycobacterial infection [122] as the increase in IL4 levels correlates with disease severity in humans [123] but not in wild boar.

Inactivated vaccines stimulate specific CD4 (+) cell populations that recognize the antigen versus a live vaccine that stimulates many T cell populations simultaneously. While antibody and IFN-gamma responses increased after vaccination in parentally BCG-vaccinated wild boar, in orally BCG and inactivated *M. bovis* vaccinated wild boar, only MUT mRNA levels correlated with protection [107]. These results are difficult to explain before further experiments help to characterize the mechanism by which vaccination with the inactivated vaccine protects against tuberculosis in wild boar. Taken together, these results suggest different protective mechanisms between parenteral and oral inactivated mycobacterial vaccines and, at least for MUT, expression levels could be a marker of protection against tuberculosis and may be used to characterize host response to BCG vaccination in wild boar.

9. Wild Boar Vaccination Biosafety Issues

Four main biosafety issues must be considered before delivery of oral baits containing live vaccines such as BCG to wild boar: (1) potential effects of high vaccine doses (e.g. ten times the normal dose) on wild boar health; (2) potential survival of *M. bovis* BCG in vaccinated wild boar; (3) potential excretion of *M. bovis* BCG by vaccinated wild boar; (4) vaccine-containing bait uptake by nontarget species, particularly by cattle.

Regarding point (1), it is highly important to determine that high doses of vaccine do not affect the animal's health since it is likely that few individuals can gain access to a high number of baits during field vaccination campaigns. In the case of wild boar, no adverse effects that can be attributed to the vaccine have been detected in vaccinated individuals [38, 107, 108]. Moreover, wild boar treated with high vaccine doses of up to 3.0×10^6 cfu did not show any adverse effect after BCG administration [38].

Concerning point (2), *M. bovis* BCG has never been isolated from tissues of vaccinated wild boar, despite the occasionally high doses used (the authors, unpublished information). However, in other species, such as brushtail possum and deer, BCG was isolated in tissues of oral BCG vaccinated animals after necropsy [124, 125].

Regarding point (3), the potential of faeces from vaccinated wild boar to lead to the accidental exposure of non-target species to BCG, shedding of BCG following bait ingestion has been tested under laboratory conditions over a period of seven days post vaccination. The analyses yielded no BCG isolates (unpublished data). In other TB hosts, BCG is detected in faeces only for a short period of time after ingestion [52, 125].

Finally, point (4), it is necessary to consider the possibility of bait consumption by nontarget species. Oral baits developed by Ballesteros et al. [36] were found highly palatable to both wild and domestic animals [55]. This fact could have negative effects in areas where cattle and wild reservoirs coexist since accidental consumption of BCG-containing baits by cattle could interfere in the TB test and slaughter campaigns. However, this risk can be reduced by using deployment strategies that assure that only target species gain access to bait such as selective feeders [10, 37]. Moreover, the scheduled preliminary field experiments are taking place in sites without cattle [37]. In addition, the research towards the development of BCG-specific blood tests for cattle, and the relatively short duration of BCG-induced reactivity in livestock contribute to limit the concerns [126].

Further information regarding BCG biosafety is needed to satisfy regulatory and licensing requirements for release of oral bait vaccines to wildlife [82, 125]. Therefore, new laboratory experiments will be conducted in order to assess the potential oral or nasal excretion of BCG by vaccinated wild boar. In addition, cattle will be exposed to BCG-containing baits under controlled conditions to assess the likeliness of developing a positive skin test. Other important information regarding biosafety will be derived from the first controlled field experiments starting soon in Southern Spain. Furthermore, the recent development of an inactivated *M. bovis* vaccine would significantly reduce the safety issues, since no viable organisms are used [107].

10. Conclusions and Future Research Directions

During the last decade, research on TB epidemiology and oral vaccine development and characterization in wild boar allowed considering oral vaccination among available TB control tools. The continued applied and basic research on integrated TB control at the wildlife-livestock interface will hopefully yield even more significant advances in the future.

Future research on TB vaccination in wild boar will include both new controlled laboratory and field experiments (Figure 2). Results obtained in experiments comparing the efficacy of inactivated *M. bovis* and BCG vaccines encourage testing combinations of these vaccine preparations. The characterization of the immune mechanisms that support protection against tuberculosis after vaccination with BCG and inactivated vaccines are essential to advance in the development of new improved vaccines and/or vaccination schemes.

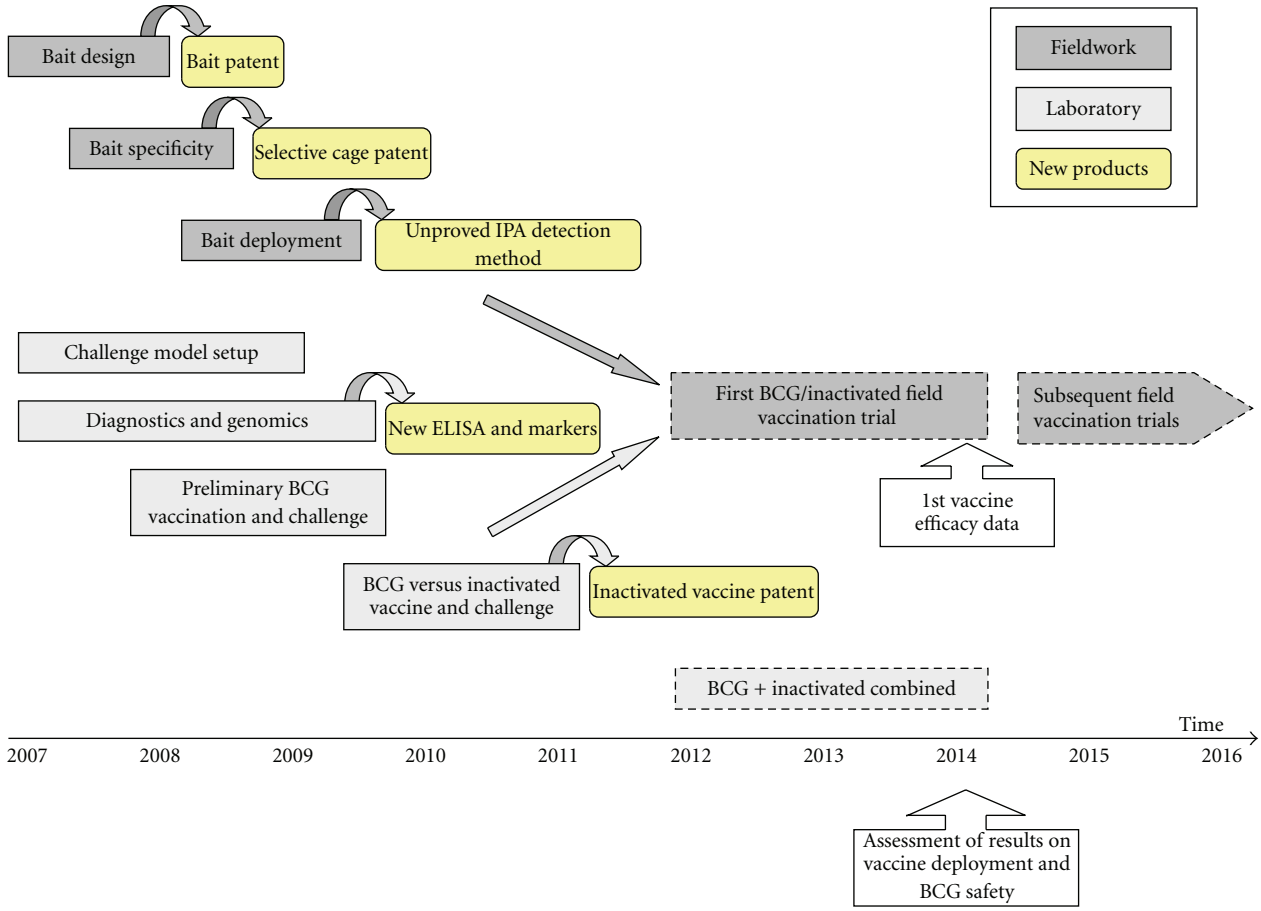


Figure 2: Flowchart of the laboratory and field research and key results towards oral vaccination of Eurasian wild boar against *Mycobacterium bovis*. Dashed boxes indicate future experiments.

A controlled and replicated experimental oral vaccination trial will start soon in southern Spain. The goals of this first field trial are assessing the response of wild boar to oral BCG and heat-killed *M. bovis* vaccination under field conditions, gathering information on safety aspects and analyzing the cost-effectiveness of vaccination for TB control in wild boar. This includes modeling the outcome of vaccination as compared to population control.

Regarding models, preliminary data gathered from individual-based models suggest that vaccinating piglets over a long-term period has the potential to successfully eradicate bTB from wild boar reservoirs in southern Spain. Further research into the transmission rates between bTB hosts and the efficacy of the vaccine itself, but also on the cost-effectiveness of wild boar vaccination as compared to population control (and their combinations) will add important reinforcements to these initial findings.

Acknowledgments

This study was supported by a Plan Nacional AGL2011-30041 grant from Ministerio de Economía y Competitividad and the EU FP7 Grant TB-STEP 212414.

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 621210, 10 pages
doi:10.1155/2012/621210

Review Article

Herd-Level Risk Factors for Bovine Tuberculosis: A Literature Review

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Received 6 January 2012; Accepted 14 April 2012

Academic Editor: Jesse M. Hostetter

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Bovine tuberculosis (TB), caused by *Mycobacterium bovis*, is one of the most challenging endemic diseases currently facing government, the veterinary profession, and the farming industry in the United Kingdom and Ireland and in several other countries. The disease has a notoriously complex epidemiology; the scientific evidence supports both cattle-cattle and wildlife-cattle transmission routes. To produce more effective ways of reducing such transmission, it is important to understand those risk factors which influence the presence or absence of bovine TB in cattle herds. Here we review the literature on herd-level risk factor studies. Whilst risk factors operate at different scales and may vary across regions, epidemiological studies have identified a number of risk factors associated with bovine TB herd breakdowns, including the purchase of cattle, the occurrence of bovine TB in contiguous herds, and/or the surrounding area as well as herd size. Other factors identified in some studies include farm and herd management practices, such as, the spreading of slurry, the use of certain housing types, farms having multiple premises, and the use of silage clamps. In general, the most consistently identified risk factors are biologically plausible and consistent with known transmission routes involving cattle-cattle and wildlife-cattle pathways.

1. Introduction

Bovine TB is a chronic disease of animals caused by infection with the slow-growing, obligate intracellular bacterium *Mycobacterium bovis* [1, 2]. This highly adapted and "successful" pathogen has a world-wide distribution and in several countries bovine TB remains a major, costly infectious disease of cattle and other domesticated, feral and wild animal populations, including badgers, possums, deer, goats, sheep, and camelids [3–5]. Bovine TB is an OIE (World Organisation for Animal Health) listed (formerly List B) disease [6, 7].

Bovine TB affects cattle health, impacts negatively on profitability and trade, and can decimate years of genetic improvement towards desirable production traits [8]. It also impacts negatively on the welfare of affected farming families [9]. Although effectively controlled by herd testing, milk pasteurization, meat inspection, health surveillance, and BCG vaccination, transmission to humans can occur and is still considered a public health risk [10–12], although some more recent opinion considers this risk to be negligible [13]. Hence, bovine TB control is currently more concerned with trade implications.

Despite sustained and costly implementation of eradication programmes since the 1950s bovine TB has not been eradicated from either the United Kingdom (UK) or Ireland. Indeed, there has been a sustained and largely unexplained increase over the last 25 years in parts of the UK [14]. Consequently, bovine TB is the most complex and difficult multi species endemic disease currently facing government, the veterinary profession, and the farming industry in the UK and Ireland [15, 16]. Bovine TB epidemiology, in the UK at least, is exceptionally complicated, and the relationship between evidence, uncertainty and risk has been difficult to communicate [17]. It is recognised as a very significant policy challenge and continues to be almost inevitably highly politicized [18].

Pathogenesis studies suggest strongly that the route of transmission of bovine TB is largely *via* the respiratory system, requiring transmission *via* infectious aerosols [19]. Consequently bovine TB is principally a respiratory infection and the majority of infections are thought to occur *via*

"direct" aerosol transmission between animals in close proximity. Although considered to be of lesser importance, oral ingestion of mycobacteria from farm environments cannot currently be excluded [1]. Whilst it is important to view bovine TB as an infectious disease which requires preventive as well as control measures, *M. bovis* infection in cattle now rarely presents as clinical disease. More commonly it appears as apparently healthy animals responding to an immunological test based on tuberculin, an entirely different scenario to that which existed when control programmes were first introduced [20].

In countries with advanced test and control programmes (a comprehensive set of surveillance and control measures to address cattle-cattle transmission) bovine TB tends to be a low-incidence infectious disease with an apparently low transmission rate. Infection appears to be relatively poorly transmitted between cattle in most, but not all, circumstances.

2. Herd Testing and Management

Monitoring of the cattle population for *M. bovis* infection depends on national programmes of herd tuberculin testing, supported by active abattoir surveillance [1, 12, 21]. The frequency of such testing is determined by the recent local incidence, ranging from annual testing (in Northern Ireland and other parts of the UK and Ireland) to four-year testing (in parts of England) [1, 21]. Limited tuberculin test sensitivity is also likely to have contributed to under- or over-estimation of the impact of several risk factors, such as, cattle contact and movement in some studies. To compound the above, multiple unreported local cattle movements and contacts are described between farms in several studies and are recognised as a factor in underestimating the role of contact and movements, particularly over short range.

Early diagnosis and intervention to interrupt transmission are the priority for control and the effectiveness of testing and removal of infectious animals will impact on transmission and depends on: how early the infections are detected, how sensitive the test(s) actually are in practice and other variables, including interoperator characteristics, test interval, and/or time to derestriction. More severe tuberculin test interpretation and/or supplementary immunological tests (cell-mediated immunity and/or humoral immunity) may be applied in defined circumstances. The herd is placed under movement restriction until all animals clear two short-interval tuberculin tests. Animals may also test "inconclusive" to the tuberculin test, a proportion of which test negative at the next short-interval test but have since been shown in an Irish study to be 12 times more likely to be TB positive at the next test than other animals [22]. Between 11.8% and 21.4% was confirmed positive at the laboratory, compared to 34–39% for standard tuberculin reactors. In summary, inconclusive reactors are at increased future TB risk [22, 23], whether they clear the next test or not and this is now reflected in policy options.

Bovine TB "reactors", and on occasions the exposed cohort, should be removed quickly from affected herds and contact tracing and testing should be implemented. Whole-herd depopulation may be indicated on rare occasions. The impact of such whole herd depopulation, for either bovine TB or bovine spongiform encephalopathy (BSE), on the recurrence of bovine TB was investigated in a recent Irish study [21]. Recurrence may reflect residual infection in cattle and/or reinfection from other sources, potentially including contiguous spread, acquired infection, infectious wildlife, and environmental contamination and may also be a consequence of imperfect test sensitivity [24]. It was concluded that future risk varied significantly by previous TB risk and reason for depopulation and that whole herd depopulation was effective in reducing future bovine TB risk. The BSE depopulated herd results were similar to a recent GB study [25] where whole herd depopulation for foot-and-mouth disease (FMD) was also not associated with reduced future bovine TB risk.

Key to understanding bovine TB epidemiology is the relationship between infection and disease (TB) and the relationship between disease and transmission. Hence, it is important to consider those risk factors, such as, infectious contacts between animals and their movements, which theoretically facilitate such transmission. The identification of risk factors and risk settings for infection and transmission is also intimately linked with those factors which affect susceptibility.

3. Risk Factor Studies

Infectious diseases in general arise from an interaction between the infectious agent, the host, and a range of covariables, which may include other infectious diseases and the environment. Risk factors (biological, behavioural, environmental, or genetic) are known to influence both transmission and susceptibility. They may operate at different scales; regional-level, herd-level, and animal-level and may vary across regions due to factors, such as, differing farm structures, farm management practices, bovine TB control and eradication programmes, regional TB incidences, wildlife densities, and the relative importance of specific risk factors by area.

The risk of a bovine TB episode is accepted to vary between herds with some herds experiencing multiple breakdowns over time, whilst others appear to remain free of infection. Also, the nature of bovine TB breakdowns is not uniform; they can be classified as "sporadic", "persistent", "recurrent", and so forth and the literature supports the view that different risk factors are likely to apply, almost on a case-by-case basis. However, some risk factors have tended to emerge in several published studies and we have discussed these here.

It is important to note that epidemiological studies may differ in the variables examined, the exact measures used (in relation to the association with wildlife, etc.), and the study size and power. Therefore, not all risk factors would be expected to be identified equally across all studies. Those risk factors which tend to converge from disparate studies would support the currently hypothesized sources of infection and routes of transmission.

There are no guarantees that such studies would identify significant risk factors, although the fundamental premise of epidemiology is that disease occurrence in populations is not random but rather is associated with identifiable factors. Not all of the studies summarized below are classical "risk factor" studies; instead they are observational studies and it is important to remember that risk factors identified in herd- and animal-level studies are *associated* with the measured outcome and are not necessarily causal. Some studies include analysis of wildlife-related risk factors, which have not been discussed in any detail here.

Summaries of the studies assessed as part of this paper have been included below in two groups. The first are classical case-control studies, the second are predominantly cohort-based studies or case-control studies focused on specific risk factors. Most of the studies listed under "other epidemiological studies" used preexisting data (e.g., cattle movement and bovine TB test data) and have addressed specific epidemiological questions, such as, the role of cattle movement.

Previous (case-control) studies, mostly in the UK and Ireland, have identified a number of risk factors associated with TB herd breakdowns. These include the purchase of cattle [26–28], the occurrence of TB in contiguous herds, and/or the surrounding area [29, 30] as well as herd size [28, 30]. The most frequently identified risks for herd-herd transmission included cattle movements and trading, where general trading or purchase from markets or herds in hot-spot areas or from infected herds have all been linked with increased risk for the receiving herd [31].

The following are among the risk factors shown to influence the potential of direct and indirect (*via* faeces and urine) exposure of cattle to the wildlife reservoir (badgers in the UK and Ireland) when at pasture; stocking regime (set-stocking), rotational *versus* strip grazing, stocking densities, farm habitat types, and livestock production intensity [31]. Sharing feed or water between cattle and wildlife when housed or at pasture, housing type and storing manure indoors are associated with the differential risk of transmission between cattle and wildlife. Results in relation to the association with the presence of badger setts or local badger density have been more variable with some studies reporting an increased risk associated with the presence of badger setts on the farm [26, 29] or local badger density [4] while others have found no association with the presence of badger setts either on the farm or the surrounding area [27, 30].

Other evidence, and in particular observational studies on badgers in GB, has suggested possible routes of transmission from badgers to cattle [32], including the possibilities of direct contact between cattle and badgers at pasture, indirect contact between cattle and infected badger faeces, urine and wound discharges, the likelihood of which is increased where cattle can access badger setts and latrines, and/or where badgers access cattle feed and/or water troughs. There is also substantial evidence in GB of badger visits to farmyards and buildings to access a range of feed sources including cattle feed in stores, maize silage, and accessing feed in troughs [33, 34], all of which would increase the likelihood of both direct and indirect transmission of infection. The physical exclusion of badgers from farm buildings has been suggested as the simplest and potentially most effective method of reducing contact between badgers and cattle [32], although issues of practicality, likely farmer uptake and compliance, require further investigation.

Many farm and management variables are likely to be highly correlated with factors, such as, herd size, herd type, and to a lesser degree herd location. Herd size, for example, was a risk factor in a number of previous studies, although it is unclear whether this variable is acting as a risk factor *per se*, as a partial summary measure of other factors or because of inherent changes in the herd level sensitivity and specificity of the test as herd size increases.

Other factors identified in some studies include farm and herd management practices, such as, the spreading of slurry [26]; the use of certain housing types [27]; farms having multiple premises [27]; the use of silage clamps [28]. In general, the most consistently identified risk factors are biologically plausible and consistent with known transmission routes involving cattle to cattle and badger to cattle pathways. Whilst many of the general risk factors for the introduction and spread of bovine TB have been identified, less is known about the practical measures that herd keepers could reasonably take to minimize their risk and the possible impact of common biosecurity practices.

4. Case-Control Studies

In bovine TB, several risk factors (e.g., cattle husbandry and environmental practices) have been suggested as predisposing farms to TB breakdowns [35]. However, they are not amenable to experimental investigation due to the large number of variables, the impracticality and cost of conducting controlled experiments on commercial livestock farms, and the need for data from a large number of representative bovine TB breakdowns. In such circumstances, a "case-control" study provides the appropriate approach [1].

The essence of a case-control study is firstly to identify if specified risk factors are statistically associated with the occurrence of a disease or condition, while controlling for confounding and interaction and secondly to estimate the magnitude of any such risk. Within a case-control design, further options exist as to whether or not cases and control should be matched. The typical output of a case-control study is a list of risk factors associated with the disease, accompanied by the statistical significance of each, the odds ratio (OR) for each, and the 95% confidence interval (CI) for the OR. For example, the study may show that a farm that purchases cattle is twice as likely to experience a bovine TB breakdown as one that does not.

The accompanying statistical significance indicates the likelihood of such a finding not being true (i.e., that such a finding has arisen in the study purely by random chance) and therefore the weight that can be applied to the finding. The 95% CI of an odds ratio indicates the likely range of the predicted risk. An estimated OR of >1.0 indicates that the factor is associated with an increased risk of a breakdown, and the greater the numerical value of the OR, the greater the risk. By contrast an OR <1.0 suggests that the factor

reduces risk and is "protective" in relation to bovine TB breakdowns.

A case-control study of risk factors for bovine TB in Northern Ireland was undertaken, based on tuberculin test reactors identified between 1990 to 1992 [29]. The study involved 427 dairy farms (excluding farms with fewer than 30 cattle and herds with reactors in purchased cattle). Variables investigated included the number and nature of farm boundaries, the number of neighbours and their bovine TB history, the number of hedgerows, the presence of badger setts, whether badger carcases had been found on the land, and the possible presence of deer. Two factors were significantly associated with bovine TB breakdowns; the presence of badger setts or carcasses on the farm (OR 2.06, 95% CI 1.27–3.33) and contiguous neighbours with confirmed bovine TB (OR 2.44, 95% CI 1.55–3.86).

A matched case-control study was undertaken in the Republic of Ireland to provide information on the role of farm management practices, environmental factors, and farmer characteristics in the epidemiology of bovine TB. Eighty dairy herds with chronic bovine TB were compared with the same number of herds which had been free of the disease for many years. A standardized questionnaire was used. The study was conducted from August to October 1990, in Counties Cork and Kilkenny. Factors which were identified as possibly contributing to recurrent outbreaks of TB included nutritional factors, cattle purchases (especially bulls), the presence of badgers, and the spreading of slurry. Overall, the findings suggested that intensively managed dairy herds were at greater risk of bovine TB outbreaks than were other herds [26].

Subsequently, a case-control study of 200 herds from East Offaly (Ireland), with cases defined as outbreaks of bovine TB detected at herd test, was undertaken [30]. Herd-level risk factors significantly associated with an increased risk of infection were herd size and the presence of TB in a contiguous herd. Differences between animal types (increased risk in cows, heifers, and bullocks compared to calves) and a reduced risk (protective) in animals purchased since the preceding herd test were found at the animal level. No significant differences were found between cases and controls in the distance to the nearest badger sett or the nearest main sett.

Herd-level risk factors for bovine TB breakdowns based on cattle farms enrolled within the GB Randomised Badger Culling Trial (RBCT), prior to the Foot-and-Mouth Disease (FMD) epidemic in 2001, were investigated [27]. The study (the TB99 study) comprised 268 farms from SW England, with questionnaires on farm management practices completed by staff from the local Animal Health Office. The strongest factors associated with an increased TB risk were movement of cattle onto the farm from markets or farm sales, operating a farm over multiple premises and the use of either covered yard or "other" housing types. Spreading artificial fertilizers or farmyard manure on grazing land was associated with a decreased risk. The presence of an active badger sett mapped to either the farm land or to within 1 km of the farm boundaries was not statistically significant.

Risk factors have been investigated in case-control studies in Europe and the USA. Historical incidence was a robust predictor of the rate of future breakdowns in UK and Irish herds, suggesting that the disease source was not adequately removed, or that some other factor(s) made them particularly susceptible. Herd size was repeatedly identified as a major risk in many studies. Large herds tend to graze larger areas and may purchase and move more cattle, increasing the probability of having contiguous herds that facilitates cattle-cattle spread. The higher production stress of intensive management has been associated with increased risk [26]. Herd breakdowns tend to recur, especially in larger herds, possibly as a result of failing to clear the source and contact with contiguous herds and infectious wildlife.

Larger herds are more likely to have at least one cow with disease. As herd size increases, the probability of at least one case increases and herds of different sizes are therefore at different risks. The observed size distribution of bovine TB-affected herds suggests that animals pose identical risks. Cattle living in different parts of the UK and Ireland probably experience different risks, and there was no consistent indication in the GB TB99 and CCS2005 data to suggest that the presence of any wildlife species, or indeed domesticated species, was associated with the risk of multireactor breakdowns [1]. There is evidence that increasing herd size for financial gain may actually contribute to increased bovine TB incidence [33].

Mathews et al. [4] examined the association between farm habitat features and other factors and the risk of bovine TB in two areas in West and SW England and involved 120 dairy herds in total (excluding herds with bovine TB breakdowns due to imported cattle). Badger road-kill records within 1 km and 5 km proximity of the farm were used as a proxy measure of badger density. The predictors found to be significant included farmland habitat, topography, and indices of badger density and herd size.

A comparative case-control study in England in which risk factors in herds with transient or persistent TB breakdowns were compared to a common set of control herds (229 herds in total) was reported [28]. Interviews with herdkeepers were conducted (March 2000–February 2003). Data on farm management practices were obtained from on-farm questionnaires, whereas the presence of badger setts and the type of habitat cover was determined by field survey of the relevant farms. The purchase of cows was a risk factor for both transient and persistent breakdown. The purchase of >50 cattle and the storage of manure for ≥6 months were risk factors for transient breakdowns, whereas the use of silage clamps increased the risk of persistent breakdown. Rather counter intuitively, decreased odds of both transient and persistent breakdown were associated with higher stocking densities (>3 cattle/ha). Running mixed herd enterprises compared to beef only or dairy only was an additional protective factor against persistent breakdown. Herd size and tuberculin testing interval were also significant risk factors for both transient and persistent breakdowns, whereas active badger sett density and regional location only affected the risk of persistent breakdowns.

Johnston et al. [31] reported the results of a matched case-control study (218 of 401 herds available for analysis) in 4 regions of England and Wales in 2005/2006, where case herds had confirmed infection. The significance of association with risk factors varied clearly by location. Overall, they report that contacts with contiguous herds (OR = 2.24), sourcing cattle from herds with a recent bovine TB history (OR = 1.90), operating a fragmented farm (OR = 2.41), feeding cattle inside housing (OR = 4.89), and presence of dead badgers on farm (OR = 3.10) were all associated with increased risk of a confirmed breakdown. Case herds were more likely to source cattle from herds with a breakdown within the last 2 years and more likely to have more direct contacts with contiguous herds with more confirmed breakdowns in the previous 2 years among contacted herds. They were also more likely to report finding dead badgers on farm. Providing feed outside of cattle housing was protective (OR = 0.41), as was the practice of not providing shelter at pasture for cattle, which may reduce the opportunities for cattle-cattle contacts. Grazing the whole pasture was associated with increased risk, possibly due to the increased potential for badger-cattle contact at pasture. They concluded that there was an increased local risk related to the occurrence of breakdowns amongst neighbours and/or contacted herds and possibly shared exposure to an external source, such as, wildlife. Risk factors tended to vary by region, so control recommendations should reflect local risk.

5. Other Epidemiological Studies

Using the GB Cattle Tracing System and VetNet data Brooks, Pollock and Keeling [36] examined the relationship between herd size and persistence of bovine TB on farms. Using a measure similar to the Critical Community Size, the VetNet data revealed that herd size was positively correlated with disease persistence. Carrique-Mas et al. [25] analysed cattle movement data and herd TB history in approximately 4,200 herds, which were restocked after-FMD. Three risk factors were identified in the study; sourcing cattle from herds that were routinely tested for bovine TB more than biennially, a history of TB breakdowns in the restocked farm (1997–2000), and increasing herd size.

Although by design a case-control study, a GB study was undertaken at the animal level and specifically examined the relationship between the selenium, copper, and vitamin B12 status of cattle, and bovine TB infection [37]. The animals involved were 200 reactors and 200 in-contact animals, selected from herds in England and Wales. The study found that lower levels of GSHPx (Selenium) and higher levels of copper were associated with an increased risk of confirmed bovine TB, but there was no association with vitamin B12.

Gilbert et al. [14] assessed the role of cattle movements in the spread of bovine TB in GB using movement records from the Cattle Tracing System data archived. Their study showed that cattle movements, particularly those from areas where bovine TB was reported, consistently outperformed environmental, topographic, and other anthropogenic variables as the main predictor of disease occurrence. Gopal et al. [38] reported on the introduction of bovine TB to NE England by bought-in cattle. Their study investigated 31 herds that experienced confirmed breakdowns between January 2002 and June 2004; nine of which had restocked after-FMD 2001. In all but one of the breakdowns the most likely source of infection was one or more purchased animals. In 17 of the breakdowns, reactor animals were traced to herds from which the same *M. bovis* genotype (spoligotype-VNTR profile) was isolated, and in five breakdowns a different genotype was isolated. Reactors in five of the breakdowns included homebred and purchased animals, providing evidence for the likely spread of the disease by cattle-cattle transmission within the herds on arrival. The lack of geographical clustering of molecular types pointed to the overwhelming source of infection being purchased cattle.

Green et al. [39] used cattle movement data to construct an individual (premises-) based model of bovine TB spread within GB, accounting for spread due to recorded cattle movements and other causes. Outbreak data for 2004 were best explained by a model attributing 16% of herd infections directly to cattle movements, with a further 9% unexplained, potentially including spread from unrecorded cattle movements. The best-fit model assumed low levels of cattle-cattle transmission. The remaining 75% of infection was attributed to local (wildlife and cattle) effects within specific high-risk areas. Green and Cornell [40] investigated herd breakdowns in four counties of England and Wales using data from the national database of bovine TB testing history (VetNet). Factors that influenced herd breakdown included calendar time, herd size, number of cattle tested, the test type, the intertest interval, and spatial grouping of farms.

The proximity of farms to badger setts was compared between Irish farms that had experienced a TB breakdown and those that had not, over the 6-year period from 1988 to 1993 [41]. The data were derived from badger removal in East Offaly, which began in 1989 and continued through 1993. By the end of 1990 approximately 80% of all badgers caught in the 6-year period had been removed. The risk of a multiple reactor TB breakdown decreased for herds at least 1 km away from an infected badger sett and increased as the number of infected badgers *per* infected sett increased. Despite the significantly reduced risk of a breakdown with increasing distance from infected badger setts, the relationship was not strong (sensitivity and specificity of the model were in the low 70s%) and explained only 9–19% of bovine TB breakdowns.

A retrospective cohort study with bovine TB test data [42] investigated breakdown severity as a predictor of future herd breakdowns in Ireland. The hazard (risk) of a future bovine TB breakdown increased directly with number of cattle in the herd, a positive history of previous bovine TB in the herd, and the local herd prevalence of bovine TB. The presence of confirmed bovine TB lesions in reactor cattle was not predictive of the future breakdown hazard when the effects of other factors were controlled.

The Irish study above contrasts with a recent GB study, which showed that ~30% of herd breakdowns extend for >8 months [43] and consume disproportional resources as well

as acting as ongoing sources of infection. Breakdown duration was a function of infection status and test performance. Potential explanations for *persistent* infection included suboptimal performance of the bovine TB tuberculin test, delay in its application, or reintroduction of infection. Skin test sensitivity has been estimated at 75.0–95.5% [12]. If the sensitivity was substantially lower [24], failure to detect and remove infected animals would create potential for within-herd persistence and onward spread.

Factors associated with breakdown *recurrence* in Ireland, where detailed animal-level data were available [44], included slurry spreading, purchase of bulls and cattle, presence of inconclusive reactors in the breakdown, and presence of badgers and nutritional status. Where only population-level surveillance data were available, factors associated with recurrence included herd size, reactor number, and recent herd bovine TB history [44–46].

In the DEFRA SE3230 research project (The Problem TB Herd: characterisation, prediction and resolution) breakdown confirmation status was by far the strongest risk factor for persistence (OR = 12.6). They used an improved case definition and concluded that this strong association may best be explained by the tendency to deploy severe interpretation of the tuberculin test in herds with confirmed status and the possibility that true prevalence was underestimated [43]. Their model could predict earlier those herds most likely to sustain persistent infection. Resources and earlier intervention could be directed at those herds. The model predicted that stopping animal movements onto the farm during the breakdown and moving salt licks indoors were associated with a small decreased risk. It is also plausible that a number of the unconfirmed herds were not actually infected.

Analysis of the GB CCS2005 epidemiological data identified that despite increased testing during and after breakdowns ~21% of breakdowns recurred within 12 months. 60% of these recurrences was disclosed at the 6-month followup, suggestive of within-herd persistence. 38% recurred within 24 months [45]. Factors associated with *recurrence* were reactor number and recent history of bovine TB in the herd, consistent with previous studies in Ireland and Northern Ireland [44, 46]. However, they found a lack of association with the confirmation status of the initial breakdown. They conclude that their data support a higher prevalence of infection than observed, residual infection, or repeated reinfection. The main risk factors associated with recurrence in this study ranked as follows: use of "other housing types" (OR = 4.6), number of contiguous farms (OR = 3.2), and borrowing animals (OR = 2.1) [45]. Protective factors associated with decreased risk of recurrence included the presence of rough grass/moorland (OR = 0.3). These recurrent breakdowns may have been reinfected from a local source, such as, wildlife or from cattle movements into the herd. As well as consuming disproportionate resources, the existence of recurrent breakdowns suggests that such herds cannot reliably be cleared of infection and undermines stakeholder confidence in the TB testing programme. They concluded that certain farm practices or characteristics may predispose to reinfection and that a combination of factors was associated with recurrence, rather than just one strong factor.

Abernethy et al. [46] used comprehensive animal-level test and movement data to investigate the effect of selected risk factors on recurrence of bovine TB in breakdown herds after derestriction in Northern Ireland. Factors associated with an increased risk included the number of reactors at the disclosing test, the number of reactors at follow-up tests, the number of follow up tests, the level of bovine TB in the district council area, herd size, the number of cattle purchased during the postoutbreak interval, and a history of bovine TB breakdown(s) within the previous two years.

Despite variation between farming practices within the British Isles, reactor number and recent history of bovine TB were consistent risks for recurrence in Irish, Northern Irish, and GB studies [45]. Whether the breakdown was confirmed or not was the major factor in the duration of breakdowns (persistence) in GB [43] but was not a factor in risk of recurrence and neither was herd size nor cattle movements [45]. This illustrates that the risk factors for different types of breakdown (sporadic, persistent, recurrent, etc.) may well be different. Either way, this could increase transmission potential to local wildlife or to local or more distant cattle herds through cattle contacts and movements, during periods when movement restrictions are not applied. The relative contribution of persistence *versus* reintroduction to recurrence is unknown [45] and although their wildlife data were relatively weak, no association was detected between badger presence and recurrence at the 6 month follow-up herd test. To increase the detection of exposed/infected cattle within herd, there have been suggestions to increase the between-test interval and the duration of herd restriction.

Olea-Popelka et al. [47] attempted to estimate the levels of badger exposure for cattle and to test the hypothesis that increased badger exposure does not increase the risk of bovine TB in selected Irish herds. They used data from the Four Areas Trial badger cull in Kilkenny (1996–1999). The specific location of cattle within each farm, and the length of time that cattle spent in each farm field during the grazing season, and in the barnyard during winter, was used to build an exposure coefficient to quantify the amount of badger exposure that cattle encountered either on pasture or in the barn. The study design was a matched case-control study in which the control herds were selected using incidence density sampling. During the 4-year study period, 543 badgers were removed and of those 96 badgers were bovine TB positive and 96 herd breakdowns occurred. There was a significant association between case herds and having a higher badger sett exposure coefficient during 1996–1998, but no significant association between case herds and having a higher exposure coefficient based on the number of badgers, or the number of bovine TB-positive badgers, during September 1997–December 1999 was found. It would be valuable to take the same approach to quantifying within-herd cattle contacts in housing and at pasture.

Porphyre et al. [48] investigated risk factors for bovine TB on New Zealand cattle farms and their relationship with possum control strategies. Study design was a retrospective cohort based on data obtained from the TB testing

surveillance programme. The model showed that, despite intensification of possum control strategies over time, proximity to forest parks (a principal possum habitat in this area) remained a significant predictor of the number of confirmed cases of TB detected *per* farm *per* year. Their analyses showed a significant, threefold increase in bovine TB risk in dairy cattle relative to beef, conditional on the size of the local possum habitat. Other factors identified included the cattle population size and the presence of previous infection.

Ramírez-Villaescusa et al. [49] examined herd- and animal-level risks associated with bovine TB tuberculin test positivity in cattle in 148 herds in RBCT areas of SW England. Data on cattle on these farms were sourced from the bovine TB VetNet database from 1996 to 2004 and from the British Cattle Movement Scheme database. Results showed that cattle were more likely to react to the bovine TB tuberculin test when they had been present at a previous bovine TB herd test(s) where other cattle had reacted. This positively correlated with age and number of tests. Cattle on restocked farms were less likely to react to the tuberculin test compared with cattle on continuously stocked farms. These results highlight the likely importance of exposure to infected cattle present at a previous test as a source of infection to cattle that subsequently became reactors. This suggests that there was a lower risk of exposure to bovine TB to cattle in newly formed herds.

Further analysis [50] examined herd and individual animal risks associated with tuberculin test positivity. Farms restocked for <12 months after-FMD had a significantly reduced risk compared to continuously stocked farms. The feeding of mineral licks and vitamin supplements was associated with reduced risk. Storing manure and slurry indoors or in a closed container, spreading manure all year, possession of dairy cattle, increased herd size, and purchase of cattle from markets as well as farm location were associated with increased risk. The authors concluded that whole herd removal might have reduced the infectious load on these premises, but this did not continue once cattle were reintroduced. The method of slurry storage or spread might has allowed *M. bovis* to persist in the environment in some cases. The increased risk associated with cattle purchase and continuous stocking *versus* restocking supports a role for undetected infection in cattle as a risk to other cattle.

6. Farm-Scale Studies

Most of the studies outlined above operate over quite large areas, larger than the individual farm scale, where model predictions would be even more useful. However, a recent study, using GB RBCT data from one year into treatment to one year after treatment, presents an analysis of spatial farm level, herd-based risk factors associated with the probability of a confirmed bovine TB breakdown [51]. Within reactive and survey-only areas, the risk of a confirmed bovine TB breakdown was associated with two factors; increasing numbers of active badger setts and having cattle herds within 1.5 km. For proactive areas, the strongest predictor of bovine TB risk was the number of *M. bovis*-positive badgers culled initially within 1.5 km, suggesting that a risk remained for those herds, which was not removed by badger culling. They provide further evidence that the local infection in cattle and badgers is linked. Within the RBCT data they found that dairy herds were more at risk than beef herds and tended to rely on one particular breed of cattle, whereas beef farms tended to use a mixture of breeds and crossbreeds. Whilst acknowledging the complex interaction of risk factors, they indicated that a breed effect might operate [52].

Further, recent, mixed modelling and event history analysis were used to investigate individual risk factors in RBCT data analysis, again at the individual farm level. Farm characteristics, in particular herd and farm size, number of land parcels and being contiguous to other breakdowns were significant and consistent risks. They also identified increased risks for those herds subjected to reactive badger culling and those with increased herd size and increased and fragmented farms [53]. In areas with previously undisturbed badger populations, risks were reduced for herds within the proactive zones, but the authors point out that they did not evaluate the effect at the cull edges or within 2 km of the cull. Risk was actually greater in reactive and survey-only areas by 23% and 18%, respectively, indicating that localized reactive culling was associated with a higher risk than not culling, and this was felt at the local farm level. Whether this risk was sustained over time since last cull remains to be reported. Farm and herd size, number of land parcels and contiguous neighbours were the most consistent risk factors, and no consistent risk due to badger- or habitat-related variables was identified at the farm level.

7. Summary

In summary, the risk factors that have been most consistently identified in relation to bovine TB, particularly in recent UK and Ireland studies include historic incidence, farm area, cattle movement, occurrence of TB on contiguous premises and/or the level of bovine TB in surrounding areas (infection pressure or force of infection), and herd size (Table 1) [54]. Other factors identified in some studies include indicators of badger density/activity, use of multiple premises, housing type, herd type, farmland habitat, fertiliser usage, mineral deficiencies, and use of silage clamps (Table 2). Herd-keeper behaviour is also likely to change during an outbreak due to increased risk perception, leading to improved biosecurity measures and risk aversion [55].

In general, the most consistently identified risk factors are biologically plausible and consistent with known transmission routes involving cattle-cattle and badger-cattle spread. It is important to note that epidemiological studies differ in the variables analysed, the exact measures used (e.g., in relation to association with badgers), and study size and power. Not all risk factors would be expected to be identified equally across different studies. Risk factors will vary across regions due to factors such as differing farm structures, farm management practices, local TB control, and the relative importance of specific risk factors within individual areas. After extensive and iterative risk factor studies on RBCT data the Independent Scientific Group (ISG) concluded that important risk factors differed between regions and hence

Table 1: The most consistently identified herd-level risk factors for bovine TB.

Herd-level risk factors most consistently identified*
Cattle movement (estimated to contribute <20% in some GB and Irish studies)
Occurrence of TB on contiguous premises and/or level of TB in surrounding areas (infection pressure)
Herd size

*It is important to note that epidemiological studies may differ in the variables examined, the exact measures used (in relation to the association with badgers etc.), and the study size and power. Therefore, not all risk factors would be expected to be identified equally across all studies.

Table 2: Other herd-level risks identified in some studies.

Other herd-level risk factors identified in some studies*
Contact with contiguous cattle
Indicators of badger density/activity
Sourcing cattle from herds with TB history
Providing cattle feed inside housing
Use of multiple premises
Housing type
Herd type
Farmland habitat
Fertiliser usage
Mineral deficiencies (selenium)
Use of silage clamps
Rotational grazing

*It is important to note that epidemiological studies may differ in the variables examined, the exact measures used (in relation to the association with badgers etc.), and the study size and power. Therefore, not all risk factors would be expected to be identified equally across all studies.

other case-control studies of bovine TB in cattle had yielded widely differing recommendations [1].

Taken together, these studies illustrate the complexity of the host/pathogen/environment interactions or "episystem" in bovine TB [56, 57] and the variation in study design and outcome. It may not be possible to reliably identify particular risk factors which could be widely adopted and predicted to lead to reduced transmission of disease to and from cattle. More insight may be achieved when risk factors are classified locally into management, wildlife, and environment factors, [1] and it should be appreciated that environmental features are rarely controllable by the herd keeper. One primary risk factor is cattle density, which increases the probability of transmission *via* aerosol between infectious and susceptible animals [35]. Regarding management factors, results suggest that cattle movements, herd contacts, use of fertilizer, housing, and feeding practices may impact on risk, although study findings identify association and not necessarily causation.

Nevertheless there is sufficient evidence that by applying the broad principles of biosecurity it should be possible to reduce the risk of cattle becoming infected by other animals, including wildlife. Account should be taken of cattle movement on and off the premises, minimising contact with other cattle and between cattle and wildlife and taking greater care with animal housing and feeding practices. In particular, studies, such as, the TB99 and CCS2005 analyses of the GB RBCT data indicate that there is no universal solution for farm management to reduce the risk of a herd breakdown. Occasionally a clear cause and effect relationship can be demonstrated by epidemiological studies, but in most cases the situation is more complex and the research tells us what factors are important concerning a specific question or a theoretical level of risk associated with a particular event, behavior, or contact. While many of the general risk factors for the introduction and spread of bovine TB have been identified, less is known about the practical measures that farmers can take to minimize their risk and the possible impact of common biosecurity practices.

8. Methodology

We searched systematically on-line resources (PubMed, Web of Science) to find appropriate peer-reviewed literature and relied on previously identified key publications. Literature was accessed until October 2011, inclusive. We purposefully selected publications that were judged most relevant for the review, with a preference for high-quality systematic reviews. Whilst publications in the last 10 years were favoured we did not exclude highly regarded older publications.

Searches were conducted using combinations of the following key words: "bovine", "tuberculosis", and "risk". In addition, open-access DEFRA R&D project web-pages were searched.

DEFRA web-pages on bovine TB, Phillips and others [58], and the Final Report of the Independent Scientific Group [1], "*Bovine TB: the Scientific Evidence*" were referenced throughout.

Acknowledgment

The authors gratefully acknowledge funding from the Department of Agriculture and Rural Development for Northern Ireland.

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 145318, 6 pages
doi:10.1155/2012/145318

Research Article

Detection of Bovine IgG Isotypes in a PPA-ELISA for Johne's Disease Diagnosis in Infected Herds

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Received 6 February 2012; Accepted 6 May 2012

Academic Editor: Michael D. Welsh

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Johne's Disease or Paratuberculosis is a chronic granulomatous enteritis disease affecting ruminants. Detection of subclinically infected animals is difficult, hampering the control of this disease. The aim of this work was to evaluate the performance of detection of IgG isotypes in a PPA-ELISA to improve the recognition of cattle naturally infected with *Map* in different stages. A total of 108 animals from Tuberculosis-free herds were grouped as follows: exposed ($n = 30$), subclinically infected ($n = 26$), clinically infected ($n = 14$), and healthy controls ($n = 38$). Receiver-operating characteristic (ROC) curves of isotypes/PPA-ELISAs were constructed and areas under the curves were compared to evaluate the performance of each test. Our study demonstrated that the conventional PPA-ELISA (detecting IgG) is the best to identify clinically infected animals with high sensitivity (92.9%) and specificity (100%). Meanwhile, IgG2/PPA-ELISA improved the number of subclinically infected cattle detected as compared with conventional IgG/PPA-ELISA (53.8 versus 23.1%). In addition, it had the maximum sensitivity (65.0%, taking into account all *Map*-infected cattle). In conclusion, the combination of IgG and IgG2/PPA-ELISAs may improve the identification of *Map*-infected cattle in different stages of disease. The usefulness of IgG2 detection in serological tests for Johne's Disease diagnosis should be further evaluated.

1. Introduction

Johne's Disease (JD) or Paratuberculosis is a chronic granulomatous enteritis disease affecting ruminants [1, 2]. It is caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) and leads to major economic losses in the dairy industry worldwide [3]. *Map* has been implicated as a possible cause of Crohn's disease, which is a chronic granulomatous ileocolitis in humans. However, its role in this pathology remains controversial [4–6].

Calves are the most susceptible category during the first months of life and become infected through ingestion of *Map*-contaminated colostrum, milk, or feces [2, 7]. Fetal transmission is also possible when dams are infected with *Map* [7, 8].

During initial infection, the immune response is predominated by a cell-mediated immune profile (Th1). Subclinically infected animals are generally low *Map* fecal shedders and have undetectable levels of *Map*-specific serum antibodies and increasing specific gamma interferon (IFN-γ) responses [9]. After a long incubation period (years), a proportion of infected animals develop to a clinical stage, which is characterized by chronic diarrhea, protein-losing enteropathy, cachexia, and eventual death. In addition, increases in bacterial shedding in feces and serum antibody titers have been described in this stage of JD, suggesting a shift of the immune response to a humoral profile (Th2) [1, 10, 11]. The humoral immune response against mycobacterial infections has been considered nonprotective [1, 2]. However, it has been demonstrated that antibodies have an active role in

Map infection *in vitro*. *Map* immune sera or purified specific antibodies enhance bacterial interaction with macrophages, improve the activation of the nuclear factor NF-kB in infected cells, and affect *Map* intracellular viability [12–14].

The control of JD has been difficult for several reasons. Fecal culture on conventional solid media is expensive, laborious and slow (requiring 6 months for assay ending), and has low sensitivity [15–17]. Detection of cellular immune response by either the skin test or IFN-γ production is useful for early diagnosis of infection, but these assays have high variability and low specificity [18, 19]. Vaccines have been demonstrated to decrease the amount of *Map* shedding, to prevent the development of the clinical stage and to reduce the impact on milk production. However, they do not prevent infection and shedding of the bacteria and interfere with Tuberculosis and JD diagnosis [20].

Although conventional ELISA (detecting IgG) has low sensitivity during the subclinical stage of the infection, it is the test most used for JD control due to its low-cost, high-throughput, standardized protocols, and correlation with *Map* fecal shedding levels [21–23]. Various antigens of *Map* have been studied, including protoplasmic antigen (PPA), lipoarabinomannan (LAM), p34 protein carboxy-terminal (P34-cx), purified protein derivative (PPDp), and heat shock proteins (Hsp), of which PPA is the one most used for diagnosis [21–23]. Production of *Map*-specific isotypes switches during the course of the disease [10, 24, 25] with Th1 responses being related to IgM and IgG2, and Th2 responses being related to IgG1 and IgA in cattle [26]. In the same way, high levels of specific IgG1 against several antigens have been detected in sera from *Map*-infected cattle at a clinical stage of the disease [13, 14, 24, 25]. In a previous study, we have shown increases in the levels of *Map*-specific IgG2 in cattle at both the subclinical and clinical stages of JD [25].

The aim of this work was to evaluate the performance of detection of IgG isotypes in a PPA-ELISA to improve the recognition of cattle naturally infected with *Map* in different stages of the disease.

2. Materials and Methods

2.1. Animals. Sera from 108 Holstein-Frisian bovines from Tuberculosis-free accredited dairy herds from the Pampas region of Argentina were used to assess the performance of IgG, IgG1, and IgG2/PPA-ELISAs.

JD diagnosis was achieved as previously described [25]. Briefly, we examined animals for clinical signs of disease and for *Map* presence in milk and fecal-isolated colonies by PCR identification of the IS*900* fragment. Milk samples were concentrated by *Map*-specific immunomagnetic beads (NEB, New England Biolabs, Ipswich, MA, USA) [27, 28]. Fecal cultures were carried out in Herrold egg yolk medium with mycobactin J (Allied Monitor Inc., Fayette, MO, USA) and pyruvate (Sigma-Aldrich Corp., St. Louis, MO, USA).

Animals were grouped as follows:

(i) exposed (E, $n = 30$): from *Map*-infected herds, without clinical signs of JD and negative to IS*900*-PCR (from feces and milk);

(ii) subclinically infected (SC, $n = 26$): from *Map*-infected herds, without clinical signs of JD and positive to IS*900*-PCR (from feces, milk, or both);

(iii) clinically infected (C, $n = 14$): from *Map*-infected herds with chronic diarrhea and positive to IS*900*-PCR (from feces, milk, or both);

(iv) healthy control (Hc, $n = 38$): from *Map*-free herds and negative to IS*900*-PCR (from feces and milk).

2.2. ELISAs. IgG, IgG1, and IgG2/PPA-ELISAs were evaluated using sera from the 108 bovines. Cross-reactive antibodies were preadsorbed with *Mycobacterium phlei* [29], which had been grown at 37°C in Middlebrook 7H9 broth (DifcoTM, BD biosciences, Franklin Lakes, NJ, and USA) containing 10% albumin-dextrose-sodium chloride and then heat-inactivated at 85°C for 30 minutes. For preadsorption, sera diluted 1:5 with PBS containing heat-inactivated *Mycobacterium phlei* (optical density (OD) at 600 nm of 1) were incubated at 37°C for 1 h with shaking, and then at 4°C for 16 h.

Flat-bottomed 96-well polystyrene plates were coated (4°C, 16 h) with 2 μg/well of PPA (Allied Monitor Inc.) in 50 μL of 0.05 M sodium carbonate buffer pH 9.6. The plates were washed three times with rinsing buffer (0.05% Tween 20 in PBS) and blocked with 10% skimmed milk in PBS. All subsequent incubations were performed at 37°C for 1 h and after each incubation, plates were washed three times with rinsing buffer. A volume of 50 μL of preadsorbed sera at a final dilution of 1:5 (for IgG2 analyses) or 1:100 (for IgG and IgG1 analyses) in 5% skimmed milk in PBS was added. The antibodies used were: HRP-conjugated goat anti-bovine IgG (KPL, Kirkegaard & Perry Laboratories Inc., Gaithsburg, MD, USA), HRP-conjugated sheep anti-bovine IgG1 (Bethyl Laboratories Inc., Montgomery, TX, USA), and mouse monoclonal anti-bovine IgG2 (Sigma-Aldrich Co.) followed by HRP-conjugated goat anti-mouse IgG (KPL). Plates were developed using ortho-phenylendiamine dihydrochloride (OPD, Sigma-Aldrich Co.) in citrate buffer (Sigma-Aldrich Co.) and read in an OpsysMR spectrophotometer (Dynex Technologies, Chantilly, VA, USA). Results are expressed as mean OD values at 490 nm.

2.3. Data Analysis. All experiments were conducted in duplicate or triplicate and repeated at least twice.

STATISTIX 8.0 (Analytical software, Tallahassee, USA) was used to analyze data of the humoral immune response against PPA. The logarithms of the mean OD values obtained were compared between groups. The levels of IgG and IgG2 were studied with ANOVA followed by Tukey's test, whereas those of IgG1 were analyzed with the Kruskal-Wallis test followed by pairwise comparisons.

CurvMedCalc Software version 12 (Mariakerke, Belgium) was used to evaluate the power of the IgG, IgG1, and IgG2/PPA-ELISAs and to build the Receiver-operating characteristic (ROC) curves of infected cattle. The sensitivity of each test was estimated as % of infected cattle (subclinically infected, clinically infected, or both) testing positive at the cut-off chosen. The specificity of each test

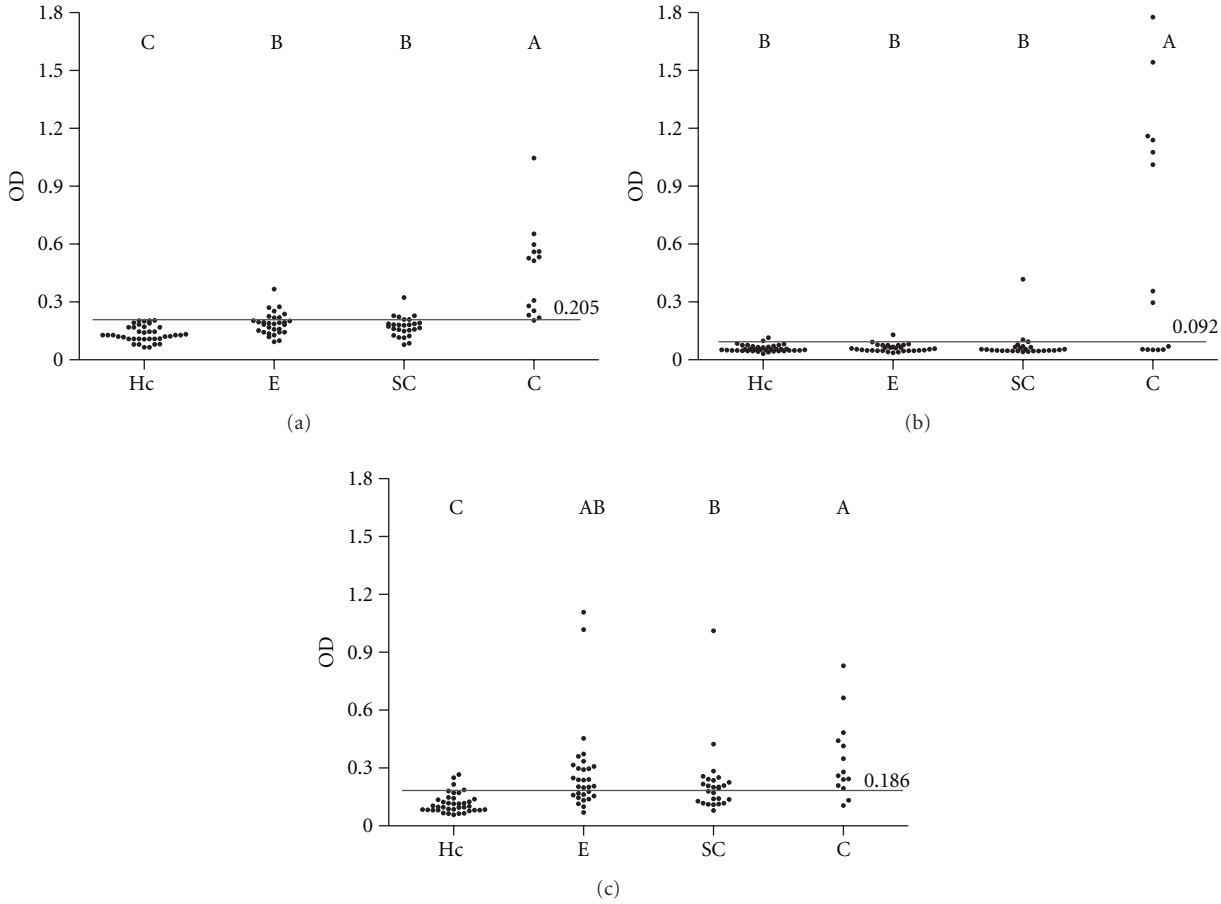

Figure 1: Results of isotypes/PPA-ELISAs. Dotplots of IgG (a), IgG1 (b), and IgG2 (c) PPA-ELISAs. Antibody responses are plotted as mean optical density (OD); lines and numbers (over lines) represent cut-off points. Letters indicate a significant difference ($P < 0.05$) between groups. Groups: healthy controls (Hc, $n = 38$), exposed (E, $n = 30$), subclinically infected (SC, $n = 26$), and clinically infected (C, $n = 14$).

was calculated as % of cattle from the healthy control group testing negative at the cut-off chosen. ROC curves for IgG, IgG1 and IgG2/PPA-ELISAs of subclinically and clinically infected cattle were constructed as plots of sensitivity versus 100 minus specificity for each possible cut-off [30, 31]. Different methods were applied to assess the cut-off points of each ELISA. The mean OD values of healthy control group ±2 standard deviation and the ROC curves of infected cattle were analyzed. Cut-off points were selected from the ROC curves in order to obtain the highest sensitivity for subclinically infected cattle with a specificity of at least 90%. The area under each ROC curve (AUC) was estimated and AUCs were compared using the method described by DeLong et al [32].

The level of significance was set at a P value < 0.05.

3. Results

Results of the isotypes/PPA-ELISAs in sera from healthy control, exposed, subclinically infected, and clinically infected cattle are shown in Figure 1 and Table 1. PPA-specific IgG was significantly increased in sera from all groups of *Map*-infected herds (exposed, subclinically infected, and clinically infected) compared with the healthy control group. In addition, the clinically infected group showed the highest values detected. When the groups were evaluated by the IgG1/PPA-ELISA, only the clinically infected group showed high levels of this isotype. Meanwhile, the levels of specific IgG2 were significantly increased in all groups from *Map*-infected herds ($P < 0.05$).

The ROC curves of the IgG, IgG1, and IgG2/PPA-ELISAs for the subclinically and clinically infected groups are shown in Figure 2. As expected, the AUCs were higher for the clinically infected group than for the subclinically infected one (Table 2).

The IgG/PPA-ELISA showed the highest specificity (100%) and sensitivity for clinically infected cattle (92.9%, Table 3). However, this test detected as positive only 6/26 of the subclinically infected animals and 8/30 of the exposed animals (Table 1).

The IgG1/PPA-ELISA demonstrated low performance and low sensitivity (27.5% of *Map*-infected cattle (subclinically and clinically infected), Figure 2, Tables 2 and 3).

The IgG2/PPA-ELISA showed 92.1% of specificity and the best performance for the subclinically infected group (AUC = 0.812) as compared with the IgG/PPA-ELISA (AUC

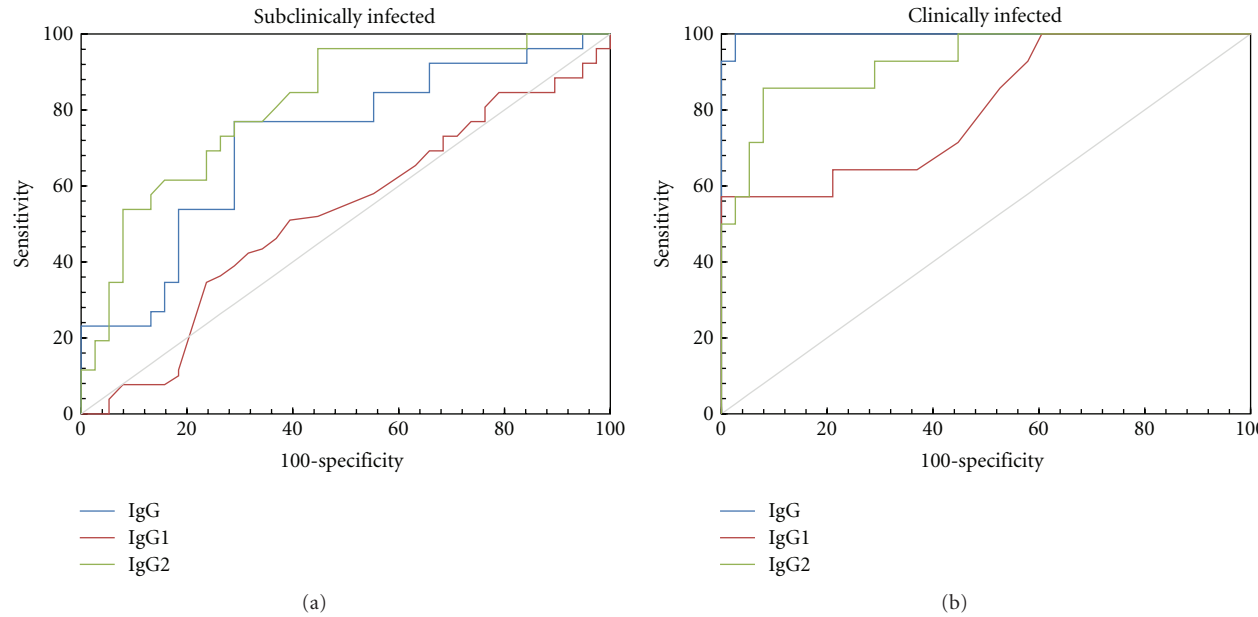

Figure 2: Performances of isotypes/PPA-ELISAs for subclinically infected (a) and clinically infected cattle (b). Receiver-operating characteristic (ROC) curves for IgG, IgG1, and IgG2/PPA-ELISAs.

Table 1: Percentages of positivity of isotypes/PPA-ELISAs.

Groups	IgG/PPA-ELISA	IgG1/PPA-ELISA	IgG2/PPA-ELISA
Hc	0.0% (0/38)	5.3% (2/38)	7.9% (3/38)
E	26.7% (8/30)	3.3% (1/30)	63.3% (19/30)
SC	23.1% (6/26)	11.5% (3/26)	53.8% (14/26)
C	92.9% (13/14)	57.1% (8/14)	85.7% (12/14)

Numbers of positive animals are shown between brackets. Groups: healthy controls (Hc, $n = 38$), exposed (E, $n = 30$), subclinically infected (SC, $n = 26$), and clinically infected (C, $n = 14$).

= 0.719) and IgG1/PPA-ELISA (AUC = 0.526), detecting 53.8% of the subclinically infected animals and 63.3% of the exposed animals (Figure 2 and Tables 1–3). In addition, the IgG2/PPA-ELISA had the maximum sensitivity (65.0%, taking into account all *Map*-infected cattle) and was able to detect 26/40 of *Map*-infected cattle. In contrast, only 19/40 were identified by the IgG/PPA-ELISA.

4. Discussion

The response of isotypes in *Map*-infected cattle has been previously studied [10, 13, 14, 24, 25]. We have described *Map*-specific isotypes detecting high levels of IgG2 in sera from *Map*-infected cattle at both the subclinical and clinical stages of the disease [13, 25]. Taking into account that PPA is the *Map* antigen most widely used [13, 22, 33], in the present work, we developed isotypes/PPA-ELISAs to evaluate their application in diagnosis of JD in cattle.

It has been described that *Map*-infected animals in the clinical stage are high shedders of bacteria in feces, and thus have the greatest potential to transmit *Map* to other animals of the herd [7, 34]. Meanwhile, subclinically infected cattle usually shed lower levels of *Map* and they are the largest part of the *Map*-infected herds, so detection of these animals is considered of great importance for JD control [35].

In this work, we detected an increase in the level of PPA-specific IgG in sera from clinically infected animals. Similar responses against other *Map* antigens have been previously reported [10, 13, 25]. We also detected increases in the levels of specific IgG in the subclinically infected group, in contrast to our previous study using *Map*-whole bacteria as antigen [25]. The IgG/PPA-ELISA demonstrated a perfect specificity (100%); this is in accordance with published studies that have described specificities from 94 to 100% [21, 36].

Although specific IgG1 against *Map*-antigens has been described as characteristic of clinically infected animals [13, 14, 24, 25], in the present study the detection of PPA-specific IgG1 did not improve the diagnosis in this stage of disease.

Interestingly, the IgG2/PPA-ELISA allowed detecting the majority of subclinically and clinically infected animals, confirming our preliminary studies [13, 25].

Although sera were preadsorbed, three animals of the healthy control group showed OD values higher than the cut-off of the IgG2/PPA-ELISA (Figure 1 and Table 1). This could be related to the lower specificity (92.1%).

Our study demonstrates that the IgG/PPA-ELISA is the best to identify clinically infected animals, with high sensitivity and specificity, in accordance with the accepted statement that conventional ELISAs mostly identify this category of infected cattle [21, 23].

On the other hand, our IgG2/PPA-ELISA improved the number of subclinically infected cattle detected as compared with conventional IgG/PPA-ELISA (53.8 versus 23.1%), maintaining high levels of specificity. Nevertheless, this sensitivity is slightly lower than that reported by Paolicchi [33] using an IgG/PPA-ELISA, although this could be related to the number of animals included (26 versus 8 animals).

Table 2: Performances of isotypes/PPA-ELISAs for subclinically infected and clinically infected cattle.

Subclinically infected				Clinically infected			
PPA ELISA	AUCs	Comparison of AUCs		PPA ELISA	AUCs	Comparison of AUCs	
IgG	0.719	IgG ~ IgG1	$P = 0.0858$	IgG	0.998	IgG ~ IgG1	$P = 0.0057$
IgG1	0.526	IgG ~ IgG2	$P = 0.2402$	IgG1	0.805	IgG ~ IgG2	$P = 0.0721$
IgG2	0.812	IgG1 ~ IgG2	$P = 0.0048$	IgG2	0.927	IgG1 ~ IgG2	$P = 0.0913$

Estimated area under the curve (AUC) of each test and pairwise statistical analysis.

Table 3: Specificity and sensitivity of isotypes/PPA-ELISAs.

PPA ELISA	Specificity	Sensitivity		
		Subclinically	Clinically	Total
IgG	100.0%	23.1%	92.9%	47.5%
IgG1	94.7%	11.5%	57.1%	27.5%
IgG2	92.1%	53.8%	85.7%	65.0%

The specificity of isotypes/PPA-ELISAs was calculated as % of cattle from the healthy control group testing negative. The sensitivity of each test was estimated as % of infected cattle (subclinically infected, clinically infected, or both) testing positive.

The sensitivity of fecal culture has been reported to be too low to define absence of *Map* infection for animals residing in known infected herds [21]. In fact, *Map*-infected cattle in the early stage may shed bacteria under detectable levels using current methods, including culture and PCR [35]. In the same way, Nielsen [37] has recently highlighted the importance of the study of *Map*-infected shedder and nonshedder animals to evaluate an immune-based diagnostic test. Thus, in the present work, we incorporated a group of exposed animals from *Map*-infected herds, excluding them from the specificity and sensitivity analysis. In this group, the use of IgG2/PPA-ELISA allowed detection of more positive animals than the other isotypes evaluated (63.3 versus 26.7 or 3.3%). Using a IgG/PPA-ELISA test, Huda et al. detected 11% of exposed animals as positive [31].

New antigens have been proposed to increase the sensitivity of JD diagnosis by IgG/ELISA [34, 36, 38]. Thus, it could be interesting to evaluate those antigens in an IgG2/ELISA.

In conclusion, our results show that IgG2/PPA-ELISA improves detection of subclinically *Map*-infected cattle or herds with animals in all stages of JD and in combination with IgG/PPA-ELISA may improve differentiation of clinical stages of disease. More studies should be conducted to better approach the utility of the IgG2/PPA-ELISA, in which, naturally and experimentally infected cattle should be included and the infection status should be supported by histopathological examination and culture of tissues. In addition, the usefulness of IgG2 detection in serological tests for Johne's Disease diagnosis should be further evaluated.

Acknowledgments

The authors would like to thank Claudia Morsella for her valuable technical assistance, and VMD Soledad Barandiaran and Maria Laura Fortuny for their helpful collaboration in field sampling. This work was supported by Grants from the Agencia Nacional de Promoción Científica y Técnica (BID PICT 2010-2672) and from Universidad de Buenos Aires (UBA-SeCyT 20020100100912).

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 563293, 7 pages
doi:10.1155/2012/563293

Research Article

Evaluation of Serodiagnostic Assays for *Mycobacterium bovis* Infection in Elk, White-Tailed Deer, and Reindeer in the United States

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Received 6 February 2012; Accepted 11 April 2012

Academic Editor: Mitchell Palmer

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In 2011, the United States Department of Agriculture conducted a project in which elk (*Cervus elaphus* spp.), white-tailed deer (WTD) (*Odocoileus virginianus*), and reindeer (*Rangifer tarandus*) were evaluated by the single cervical tuberculin test (SCT), comparative cervical tuberculin test (CCT), and serologic tests. The rapid antibody detection tests evaluated were the CervidTB Stat-Pak (Stat-Pak), and the Dual Path Platform VetTB (DPP). Blood was collected from presumably uninfected animals prior to tuberculin injection for the SCT. A total of 1,783 animals were enrolled in the project. Of these, 1,752 (98.3%) were classified as presumably uninfected, based on originating from a captive cervid herd with no history of exposure to TB. Stat-Pak specificity estimates were 92.4% in reindeer, 96.7% in WTD, and 98.3% in elk and were not significantly different from SCT specificity estimates. Using the DPP in series on Stat-Pak antibody-positive samples improved specificity in the three species. Thirty one animals were classified as confirmed infected, based on necropsy and laboratory results, and 27/31 were antibody positive on Stat-Pak for an estimated sensitivity of 87.1%. The study findings indicate that rapid serologic tests used in series are comparable to the SCT and CCT and may have a greater ability to detect TB-infected cervids.

1. Introduction

Mycobacterium bovis has been detected sporadically in captive cervids in the United States and is enzootic in free-ranging white-tailed deer (WTD) (*Odocoileus virginianus*) in a small geographic area of Michigan [1, 2]. A multistate outbreak of *M. bovis* involving 37 captive cervid herds occurred in the United States during 1990–1999 [3]. The cervid species involved in this outbreak included elk (*Cervus elaphus* spp.), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and sika deer (*Cervus nippon*). *M. bovis* was detected again, beginning in 2009, in elk, red deer, and fallow deer in 4 herds located in Nebraska and Indiana [4, 5].

Testing captive cervids for *M. bovis* in the United States is conducted as part of an official disease eradication program administered by the United States Department of Agriculture (USDA). The single cervical tuberculin skin test (SCT) was first evaluated for use in elk in the United States in 1991 [6]. Captive cervids were not routinely tested for bovine tuberculosis (TB) until these species were officially brought into the federal tuberculosis program in 1994 [7]. Currently, testing for TB in cervids is performed using tuberculin skin tests. The SCT is the initial test and consists of the intradermal administration of bovine-purified protein derivative (PPD) tuberculin in the midcervical region [8]. Animals with any detectable responses are tested by the comparative cervical tuberculin skin test (CCT), in which

balanced bovine and avian PPD tuberculins are injected, the pre- and postinjection skin thickness is measured, and the measurements are plotted on a graph [8]. Areas of the graph classify the animal as negative, suspect, or reactor. In cervids, the CCT must be administered within 10 days or after 90 days of the SCT tuberculin injection.

Limited and at times conflicting information is available regarding the performance of TB skin testing in captive cervid species. A comprehensive evaluation of cervid TB testing estimated the individual animal specificity of the SCT and CCT used in series to be 87.1 and 90.4% for deer and elk, respectively [9]. Individual animal sensitivity of the SCT and CCT in series could not be estimated; however, 14 elk from 12 infected herds detected between 1991 and 1996 were CCT test negative and subsequently confirmed to be infected with *M. bovis*. More recently, 25/28 confirmed infected Nebraska elk had a false negative result on the SCT [4]. The estimated specificity of the CCT in reindeer (*Rangifer tarandus*) is significantly different than nonreindeer cervids, with reindeer approximately four times more likely to test false positive on the CCT [10]. Eleven experimentally infected reindeer tested positive by the CCT at three and eight months after infection [11]; however, estimates of sensitivity of CCT in naturally infected reindeer are not possible as TB is extremely rare in this species.

In addition to concerns regarding TB skin test performance, animal handling challenges resulting in animal morbidity and mortality are not uncommon. With TB skin testing, captive cervids may be required to be captured and restrained for testing up to four different times depending on test results. Serologic testing offers the advantage over skin testing of reduced animal handling, with a reduction in the associated morbidity and mortality. An additional advantage is eliminating the subjectivity of interpreting the skin response at the tuberculin injection site. For these reasons, there is potential for improved surveillance in captive cervids using a serologic test with adequate performance.

In 2009, the CervidTB Stat-Pak lateral flow test (Stat-Pak, Chembio Diagnostic Systems, Inc., Medford, New York) was licensed for use in elk and red deer. The Stat-Pak is a rapid point-of-care test that utilizes single-directional lateral-flow serological antibody detection technology and a cocktail of recombinant *M. bovis* antigens [12]. In addition to the Stat-Pak, Chembio has two additional antibody detection assays for TB, the multiantigen print immunoassay (MAPIA) and the recently developed dual path platform VetTB (DPP) assay [13]. The MAPIA is performed in the laboratory as a confirmatory testing method for samples with antibody-positive results on the Stat-Pak. The DPP is a new-generation point-of-care test format that offers improved specificity compared to the Stat-Pak assay with similar sensitivity in red deer, elk, and fallow deer [4, 14].

The primary objective of this study was to evaluate the Stat-Pak as a primary test for use in the diagnosis of TB in captive and free-ranging elk, WTD, and reindeer. In addition, the DPP was evaluated as a followup test for Stat-Pak antibody-positive samples, and the two serologic test results were compared to TB skin test results.

2. Methods

Blood samples from two sources were tested as part of the project: blood collected prospectively during triennial herd TB accreditation testing and banked serum. The species evaluated included elk, WTD, and reindeer. Banked serum was from the TB serum bank located at the National Veterinary Services Laboratories (NVSL) in Ames, Iowa.

Banked serum samples were derived from animals sampled during 2008–2010 and included samples from elk, WTD, and reindeer with corresponding SCT results. Most banked samples were from captive cervid herds with no history of exposure to TB with the exception of one infected elk herd that was detected and depopulated in 2009. Some samples reported by Waters et al. [4] were retested for the sensitivity portion of this project. Prospectively enrolled animals were sampled during calendar year 2011. Captive cervid producers were recruited to enroll animals in the project through producer organizations. In addition, free-ranging cervids being TB tested (such as for wildlife restoration projects) were enrolled. The animals sampled prospectively came from 51 separate premises, including 16 for elk, 3 for reindeer, and 32 for WTD herds.

Accredited or regulatory veterinarians collected blood samples. Blood was collected into 10 mL serum collection tubes or serum separator tubes on the day of tuberculin injection for the SCT. An exception to these methods occurred with the infected elk herd; SCT testing was conducted three months prior to the animals being euthanized and necropsied, and blood collection was done immediately after postmortem at necropsy. Blood samples were centrifuged, separated, and shipped to NVSL. Alternatively, unseparated blood was cooled and shipped overnight to NVSL, where the serum was separated. Copies of the completed SCT results were submitted with blood or serum [8].

Prospectively sampled animals that were antibody positive on the Stat-Pak were further evaluated by the CCT and were later necropsied. However, for some cases, the owners chose not to have their animals CCT tested and euthanized. Animals that responded to the SCT test were administered a CCT according to current USDA TB program requirements, regardless of Stat-Pak results (Table 1) [15]. The USDA TB program requires that CCT-positive animals are euthanized and necropsied, with tissue samples taken for histopathology and bacteriologic culture; CCT-negative animals are released from quarantine.

Tissue samples from necropsied animals were divided, and the two sections were submitted separately in 10% buffered formalin and a saturated sodium borate solution for histopathology and bacteriologic culture, respectively. Head and thoracic cavity lymph nodes and any lesioned tissues were submitted from necropsied animals. No followup was conducted on animals testing negative on the Stat-Pak, although some reindeer sampled in 2011 were subsequently slaughtered and inspected. Histopathology and bacteriologic culture were conducted at the NVSL.

Tissues submitted in 10% buffered formalin were initially evaluated for lesions and the presence of acid-fast bacilli by histopathology. Tissues were diagnosed as compatible

Table 1: Decision algorithm for supplemental testing.

	Single cervical tuberculin (SCT) test result	
Stat-Pak result	SCT negative	SCT positive
Negative	No additional testing	Follow USDA TB program regulations [15]
Positive	MAPIA and DPP, purchase with indemnity	MAPIA and DPP, follow USDA TB program regulations

with mycobacteriosis when granulomas were identified that contained acid-fast bacilli. Tissues for culture were prepared as previously described [16]. All submitted tissues were cultured using at least one liquid media system (BACTEC 460 or MGIT 960) and 2 tubes of a modified Middlebrook 7H11 supplemented with calf serum, hemolyzed blood, pyruvate, and malachite green.

The Stat-Pak was performed at NVSL per the manufacturer's instructions. Serologic and TB testing in the infected elk herd have been previously described [4]; however, in this study, serum samples from this herd were tested by the Stat-Pak at NVSL. All sera with an antibody-positive result on the Stat-Pak were further tested via the MAPIA and DPP at Chembio, Inc., in Medford, New York as described previously [12–14].

Animals that are necropsied as a result of official TB testing or antibody-positive Stat-Pak results and have microscopic lesions that are compatible for mycobacteriosis, and/or *M. bovis* is isolated from tissue, were classified as TB infected. Animals were classified as presumably uninfected if they were from herds with no history of exposure to TB and resided in states declared free of TB in cattle. Animals that did not meet the case definition for confirmed TB infected but were from herds with infected animals were classified as exposed and not included in the analysis.

The sample size estimates were derived from Greiner and Gardner [17]. Sensitivity was defined as test positives/test positives + test negatives using the sample of animals that met the case definition for confirmed TB infected. Specificity was defined as test negatives/test negatives + test positives for animals meeting the case definition for presumably uninfected. Ninety five % confidence intervals for proportions were calculated using the *F* statistic function in Excel (function = F.INV.RT, Microsoft, 2010). Tests of statistical significance were performed using the chi-square, Fischer exact and mid-p exact, and were calculated online using Open Epi (http://www.openepi.com/OE2.3/Menu/OpenEpiMenu.htm). Results were considered statistically significant for P values < 0.05.

3. Results

A total of 1,783 animals were tested by the Stat-Pak, including 873 elk, 725 WTD, and 185 reindeer. Of these, 1,752 (98.2%) were classified as presumably uninfected, and 31 animals (elk from one affected herd) were classified as confirmed TB infected.

3.1. Presumably Uninfected Animals. Of the samples from presumably uninfected animals, 52/1752 (3.0%) were antibody positive by the Stat-Pak assay, including 14/842 elk (1.7%), 24/725 WTD (3.3%), and 14/185 reindeer (7.6%) (Table 2). Twenty six of 853 (3.1%) samples from banked serum and 26/899 (2.9%) samples collected prospectively were antibody positive by the Stat-Pak assay. Of the 26 antibody-positive animals sampled prospectively, 9 were elk, 13 were WTD, and 4 were reindeer. Of these, a necropsy was completed on 7 elk, 4 WTD, and 1 reindeer with no evidence of *M. bovis* infection. For the remaining 14 animals, the owners declined to have the animals euthanized for necropsy. One reindeer and one elk had a histopathologic diagnosis of microgranuloma. Two elk had a histopathologic diagnosis of lymphoid hyperplasia. *Mycobacterium intracellulare* and an unidentified atypical mycobacterium were isolated from the elk with lymphoid hyperplasia; *M. intracellulare* was isolated from an elk with no significant findings on histology.

The estimated specificity of the Stat-Pak by species is 98.3% in elk, 96.7% in WTD, and 92.4% in reindeer in this study (Table 2). Specificity was significantly different for the three species ($P = 0.00008$, uncorrected chi-square). SCT data from USDA TB Program official testing during FY 2009 provided an estimated specificity of 98.5% for elk, 97.4% for WTD, and 82.7% for reindeer, from a sample of over 6,700 presumably uninfected animals (Table 3). For elk and WTD, the estimated specificity of the Stat-Pak compared to the FY 2009 SCT was not significantly different ($P > 0.05$, mid-p exact). In reindeer, significantly fewer animals were antibody positive by the Stat-Pak compared to responders on the SCT ($P = 0.024$, mid-p exact). The USDA TB Program testing requires that SCT responders be administered the CCT test. During routine USDA TB Program testing during FY 2009-2010, 5/123 (4.1%) elk were positive on the CCT, and 118 were negative. For WTD, 6/119 (5.0%) animals were positive on the CCT, and 113 animals were classified negative. In reindeer, 19/19 CCT tested animals were negative. The specificity of the SCT and CCT tests in series could not be calculated.

USDA has reported the estimated specificity of the SCT and CCT used in series to be 87.1% (95% CI 84.5–89.4) in deer and 90.4% (95% CI 87.4–92.9) in elk and red deer [9]. This was significantly lower than the estimated specificity of the Stat-Pak and DPP used in series for WTD and elk ($P < 0.0001$, mid-p exact). A second study by Norden et al. [18] reported higher specificity estimates for the SCT and CCT in series of 98.3% (95% CI 96.0–99.5) in deer and 99.5% (95% CI 95.4–98.6) in elk and red deer, in which animals classified as "suspects" (weak positives) on the CCT were reclassified as negative. Comparing the 1997 SCT and CCT in series results to the current study, the specificity estimates for the Stat-Pak and DPP in series were not significantly different for WTD but were different for elk ($P = 0.000036$, mid-p exact), with the Stat-Pak/DPP combination having a higher specificity than the SCT/CCT. Test performance for reindeer was also reported in Norden et al. [18], where 29/29 reindeer were negative when tested in series by the SCT and CCT, compared to 182/185 Stat-Pak/DPP test negatives in the present study.

Table 2: Stat-Pak results in presumably uninfected animals.

Species	Sex	Stat-Pak Negative	Stat-Pak Antibody Positive	Total Tested	Percent Positive	95% Confidence Interval
Elk	Female	428	7	435	1.6%	0.6–3.3%
	Male	338	3	341	0.9%	0.2–2.5%
	Not known	62	4	66	6.1%	—
Elk Total		828	14	842	1.7%	0.9–2.8%
Reindeer	Female	138	12	150	8.0%	4.22–13.6%
	Male	33	2	35	5.7%	0.7–19.2%
Reindeer Total		171	14	185	7.6%	4.2–12.4%
WTD*	Female	391	17	408	4.2%	2.4–6.6%
	Male	233	6	239	2.5%	0.9–5.4%
	Not known	77	1	78	1.3%	—
WTD Total		701	24	725	3.3%	2.1–4.9%
Grand Total		1700	52	1752	3.0%	2.2–3.9%

* WTD = white-tailed deer.

Table 3: Single cervical tuberculin test response fraction in elk, WTD and reindeer in federal fiscal year (FY) 2009.

Species	Total Tested	Responders	Percent Responders	95% Confidence Interval
Elk	3,223	47	1.5%	1.1–1.9
WTD*	3,421	89	2.6%	2.1–3.2
Reindeer	81	14	17.3%	9.8–27.30
Total	6,725	150	2.2%	1.9–2.6

* WTD = white-tailed deer.

Table 4: Comparison of single cervical tuberculin skin test (SCT) and the Stat-Pak, presumably uninfected animals, by species in 2011.

SCT Result*	Stat-Pak Negative	Stat-Pak Positive	Total
Elk			
Negative	330	7	337
Positive	5	2	7
Elk, total	335	9	344
White-tailed deer (WTD)			
Negative	468	13	481
Positive	13	0	13
WTD, total	481	13	494
Reindeer			
Negative	29	2	31
Positive	7	3	10
Reindeer, total	36	5	41
Total	852	27	879

* Includes only animals sampled prospectively during 2011.

Nearly 62% of the animals evaluated were female (993/1608). The highest percentage of female animals occurred in reindeer (150/185 female) (Table 2). Gender was not reported for 144 animals. When stratified by species, test performance was not significantly different between females and males in this study ($P = 0.575$, elk; $P = 0.969$, reindeer; $P = 0.282$, WTD; mid-p exact).

Table 5: Results of multi-antigen print immunoassay (MAPIA) and dual-path platform VetTB (DPP) for Stat-Pak antibody positive samples.

DPP Results	MAPIA Results*						
	Elk		Reindeer		WTD		Total
	Neg	Pos	Neg	Pos	Neg	Pos	
Negative	13	1	10	1	13	6	44
Positive	0	0	0	3	0	5	8
Total	13	1	10	4	13	11	52

* Neg = negative, Pos = positive, WTD = white-tailed deer.

Samples collected during 2011 were used to compare the performance of the Stat-Pak with the SCT (Table 4). A total of 879 animals had results for both the SCT and the Stat-Pak. For elk, 7/344 (2.0%) animals were SCT positive, while 9/344 (2.6%) were antibody positive on the Stat-Pak. For WTD, 13/494 (2.6%) and 13/494 (2.6%) animals were SCT positive and Stat-Pak antibody positive, respectively. SCT results were available for 41 reindeer. Of these, 9/41 (22.0%) and 4/41 (9.8%) were SCT positive and Stat-Pak antibody positive, respectively. Three reindeer and two elk were SCT positive and antibody positive on the Stat-Pak. In all other cases, the Stat-Pak antibody-positive animals were different individuals than the SCT-positive animals. CCT tests were administered to 11 Stat-Pak antibody-positive animals. Three elk, four reindeer, and two WTD were CCT negative. Two WTD were classified as positive on the CCT.

Fifty two samples antibody positive on Stat-Pak were tested by the DPP and MAPIA (Table 5). Both assays use an expanded panel of antigens in addition to the antigens included in the Stat-Pak, to improve specificity. The largest improvement in specificity was observed in elk, where 13/14 samples (each antibody positive on Stat-Pak) were negative by the MAPIA, and only one sample was positive (Table 5). In WTD, 13/24 (54.2%) antibody positive on the Stat-Pak were negative on the MAPIA. In reindeer, 11/14 (78.6%)

antibody positive on the Stat-Pak were negative on the MAPIA. The DPP had similar results to the MAPIA. The increase in specificity estimates from the Stat-Pak only to the combination of the Stat-Pak and DPP tests used in series were 98.3% to 100.0% (842/842, 95% confidence interval (CI) 99.6–100.0) in elk; 96.7% to 99.3% (720/725, 95% CI 98.4–99.8) in WTD; 92.4% to 98.4% (182/185, 95% CI 95.3–99.7) in reindeer.

3.2. Serology Test Performance in a TB-Affected Elk Herd. A total of 34 animals from a known TB-affected Nebraska elk herd were evaluated for the project. Thirty one animals met the case definition for confirmed TB infected and had a serum sample available for inclusion in the project. Of these, 27 (87.1%) were antibody-positive by the Stat-Pak, and followup testing resulted in 26 and 27 antibody positive samples by the MAPIA and DPP, respectively. Twenty of the 31 confirmed infected animals were SCT tested with negative results three months prior to being necropsied. Among these 20 SCT negative animals, 18 (90.0%) were Stat-Pak antibody positive, and 2 were negative. Three animals from the infected elk herd did not meet the definition for confirmed TB infection; these animals were considered exposed to TB and were excluded from the analysis. Lesioned tissues from these 3 animals were compatible for mycobacteriosis by histopathology, but *M. bovis* was not isolated. For these 3 animals, two serum samples were negative, and one was antibody positive on the Stat-Pak and the DPP.

4. Discussion

The estimated specificity of the Stat-Pak from this study was not significantly different from the SCT in elk and WTD and was higher in reindeer compared to the SCT. This finding is similar to a study in which significant differences in SCT test performance between cervid species were found [9]. A substantial improvement in specificity was observed when the Stat-Pak was used in series with either the DPP or MAPIA. The increase in specificity varied by species with the largest improvement occurring in elk and the lowest in WTD. In this study, the specificity estimates for the Stat-Pak and DPP used in series for WTD and elk are significantly higher than estimates of the SCT and CCT used in series in deer and elk [9]. However, the 1996 estimate for deer may not accurately reflect test performance in WTD, as multiple species were included as deer and could have included reindeer, roe, fallow, and other species, in addition to WTD.

In this study, the specificity estimates for the Stat-Pak and DPP were similar to other reports. Waters et al. [4] reported an estimated specificity of the DPP of 98%, while in New Zealand red deer, a species closely related to elk, the estimated specificity was 98.3% [14]. A previous study of the Stat-Pak found an estimated specificity in WTD of 98.9% [12].

While this project was primarily focused on specificity because of the limited number of samples from infected cervids, sensitivity is also an important consideration. In this study, the sensitivity of the Stat-Pak was 87.1%; however, this estimate was determined from a relatively small number of animals in a single herd. Buddle et al. [14] reported a sensitivity for the Stat-Pak and DPP of 75% each (not used in series) in naturally infected red deer. Waters et al. [4] noted that the SCT performed poorly in detecting TB, as only 3/28 confirmed infected elk were SCT positive. The failure of the skin test to detect infection in elk has been documented previously; the CCT was negative in 14 elk that were subsequently confirmed to be infected with *M. bovis* [9]. In the present study, three months elapsed between the time the SCT was administered and blood samples were collected for serologic testing. It is possible that the SCT-negative results occurred in animals that were not yet infected; however, at necropsy many animals in the herd had advanced clinical disease [4], making this explanation less likely. However, Norden et al. [9] described that several of 14 confirmed infected elk that were initially CCT negative were CCT positive when tested again at a later date.

Samples from naturally infected WTD and reindeer were not available for this project; however, published studies have reported Stat-Pak sensitivity ranging between 55% and 67% in naturally infected, free-ranging WTD, and 79% in experimentally infected animals [12, 19]. The estimated sensitivity of the CCT administered as the only skin test in experimentally infected WTD was 97% [20]. An evaluation of 11 experimentally infected reindeer found that *M. bovis*-specific antibody was found as early as four weeks after infection [11]. No naturally infected reindeer have been detected in the US.

Animals that were positive on the Stat-Pak were different than the animals that responded to the SCT, with only a few exceptions. One possible explanation for this finding may be the basic differences between these tests, with one measuring humoral, and the other, cell-mediated immunity. Additionally, the serologic tests use specific antigens, while PPD tuberculins are a complex mixture of many cross-reactive antigens. This finding has important implications for choosing a supplemental test. The CCT may not be an appropriate supplemental test to use in series with the Stat-Pak, because some of the sensitivity gained using the serologic test could be lost using the skin test. Alternatively, measuring different aspects of immunity may be more robust at detecting infection than two tests that measure the same immune response components. For example, the humoral immune response may not be subject to anergy, which is known to cause false-negative skin test results.

The primary study limitation was that animals testing negative on the Stat-Pak were not necropsied to confirm that they were not infected with *M. bovis*, with the exception of slaughter inspection conducted in some reindeer sampled prospectively. Given the recent findings of *M. bovis* in several US captive cervid herds, there is a small risk that infected animals classified as presumably uninfected were included in the study. Additionally, the samples included in this study were a convenience sample of animals being TB tested for routine purposes and subject to producers volunteering to participate. For example, a number of producers that originally agreed to participate in the study declined after learning of the requirement to euthanize animals testing positive on the Stat-Pak and a maximum

indemnity payment of $3,000. Only 12/26 prospectively sampled antibody-positive animals were necropsied because producers declined to have their animals euthanized and necropsied. To avoid these limitations in the future, studies would need adequate funding and industry cooperation to more thoroughly evaluate the disease status for both test-negative and -positive animals.

In addition to the potentially higher diagnostic accuracy of antibody detection methods evaluated in the present study, point-of-care serologic testing by simple and rapid assays offers the advantage over skin testing of reduced animal handling and the associated morbidity and mortality and eliminates the subjectivity associated with evaluating the tuberculin injection site for a response. It is anticipated that growing acceptance of this approach by producers will result in gradually expanded TB testing to further improve overall TB surveillance in captive and free-ranging cervid populations.

Acknowledgments

The authors greatly appreciate the work of (1) Drs. Roger Krogwold (USDA, APHIS, VS, Ohio), Robert F. Gerlach (Alaska Division of Environmental Health), James Meronek (USDA, APHIS, VS, Wisconsin), David Barber (USDA, APHIS, VS, Illinois), Sherry Shaw (USDA, APHIS, VS, Minnesota), Dan Love (Colorado Department of Agriculture), Don Evans (USDA, APHIS, VS, Kansas), Kate Bowers (USDA, APHIS, VS, Pennsylvania), Jodi Lovejoy (Indiana Board of Animal Health), Aaron Hecht (Kentucky Department of Fish and Wildlife), John M. Horn (USDA, APHIS, VS, Texas), George Merrill, and Patrina Ashley (New York State Department of Agriculture and Markets), for postmortem examinations, sample collection, preparations, shipping, and record keeping; (2) numerous others from VS APHIS USDA and state boards of animal health and agriculture departments for recruiting producers to participate in the project and assisting with postmortem examinations and sample collection; (3) Drs. Konstantin Lyashchenko (Chembio Diagnostic Systems, Inc., Medford, New York), W. Ray Waters (National Animal Disease Center, Agricultural Research Service, USDA, Iowa), and Dr. Alecia L. Naugle (Ruminant Health Programs, Veterinary Services, USDA, Maryland) for reviewing this paper; (4) the elk, WTD, and reindeer industry representatives and producers across the country for supporting the project through volunteering their time and animals to participate in the project. 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 US Department of Agriculture.

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 860362, 9 pages
doi:10.1155/2012/860362

Research Article

Search for *Mycobacterium avium* Subspecies *paratuberculosis* Antigens for the Diagnosis of Paratuberculosis

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Received 3 February 2012; Accepted 1 May 2012

Academic Editor: Jesse M. Hostetter

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The aim of this study was to evaluate a wide panel of antigens of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) to select candidates for the diagnosis of paratuberculosis (PTB). A total of 54 recombinant proteins were spotted onto nitrocellulose membranes and exposed to sera from animals with PTB ($n = 25$), healthy animals ($n = 10$), and animals experimentally infected with *M. bovis* ($n = 8$). This initial screening allowed us to select seven antigens: MAP 2513, MAP 1693, MAP 2020, MAP 0038, MAP 1272, MAP 0209c, and MAP 0210c, which reacted with sera from animals with PTB and showed little cross-reactivity with sera from healthy animals and animals experimentally infected with *M. bovis*. The second step was to evaluate the antigen cocktail of these seven antigens by ELISA. For this evaluation, we used sera from animals with PTB ($n = 25$), healthy animals ($n = 26$), and animals experimentally infected with *M. bovis* ($n = 17$). Using ELISA, the cocktail of the seven selected MAP antigens reacted with sera from 18 of the 25 animals with PTB and did not exhibit cross-reactivity with healthy animals and only low reactivity with animals with bovine tuberculosis. The combined application of these antigens could form part of a test which may help in the diagnosis of PTB.

1. Introduction

PTB is a prevalent and economically important disease that affects cattle and thus impacts on the cattle industry. It is caused by MAP.

Clinical PTB is characterized by chronic granulomatous enteritis with clinical signs of diarrhea, weight loss, decreased milk production, and mortality. However, most infected cattle show no clinical signs during the prolonged incubation stage of infection [1].

On the other hand, a number of theories have proposed that the principal infective agent of Crohn's disease, a chronic enteropathy in humans, is MAP [2–4]. The economic impact and possible link to Crohn's disease highlights the importance of the development of control programs at the herd level. To this end, it is necessary to improve the diagnostic methods of PTB.

Cattle are most often infected as young calves, before 6 month of age, but some studies have shown that infection may also occur in adult cattle. Fecal shedding of MAP generally starts after 2 years, and clinical symptoms appear after an incubation period of 2–10 years. In addition, the elimination of the agent through the stool is very variable [5].

Cell-mediated immune response wanes with progression of the disease and when this occurs, a humoral immune response becomes measurable. It has been shown that cattle are more likely to have a combined antibody and cellular response rather than a switch from cellular to antibody response [6–8]. Among tests to detect serum antibody to

MAP, ELISAs are the most widely used. Several commercial ELISA kits for bovine PTB are currently available, and multiple studies have compared their accuracy [9, 10]. Comparative studies of ELISAs with different antigens have shown discrepancies in the ability of these tests to identify all infected animals [11]. Some authors have suggested that this may be due to the lack of representation of the entire range of immunodominant antigens for MAP in a given ELISA test [12]. Then, one of the crucial components of this test is the antigen used for the preparation of the ELISA test. The antigen most widely used for the serological diagnosis of PTB is PPA-3, which is the *M. avium* strain 18 protoplasmic antigen. Currently, antigen-based tests to detect MAP with a mixture of proteins include whole-cell sonicated extract, parcel purified antigen, and protoplasmic antigens. These antigens show variability in potency and cross-reaction. This diagnostic method has drawbacks due to the cross-reaction with animals sensitized with this mycobacterium or other pathogens antigenically related to MAP [11]. Since the sequencing and analysis of the entire MAP genome was obtained [13], several specific proteins have been detected in the genome of MAP and the immunoreactivity of these proteins investigated [14]. Bannantine et al. [15] developed a spot protein array for initial antigen screening. Available diagnostic MAP antigens are reviewed in Mikkelsen et al. [16]. However, individual antigens are able to identify only a subset of PTB-infected animals. Then, a mix of antigens could be a good candidate for serological diagnosis.

In the present work, MAP antigens were obtained after fractionating proteins from the whole cell or membrane or secreted fraction, resolved with two-dimensional gels, printed in line onto nitrocellulose membranes, and analyzed with sera from animals with PTB.

MAP proteins recognized specifically by sera from PTB-infected animals were used to develop a cocktail of selected antigens to be evaluated by ELISA.

2. Material and Methods

2.1. Animals. Sera from a total of 43 animals from different groups (a) cattle coming from PTB-free herds and with MAP negative fecal cultures (negative control animals) ($n = 10$), (b) cattle naturally infected, with MAP-positive fecal cultures or ELISA-positive tests ($n = 25$), and (c) animals experimentally infected with *M. bovis* with lesions ($n = 8$) were used to evaluate the 54 recombinant proteins of MAP.

The final screening using a cocktail of seven selected antigens was carried out with sera from 68 cattle: 25 cows with MAP positive fecal culture or ELISA-positive tests, 26 cows from negative herds with no suspected cases of PTB and negative tests (fecal cultures, serology and INF-g), and 17 from animals experimentally infected with *M. bovis* and with lesion at the time of necropsy.

2.2. Selection of Antigens. With the aim of identifing and characterizing immunoreactive proteins for their possible use in diagnosis, we extracted proteins of MAP by treatment of cells with sodium dodecyl sulfate at 50°C [17]. The proteins were resolved by one- or two-dimensional SDS-PAGE, carried out in duplicate. Both duplicates were stained with colloidal Coomassie blue and the other was transferred to a nitrocellulose membrane (Amersham Hybond-ECL, GE Healthcare Life Sciences, Buckinghamshire, UK) to perform a Western blot with a PTB-positive bovine serum sample. Six proteins were immunoreactive with sera from animals with PTB and as nonimmunoreactive with sera from animals infected with *M. avium*. These proteins were cut from the gel and identified by MALDI TOF. This study identified these proteins as encoded by MAP 1962 (Glutamine synthetase), MAP 4143 (Elongation factor tu), MAP 0187c (SodA), MAP 3194, MAP 3205, and MAP 3206, as potential diagnostic antigens.

The other 48 proteins here evaluated were from CIDC-Lelystad, Central Institute for Animal Disease Control Department Bacteriology, and TSEs, Lelystad, the Netherlands.

2.3. Recombinant Antigens. The 54 proteins were produced as recombinant. Briefly, they were cloned and purified essentially as described previously [18], where all proteins were PCR-cloned using 5′- and 3′-primers amplifying DNA fragments encoding the mature protein except for secreted proteins, which were cloned without the signal peptide. The DNA fragments were cloned into expression vectors. The antigens were cloned into the pET33b (Novagen Inc., Madison, WI, USA) and pRSET (Invitrogen Corp., Carlsbad, CA, USA) vectors, and expression was induced by the production of T7 RNA polymerase in BL21 (DE3) *E. coli*. These cells also produce T7 lysozyme to reduce the basal expression of the target genes. The secreted antigens described in Willemsem et al. [18] (MAP 2609, MAP 2942c and MAP 0210c) were cloned in pQE80 (Qiagen, Germantown, MD, USA). Antigens were purified using their histidine-tagged N-terminal region and Nickel-affinity columns, (1 mL HisTrap HP columns) (GE Healthcare Life Sciences, UK). Before that, solubilization of the recombinant antigen was established by a buffer containing 6 M Guanidine, 20 mM Tris (pH 8.0), 0.5 M NaCl, or 50 mM Imidazol, 0.25% CHAPS, 1 mM DTT, 0.5 mM PMSF, and 1% iso-propanol to solve proteins from inclusion bodies (Guanidine), reduce nonspecific binding (Imidazol), and decrease the amount of LPS (iso-propanol) or 8 M Urea. After affinity-purification, the antigens were dialyzed (10 kDa cut-off, except for Map 4000c, for which a 1 kDa cut-off was used) against a buffer containing 0 to 6 M Urea, 10 mM Tris (pH 8.0), 25% glycerol, 1 mM DTT, 0.5 mM PMSF, 1% iso-propanol or 0 to 7 M Urea, 10 mM Tris (pH 8.0), 10% glycerol, 1 mM DTT, 0.5 mM PMSF, and 1% iso-propanol. The urea concentration was the minimal concentration needed to keep the proteins solubilized and determined empirically.

The recombinant proteins were analyzed by Coomassie-stained SDS-PAGE to test purity.

2.4. Coomassie Blue Staining. A gel containing six of the recombinant purified proteins included in the cocktail is shown in Figure 1. The protein encoded by MAP 0209c

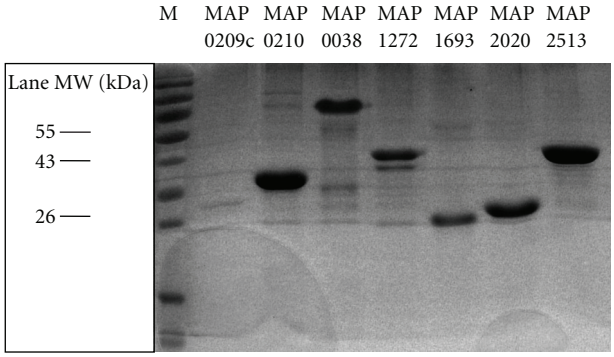


Figure 1: Recombinant proteins analyzed by coomassie-stained SDS-PAGE to test purity.

(of 56.5 kDa) is shown on the first lane but is not visible because it is very close to the front of the run. The proteins were run in a 15% polyacrylamide gel and then incubated in a Coomassie blue solution 0.05% (Coomassie brilliant blue R250 0.05%, methanol 50%, acid acetic 10%) for 1.5 hours with agitation. The Coomassie blue solution was then removed and bleached with bleaching solution (methanol 50%, acetic acid 10%).

2.5. Evaluation of Humoral Response by a Line Print Immunoassay. The panel of 54 proteins was evaluated as follows: 20 μL of each antigen was applied to a nitrocellulose membrane at a concentration of 100 μg/mL, using a semi-automatic aerosolizer (Camag Scientific Inc., Wilmington, Delaware). The membranes were blocked with 50 mL of blocking solution (5% milk TBS) for 1 h. The membranes were then placed in a "miniblotter" (Isogen BioSolutions, the Netherlands). This allowed parallel analysis of 45 sera. We evaluated serum dilutions of 1 : 100. After 1 h incubation, the serum samples were aspirated and washed three times for 10 min with TBS 1x. The membrane was then incubated for 1 h with protein G conjugated to peroxidase (1 : 1500). The membrane was washed three times for 10 min with TBS 1X and revealed by chemiluminescence (Pierce ECL western blotting, Thermo Scientific, USA).

Line print immunoassay generated macroarrays of recombinant proteins which were evaluated with sera from animals with PTB. In order to identify potentially cross-reactive proteins, the macroarrays were also used to probe against sera from healthy animals and from animals experimentally infected with *M. bovis*. The macroarrays were subjected to densitometry analysis to provide quantification for reactivity at each spot, reported as spot intensities.

2.6. Bioinformatic and Statistical Análisis. The panel of 54 recombinant proteins included in the present study was characterized *in silico*: molecular weight, location prediction, and homology with other mycobacterial proteins (Table 1). PSORTb analysis was used to predict protein localization based on a number of factors including transmembrane helices, signal peptide, motif search, and similarity to proteins with known subcellular location. (http://www.psort.org/psortb/). According to PSORTb prediction, the set of recombinant proteins contains 28 cytoplasmic proteins, 7 cytoplasmic and membrane proteins, 6 extracellular proteins, and 13 proteins with uncertain localization (Table 1).

BLAST similarity searches were performed locally on coding sequences by comparison with the GenBank nonredundant protein database (Table 1).

2.7. Measurement of Spot Intensities. Quantitative spot intensities were obtained by performing a densitometric scan of the membrane. For the analysis of the results, the intensities of the points were measured with the ImageQuant TL Array Version 7.0 Software, (GE Healthcare, Pittsburgh, PA).

This quantification software processes spot intensities on the array and determines the mean intensities of pixels within a spot as well as those of the background pixels around the spot. These local-background intensities are subtracted from the raw signals to obtain the local-background-corrected levels. The measured diameter that was selected for scans of different arrays was consistent for all the arrays in this study. Adjusted intensities were obtained following normalization of each spot. Five proteins (MAP 0038, MAP 1272, MAP 1693, MAP 0210c, and MAP 0209c) showed a stronger mean intensity with sera from MAP-infected animals than with sera from non-MAP-infected animals and were thus selected for a cocktail of antigens (Table 2).

MAP 2020 and MAP 2513 were also selected because although they did not show a strong intensity with sera from MAP-infected animals, they recognized nine and six animals with PTB, respectively (Table 3), and because these proteins had not been previously evaluated or reported in the literature.

2.8. ELISA. The antigens used for the ELISA test were PPA-3 (Allied Monitor, Inc. USA) and a cocktail with the seven antigens selected (MAP 2513, MAP 1693, MAP 2020, MAP 0038, MAP 1272, MAP 0209c, and MAP 0210c). The cocktail was prepared with 30 μg of each antigen for 1 mL of the mixture. The microtiter plates were coated at 4°C overnight with 100 μL of 40 μg/mL PPA-3 or 20 μg/mL of the cocktail in carbonate buffer (pH: 9.6). Then, the plates were saturated with 100 μL of PBS/0.5% w/v gelatin for 1 h at 37°C, then washed five times with PBS/0.1% Tween20 (PBS/T), and incubated for 1 h at 37°C with 100 μL of 100-fold dilution of sera in PBS/T containing 0.5% (w/v) gelatin. The plates were then washed five times with PBS/T and incubated for 30 min at 37°C with 100 μL of 1500-fold dilution of peroxidase-conjugated protein in PBS/T containing 0.5% (w/v) gelatin. Plates were washed five times with PBS/T, and 50 μL of peroxidase substrate was added. Optical density (OD) was measured at 405 nm.

3. Results

54 proteins were evaluated by Line print immunoassay with sera from healthy animals, animals with PTB, and animals

Table 1: Characterization *in silico* of a panel of 54 recombinant proteins, included in the present study.

Antigen (ORF no.)	Predicted localization	Theoretical MW	Homology with other mycobacteria
MAP 0011	Cytoplasmic	19,196 kDa	Iron-regulated peptidyl-prolyl cis-trans isomerase A in *Mycobacterium tuberculosis* H37Rv
MAP 0034	Cytoplasmic-Membrane	44 kDa	P44 protein in *Mycobacterium avium* subsp. *avium*
MAP 0047c	Extracellular	41,1 kDa	Lpp-LpqN family conserved in Mycobacteriaceae
MAP 0038	**Unknown**	**48,7 kDa**	**Hypothetical protein Mb0027 in *Mycobacterium bovis* AF2122/97**
MAP 0187	Extracellular	23 kDa	Superoxide dismutase in *Mycobacterium bovis*
MAP 0209c	**Extracellular**	**56,5 kDa**	**Protein potentially involved in peptidoglycan biosynthesis in *Mycobacterium avium* subsp. *avium***
MAP 0210c	**Cytoplasmic-Membrane**	**30,7 kDa**	**Secreted antigen P36/P34 precursor in *Mycobacterium bovis*, maxima ident 60%**
MAP 0211	Cytoplasmic	46 kDa	UDP-galactopyranose mutase glf in *Mycobacterium tuberculosis*
MAP 0297	Cytoplasmic	55,23 kDa	Hypothetical protein Mb1161 *Mycobacterium bovis* AF2122/97
MAP 0334	Unknown	34,386 kDa	Oxidoreductase in *Mycobacterium bovis* AF2122/97
MAP 0900	Cytoplasmic-Membrane	29,6–34 kDa	Antigen 34 kDa in *Mycobacterium tuberculosis* CDC1551
MAP 0946c	Cytoplasmic-Membrane	33,5 kDa	Sigma factor in *Mycobacterium avium* 104
MAP 1012c	Cytoplasmic	37,374 kDa	Hypothetical protein TMAG_01006 in *Mycobacterium tuberculosis*
MAP 1050c	Cytoplasmic	33 kDa	Peptidyl-prolyl cis-trans isomerase, cyclophilin-type conserved in Mycobacterium
MAP 1272	**Extracellular**	**33,4 kDa**	**NLP/P60 family protein in *Mycobacterium tuberculosis* CDC1551**
MAP 1293	Unknown	49,24 kDa	Histidinol dehydrogenase his D in *Mycobacterium tuberculosis*
MAP 1308	Unknown (This protein may have multiple localization sites.)	46 kDa	Prolipoprotein diacylglyceryl transferase lgt in *Mycobacterium tuberculosis*
MAP 1564c	Unknown	23,01 kDa	Short chain dehydrogenase in *Mycobacterium bovis*
MAP 1589c	Cytoplasmic	21,60 KDa	Alkyl hydroperoxide reductase subunit C in *Mycobacterium tuberculosis*
MAP 1653	Unknown	16,7–20 kDa	Thiol peroxidase tpx in *Mycobacterium tuberculosis* T17
MAP 1693c	**Unknown**	**18,30 kDa**	**Peptidyl-prolyl cis-trans isomerase domain-containing protein conserved in Mycobacterium**
MAP 1754c	Cytoplasmic-Membrane	30,84 kDa	Hypothetical protein Rv2005c in *Mycobacterium tuberculosis* H37Rv
MAP 1889c	Cytoplasmic	28 kDa	Wag31 protein in *Mycobacterium avium* 104
MAP 1962	Cytoplasmic	53,68 kDa	Glutamine synthetase glnA1 in *Mycobacterium tuberculosis*
MAP 2020	**Unknown**	**26,90 kDa**	**Cutinase in *Mycobacterium bovis* AF2122/97**
MAP 2167	Extracellular	17 kDa	Low molecular weight protein antigen cfp2 in *Mycobacterium tuberculosis*
MAP 2182	Cytoplasmic-Membrane	16 kDa	Deazaflavin-dependent nitroreductase family protein in Mycobacterium
MAP 2513	**Cytoplasmic**	**36,50 kDa**	**Alkanal monooxygenase alpha chain in *Mycobacterium avium* 104**
MAP 2609	Cytoplasmic-Membrane	11,40 kDa	Low molecular weight T-cell antigen TB8.4 in *Mycobacterium tuberculosis*
MAP 2676c	Cytoplasmic	13,89 kDa	Hypothetical protein MAV_1246 in *Mycobacterium avium* 104
MAP 2685	Unknown	21,20 kDa	Hypothetical protein BCG_1169c in *Mycobacterium bovis* BCG str. Pasteur 1173P2
MAP 2878c	Cytoplasmic	25,43 kDa	Dihydrodipicolinate reductase in *Mycobacterium bovis* BCG str. Pasteur 1173P2
MAP 2942c	Extracellular	18,30 kDa	Soluble secreted antigen MPT53 in *Mycobacterium tuberculosis*
MAP 2956	Cytoplasmic	30,02 kDa	30S ribosomal protein S2 in *Mycobacterium tuberculosis* H37Rv
MAP 3175c	Cytoplasmic	41,38 kDa	Peptide chain release factor 2 in *Mycobacterium tuberculosis* H37Rv
MAP 3194	Cytoplasmic	30,46 kDa	Pyruvate carboxyl transferase in *Mycobacterium avium*

Table 1: Continued.

Antigen (ORF no.)	Predicted localization	Theoretical MW	Homology with other mycobacteria
MAP 3205	Cytoplasmic	27 kDa	nuoE NADH dehydrogenase subunit E in *Mycobacterium avium* 104
MAP 3206	Cytoplasmic	48-49 kDa	nuoF NADH-Quinone oxidoreductase subunit F in *Mycobacterium tuberculosis*
Mb 3341c	Unknown	10,63 kDa	Equivalent to Rv3312A, len: 103 aa, from *Mycobacterium tuberculosis* strain H37Rv
MAP 3402	Cytoplasmic	33,28 kDa	Thiosulfate sulfurtransferase in *Mycobacterium bovis* AF2122/97
MAP 3457	Cytoplasmic	47,61 kDa	O-acetylhomoserine sulfhydrylase metC in *Mycobacterium tuberculosis* T92
MAP 3491	Cytoplasmic	28,16 kDa	Hydrolase in *Mycobacterium tuberculosis* SUMu003
MAP 3527	Unknown	35,70 kDa	Serine protease PepA in Mycobacterium tuberculosis H37Rv
MAP 3627	Cytoplasmic	37,37 kDa	O-methyltransferase in *Mycobacterium tuberculosis* H37Rv
MAP 3651	Cytoplasmic	44 kDa	Acyl-CoA dehydrogenase fadE3 in *Mycobacterium bovis* BCG
MAP 3692	Cytoplasmic	47 kDa	fabG 3-ketoacyl-ACP reductase in *Mycobacterium tuberculosis* H37Rv
MAP 3840	Cytoplasmic	67 kDa	Heat shock protein 70, molecular chaperone DnaK in Mycobacterium
MAP 3841	Unknown	23,57 kDa	Heat shock protein GrpE in *Mycobacterium bovis* AF2122/97
MAP 3857	Cytoplasmic	18,73 kDa	Orotate phosphoribosyltransferase phosphoribosyltransferase in *Mycobacterium tuberculosis* CDC1551
MAP 3936	Cytoplasmic	57 kDa	Heat shock protein 65, GroEL in Mycobacterium sp
MAP 4000c	Unknown	12 kDa	Esat-6 like protein esxF in Mycobacterium and hypothetical protein Mb3935c in *Mycobacterium bovis*
MAP 4143	Cytoplasmic	43,77 kDa	Iron-regulated elongation factor tu in *Mycobacterium tuberculosis*
MAP 4147	Cytoplasmic	42,09 kDa	Ferredoxin reductase in *Mycobacterium tuberculosis* H37Rv
MAP 4227c	Cytoplasmic	30,14 kDa	Hypothetical protein Rv3463 in *Mycobacterium tuberculosis* H37Rv

experimentally infected with *M. bovis*. The stronger mean intensity values are listed in Table 2. Serum samples with density values higher than the mean obtained with the control (PBS) were considered positive and the numbers of animals reactive with each protein are shown in Table 3.

The antigens selected were those that showed stronger intensity with sera from MAP-infected animals than with sera from non-MAP-infected animals. These antigens were MAP 0038, MAP 0210c, MAP 1272, MAP 1693c, and MAP 0209, shown in bold in Tables 2 and 3. In addition, we selected two antigens, MAP 2020 and MAP 2513, because they recognized nine and six animals with PTB (Table 3), respectively, and because they had not been previously evaluated or reported in the literature.

These results contributed to the development of an antigen mixture with seven antigens (MAP 0038, MAP 0210c, MAP 1272, MAP 1693c, MAP 2020, MAP 2513, and MAP 0209c).

The ORF of MAP 1272 codes for a protein that possesses an NLP/P60 domain of unknown function that is found in several lipoproteins. MAP 0210 codes for the P36/Erp protein of *M. bovis*, which has been studied in our laboratory [19]. PSORTB analysis software predicted that MAP 2513 is localized in the cytoplasm, MAP 0210c in the cytoplasm-membrane, and that MAP 1272 and MAP 0209c are extracellular. The remaining proteins were of unknown localization. All seven of these proteins have not been previously evaluated or reported in the literature, except for MAP 0210c, which has been studied by Willemsen et al. [18].

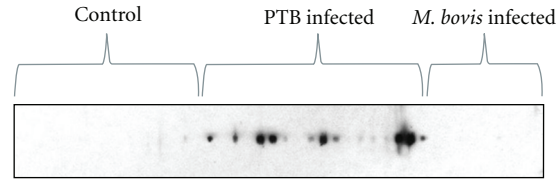


Figure 2: Evaluation of the cocktail by a Line print immunoassay. Result of the cocktail with seven antigens (MAP 0038, MAP 0210c, MAP 1272, MAP 1693, MAP 2020, MAP 0209c and MAP 2513) printed in a membrane of nitrocellulose and evaluated with the sera from healthy animals and animals infected with MAP and *M. bovis*.

The cocktail of seven antigens was printed in the nitrocellulose membrane and evaluated by Line print immunoassay with sera from animals with PTB and healthy controls and animals experimentally infected with *M. bovis* (Figure 2). By line print immunoanalysis, 14 out of 25 of the sera from animals with PTB developed antibody response to the cocktail. This cocktail was not recognized with the sera from animals experimentally infected with *M. bovis* here evaluated and two serum samples from healthy animals gave a very weak signal (Figure 2).

Table 2: Spot intensities for protein macroarrays.

Antigen assayed	Mean spot intensity of proteins exposed to sera		
	control ($n = 10$)	PTB infected ($n = 25$)	TB infected ($n = 8$)
MAP 0011	112	135	0
MAP 0034	240	247	0
MAP 0038	**141**	**860**	**0**
MAP 0047	344	309	0
MAP 0187c	184	0	0
MAP 0209c	**367**	**499**	**0**
MAP 0210c	**0**	**398**	**0**
MAP 0211	*0*	*572*	*0*
MAP 0297	117	225	130
MAP 0334	108	167	1
MAP 0900	*0*	*749*	*0*
MAP 0946	*218*	*900*	*295*
MAP 1012	280	0	0
MAP 1050	268	275	0
MAP 1272	**223**	**733**	**0**
MAP 1293	585	481	396
MAP 1308	163	894	0
MAP 1564	259	344	0
MAP 1589c	323	682	466
MAP 1653	398	844	461
MAP 1693	**226**	**967**	**209**
MAP 1754	0	142	27
MAP 1889c	280	180	494
MAP1962	341	270	354
MAP 2020	**0**	**150**	**228**
MAP 2167	315	0	0
MAP 2182c	223	117	192
MAP 2513	**41**	**164**	**291**
MAP 2609	137	308	0
MAP 2676	201	138	0
MAP 2685	317	323	0
MAP 2878	307	323	0
MAP 2942	136	251	0
MAP 2956	345	119	424
MAP 3175	343	130	0
MAP 3194	0	100	0
MAP 3205	0	0	0
MAP 3206	100	200	0
Mb 3341	159	171	178
MAP 3402	205	178	250
MAP 3457	447	365	325
MAP3491	286	454	177
MAP 3527	528	414	635
MAP 3627	207	265	0
MAP 3651	1000	906	958
MAP 3692c	305	212	0
MAP 3840	0	198	712
MAP 3841	707	678	457
MAP 3857	373	267	265
MAP 3936	381	544	691
MAP 4000c	134	242	0
MAP4143	413	397	439
MAP 4147	1000	556	0
MAP 4227	138	101	0
PPDA	208	105	256
PPDB	346	531	1468
PPA-3	131	609	108
Cocktail	**140**	**1460**	**0**

Intensities were obtained using ImageQuant TL Array Version 7.0 Software, (GE Healthcare, Pittsburgh, PA). Antigens selected to evaluate as cocktail are shown in bold. PPA-3: Paratuberculosis protoplasmatic antigen (Allied Monitor, Inc. USA); PPDA and PPDB: avian- and bovine-derivative protein purified, respectively (Prionic Switzerland). Cocktail: mix of the 7 antigens shown in bold.

In addition, ELISAs with PPA-3 and with the cocktail with seven antigens were evaluated with sera from animals with PTB ($n = 25$), healthy animals ($n = 26$), and animals experimentally infected with *M. bovis* ($n = 17$).

The ELISA-PPA-3 test recognized 16 out of the 25 animals with PTB (64%) but also 12 of the 17 animals experimentally infected with *M. bovis*, while the ELISA-cocktail detected 18 of 25 animals with PTB (72%) and only 3 of the 17 animals experimentally infected with *M. bovis* (Figure 3). Both ELISAs did not have a reaction with sera from non-infected controls; however, when using sera from *M. bovis*-infected, ELISA-PPA-3 test recognized (12/17) 70,5% of these animals and ELISA-cocktail (3/17) 17,6% of these animals.

This new ELISA for bovine PTB showed 72% of sensitivity and had higher specificity than the ELISA with PPA-3 as antigen, using animals experimentally infected with *M. bovis*.

4. Discussion

The early and specific diagnosis of PTB is still a challenge. It has generally been believed that the early immune response to infection with MAP consisted primarily of a cellular immune response characterized by interferon gamma production, and this response would later be replaced by antibody production. However, some studies have shown that antibodies appear much earlier and therefore ELISA could be used as an early diagnostic tool [6–8]. Then it is necessary to characterize MAP antigens to increase the sensitivity and specificity of the ELISA test for PTB diagnosis. The MAP genome sequencing represented a significant

Table 3: Reactivity of bovine sera to the panel of selected antigens from MAP.

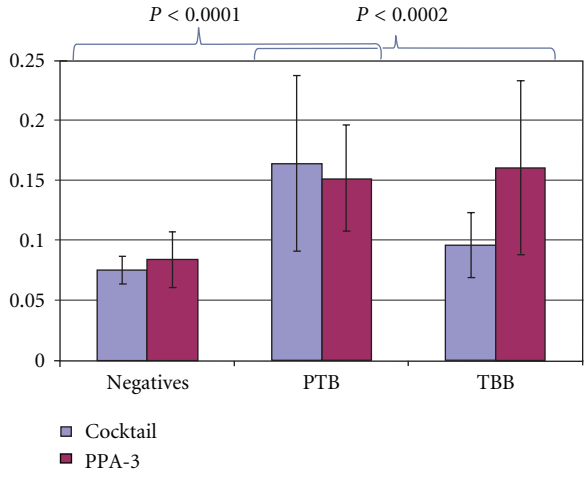
Antigen assayed	No. of sera with positive recognition		
	control ($n = 10$)	PTB infected ($n = 25$)	TB infected ($n = 8$)
MAP 0011	0	5	4
MAP 0034	5	5	0
MAP 0038	**3**	**8**	**0**
MAP 0047	3	4	0
MAP 0187c	1	1	0
MAP 0209c	**4**	**9**	**0**
MAP 0210c	**0**	**8**	**0**
MAP 0211	0	6	0
MAP 0297	2	4	1
MAP 0334	2	15	1
MAP 0900	0	3	0
MAP 0946	1	8	0
MAP 1012	2	0	0
MAP 1050	4	9	0
MAP 1272	**2**	**8**	**0**
MAP 1293	10	13	1
MAP 1308	2	2	0
MAP 1564	5	1	0
MAP 1589c	2	9	2
MAP 1653	10	20	8
MAP 1693	**3**	**12**	**2**
MAP 1754	0	5	4
MAP 1889c	7	7	3
MAP 1962	3	16	5
MAP 2020	**0**	**9**	**2**
MAP 2167	3	0	0
MAP 2182c	5	1	2
MAP 2513	**1**	**6**	**2**
MAP 2609	2	4	0
MAP 2676	1	4	0
MAP 2685	10	2	0
MAP 2878	10	2	0
MAP 2942	2	7	0
MAP 2956	1	1	1
MAP 3175	4	4	4
MAP 3194	0	1	0
MAP 3205	0	0	0
MAP 3206	1	5	0
Mb 3341	4	6	3
MAP 3402	2	20	1
MAP 3457	10	8	4
MAP 3491	7	7	1
MAP 3527	6	25	8
MAP 3627	3	4	0
MAP 3651	10	14	8
MAP 3692c	5	8	0
MAP 3840	0	3	3
MAP 3841	10	14	1
MAP 3857	10	4	4
MAP 3936	5	8	4
MAP 4000c	3	5	0
MAP 4143	8	16	7
MAP 4147	1	3	0
MAP 4227	1	1	1
PPDA	6	1	4
PPDB	4	16	3
PPA-3	1	18	2
Cocktail	**2**	**14**	**0**

20 uL of antigens were applied to a nitrocellulose membrane (Amersham Hybond-ECL, GE Healthcare Life Sciences, UK) at a concentration of 100 ug/mL, using a semiautomatic aerolizer (Camag Scientific Inc., Wilmington, Delaware). Membranes were evaluated by immunoblotting using sera from healthy and infected animals. Numbers of sera with antibody response are indicated. Samples with intensities values higher than the media obtained with the control (PBS) were considered positive. Antigens selected to evaluate as cocktail are shown in bold. PPA-3: Paratuberculosis protoplasmatic antigen (Allied Monitor, Inc. USA); PPDA and PPDB: avian- and bovine-derivative protein purified (Prionic Switzerland); Cocktail: Mix of the 7 antigens shown in bold.

advance and will most likely contribute with new tools for diagnosis. The evaluation of a specific panel of antigens such as that studied in the present work is the first step in the selection of candidates to be studied at different herds with PTB of our country. This is an important area, since novel antigens that could improve the diagnosis of MAP-infected cattle are needed.

The proteomic approach has been used to define specific antigens by 2D fraction of MAP proteins by several researchers [20–24]. Another approach to obtain specific antigens is to express recombinant proteins from cloned MAP-coding sequences and use them to construct a protein array [15]. Here we selected MAP proteins by 1D or 2D electrophoresis and developed macroarrays by line print of these proteins. These macroarrays were probed with sera from animals with PTB and healthy controls and animals experimentally infected with *M. bovis*.

After evaluation of 54 proteins of MAP with sera from PTB-infected, experimentally infected with *M. bovis* and healthy animals, we selected seven proteins, which were incorporated in an ELISA to develop an antigen-based diagnostic test (ELISA-cocktail). This new ELISA for bovine PTB showed 72% of sensitivity and had higher specificity than the ELISA with PPA-3 as antigen (Figure 3). This sensitivity was based on 25 animals from MAP fecal culture-positive or



Antigen assayed by ELISA	No. of sera with positive recognition		
	Control ($n = 26$)	PTB infected ($n = 25$)	*M. bovis* infected ($n = 17$)
Cocktail	0	18	3
PPA-3	0	16	12

Figure 3: Reactivity of bovine sera to the cocktail by ELISA. Comparison of the diagnostic performance of using PPA-3 (Allied monitor) and the antigen cocktail (MAP 0038, MAP 0210c, MAP 1272, MAP 1693, MAP 2020, MAP 0209c, and MAP 2513).

ELISA-positive cattle, because naturally infected cattle may represent various stages of MAP infection and serum samples only from culture-positive animals can express differentiated antigen patterns [25]. In addition, the use of only culture-positive animals to estimate the sensitivity of ELISA may increase the values of the test because most culture-positive animals are also ELISA-positive [11].

The results presented here suggest that several specific antigens can improve the detection of MAP infection. In fact, the profiles of antibody response varied considerably and then the antibody response to single antigens was not prominent, while simultaneous usage of several recombinant antigens is able to recognize the ongoing antibody response over time in the course of infection.

In summary, here we identified novel antigens of MAP by using multiple antigen print immunoassay. Based on this knowledge, we developed an antigen cocktail, which increased the correct diagnosis of MAP-infected animals in comparison with the results of ELISA-PPA-3. The study presents an antigen cocktail, which could be of diagnostic significance for further researches. However, the cocktail needs to be evaluated by larger sample sizes in order to estimate its sensitivity and specificity. In addition, the results here shown with the 54 proteins, indicated that other proteins not included were also good candidates. Then, new cocktails should be incorporated and evaluated to increase the sensitivity and specificity of the ELISA test for diagnosis of PTB.

Acknowledgment

This study was financially supported by FONARSEC and PICT of the Agencia Nacional de Promociòn Cientìfica y Tecnológica of Argentina.

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 236205, 17 pages
doi:10.1155/2012/236205

Review Article

Mycobacterium bovis: A Model Pathogen at the Interface of Livestock, Wildlife, and Humans

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Received 6 February 2012; Accepted 11 April 2012

Academic Editor: Jesse M. Hostetter

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Complex and dynamic interactions involving domestic animals, wildlife, and humans create environments favorable to the emergence of new diseases, or reemergence of diseases in new host species. Today, reservoirs of *Mycobacterium bovis*, the causative agent of tuberculosis in animals, and sometimes humans, exist in a range of countries and wild animal populations. Free-ranging populations of white-tailed deer in the US, brushtail possum in New Zealand, badger in the Republic of Ireland and the United Kingdom, and wild boar in Spain exemplify established reservoirs of *M. bovis*. Establishment of these reservoirs is the result of factors such as spillover from livestock, translocation of wildlife, supplemental feeding of wildlife, and wildlife population densities beyond normal habitat carrying capacities. As many countries attempt to eradicate *M. bovis* from livestock, efforts are impeded by spillback from wildlife reservoirs. It will not be possible to eradicate this important zoonosis from livestock unless transmission between wildlife and domestic animals is halted. Such an endeavor will require a collaborative effort between agricultural, wildlife, environmental, and political interests.

1. Introduction

Zoonotic diseases are responsible for most (60.3%) emergent diseases of humans. Moreover, the preponderance (71.8%) of emerging pathogens are of wildlife origin or have an epidemiologically important wildlife host [1]. The emergence of newly recognized diseases in wildlife is the result of complex, and sometimes unintended, interactions between wildlife, domestic animals, and humans, in terms of host ecology, pathogen, and environment [2, 3]. These interactions include factors such as translocation or introduction of wildlife to new ecosystems, encroachment of human populations on traditional wildlife habitat, artificial feeding of wildlife, and transmission of livestock diseases to wildlife [2]. Wild animals are susceptible to infection with many of the same disease agents that afflict domestic animals and transmission between domestic animals and wildlife can occur in both directions. For veterinarians, diseases in wildlife that are notifiable, eradicated, or near eradication in domestic animals are most problematic. A single case of a reportable disease in livestock can result in serious economic consequences for the producer, public, and government [4].

Transmission of *Mycobacterium bovis* from domestic animals to wildlife (spillover) and subsequent transmission from wildlife back to domestic animals (spillback) is a theme common in several regions of the world attempting eradication of *M. bovis* infection. In most cases, both spillover and spillback have been facilitated by anthropogenic factors such as encroachment on wildlife habitat, animal translocation, or supplemental feeding of wildlife. The scrutiny of wildlife reservoir hosts is essential in control or elimination of *M. bovis* from livestock. Total eradication of any disease is impossible if wildlife maintain a reservoir of infection.

Critical to control of tuberculosis is the understanding of spillover hosts and maintenance hosts. Among spillover hosts, disease does not persist without an external source of reinfection. This external source of infection is often a separate population of susceptible hosts, wild or domestic. In most cases, *M. bovis* was originally introduced by spillover from domestic cattle (maintenance host) to a susceptible wild population (maintenance or spillover host). By definition, disease in spillover hosts will disappear as disease is eliminated from the source of infection. Spillover hosts may be dead end hosts and play no role in disease transmission, but disease may persist for a limited time. In contrast, among maintenance hosts, disease persists without an external source of reinfection. Maintenance hosts may be domestic or wild. There is no sharp demarcation between spillover and maintenance hosts but rather there is a continuum of persistence and transmission efficiency between members of the host populations. For example, the ferret (*Mustela furo*) in New Zealand is a an inefficient spillover host as the disease disappears rapidly from the population due to ineffective intraspecies transmission, but where population density is high, they can act as a maintenance host [5]. Maintenance hosts are critical in disease epidemiology and control because without intervention, disease will persist indefinitely. Hence, the most efficient disease control efforts are aimed at maintenance hosts.

There is general acceptance that among wildlife species the badger (*Meles meles*) in the United Kingdom (UK) and the Republic of Ireland, the brushtail possum (*Trichosurus vulpecula*) in New Zealand, the European wild boar (*Sus scrofa*) in Iberian Peninsula, and the white-tailed deer (*Odocoileus virginianus*) in Michigan, United States (US) represent true maintenance hosts and a source of infection for other species. These maintenance host reservoirs have in common, high population density, and continuous interspecies interaction at the wildlife-domestic animal interface, both of which facilitate disease persistence [6]. Host species alone does not necessarily designate spillover host or maintenance host assignation. In one ecosystem, a particular species may act as maintenance host (i.e., white-tailed deer in Michigan and wild boar in the Iberian Peninsula) [7, 8] while in another ecosystem the same species may act as a spillover host (i.e., white-tailed deer in Minnesota, US and feral pigs in New Zealand and Australia) [6, 9–11]. These differing roles are likely the result of many factors including animal density, environment, and contrasting agricultural and cultural practices.

In the early part of the 20th century, there were large numbers of tuberculous cattle in industrialized regions of North America, Europe, and Australia. Often an association was made between the number of *M. bovis*-infected humans and the prevalence of tuberculosis in the local cattle population. Infected cattle were generally considered the source of human infection with *M. bovis*; transmission being through ingestion of unpasteurized dairy products [12, 13]. Additionally, abattoir workers and veterinarians were infected during slaughter or postmortem examination of cattle [14–16]. More recently, exposure to tuberculous elk (*Cervus canadensis*), white-tailed deer, and possums has resulted in human infection [17–20]. In developed countries, mandatory pasteurization of milk combined with tuberculin testing and slaughter of infected cattle resulted in dramatic declines in the incidence of human tuberculosis due to *M. bovis*. Notwithstanding, in 1995, it was estimated that worldwide 50 million cattle were infected with *M. bovis,* at a cost to the agricultural community of US $3-4 billion per annum [21]. In developing countries, *M. bovis* infection is still widespread, in both cattle and humans. Even in developed countries, successful eradication of disease from livestock is hampered by the presence of wildlife reservoirs of *M. bovis*. In general, countries with a wildlife reservoir of *M. bovis* have not been able to eradicate *M. bovis* infection from livestock. The following examples illustrate the complex interaction of wildlife, domestic animal, and human factors in the creation and maintenance of wildlife reservoirs of tuberculosis.

2. White-Tailed Deer in the United States—Supplemental Feeding of Wildlife

Prior to 1994, there had been isolated case reports of tuberculosis in white-tailed deer in the US [22–25]. All reports involved 1 or 2 animals and were seen in captive deer, hunter-killed deer, or cases of accidental death. At the time, it was postulated that *M. bovis* had spilled over from tuberculous livestock in the region; however, no followup surveys were conducted and no strain comparisons were made to confirm such a hypothesis. In 1975, a free-ranging white-tailed deer in northern Michigan was diagnosed with tuberculosis due to *M. bovis* [26]. The tuberculous white-tailed deer was thought to be an anomaly and no followup surveys of free-ranging deer were conducted. Meanwhile, Michigan was granted TB-free status by the US Department of Agriculture (USDA) in 1979.

In 1994, a hunter-killed white-tailed deer was identified with tuberculosis due to *M. bovis*. This deer was found only 13 km from the site where the tuberculous deer had been identified in 1975. Subsequent surveys identified a focus of *M. bovis* infection in free-ranging white-tailed deer in northeast Michigan [26]. This represented the first known reservoir of *M. bovis* in free-living wildlife in the US and the first known epizootic of tuberculosis in white-tailed deer in the world. Several factors are thought to have contributed to the establishment and persistence of *M. bovis* in Michigan white-tailed deer. It is postulated that *M. bovis* was transmitted from cattle to deer during the early to mid 1900 s when the prevalence of *M. bovis* in Michigan cattle was high [27]. Statistical models estimate that spillover from cattle to deer occurred around 1955 [28]. During this same period, Michigan's deer population was steadily increasing beyond normal habitat carrying capacity. In 1930 there were an estimated 592,000 deer in Michigan and by 1998, the number of deer had grown to over 1.7 million with focal concentrations of 19 to 23 deer per km^2. Regions of highest deer density were later found to be the center of the current tuberculosis outbreak [26, 29, 30]. Transmission and maintenance of *M. bovis* among deer in Michigan was facilitated, not only by high deer density, but also by the common practice of long-term winter-feeding of large volumes of sugar beets,

carrots, corn, apples, pumpkins, and pelleted feed to deer by the public. Supplemental feeding was intended to decrease winter mortality and prevent migration in order to preserve high deer numbers for hunting purposes [26]. High deer density, combined with prolonged crowding of deer around feeding sites provided opportunity for deer to deer contact and enhanced transmission of tuberculosis [31]. Epidemiologically, supplemental feeding has been documented as a contributing factor to *M. bovis* infection in deer [29]. Specific factors associated with increasing risk of tuberculosis were location of a feeding site near hardwood forest, number of deer fed per year, presence of other nearby feeding sites, and the quantity of grain, fruits or vegetables fed. DNA fingerprinting through restriction fragment length polymorphism (RFLP) analysis of *M. bovis* isolates from Michigan white-tailed deer showed that the majority of deer were infected with a common strain of *M. bovis* suggesting a single source of infection [32]. By 2010, over 188,000 deer had been tested by gross necropsy, bacteriologic culture, and histopathology, and of these, 687 confirmed cases of *M. bovis* infection had been identified in 12 counties in northern Michigan.

2.1. Pathology. Tuberculous white-tailed deer most commonly develop lesions in retropharyngeal lymph nodes, followed by the lung and associated lymph nodes [26, 33, 34]. Similar to other species of deer, lesions may grossly resemble abscesses due to other organisms making differential diagnosis important. Unlike red deer (*Cervus elaphus*), elk (*Cervus canadensis*), and fallow deer (*Dama dama*), draining fistulae from superficial lymph node lesions have not been reported in white-tailed deer [35–38]. In these other species, such lesions may be important in disease transmission.

Microscopically, lesions consist of foci of caseous necrosis with or without mineralization, surrounded by infiltrates of epithelioid macrophages, lymphocytes, and Langhan's type multinucleated giant cells. Lesions are often surrounded by variable amounts of fibrous connective tissue with low numbers of acid-fast bacilli (AFB) present within the caseum, macrophages, or multinucleated giant cells. Microscopically, lesions in white-tailed deer are similar to those seen in cattle, although lesions in cattle are generally surrounded by greater amounts of fibrous connective tissue.

2.2. The Role of Artificial Feeding of Wildlife. Artificial feeding is broadly defined as placing natural or artificial food into the environment that supplements food in the natural home range of a given wildlife species. Both supplemental feeding and baiting (use of food as an attractant for hunting purposes) of wildlife have been associated with increased transmission of infectious diseases such as tuberculosis and chronic wasting disease [39]. Contact can be direct through physical (nose-to-nose) contact or transmission through infectious aerosolized respiratory droplets, or indirect as occurs when two animals share the same feed or feeding site. Although increased potential for disease transmission is perhaps of greatest concern, feeding and baiting can also disrupt animal movement patterns, spatial distribution, social structure, and result in habitat degradation [40]. Stress from overcrowding around feeding sites can negatively affect immune protection of individual animals, exacerbating disease and increasing the likelihood of disease transmission.

2.3. Interspecies Transmission Including Zoonotic Potential. The presence of *M. bovis* in wildlife is not only detrimental to the health of the wildlife population but also represents a serious threat to livestock and a risk to human health. Over 50 *M. bovis*-infected cattle herds have been identified in Michigan since the identification of tuberculosis in white-tailed deer in 1994. RFLP analyses suggest that cattle, deer, and other wildlife are infected with a common strain of *M. bovis*. Cattle probably become infected through direct or indirect contact with free-ranging white-tailed deer [32]. By 2010, estimates suggested the overall cost to Michigan of the presence of *M. bovis* in deer and cattle had been greater than US $100 million [41]. Surveys of carnivores and omnivores in Michigan have confirmed spillover of *M. bovis* infection to coyotes (*Canis latrans*), bobcats (*Felis rufus*), red foxes (*Vulpes vulpes*), black bears (*Ursus americanus*), opossums (*Didelphis virginiana*), raccoons (*Procyon lotor*), and domestic cats (*Felis catus*) [42–44]. Non-deer wildlife are likely to have been infected through scavenging of dead deer carcasses. Infection in nondeer wildlife is characterized by limited lesion development suggesting that they are dead-end spillover hosts and unimportant in maintenance of the epizootic in deer or transmission to other susceptible hosts [45, 46].

In tuberculous humans, aerosol transmission via respiratory secretions containing *Mycobacterium tuberculosis* is the primary means of human-to-human spread. Minute ($<5\,\mu$m) aerosolized droplets known as droplet nuclei can be generated by talking or coughing [47, 48]. Such nuclei remain airborne for prolonged periods while larger droplets quickly come to rest within a short distance of the host. Some droplet nuclei carry *M. tuberculosis* and once inhaled pass deep in to the bronchi and bronchioles where they can establish infection and initiate the disease process. Both aerosol and oral transmission of *M. bovis* between deer can occur as deer congregate around artificial feeding sites. One study found that *M. bovis*-infected deer were more closely related genetically, than noninfected deer, suggesting that contact within family groups was important in disease transmission [49]. Indeed, deer in family groups are more likely to share feed from the same sources, participate in mutual grooming, and spend time within distances favorable to aerosol transmission.

Aerosol transmission between deer and cattle is less likely to occur as deer are seldom in close proximity to cattle. One study on deer-cattle interactions, within the TB-endemic zone of Michigan, found direct deer-cattle interactions (deer within 5 m of cattle) to be exceedingly rare; however, deer were commonly seen in feed storage areas eating out of hay racks and feeding troughs [50]. Accordingly, most deer to cattle transmission is believed to be indirect through sharing of feed. White-tailed deer experimentally infected with *M. bovis* shed tubercle bacilli in saliva and nasal secretions [51, 52]. Research shows that experimentally infected deer can transmit *M. bovis* to other deer or cattle through both direct (cohoused) and indirect contact such as sharing of

feed with no opportunity for direct contact or aerosol transmission [31, 52, 53]. Feed contaminated with saliva and nasal secretions containing *M. bovis* can be a source of infection for other animals.

Mycobacterium bovis is relatively resistant to environmental factors and under appropriate conditions (e.g., cool and protected from sunlight) may persist in the environment for weeks or months, prolonging the likelihood of transmission by ingestion [54–58]. A study on environmental survivability of *M. bovis* under natural weather conditions in Michigan found *M. bovis* survived up to 88 days in soil, 58 days in water and hay and 43 days on corn [55]. Although capable of surviving for many weeks in the environment, the risk from environmental *M. bovis* is mitigated by the location of the bacilli in soil or water making the tubercle bacilli less accessible to hosts. Survival on feedstuffs commonly used as supplemental feeds provides a more conceivable route of indirect transmission. The dose required for indirect transmission through the sharing of feedstuffs is unclear, but is likely higher than that required for transmission through direct contact (nose to nose) or aerosol transmission. In utero transmission has not been documented in white-tailed deer; however, potential transmission from doe to nursing fawns has been suggested experimentally, with 3 of 5 fawns infected through the consumption of milk containing 1×10^4 colony forming units (CFU) of *M. bovis*. The frequency of such doe to nursing fawn transmission in nature is likely low and probably not important in the maintenance of disease as mammary gland lesions in naturally infected deer have been rarely reported [59, 60].

Epidemiologic modeling suggests a 2-stage model of transmission within deer populations. Stage 1 involves transmission within matriarchal family groups, allowing disease to persist in the population at a low level [30]. Family groups consisting of a matriarchal doe and several generations of her daughters and their fawns characterize the social structure of white-tailed deer. Fawns from the previous year leave the dam when she nears parturition in spring. Yearling does often rejoin their dam and her fawns in the fall. Stage 2 involves both supplemental feeding, with resultant increased deer density, and dispersal of male fawns to join male groups that travel together at all times except during breeding season [30]. Higher disease prevalence has been observed in adult male deer [8]. Shifting group membership by male deer results in temporary association with several different groups and increased contact with numerous susceptible animals.

Although *M. bovis* is a recognized zoonotic agent, no change in incidence of *M. bovis* infections in Michigan's human population has been detected since the epizootic was recognized [19]. However, two cases of *M. bovis* infection in humans have been linked to *M. bovis* found in free-ranging deer [20]. One of the two cases was cutaneous tuberculosis in a hunter, the result of an injury sustained during field dressing of a tuberculous white-tailed deer. In spite of the paucity of cases, there are potential risks as hunters are exposed to *M. bovis* during the field dressing of deer or the consumption of undercooked venison products. Michigan's Departments of Community Health, Natural Resources, and Agriculture have worked cooperatively to educate hunters, farmers, and Michigan residents on the identification of tuberculosis in deer, recommended personal protective measures, and the importance of thorough cooking of venison prior to consumption [19].

2.4. Disease Control Effort. In Michigan, wildlife and domestic animal health authorities have adopted control measures that (1) reduce deer density and population through increased hunting, (2) restrict or eliminate supplemental feeding of deer, and (3) monitor both wildlife and livestock through hunter-killed deer surveys, carnivore and omnivore surveillance, and whole-herd tuberculin testing of cattle. These control measures appear to have succeeded in preventing an increase in prevalence and geographic spread of tuberculosis in white-tailed deer in Michigan. In 1998, supplemental feeding was banned in counties where tuberculous deer had been identified. Enforcement has been problematic and universal compliance has not been achieved. Public and political pressures have resulted in an easing of prohibitions that allow baiting in previously restricted areas. Deer numbers have been reduced by 50% in the endemic areas through increased hunting pressure and unlimited harvesting of female deer. However, progress towards eradication will likely require additional actions and more time. Epidemiological modeling suggests that further decreases in deer density and a strictly enforced ban on supplemental feeding will be required to eradicate *M. bovis* from Michigan wildlife and cattle.

2.5. Vaccination. Mycobacterium bovis strain bacilli Calmette-Guerin (BCG) has been used as a vaccine and showed protection in cases of naturally occurring tuberculosis in sika deer (*Cervus nippon*) [61], and in experimental infections of red deer [62, 63]. Using experimentally infected white-tailed deer, both BCG strains Pasteur and Danish provided protection in the form of decreased lesion severity [64–66]. In vaccinates, there were fewer, smaller, and less extensive lesions compared to nonvaccinates. Lesions in vaccinates were characterized by less necrosis and fewer AFB than in nonvaccinates. One study showed that oral vaccination provides equivalent protection when compared to subcutaneous vaccination [64].

BCG can persist in tissues of vaccinated deer. Studies to examine BCG persistence demonstrate that after oral or subcutaneous administration, BCG persisted in tissues for up to 3 and 9 months respectively [67]. Shedding of BCG by vaccinates was assumed to have occurred as nonvaccinates sharing the same pen became infected with BCG [65, 66, 68]. Vaccine shedding to nonvaccinated animals has not been described in BCG vaccination studies in cattle or red deer. However, studies to examine transmission from vaccinated deer to nonvaccinated cattle through indirect contact have been unsuccessful [68].

2.6. The Minnesota Experience. In 1971, Minnesota was considered free of bovine tuberculosis and was granted TB-free status by the USDA. However, in 2005, a beef cow infected with *M. bovis* was discovered through meat inspection surveillance [6]. The cow originated from northwestern

Minnesota. Testing of remaining cattle in the herd revealed a prevalence of 1.2%. Epidemiological investigations identified 4 other herds in the region with tuberculous cattle. The discovery of bovine tuberculosis prompted surveillance of local free-ranging deer. Harvesting of 474 deer yielded 1 deer infected with *M. bovis*. The response by Minnesota animal health and wildlife authorities was aggressive and included statewide testing of all cattle, dramatically decreasing deer density in the region through increased removal by hunters, landowners, and government officials, removal of many of the cattle in the area through a voluntary buy-out program, and fencing of feeding areas on remaining farms with cattle herds. Over 6200 head of cattle were removed from the region at a cost of US $4.6 million. Between 2005 and 2009, *M. bovis* was found in 27 deer and 12 cattle herds [6]. In 2010, no *M. bovis*-infected deer or cattle herds were found within the state, and as of November 2011, Minnesota regained TB-free status by the USDA. The effort at preventing the establishment of a wildlife reservoir of *M. bovis* in Minnesota's white-tailed deer was aggressive, but not without cost. The costs to USDA were estimated at US $70 million, Minnesota Board of Animal Health US $12.5 million, and Minnesota Department of Natural Resources US $3.5 million.

3. Badgers in the United Kingdom and Ireland—Spillover and Spillback

Mycobacterium bovis is endemic among badgers in southwest England, south Wales, and Ireland. It is hypothesized that badgers are a source of infection for cattle and responsible for an increase in tuberculosis among domestic cattle herds in the UK. *Mycobacterium bovis* was first isolated from badgers in Switzerland in 1957 [69]. In 1971, the first tuberculous badger was identified in England [70] and in 1975 the first infected badger was reported in Ireland [71]. It is believed that badgers became infected with *M. bovis* by spillover of infection from cattle during the late 19th and early 20th centuries when a large percentage of British and Irish cattle were infected with *M. bovis.* By the 1970s, bovine tuberculosis had been removed from large areas of the UK, and animal health authorities anticipated eradication. In 1981, the Wildlife and Countryside Act provided protection of badger populations in the UK, and in Ireland protection was granted in 1976. Protection has resulted in a large increase in badger populations in both countries. Over the past decades, the UK has experienced a rising incidence of bovine tuberculosis but herd incidence rates have remained constant in Ireland. In 1998, fewer than 6% of herds in the UK were under movement restriction due to bovine tuberculosis, this figure had increased to more than 13% by 2010 [72].

3.1. Badger Ecology. The badger is a mustelid, a family of carnivorous mammals. They are nocturnal and live in social groups of mixed ages and sexes in underground setts. Setts are elaborate structures of multiple interconnecting tunnels and nest chambers with numerous entrances that can be found throughout the territory of a social group [73]. They are used for resting, breeding, protection from predators, shelter from harsh weather, and emergency refuge [73]. In areas of high population density, as in southwest England, social groups may consist of 8–20 individuals [74, 75] but in low density areas, as in Ireland, social groups are smaller and consist of only 2 to 3 individuals [76]. Social groups are territorial. Territories are well defined and stable over time in areas of high population density, as in southwest England [77], but are less clearly demarcated in low-density areas [78, 79]. There is a constant level of intergroup mingling but during the breeding season territories are fiercely defended resulting in high levels of intergroup aggression. In areas of low density, there appears to be proportionately more intergroup mingling [78].

The badger's natural habitat is such that it lives on or near pastures used by cattle where it seeks carrion and digs for earthworms, frogs, and insect larvae [80]. Setts provide ideal conditions for the spread of respiratory diseases. In southwest England, where the highest density of badgers is found, badger density can be as high as 25.3 adults per km^2; but in Ireland the density is 1-2 adults per km^2 [81]. There appears to be no direct correlation between badger density and the prevalence of *M. bovis* infection among badgers [75, 82].

3.2. Pathology. Tuberculosis is a chronic infection that progresses slowly with infected badgers maintaining a normal life expectancy. Badgers are very susceptible to infection with *M. bovis*, with infection established by endobronchial instillation of doses below 10 CFU, yet they are able to control infection with higher doses ($\sim 10^4$ CFU) [83]. Latent tuberculosis infection, that is, infection in the absence of gross lesions, is found in 50% to 80% of naturally infected badgers in wild populations [84]. Both naturally infected and experimentally infected badgers have few sites of infection but lesions can be found in a wide range of anatomical locations. In badgers infected by natural transmission, the most frequent sites of infection are the lungs, lung-associated lymph nodes, and medial retropharyngeal and axillary lymph nodes, while renal infection is infrequent [85]. The frequency of infection and the anatomical sites affected is considerably greater than can be appreciated from the distribution of gross lesions [86, 87]. Tuberculous granulomas in badgers are composed predominantly of epithelioid cells, macrophages, and few lymphocytes. They are highly cellular and proliferative, with little necrosis, mineralization, or fibrosis and have the same general appearance in all tissues [88]. The lesions are interstitial and expansive, compressing surrounding parenchyma. Histological features that are characteristic of tuberculosis in other species, such as caseation, fibrous tissue encapsulation, cavitation, abscessation, or Langhan's multinucleated giant cells are not seen in badgers. The histopathology of tuberculosis in badgers resembles that seen in other carnivores [89].

3.3. Intraspecies and Interspecies Transmission Including Zoonotic Potential. Badger to badger transmission is most likely through aerosols and to a lesser extent through bite wounds [82]. Experimental studies demonstrate that badgers can transmit *M. bovis* to cattle [90]. The exact route of transmission is unclear; nevertheless, based on excretion patterns

it is probably via infectious aerosols. Infected badgers shed *M. bovis* in respiratory secretions and exudates from draining superficial lesions [87]. Shedding in urine and feces only occurs in the small proportion of badgers with generalized disease [85]. As infected badgers have been observed to live for 3-4 years after shedding of *M. bovis* was first detected, they are an excellent maintenance host of *M. bovis* with even mildly infected badgers constituting an ongoing risk. [91]. It is suggested that cattle may become infected by inhalation and less likely ingestion of fodder contaminated with infected badger sputum, urine, feces, or exudates from superficial lesions [92]. Badgers mark territory boundaries at localized areas used for urination and defecation known as latrines and likewise mark travel pathways with urine [93]. Both latrines and pathways are generally accessible to cattle making them areas of risk for cattle. High doses of *M. bovis* are required to infect cattle by ingestion; however, excretion of high numbers of tubercle bacilli in urine and feces is uncommon in badgers [85]. Experimentally, calves have been infected when housed with experimentally infected, as well as naturally infected badgers [90]. Five of nine calves became infected after being housed with infected badgers that were shedding *M. bovis*; exposure was for periods of 6 to 12 months and all infected calves had lung and thoracic lymph node lesions with one having additional lesions in the medial retropharyngeal and mesenteric lymph nodes. Epidemiological studies show that areas with the greatest density of badgers have the highest incidence of tuberculosis among cattle [70, 82, 94]. Consequently, in Ireland, cattle have been shown to be effective sentinels for tuberculosis in badgers [95].

Recently, since the resurgence of disease in the UK, the first documented cases of spillover of *M. bovis* from animals to humans were reported [96]. Two siblings residing on a farm were diagnosed with tuberculosis due to *M. bovis*. Cattle on the farm had also been diagnosed with *M. bovis*. The cattle isolate was indistinguishable from the isolates from the 2 siblings when examined by RFLP analysis, spacer oligonucleotide typing (spoligotyping), and variable number tandem repeat (VNTR) analysis, suggesting transmission between cattle and humans. Moreover, the farm supported a large badger population where *M. bovis* infection had been previously diagnosed. It is suggested, although not proven, that cattle became infected through contact with badgers and that humans became infected through contact with cattle.

3.4. Disease Control Effort. Badgers are an ideal host for *M. bovis*; infection in populations is usually endemic, tuberculosis kills few badgers, and their deaths do not significantly perturb population density nor the size and structure of social groups. Badgers may survive for long periods while suffering from overt disease; however, the majority of infected badgers remain clinically healthy, with a high proportion (50–80%) having latent infection. Tuberculous females often continue to produce cubs [82]. The removal of infected badger populations from cattle farming areas has resulted in a decline in bovine tuberculosis in cattle. However, reports of the efficacy of culling in decreasing the risk of spread of infection from badgers to cattle differ between those of Ireland and the UK.

Evaluation of badger culling trials conducted in the UK from the 1970s to the mid-1990s failed to provide clear outcomes due to their small size and lack of controls [94]. In the UK, following the first suggested links between badgers and bovine tuberculosis, farmers were allowed to cull all badgers in individual setts. This type of proactive culling was later replaced with a strategy to identify and remove clusters of infected badgers (reactive culling). Over a 6-year period, more than 20,000 badgers were culled in an attempt to control escalating rates of bovine tuberculosis [97]. From 1986 to 1998, reactive culling occurred only on land where tuberculin-test-positive cattle were present [98]. Increasing spread of tuberculosis in cattle led to the suspension of badger culling in England and Wales and the appointment of an independent advisory committee, the Independent Study Group on Cattle TB (ISG), with the broad charge of examining the role of badgers in the epidemiology of tuberculosis in animals and to investigate options for badger control [99]. In 1998, a large experiment was implemented, under the direction of the ISG, known as the Randomized Badger Culling Trial (RBCT). The RBCT was designed to determine the role of badgers as a reservoir of *M. bovis* and to compare the effects of three different control strategies; no culling of badgers, localized selective culling of badgers in response to identified cases of tuberculosis in cattle (reactive culling), and removal of all badgers across entire trial areas (proactive culling). The trial clearly demonstrated that infected badgers were a reservoir of infection for cattle [100]. The reactive culling component of the RBCT was prematurely curtailed when analysis of data from the first few years suggested that reactive culling had increased disease risk in cattle herds [98]. Little useful results have been generated from the reactive culling component of the RBCT and the analysis leading to the cessation of reactive culling has been severely criticized [101, 102]. In contrast, after 5 years of proactive culling, there was a 23% reduction in the incidence of cattle tuberculin reactors inside the culling area and an ongoing beneficial effect, with a 54% decrease 1-2 years after the last proactive cull [99].

In Ireland, a national bovine tuberculosis eradication policy was initiated in 1954. Progress in the form of decreasing prevalence was good through the mid-1960s. In the first 11 years of the program, overall prevalence of bovine tuberculosis decreased from 17% to 0.3% [103]. As in England and Wales, tuberculosis is endemic in the badger population in Ireland and the *M. bovis* strains from badgers and cattle are identical by RFLP and spoligotyping analysis [104]. Although badgers were given legal protected status Ireland in 1976, proactive culling of badgers for research and focal (reactive) culling are allowed through licenses granted by the National Parks and Wildlife Service. In Ireland, focal (reactive) culling in response to a herd outbreak is undertaken only after an epidemiological investigation has eliminated all other sources of infection [105]. Culling remains an interim strategy while research on alternative control options are investigated [106]. The case for culling is supported by research conducted in Ireland. Epidemiological reports of tuberculin reactors in cattle in 1988 found evidence of badger involvement in 14% of cases [107]. That infected

badgers pose a significant risk to cattle has been shown in two studies, the East Offaly study (1988) and the "Four Areas" (1997–2002) study [103]. In both studies, proactive badger removal was shown to significantly decrease herd incidence of bovine tuberculosis. The East Offaly study showed that control herds, where no badgers were removed, were twice as likely to have cattle movement restricted due to bovine tuberculosis than herds in the badger removal areas [107]. The Four Areas study was conducted between 1997 and 2002 in matched removal and reference areas (average area 245 km^2), in four counties with differing agricultural land types and farming practices. Proactive badger culling was intensive and thorough in removal areas, but in the reference areas, reactive culling was only done in response to a severe tuberculosis outbreak in cattle. During the study, the odds and hazard ratios of a herd movement restriction due to bovine tuberculosis in the removal areas were significantly lower than in matched reference areas [103]. Reactive culling has also been shown to have a broader beneficial impact than just protection of the herd at the center of the culling. In County Laois between 1989 and 2005, reactive badger removal had a significant beneficial impact on the risk of future outbreaks in herds surrounding the area where badgers were removed [108].

Why were the experiences in the Republic of Ireland and the UK so different? The seemingly conflicting findings from the badger culling studies in Ireland and England may be partially due to the presence of geographical barriers in the Irish study that could have impeded badger movement, such as coastline, sea inlets, mountain ranges and rivers, differences in badger density, and trapping efficacy [97, 103]. As badger immigration was noted as a compounding variable in the East Offaly study, trial locations in the "Four Areas" study were intentionally selected to maximize natural boundaries (e.g., coast, rivers, mountains) so as to minimize the effect of badger immigration. Indeed, the success of the Irish "Four Areas" study may have resulted from a combination of low badger population density, limited immigration, and effective badger removal over a geographical area larger than the RBCT [97]. In the "Four Areas" study, researchers sought to achieve as complete removal as possible over a large area, and to sustain this effort throughout the 5-year study period [103].

Badgers have complex social structures, the stability of which varies depending on population density [78]. Examination of the RBCT culling areas, which support some of the highest badger densities ever recorded, revealed that culling resulted in social restructuring and increased home range of remaining badgers. Increased ranging behavior likely resulted in increased contact with other badgers, as well as cattle [109]. In the UK studies, social restructuring and increased badger movements have been correlated with an increased incidence of *M. bovis* infection among badger populations [75]. In the low-density Irish badger population, culling may have resulted in less social disruption.

One theoretically effective means of preventing transmission of infection is segregation of cattle from badgers. Accordingly, cattle husbandry practices aimed at separating cattle and badgers have been proposed as a means of tuberculosis control. Exclusion of badgers from cattle housing and feeding areas is a feasible control measure. Keeping cattle away from badger setts, urination trails, and latrines may be beneficial, but keeping badgers away from cattle at pasture would be expensive and result in disruption of normal badger behavior patterns [110]. Whereas public attitudes in the UK are not in favor of badger culling and surveys show public preference of conservation and animal welfare over disease control, in Ireland, culling is accepted as an interim policy and is under continuing review by the National Parks and Wildlife Service.

3.5. Vaccination. Complete removal of any wildlife reservoir of infection would be extremely difficult, unethical, and would contravene commitments by the UK and Ireland to the Bern Convention on the Conservation of European Wildlife, which promote responsible conservation of native species. In the long term, most believe the best prospect for control of bovine tuberculosis in the UK and Ireland is through vaccination of cattle or wildlife, combined with improved diagnostic tests to distinguish vaccinated from infected cattle [94, 106]. BCG is the most likely candidate for a badger tuberculosis vaccine as it induces protection after being administered by subcutaneous, conjunctival, intranasal, and intramuscular routes [111]. The first demonstration of BCG vaccine-induced protection in badgers was reported in 1988 using the intradermal route of administration [112]. Vaccinates lived longer and shed fewer tubercle bacilli than the nonvaccinates. BCG has also been found to be protective in badgers when delivered by either mucosal [111] or oral [88] routes [113, 114]. For BCG vaccination of wildlife, an oral bait is the most practical means of delivery [115]. For oral administration, BCG was encapsulated in a lipid matrix that provided protection of the bacilli from the lethal effects of gastric secretions [116]. In a UK field trial, BCG vaccinated badgers had a lower rate of seroconversion (a useful indicator of *M. bovis* infection in badgers) than controls [113]. Testing of the lipid encapsulated oral BCG vaccine in a large-scale field trial is currently underway in Ireland [117], while in March 2010, the UK veterinary medicines licensing body granted approval for use of *M. bovis* BCG strain Danish 1331, in badgers (intramuscular use only) [99], where it will be used in a vaccine deployment project (http://www.defra.gov.uk/fera/bvdp/).

3.6. Other Wildlife. In 2004, the results were released of a study to examine numerous species of wildlife in the UK for tuberculosis. Over 4700 animal carcasses were examined and tissue samples processed for isolation of *M. bovis*. Infection was confirmed in foxes, stoat (*Mustela erminea*), polecat (*Mustela putorius*), common shrew (*Sorex araneus*), yellow-necked mouse (*Apodemus flavicollis*), wood mouse (*Apodemus sylvaticus*), field vole (*Microtus agrestis*), grey squirrel (*Sciurus carolinensis*), roe deer (*Capreolus capreolus*), red deer, fallow deer, and muntjac deer (*Muntiacus reevesi*). Sample size varied widely, but the highest prevalence was seen in polecats (4.2% of 24), stoats (3.9% of 78), foxes (3.2% of 756), yellow-necked mouse (2.8% of 36), common shrew (2.4% of 41), field vole (1.5% of 67), roe deer (1.0%

of 885), red deer (1.0% of 196), fallow deer (4.4% of 504), and muntjac deer (5.2% of 58). A qualitative risk assessment based on prevalence, likelihood of excretion, likelihood of contact with cattle, and animal biomass identified fallow deer and red deer as the highest risk for transmission of *M. bovis* to cattle [118]. This survey demonstrates that deer may pose a significant risk to cattle, especially in regions where deer density is high. However, with regional tuberculosis, prevalence as high as 20.5% in badgers, badgers remain a primary concern for tuberculosis control in the UK. Recently, *M. bovis* was reported in a free-ranging wild boar in the UK. Boar in the wild have not been present in England for several centuries; however, escapes from captive facilities and deliberate releases have resulted in small feral populations. *Mycobacterium bovis* has been isolated from captive boar on only 3 occasions since 1997.

4. Brushtail Possum in New Zealand—Wildlife Introduction and Translocation

Although in New Zealand *M. bovis* infection has been found in 14 different domestic and wild animal species, the most significant wild maintenance host is the brushtail possum with deer species, particularly red deer, and ferrets being spillover hosts [5]. Prior to arrival of the first humans, the only mammals present in New Zealand were 2 species of bats [119]. Early European settlers introduced cattle approximately 200 years ago and these settlers were responsible for clearing large areas of forest to accommodate pastoral farming. Europeans introduced 31 other mammal species to New Zealand including brushtail possums, ferrets, and seven deer species [120].

Brushtail possums were first introduced to New Zealand from Australia in the mid-19th century to establish a fur trade. Between 1837 and 1922, over 30 groups of possums were imported, maintained in captivity for breeding, and released at over 160 different sites around New Zealand [119]. Lack of natural predators combined with abundant food sources resulted in a rapid increase in possum numbers. Currently possums occupy over 90% of New Zealand land area with an estimated 60–70 million possums nation-wide. Possum density estimates range from 1.5 to 25 per hectare and in some areas possum density is 20 times greater than that typically seen in Australia [119].

Seven species of deer were introduced: red deer, sika, white tailed deer, fallow deer (*Dama dama*), elk, sambar deer (*Rusa unicolor*), and rusa deer (*Rusa timorensis*). These deer species were introduced at various times between 1864 and 1907 for recreational hunting purposes. By the middle of the 20th century, red deer numbers had climbed to such levels that they were considered nuisance pests. Farming of red deer began in the 1970s when wild deer were captured to establish breeding herds [119].

Mycobacterium bovis was likely introduced to New Zealand with the importation of cattle in the 19th century. By the early 20th century, tuberculosis was recognized as a serious animal and human health problem. Tuberculosis was first diagnosed in farmed deer in 1978 and subsequently spread by movement of untested farmed deer and capture of infected wild deer. The first reported case of tuberculosis in a wild possum in New Zealand was in 1967 [121]. However, the susceptibility of brushtail possums to infection with *M. bovis* had been determined much earlier [122]. Epidemiological evidence links possum tuberculosis and tuberculosis in cattle [123]. Tuberculosis has never been identified in possums in Australia, the original source of New Zealand's possums; therefore, it is likely that possums in New Zealand acquired *M. bovis* from other animals, most likely cattle.

4.1. Pathology. *Mycobacterium bovis* infection in possums is typically a fulminating, rapidly fatal pulmonary infection [124] with cases of self-cure being rare exceptions [125]. After infection by natural transmission, death ensues in 3–8 months [126]. Tuberculous possums often develop disseminated disease, with lungs, mediastinal lymph nodes, axillary lymph nodes, and liver being the most common sites of infection. Infection of the axillary lymph nodes frequently leads to formation of discharging sinuses [127]. Lesions are also seen in the spleen, kidneys, adrenal glands, and bone marrow suggesting generalized hematogenous spread of bacilli [128]. In contrast to lesions in cattle, fibrosis, mineralization, and Langhan's type giant cells are uncommon, while AFB are numerous. These characteristics suggest an ineffective host response to infection, leading to an inability to sequester infection, thereby allowing rapid hematogenous dissemination. In spite of disseminated disease, the clinical appearance, body condition, and behavior remain within normal bounds until the latter stages of disease [128]. In contrast, terminally ill possums show a profound change in behavior. The disseminated nature of the disease, with pulmonary lesions, superficial draining sinuses, and large numbers of AFB, combined with the limited effect on behavior for most of the period of infection, make possums an ideal maintenance host capable of efficient transmission to other susceptible hosts.

4.2. Epidemiology. Horizontal transmission between possums is principally by infectious aerosol leading to lower respiratory tract infection. Infected possums shed *M. bovis* primarily in respiratory secretions and exudates from superficial draining sinuses. [129]. There is pseudovertical transmission between mother and offspring by aerosol and limited occurrence of pseudovertical transmission by ingestion from tuberculous mastitis [129]. Aerosol transmission may occur at the time of mating and through the sharing of dens. Direct and indirect transmission may also occur through mutual grooming or from a contaminated environment. In studies using captive possums, den sharing provided the greatest risk of transmission between possums [124]. Den sharing has not been commonly observed in free-living possums; however, sequential den use by different possums has been observed [130] and as *M. bovis* can survive inside possum dens for 7–28 days this mode of transmission is possible [56]. *Mycobacterium bovis* remains viable inside possum carcasses for up to 3 days in summer and 27 days in winter and live possums have been observed interacting with possum carcasses. Consequently, transmission from dead infected to live susceptible possums can occur by ingestion, but this route is unlikely to

be a significant mode of transmission [131]. The dynamics of possum to possum transmission of *M. bovis* are complex and may involve individual possum social status. Evidence of this was found in captive possum studies where possums infected by natural transmission were those central and prominent in the social hierarchy. Furthermore, when socially dominant possums were experimentally infected, it resulted in higher levels of disease transmission than when possums lower in the societal structure were experimentally infected [132].

Healthy possums generally avoid contact with cattle and deer [133]. However, terminally ill possums exhibit abnormal behavior such as increased daytime activity, stumbling, rolling, and falling, actions that attract the attention of inquisitive cattle and deer. Studies using sedated possums, to simulate terminally ill possums, demonstrated that both deer and cattle exhibit a profound interest in possums behaving abnormally. Cattle were attracted from as far as 50 m to investigate sedated possums [133]. Both deer and cattle were shown to spend significant amounts of time within a distance compatible with aerosol transmission (approximately 1.5 m) and to even sniff, touch, lick, roll, lift, chew, and kick the possum creating an opportunity for direct transmission [130, 134]. In studies where cattle have been excluded from areas used as dens by tuberculous possums, decreased transmission of *M. bovis* from possums to cattle has been demonstrated. In contrast, where cattle are allowed to graze areas used for dens by tuberculous possums transmission to cattle continues unabated [130].

4.3. Disease Control Effort. The core of tuberculosis control in New Zealand has been to conduct both an intensive test and slaughter program in cattle and farmed deer, along with equal emphasis to identify and control infected possum populations. Of the wild animals that have been found infected with *M. bovis,* only possums have been targeted for widespread population control, with the intention of reducing possum density and the probability of intraspecies and interspecies transmission. This level of control has approached eradication of possums in some locations [5]. Limited focal culling of ferrets has also been undertaken. No widespread eradication of a vertebrate host has ever been successful on mainland New Zealand, but it has been achieved on some large offshore islands. Social attitudes towards possums in New Zealand differ from those of other wildlife reservoirs of tuberculosis in other countries. In New Zealand, possums are nonnative, invasive pests that cause widespread ecological damage to New Zealand forests and, therefore; widespread removal of possums is desirable for many reasons apart from tuberculosis control. Possums have had a disastrous impact on native flora and fauna. Every night an estimated 70 million possums consume approximately 21,000 tonnes of green shoots, leaves, and berries. Possums are omnivorous and also consume bird's eggs, chicks, small reptiles, and insects. While browsing in the forest canopy on fruits and flowers, possums are in direct competition with native birds. While on the ground, possums compete with native kiwi for dens and have been seen eating kiwi eggs. Theoretically, widespread removal of possums from New Zealand's ecosystem would be more socially palatable than removal of native wildlife reservoirs of tuberculosis in other countries.

It is clear that the key underlying wildlife reservoir of tuberculosis in New Zealand is the infected possum population [5]. Systematic and widespread control of possum populations using poisons and traps has significantly reduced the risk of transmission of tuberculosis to cattle and deer, and the ecological impact of possums. Early control measures included a bounty system on possums that was minimally effective, as it did not allow for prioritization of control efforts, and although many possums were removed, they were generally not removed from essential tuberculosis control areas. The principle method for controlling infected possum populations across large tracts of land is aerial distribution of baits containing 1080 poison (sodium monofluoracetate). An effective poison, 1080 causes cardiac or respiratory failure in possums. Other poisons that have been used to control possums include brodifacoum, pindone, cyanide, and cholecalciferol. Elimination of tuberculosis from possum populations is based on maintaining possum densities at very low levels for a minimum of 5 years [5]. In areas where 1080 baits have been used temporarily to decrease possum numbers for only a limited period of time, tuberculin reactor rates in cattle herds, and numbers of tuberculous possums initially decreased but returned to elevated levels in 8–10 years, with possum numbers recovering through breeding and immigration from surrounding areas [135, 136]. Long-term (>10 year) maintenance of possum populations below 40% of precontrol densities over widespread areas may be required to affect significant change in cattle tuberculin reactor rates and eradicate tuberculosis from possum populations [137]. Reduction in possum populations in *M. bovis* endemic regions has also reduced prevalence in spillover hosts such as feral pigs (*Sus scrofa*) [11].

4.4. Vaccination. Although widespread removal of possums through poisoning may decrease the prevalence of tuberculosis in cattle, complete removal of possums from New Zealand may be impractical. It has been suggested that the most promising option for long-term control of tuberculosis in possums may involve targeted vaccination combined with strategies for limited population control or biological control of possums. *Mycobacterium bovis* BCG induces protection and has been administered to possums by subcutaneous, intranasal, intraduodenal, and oral routes [138–142]. All routes provide evidence of protection against aerosol challenge with virulent *M. bovis*, with vaccinates having reduced disease severity, reduced loss of body weight, fewer lung lesions, and decreased bacterial colonization. Two field studies in free-ranging possums in New Zealand have demonstrated that BCG vaccine is protective and can prevent infection. In the first trial, possums were vaccinated by a combination of intranasal aerosol and conjunctival instillation. Vaccine efficacy was estimated at 69% [143]. In the second trial, using oral vaccination, efficacy was estimated at 95%-96% [144].

There are a number of ways that a vaccine could be delivered to free-ranging wild animals, but oral baits are favored as the most practical and cost-effective method [145].

A lipid-based bait has been developed for oral delivery of BCG. Lipid serves to protect viable BCG from degradation in the stomach, allowing lipolytic enzymes of the small and large intestines to liberate BCG, enhancing uptake by gastrointestinal associated lymphoid tissue (GALT) [146]. BCG vaccine delivered in such a fashion persists in mesenteric lymphoid tissue of the GALT for up to 8 weeks [147]. Possums vaccinated with the lipid formulated BCG shed viable BCG in feces for up to 7 days after vaccination, but always in low numbers ($<10^3$/gm feces) [147]. Vaccine persistence and shedding are important to evaluate as nontarget species such as scavengers, predators, and even cattle may be exposed to persistent BCG in tissues, feces, or the environment. Notwithstanding, persistence in host tissue, to some degree, is vital for initiation of protective immune response. When administered as lipid formulated BCG to mice, viable BCG persists in lymphoid tissue for up to 30 weeks after vaccination, compared to survival of only 12 weeks when nonlipid formulated BCG was administered [148]. In experimental vaccination and challenge studies, vaccinates showed decreased weight loss, lower lung-body weight ratio, fewer extrapulmonary lesions, and lower lung and spleen bacterial counts compared to nonvaccinates [149]. BCG is avirulent in all animal species so far tested [150], consequently; the risk from BCG persistence in tissues, or excretion in feces, is of little importance, with the exception of livestock. Exposure to BCG could interfere with current diagnostic testing for *M. bovis* infection, making the differentiation of vaccine-exposed cattle from *M. bovis*-infected animals difficult.

4.5. Other Wildlife Species. Other species such as red deer, feral pigs, feral cats (*Felis catus*), ferrets, stoats, goats (*Capra hircus*), rabbits (*Oryctolagus cuniculus*), hares (*Lepus europaeus*), and hedgehogs (*Erinaceus europaeus*) have been found infected with *M. bovis* [11, 136, 151]. In New Zealand, research shows feral pigs to be spillover hosts. Exploiting this observation, feral pigs have been used as sentinels in the surveillance for tuberculosis in possums [11, 152]. In contrast, some high-density populations of feral red deer and ferrets are considered maintenance hosts [5]. Other species are inconsequential in the epidemiology of bovine tuberculosis in New Zealand [5].

5. Wild Boar in the Iberian Peninsula (Portugal and Spain)—Management of Wild Populations for Commercial Hunting

Increased interest in commercial hunting of animals such as the Eurasian wild boar has resulted in increased fencing of hunting estates. This in turn is linked with increasing population densities, artificial feeding/watering, and translocations, [153] contributing to the widespread distribution of *M. bovis* infection in wild boar in the southern Iberian Peninsula [154]. The experience in Spain is unique compared to other countries in that the prevalence of *M. bovis* infection in wild boar is 100% in some areas, higher than for any other wild ungulate in the Peninsula, or for any other wildlife reservoir in the world [154, 155]. Remarkably, in some areas, this high prevalence of infection exists even in the absence of confounding anthropogenic factors such as artificial feeding [155]. The overall prevalence in red deer is also high at 14% with some areas reaching 50% prevalence in the Iberian Peninsula [154].

Nation-wide, the first efforts to eliminate bovine tuberculosis in Spain began in 1956 [156]. Financial government assistance in the program began in 1965. Early efforts focused on dairy cattle where the prevalence of bovine tuberculosis was estimated to be 20% [156]. In 1991, the prevalence of bovine tuberculosis in Spain was >10%, the highest in the European Community [7, 157]. By 2005, test and slaughter policies had dramatically reduced the prevalence to approximately 0.3% [7]. Restriction fragment length polymorphism analysis and spoligotyping have shown that many of the strains isolated from wild boar are identical to isolates obtained from cattle in the same region [158]. The exact means of interspecies transmission is unclear; however, it is speculated that wild boar contaminate pastures, feed, and sources of water, and thus transmit disease to cattle. Juvenile wild boar are the dispersing age group [159] and may, therefore, contribute to much of the geographical spread of tuberculosis. Philopatric females associate in matriarchal groups, consisting of dam and female offspring. In this environment, intimate contacts between individuals during social and foraging activities are frequent and facilitate pathogen transmission directly and indirectly. Male boar begin to disperse as they become sexually mature (approx, 11 months of age). Dispersal distance increases with age, peaking at 13 months of age and ceasing at 16 months of age, with some males dispersing more than 50 km [159]. The home ranges of adult male boar overlap, except during breeding season when competition for females intensifies. Increased movement of younger males results from aggressive interactions with older males. Extensive tuberculosis lesions in more than one anatomical region have been found in a high proportion of juvenile wild boar that probably represent the main source of mycobacterial excretion [160].

Similarly, *M. bovis* has been identified in other Iberian wildlife, including red deer, fallow deer, Iberian lynx (*Lynx pardinus*), and hare. Again, transmission between cattle and wildlife is implicated due to similar spoligotype patterns of isolates from both livestock and wildlife [161].

One well-studied region is Doñana Biosphere Reserve, a conservation area that is home to a large number of animal species. Hunting and trapping are not allowed within the reserve; however, cattle have been grazed within the reserve for centuries. The first recognized cases of *M. bovis* infection in wildlife within the reserve date back to 1980s. Cattle densities differ between regions within the reserve, but are known to have been as high as 24 per km^2 [155]. Tuberculin skin testing of cattle in 2006 revealed a reactor rate of 9.4% [155]. In 2006 and 2007, sampling of wild ungulates in the reserve showed *M. bovis* in 52.4% of samples from wild boar, 27.4% from red deer, and 18.5% of fallow deer [155]. A causal link between wildlife tuberculosis and domestic cattle tuberculosis has been shown as removal of wild boar from this site resulted in a corresponding decrease in cattle tuberculosis. In contrast to New Zealand and Australia, wild

boar in the Iberian Peninsula are considered one of the main maintenance host species of *M. bovis* [7] and can maintain disease transmission cycles lasting many years [162]. Moreover, data suggest that significant intraspecies transmission likely occurs during the mating season [162].

5.1. Pathology. Lymph nodes, principally the paired mandibular lymph nodes are the most commonly affected tissues (>90%) [160]. Greater than 40% of animals have lesions restricted to the mandibular lymph node. Retropharyngeal and parotid lesions are also seen, but generally not without accompanying lesions in the mandibular lymph node [160]. Lymph node lesions generally contain few AFB [160]. Careful microscopic examination of the lungs revealed pulmonary lesions in 38% of the cases [160]. Larger lesions (i.e., 10 cm to 15 cm) were more common in juveniles and those animals with generalized tuberculosis. In addition to cranial lymph nodes and lungs, microscopic lesions are seen in tonsil of the soft palate and ileocecal valve. Microscopically, granulomas are composed of a mixture of epithelioid macrophages and multinucleated giant cells surrounded by infiltrates of lymphocytes, plasma cells, and macrophages. Larger granulomas can contain central regions of caseonecrotic debris that may be mineralized. Multiple bands of fibrous connective tissue may surround larger granulomas. Larger granulomas with more extensive caseonecrosis and mineralization were more common in young animals. In general, intralesional AFB are low in number with the exception of the lung, in which granulomas can contain high numbers of AFB [160].

Over 50% of wild boar have generalized disease. Common involvement of cranial lymph nodes in many animals combined with large numbers of animals showing generalized disease with pulmonary involvement suggests that both oral and aerosol routes are important means of transmission. Contrastingly, in feral pigs in Australia generalized lesions of tuberculosis were seen in <7% of animals and pulmonary lesions were uncommon, supporting the belief that feral pigs in Australia are dead end hosts and transmission from pig to cattle is improbable [9].

In various surveys an extraordinary proportion of both red deer and wild boar in the Iberian Peninsula have presented with disseminated tuberculosis involving numerous organs [154, 155]. Wild boar routinely scavenge carrion, which may explain most of the deer to boar transmission [153]. Monitoring of deer carcasses with motion-activated infrared digital cameras shows carcasses consumed by wild boar, alone or in large packs consisting of males, females, and piglets [155]. Efficient scavengers consuming highly infectious material create an ideal environment for transmission. Both wild boar and deer are known to congregate at watering and feeding sites, especially in managed estates of private land. The aggregation of boar at watering sites is significantly associated with the presence of tuberculosis in both boar and deer. Indeed, aggregation of boar at feeding sites significantly increases the risk of tuberculosis in deer [153]. Gathering of animals at watering and feeding sites increases inter- and intraspecific contact and results in a more heavily contaminated environment where indirect transmission is more likely to occur. Models have shown that interspecific transmission at watering and feeding sites is more likely to be boar to deer rather than deer to boar [153].

5.2. Disease Control Effort. The starting point for wildlife disease control is proper disease monitoring. Basic requirements for such monitoring include (1) ensuring the disease is monitored in the relevant domestic animals; (2) ensuring that background information on wildlife host population ecology is available to maximize the benefits of the monitoring effort; (3) selecting the appropriate wildlife hosts for monitoring; (4) selecting the appropriate methods for diagnosis and for time and space trend analysis; (5) deciding which parameters to target for monitoring; finally (6) establishing a reasonable sampling effort and a suitable sampling stratification to ensure detecting changes over time and changes in response to management actions [163].

Three main options exist for control of *M. bovis* transmission at the wild boar-livestock interface: improved biosafety, population control, and vaccination. The first option is being addressed through applied research on cattle feed and waterhole protection, as well as carrion consumption by wild boar, and the risk of leaving behind hunting remains (i.e., viscera not for human consumption). Regarding the second option, in Spain lethal means of population control other than shooting (e.g., poison) are not allowed [164]. Significantly reducing wild boar density through increased hunting reduces tuberculosis prevalence in wild boar and result in lower incidence of tuberculosis in sympatric ruminants. Nevertheless, other means of population control such as feeding bans or contraception remain to be tested. Additionally, newly developed blood tests may allow test-and-cull schemes for removal of infected boars [165, 166].

5.3. Vaccination. The third option is BCG vaccination of wild boar to reduce infection prevalence. In controlled laboratory experiments, oral delivery of baits containing BCG reduced *M. bovis* infection and lesion scores by ≥50% in wild boar piglets. Thoracic lesions that correlated with presence and potential excretion of viable *M. bovis* were reduced by 70%, and the serum antibodies against *M. bovis*, which correlate with lesion scores, were reduced by 79%. At the molecular level, oral BCG immunization of wild boar resulted in upregulation of various immunoregulatory genes that may be associated with a protective response to *M. bovis* infection [167, 168]. Recently, a new heat-inactivated *M. bovis* vaccine showed protection and produced wild boar immune responses similar to those seen in BCG-vaccinated boar, suggesting an alternative to use of live BCG in the field [168].

The effective and efficient field vaccination of wildlife species requires the development of stable, species-specific oral baits as delivery vehicles for use in appropriate baiting strategies. Oral baits suitable to deliver pharmaceuticals to free-living wild boar have been developed, as well as techniques to improve specificity and uptake rate in overabundant wild boar populations [164, 167]. Safety experiments yielded no shedding of BCG in feces, even after oral delivery of high doses [169]. Marker studies revealed no bait uptake

by nontarget species [170]. In 2010, the Spanish Ministry of Agriculture listed research on wildlife vaccination as a priority in the tuberculosis control strategy. Controlled field experiments with live BCG and the heat-inactivated *M. bovis* vaccine are scheduled for 2012.

5.4. Other Species. Although the single most important *M. bovis* maintenance host in the Iberian Peninsula is the wild boar. In actuality, maintenance of *M. bovis* involves a complex, multihost system in which transmission occurs among multiple wild species (wild boar, red deer, fallow deer, and badgers), cattle, and to a lesser extent livestock such as pigs and goats [171].

6. Conclusions

Transmission of *M. bovis* across the wildlife-domestic animal interface represents a significant obstacle to bovine tuberculosis eradication efforts in many countries around the world. In spite of long-standing, expensive, and somewhat successful efforts over many decades, animal health officials have found that traditional test and slaughter methods, the centerpiece of most bovine eradication programs, of limited success when affected cattle herds are surrounded by wildlife infected with *M. bovis*. A few countries have implemented various programs intended to decrease the relevant wildlife population density to such a level that interspecies and intraspecies transmission is virtually extinguished. Another commonly used tool is the creation of physical barriers (e.g., fences, animal housing) between cattle and wildlife to mitigate direct and indirect contact. In some cases this requires changing traditional agricultural practices (e.g., methods or location of livestock feeding) that have existed for decades. Such measures have been moderately successful, but in each case animal health officials have concluded that more tools are needed. Wildlife vaccination may be that much needed tool. Significant research will be required on vaccine delivery, safety, and efficacy. As each country battles a different wildlife host, research on each of the reservoir maintenance hosts will be required, as extrapolation of information from one species to another may not be appropriate nor relevant. As such there is a continued need of knowledge of the biology, immunology, and pathology of tuberculosis in each host. Development of successful mitigation strategies will likely require combined efforts and input from the global tuberculosis research community, federal, and regional policy makers (including both animal health and wildlife disease experts), as well as livestock and natural resources stakeholders.

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 931948, 8 pages
doi:10.1155/2012/931948

Research Article

Longitudinal Pathogenesis Study of Young Red Deer (*Cervus elaphus*) after Experimental Challenge with *Mycobacterium avium* subsp. *paratuberculosis* (MAP)

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Received 9 December 2011; Accepted 15 April 2012

Academic Editor: Michael D. Welsh

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Paratuberculosis progresses more quickly in young red deer than in sheep or cattle. This study describes the clinical, immunological and pathological changes over a 50-week period in fourteen 4-month-old red deer that received heavy oral challenge with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). At 4 and 12 weeks post challenge they were anaesthetized and a section of jejunal lymph node was surgically removed for culture, histopathology, and genetic studies. All 14 deer became infected, none were clinically affected, and they had varying degrees of subclinical disease when killed at week 50. Week 4 biopsies showed no paratuberculosis lesions, but MAP was cultured from all animals. At weeks 12 and 50 histopathological lesions ranged from mild to severe with corresponding low-to-high antibody titres, which peaked at 12–24 weeks. IFN-γ responses peaked at 8–15 weeks and were higher in mildly affected animals than in those with severe lesions.

1. Introduction

Paratuberculosis (Johne's disease), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), can cause significant loss of production in red deer (*Cervus elaphus*) on farms in New Zealand [1, 2]. Young deer are particularly susceptible [3] and clinical disease often occurs in 8–27-month-old animals [1], compared with 2–4 years in sheep and cattle. Clinically affected young deer typically have diarrhoea, lose weight, and have some thickening of the small intestines and grossly enlarged mesenteric lymph nodes, often with tuberculosis-like caseous lesions [2].

Although early immune responses have been studied in cattle [4, 5] and in sheep [6], there is little published information about the progression of disease and immunological responses over the 12 months after challenge with MAP in red deer. Early innate and acquired immune responses are thought to play important roles in determining the pathogenesis and severity of the disease.

An experimental paratuberculosis challenge model for red deer has been developed to reproduce a typical range of disease outcomes from mild subclinical infection to clinical disease [7]. This challenge model has been used to study the dose response and differences in susceptibility of deer to ovine and bovine strains of MAP [8], protective efficacy of vaccines [9], and age susceptibility [3].

The two main objectives of this study were to monitor the clinical, immunological, and pathological changes over the course of a year and to collect biopsy samples of jejunal lymph node at three time points for future study of gene expression related to resistance/susceptibility MAP.

2. Materials and Methods

2.1. Animals. Seventeen red deer calves were born to 22 randomly chosen mixed-age seronegative hinds that had been inseminated with frozen semen from two unrelated red stags (7 to sire 1, 10 to sire 2) on a farm which had no previous history of clinical paratuberculosis and has been tested free of bovine tuberculosis for 30 years. These calves were weaned in late February when they were ~3 months of age. They were treated in early March with pour-on moxidectin (Cydectin; Fort Dodge Animal Health Ltd., Auckland, NZ), dosed with copper oxide wire particles (4 g Copper Capsule; Bayer NZ Ltd., Auckland, NZ), and vaccinated with Yersiniavax (AgVax Developments Ltd., Upper Hutt, NZ). This study was approved by the AgResearch Invermay Ethics Committee (AEC 11425).

2.2. Challenge. In late March 2008, the 17 deer, which were all Paralisa test negative, were challenged with MAP using a standard infection model described previously [7, 8]. Briefly, each animal received an oral challenge dose daily for four days of ~10^9 cfu of a virulent bovine strain of MAP harvested directly from the jejunal lymph nodes (JJLNs) of clinically affected deer. The estimated number was based on BACTEC 12B liquid culture of serial dilutions.

2.3. Clinical Monitoring. The deer were grazed together at pasture throughout the 50-week study and their diet was supplemented with hay and barley over the winter. Their physical condition was monitored daily and they were weighed monthly in April, May, and June, two weekly in July and weekly from August to early March. The protocol required that any deer that developed early clinical signs of paratuberculosis (loss of 5–10% body weight over a two week period, loss of muscle mass, ± diarrhoea) would be euthanised.

2.4. Jejunal Lymph Node Sampling. At 4 and 12 weeks post challenge (pc) each deer was anaesthetized using intravenous fentanyl citrate, xylazine, and azaperone (Fentazin 5; Bomac Laboratories Ltd., Manukau), intubated and maintained on halothane (Halothane-Vet; Merial Ancare, Manukau) and oxygen. The animal was placed in dorsal recumbency and the abdomen was clipped, prepped, and draped. A 15 cm long midline laparotomy was performed and the gross appearance of the intestines, mesenteric lymph nodes, and mesenteries noted. A 40–50 mm piece of posterior JJLN was excised and cut into five pieces; three pieces were placed in cryotubes and immediately frozen in liquid nitrogen for future gene expression studies, one piece was fixed in 10% buffered formalin for histopathological examination, and one piece was cultured for MAP. The incision was closed with Maxon sutures (Davis & Geck, USA) in the linea alba and Vicryl sutures (Ethicon, USA) in the skin. The animals were injected with long-acting penicillin (Norocillin LA; Norbrook NZ Ltd., Rangiora) to prevent postoperative infection and with meloxicam (Metacam 20; Boeringer Ingelheim NZ Ltd., Auckland) for postoperative pain relief. The anaesthetic was reversed with yohimbine and naloxone (Contran H; Bomac Laboratories Ltd., Manukau). The deer were monitored closely for two weeks following each biopsy session.

All surviving deer were euthanised 50 weeks pc and necropsied and samples were taken from the anterior, mid, and posterior jejunum (JJ), ileocaecal valve (ICV), and associated lymph nodes and processed as for the biopsies. At biopsy and necropsy any gross lesions of paratuberculosis in the JJ, ICV, JJLN, ileocaecal lymph node (ICLN), and mesenteries were described and graded according to the following gross lesion scores: 0, no visible lesions (NVLs); 1, slightly enlarged JJLN/ICLN; 2, moderately enlarged JJLN/ICLN; 3, very enlarged JJLN/ICLN; 4, enlargement plus a single caseogranulomatous JJLN or ICLN lesion; and 5, multiple JJLN and ICLN lesions.

2.5. Immune Monitoring. Blood samples were taken at regular intervals during the trial and tested with the Paralisa test and gamma interferon (IFN-γ) assay and a comparative cervical tuberculin test (CCT) was carried out two weeks prior to slaughter. The Paralisa test is an IgG_1 antibody class-specific ELISA test using two antigens, namely, MAP protoplasmic antigen (PPA) and MAP purified protein derivative (PPDJ) and uses an in-house anti-red deer antibody [10, 11]. The results are expressed as ELISA units (EUs) calculated by $(OD_{sample} - OD_{negcontrol}) \times 100$ and if an animal has a titre >50 EU to either antigen, it is considered positive. The IFN-γ assay uses an in-house ELISA assay [12] to measure IFN-γ responses in heparinised blood samples stimulated with PPDj. Briefly, 96-well flat-bottomed microtiter plates were coated with IFN-γ capture antibody (Serotec). Wash buffer only was added as a negative control, and recombinant cervine IFN-γ was added as a positive control. The detection system used biotinylated antibody (Serotec) and streptavidin-horseradish peroxidase conjugate. Plates were read in an ELISA reader at 450 nm with reference at 650 nm. The results were expressed as (PPDj − neg control) in ELISA units. For the CCT, intradermal injections of 0.1 mL of avian tuberculin (2500 IU) and bovine tuberculin (5000 IU) were given at two closely clipped sites on the neck. Skin thickness measurements were taken before injection and ~72 hours later and the increase in skin thickness was calculated.

2.6. Culture. All tissue and faecal samples (5 g) were cultured for MAP using BACTEC 12B liquid culture medium containing egg yolk and mycobactin [13, 14], which gives semiquantitation according to the speed at which the samples turn positive; that is, the number of days to positive (dtp), with the cultures read every 5–7 days.

The JJLN biopsy samples taken at week 4 and week 12 pc were chilled and cultured within 48 hours of collection. At week 50 a pool of four intestinal samples (anterior, mid and posterior JJ and ICV) and a pool of four corresponding lymph node samples (JJLN and ICLN) from each animal were collected immediately after death, chilled overnight, and cultured the next day. Faecal samples collected from the rectum at week 50 pc were chilled overnight, processed using the double incubation method [15], and then cultured.

2.7. Histopathology. Sections of fixed samples from each animal were stained with H&E and Ziehl-Neelsen (ZN) and the histopathological lesion severity scores (LSSs) were assessed using a modified 7-point scale for both the mesenteric lymph nodes (MLN) and the enteric lesions. The week 50 MLN and enteric scores were added together for a total LSS score on a 14-point scale [16, 17]. Sections were examined without knowledge of animal breeding or gross lesions. For MLN and enteric lesions, LSSs of 1 and 2 were regarded as very mild or nonspecific, 3 as suggestive of very mild paratuberculosis, 4 as mild, 5 as moderate, 6 as moderately severe, and 7 as severe paratuberculosis. For the total LSS additive scores of 4–8 were mild, 9–11 were moderate, and 12–14 were severe paratuberculosis. Lesions were also described as no acid-fast organisms (AFOs), paucibacillary (PB), or multibacillary (MB) [16].

2.8. Statistical Analysis. There were insufficient animals in this study for meaningful statistical analysis.

3. Results

3.1. Clinical Outcomes. No animals showed signs of clinical paratuberculosis and the deer all gained weight throughout the study, with hinds averaging 35 kg and stags 56 kg weight gain over the 50 week study, and there was no significant difference in weight gain between Sire 1 and Sire 2 offspring. Three deer died of misadventure unrelated to paratuberculosis.

3.2. Gross Lesion Score. There were no gross lesions apparent in the JJLN at week 4, but by week 12 half the deer had slightly or moderately enlarged JJLN (Table 1). At week 50 four deer had very enlarged JJLN and one of these animals had multiple caseous foci, while the other ten deer had no or slight enlargement of the JJLN.

3.3. Histopathological Lesion Severity Score. There were no specific lesions seen at Week 4, but by Week 12 MLN LSSs ranged from mild to severe (4–7) (Table 1), although these were scored conservatively because they were based only on a small piece of posterior JJLN. At Week 50 MLN LSSs ranged from 2 to 7, and total LSS, from 2 to 14. One animal had no visible AFO, three had no AFO but were classed as PB, one was MB, and the rest were PB.

Over the period from Week 12 to Week 50 the following changes in MLN LSS occurred; 11 of 14 reduced, one increased and two remained the same. Of those that became less severe, one went from severe to very mild, two from severe to mild, three from severe to moderate, one from severe to moderately severe, one from moderate to mild/nonspecific, two from moderate to mild, and one mild went to nonspecific. The one that increased in severity went from mild to moderate. One severe and one moderately severe remained the same. At Week 50, MLN and enteric LSS generally had similar scores.

Interestingly, an increased concentration of AFO in calcified tissue present in two of the animals at Week 50 was associated with PB, as per criteria described by Clark et al. in 2010 [16]. The same animals were MB at week 12.

There was no apparent difference in LSS, the changes in LSS with time or PB/MB ratio between offspring of the two sires throughout the study.

3.4. Culture. MAP was isolated from all JJLN biopsies at weeks 4 and 12 and from pooled LNs at Week 50 of all 14 deer (Table 2). Culture times for JJLN of 28–42 dtp at Week 4 suggest small numbers of MAP present, but by Week 12 this had reduced to 12–21 dtp, indicating an increase in numbers of MAP, with the three least affected animals averaging 19 dtp compared with 13 dtp for the most severely affected. By Week 50, the least affected had increased to 31 dtp, and the worst affected increased slightly to 18 dtp. At Week 50 the dtp for JJ + ICV tended to be higher than that for JJLN + ICLN. At slaughter only 4/14 were faecal-culture positive (31–37 dtp) indicating relatively low numbers of MAP present.

3.5. Serology. Over the course of the 50 week study, four of the animals (506, 509, 511, and 512) had low titres to Johnin throughout and all peaked at <60 EU (Figure 1(a)) and three of these had mild-to-moderate lesions (total LSS 2–8). By contrast, four animals (508, 513, 510, and 507) had high titres 151–172 EU that peaked 15–20 weeks pc and tended to remain high (Figure 1(a)) and had moderate-to-severe lesions (total LSS 8–12). The remaining six animals had intermediate titres 99–146 EU that peaked 12–26 weeks pc and tended to decline to much lower levels (Figure 1(b)) and had mild-to-severe lesions (total LSS 6–12). Animals with higher titres (504, 508, 510, 514, and 517) tended to rise at Week 50, two weeks after the skin test at Week 48, while animals with the lowest titres (500, 501, 502, 506, 509, 511, 512) do not rise.

3.6. IFNγ. The IFNγ of the least affected animals (total LSS 2–7) rose earlier and peaked higher than those of the more severely affected animals (total LSS 11-12) during the 50-week study (Figure 2). The IFNγ responses peaked twice, with the first peak at 12–15 weeks pc, a decline to a low at 24 weeks, and a second peak at 30–36 weeks. Only the LSS 2–7 group had an increase in IFNγ at Week 50, 2 weeks after the skin test.

3.7. CCT. The increase in skin thickness at the avian and bovine sites averaged 9.7 mm (1.8–13.4) and 3.1 mm (0.6–8.4), respectively, with $A > B$ in all animals; that is, negative for bovine Tb. The average avian site increase for the three most severely affected deer was 8.2 mm (6.5–10) compared with 3.5 mm (1.8–5) for the least affected.

4. Discussion

The red deer MAP infection model allowed a number of variables to be controlled so that their immune response to challenge could be studied efficiently. The offspring were all the same age, having been bred on the same day by artificial insemination of synchronised hinds, which calved

Table 1: Gross lesion score (Weeks 12 and 50), histopathological lesion severity scores (LSSs) for mesenteric lymph node (MLN), enteric (ENT) and total, and description of acid fast organisms (AFO) in the lesions as none, paucibacillary (PB), or multibacillary (MB) for samples biopsied at weeks 4 and 12 and taken at week 50 necropsy of the offspring of two unrelated sires.

Sire	Tag	Week 4		Week 12			Week 50				
		Sex	MLN LSS	Gross	MLN LSS	AFO	Gross	MLN LSS	ENT LSS	Total LSS	AFO
1	506	F	2	0	5	PB	0	2	0	2	None
1	511	F	0	0	4	PB	0	2	2	4	PB
1	513	M	0	2	7	MB	0	4	4	8	PB
1	508	F	0	2	7	MB	4	6	6	12	PB
1	507	M	0	2	7	MB	5	7	5	12	MB
2	514	M	0	2	7	PB	4	4	2	6	PB
2	504	F	0	1	7	PB	0	3	4	7	PB
2	509	M	0	0	5	PB	0	4	4	8	PB
2	502	M	0	2	7	PB	3	5	4	9	PB
2	501	M	0	0	7	MB	0	5	5	10	PB
2	500	F	0	0	5	MB	0	4	6	10	PB
2	512	M	0	0	5	MB	1	5	6	11	PB
2	510	M	2	2	7	MB	1	5	6	11	PB
2	517	F	0	0	5	MB	0	6	6	12	PB

Gross lesion scores: 0, no visible lesions (NVLs); 1, slightly enlarged jejunal lymph nodes (JJLNs) and/or ileocaecal lymph nodes (ICLNs); 2, moderately enlarged JJLN/ICLN; 3, very enlarged JJLN/ICLN; 4, enlargement plus a single caseogranulomatous JJLN or ICLN lesion; 5, multiple JJLN and ICLN lesions. MLN and ENT LSSs of 1 and 2 were regarded as very mild nonspecific, 3 as suggestive of very mild paratuberculosis, 4 as mild, 5 as moderate, 6 as moderately severe and 7 as severe paratuberculosis. Lesions were also described as no AFO, paucibacillary (PB), or multibacillary (MB).

Table 2: Summary of BACTEC culture results in terms of the number of days to positive (dtp) for jejunal lymph node (JJLN) samples taken at Weeks 4 and 12, and for a pool of three samples of JJLN plus ileocaecal lymph node (ICLN) and a pool of three jejunum and ileo-caecal valve samples (JJ + ICV) and faecal samples (FSs) at week 50, compared with MLN lesion severity scores (LSSs) at each time point, with the animals sorted by total LSS at week 50 and designated as least or most affected.

Tag	Sire	Week 4		Week 12			Week 50			
		MLN LSS	JJLN	MLN LSS	JJLN	MLN LSS	JJLN + ICLN	JJ + ICV	FS	Affected
506	1	2	36	5	21	2	34	34	Neg	Least
511	1	0	36	4	21	2	34	34	Neg	Least
509	2	0	36	5	15	4	26	26	Neg	Least
Mean			**36**	5	**19**	**3**	**31**	**31**		
514	2	0	28	7	15	4	34	26	Neg	
504	2	0	32	7	15	3	26	21	Neg	
513	1	0	32	7	15	4	18	18	31	
502	2	0	36	7	12	5	18	26	Neg	
501	2	0	42	7	12	5	18	34	37	
500	2	0	35	5	12	4	21	18	Neg	
510	2	2	36	7	12	5	18	26	Neg	
512	2	0	36	5	12	5	18	34	31	
Mean			**35**	**6**	**13**	**5**	**21**	**25**	**33**	
517	2	0	36	5	12	6	18	26	Neg	Most
508	1	0	32	7	15	6	18	26	Neg	Most
507	1	0	36	7	12	7	18	18	37	Most
Mean			**35**	7	**13**	**6**	**18**	**23**	**37**	

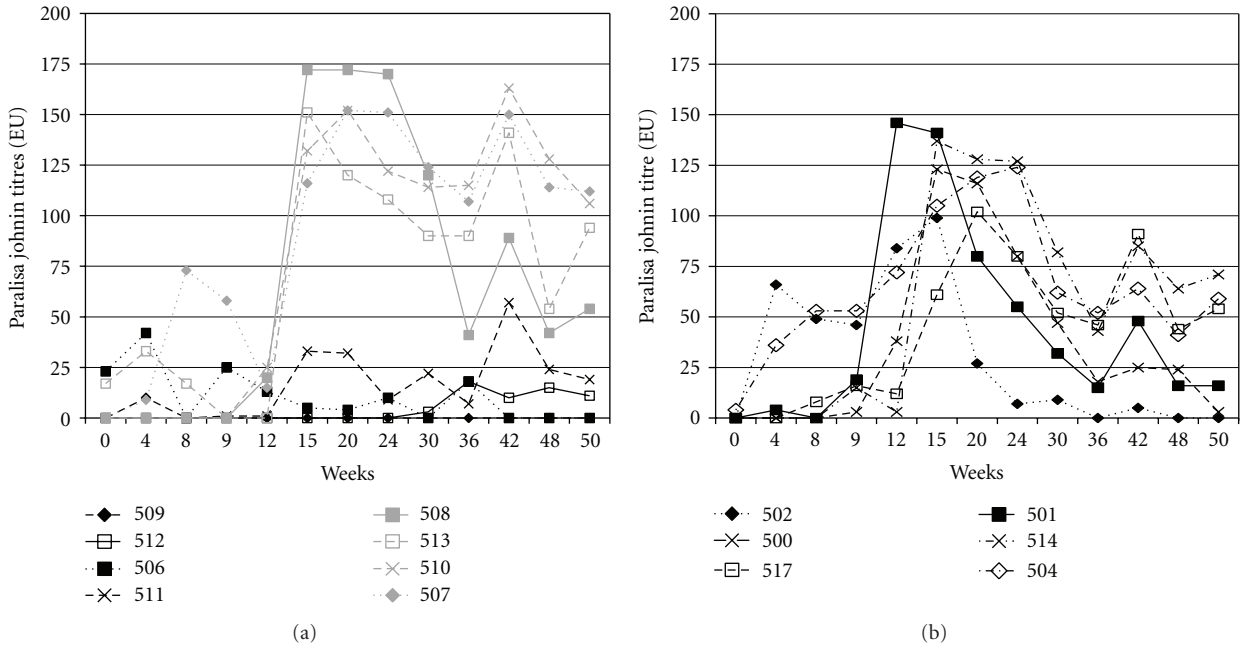

Figure 1: (a) Paralisa titres (PPA antigen) in ELISA units (EU) over the 50 week study for the four deer (506, 509, 511, 512) that had the lowest serological response to the Paralisa Johnin antigen (black lines), and the four deer (507, 508, 510, 513) that had the highest and most sustained serological response to Johnin (gray lines). (b) Paralisa titres (PPA antigen) in ELISA units (EU) over the 50 week study for the six deer that had an intermediate serological response to the Paralisa Johnin antigen.

within a few days of each other. The animals were all run together in one mob from birth, treated regularly with anthelmintics, vaccinated against yersiniosis, treated with mineral supplements, grazed at pasture, and given the same infective dose of MAP on the same days. The only common variable that was not controlled was gender, but there was no obvious gender bias in the results. In previous studies a proportion of young red deer challenged by MAP have developed clinical disease [3, 8, 9]. In this study, although the age of animal and the strain of MAP were the same as previously, there were no clinically affected animals and few had severe disease, suggesting that the offspring of both sires had intermediate resistance to MAP, or the inoculum had fewer MAP than previously.

The use of the infection model together with the sampling of jejunal lymph nodes at 4, 12, and 50 weeks pc and periodic serology and IFN-γ assays enabled us to gain insights into the pathogenesis of MAP infection as well as longitudinal information about the immune responses. Deer appear to be relatively susceptible to paratuberculosis and the disease progresses much more quickly in susceptible deer than that in sheep and cattle. Under natural farm conditions, clinical cases commonly occur in red deer between 8 and 15 months of age [1], whereas clinical disease is more commonly seen in sheep and cattle aged 3-4 years, which make natural disease harder to study in a reasonable time frame. The scoring of lesion severity [16, 17] based on histopathological examination of biopsy and necropsy samples, together with MAP culture times, enabled objective longitudinal observations of disease within and between animals over time. The scoring of lesion severity for the biopsy samples at Weeks 4 and 12 was conservative because it was only based on a single piece of posterior JJLN, whereas the samples taken at necropsy were based on four samples of anterior, mid, and posterior JJ and ICV plus their associated lymph nodes.

There have been a small number of studies in sheep and cattle that have used surgical biopsies of small intestine and mesenteric lymph nodes to diagnose paratuberculosis [18–20] or to follow the course of infection and to attempt to associate these changes with immunological measurements [6, 21, 22]. Gilmour et al. biopsied five experimentally infected Cheviot x Suffolk sheep on four occasions 5, 11, 17, and 23 months and at necropsy 27 months pc while Dennis et al. monitored a total of 77 naturally infected Merino sheep and biopsied 20 of these at 12, 18, and 24 months of age. Clinically affected sheep were euthanised and necropsied, as were all the remaining sheep 36 months of age. Gilmour et al. also killed groups of sheep at intervals and necropsied them as well as clinically affected animals. Both these groups obtained a wide range of disease outcomes over a 27–36-month period. Wu et al. infected one-month-old calves and then monitored disease progression by taking biopsies of the small intestine and mesenteric lymph node at 1-2-month interval and calves were killed at 6–9 months of age. There were no signs of clinical disease, although they detected changes in the inflammatory responses and the levels of cytokines indicated Th1-type-associated cellular, but not Th2-type-associated humoral responses. In contrast the deer model used in this study has the advantage of reproducing a range of natural disease outcomes in a one-year study.

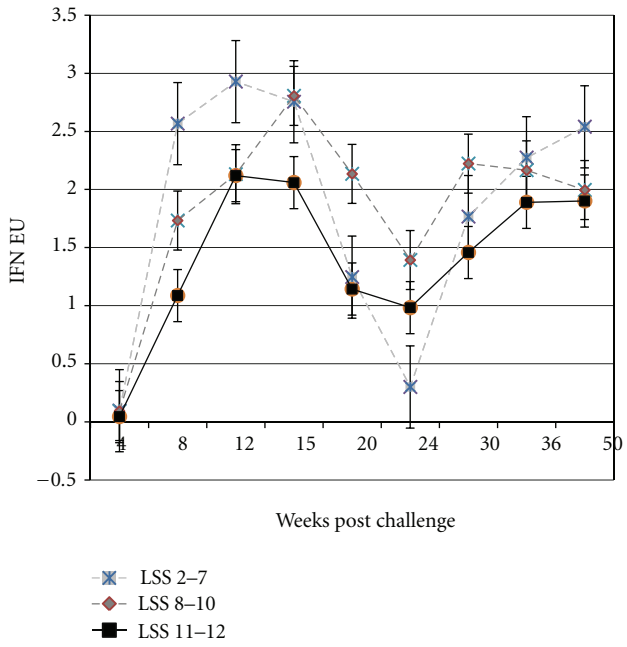

Figure 2: Mean gamma interferon (IFN-γ) assay results in ELISA units (EUs) for the 14 deer sorted into three groups based on their total lesion severity score (LSS) at week 50 after MAP challenge at day 0.

An interesting outcome of this study was the spectrum of changes in disease severity that took place over the course of a year. At Week 4 MAP was cultured from the JJLN of all animals but there were no paratuberculosis-specific lesions seen on histology. Between Weeks 4 and 12 all the deer showed an increase in MAP numbers and they developed JJLN lesions ranging from mild to severe. However, MAP numbers increased more in the animals that went on to become more severely affected, suggesting that differences in relative resistance/susceptibility were influencing the development of lesions and rate of MAP multiplication. Between Week 12 and Week 50, some animals remained mildly affected, some remained severely affected, while some improved and others got worse. About half the animals managed to suppress the multiplication of MAP and limit the extent of disease, such that the LSS and the number of MAP in the JJLN declined, as indicated by an increase in dtp. In contrast, some of the animals appeared to be unable to suppress MAP multiplication, their LSS worsened, and number of MAP present increased or stayed the same over this 9-month period. Two animals that were MB at Week 12 were found to be PB at Week 50 but had a few foci of high numbers of AFO in mineralized tissue. This type of lesion has been observed previously in naturally infected animals [16] and the pathogenesis appears to relate to a previous MB lesion phase, which is a new finding.

The most severely affected animals also had the highest antibody titres, while the two least affected animals remained seronegative throughout the study and had higher earlier IFN-γ levels than the more severely affected animals (LSS 5–7). These findings support the view that the more resistant animals have a more effective Th1 cell-mediated immune response, while the more susceptible animals switch to a Th2 humoral response earlier [23]. The results suggest that innate immunity may have played only a minor role in the killing or suppression of multiplication of MAP in the first 4 weeks pc and that acquired immunity played a major role in the more resistant animals. It is interesting to note that even the relatively resistant animals still took some months to get on top of the infection and to reduce the number of MAP as well as lessen the apparent severity of the lesions. It is likely that the migration of infected macrophages and dendritic cells from the small intestine to the draining lymph node is essential for efficient local T-cell activation in paratuberculosis, in the same way that dendritic cell migration from the lungs to the draining lymph nodes is thought to be critical in T-cell activation in TB [24]. This appears to have occurred within a few weeks of MAP challenge in deer. It was not practical in this study to biopsy the small intestine at the same time as the JJLN, but monitoring the changes in the JJLN gave useful longitudinal insight into the immunological and pathological changes over the 50 weeks.

Similar observations have recently been reported in naturally infected sheep that were monitored over a 36-month period using lymph node and intestine biopsies [6]. Sheep that developed severe multibacillary enteritis never improved over time, and the affected sheep expressed clinical signs within 12 months of showing these severe lesions. However, six of the 46 (8.7%) sheep that had MAP culture positive biopsies at some stage, later had negative cultures at necropsy, suggesting recovery from infection. The study also showed that infection was never detected in 40.3% of the sheep in this flock, which were exposed to heavy natural challenge, suggesting that some animals were resistant to infection.

The longitudinal changes in lesion severity and number of MAP in this study are similar although not as extreme as those seen in a subsequent study undertaken in 2009, which also showed significant heritable resistance/susceptibility to experimental paratuberculosis challenge in the offspring of two sires that had displayed differences in resistance/susceptibility to paratuberculosis [25].

5. Conclusions

The longitudinal monitoring of histopathology and culture of the jejunal lymph nodes, as well as serology and IFN-γ responses, have given some insight into the pathogenesis of subclinical paratuberculosis in young red deer. Heavy oral challenge resulted in all animals becoming infected, but the course of infection varied greatly. MAP numbers increased in all animals up to 12 weeks pc and MLN lesions were mild to severe. However, MAP numbers increased more in the animals that went on to become more severely affected, suggesting that differences in relative resistance were influencing the development of lesions and rate of MAP multiplication. Between Week 12 and Week 50, half the animals managed to largely suppress the multiplication of MAP and limit the amount of disease, while others appeared unable to suppress MAP multiplication and their lesions

became more severe. The most severely affected animals also had the highest antibody titres, while the two least affected animals remained seronegative throughout the study. This, together with the observation that the least affected animals had earlier and higher IFN-γ responses, supports the view that the more resistant animals had a more effective Th1 cell-mediated immune response. Innate immunity did not prevent infection in any of these animals. However, the decline in the severity of disease between 12 and 50 weeks pc suggests that acquired immunity played a major role in the more resistant animals.

Acknowledgments

The authors wish to acknowledge the assistance of staff at AgResearch Invermay, the Disease Research Laboratory, Otago University, and the Wallaceville Tb Laboratory for field work and laboratory work in carrying out this study. The study was funded by the Johne's Disease Research Consortium.

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 674238, 13 pages
doi:10.1155/2012/674238

Review Article

Opportunities for Improved Serodiagnosis of Human Tuberculosis, Bovine Tuberculosis, and Paratuberculosis

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Received 29 January 2012; Accepted 2 April 2012

Academic Editor: Mitchell Palmer

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Mycobacterial infections—tuberculosis (TB), bovine tuberculosis (bTB), and Johne's disease (JD)—are major infectious diseases of both human and animals. Methods presently in use for diagnosis of mycobacterial infections include bacterial culture, nucleic acid amplification, tuberculin skin test, interferon-γ assay, and serology. Serological tests have several advantages over other methods, including short turn-around time, relatively simple procedures, and low cost. However, current serodiagnostic methods for TB, bTB and JD exhibit low sensitivity and/or specificity. Recent studies that have aimed to develop improved serodiagnostic tests have mostly focused on identifying useful species-specific protein antigens. A review of recent attempts to improve diagnostic test performance indicates that the use of multiple antigens can improve the accuracy of serodiagnosis of these mycobacterial diseases. Mycobacteria also produce a variety of species-specific nonprotein molecules; however, only a few such molecules (e.g., cord factor and lipoarabinomannan) have so far been evaluated for their effectiveness as diagnostic antigens. For TB and bTB, there has been recent progress in developing laboratory-free diagnostic methods. New technologies such as microfluidics and "Lab-on-Chip" are examples of promising new technologies that can underpin development of laboratory-free diagnostic devices for these mycobacterial infections.

1. Introduction

Mycobacterial infections are a leading cause of health concerns in humans and animals worldwide. *Mycobacterium tuberculosis* (MTB), *Mycobacterium bovis* (MB), and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) are the causative agents of human tuberculosis (TB), bovine tuberculosis (bTB), and Johne's disease (JD), respectively. In 2009, more than 9 million cases of TB were reported, causing 1.8 million deaths [1]. Multidrug-resistant TB strains and coinfections of TB and HIV are emerging problems globally [2–4]. Despite much progress in eradicating bTB in developed countries, this disease is responsible for US$ 3 billion economic losses globally [5] and remains prevalent in some wild species [6, 7]. MAP is present in 68% of US dairy herds [8], with JD responsible for an annual $220 million economic loss to the US dairy industry [9].

Control measures for these mycobacterial diseases revolve around understanding their epidemiology and improving treatment/vaccination protocols; however, a major bottleneck has been the lack of efficient diagnostic methods [2, 10–12]. Consequently, there would be much benefit to the development of rapid and accurate diagnosis of TB at point-of-care [3] (In this paper, point-of-care diagnosis is defined as diagnostic methods that can be conducted on-site (e.g. field, bed-side), with or without a requirement for laboratory facilities. Laboratory-free (lab-free) diagnosis is defined as point-of-care diagnostic methods that does not require any laboratory facility). Similarly, the most common current diagnostic test for bTB, the tuberculin skin test (TST), is not practical for controlling bTB in wild animals, so a lab-free diagnostic device would also be helpful in this context. Diagnosis of JD is currently conducted annually or biannually in diagnostic laboratories. If a lab-free diagnostic

device became available, it would reduce the long time interval and cost of diagnosis. Thus, there would be a great value in lab-free diagnostic technologies for TB, bTB, and JD [13, 14].

Unfortunately, efficient lab-free diagnostic devices for these diseases are not yet available [14, 15]. Here, therefore, we briefly review currently available and recently developed diagnostic methods for these three mycobacterial diseases and highlight the potential benefits of lab-free diagnosis. Since serodiagnosis has been the most favored format for development of lab-free diagnostic method, we focus in this paper on methods of serodiagnosis over other diagnostic methods such as bacterial culture and nucleic acid amplification that are necessarily laboratory based.

2. Human Tuberculosis

2.1. Background. Human tuberculosis (TB) is caused primarily by MTB and occasionally by MB and *M. africanum* (in this paper we focus on MTB). TB is a leading cause of human morbidity and mortality throughout the world [16]. One-third of the world's population is infected by MTB [1], although only 5–10% of infected individuals develop an active, life-threatening form of the disease. In 2009, 9.4 million cases of TB were reported with 1.8 million deaths worldwide [1, 2, 17].

Depending on the pathogenesis, infectivity, immune response, and effectiveness of treatment, TB can be divided into 3 major forms. The first is the active form of TB (TBA), which results in a rapid development of clinical signs in patients following contact with MTB. TBA develops in only 5% of individuals infected with MTB; the remainder develops a strong acquired immune response showing no clinical signs, termed latent TB (TBL) [18]. The third form is multidrug-resistant TB (MDRTB), which constitutes approximately 5% of TBAs [19]. MDRTB is caused by organisms resistant to, at least, isoniazid and rifampin [20]. The overall prevalence of MDRTB in developed nations is much lower than that in developing nations, but can be high in immigrant populations and among prisoners and immunocompromised individuals [21, 22]. During the past two decades, the emergence of HIV infection has led to the recognition that TB/HIV coinfection promotes both the reactivation of TBA from TBL and also the rapid progression of primary TB following recent exposure to MTB [23].

Controlling TB depends on the following factors: case detection, treatment of individuals with TBA, improving anti-TB therapy to prevent resistance, identification of TBL, and better vaccination strategies for susceptible individuals [16]. All these factors would benefit from a better understanding of the epidemiology of the TB infection [21] and the development of more cost-effective, evidence-based approaches for its diagnosis [22]. Efficient diagnosis of TB is particularly important in third world nations that presently lack adequate diagnostic resources at primary health care centers. In these nations, TBL and MDRTB often remain undiagnosed, which facilitates further transmission.

Presently, there are a number of alternative diagnostic approaches towards diagnosis of TB and of TB coinfection with other emerging infectious diseases; these are reviewed briefly here.

2.2. Imaging and Microscopic Techniques. Radiographic imaging is still widely used to diagnose TB; however, there are no definitive diagnostic patterns, so that the method can be used only for screening of TB cases. Further bacteriological examinations are required for confirmation [64, 65]. Smear microscopy of stained sputum or other clinical material is the most common test for TBA. This relatively inexpensive method can be carried out rapidly in low-resource settings; however, it lacks sensitivity and requires a large number of bacilli (5,000–10,000 organisms/sample) [64, 66] in the clinical specimen, which is often not the case in children, advanced-stage TBA patients, and individuals coinfected with HIV. Fluorescent microcopy is more sensitive, but its application is limited by high cost and by issues relating to the use of mercury vapor lamps in conventional fluorescent microscopes [67]. Nucleic acid amplification (NAA) assays have been found useful for diagnosis of TBA and MDRTB infections, as they have high specificity and sensitivity and can provide results within a few hours. Unfortunately, these assays are costly, require a laboratory with trained staff, and suffer from poor specificity under field conditions [64, 68, 69].

2.3. Bacterial Culture and Cell-Mediated Immune-Response-Based Testing. Bacterial culture is considered the gold standard for TBA diagnosis, having close to 98% specificity, and is also useful in diagnosis of MDRTB. However, the bacterial culture method suffers from low sensitivity (26–42%), delayed results (6–8 weeks are required for culture growth), a need for trained personnel and culture facilities, and the high cost of the culture examination. The need for technical expertise can be particularly problematic in developing nations. Parsons et al. have recommended new technologies including urine antigen detection, assays based on volatile markers, bead-based, and flow-cytometric-based assays [3]—to help address these problems, but these assays await optimization and establishment of clinical utility.

The tuberculin skin test (TST)—based on detection of delayed-type hypersensitivity after an intradermal injection of purified protein derivative (PPD) extracted from heat killed MTB—has been in use for almost a century. The primary roles of TST are to identify TBL individuals and to monitor recent infection in high-risk groups. Some limitations of TST include a high frequency of false reactions, the need for a follow-up visit after 2-3 days of PPD inoculation, misleading results due to confounding factors (e.g., age, HIV infection, and infection with other mycobacterial species or cancer), and positive reactions in TBA patients [64, 67, 70]. Based on the identification of MTB-specific antigens using molecular techniques, detection of cell-mediated immune (CMI) response against MTB infection has improved the diagnosis of TB. These assays measure the production of cytokines (mainly interferon-gamma [IFN-γ]) produced by T cells of MTB-infected individuals. Initial IFN-γ assays were based on PPD antigen, but later the antigen was replaced by MTB-specific antigens, such as early-secreted

antigenic target (ESAT-6) and culture filtrate protein (CFP-10) [71]. IFN-γ assays do provide an improved diagnosis of TBL; however, since they detect the presence of the host's CMI response towards MTB antigens, fresh blood samples are required for the test. Inability to differentiate between TBA and TBL, poor reproducibility, and reduced efficacy in children are additional problems of the CMI-based diagnostic tests [72]. In developing countries, TST is still preferred over IFN-γ assay due to its lower cost but suffers from low efficacy in children, poor reproducibility, and reduced diagnostic accuracy for TBL [72–74].

2.4. Humoral-Immune-Response-Based Testing. In circumstances where medical resources (facilities and health care providers) are limited, serodiagnostic methods for detection of anti-MTB antibodies have some advantages (i.e., simplicity, low cost, and requirement of minimum medical resources) over aforementioned diagnostic methods [75]. Several target molecules (antigens) have been used to detect the humoral responses (anti-MTB antibodies) in TB patients. Early assays used PPD or other crude extracts as antigens for capturing anti-MTB antibodies; however, these showed poor specificity as dominant antibody responses are against cross-reactive antigens (i.e., antigens commonly found in MTB and also in other mycobacteria) [24]. As molecular techniques have improved, many antigens have been evaluated in serological tests, especially in the format of the enzyme-linked immunosorbant assay (ELISA). Some major antigens used in such tests are discussed below.

Antigen 5, also known as 38 kDa antigen, is the best studied and most available antigen for MTB diagnosis due to its expression in the *E. coli* system. Many attempts to develop an improved serological assay for TB have used this antigen [30, 76]. Early studies reported 89% sensitivity and 100% specificity in TBA patients [31]. Later studies showed even higher sensitivity and demonstrated a correlation between antibody level and bacterial load [77–80]. As summarized in a review article [81], detection of antibodies against Antigen85 complex in ELISA formats achieves 50% sensitivity; however, this complex is highly cross-reactive and often generates false-positive results in individuals infected with atypical mycobacteria. A cell wall component, called a cord factor (trehalose-6,6′-dimycolate), used as antigen in ELISA format achieved 84% sensitivity with 100% specificity [32]. However, in a subsequent study, it was shown that anticord factor antibodies decline after antituberculous chemotherapy, which makes it difficult to determine the status of the infection in such patients [33]. Studies of the serodiagnostic potential of ESAT-6 [34, 35] and CFP-10 [34, 35, 39, 40] have also been conducted. One showed low sensitivity (67%) and specificity (51%) for ESAT-6 [34]. Low sensitivity (48–63%) also has been reported for CFP-10 [34, 82]. In high incidence areas, neither ESAT-6 nor CFP-10 antigens are useful in differentiating between TBA and TBL [34]. Another antigen, Kp 90, has been used in ELISA format to detect IgA antibodies against the protein; the results, when compared with NAA and other serological assays, indicated that anti-Kp 90 antibodies were detected in 78% of serum samples and 69% of samples from synovial, cerebrospinal, and abscess body fluids [41].

Antigen 60 (A60) is the main thermostable component of PPD [83, 84]. Many studies have used this antigen and found almost 100% specificity [42], with sensitivity ranging from 68 to 91% [43, 85]. Unfortunately, this molecule has also been found in nonpathogenic *Nocardia* and *Corynebacterium* species [83]. A 30 kDa antigen (isolated from a culture filtrate of MTB, Antigen 85B) was used in dot immunoassay, and the result was compared with that of standard plate ELISA. The specificities of the dot immunoassay and ELISA were 92% and 97%, respectively, and the sensitivities in the assays were 69% and 78%, respectively [44]. Further studies showed that this antigen not only diagnosed TBA but also detected the nonprotective immune response of a healthy household contact group [86].

Malate synthase (MS), a 81 kDa protein (present in MTB culture filtrates, cell wall, and cytoplasmic subcellular fractions) is an enzyme of the glyoxylate pathway used by MTB during intracellular replication in macrophages [50]. Studies with an MS-based assay have shown a sensitivity of 73% and specificity of 98% in smear positive patients, suggesting that MS is a potential candidate for TB diagnosis [82, 87]. The cell wall of MTB also contains lipoarabinomannan (LAM); however, its use as antigen in diagnostic tests is limited due to immune complex formation [3]. LAM antigen is found in urine of TBA patients, and tests based on detecting the LAM in urine samples have been developed [46, 88, 89].

Steingart et al. conducted an intensive meta-analysis of 67 studies published in 1990–2006 on commercial serological tests for TBA (e.g., Detect-TB, and a-TB ELISA, ICT TB test) [75]. Antigens used in the commercial tests include Antigen 60, 38-Kda protein, LAM, and Kp-90. The meta-analysis revealed that estimated diagnostic sensitivities (0–100%) and specificities (31–100%) in the studies were inconsistent and imprecise, which is consistent with a WHO report in 2008 [90].

In patients coinfected with HIV and MTB, the level of antibody production to TB antigens differs from that of HIV-negative TB patients. For example, an ELISA based on MS/MPT51 antigens showed positive reactions in approximately 80% of HIV-positive, TB-positive patients and in 42% of HIV-negative, TB-positive patients [51]. Wanchu suggested that better diagnosis of TB will require a focus on development of multi-antigen-based tests and identification of novel MTB proteins that increase in HIV patients [91].

2.5. Point-of-Care Diagnosis and Future Directions. The studies described above indicate the need for an improved diagnostic test that is better able to differentiate the three forms of TB infection and to diagnose TB in the presence of HIV infection. Furthermore, since most deaths due to TB occur in developing countries that lack proper laboratory facilities and specialist training, it is important to develop a simple, rapid, and cost-effective test. The Xpert MTB/RIF assay has been recently used as point-of-care diagnosis for MDRTB and drug-sensitive TB [92, 93]. Although simple to perform and highly sensitive, this assay is costly [94]. McNerney and Daley have summarized the importance of

point-of-care diagnosis [95] and suggest three important areas in which progress should be made to achieve better point-of-care for TB. The first is through identification of biological, metabolic, and pathogen-derived markers that will assist in understanding the disease. The second is the development of effective technologies like immunochromatography and nanotechnology. The third is to better understand the economical and logistic constraints on the implementation of new tests [95]. In summary, there is an urgent need to develop a lab-free diagnostic device for TB that will decrease disease transmission rate, reduce death rates, and permit faster initiation of treatment.

3. Bovine Tuberculosis

3.1. Background. Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* (MB), is an infectious, chronic but progressive disease characterized by the formation of granulomatous lesions with varying degrees of necrosis, calcification, and encapsulation [11]. MB is known to infect and cause tuberculosis in a wide range of wild animals, livestock animals, and humans. Although bTB has been mostly eradicated in the livestock industry of developed countries, the disease in wildlife still poses a risk to livestock, tourism economy, and wildlife conservation [11]. Infected wildlife species include white-tailed deer (*Odocoileus virginianus*) in several states of the USA, Eurasian badgers (*Meles meles*) in Great Britain and the Republic of Ireland, and brushtail possums (*Trichosurus vulpecula*) in New Zealand [6]. Global economic losses from bTB total US$ 3 billion annually [5]. In the USA, US$ 40 million and in Great Britain £100 million were spent on bTB management in the year 2008-2009 alone [5]. In developing countries, bTB still causes serious concerns not only for wildlife, but also for public health, food safety, and the economy of livestock industries. More accurate diagnosis of bTB would reduce the unnecessary sacrifice of healthy animals and would also help to more effectively control bTB. At present, postmortem diagnosis based on examination of gross lesions, followed by histopathology and culture, is widely used for surveillance of bTB in wild animals, but this method is time-consuming and cannot diagnose an early infection [96].

3.2. Cell-Mediated Immune-Response-Based-Testing. The ante mortem diagnostic method currently prescribed by OIE is the intradermal tuberculin skin test (TST) [97]. The TST is by far the most effective test used in the eradication of bTB in the developing countries. The test is performed by injecting a small volume of bovine tuberculin in the skin of the animal and palpating a change in the thickness of the skin at the site of injection after 48–72 hours. The tuberculin used in most of the countries is derived from cultures of MB AN5, a field strain isolated in England circa 1948 [5, 26, 96]. The TST is, however, susceptible to causing false-positive reactions due to exposure of some animals to environmental mycobacteria such as *M. avium* and MAP [96, 98–100]. TST can also cause false-negative reactions due to immunosuppression, desensitization towards tuberculin, subpotent use of tuberculin, and lengthy exposure to a field strain [96]. Steps have been taken to improve specificity by using specific antigens, such as ESAT-6 [101] and a cocktail of ESAT-6/CFP-10/MPB83; however, these studies still need to be validated at a larger scale [5].

Revisiting the animal after 2-3 days application of the TST to check their reaction is labor intensive (and usually impractical for free-ranging wildlife). The alternative IFN-γ assay is an *in vitro* blood test based on measuring the CMI response of the animals infected with MB [102]. The IFN-γ assay is usually performed using PPD as antigen, although recent studies have evaluated ESAT-6 and CFP-10 [103–106]. A problem with the IFN-γ assay is that it is a costly process that requires well-trained personnel to carry out the test [26, 107]. Bacteriological culture of clinical samples (i.e., milk, blood, nasal swab, and cattle tissues) is considered to be the gold standard for bTB diagnosis but, the test requires a minimum of several weeks [96, 108]. Nucleic acid amplification methods (e.g., PCR) have been also used for bTB diagnosis, but these methods are costly, less sensitive than the bacteriological culture test and again require a trained technician to perform the test [96, 108–110].

3.3. Humoral Immune-Response-Based Testing. Another type of immunological test is based on detection of humoral immune response (i.e., antibody production). The major advantages of the antibody-detection tests are that they are inexpensive and relatively easy to perform. However, low sensitivity of the antibody-detection tests remains a concern. Several attempts have been made to develop ELISA tests for detection of antibody response against MB infections. PPD was used as an antigen to measure antibody response in animals with MB infection [111, 112], but the cross reactivity of PPD with closely related mycobacterial species has always been a concern. Auer [113] used a sonicated preparation of MB as antigen and reported low specificity [113]. Further studies used a specific protein isolated from MB bacillus Calmette-Guerin (BCG) strain, MPB70, as an antigen for developing assays for the diagnosis of bTB. The use of MPB70 achieved better specificity (96.4%) but had poor sensitivity (18.1%) [114–116]. Ag85 complex consists of the major secretion products of MB BCG strain and has 3 major components: 85A (31 kDa), 85B (30 kDa), and 85C (31.5 kDa). This complex is strongly immunogenic and has been used for the development of assays to diagnose TB and bTB. However, low sensitivity was reported from studies using Ag85 in ELISA format and attributed to false-positive reactions caused by infections with environmental mycobacteria [54, 81, 115]. MPB83 has been used as antigen in many studies and is a very promising candidate for bTB serodiagnosis [53]. As discussed in the TB section, LAM, ESAT-6, and CFP-10 have also been used as antigens to detect antibody response against MB [117–122]. Further, as molecular biology tools have improved, recombinant proteins have come to be used as antigens for diagnosis of bTB. Since recombinant proteins can be produced at large scale, they are cost-effective and provide consistency in their quality as diagnostic antigen [55, 123, 124].

3.4. Point-of-Care Diagnosis and Future Directions. One of the promising antibody-based detection assays, Multi-Antigen Print Immuno-Assay (MAPIA), is based on immobilization of antigens onto nitrocellulose membranes by semiautomated microspraying, followed by standard chromogenic immune development. This serodiagnostic test uses a cocktail of multi-antigens, such as MPB83/70, ESAT-6, and CFP10 [36]. In a recent study, seroreactivity with MPB83 in deer was 89%; however, MAPIA showed that 26% of these were false positives [37]. Based on these MAPIA results, a new version of an immunochromatographic test format for rapid diagnosis of MB infection, called rapid test (RT), was developed using colloidal gold conjugated to protein A. RT uses recombinant proteins of MPB83 and TBF10 printed onto a membrane either separately as two bands or as a combination of the two antigens in one test line [56]. Diagnostic sensitivity of the RT in experimentally infected deer was 79%, whereas that in naturally infected deer was 67% [37]. Jaroso et al., [125] compared the RT with the comparative cervical skin test (CCT) and found similar sensitivities of 80.1%. They also found that by combining the results from both RT and CCT, the sensitivity was 100%. It was suggested that the combined uses of RT and CCT would maximize sensitivity of bTB detection [125]. Some recent studies have concluded that ESAT-6 and CFP10 (used either individually or as cocktail) are better candidates for diagnosis of bTB [126–128].

MAPIA and RT can be conducted in field situations and so can contribute to effective testing/control of bTB, especially in wild animals. However, interpretation of the test results in MAPIA and RT relies on observation of color development on a strip, which may vary depending on examiners. Higher accuracy and consistency could be achieved via a lab-free diagnostic device that outputs numerical data based on level of antibody binding to MB antigen(s). Further, as we discussed above, effort needs to be directed towards identifying a better antigen (or a combination of antigens) to further improve diagnostic sensitivity and specificity.

4. Johne's Disease

4.1. Background. Johne's disease (JD) or paratuberculosis is a chronic infectious enteritis of domestic and wild ruminants, causing reduction in milk production, malnutrition, weight loss, and eventually death [129, 130]. JD is prevalent worldwide and has a significant impact on global animal husbandry. In the USA, the causative agent of JD, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is found in 68% of dairies [8], with average herd-level prevalence of JD estimated to be 22%. The annual loss in the US dairy industry caused by MAP infection has been estimated at $220 million [9]. Economic losses associated with JD arise from decreased milk production, reduced fertility, and higher rate of culling [131]. In addition to the economic impact of JD to dairy industry, it is possible that MAP plays a role in Crohn's disease, which is an inflammatory bowel disease in humans [132]. These economic and possible health concerns create an urgent need for improved control of JD. As no practical treatment is available for JD, a better understanding of the transmission, detection, and management of the disease are the recommended procedures for its control [133].

4.2. Bacterial Culture and Cell-Mediated Immune-Response-Based Testing. Diagnostic tests to detect infection with MAP can be categorized as those that identify the organism and those that identify the immunological response to the organism. Fecal culturing for MAP using Herrold's egg yolk medium (HEYM) has been considered as a gold-standard test for JD diagnosis; however, it takes as long as 16 weeks to see an observable growth. Other approaches, such as the use of BACTEC radiometric liquid culture [134, 135] and MGIT culture medium [136], have been examined to reduce the culture time but these approaches require a specialist and are relatively expensive. Polymerase-chain-reaction- (PCR-) based diagnosis using IS900 insertion sequence [137], HspX [138], or F57 DNA fragment [139], on the feces of suspect animals can also be used. This PCR-based approach is much faster but is less sensitive than the culture test because PCR reaction can be inhibited by substances in the feces. Animals develop both CMI and humoral responses against MAP. A CMI-based diagnostic test, the IFN-γ assay, has been evaluated using blood samples of experimentally infected cattle. The study demonstrated that the IFN-γ assay could detect MAP infections in early stage of JD [140, 141]; however, IFN-γ assay is affected by antigen stimulation and blood sampling-storage conditions [142, 143]. This suggests that the IFN-γ test requires further optimization.

4.3. Humoral Immune-Response-Based Testing. Three different tests are used to measure antibody response in JD: complement fixation, agar gel immunodiffusion, and ELISA. The complement fixation and agar gel immunodiffusion tests both suffer poor sensitivity [144], and so a recent report has suggested that ELISAs are the best of the three methods for controlling JD in dairy and beef herds [133]. Diagnoses of JD using ELISA have been reported in many previous studies using different antigens [28, 29, 48, 63, 141, 145–151]. The antigens used in these studies have used protoplasmic antigen (PPA) [28, 29, 146, 147, 149, 150], lipoarabinomannan (LAM) [48], culture filtrate of MAP [63], and MAP proteins-1152 and 1156 [151] for testing antibodies against MAP. Beam et al. described a crude antigen mixture termed PPA, which is prepared by thorough physical disruption of mycobacterial bacilli followed by removal of cell debris and cell wall components [152]. Although many investigators have prepared PPA using various preparation protocols, it contains proteins very similar to proteins commonly found in closely related mycobacteria species. LAM is one of the components of the cell wall of mycobacteria species [120], and its core structure is shared among mycobacterial species [153].

Sweeney et al. tested milk and serum samples in an LAM-based ELISA to detect antibodies for JD diagnosis and found that sensitivity and specificity of the ELISA were similar regardless of the tested samples (i.e., milk and serum) [48]. McKenna et al. [49] compared diagnostic performance of PPA-based ELISA and LAM-based ELISA using fecal

culture test as a gold standard. Sensitivity and specificity of the PPA-based ELISA were higher than that for the LAM-ELISA [49]. PPA and LAM both contain structures common in mycobacterial species, so the use of these molecules as diagnostic antigen can cause false-positive reactions in animals infected with environmental mycobacteria other than MAP [154].

Bannantine et al. tested 18 purified recombinant proteins in ELISA format for serodiagnosis of ovine paratuberculosis. They found that MAP proteins 0862 and 3786 demonstrated the strongest antibody response and MAP protein 2116c the weakest [58]. Shin et al. used culture filtrate of an MAP strain, JTC, in ELISA format for JD diagnosis and named the method JTC-ELISA [63]. JTC-ELISA showed significantly higher sensitivity (56.3%) than that of commercial ELISA tests (28–44%) and performed effectively on both serum and milk samples. As mentioned above, the recommended control measure for JD is testing herds by ELISA methods but the current ELISA tests have low sensitivity (28–44.5%) [29]. We have previously reported that the surface antigens of MAP are capable of detecting anti-MAP antibodies in serum at early stages of JD [59, 60]. Since mycobacteria are known to express species-specific lipidic molecules on their surface, surface antigens were extracted by gently mixing MAP with various organic solutions and tested for antibody binding in ELISA format [61]. Antigens extracted from MAP by using 80% ethanol showed the greatest differentiation between antibody binding in JD-negative and JD-positive serum samples [61]. An ELISA test developed using the ethanol extract has been named ethanol vortex ELISA (EVELISA). The results from EVELISA showed that 98.4% of the JD-positive samples had higher antibody binding levels than those of JD-negative samples, whereas the percentage of positive antibody binding in a commercial ELISA test was 50% [61]. By using thin layer chromatography, species-specific lipidic molecules were detected in the ethanol extract (unpublished data). Eckstein et al. reported that species-specific antigenic lipopeptides (e.g., Para-LP-01) exist on the surface of MAP [155], and the high sensitivity of the EVELISA may be attributed to these lipopeptides.

ELISA, as well as other methods for JD diagnosis, needs to be conducted in diagnostic laboratories employing staff with expertise in microbiology, molecular biology, and immunology. This requires a labor-intensive process involving collecting samples into proper containers, indexing, packing, and shipping. Furthermore, cost per sample is relatively high—testing a sample by current fecal culture, PCR, and ELISA tests cost $16–19, $25, and $5-6, respectively, and this does not include costs associated with site visits and sample collections and shipping [133]. Because of the labor and cost for the current JD diagnosis, screening of cattle herds for JD is generally conducted at an interval of 6–12 months. During this interval, nonshedding animals can become shedders and low-shedding animals can become high shedders, thereby spreading MAP infection widely in the herd. This relatively long time interval between JD screening tests, in combination with low sensitivity of current diagnostic tests, may have been a reason that MAP infections remain so widespread in the US dairy and beef industries.

4.4. Point-of-Care Diagnosis and Future Directions. Controlling JD requires a better understanding of the spread of MAP in a dairy herd, which can be achieved by continuous monitoring of the infection using a lab-free diagnostic device. For development of a lab-free diagnostic device, microfluidic technology has begun to be employed in the last decade [15]. Microfluidic devices are state-of-the-art tools for biochemical and immunological analysis that have high sensitivity, require only short periods of time, small amounts of reagents, and do not require an expert operator [13, 14, 156]. In our recent study, we developed a prototype of lab-free diagnostic device for JD by using a microfluidic technology and the antigen used in the EVELISA test [157]. The device is composed of microfluidic channels/chamber with electrodes, light source for fluorescence excitation, and light detector. The EVELISA antigen was immobilized in the microchannel and reacted sequentially with bovine serum sample and fluorescently labeled secondary antibody. Liquid flow was controlled by applying AC signals to the electrodes in the microchannel. Further, antibody-antigen interaction was accelerated by creating liquid vortices by applying AC signals to the reaction chamber. The major advantages of this system are its low cost, ultraportable, and disposable immunoreactions chip, and the ability to detect antibodies within 20 min [157].

5. Conclusion

Among the diagnostic methods used for TB, bTB, and JD, serological methods have some compelling advantages that include short turn-around time, simple procedure, and low cost. However, as summarized in Table 1, previous reports on serodiagnosis indicated a lack of diagnostic accuracy and/or insufficient-tested samples for validation of the estimated diagnostic accuracy. The low diagnostic accuracy of the current serodiagnosis for the mycobacterial infections may be due to the false-positive reactions (causing low specificity), arising from exposure of some tested individuals to other nonpathogenic environmental bacteria. Recent studies have indicated that the use of multiple species-specific antigens may improve diagnostic accuracy of the serodiagnosis of the mycobacterial diseases. Some nonprotein molecules (cord factor and lipoarabinomannan) were also evaluated for serodiagnosis of mycobacterial infections. Since mycobacteria are known to produce a variety of species-specific non-protein molecules, further efforts to identify non-protein diagnostic antigens may be a useful contribution to the development of more specific tests for TB, bTB, and JD.

Most, if not all, of the current diagnostic tests for mycobacterial infections are carried out in a diagnostic laboratory, causing cost for sample processing and/or long turn-around time. Lab-free diagnostic devices would be valuable in understanding the epidemiology of the mycobacterial infections and would facilitate their control. The emergence of new technology, microfluidic lab-on-a-chip (LOC), holds considerable promise for accelerating

Table 1: Summary of humoral immune response based assays.

Target antigen	MTB/MB/MAP	Method of testing	Se (%)	Sp (%)	*P*	*N*	Species tested	Reference
PPD	MTB	ELISA	89	87	18	83	Human	[24, 25]
PPD	MB	ELISA	68–95	96–99	120	223	Cattle	[26, 27]
PPD	MAP	ELISA	29–72	99	359	2094	Cattle	[28, 29]
Antigen 5 (38 kDa)	MTB	ELISA	89	94–100	82	30	Human	[30, 31]
Cord factor	MTB	ELISA	81–84	96–100	65	66	Human	[32, 33]
ESAT-6	MTB	ELISA	67	51	100	100	Human	[34, 35]
ESAT-6	MB	ELISA	49–59	84–95	522	1489	Cattle	[36–38]
ESAT-6	MB	MAPIA	67	98	9	98	Deer	
CFP-10	MTB	ELISA	48–63	51–71	100	100	Human	[34, 39, 40]
CFP-10	MB	ELISA	49–59	84–95	522	1489	Cattle	[36–38]
CFP-10	MB	MAPIA	56	99	9	98	Deer	
Kp90	MTB	ELISA	78	82	51	71	Human	[41]
Antigen 60	MTB	ELISA	68–91	100	337	131	Human	[42, 43]
30 kDa antigen	MTB	ELISA	84	96.7	175	150	Human	[44, 45]
LAM	MTB	ELISA	17.8	87.7	47	153	Human	[27, 46–49]
LAM	MB	ELISA	60	na	120	—	Cattle	
LAM	MAP	ELISA	66	88	167	216	Cattle	
MS	MTB	ELISA	73–75	97-98	35	17	Human	[50–52]
MPT51	MTB	ELISA	80	na	53	—	Human	[51]
MPB70	MB	ELISA	73	88	120	223	Cattle	[27, 36–38, 53]
MPB70	MB	MAPIA	44	100	9	98	Deer	
Antigen 85 complex	MB	ELISA	48	89	208	54	Cattle	[54, 55]
MPB 83	MB	ELISA	49–59	84–95	522	1489	Cattle	[36, 53, 56]
MPB83	MB	MAPIA	89	99	9	98	Deer	[36–38, 53, 56, 57]
MPB83	MB	RT	60	96	25	25	Cattle	
MAP proteins 0862 and 3786	MAP	ELISA	81	na	11	—	Sheep	[58]
Ethanol extract	MAP	ELISA	97.4	100	64	38	Cattle	[59–62]
JTC	MAP	ELISA	56.3	99	444	412	Cattle	[63]

Se, Sensitivity; Sp, Specificity; *P*, no. of positive samples tested; *N*, no. of negative samples tested.

the development of lab-free diagnostic devices for these mycobacterial infections. LOC refers to miniaturized devices that can perform single or multiple laboratory procedures on a chip with a footprint of only a few inches in size [158]. Various LOCs have been developed for biochemical assays, detection of small particles (cells and bacteria), single-cell analysis, immunoassays, and so forth. Because of its small size and capability of automation, the technology offers opportunities for the development of point-of-care diagnostic devices for various diseases and physiological conditions. In the last decade, LOC technology has been employed for development of antibody detection assays [159, 160]. The principle of the immunoassay is same as conventional serological tests—detection of antibody binding to immobilized diagnostic antigen. However, whole assay processes (antibody reaction, washing, and detection) are carried out in a microfluidic system (microchannels). Liquid flow in the microchannel is controlled by electric fluid handling, pressure-driven fluid handling, or passive capillary force fluid handling [160]. Detection of antibody binding in LOC is based on either optical or nonoptical detection methods [159]. The most common types of optical detection systems are fluorescence detection and surface plasmon resonance. Fluorescence detection is highly useful technique due to its high sensitivity and the ease of integrating a label to the marker [159]. Surface plasmon resonance technology is based on measurement of the change in plasmon mode due to binding of biomolecules (antibody) to the surface (immobilized antigen) [161]. The nonoptical detection system is based mainly on measurement of change in the electrochemical properties due to molecular interactions on the reaction surface. This approach (i.e.,

a label-free sensor) does not require cumbersome detection system and therefore makes the LOC device relatively small and inexpensive [161]. Although development of lab-free diagnostics for mycobacterial diseases is in its infant stage, a recent study demonstrated the detection of MTB using fluorescent markers [95, 162]. Also, we recently reported that a prototype of LOC-based system could detect antibodies in JD-positive serum in 20 min [157]. Further, the system was converted to a label-free system using an electrochemical detection, reducing the detection time to 2 min (unpublished data).

A combination of species-specific (multi) antigens and LOC technology may lead to development of an accurate on-site (in-field) diagnostic device and thereby contribute to effective control of mycobacterial infections.

Acknowledgments

Our projects related to this review were supported by a 2011 UT AgResearch and Extension Innovation Grant (S. Eda), a UT Research Foundation Technology Maturation Fund (S. Eda), a UT M-CERV seed grant (S. Eda), NIMBioS support to G. J. Hickling, and a NIMBioS Graduate Research Assistantship (A. Wadhwa).

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 192926, 12 pages
doi:10.1155/2012/192926

Research Article

Differential Gene Expression Segregates Cattle Confirmed Positive for Bovine Tuberculosis from Antemortem Tuberculosis Test-False Positive Cattle Originating from Herds Free of Bovine Tuberculosis

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Received 6 January 2012; Revised 20 March 2012; Accepted 2 April 2012

Academic Editor: Mitchell Palmer

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Antemortem tests for bovine tuberculosis (bTB) currently used in the US measure cell-mediated immune responses against *Mycobacterium bovis*. Postmortem tests for bTB rely on observation of gross and histologic lesions of bTB, followed by bacterial isolation or molecular diagnostics. Cumulative data from the state of Michigan indicates that 98 to 99% of cattle that react positively in antemortem tests are not confirmed positive for bTB at postmortem examination. Understanding the fundamental differences in gene regulation between antemortem test-false positive cattle and cattle that have bTB may allow identification of molecular markers that can be exploited to better separate infected from noninfected cattle. An immunospecific cDNA microarray was used to identify altered gene expression ($P \leq 0.01$) of 122 gene features between antemortem test-false positive cattle and bTB-infected cattle following a 4-hour stimulation of whole blood with tuberculin. Further analysis using quantitative real-time PCR assays validated altered expression of 8 genes that had differential power (adj $P \leq 0.05$) to segregate cattle confirmed positive for bovine tuberculosis from antemortem tuberculosis test-false positive cattle originating from herds free of bovine tuberculosis.

1. Introduction

Bovine tuberculosis (bTB) caused by *Mycobacterium bovis* (*M. bovis*) occurs worldwide and has been estimated to cause annual losses of three billion dollars to global agriculture [1, 2]. In addition to being an important pathogen of cattle, *M. bovis* may infect many other domestic and wildlife species, and it infects humans [3–5]. The zoonotic aspect of bTB is underappreciated, as documented by The World Health Organization recently listing bTB as a neglected zoonotic disease [6]. Thus, control of bTB is a continuing effort that is necessary to protect livestock, wildlife, and human populations. In many developed countries, control of bTB is based on "test and slaughter" programs. Field and/or laboratory diagnostic tests are used to identify potentially infected cattle herds for quarantine, which may be followed by additional diagnostic testing and slaughter of all cattle that show positive test reactions. Although proven effective, "test and slaughter" programs are costly and have not been adopted by most developing countries.

The bTB control program in the United States (US) has reduced the prevalence of bTB-infected cattle herds from an estimated 5% of all herds in 1917 to an estimated infection rate of <0.001% for all herds [7]. Most antemortem diagnostic tests currently approved in many countries for detection of bTB measure cell-mediated immune responses. In the US, approved tests include the caudal fold tuberculin skin test (CFT), as the primary screening test, and either the comparative cervical tuberculin skin test (CCT) or the whole blood interferon-gamma (IFN-γ) ELISA assay as secondary

tests. Cattle that show positive reactions in successive primary and secondary tests usually are culled for postmortem examination. Predictably, as prevalence of bTB-infected cattle decreases, the proportion of antemortem test-false positive cattle culled for postmortem examination increases [8]. The prevalence of bTB in the state of Michigan is low, and only 1-2% of cattle that show positive responses on two successive antemortem tests are confirmed as positive for bTB at postmortem examination [9]. Thus, there is a need for ancillary antemortem tests for bTB that improve the positive predictive value of the testing process.

DNA microarray technologies facilitate rapid and large-scale examination of global gene expression profiles. This allows efficient detection of target genes that might be affected by a treatment or a disease process. This approach for identification of altered gene expression is particularly useful in studies of host response to various infections [10–13]. Altered gene expression profiles may show common patterns of response across different host cell types with different pathogens [10, 11]. Also, unique gene expression signatures can be identified in a host's response to a specific pathogen. This may be attributed to pathogen-driven differences in reprogramming of host gene transcription at the host-pathogen interface [12–14]. Thus, mining differential host transcriptome response to identify molecular events associated with pathogenesis offers an opportunity for discovery of diagnostic molecular markers predictive for specific infectious, metabolic, or genetic diseases [15–18]. Recently, microarray platforms of bovine genes have been used to study pathogenic processes and to identify molecular markers of infection, for two mycobacterial pathogens of cattle; *M. avium* subspecies *paratuberculosis* (MAP) and *M. bovis* [15, 16, 19–24].

Peripheral blood transcriptome profiles are particularly useful for identification of pathogen-associated immune response signatures, which can be used to develop diagnostic tools [10, 12, 14, 23]. Previous studies of bTB by Meade et al. [16, 21, 22] compared peripheral blood mononuclear cells (PBMCs) of cattle that had bTB with normal healthy cattle that tested negative for bTB. In the current study, we compared differential gene expression in cattle that had bTB with cattle that were single or double antemortem test-false positive for bTB. The intent was to identify differential gene expression profiles among cattle that have similar reactions in antemortem bTB tests but differ in their infection status at postmortem examination. Using this approach is critical because cattle that are antemortem test-false positive for bTB currently are not differentiated from truly infected cattle until a time-consuming and expensive postmortem diagnostic process is completed. Our overall objective was to identify gene targets that can be used for differentiation of antemortem test-false positive cattle from cattle that have bTB.

2. Materials and Methods

2.1. Experimental Animals and bTB Infection Status. Cattle used in this study were submitted for postmortem examination because of positive reactions in antemortem diagnostic tests for bTB. Cattle were transported to the Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University (MSU) the day before humane euthanasia and postmortem examination were performed. A presumptive positive or negative diagnosis was made at the DCPAH based on the presence of gross and/or microscopic lesions. Regardless of the presumptive diagnosis, samples from all cattle examined were submitted to United States Department of Agriculture's National Veterinary Services Laboratories (NVSLs). Final diagnosis was made by the NVSL based on results of polymerase chain reaction (PCR) assays and/or mycobacterial culture.

Three study groups of cattle were used for microarray analysis. The study groups included bTB positive cattle (bTB, $n = 4$), which were positive for lesions of bTB at postmortem examination and confirmed positive by the NVSL; double antemortem test-false positive cattle (DFP, $n = 4$), which showed positive reactions on primary and secondary diagnostic tests for bTB but were negative for lesions of bTB at postmortem examination, and were confirmed negative for bTB by the NVSL; and single antemortem test-false positive cattle (SFP, $n = 7$), which were positive on the CFT but were negative on the CCT or IFN-γ assay, were found negative for lesions of bTB at postmortem examination and were confirmed negative for bTB by the NVSL. The number of cattle in each group was expanded to 10 for validation of altered gene expression, using quantitative real-time PCR (qPCR) assays. Finally, healthy cattle from long-term bTB negative farms ($n = 12$) with recent negative test records for bTB, MAP, bovine leukosis virus, and bovine viral diarrhea virus were used as blood donors to obtain a reference pool of control RNA for the study.

2.2. Blood Collection, Antigen Stimulation, and RNA Extraction. Blood (~45 mL) was collected from each animal in the bTB, DFP, and SFP groups into 10 mL heparin-containing evacuated tubes (Vacutainer, BD Diagnostics, Franklin Lakes, NJ) immediately before euthanasia for postmortem examination. Within 3 hours of collection, the blood from each animal was pooled into individual sterile 50 mL conical tubes and stimulated with purified protein derivative prepared from heat-killed cultures of *M. bovis* (bPPD) (Prionics AG, Switzerland) at 20 μg bPPD/mL of blood. The blood was incubated at 38 ± 1°C for 4-hours prior to harvest. Blood samples from the 12 healthy cattle were similarly collected, processed, and stimulated. The 4 hour period for antigen stimulation was chosen because it was considered the maximal time that could be used if receipt of sample, stimulation of blood, and extraction of RNA were to occur during a 10-hour diagnostic laboratory work day.

After stimulation, the blood was centrifuged at 1200 ×g for 15 minutes at 18°C to form layers of plasma, buffy coat cells and red blood cells. The buffy coat layer of cells, and 2 mL of red blood cells immediately below the buffy coat layer were harvested by aspiration and transferred to a new 50 mL conical tube. Two rounds of hypotonic lysis of red blood cells were performed by addition of ice-cold diethylpyrocarbonate (DEPC) treated-sterile deionized water for 2 minutes, followed by addition of an equal volume of ice-cold

DEPC-treated sterile 2X saline (1.7% w/v NaCl). Intact cells were pelleted by centrifugation at 1200 ×g for 15 minutes at 18°C after the first round of hypotonic lysis, then at 190 ×g for 10 minutes at 4°C after the second round. After the second round of hypotonic lysis, the supernatant was decanted, and 1 mL TRIzol Reagent (Invitrogen, Carlsbad, CA) was added to the loose cell pellet for each 9 mL beginning volume of whole blood. This mixture was frozen at −84°C until use. For isolation of RNA, the mixture was thawed on ice and subjected to 10 passages through a 20-gauge needle. The resulting homogenate was divided into 1 mL aliquots, and the remainder of the RNA extraction procedure was performed according to the manufacturer's recommendations. The total cellular RNA from each animal was then pooled into a single tube and treated with RQ1 RNase-Free DNase (Promega, Madison, WI) according to manufacturer's recommendations. The treated RNA was extracted again using equal volumes of phenol-chloroform, followed by purification using MEGAclear Purification Kit (Ambion, Austin, TX). The purified RNA was immediately stored at −84°C until use.

Before use, the RNA from the study cattle was thawed on ice, and the integrity and concentration of the RNA was determined using the Agilent 2100 Bioanalyzer and RNA Nano 6000 Kit (Agilent Technologies, Santa Clara, CA). The RNA from the 12 healthy cattle was mixed to form a homogenous control reference pool and the integrity and concentration of that pooled RNA was similarly determined.

2.3. Experimental Design, cDNA Synthesis, and Microarray Hybridization. The BOTL-5 cDNA microarray used in this study was the 5th generation of a previously described bovine total leukocyte immunospecific microarray [25, 26]. The gene content and sequence information for BOTL-5 microarray are available at the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO, platform number GPL5751). Briefly, BOTL-5 contains 3,888 features including 1,391 bovine expressed sequence tag (EST) cloned inserts and PCR amplicons derived from known sequences of bovine immune response genes, spotted in duplicate along with multiple replicates of microarray specific control features. A common reference design was used for microarray hybridization in this study. That design was selected because the cattle used were submitted for postmortem examination over a two-year period. The use of a common reference on each microarray allowed comparison of gene expression of individual samples obtained over a large time span, and allowed comparison of gene expression across the various study groups [27–29].

The cDNA synthesis and dye labeling were performed with 15 μg aliquots of total RNA using the SuperScript III Fluorescent Labeling Kit containing Cy5 and Cy3 dyes (Cat no. L101401; Invitrogen Corp., Carlsbad, CA) following the manufacturer's recommendations. RNA from the study cattle was labeled with the Cy3 dye and cohybridized with the control reference pool of RNA labeled with Cy5 dye. For each microarray experiment, the Cy3-labeled sample and Cy5-labeled reference pool were combined and concentrated, using a Microcon 30 centrifugal filter unit (Millipore, Billerica, MA). The labeled cDNA mixture was eluted in 110 μL of SlideHyb buffer no. 3 (Ambion, Austin, TX) and heated for 5 minutes at 70°C prior to hybridization. The hybridization was done using a GeneTAC HybStation (Genomic Solutions Inc., Ann Arbor, MI) and an 18-hour step-down protocol (3 hours at 60°C, 3 hours at 55°C, 12 hours at 50°C). Immediately following hybridizations, the slides were subjected to 5 washes of 30 seconds each at 50°C with 2x SSC containing 0.1% SDS, 5 washes of 30 seconds each at 42°C with 0.2x SSC containing 0.1% SDS and 5 washes of 30 seconds each at 42°C with 0.2x SSC. After removal from the hybridization unit, the microarray slides were rinsed once in 2x SSC and once in double-distilled water and then dried by centrifugation for two minutes at 1,200 ×g. Hybridized cDNA microarrays were scanned immediately using a GeneTAC LS IV microarray scanner and GeneTAC LS software (Genomic Solutions Inc., Ann Arbor, MI).

2.4. Data Processing, Normalization, and Analysis. Microarray images were processed using GenePix Pro 6.0 software (Molecular Devices, Downingtown, PA) to generate spot intensity files. The output files were analyzed using the LIMMA (LInear Models for MicroArray) software package [30] implemented in the R language and environment (http://www.r-project.org/) [31]. Briefly, background correction [32] and within microarray normalization [33] were performed prior to linear regression analysis. Prior to and after normalization, MA plots of data were generated for visual assessment of the normalization effect. Log-ratios of median fluorescence intensities were used for data analysis. The empirical Bayes moderated T statistic [34] was used to verify altered expression of gene features within each group of cattle (SFP, DFP, and bTB) and between groups of cattle (bTB versus SFP, bTB versus DFP, and DFP versus SFP).

2.5. Quantitative Real-Time PCR (qPCR) Validation of Differential Gene Expression. Twelve potential reference genes were evaluated for stability of expression level within and between the study groups of cattle, using 3 available programs (*BestKeeper, NormFinder,* and *geNorm*) [35]. Succinate dehydrogenase complex subunit A (SDHA) was determined to be the most suitable reference gene for this study (data not shown). The list of 33 genes selected for validation of expression using qPCR assay, PCR primer sequences, primer concentration, PCR efficiency, and amplicon size are given in Table 1. The PCR primers for the gene targets were designed using Clone Manager Suite 7.0 (Sci-Ed Software, Cary, NC) or Primer Express 3.0 software (Applied Biosystem, Foster City, CA) and were synthesized by Integrated DNA Technologies (Coralville, IA). All primers were tested for amplification with the control reference pool of RNA and with a no template control (NTC). Optimal primer concentration for qPCR was empirically determined (data not shown).

Validation of altered gene expression using qPCR was done for 17 gene features selected from the microarray data. Those gene features showed substantial altered expression among cattle or showed unique regulation within a group of

Table 1: Genes selected for qPCR analysis, the nucleic acid sequence and concentration (nM) of PCR primers for those genes (forward primer (F) and reverse primer (R)), the PCR efficiency (E), and the PCR amplicon sizes (bp).

	Gene symbol	Gene name	Primer (5′-3′)	Primer conc. (nM)	PCR eff. (E)	Amplicon size (bp)
#	ARF3	ADP-ribosylation factor 3	F: TTGCCTAATGCCATGAATGC R: CACAGGTGGCCTGAATGTA	300	1.817	91
#	BOLA-DMA	Major histocompatibility complex, class II, DM α-chain	F: TTGTTGGCTTGGTCCTCTTC R: ACACCTCCTGCTTGGATGG	300	1.975	105
#	BOTL08_C07	Unknown	F: ATCACTTCCCGCCTCCTTAG R: AGGCAGGTGACCAAGGAAAC	600	1.925	92
#	CXCL2	C-X-C ligand 2 (GRO-alpha)	F: AACAAGGCTAGTGCCAACTG R: CCACTGAGGCTGCTGGAG	300	1.912	68
#	DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	F: AGAGATCTGGTGGGTAGCTTTA R: ACCCTATCCTCTCCTTGCAAAC	300	1.917	79
#	IL-4	interleukin-4	F: GCCACACGTGCTTGAACAAA R: TGCTTGCCAAGCTGTTGAGA	450	1.910	63
#	LTBR	Lymphotoxin beta receptor (TNFR superfamily member3)	F: CCGGAGTGACGAGGAAGAC R: CAAAACTCGCCCTTATACCTTG	450	1.859	104
#	PPP2R5B	Protein phosphatase 2, regulatory subunit B′, beta isoform	F: GTGGTCCTGGCAACAGAAC R: CTGGAGCCCAGCTTTGTG	300	1.895	110
#	PRKCI	Protein kinase C, iota	F: CAAGGACCCAAAGGAACGATT R: ACCACCTGCTTTTGCTCCAT	300	1.897	114
#	PTGS2	Prostaglandin-endoperoxide synthase 2(cyclooxygenase-2)	F: CGACACCAAGAACGTATTCCTA R: GAGATGTGGAAAAGAAGCATTG	300	1.930	105
*	BOLA-DRA	MHC class II DR alpha	F: GCTCTGGTGGGCATCATTG R: CCTCGGCGTTCAACGGTG	300	1.910	77
*	TPRA1	Transmembrane protein, adipocyte associated 1	F: GTGCGCAGACATCATTGAG R: GGCGCAAAGAAGCTGAAG	450	1.974	72
*	TRIM13	Tripartite motif-containing 13	F: CTGGCACGTTCATTAGCAAG R: GGCCAAGCAGAATGACCAC	300	1.962	69
*	FCGRT	Fc fragment of IgG, receptor, transporter, alpha	F: GGCCCGAATCGTTGTGTT R: GAAGCCCAAGGCTTACACC	450	1.822	81
*	TMX4	Thioredoxin-related transmembrane protein 4	F: ACCTTGACTTGTGCTCACTT R: TGGAGGTACCACTGGAACTG	300	1.993	85
*	BOTL11_A05	Unknown	F: CACACTCTATGGCGCAAATC R: CCCTGGACCACCACCTCTA	300	1.903	75
*	RPL19	Ribosomal protein L19	F: GGCTCCAGGCCAAGAAAG R: AATTGCCGAGGCCACTATG	300	1.972	106
§	CSF3	Colony stimulating factor 3 (granulocyte)	F: CTGGGTGAGACTGGGAAATG R: TCTCTCACACCCCGTCACA	300	1.959	62
§	GCP2	Granulocyte chemotactic protein 2 (CXCL6)	F: CATTGGAATGCTGTATATGGAGAT R: TCTTCCAAAGGTCAAGAGTAAGA	300	1.874	122
§	IL-10	Interleukin-10	F: CTTGTCGGAAATGATCCAGTTTT R: TCAGGC CCGTGG TTCTCA	300	1.948	66
§	IL-10RA	Interleukin-10 receptor A	F: GTCACCCTGCCACTGATCAC R: GGCAGCGTGCAGCTGAAATC	300	1.828	84
§	IL-6	Interleukin-6	F: GGCTCCCATGATTGTGGTAGTT R: GCCCAGTGGACAGGTTTCTG	300	1.873	64
§	IL-12p40	Interleukin-12, p40 subunit	F: CAAACCAGACCCACCCAAGA R: GACCTCCACCTGCCGAGAA	300	1.896	64
§	IL-15	Interleukin-15	F: GGCTGGCATTCATGTCTTCA R: CATACT GCCAGT TTGCTTCTGTTT	300	1.850	74

Table 1: Continued.

	Gene symbol	Gene name	Primer (5′-3′)	Primer conc. (nM)	PCR eff. (E)	Amplicon size (bp)
§	IL-18	Interleukin-18	F: GAAAATGATGAAGACCTGGAATCA R: ACTTGGTCATTCAAATTTCGTATGA	300	1.896	84
§	IL-1b	Interleukin-1 beta	F: AAGCAGGCGCATCTGTGAA R: ATGGCACTCTAACCCGGAAA	450	1.915	70
§	IL1R2	Interleukin-1 receptor 2	F: ATACCTGTGCCATGACGTATGC R: CGGAGTTTGATATTCCTGGTGAT	300	1.923	67
§	IL2	Interleukin-2	F: TGATGCAACAGTAAACGCTGTAG R: GAGAGGCACTTAGTGATCAAGTC	450	1.928	95
§	IL-1α	Interleukin-1 alpha	F: TTGGTGCACATGGCAAGTG R: GCACAGTCAAGGCTATTTTTCCA	450	1.948	72
§	IL-8	Interleukin-8	F: GGAAAAGTGGGTGCAGAAGGT R: GGTGGTTTTTTCTTTTTCATGGA	100	1.888	80
§	INF-γ	Interferon, gamma	F: TGGCATGTCAGACAGCACTTG R: CCTGAAGCGCCAGGTATAAGG	450	1.932	96
§	TGFβ	Transforming growth factor, beta	F: CTGAGCCAGAGGCGGACTAC R: TGCCGTATTCCACCATTAGCA	300	1.897	63
§	TNFα	Tumor necrosis factor, alpha	F: TCTACCAGGGAGGAGTCTTCCA R: GTCCGGCAGGTTGATCTCA	300	1.871	68
	SDHA	Succinate dehydrogenase complex subunit A	F: CCACGCCAGGGAGGACTTC R: CGTAGGAGAGCGTGTGCTTC	300	1.879	116

#Genes that showed substantial altered expression within a group of cattle in microarray studies.
*Genes that had differential power between groups of cattle using microarray expression data analyzed with MAANOVA.
§Genes that were selected from the literature as being relevant to the bTB infection.

cattle (Table 1). An additional 16 genes coding for cytokines or chemokines were selected from the literature for qPCR analysis (Table 1). The cytokines or chemokines produced by those genes are reported as important mediators in bovine and human TB infections. Samples of RNA from 30 cattle (10 cattle per study group) were used to assess altered expression of the 33 selected genes. The samples of RNA subjected for qPCR included some of the original samples used in the microarray experiments plus new samples of RNA from additional cattle that met the criteria for each study group. Synthesis of cDNA was performed with 2 μg of total RNA from each study animal and from the common reference pool of RNA, using commercially available reagents (Superscript II Reverse Transcriptase and Oligo $(dT)_{12–18}$ Primer, Invitrogen, Carlsbad, CA) and the manufacturer's recommended protocol. Upon completion of cDNA synthesis, the RNA template in each reaction was removed with 1U of RNase H (Invitrogen, Carlsbad, CA). The cDNA was purified using QuickClean enzyme Removal Resin (Clontech Laboratories, Mountain View, CA). Finally, the concentration of purified cDNA was measured by spectrometry (ND-1000, NanoDrop Technologies, Wilmington, DE) and diluted to final concentration of 10 ng/μL. The cDNA was stored at −20°C until use in qPCR assays.

The qPCR assays were performed in triplicate using SYBR Green PCR Master Mix and an ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA). Each 20 μL reaction consisted of 1x SYBR Green PCR master mix, 30 ng of cDNA, and a pair of primers at predetermined optimal concentrations (Table 1). The reaction conditions were 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, and 58°C for 1 minute. Dissociation curve analysis was done for each reaction.

2.6. PCR Efficiency Determination and qPCR Data Analysis. The magnitude of the normalized reporter signal (delta Rn) data and the cycle threshold (Ct) data exported from the ABI 7500 SDS software were used to verify that acceptable PCR efficiency (>1.8) was achieved and to calculate the relative expression level of the targeted genes, respectively. The efficiency of each qPCR reaction was determined based on the slope of the exponential phase of the reaction, using the LinRegPCR program [36]. The mathematical model for calculation of relative gene expression proposed by Pfaffl et al. was used for qPCR analysis [37]. The SDHA gene was used as the reference/normalizer gene, and the common reference pool (as in the microarray experiments) was used as the calibrator. For each animal, the mean Ct value from triplicate reactions for each gene target was used to determine the relative gene expression value. To calculate the mean differential expression of a gene target for an entire study group, the relative gene expression values of the gene target for each animal within the group were averaged. Thus, the calculated mean differential expression represents altered expression of a gene target among cattle in a study group relative to the common reference pool of RNA. The Student's t-test was used to determine the statistical significance of altered expression of gene targets within each study group (SFP, DFP, and bTB).

The relative gene expression values of each gene target for each animal in a group were used to determine the significance of differential expression of a gene target among groups of cattle (bTB versus SFP, bTB versus DFP, and DFP

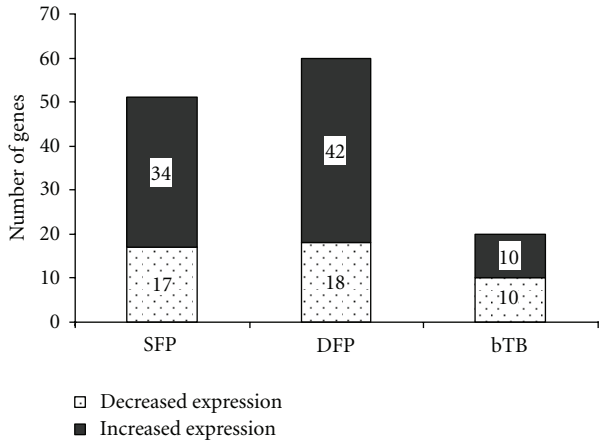

Figure 1: Number of genes from microarray analysis that were differentially expressed ($P \leq 0.01$) within single antemortem test-false positive (SFP), double antemortem test-false positive (DFP), and bTB-infected (bTB) groups of cattle. The RNA used for microarray analysis was harvested after a 4-hour stimulation of whole blood with tuberculin, and comparison of gene expression levels was with a reference pool of mRNA harvested from the blood of healthy cattle after 4-hour stimulation with tuberculin. The number of genes for each group of cattle that showed increased expression (solid box) or decreased expression (shaded box) relative to the reference pool of RNA is indicated by the figure in the boxes.

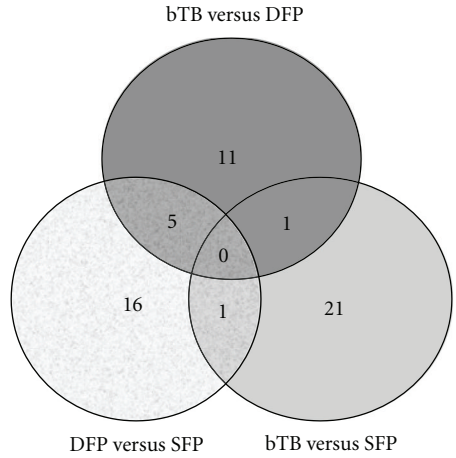

Figure 2: Numbers of gene features identified from analysis of microarray data that were differentially expressed ($P \leq 0.01$) among single antemortem test-false positive (SFP), double antemortem test-false positive (DFP), and bTB-infected (bTB) groups of cattle. The numbers of genes common to or unique for the groups of cattle are shown in a Venn diagram.

versus SFP). The analysis was performed with the ANOVA test based on linear fixed effect models [38], implemented in the MAANOVA software package [39]. Simultaneous fitting of multiple linear models was done with 5000 permutation tests and with the jsFDR method for false discovery rate (FDR) adjustment [40].

3. Results

3.1. Identification of Altered Gene Expression Profiles from Microarray Data. A total of 1,391 gene features on the BOTL-5 microarray were analyzed, of which, 122 gene features were differentially expressed ($P \leq 0.01$) in one or more groups of cattle. Only 9 of the 122 gene features were shared by two groups of cattle; the remaining 113 genes were uniquely regulated within individual groups of cattle (SFP, DFP, and bTB). Overall, we found more genes with altered expression in each group of antemortem test-false positive cattle than in the bTB positive cattle. In both the SFP and DFP groups of cattle, the ratio of gene features showing increased expression levels to those showing decreased expression levels was at least 2 : 1. In contrast, the ratio of increased expression to decreased expression was 1 : 1 in the bTB group of cattle (Figure 1). The complete list of differentially expressed gene is provided in Supplementary Table S1 (see Table S1 in Supplementary Material available online at doi: 10.1155/2012/192926).

The objective of this study was to find molecular markers that can differentiate antemortem test-false positive cattle from bTB infected cattle. When a comparison of gene expression data was done between groups of cattle, only 55 gene features showed significant statistical power to differentiate the study groups from each other (bTB versus SFP, bTB versus DFP, and DFP versus SFP). The complete list of those genes is provided in Supplementary Table S2. Using the data generated from analysis of the microarrays, differentiation of a particular group of cattle from each of the other two groups was possible, but only a few gene features were useful for that purpose (Figure 2). The DFP group of cattle could be differentiated from the bTB and the SFP groups using the altered expression levels of 5 gene features. Those genes were thioredoxin-related transmembrane protein 4 (TMX4); transmembrane protein, adipocyte associated 1 (TPRA1); major histocompatibility complex, class II, DM alpha-chain (BOLA-DMA); Fc fragment of IgG, receptor transporter alpha (FCGRT); ribosomal protein L19 (RPL19). The altered expression level of only one gene feature, tripartite motif-containing 13 (TRIM13), was useful for differentiating the SFP group of cattle from the bTB and DFP groups. Similarly, altered expression of only one gene feature, clone BOTL0100011_A05 (a gene feature of unknown function), was useful for differentiation of the bTB group of cattle from the SFP and DFP groups.

3.2. Group Level Gene Expression Profiling with Quantitative Real-Time PCR (qPCR) Data. With the extended panel of 10 cattle per study group, the statistical significance of the mean altered gene expression within each group of cattle could be assessed with greater accuracy. The qPCR assays identified many genes that showed considerable variation in expression level among cattle within all study groups. Seven genes showed increased expression in all 3 study groups, and 12 genes showed decreased expression in all 3 study groups (Figures 3(a) and 3(b)). The gamma interferon (IFN-γ) gene showed the greatest increase in expression in all study groups

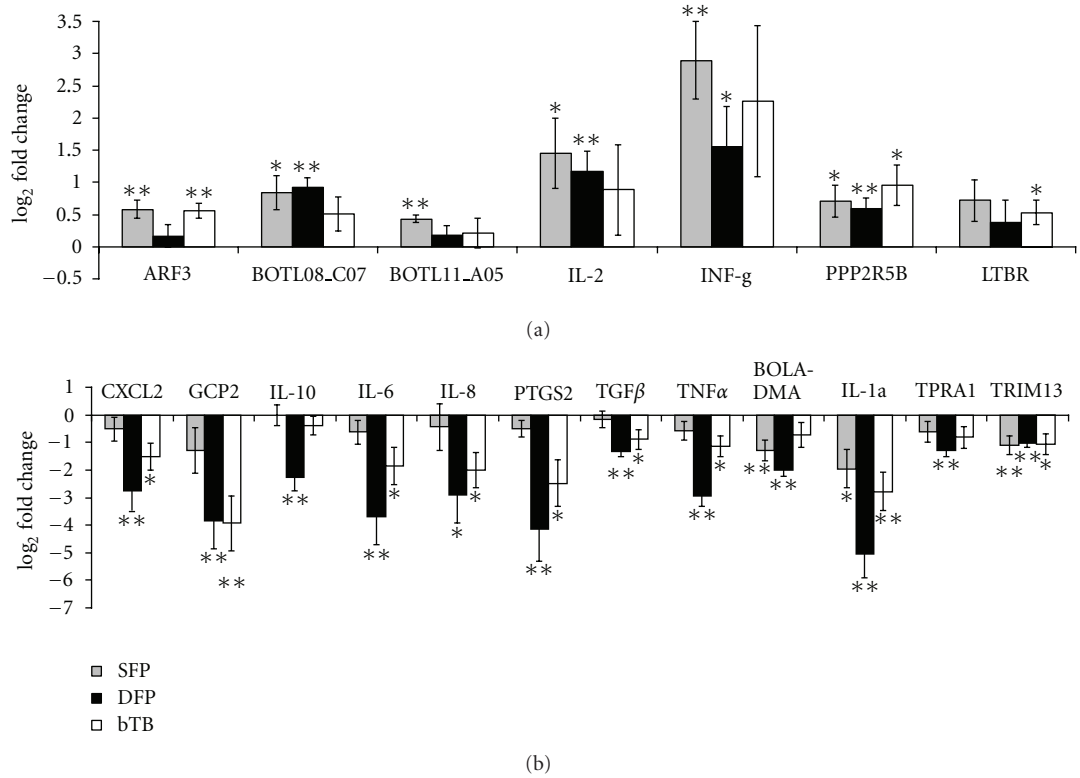

Figure 3: The relative gene expression levels compared with the reference pool of RNA from healthy cattle, as determined by qPCR assays, for the single antemortem test-false positive (SFP, shaded box), double antemortem test-false positive (DFP, solid box), and bTB-infected (bTB, clear box) groups of cattle. Gene expression levels (in $\log_2$ fold change) were calculated using the published mathematical algorithm [37] in which the reference pool of RNA was set as baseline (0 value at Y-axis) and used as the calibrator. Statistically significant differences were determined using Student's t-test and are shown at $P \leq 0.05$ (*) and $P \leq 0.01$ (**). The error bars represent the standard error of the mean expression level for a group of cattle. (a) Genes with increased expression in all groups of cattle. (b) Genes with decreased expression in all groups of cattle.

(2.92- to 7.42-fold). Interleukin-2 (IL-2) also showed a marked increase in expression (1.84- to 2.74-fold). Other genes that showed increased expression in all groups of cattle were serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit beta isoform (PPP2R5B), lymphotoxin beta receptor (LTBR), ADP-ribosylation factor 3 (ARF3), and 2 clones with unknown function (BOTL0100008_C07 and BOTL0100011_A05).

Proinflammatory cytokines were among the downregulated genes, including interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and interleukin-1 alpha (IL-1α). Also downregulated were anti-inflammatory cytokines, including interleukin-10 (IL-10) and transforming growth factor beta (TGF-β), along with several chemokines, including interleukin-8 (IL-8), chemokine (C-X-C motif) ligand 2 (CXCL-2), and chemokine (C-X-C motif) ligand 6 (GCP2). Decreased expression of many of those genes was especially evident in cattle from the DFP group. Other genes that showed decreased expression included the major histocompatibility complex Class II molecule (BOLA-DMA), prostaglandin-endoperoxide synthase 2 (PTGS2), tripartite motif-containing 13 (TRIM13) and transmembrane protein, adipocyte associated 1 (TPRA1).

3.3. Analysis of Altered Gene Expression in Individual Cattle. The data from qPCR assays were analyzed at the individual animal level using the ANOVA test to identify genes that could significantly differentiate individual cattle within a group from cattle in the other groups. Of the 33 genes selected for qPCR assay, 17 were found to have differential power at adj $P \leq 0.05$. The expression levels of 16 of those genes could be used to differentiate DFP cattle from SFP cattle (Figure 4(a)). Only 5 genes differentiated bTB cattle from DFP cattle, and only 1 gene significantly differentiated bTB cattle from SFP cattle. These results suggest that the gene expression profile of the SFP cattle was more similar to that of the bTB cattle than the DFP cattle. This finding was unexpected, because it was anticipated that the SFP and the DFP groups of cattle would be closer in expression profile to each other than to the bTB cattle.

All 10 SFP cattle and 4 of 10 cattle in the DFP group originated from bTB-positive herds and may have been exposed with *M. bovis*. Exposure with *M. bovis* might have influenced the gene expression profiles. The remaining 6 cattle in DFP group that did not have a history of bTB exposure were designated as double antemortem test-false positive non-bTB exposed (DFP-non-ex, $n = 6$), and the gene expression data

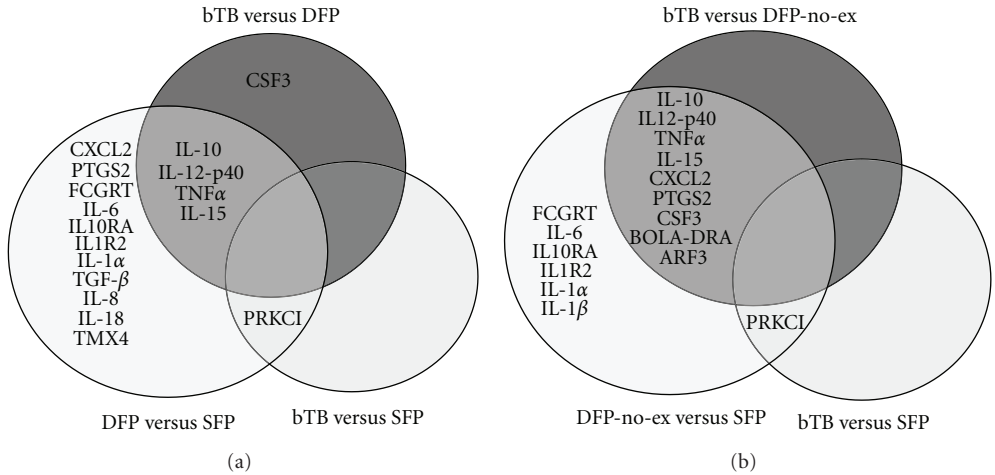

Figure 4: Venn diagrams showing the statistically significant (adj $P \leq 0.05$) differentially expressed genes that were unique to or common among (a) single antemortem test-false positive (SFP), double antemortem test-false positive (DFP), and bTB-infected (bTB) groups of cattle in initial analysis of 30 cattle and (b) single antemortem test-false positive (SFP), double antemortem test-false positive non-bTB-exposed (DFP-non-ex), and bTB-infected (bTB) groups of cattle after removal of bTB-exposed cattle from the DFP group. The gene expression levels were determined by qPCR assay, where each animal was calibrated relative to the reference pool of RNA from healthy cattle; differential expression between 2 groups of cattle (i.e., X versus Y) was determined using ANOVA analysis.

from the qPCR assays were reanalyzed for this new group. Overall, removal of bTB-exposed cattle from the DFP-non-ex group did not have a statistically significant (adj $P \leq 0.05$) effect on the mean expression values for most of the genes previously identified. However, the mean expression levels of 8 down regulated genes in the DFP-non-ex group were further down regulated by >2-fold change. Those genes are CXCL2, GCP2, IL-10, IL-1α, IL-1R2, IL-6, IL-8, and PTGS2. In addition, the mean expression levels of IFN-γ and PPP2R5B were no longer statistically significant for the DFP-non-ex group. The loss of statistical significance was attributed to the wide range of expression levels for those genes among the cattle and the reduction in the size of the group from 10 to 6 cattle.

Removal of the bTB-exposed cattle from the DFP group also affected the analysis at the individual animal level, using the ANOVA test. The differential power of 4 genes (TGF-β, IL-8, IL-18, and TMX4) was no longer statistically significant, but 2 additional genes (BOLA-DRA and ARF3) had statistically significant differential power among 2 or more groups of cattle. Thus, altered expression level of 15 genes was deemed significant (adj $P \leq 0.05$) for differentiation of the 3 groups of cattle (Figure 4(b)). After removal of the bTB-exposed cattle, the number of genes that could be used to differentiate cattle in the DFP-non-ex group from cattle in the bTB group increased from 5 to 8 (Table 2).

Table 2: Genes that showed differential power (adj $P \leq 0.05$) between cattle that were double antemortem test-false positive with no bTB-exposure history (DFP-non-ex) and cattle that were bTB infected (bTB), as determined by qPCR analysis. The differential expression level ($\Delta\log_2$ FC) of the bTB and DFP-non-ex groups of cattle (bTB minus DFP-non-ex) was determined using ANOVA analysis.

Gene	$\Delta\log_2$ FC (bTB versus DFP-non-ex)	adj P
IL-10	1.852	0.0314
IL12-p40	1.975	0.0267
TNFα	1.809	0.0217
PTGS2	3.413	0.0183
CSF3	2.199	0.0175
CXCL2	2.498	0.0175
BOLA-DRA	1.010	0.0175
ARF3	0.736	0.0175

4. Discussion

Currently, the OIE-approved bTB tests for international trade of cattle are the tuberculin skin tests (TSTs), which are based on a physically measurable cell-mediated inflammatory response against tuberculin antigen injected into either the skin of the neck or the caudal fold of the tail, and the IFN-γ ELISA assay, which measures IFN-γ secreted into plasma after stimulation of whole blood with tuberculin antigen [2, 41]. The TSTs are most commonly used to screen for bTB and normally are effective for control of bTB. However, limitations in sensitivity and specificity of TST have long been recognized [2, 42]. To increase diagnostic sensitivity, the IFN-γ ELISA assay is used in some countries in parallel with, or sequential to, various applications of the TST [43, 44]. Regardless of testing schemes employed, false positive and false negative test results remain an issue for bTB control programs. In Michigan, the current rate of bTB-infection is extremely low, which leads to far more antemortem test-false positive cattle being culled as bTB suspects than the number of bTB infected cattle identified at

postmortem examination. This has driven our interest in comparing the gene expression profiles of bTB-positive and bTB antemortem test-false positive cattle. We hypothesized that altered transcription levels of select genes could discriminate between cattle infected with bTB and cattle that were antemortem test-false positive using currently approved diagnostic assays.

To test our hypothesis, mRNA expression levels were evaluated by microarray analysis that made use of a common reference design. The common reference used in the current study was a pool of RNA extracted from PBMC of healthy cattle after samples of whole blood from those cattle were stimulated for 4 hours with bPPD. The methods for antigen-stimulation and for RNA extraction from PBMC of healthy cattle were identical to those used for the 3 study groups of SFP, DFP, and bTB cattle. Previous studies have shown that bPPD stimulation of PBMC from healthy cattle will induce altered gene expression [16, 22]. By normalizing each microarray with a pool of RNA from antigen stimulated cells obtained from healthy cattle, it was hoped that any changes in gene expression that were due to nonspecific stimulation caused by bPPD would be filtered out.

After 2–4 hours of antigen stimulation, comparable microarray studies on cattle infected with MAP [45] or bTB [16, 22] have shown rapid changes in gene expression profiles of PBMC. Importantly, a marked increase was reported in the number of differentially expressed genes in bTB-positive cattle compared with TST-negative cattle following a 3-hour stimulation of whole blood with bPPD [16]. In the current study, we also found rapid changes in gene expression in bTB-positive cattle (bTB group) and in antemortem test-false positive cattle (SFP and DFP groups), after stimulation of blood with bPPD for 4 hours. At 0.01 level of significance, the number of genes showing altered expression was similar among the SFP ($n = 51$) and DFP groups ($n = 60$). In comparison, the numbers of genes showing altered expression in the bTB group were substantially less ($n = 20$). Most genes that showed altered expression were unique to the individual groups of cattle, and only a few genes were shared among 2 or more groups of cattle.

Although microarray hybridization analyses are useful as a general screening tool for identifying genes that show altered expression [13, 23], qPCR is accepted as the more sensitive and accurate assay for quantifying differential gene expression [46, 47]. Thus, qPCR was used to validate altered expression levels for select genes. The qPCR assays conducted in the current study confirmed that there were differences in gene expression between the SFP, DFP, and bTB groups of cattle (Figure 3). Compared with the reference pool of RNA, the expression of many genes was decreased at 4 hours after stimulation, especially in the DFP group of cattle. This finding was consistent with other studies that report a temporal decrease in level of gene expression following antigen stimulation of PBMC [22, 45]. The greatest increase in gene expression was observed for the cytokine IFN-γ, an essential event for the whole blood IFN-γ ELISA assay for bTB [48]. However, the IFN-γ gene was not useful for separating infected from noninfected cattle in the current study because the expression levels for that gene varied considerably among animals both within and between groups. Similarly, variability was observed in the amount of IFN-γ detected using the whole blood IFN-γ ELISA assay on samples of blood obtained from cattle in the current study (data not shown). Furthermore, the levels of altered expression of mRNA did not correlate with optical density readings from plasma in the IFN-γ ELISA assay.

The gene expression data from qPCR assays were analyzed to identify gene targets that might differentiate antemortem test-false positive cattle from the true bTB-infected cattle. Differentiation of the DFP cattle from the bTB group and the SFP groups of cattle was possible based on the expression levels of 5 and 16 genes, respectively (Figure 4(a)). However, only one of the genes subjected to qPCR assay could differentiate the bTB group of cattle from the SFP group. The origin of the cattle in the SFP group suggested a possible explanation for that finding. All of the cattle in the SFP group were exposed to *M. bovis* infected cohorts; thus, it was possible that some of the cattle in the SFP group had been infected with *M. bovis*. Similarly, a few of the cattle in the DFP group were exposed to *M. bovis* infected cohorts. We could not reevaluate the SFP group; however, we could reevaluate the DFP group by forming a new group of cattle (DFP-non-ex) that consisted of cattle with no known exposure to *M. bovis*. When the data from the qPCR were reanalyzed using the new groups of cattle, the list of genes that had statistically significant differential power to separate the DFP non-ex-group from the bTB group increased in number from 5 to 8 (Figure 4(b)).

The process of postmortem examination is not perfect; infections with *M. bovis* prior to lesion development may not be detected. It is thought that up to 30% of cattle in an infected herd can become infected with bTB [49]. Cattle with an effective innate immune response may clear an infection with *M. bovis*. In that event, those cattle might test positive by TST and/or IFN-γ ELISA assay but lack lesions at postmortem examination and be negative for *M. bovis* on bacterial culture [50]. Similarly, cattle in an early stage of infection with *M. bovis* may test positive by TST and/or IFN-γ ELISA assay but lack lesions at postmortem examination and be negative on cultures for *M. bovis* [51, 52]. Latent infection is known to occur in humans infected with *M. tuberculosis,* and it is believed that latent bTB infection can occur in cattle [53–56]. It is likely that some latently infected cattle would test positive by TST and/or IFN-γ ELISA assay but lack lesions at postmortem examination and be negative on cultures for *M. bovis* [53, 54]. Thus, failure to identify genes with altered expression that can differentiate bTB-infected cattle from antemortem test-false positive-bTB-exposed cattle may have been due to use of "non-infected" cattle that were, or had been, infected with bTB.

The current study examined altered expression of genes in PBMC at 4 hours following stimulation with bPPD. The gene expression profiles of the DFP-non-ex group of cattle were clearly different than those of the bTB group of cattle. That finding provides support for the hypothesis that detection of altered expression of a few genes could be used to differentiate bTB-infected cattle and antemortem test-false positive cattle from bTB-free herds. Temporal studies that

used antigen stimulation of PBMC from cattle infected with bTB have shown that there is a rapid and transient burst of gene expression that occurs within hours of antigen stimulation. A second burst of altered gene expression occurs at 12 to 24 hours after stimulation [16, 22, 23]. The current study tried to capitalize on the early burst of altered gene expression. We recently conducted a similar study analyzing gene expression profiles after overnight stimulation of PBMC with antigen. As expected, it was observed that many of the genes that showed altered expression after 4 hours of antigen stimulation in the current study did not show altered expression after overnight incubation with antigen. Instead a new set of genes with altered expression were identified that may be evaluated for use as molecular markers for segregation of bTB-infected cattle from noninfected cattle. The results of the current study indicate that monitoring altered expression of genes with differential power has potential to separate bTB-infected cattle from antemortem test-false positive cattle in bTB-free herds. However, further studies are needed to evaluate the gene expression profiles of the antemortem test-false positive cattle from both low-risk herds for bTB exposure and cattle from bTB-infected herds.

5. Conclusions

The results from differential gene expression analyses reported here clearly showed that gene expression profiles differed between the DFP-non-ex group of cattle and the bTB group of cattle. However, differentiating the bTB-infected cattle from the antemortem test-false positive cattle that had been exposed to bTB infected cattle in the field was problematic. Therefore, further work is needed to gain a better understanding of the distinct differences in gene expression profiles of these cattle. The results from this study are encouraging for use of altered gene expression profiles in the development of ancillary tests for bTB that can improve the diagnostic process and reduce the unnecessary destruction of antemortem test-false positive cattle from bTB-free herds.

Acknowledgments

The authors would like to thank the staffs of Michigan Department of Agriculture for assistance with blood sample collections, the Michigan State University Research Farms for providing blood samples from healthy cattle, the staffs at the MSU Center for Animal Functional Genomics for excellent technical assistance, and the United States Department of Agriculture for funding support of this study.

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 842861, 5 pages
doi:10.1155/2012/842861

Research Article

Experimental Aerosol Inoculation and Investigation of Potential Lateral Transmission of *Mycobacterium bovis* in Virginia Opossum (*Didelphis virginiana*)

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Received 9 January 2012; Revised 2 April 2012; Accepted 15 April 2012

Academic Editor: Michael D. Welsh

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An endemic focus of *Mycobacterium bovis* (*M. bovis*) infection in the state of Michigan has contributed to a regional persistence in the animal population. The objective of this study was to determine if Virginia opossums (*Didelphis virginiana*) contribute to disease persistence by experimentally assessing intraspecies lateral transmission. One wild caught pregnant female opossum bearing 11 joeys (young opossum) and one age-matched joey were obtained for the study. Four joeys were aerosol inoculated with *M. bovis* (inoculated), four joeys were noninoculated (exposed), and four joeys plus the dam were controls. Four replicate groups of one inoculated and one exposed joey were housed together for 45 days commencing 7 days after experimental inoculation. At day 84 opossums were sacrificed. All four inoculated opossums had a positive test band via rapid test, culture positive, and gross/histologic lesions consistent with caseogranulomatous pneumonia. The exposed and control groups were unremarkable on gross, histology, rapid test, and culture. In conclusion, *M. bovis* infection within the inoculated opossums was confirmed by gross pathology, histopathology, bacterial culture, and antibody tests. However, *M. bovis* was not detected in the control and exposed opossums. There was no appreciable lateral transmission of *M. bovis* after aerosol inoculation and 45 days of cohabitation between infected and uninfected opossums.

1. Introduction

Numerous wildlife species have proven to be a significant reservoir of *Mycobacterium bovis* (*M. bovis)* some examples include: the Eurasian Badger (*Meles meles*) in Great Britain, the African Buffalo (*Syncerus caffer*) in South Africa, the Brushtail possum (*Trichosurus vulpecula*) in New Zealand, Eurasian wild boar (*Sus scrofa*) in some regions of Spain, and the White-Tailed Deer (*Odocoileus virginianus*) in the United States [1–9]. *Mycobacterium bovis* has the ability to produce disease within a wide range of mammal species including humans, thus making collaborative research, surveillance, and control essential to understanding the epidemiology of this disease. Virginia opossum (*Didelphis virginiana*), family Didelphidae and the Brushtail possum, family Phalangeridae belong to the same order, Marsupialia; however distant, these relatives share similar behavioral traits that may contribute to the spread of tuberculosis [7, 10]. It has been established that Brushtail possums are an ideal host for tuberculosis due to the fact that they are highly susceptible to *M. bovis*, shed the organism through multiple routes and have shared dens [5, 7, 11]. Virginia opossum is a known natural host of tuberculosis in the state of Michigan in the United States and previous studies have shown them to be susceptible

to *M. bovis* by aerosol inoculation [5, 6, 12]. Additionally, Virginia opossum utilizes shared dens, and in the state of Michigan, has a high potential for significant interaction with other animals harboring tuberculosis [8]. The aim of the present study was to determine whether the Virginia opossum may contribute to disease spread by characterizing the intraspecies lateral transmission after aerosol inoculation and 45 days of cohabitation.

2. Materials and Methods

2.1. Virginia Opossum. One wild caught pregnant female Virginia opossum bearing 11 joeys, approximately 10 weeks old, plus one age matched joey from outside of the litter were obtained. Animals were assessed and clinically judged to be in good health at Michigan State University, College of Veterinary Medicine, Zoo and Wildlife Services. The dam was prophylactically treated with oral fenbendazole (50 mg/kg). Animals were monitored daily and offered a commercially available dry cat food and water ad lib with weekly supplements of granny smith apples or moist canned cat food. Institutional Animal Care and Use Committee (IACUC) approved guidelines were implemented.

The stock *M. bovis* isolate was obtained from the Michigan Department of Community Health (MDCH), Lansing, Michigan, USA, animal 08 TB 883 AF 327 DEER 269398. This pure culture was quantified by plating 100 uL of culture onto Middlebrooks 7H10 agar and incubated at 37°C. The undiluted stock culture was estimated to have 300,000 cfu/mL, aliquots were diluted to the desired concentration of 1×10^6 colony forming units (cfu) per mL [5]. Sedation of the joeys was achieved by intramuscular injection of Telazol (Fort Dodge Animal Health) 100 mg/kg. Four sedated joeys received aerosol inoculation of *M. bovis* (inoculated group), four served as noninoculated in-contact joeys (exposed group), and three joeys, the dam, and the additional age matched joey from outside of the litter served as the control group. *Mycobacterium bovis* was administered to the joeys in the designated inoculated group at a concentration of 1×10^6 cfu via nebulization for a total of 10 minutes [6]. Inoculated joeys were ear-notched for identification purposes. Inoculated and noninoculated (exposed) joeys were housed individually for one week prior to the forty-five days of cohabitation in a BL-3 Horsfall isolator [6]. One noninoculated (exposed) joey was housed with one inoculated joey making four replicate cohabitation groups. The control animals were individually housed in a separate containment room.

2.2. Gross and Histopathology. Individual weight measurements were taken every two weeks until the joeys were sacrificed. At day eighty-four after inoculation or after exposure, joeys were sacrificed by initial sedation with an intramuscular injection of Telazol (100 mg/kg) and subsequent intracardiac exsanguination. Immediately after exsanguination the whole blood samples were clotted at 4°C for 1 hour, centrifuged at 5,000 time gravity for 5 minutes, and serum was then separated into sterile tubes and frozen at −20°C until all samples were collected for the entirety of the study.

A complete postmortem examination was performed. Brain, eye, nasal turbinates, trachea, lungs, heart, liver, kidney, spleen, stomach, pancreas, gonad, adrenal gland, small intestine, large intestine, tonsil, lymph nodes (cranial, thoracic, and abdominal), urinary bladder, skeletal muscle, and pinea were harvested, fixed in 10% neutral-buffered formalin, and trimmed for histopathology. All major organs (lungs, liver, kidney, and spleen) were individually weighed and collected for *M. bovis* culture. Slides were stained with hematoxylin and eosin and Ziehl-Neelsen's acid-fast stain followed by light microscopy examination.

2.3. Bacteriology. Tissues were processed for *M. bovis* isolation at Michigan Department of Community Health (MDCH) utilizing standardized protocols [6]. Four tissue groups were pooled for culture. Pool A: cranial lymph nodes and tonsil, Pool B: thoracic lymph nodes and lungs, Pool C: liver, kidney, spleen, abdominal lymph nodes, and Pool D: small intestine and large intestine.

2.4. Serology Assay. Serum was sent to a commercial laboratory for rapid test analysis (Chembio Diagnostics Systems Inc., Medford, NY, USA). The rapid test is a lateral-flow, blue latex bead signal-based, qualitative antibody detection assay that utilized a cocktail of selected *M. bovis* antigens (ESAT-6, CFP10, MPB83). The assay uses a ready-to-use plastic cassette containing a nitrocellulose membrane impregnated with the cocktail of test antigens. Thirty microliters of test serum and 3 drops of diluent buffer were added to the test well and the result of the reaction was read by visual evaluation after 20 minutes [13]. An antibody positive sample was indicated by a visible band at both the test and control lines, while an antibody negative sample was indicated by a visible band at the control line but no band at the test line [13].

2.5. Statistical Analysis. The two-sample t-significance test was calculated on all data sets to determine difference between the inoculated, exposed and control groups. The Student's t-test was chosen based on the minimal sample size and distribution of values [14]. The t-statistic obtained from the data was compared to the t distribution critical values table using the smallest degrees of freedom and P value of 0.05 for a one-sided test and 0.025 for a two-sided test [14].

3. Results

3.1. Gross and Histopathology. All of the joeys gained weight during the extent of the study. The average biweekly weight gain between the three groups of joeys was not remarkably different, inoculated (425 g), exposed (385 g), and controls (502 g), and no significant difference was noted for total body weight gain. Additionally, there was no significant difference noted for any of the major organs across any of the groups. There was no significance noted when comparing total body weight gain of control versus the inoculated opossums, controls versus exposed opossums, and inoculated versus

Table 1: *Mycobacterium bovis* culture group results. The column to the left indicates the opossum group as *M. bovis* inoculated, exposed, or control. The first row indicates the four pools that were created for culture. Pool A: cranial lymph nodes and tonsil, Pool B: thoracic lymph nodes, Pool C: liver, kidney, spleen, and abdominal lymph nodes, and Pool D: small intestine and large intestine. The body of the table is split into boxes indicating the positive (Pos) and negative (Neg) *M. bovis* culture results.

Inoculation group	Pool A upper respiratory		Pool B lower respiratory		Pool C systemic		Pool D alimentary	
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Inoculated	0	4	4	4	2	4	0	4
Exposed	0	4	0	4	0	4	0	4
Controls	0	4	0	4	0	4	0	4

exposed opossums. There was no significance noted when comparing major organ weight of controls versus inoculated opossums for lung, liver, kidney, and spleen. There was no significance noted when comparing major organ weight of controls versus exposed opossums for lung, liver, kidney, and spleen. And finally, there was no significance noted when comparing major organ weight of exposed versus inoculated opossums for lung, liver, kidney, and spleen.

On gross examination, the lungs of all four inoculated opossums were characterized by marked multifocal to coalescing, raised, white, firm caseogranulomatous nodules distributed throughout all lung lobes which on histological examination were characterized by multifocal caseogranulomatous pneumonia (Figures 1 and 2). No gross or histologic lesions were noted in the exposed or control opossums.

3.2. Bacteriology. The isolation of *M. bovis* from pulmonary tissue was successful in all the inoculated opossums. *M. bovis* was isolated from pooled samples of liver, kidney, and spleen in half of the inoculated group (see Table 1). Bacterial cultures for *M. bovis* were negative for all control and exposed opossums.

3.3. Serology. The rapid test identified positive results in all of the inoculated opossums. The exposed and control opossums were rapid test uniformly negative (Figure 3).

4. Discussion

This study investigated the potential for intraspecies lateral transmission of *M. bovis* in Virginia opossum. Part of the justification for this investigation was the well-established role of the Brushtail possum, a distant relative of the Virginia opossum, as a reservoir host of *M. bovis* and their role in the epidemiology of animal tuberculosis in New Zealand [7, 11]. There is little information on the potential of the Virginia opossum population as a reservoir of *M. bovis* or spread of infection within the population [5, 6].

This study failed to demonstrate any detectable horizontal transmission from opossums infected by aerosol with *M. bovis* to exposed opossums. All of the inoculated animals had gross, histologic, bacterial culture, and serologic positive tests for tuberculosis, whereas the exposed and control groups had no gross or histologic lesions and remained serologically negative. Typical gross and histologic lesions of multifocal caseogranulmatous pneumonia were noted within all of the inoculated opossums. All inoculated opossums were culture positive for *M.bovis* from the respiratory tissue (pooled thoracic lymph nodes and lung) and half of these were also positive from systemic tissue (pooled liver, kidney, spleen, and abdominal lymph nodes). By day 84 after inoculation, the disease was widely disseminated in half of the inoculated opossums but these opossums did not show any clinical signs of illness, emaciation, or draining tracts. This is in contrast to the Brushtail possums with natural *M. bovis* infection, where

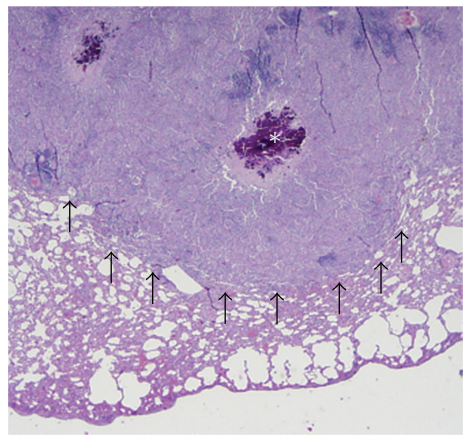


Figure 1: Photomicrograph of a pulmonary tubercle obtained from a *M. bovis* inoculated opossum (2x magnification). Light microscopic features included marked, multifocal, caseogranulomatous pneumonia (outlined by the arrows) with variable amounts of central mineralization (*).

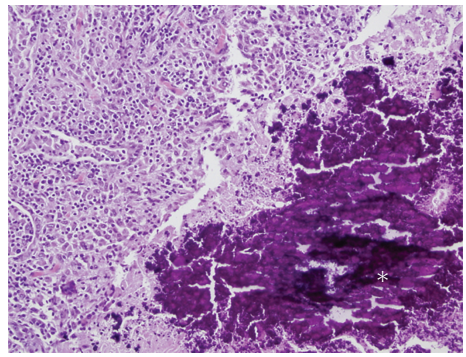
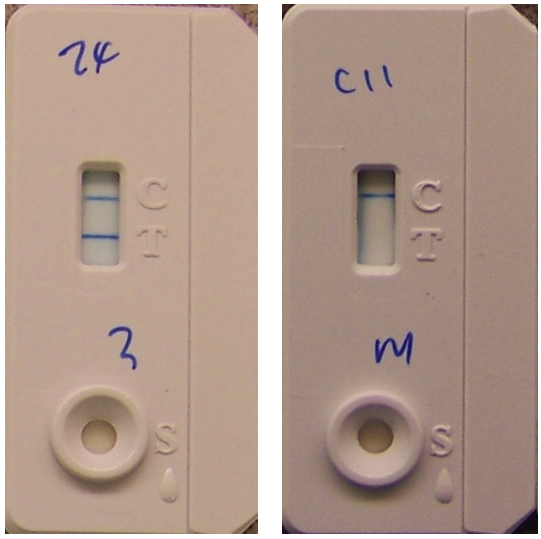


Figure 2: Photomicrograph of a pulmonary tubercle obtained from the opossum described in Figure 1 (40x magnification). Higher magnification of a representative *M. bovis* inoculated opossums characterized by marked, multifocal, caseogranulomatous pneumonia with variable amounts of central mineralization (*).



(a) (b)

Figure 3: Representative results for the rapid test. (a) The cassette to the left displays a positive band at the control (C) and test (T) window, representing a positive *M. bovis* result. (b) The cassette to the right only displays a positive band at the control (C) window and no band at the test (T) window, representing a negative *M. bovis* result.

the disease is highly progressive and fatal. The mean survival time of Brushtail possums with natural tuberculous is 4.7–14 months and with experimental tuberculosis is 8 weeks after inoculation by intratracheal inoculation [4, 5, 11]. The present study did not address the clinical manifestations of chronic disease progression or bacterial shedding in Virginia opossums; this should be investigated in future studies.

Recent advances in development of serologic assays for antemortem detection of *M. bovis* infection in multiple-host species include the Chembio rapid test [13]. In the present study, this serodiagnostic method was able to identify all inoculated opossums as positive and the exposed and control opossums as negative. Interestingly, two of the four infected animals in which *M. bovis* cultures were isolated from both the respiratory tissues and the systemic tissues showed very prominent test bands on the rapid test. This observation suggests that the disease burden may be associated with antibody levels; further assessment of disease burden in the context of the infectious dose should be addressed in future studies to fully characterize this potential association. The intradermal tuberculin test is a traditional method that is often performed to determine tuberculosis status in live animals [3, 8, 12, 15, 16]. In our experiment we attempted to evaluate intradermal pinnal injections of bovine purified protein derivative (bPPD) but this procedure was difficult and was subsequently discontinued (data not shown).

In conclusion, experimental *M. bovis* aerosol infection of Virginia opossums produced pathological, bacteriological, and serological evidence of tuberculosis. However, *M. bovis* was not detected in exposed opossums after 45 days of cohabitation between aerosol-infected suggesting no appreciable lateral transmission of *M. bovis*. Future studies may be warranted to assess shedding patterns and chronic disease progression in Virginia opossum involving a longer exposure period.

Acknowledgments

Authors would like to acknowledge Janet Hengesbach, Jean Gaymer, Danielle Ferguson, and the MSU animal containment facility staff for help in animal care and restraint, and RoseAnn Miller for help in data analysis. Partial funding for this study was provided by the Michigan Department of Agriculture (MDA) and the United State Department of Agriculture (USDA).

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 457872, 6 pages
doi:10.1155/2012/457872

Research Article

T-Cell and Antibody Responses to Mycobacterial Antigens in Tuberculin Skin-Test-Positive *Bos indicus* and *Bos taurus* Cattle in Ethiopia

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Received 24 January 2012; Revised 4 March 2012; Accepted 20 March 2012

Academic Editor: Jesse M. Hostetter

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Higher IFN-γ responses to mycobacterial antigens were observed in *Bos taurus* (Holsteins) than in *Bos indicus* (Zebu) cattle which could due to differences in antigen recognition profiles between the two breeds. The present study was conducted to evaluate mycobacterial antigen recognition profiles of the two breeds. Twenty-three mycobacterial antigens were tested on 46 skin test positive (24 Zebu and 22 Holstein) using enzyme-linked immunospot assay (ELISPOT) and multiple antigen print immunoassay (MAPIA). Herds from which the study cattle obtained were tested for Fasciola antibody. The T cells from both breeds recognized most of the mycobacterial antigens at lower and comparable frequencies. However, antigens such as CFP-10, ESAT-6, Rv0287, Rv0288, MPB87, Acr-2, Rv3616c, and Rv3879c were recognized at higher frequencies in zebu while higher frequencies of T cell responses were observed to Hsp65 in both breeds. Furthermore, comparable antibody responses were observed in both breeds; MPB83 being the sero-dominant antigen in both breeds. The prevalence of Fasciola antibody was 81% and similar in both breeds. This piece of work could not lead to a definitive conclusion if there are differences in mycobacterial recognition profiles between the two breeds warranting for further similar studies using sound sample size from the two breeds.

1. Introduction

Historical reports indicated that *Bos taurus* (the group to which Holsteins belong) are more susceptible to bovine tuberculosis (TB) than *Bos indicus* (zebu) [1]. Experimental studies have also showed difference in susceptibility to bovine TB between *Bos taurus* and *Bos indicus* breeds [1]. It has also recently been found that there is higher prevalence of bovine TB in Holstein than in zebu kept under identical husbandry conditions in Ethiopia [2]. In addition, the severity of pathology in bovine TB was significantly higher in Holstein than in Zebu under a similar field cattle management in central Ethiopia [2]. Similarly, it was observed that IFN-γ responses to mycobacterial antigens were higher in Holstein than those in Zebu [3]. One of the possible reasons for the lower IFN-γ responses in Zebu could be a difference in antigen recognition profiles between Holstein and Zebu. Human and mouse studies have also shown that the immune response to particular mycobacterial antigens varies with the genetic background of the subjects involved [4, 5]. This hypothesis is being addressed in this study. To assess repertoire difference between Holstein and Zebu, peripheral blood mononuclear (PBMC) and sera from the two breeds were investigated for their specificities and intensities in respond to different mycobacterial antigens.

2. Materials and Methods

2.1. Study Animals. For the assessment of T-cell responses 30-skin-test-positive cattle (14 Holstein and 16 Zebu) that were managed under the same field condition were used. These animals were recruited from herds in which the two breed types were kept under identical husbandry conditions by traditional farmers. They were screened by applying

Table 1: List of mycobacterial antigens used the comparative study of immune response between *B. indicus* (Arsi breed) and *B. taurus* (Holstein) in Ethiopia.

Antigens	Protein	Peptides	Working concentration Final concentration at well (μg/mL)	Supplier
(1) *ESAT-6-like:*				
CFP-10	X		5	Lionex[1]
ESAT-6	X		5	Lionex
Rv0288	X		5	Protix[2]
Rv0287	X		2	Dr Mustafa[3]
Rv3019c	X		5	Lionex
(2) *Heat-shock proteins:*				
HSP65	X		5	Lionex
HSP70	X		5	Lionex
Acr1	X		5	Lionex
Acr2	X		5	Protix
(3) *Secreted antigens/lipoproteins:*				
Ag85A	X		5	Lionex
Ag85B	X		5	Lionex
MPB83	X		5	Lionex
MPB70	X		5	Lionex
19 kDa antigen	X		5	Lionex
38 kDa antigen (Pst-1)	X		5	Lionex
(4) *Misc.*				
Rv0909		X	10	Pepscan[4]
Rv3616c		X	10	Pepscan
Mb2555	X		5	VSD[5]
Mb2890	X		5	Lionex
Mb3895		X	10	Pepscan
Rv3879c	X		5	VSD
Rv1196	X		2	Dr Mustafa
Rv1769	X		5	Lionex

[1]Lionex, Braunschweig, Germany; [2]Protix Prague, Czech Republic; [3]Dr A. Mustafa, Kuwait University, Kuwait; [4]Pepscan, Lelystad, The Netherlands; [5]Veterinary Science Division (VSD), Belfast, Northern Ireland.

the single intradermal comparative skin test using avian PPD (aliquots of 0.1 mL of 2500 IU/mL; Veterinary Laboratories Agency, UK) and bovine PPD (aliquots of 0.1 mL of 2500 IU/mL; Veterinary Laboratories Agency, UK), and tested positive applying the standard interpretation of this test (responses to bovine PPD were more than 4 mm larger than those to avian PPD). Blood was collected 6 weeks after skin testing. For the evaluation of antibody responses, 16 cattle (8 Holstein and 8 Zebu) of similar age and having strong reactions to the TB StatPak lateral-flow assay (Chembio, NY, USA) were tested by multiantigen print immunoassay (MAPIA) as described earlier [6]. Although the cattle used for the evaluation of antibody were different from those cattle used the assessment of T-cell response, both groups of cattle were kept homogenously on pasture by the same traditional farmers in the same area.

2.2. Mycobacterial Antigens. Avian and bovine PPDs were used for the skin test and *in vitro* immunological assays. Additionally, 23 mycobacterial antigens were used. Detailed information regarding these antigens is presented in Table 1.

2.3. Enzyme-Linked Immunospot (ELISPOT) Assay. The ELISPOT assay was performed following the procedure used by other researchers earlier [7]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated using ficoll-Histopaque-1077 (Sigma-Aldrich, Poole, UK) gradient centrifugation at 800 ×g for 45 min. The PBMCs were washed 2 times in HBSS (Gibco, Paisley, UK), supplemented with Heparin (Leo, Ballerup, Denmark) by centrifugation for 5 min at 500 ×g. The cells were resuspended at 2×10^6 cell/mL in RPMI) 1640 medium supplemented with 10% foetal calf serum (FCS), penicillin/streptomycin, 2-mercaptoethanol, nonessential amino acids (all Sigma-Aldrich) (complete medium). Polyvinylidene difluoride (PVDF) microplates were coated at 4°C with 100 μL/well (10 μg/mL) anti-bovine IFN-γ capture monoclonal antibody (mAb) 5D10 (Bioscience, Wheatley, UK) diluted 1 : 600 in sterile coating buffer. The

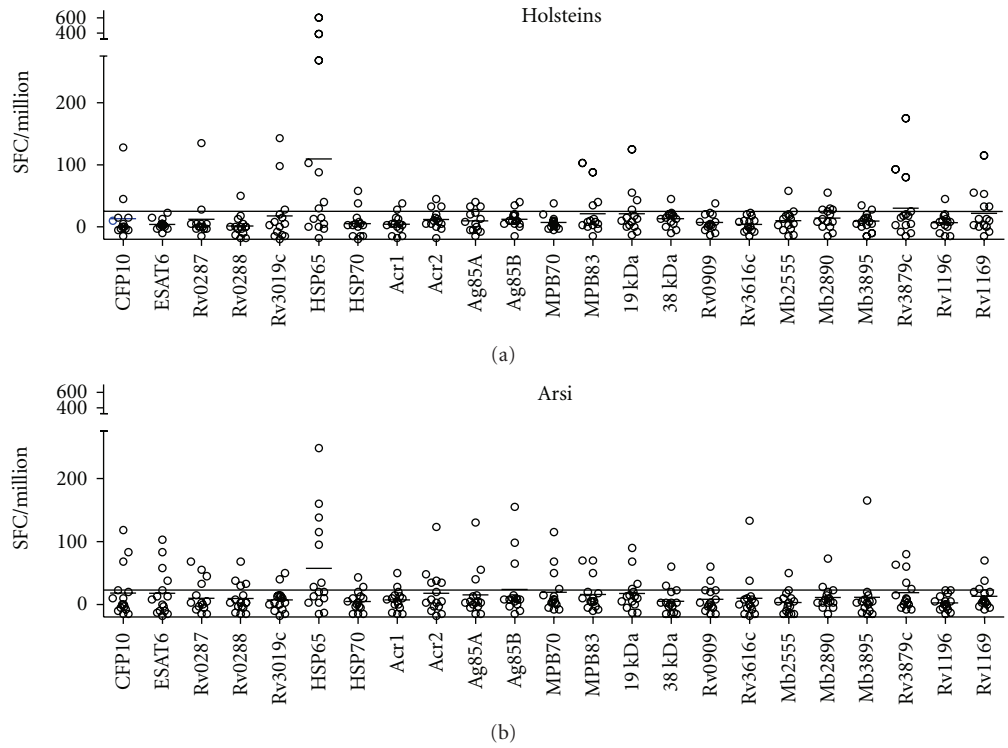

Figure 1: IFN-γ producing T cells. Enzyme immunospot assay (ELispot) was used for the assessment of IFN-γ producing T cell in response to novelmycobacterial antigens in 30 cattle. Twenty-three mycobacterial antigens were used to assess the response in each breed. Horizontal line = cutoff for positivity (25 SFC/million PBMC) used to calculate responder frequencies for figure.

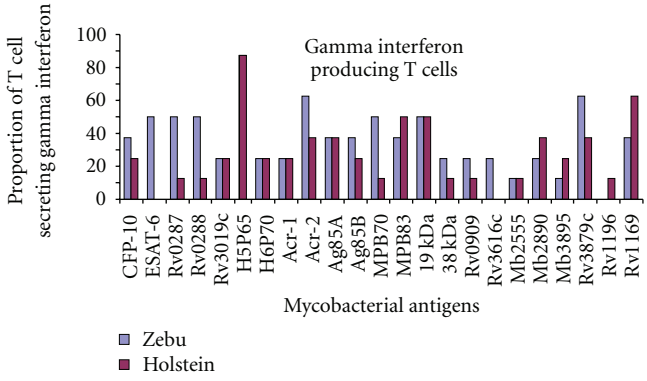

Figure 2: Proportion of IFN-γ producing T-lymphocytes in Holstein and zebu cattle. Enzyme immunospot assay (ELispot) was used for the assessment of IFN-γ producing T cell in response to novel mycobacterial antigens. Responder frequencies for Holstein and zebu cows were calculated using a cutoff for positivity >25 SFC/million). Although both breeds recognized the majority antigens studied at comparable frequencies, antigens such as CFP-10, ESAT-6, Rv0287, Rv0288, MPB87, Acr-2, Rv3616c, and Rv3879c were recognized at higher frequencies in Zebu than in Holstein.

plates were then washed twice with RPMI-1640 and blocked with RPMI-1640/10% FCS for 2 h at 37°C. After removing the blocking solution, 100 μL (2×10^6 cell/mL) PBMC in complete medium was added into each well and stimulated with 100 μL of each of the antigens at the concentrations indicated in Table 1. The plates were incubated at 37°C and 5% CO_2 for 24 h and washed twice with distilled water and three times with phosphate buffer solution in Tween 20 (PBS-T, 0.05% Tween in PBS). Thereafter, 100 μL rabbit anti-bovine IFN-γ diluted 1 : 100 in serum albumin (BSA, Sigma-Aldrich) was added into each well and incubated at room temperature for 1 h. The plates were then washed four times with PBS-T, which was followed by the addition of 100 μL monoclonal anti-rabbit IgG alkaline phosphatase conjugated to streptavidin (Sigma clone R696 diluted 1 : 2000 in PBS-T/BSA) and incubated for 1 h at room temperature. Bromo-4-chloro-3-indolyl phosphate (BCIP) nitroblue tetrazolium (NBT) substrate was prepared by vortexing one buffered BCIP/NBT 6 substrate tablet (Sigma-Aldrich) in 10 mL distilled water 2-3 minutes. This was followed by the addition of 100 μL substrate solution and incubation for 10 minutes in the dark. Thereafter, the substrate was flicked off, the plastic manifold was removed, and the plates were washed with water on the front and back. Then, the plates were air-dried and read using the automated AID ELISPOT Reader (AID, Strassberg, Germany).

2.4. Multiantigen Print Immunoassay (MAPIA). MAPIA was performed as described previously [6] on sera of 16 cattle. A panel of 12 mycobacterial antigens were immobilized on nitrocellulose membranes (Schleicher & Schuell, Keene NH)

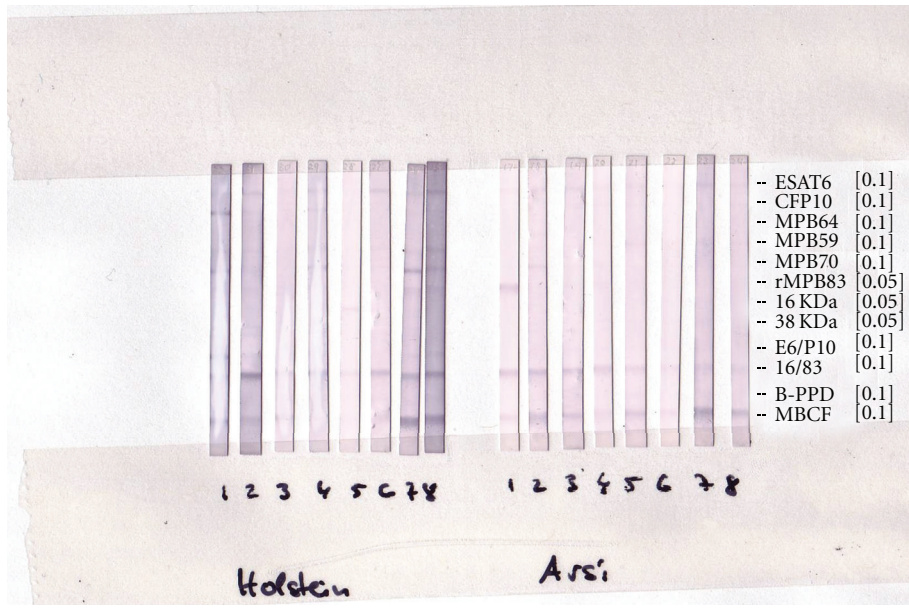

Figure 3: Antigen recognition pattern by antibody from Holsteins and zebus kept on pasture homogenously. Serological reactivity to *M. tuberculosis* complex antigens was determined by MAPIA for the two breeds. Antigens are indicated on the right margin, while the numbers on the bottom indicate animals (eight animals from each breed). *M. bovis* culture filtrate (MBCF) and 16 kDa alpha-crystallin/MPB83 fusion proteins (16/83) were serodominant antigens in both breeds. E6P10 refers to the ESAT6 : CFP10 fusion protein; PPDb refers to *M. bovis* PPD.

at a protein concentration of 0.05 mg/mL using a semi-automated airbrush-printing device (Linomat IV, Camag Scientific Inc, Wilmington, DE). The membrane was cut perpendicular to the antigen pads into 4 mm wide strips. Strips were blocked for 1 h with 1% nonfat skimmed milk in PBS with 0.05% Tween 20 and then incubated for 1 h with serum samples diluted 1 : 50 in blocking solution. After washing, the strips were incubated for 1 h with horseradish peroxidase-conjugated protein G (Sigma) diluted 1 : 1000 for IgG detection or with peroxidase-labeled antibody to bovine IgM (Kikegaard and Perry Laboratories) diluted 1 : 500, followed by another wash step. Bovine antibodies bound to printed antigens were visualized with TMB membrane peroxidase substrate (Kirkegaard &Perry Laboratories, Gaithersburg, MD). Results were evaluated by visual observation for the formation of band against of the antigens used.

2.5. Antibody Detection for Fasciola. Fasciola seroprevalence was estimated in randomly selected 263 (67 skin test positives and 196 skin test negative) cattle. Sera samples were submitted to the Veterinary Laboratories Agency (VLA) for the examination for the presence of antibodies against Fasciolosis using enzyme-linked immunosorbent assay following the standard operating procedures of VLA.

3. Results

3.1. Mycobacterial Epitope Recognition Patterns of T Cells in Grazing Zebu and Holsteins. ELISPOT analysis was performed for the assessment of the specificities and intensity of IFN-γ producing T cell in response to the defined mycobacterial antigens listed in Table 1. Figure 1 shows T-cell responses of individual animals. Whilst hasp65 was recognized most frequently and most strongly, a number of additional antigens were also recognized at comparable frequencies by T cells from both breeds.

When responses were stratified according to breed (Figure 2), although both breeds recognized the majority antigens at comparable frequencies, antigens such as CFP-10, ESAT-6, Rv0287, Rv0288, MPB87, Acr-2, Rv3616c, and Rv3879c were recognized at higher frequencies in zebu than in Holstein.

3.2. Mycobacterial Antigen Recognition Patterns of Antibodies in Grazing Zebu and Holstein. In order to investigate possible differences in antibody repertoire between Zebu and Holsteins cattle, MAPIA was performed. Holstein and Zebu exhibited similar antibody recognition profiles; with MPB83 being serodominant antigen in both breeds (Figure 3).

4. Discussion

In this study, the responses of T cell and antibody to mycobacterial antigens were compared in Holsteins and zebus kept under identical cattle management. In the ELISPOT assay, both Holsteins and zebus exhibited similar frequencies of T-cell responses to most of the mycobacterial antigens included in this study; a stronger T-cell response was observed to Hsp65 in both breeds. Previous studies [7] indicated that vaccination of calves with Hsp65 resulted in

enhancement of lymphocyte proliferation following stimulation with Hsp65. The same workers have reported that PBMC from unvaccinated calves neither proliferate nor release IFN-γ following *in vitro* stimulation with Hsp65, whilst experimentally with *M. bovis*-infected cattle showed responses to this antigen [8, 9]. However, Hsp65 is an ubiquitously expressed antigen across the genus *Mycobacterium*, which could also result in cross-reactivity after exposure to environmental mycobacteria. However, higher responses to some of the specific antigens (ESAT-6, CFP-10, and MPB70) were observed in zebu than in Holstein. Nonetheless, as this experiment is preliminary, it needs further confirmation using sufficient number of cattle from both breeds.

In addition, a comparable recognition of mycobacterial antigens by antibodies from both Holstein and zebu breeds was also observed. *M. bovis* culture filtrate (MBCF) and 16 kDa alpha-crystallin/MPB83 fusion protein (16/83) were the serodominant antigens in both breeds. MBCF is a complex mixture of proteins and lipids, with a principal protein component of MPB70. The latter is a secreted antigen of *M. bovis* with high sequence homology with MPB83 [10, 11]. MPB70, ESAT-6, and CFP-10 were less frequently recognized by both breeds. A previous study [12] has also reported the sero-dominance of MBCF and 16/83 antigens in cattle infected with *M. bovis* experimentally. Waters et al. [12] further reported increment of the intensity of the antibody response to these antigens over time after inoculation of *M. bovis*. Similarly, in badger study, MBCF and MPB83 were the most frequently recognized antigens during infection with *M. bovis* [13].

However, in both breeds the intensities of the T-cell responses to mycobacterial antigens was generally low, which could be due to coinfection with helminthes that leads to a Th2 bias response thereby downregulating the T-cell response [14]. In our study, we found high seroprevalence of Fasciola in grazing cattle, which could result in generation of Th2 response resulting in inhibiting Th1 response [14]. Thus, Fasciola seropositivity was found in 81% ($n = 213$) in the population of cattle from which the study animals were selected. When these results were analyzed according to cattle breed, the prevalences of Fasciola antibody were similar ($\chi^2 = 4.26$; $P > 0.05$) in Holstein (82.4%, $n = 159$) and in Zebu (78.8%, $n = 104$) cattle. Even within grazing cattle the higher IFN-γ response was reported in Holsteins compared to Zebu [3]. This could be attributed to the higher severity of pathology of bovine TB in Holsteins as compared to Zebu as observed in our earlier observation [2]. A positive correlation has been reported between the intensity of IFN-γ responses to mycobacterial antigens and severity of pathology of bovine TB under experimental conditions [15–17]. However, an ELISA readout was used in the previous study, and it is possible that the more responses were revealed in the more sensitive ELISPOT assay that we employed in the present study.

As this piece of work could not lead to a definitive conclusion if there is a difference in mycobacterial recognition profiles between Holstein and zebu cattle, further studies on the cytokine profiles and T-cell subset distribution in the two breeds are recommended.

Acknowledgments

This work was financially supported by the Wellcome Trust. Surane Gemeda and Nega Nigussie was acknowledged for their technical support.

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 742478, 6 pages
doi:10.1155/2012/742478

Research Article

DNA Typing of *Mycobacterium bovis* Isolates from Badgers (*Meles meles*) Culled from Areas in Ireland with Different Levels of Tuberculosis Prevalence

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Received 20 December 2011; Revised 13 February 2012; Accepted 29 February 2012

Academic Editor: Jesse M. Hostetter

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Badgers (*Meles meles*) have been implicated in the transmission of *Mycobacterium bovis* infection to cattle in Ireland and UK. Recent studies in Ireland have shown that although the disease is endemic in badgers, the prevalence of disease is not uniform throughout the country and can vary among subpopulations. The extent to which the prevalence levels in badgers impact on the prevalence in cattle is not known. Previously, DNA fingerprinting has shown that *M. bovis* strain types are shared between badgers and cattle, and that there are a large number of strain types circulating in the two species. In this study we have carried out spoligotyping and variable number tandem repeat (VNTR) analysis of *M. bovis* isolates from two groups of badgers, representing a wide geographic area, with different tuberculosis prevalence levels. The results of the typing show that there is no geographic clustering of strain types associated with prevalence. However, two VNTR profiles were identified that appear to be associated with high- and low-prevalence *M. bovis* infection levels, respectively. In addition, spoligotyping and VNTR analysis has provided evidence, for the first time, of multiple infections of individual badgers with different *M. bovis* strains.

1. Introduction

In the Republic of Ireland (RoI) and the UK tuberculosis (TB) is present in badger populations [1]; infected badgers are considered as a maintenance host and are directly implicated in the transmission of *Mycobacterium bovis* to cattle [2]. In RoI, as part of a medium-term strategy for the control of TB in cattle, badgers are removed (focal culling) when an epidemiological investigation associates a cattle herd TB breakdown with the presence of infected badgers. Studies using comprehensive postmortem examination and bacterial culture of tissues have found an infection prevalence of 36–50% in these culled badgers [3] (Corner, unpublished).

In a recent comparative study we determined the infection prevalence in badgers in areas with historically and consistently low prevalence of infection in cattle [4]. Badgers were removed from geographically dispersed sites and all were examined using detailed postmortem and bacteriological procedures. A significantly lower prevalence of *M. bovis* infection was found in these badgers than in badgers removed during focal culling. While the results validated the use of cattle as sentinels for TB in badgers, they also raised questions on the nature of *M. bovis* infection in badgers in high prevalence and low prevalence populations. One possibility for the different prevalence rates is that the strains of *M. bovis* circulating in these populations differ in virulence and in their potential for transmission from badger to badger, and badger to cattle. Although very little is known about the virulence potential of field isolates of *M. bovis*, DNA fingerprinting of strains has been used to study the dynamics of TB in animals and to investigate links between infections in farmed and wild species [5, 6]. These studies have revealed many different strains circulating in cattle populations in RoI and the UK although detailed

analysis has revealed that over 99% of strains originate from a single clonal complex named Eu1 [7]. In previous studies, the genotyping of 452 isolates of *M. bovis* by restriction fragment length polymorphism (RFLP) analysis in RoI revealed that the most prevalent RFLP types were widely distributed and present in both cattle and badgers [8]. The relationships between the strains isolated from cattle and badgers over large areas revealed that badgers and cattle tended to have similar strains, consistent with the sharing of *M. bovis* strains within an area and providing evidence of cross-species transmission [9].

The development of molecular strain typing techniques for the differentiation of *M. bovis* strains has greatly enhanced the ability to conduct epidemiological investigations of disease transmission in wildlife and livestock [10]. In RoI the combination of spoligotyping and MIRU-VNTR typing has proved to be superior to either test alone in revealing the diversity of *M. bovis* strains circulating in cattle and badgers [11]. In the present study we applied a combination of spoligotyping and MIRU-VNTR typing of *M. bovis* isolates from badgers in high-and low-prevalence areas of cattle TB; the objective was to investigate whether the same or dissimilar strain types were associated with the infection prevalence level in these populations.

2. Materials and Methods

2.1. Selection of Badgers from Different Prevalence Populations. Low Prevalence Group (LP). The detailed selection process to identify badger setts in the areas of the country with the lowest cattle TB herd prevalence has been described elsewhere [4]. Briefly, the process exploited the Irish national bovine disease database (animal health computer system, AHCSI), the Land Parcel Identification System (LPIS) geographic database, and Geographical Information Systems (GIS) analytical software. Badgers were identified in areas with herds where historically there was a low prevalence of TB in cattle (<2 standard reactors in the previous 5 years). Because the badger is a protected species in Ireland, the opportunity to cull animals is severely restricted in the absence of strong indications of the presence of disease. As LP areas do not qualify for routine badger culling, we were only granted access to LP area badgers on a very limited basis, and we were restricted to collect one badger per site with active setts.

Areas ($n = 198$ low prevalence sites) were identified in 24 different counties. The areas were surveyed for badger setts or signs of badger activity, and 138 areas were deemed to be active. Trapping was conducted in these areas, and a single badger was removed from 101 sites in the vicinity of setts with badger activity. The badger was then euthanized and subjected to a detailed necropsy with the collection of pools of tissues for bacterial culture. Infected badgers were identified in 10 different counties giving an infection prevalence of 15.8% in these badger populations.

High Prevalence Group (HP). For comparative purposes, badgers ($n = 215$) were obtained from culling operations associated with cattle herd breakdowns and were sourced from areas across 16 different counties in RoI [3]. The social group sizes ranged from 1 to 7 with a mean of 1.62 and a median of 1. There was no significant difference in infection prevalence between social groups of different sizes [3]. The *M. bovis* infection prevalence in this population was 36.3%.

2.2. Postmortem Examination. Captured badgers were anaesthetised with ketamine hydrochloride (0.1 mL/kg) and medetomidine (Domitor; 0.1 mL/kg) and then were euthanased with an overdose of intravenous sodium pentabarbitone. The badgers were subjected to a detailed necropsy including examination for bites or wounds. For the post mortem examination, the carcase was placed in dorsal recumbency on a downdraft table (Astec Microflow). To minimise the risk of cross-contamination, separate sterile instruments were used to expose lymph nodes, to open the abdominal and thoracic cavities, and to expose and dissect free-lymph nodes from surrounding fat and connective tissue, and to collect sections of visceral organs. From each badger, 20 separate lymph node samples and samples of kidney, spleen and liver were cultured as pools (head, carcase and thoracic lymph nodes, abdominal lymph nodes and organs, and lungs) and any bite wounds, subcutaneous abscesses or suspect gross lesions were cultured separately [3, 4]. All samples were stored at −20°C prior to culture.

2.3. Culture of M. bovis, DNA Extraction, and Spoligotyping. Samples were cultured on selective mycobacterial media as described by Murphy et al. [3, 4]. For the purpose of this study, a badger was considered infected when *M. bovis* was isolated by bacterial culture from any sample. Multiple colonies generated from each tissue sample were scraped from the solid media and transferred to a single microtube containing 500 μL of phosphate-buffered saline with Tween 20 (PBS-Tw) (Sigma Aldrich, Wicklow, Ireland). DNA was extracted by heat lysing of cells as described by McLernon et al. (2010) [11]. DNA template was stored at −20°C. Spoligotyping was performed according to the method described by Kamerbeek et al. [12] except that a digoxigenin labeling and detection system (Roche Diagnostics, West Sussex) was used. Spoligotype patterns were given the names assigned in the *M. bovis* spoligotyping database at http://www.mbovis.org/.

2.4. VNTR Typing. VNTR typing was performed using the six loci; 2163a (alternate name, QUB 11a), 2163b (alternate name, QUB 11b), 2165 (alternate name, ETR A), 2996 (alternate name, MIRU 26), 4052 (alternate name QUB26) and 1895. The six genomic loci were amplified separately using the primers and polymerase chain reaction (PCR) procedures described by McLernon et al. [11].When the PCR was complete, the amplified products were stored at −18°C until analysed using the MegaBACE 1000 (GE Healthcare Life Sciences) as described by McLernon et al. [11].

2.5. Statistical Analysis. The comparisons of the strains in badgers were made between the areas, and not the individuals within the areas. The data were analysed using GraphPad Prism (GraphPad Software, USA,

http://www.graphpad.com/). Chi squared test was used to analyse the geographic distribution of spoligotypes.

3. Results

3.1. Geographic Distribution of Badgers and Distribution of Infection within Badgers. In both high (HP) and low (LP) prevalence populations, infected animals were obtained in the vicinity of active setts over a wide geographic area, 16 counties for the HP group and 10 counties for the LP population. This minimized the impact of geographic clustering effects of infection and particular strain types. A total of 51 isolates were typed from 36 badgers in the HP population (average 1.4 isolates per badger, range 1–3) and 42 isolates from 16 badgers in the LP population (average 2.6 isolates per badger, range 1–5). Two or more isolates were obtained from 20 badgers: 2 tissue pools were positive in 11 badgers, 3 pools in 2 badgers, and ≥4 pools in 7 badgers. Five badgers were coinfected with two different strains, 3 from the HP group, and 2 from the LP group.

3.2. Spoligotyping of M. bovis Isolates in HP and LP Badgers. There were 9 different spoligotypes identified among the badgers, 7 types in the 36 HP badgers, and 4 among the 16 LP badgers. SB0140 was the most common spoligotype across both badger populations and apart from SB0130 the only spoligotype common to the two groups (Table 1). There was no evidence of geographic clustering of SB0140 as it was identified in all regions where the badgers for each group were sourced. There was no significant difference (χ^2 test, $P > 0.05$) in the proportion of badgers infected with this spoligotype between the groups, it being present in 13 of the 16 (81.3%) badgers of the LP population, and 24 of the 36 (66.7%) badgers of the HP group.

3.3. VNTR Analysis of M. bovis Isolates. The panel of 6 VNTR loci subdivided the 93 isolates into 22 strain types, there were 17 VNTR types in the badgers of the HP group and 9 VNTR types in the LP group. Among the group of strains bearing spoligotype SB0140, there were a total of 17 different VNTR types identified (Table 2). Thirteen of these VNTR profiles were identified in 24 badgers belonging to the HP group, while 7 were present in 13 badgers of the LP group. Two of these VNTR types were differentially represented in the HP and LP groups. VNTR type 11 3 7 5 4 4, was found in 8/13 (61.5%) badgers with SB0140 in the LP group, whereas, it was found in only 1/24 (4.1%) badgers infected with SB0140 in the HP group. VNTR type 11 4 7 5 4 4 was identified in 9/24 (37.5%) of badgers in the HP group infected with SB0140, but in only 1/13 (7.7%) of LP badgers infected with SB0140. The remaining VNTR types belonging to SB0140 and other spoligotypes were present in low numbers of badgers from each group. There was no evidence of geographical clustering of the two dominant VNTR types, both were found in badgers over a wide geographic area.

3.4. Coinfection of Badgers with Multiple M. bovis Strain Types. Five badgers were coinfected with two different strains, 3 from the HP group and two from the LP group (Table 3). In two of the badgers the strains were differentiated by spoligotyping, while the remaining three were co-infected by strains bearing spoligotype SB0140 that were differentiated by VNTR analysis. There was no spatial relationship between any of the co-infected badgers, each animal originated from a different area. In one badger belonging to the LP group, one of the co-infecting strains was isolated from a lesion, described as an "enlarged submandibular lymph node." The distribution of infected tissues from which the co-infecting strain was isolated (Table 3) did not reveal any predilection sites.

Table 1: Number of badgers infected with each *M. bovis* spoligotype. *One HP badger was coinfected with SB0130 and SB0275. **One LP badger was coinfected with SB0263 and SB0140.

Spoligotype	HP	LP
SB0130	5	2
SB0140	24	13
SB0142	1	0
SB0144	1	0
SB0145	1	0
SB0146	0	1
SB0263**	0	1
SB0275*	2	0
SB0978	3	0

Table 2: VNTR profiles of *M. bovis* strains bearing the SB0140 spoligotype. Differentially represented strains are highlighted/italicised. *VNTR types were not unique to individual badgers (see Table 3).

VNTR	HP	LP
3 4 7 5 4 4*	3	0
3 5 7 5 4 4*	2	0
9 4 7 5 3 4	1	0
9 4 7 5 4 4	1	0
10 3 4 5 4 3	1	0
10 3 5 5 4 3	1	0
10 4 5 5 4 3	0	1
10 4 7 5 4 4*	0	1
11 3 7 5 4 4*	1	8
11 4 5 5 3 4	1	0
11 4 6 5 4 4	2	0
11 4 7 5 2 4	1	1
11 4 7 5 3 4	1	0
11 4 7 5 4 4*	9	1
11 4 7 6 3 4	2	0
6 3 7 5 4 4	0	1
6 4 7 5 4 4	0	1

Table 3: Coinfection of badgers with *M. bovis* strains. Tissues from which *M. bovis* was isolated: H: head, T: Thorax, C: Carcase, L: Lung, Les: lesion, A: Abdomen. Co-infecting strains are highlighted/italicised. VNTR loci are listed in order 2163a, 2163b, 2165, 2996, 4052, and 1895.

Badger	Tissue	Spoligotype	VNTR
HP95	H	SB0275	10 3 7 5 4 4
HP95	T	**SB0130**	**3 3 7 3 3 2**
HP95	C	SB0275	10 3 7 5 4 4
HP108	H	SB0140	3 4 7 5 4 4
HP108	T	**SB0140**	**3 5 7 5 4 4**
HP108	C	SB0140	3 4 7 5 4 4
HP212	C	**SB0140**	**3 4 7 5 4 4**
HP212	H	**SB0140**	**11 4 7 5 4 4**
LP240	H	SB0140	10 4 7 5 4 4
LP240	L	SB0140	10 4 7 5 4 4
LP240	C	SB0140	10 4 7 5 4 4
LP240	Les	**SB0140**	**11 3 7 5 4 4**
LP307	T	SB0140	6 4 7 5 4 4
LP307	H	SB0140	6 4 7 5 4 4
LP307	L	SB0140	6 4 7 5 4 4
LP307	C	SB0140	6 4 7 5 4 4
LP307	A	**SB0263**	**10 4 7 5 4 4**

4. Discussion

In RoI, prevalence studies in badgers conducted using a detailed postmortem and bacteriological examination have shown that the prevalence of infection with *M. bovis,* in areas associated with high prevalence of infection in cattle, is significantly higher than in areas with a low prevalence of infection in cattle [3, 4]. While there may be various epidemiological factors responsible for these differences in prevalence, most of which are not readily apparent, one question that can be resolved is the possibility that differences in prevalence are associated with the strains circulating in the badger population. In this study *M. bovis* strains bearing the spoligotype SB0140 were predominant among the HP and LP badger groups. In a previous study approximately 50% of 386 strains, mainly from cattle and badgers, had this spoligotype [11]. In the present study the proportion of strains bearing spoligotype SB0140 was higher especially among the LP group of badgers where 81.3% of strains were of this type. However, these differences could have been due to sampling factors and a result of the small number of isolates available for typing. Spoligotype SB0140 has a widespread distribution and is long established in RoI [11], and being found in equally high frequencies in both high and low prevalence areas, it appears not to be a defining factor in the prevalence of infection in badgers.

When VNTR analysis was used to further differentiate the strains, the highest number of VNTR profiles was found within the SB0140 strains, followed by the SB0130. This is consistent with the analysis of a much larger number of isolates from cattle, badgers, and deer [11]. The most intriguing result from the current study was the unequal representation of two VNTR profiles of the SB0140 strains between the HP and LP groups of badgers. The observation that these profiles have a wide geographic distribution suggests that their frequency of occurrence is not due to a recent local clonal expansion of a particular strain type. It is tempting to speculate that their overrepresentation in each prevalence group is indicative of virulence properties of the strains that impact on prevalence of infection in the two populations. Analysis of a much larger sample size should help clarify the national distribution of each strain type and may resolve their association with prevalence of infection in badgers. The VNTR profiles from a large sample of cattle isolates show both VNTR types were present in cattle, so the profiles are not unique to infected badgers (unpublished results). In Spain, similar strain typing studies carried out in wild animals (deer, wild boar, Iberian lynx, and fox) and cattle showed that while many *M. bovis* spoligotypes were shared between wild animals and cattle, there were spoligotypes uniquely isolated from cattle [13].

The finding of badgers coinfected with different strains *M. bovis* has not been described previously and raises questions on the pathogenesis of infection and the source of the infections. One possibility is that the strains had evolved in the badger following infection with a single strain. However, in only two of the badgers (HP108 and HP212, Table 3) could this have been possible, as there was a difference at only one VNTR locus. The spoligotypes in HP95 could not have been derived from each other by a single genetic change, and the VNTR profiles also differed at four loci. In addition, the VNTRs for LP240 differed at two loci and there were differences in both spoligotypes and VNTRs for LP307. The evidence strongly points to coinfection as the most likely explanation for multiple strains.

Coinfection with different strain types may not be related to population prevalence as it was found in each prevalence group. A badger harbouring multiple strains is probably the result of multiple transmission events as aerosol infections are the result of single bacterial clones [14]. The observation that animals are coinfected also suggests that there is limited immunity afforded by infection with the original strain that does not prevent subsequent infection. Hence, repeat exposure by either of the common routes of infection may lead to initiation of infection with additional strains. We could only identify multiple infections through differences in strain types, and multiple infections may have been more prevalent than we have reported because strains may have been indistinguishable, and badgers may have been reinfected with the strains of the same spoligotype or VNTR profile as the original infection. In addition, as samples were initially pooled for culture, any co-infecting strain present at very low levels within a pool may not have been detected.

The source of multiple infections was probably transmission from other badgers of the same social group or from immigrant badgers. In areas where badgers have been culled in successive years, it has been observed that RFLP strain profiles of *M. bovis* at particular setts can change from year to

year and are most probably a consequence of inward migration of new badgers. As the LP group was obtained from areas with historically low levels of TB in cattle, it is most likely that infection resulted from badgers to badger transmission as in RoI there have been very few reports of *M. bovis* infection in other wild animals, only in wild deer [15, 16]. Given the prevalence of infection in both prevalence groups, the most plausible explanation is that multiple infections arise from contact of badgers from different territories as a result of movement between territories. While the VNTR analysis reveals the large diversity of strains in the populations, the finding of multiple strains in individual badgers might reflect the high numbers of interactions between badgers. Coinfection of wildlife (red deer, fallow deer, and wild boar) with multiple strains of *M. bovis* has been recorded in Spain, highlighting the complexity of multihost interactions and transmission of multiple strains [17]. Infection with *M. bovis* is most frequently seen in its latent form in badgers [3], and as this form of infection will have minimal impact on a badger's behaviour, the risk of acquiring new infections is not likely to be influenced by previous infections.

In infected badgers, the high prevalence of lung infection strongly supports the lungs as the principal site of primary infection and that inhalation of infectious aerosol particles is the principal mode of transmission. However, other routes including transmission *via* infected bite wounds are known to occur [3]. The distribution of different VNTR types in each co-infected badger did not provide information to deduce the possible route of infection. The transmission and maintenance of *M. bovis* in badger populations is a complex process where many factors influence within-population prevalence and rates of transmission. It is likely the infective dose of each strain may have determined its level of dissemination within the body.

The results of this study provide further evidence of extraterritorial movement of badgers and the discrimination of strains by spoligotyping, and VNTR analysis demonstrates that the interactions between badgers can result in co-infections of individual badgers with different strains. The identification of diverse VNTR profiles also suggests that different strains may be associated with the local prevalence of infection. A field vaccination trial of badgers with the BCG vaccine is currently underway [18]. Although it has been demonstrated that the BCG is effective in protecting captive badgers against experimental *M. bovis* infection, it remains to be determined if the vaccine is equally effective against the many *M. bovis* strain types found in badgers [19].

Acknowledgments

This work was funded by a European Union Framework 7 Project Grant, no. KBBE-2007-1, TB-Step) with assistance from the Department of Agriculture, Food and the Marine Food ERAD program. The authors wish to acknowledge the support provided by Elvira Ramovic and staff from the Central Veterinary Research Laboratory (CVRL), Backweston, Co. Kildare, Ireland.

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 674368, 6 pages
doi:10.1155/2012/674368

Research Article

An Evaluation of MAPIA in Michigan as an Ante-Mortem Supplemental Test for Use in Suspect Tuberculosis Cattle

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Received 19 December 2011; Accepted 13 February 2012

Academic Editor: Mitchell Palmer

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The objective of this study was to make use of bovine tuberculosis suspect cattle from the state of Michigan to validate a multiantigen print immunoassay for use on sera to serve as an improved supplementary ante-mortem test to increase specificity of current tuberculosis testing methods. Over a 27-month period, 234 sera were collected and tested by MAPIA method, which was evaluated using four different interpretation criteria. These results were subsequently compared to final mycobacterial culture and PCR results obtained by the National Veterinary Services Laboratories, Ames, IA, which served as the true indicator of the cattle's tuberculosis infection status. This study indicates that an interpretation criterion which includes 3 or more positive reactions to the 11 different mycobacteria antigens utilized provided both an acceptable sensitivity (69.39%) and a high specificity (90.27%). This MAPIA technique shows potential for eventual application as a supplementary ante-mortem tuberculosis serologic test following one of the various current or soon-to-be-approved whole herd screening assays as part of a tuberculosis eradication program.

1. Introduction

Since the state of Minnesota has been reclassified as bovine tuberculosis accredited free in October, 2011, there are few USA states remaining which have endemic bovine tuberculosis in either its domestic cattle or a free-ranging wildlife reservoir host [1]. Presently, only Michigan and California are not classified as state-wide bovine tuberculosis accredited-free states. Therefore, these states are the logical locations in which to conduct trials on new or alternative bovine tuberculosis assays.

The ongoing decline in bovine tuberculosis in the USA, coupled with the ongoing economic recession, has led the USDA to reevaluate its current approaches for bovine tuberculosis surveillance and eradication [2]. The USDA will now be increasing the options for managing tuberculosis-infected herds, and developing alternative control strategies other than whole-herd depopulation. As part of this process, the USDA is also accelerating development of new diagnostic tests for ante-mortem cattle testing. The rapid test or lateral flow assay is one of those new diagnostic tests for use on bovine serum which is currently in final stages of validation prior to USDA licensure and market introduction [3]. One of the needs for this new assay is development of a supplemental assay to be used as a followup on cattle which are considered as suspects or reactors to the initial rapid test, much as the comparative cervical test was used for decades as a supplemental assay on cattle which reacted to the caudal tail fold test.

Over the last three years, our laboratory has been standardizing, validating, and applying the multiantigen print immunoassay (MAPIA) which was initially developed by Lyashchenko and others [4]. This ante-mortem serum-based Western blot assay utilizes several of the specific antigens which will be included in the rapid test, therefore making it a logical choice as the supplemental assay. All current approved ante-mortem cattle tuberculosis tests (caudal fold test, comparative cervical test, gamma-interferon assay) are all based on testing the cellular immune response. The MAPIA is a serologic assay which tests the humoral immune response [5]. Currently, Michigan is still in the process of dealing with

bovine tuberculosis endemic in its wild white-tailed deer, which annually spills over into multiple domestic cattle herds each year, and so Michigan is a natural location in which to investigate the specific capabilities of this assay under field conditions, utilizing sera from both suspect and exposed cattle. Furthermore, while current specificity of the approved caudal fold test and comparative cervical assays have always been reported to be in the high 90 percentiles, we found our diagnostic laboratory was processing 10, 20, even 30 or more indemnified suspect cattle to obtain a single positively infected individual [5]. Our goal in this study was to try to develop and evaluate a supplemental ante-mortem assay which could significantly reduce the false positives (i.e., increase specificity) that current screening methods were producing, while still maintaining a high enough sensitivity to move the detection and eradication process for bovine tuberculosis forward.

2. Materials and Methods

2.1. Cattle. Sera were collected from live cattle submitted to the Diagnostic Center for Population and Animal Health, Michigan State University, by the Michigan Department of Agriculture for tuberculosis testing as part of their ongoing tuberculosis eradication program. Using the USDA Uniform Methods and Rules, these cattle were classified as caudal fold test suspects or reactors (CFT Suspects), comparative cervical test suspects (CCT Suspects), gamma-interferon assay suspects (IFN-γ Suspects), gamma interferon assay failed (IFN-γ Failed), reactor cattle not otherwise specified to the initial assay utilized (Reactor NS), traceback cattle originating from a tuberculosis positive herd (Traceback), or cattle exposed to a another known positive animal (Exposed) [6]. Cattle have generally been field testing using tuberculin between 3 and 6 weeks prior to their submission to the Diagnostic Center, although some cattle my take as long as 2 to 4 months after field testing before being submitted. According to current methods, neither traceback nor exposed cattle are required to undergo any ante-mortem tuberculosis testing prior to removal and necropsy/slaughter. Following blood collection, cattle were humanely euthanized and underwent complete necropsy including collection of all major lymph nodes from their head, thorax, and abdomen for routine histopathology, acid-fast staining, and mycobacterial culture, and PCR at the National Veterinary Services Laboratories, Ames, IA [7]. The culture and PCR results provided by NVSL served as the definitive gold standard as to whether cattle were infected with *Mycobacterium bovis* or not. These cattle were all sampled between July 1, 2009, and September 30, 2011. Exceptions to this included two cattle previously sampled on April 30, 2001, which were in an advanced state of tuberculosis and served as known positives for initial assay standardization; 20 known positive sera from other states (Nebraska, Texas, South Dakota, Colorado, and Indiana) purchased from the USDA Tuberculosis Serum Bank to increase the number of positive samples in the study; and 11 beef cattle with gross lesions from Michigan which went directly to slaughter but for which the sera were collected and the same lymph node histopathology, culture, and PCR testing were performed at NVSL.

2.2. MAPIA. The MAPIA assay was modified slightly from the technique previously described [4, 8]. Briefly, sera were stored frozen at −20°C, thawed, and diluted 1 : 20. Eleven antigens were diluted to 50 μg/mL in PBS, then applied by a semiautomated airbrush-printing device (Linomat 5, Camag Scientific Inc., Wilmington, NC, USA) onto nitrocellulose membranes Protran Nitrocellulose Membrane (Whatman, Dassel, Germany) in eleven 12 cm long parallel strips. A blue stain (Coomassie Blue R350, GE Healthcare, Piscataway, NJ, USA) was also applied to the membranes to serve as an indicator of the correct up-side of the membrane, to indicate the bottom end of the membrane, and to serve as a standard reference on each membrane with which to compare the strength of the antigen-antibody reactions. After drying, the membranes were cut perpendicular to the antigen strips at approximately 4 mm widths, creating test strips with 11 different antigen lanes plus the Coomassie Blue band. Strips were blocked for 1 hr with 1% skim milk in phosphate buffered saline (PBS) with 0.05% Tween (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA), incubated with each serum sample diluted 1 : 20 in PBS (Kirkegaard and Perry Laboratories) for 2 hrs, washed three times with PBS, reacted with Protein G (Sigma-Aldrich, St. Louis, MO, USA) for 1 hr, washed 3 times, and finally reacted with 3,3′,5,5′-tetramethylbenzidine peroxidase (TMB 1-Component Membrane Peroxidase Substrate, Kirkegaard and Perry Laboratories) for 5 min. Strips were rinsed in cold water 3 times to stop the reaction and then air-dried overnight before being read for results. Strip results were read by unaided eye as either negative, weak positive if the line of reactivity was thin and less intense than the control band of Coomassie Blue stain, or strong positive if the line of reactivity was as thick and of similar intensity as the Coomassie Blue stain band.

2.3. Antigens. Antigens selected for use had been previously reported as regularly occurring in *Mycobacterium bovis* or *M. tuberculosis*, or as inducing significant antibody responses in cattle with bovine tuberculosis infections [9]. These recombinant antigens included ESAT-6 [10, 11] (Statens Serum Institut, Copenhagen, Denmark); ESAT-6/CFP10 fusion protein [12] and MPB83 [13, 14] (provided by a collaborator at National Animal Disease Center, Ames, IA); Acr1 [15], 38 kDa [16], 45 kDa [17], Ag85B [18], GroES [19] (all from TB Vaccine Testing and Research Materials Contract, Colorado State University, Fort Collins, CO, USA), and MPB59, MPB64, and MPB70 [20] (provided by a collaborator at Agri-Food and Biosciences Institute, Belfast, Northern Ireland).

2.4. Statistical Analysis. MAPIA results were interpreted using the NVSL mycobacterial culture and PCR results as the true tuberculosis status of the tested cattle. Four different criteria for assay interpretation were developed as follows. Criterion one is a positive reaction to any single antigen; criterion two is positive reactions to any two antigens;

Table 1: Sensitivity, specificity, positive predictive value, and negative predictive value of the MAPIA test, using four different interpretation criteria.

MAPIA test criteria*	Results	True status		Test performance		Predictive value	
		Positive ($n = 49$)	Negative ($n = 185$)	Sensitivity	Specificity	Positive	Negative
(1) Any positives	Positive	48	120	97.96	35.14	28.57	98.48
	Negative	1	65				
(2) Two positives	Positive	46	52	93.88	71.89	46.94	97.79
	Negative	3	133				
(3) Three positives	Positive	34	18	69.39	90.27	65.38	91.76
	Negative	15	167				
(4) Any strong positive	Positive	29	22	59.18	88.11	56.86	89.07
	Negative	20	163				

*Test criteria.
(1) Any positives: weak or strong positive reaction to at least one antigen
(2) Two positives: weak or strong positive reaction to at least two antigens
(3) Three or more positives: weak or strong positive reaction to at least three antigens
(4) Any strong positive: strong positive reaction to at least one antigen.

Table 2: Sensitivity, specificity, positive predictive value, and negative predictive value of a weak or strong positive reaction to at least three antigens (criterion 3) in the MAPIA test, by different antecedent tests.

MAPIA test criteria*	Results	True status		Test performance		Predictive value	
		Positive ($n = 49$)	Negative ($n = 185$)	Sensitivity	Specificity	Positive	Negative
CFT suspect/reactor	Positive	11	2	84.62	92.31	84.62	92.31
	Negative	2	24				
CCT suspect	Positive	1	2	50.0	90.91	33.33	95.24
	Negative	1	20				
IFN-γ suspect	Positive	12	10	70.59	81.82	54.54	90.0
	Negative	5	45				
Traceback	Positive	0	4	—	80.0	—	100.0
	Negative	0	16				
Exposed	Positive	0	0	—	100.0	—	100.0
	Negative	0	58				

criterion three is positive reactions to any 3 or more antigens; and criterion four is a strong positive reaction to any single antigen. Each of these was then calculated for sensitivity, specificity, and positive or negative predictive values. In addition, each individual antigen was evaluated for sensitivity, specificity, positive and negative predictive values.

3. Results

Of the 234 cattle tested for this study, 49 were true positive cattle based on mycobacterial positive cultures and PCR positive results for *M. bovis* at the NVSL, Ames, IA. The authors acknowledge that some positive cattle in very early stages of infection may not have exhibited gross or histologic lesions of tuberculosis and may not have been detected by current culture methods; however, for the purposes of this study true positive cattle must have positive mycobacterial culture or PCR results. The remaining 185 cattle were true negatives based on negative PCR and culture results.

Four criteria were used to evaluate the MAPIA results as previously described in the methods. Of these, criteria 1, 2, and 3 all provided good sensitivities compared to the true positive status of the individual cattle tested (see Table 1). But criterion 3 provided by far the best specificity of 90.27%, which was significantly better than either criterion 1 or 2. In addition, using positive predictive value, criterion 3 was the best criteria correctly identifying nearly two-thirds of all true positive animals as positive, while both criteria 1 and 2 had significantly lower positive predictive values. Therefore, for our current situation in Michigan, criterion 3 proved to be the best method for using the MAPIA assay.

Table 2 illustrates how criterion 3 correlates with each of the antemortem categories of cattle (CFT suspects, CTT suspects, IFN-γ suspect, etc.). This criterion correlates best with CFT suspect/reactors and IFN-γ suspects, while it does not correlate as well with CCT suspects. There were no true positive cattle in the traceback and exposed cattle, so the method cannot be meaningfully evaluated for its performance in these categories. Nor were cattle in this study included in

Table 3: Sensitivity, specificity, positive predictive value, and negative predictive value of weak or strong positive reactions to individual antigens used in MAPIA.

Antigen	Results	True status		Test performance		Predictive value	
		Positive ($n = 49$)	Negative ($n = 185$)	Sensitivity	Specificity	Positive	Negative
ESAT-6	Positive	41	97	83.67	47.57	29.71	91.67
	Negative	8	88				
ESAT-6/CFP10	Positive	43	55	87.76	70.27	43.88	95.59
	Negative	6	130				
Acr1	Positive	13	9	26.53	95.14	59.09	83.02
	Negative	36	176				
38 kDa	Positive	2	1	4.08	99.46	66.67	79.65
	Negative	47	184				
45 kDa	Positive	24	21	48.98	88.65	53.33	86.77
	Negative	25	164				
Ag85B	Positive	5	3	10.20	98.38	62.50	80.53
	Negative	44	182				
GroES	Positive	14	8	28.57	95.68	63.64	83.49
	Negative	35	177				
MPB83	Positive	3	0	6.12	100.0	100.0	80.09
	Negative	46	185				
MPB59	Positive	1	0	2.04	100.0	100.0	79.40
	Negative	48	185				
MPB64	Positive	2	0	4.08	100.0	100.0	79.74
	Negative	47	185				
MPB70	Positive	24	3	48.98	98.38	88.89	87.92
	Negative	25	182				

Table 2 if their initial method of field testing (CFT, CCT, IFN-γ) were not known.

Table 3 compares the sensitivity, specificity, and positive and negative predictive values for each of the 11 specific antigens used in our MAPIA assay. While ESAT-6 and the ESTA-6/CFP10 fusion protein had the highest sensitivities (83.67% and 87.76% resp.), these two antigens also demonstrated the lowest specificities (47.57% and 70.27% resp.). The other 9 remaining antigens tested exhibited significantly lower sensitivities, but also uniformly higher specificities.

4. Discussion

MAPIA assay in our laboratory, compared to known positive and negative bovine tuberculosis infected cattle primarily from the state of Michigan, offers promise as a supplemental test. The MAPIA assay requires some specialized equipment, some moderately expensive reagents, and approximately 4.5 hours of time to run. The time, equipment, cost all make this assay less than optimal for whole herd screening. But as a follow-up or supplemental test, especially for the CFT, this assay when interpreted using criterion 3 offers high sensitivity (84.62%) and high specificity (92.31%). This in turn can lead to large monetary savings to state and federal agencies by significantly reducing the total number of cattle indemnified, transported to necropsy facilities, and undergoing extensive post-mortem testing.

For example on the cost savings, and using the 234 cattle included in this study as an example, if we had run the MAPIA before sacrificing these cattle, only 78 would have been considered suspects and sent to necropsy using criterion 3. The MAPIA costs between \$100 and \$150 to run including reagents, technician time, and so forth. Multiplying 234 cattle sera by \$150 results in an additional cost of doing this supplemental test of \$35,100. Now we calculate the cost of sacrificing those other 156 cattle which the MAPIA would have classified as not suspects. Maximum indemnity in Michigan is currently \$3500 per cow; we will take \$1750 as an estimate at the average indemnity cost of a cow. Add in the state of Michigan paying for a 4-hour hauling charge from the endemic tuberculosis area to the laboratory, estimated at \$150 per cow, the Diagnostic Laboratory charge of \$250 per cow for full tuberculosis surveillance workup, and the additional charges incurred by the USDA for mycobacterial culture, PCR, and histopathology on the harvested lymph

nodes estimated at $450 per cow. This totals to an average cost of $2,600 per cow. Multiplying 156 cattle by the average cost of $2,600 we get an additional cost of $405,600. The extra cost of $35,100 for the additional MAPIA testing, is more than offset by the additional cattle costs of $405,600, resulting in a net savings of $370,500. This is not a perfect result as 12 true positive cattle would have remained on their farms since MAPIA criterion 3 did not call them positive. However, this assay can be interpreted by various criteria. Criterion 1 would have only missed one positive cattle, and criterion 2 would have only missed three positive cattle; but both criteria would have required the sacrifice of additional cattle, resulting in lower total monetary savings. Remember that each state could select the MAPIA interpretation which makes the most sense for their situation. In Michigan, you might reasonably select criterion 1 to miss the lowest number of positive cattle since tuberculosis is present in both the cattle and wildlife populations. Most of the US states are free of tuberculosis and could select a more specific interpretation criterion by which to use the MAPIA. The interpretation could also be adjusted depending on the area the cattle were in (accredited free, modified accredited free, etc.), or if the farm was known to have other currently infected animals or if the farm had previously contained infected animals then more sensitive criterion could be used.

Several researchers have indicated that ESAT-6 or ESAT-6/CFP10 are among the best antigens to be used for ante-mortem serologic testing for tuberculosis [9–12, 21]. Our data indicates that these two antigens do detect the highest percentages of true positive tuberculous cattle and result in the highest sensitivity. However, our results also show relatively low specificity for both antigens due to many false positives. These antigens may not be as specific for *Mycobacterium bovis* as previously believed. Alternatively, they are secreted so early in the immune response that they may increase rapidly following the intradermal injection of tuberculin utilized in the CFT or CCT tests, therefore resulting in false positives. One recent study actually documented a significant boost in BCG-vaccinated cattle following the use of the intradermal tuberculin test, resulting in increased immunoglobulin levels of eight different mycobacterial antigens when measured by the MAPIA [9]. Whatever the reason, all 9 of the other antigens utilized in this study had significantly higher specificities (ranging from 88.65% up to 100%), but also significantly lower sensitivities (ranging from 2.04% to 48.98%). It is interesting to note of the 9 antigens evaluated other than ESAT-6 and ESTA-6/CFP10, the two antigens showing the highest sensitivity were 45 kDA and MPB70 (both had sensitivities of 48.98%). MPB70 has been previously shown to have high sensitivity in detecting tuberculous cattle in surveys conducted in a number of countries [21]. Therefore, by using a criterion for MAPIA interpretation which combines multiple antigen reactions, one gains increased specificity while maintaining high overall sensitivity.

Looking for stronger positive reactions as in criterion 4 in the MAPIA did not result in increased sensitivity, but actually decreased sensitivity (59.18%) to the lowest of all four criteria used. This may reflect the less important role of antibody response in tuberculosis infection compared to cell-mediated immunity; therefore, infected cattle may not necessarily develop the highest antibody response against mycobacterial antigens. For whatever reason, strength of antibody-antigen reaction was not highly correlated with true tuberculosis status. For this reason, the authors chose to simplify the reading and reporting of the assay to three simple responses, negative, weak, or strong and not to include quantitative optical density measurements for each antigen-antibody reaction within the MAPIA assay as in some previous studies [4].

One interesting side note is how this MAPIA assay was performed in cattle which were negative for *M. bovis*, but from which environmental mycobacteria outside the *M. tuberculosis*-group were isolated. Only four cattle out of 234 were culture positive for either *M. avium* (2 isolates) or non-*M. tuberculosis* group (2 isolates), not otherwise specified. So the numbers are too low to make any generalizations about how the MAPIA assay performs. However, these 4 cattle were uniformly interpreted negative on MAPIA testing by criteria 2, 3, and 4; while 2 of 4 cattle (both non-*M. tuberculosis* group individuals) were interpreted positive by criterion 1. This reinforces the value to utilizing an interpretation criterion that includes more than one positive response as a method to increase the test specificity.

Ideally, our evaluation of MAPIA will continue, with testing of additional known positive and negative cattle. In addition, if this assay is to someday be approved by USDA as a supplemental test, we would ideally like to run all our banked sera samples by the rapid test—lateral flow assay. That assay—as previously stated—is in the approval process for validation and licensure for use in the US [3]. Since that assay utilizes several of the same antigens as the MAPIA, and is an ante-mortem serologic assay, it would be important to compare these two assays performance on the same set of sera. However, since the rapid test is not yet approved, and therefore not commercially available, we have been unable to complete this important validation step to date.

The MAPIA assay requires some specialized equipment, some moderately expensive reagents, and approximately 4.5 hours of time to run. However, with the proper application of methods, selection of test antigens, and correct interpretation criteria, it shows remarkable promise for use as limited supplemental test following initial ante-mortem field screening tests. While the MAPIA is currently too expensive to use as a primary screening test for tuberculosis in cattle, its use to either increase sensitivity or specificity depending on the specific needs of the state, area or farm, when used as a supplemental test, could prove valuable as an epidemiologic tool, and potentially result in significant cost savings for the national tuberculosis eradication program.

Acknowledgments

The authors thank Dr. James McNair of the Agri-Food and Biosciences Institute, Belfast, Northern Ireland, for generously providing them with the MPB59, MPB64, and MPB70 antigens for use in this study, as well as Dr. Ray Waters, Bovine Tuberculosis Laboratory, National Animal Disease

Center, United States Department of Agriculture, Ames, IA, for generously providing them with the ESAT-6/CFP10 fusion protein and MPB83 antigens. They also wish to thank the many Michigan Department of Agriculture and USDA veterinarians who helped with the ante-mortem field testing, transportation, and bleeding of the cattle which made up the bulk of the animals tested in Michigan. They wish to thank the many veterinary pathologists, anatomic pathology residents, and laboratory technicians who assisted with the final necropsy and sample collection from Michigan cattle at the Diagnostic Center for Population and Animal Health, Michigan State University; without their help the authors would not have the definitive information on which cattle were positive and negative for tuberculosis. Finally they acknowledge financial support of this study by a grant from the United States Department of Agriculture which helped to make this study possible.

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 642145, 6 pages
doi:10.1155/2012/642145

Research Article

Pulmonary Disease due to *Mycobacterium tuberculosis* in a Horse: Zoonotic Concerns and Limitations of Antemortem Testing

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Received 21 December 2011; Accepted 3 February 2012

Academic Editor: Mitchell Palmer

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A case of pulmonary tuberculosis caused by *Mycobacterium tuberculosis* was diagnosed in a horse. Clinical evaluation performed prior to euthanasia did not suggest tuberculosis, but postmortem examination provided pathological and bacteriological evidence of mycobacteriosis. In the lungs, multiple tuberculoid granulomas communicating with the bronchiolar lumen, pleural effusion, and a granulomatous lymphadenitis involving mediastinal and tracheobronchial lymph nodes were found. Serologic response to *M. tuberculosis* antigens was detected in the infected horse, but not in the group of 42 potentially exposed animals (18 horses, 14 alpacas, 6 donkeys, and 4 dogs) which showed no signs of disease. Diagnosis of tuberculosis in live horses remains extremely difficult. Four of 20 animal handlers at the farm were positive for tuberculous infection upon follow-up testing by interferon-gamma release assay, indicating a possibility of interspecies transmission of *M. tuberculosis*.

1. Introduction

Tuberculosis (TB) is a zoonotic disease caused by *Mycobacterium tuberculosis, M. bovis,* or other members of the *M. tuberculosis* complex in a broad range of mammal hosts [1, 2]. Although natural susceptibility to infection may vary among humans and animals, interspecies transmission is not uncommon, especially in captive wildlife populations in which multi-host TB outbreaks have been reported [3, 4]. Horses are believed to be more resistant to mycobacterial infections compared to other livestock species [5–8]. The current incidence of TB in horses is extremely low, especially in countries with established control programs [9]. Nevertheless, equine cases of clinical disease due to *M. bovis*, *M. avium*, or *M. tuberculosis* have been described [7, 10–12]. Among the tuberculous mycobacteria, *M. bovis* has historically been the predominant causative agent, whereas *M. tuberculosis* is less commonly isolated from horses [9, 13]. However, effective bovine TB control programs in many countries have further reduced the incidence of *M. bovis* infection in horses over the last 20 years. Recently, an isolated case of TB due to *M. bovis* was reported by Keck et al. [14]

in a horse living in close contact with infected cattle in the Camargue region of France, an area known for *M. bovis* infection in fighting bulls [15].

Diagnosis of mycobacterial infections in horses is confounded by the atypical morphology of lesions, diverse clinical signs of infection, and limited options available for antemortem testing [9]. The most frequent clinical signs are lethargy and chronic weight loss. Terminal signs of pulmonary TB in horses are fever, dyspnea, and cough. In most countries, tuberculin skin test is not recommended due to its poor accuracy in this species [4, 16]. Definitive diagnosis of equine TB relies on postmortem examination with histopathological assessment of affected tissues including acid fast staining and PCR for *IS 6610* (which identifies *M. tuberculosis* complex organisms) and culture for *Mycobacterial* spp.. Currently, no blood-based TB tests are described for use in horses.

Several antibody assay platforms using recombinant proteins of *M. tuberculosis* or *M. bovis* have recently been developed for rapid TB detection in multiple-host species including free-ranging and captive wildlife as well as in domestic animals [17–21]. As these assays have shown potential for use in a wide range of species (e.g., cattle, deer, camelids, elephants, badgers, wild boar, etc.), they may also be useful for antemortem TB diagnosis in horses. The present report describes a case of pulmonary TB caused by *M. tuberculosis* in a horse, the ensuing investigation of exposed animals and animal handlers at the farm, and evaluation of serologic response in the index case.

2. Materials and Methods

2.1. Animals. The index case was a 20-year-old Romanian Warmblood gelding (Orlov horse) imported from Poland to Switzerland in 1993. Fourteen other horses were cohoused with the index case within a stable. Fifty-eight animals including 14 alpacas, 6 donkeys, 29 goats, 5 sheep, and 4 horses lived on a pasture adjacent to the horse stable and were all taken care of by the same staff. Four dogs had free access to the stable and the pasture. In addition, 5 horses from known TB-free area in the United States were included in the study as a negative control group for serology evaluation.

2.2. Identification of M. tuberculosis. Mycobacteria were identified by PCR on formalin-fixed, paraffin-embedded tissue samples of lung and pulmonary lymph nodes as previously reported [22]. DNA was extracted, and spoligotyping was performed as described [23]. The data was compiled in Microsoft Excel and analyzed by using the MIRU-VNTR plus online analysis tool (http://www.miru-vntrplus.org) [24]. Differentiation of the *M. tuberculosis* complex members was carried out by the GenoType 1 MTBC assay (Hain Lifescience GmbH, Nehren, Germany).

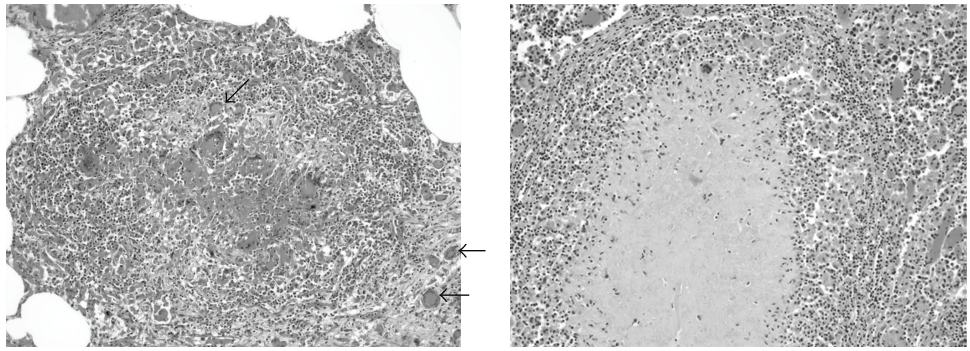
2.3. Tuberculin Skin Test (TST). Goats and sheep were tested by single cervical tuberculin skin test, which was performed by intradermal injection of 2000 IU of bovine purified protein derivative (Bovituber, Synbiotics Europe, Lyon, France).

2.4. Serology. Three serological tests developed for rapid detection of TB in various host species included VetTB STAT-PAK, multiantigen print immunoassay (MAPIA), and dual path platform (DPP) VetTB test (Chembio Diagnostic Systems, Inc., Medford, NY, USA). The immunoassay procedures were performed using animal serum samples in accordance with the manufacturer's instructions as previously described [19]. VetTB STAT-PAK kit (also known as rapid test (RT)) and DPP VetTB assay used several recombinant antigens of *M. tuberculosis* or *M. bovis* including ESAT-6, CFP10, and MPB83 protein. Additionally, we applied lipoarabinomannan (LAM) for antibody detection in a DPP assay. MAPIA employed a panel of 14 mycobacterial antigens [20] to detect serum IgG antibody using peroxidase-labeled streptococcal protein G (Sigma, St. Louis, MO, USA) diluted 1 : 1000 or to detect serum IgM antibody using peroxidase-conjugated antibody to horse IgM (Kirkegaard Perry Laboratories, Gaithersburg, MD USA) diluted 1 : 1000, along with substrate 3,3′,5,5′-tetramethylbenzidine (Kirkegaard Perry Laboratories, Gaithersburg, MD USA). Test results were read visually and by using a DPP reader device to measure reflectance in relative light units (RLUs) or densitometry to measure intensity of MAPIA bands in arbitrary units as described earlier [19].

2.5. Interferon-Gamma Release Assay (IGRA). The T-SPOT.TB assay (Oxford Immunotec, Oxford, UK) was used in accordance with the manufacturer's recommended procedure for screening of the farm personnel for latent TB infection (LTBI). The employees were additionally tested by chest X-ray to rule out active pulmonary TB. Two months after the euthanasia of the infected horse, a total of 20 persons including the owner and his companion, the veterinarian, 2 pathologists, 7 workers in the stable, and 8 employees of the farrier were evaluated. Five subjects originated from countries with moderate incidence of human TB (3 from Bosnia, 1 from Portugal, 1 from Turkey), whereas 15 subjects were from low-incidence countries (11 Swiss, 2 French, 2 Italian). Eighteen individuals were evaluated by chest X-ray (all employees, except for the 2 pathologists), and 17 persons had the T-SPOT.TB assay. The IGRA testing was not performed for the 3 oldest subjects (aged 65, 79, and 93) because of the low sensitivity demonstrated for this test in senior population [25] and generally higher probability of harboring previously acquired infection [26]. Subjects with a positive test result had a medical visit including history interview and clinical examination.

3. Results and Discussion

3.1. Clinical Findings. The index case, a 20-year-old Romanian Warmblood gelding, presenting with symptoms of cardiac insufficiency and ventral edema in July 2010 was referred to the Equine Clinic of the University of Berne with a suspicion of pericarditis and signs of azotemia. Prior to referral,



(a) (b)

Figure 1: Histology of lung (a) and pulmonary lymph node (b) lesions. Typical tuberculoid granulomas demonstrating central necrosis (particularly apparent in the lymph node lesion) surrounded by epithelioid macrophages and multinucleated, Langhans-type giant cells (examples noted with arrows) admixed with lymphocytes.

the horse had been febrile (40.0°C) and showed edema in the pectoral and ventral regions for 4 days. The gelding had been treated by the referring veterinarian with antibiotics and nonsteroidal anti-inflammatory drugs. Upon presentation to the Equine Clinic of the University of Berne, the horse was depressed with a heart rate of 60 beats per minute, a respiratory rate of 40 breaths per minute, and a rectal temperature of 38.3°C. The mucous membranes were moist and slightly red with a capillary refill time of 2s. The complete blood count revealed a packed cell volume of 39%, a clear hyperproteinemia of 80 g/L, a marked leucocytosis ($33 \times 10^3/\mu$L), and eosinophilia ($18.3 \times 10^3/\mu$L). Fibrinogen was within normal range at 2 g/L. Urea and creatinine were well above referenced range with 18 mmol/L and 233 μmol/L, respectively. A thoracic ultrasound examination was performed and revealed severe bilateral pleural and pericardial effusion. Measured lactate in the drained pleural effusate was not different to serum lactate levels. A pericardiocentesis was not performed. Radiographs, acquired after draining the pleural effusion, showed a dense bronchiointerstitial pattern. No abnormalities of the esophagus or stomach were detected on gastroscopy. The clinical differential diagnoses included intra-thoracicneoplasia and infectious pneumonia with pleuritis. Twenty four hours later, the clinical condition deteriorated rapidly with increasing tachypnea (respiratory rate of 40 breaths per minute) and quickly recurring pleural effusion. No etiological diagnosis had been made at this point; yet due to the deteriorating clinical progression and a guarded prognosis, the owner elected to have the horse euthanized.

3.2. Necropsy Findings. Tan firm nodules ranging from 2 to 3 mm were disseminated throughout the pulmonary parenchyma. The tracheobronchial and mediastinal lymph nodes were moderately enlarged, with irregular, often multinodular surface. On cut surface the lymph nodes showed multiple nodules with a necrotic core. A severe acute fibrinous pericarditis and an acute to subacute fibrinous pleuritis with abundant pleural exudation and multiple pleuro-diaphragmatic and pleuro-pericardial adherences were also present. Additional findings included mild diffuse subcutaneous edema, endocardial fibroelastosis and acute ulcers along the margoplicatus of the squamous portions of the gastric mucosa.

Histologically, typical tuberculoid granulomas with central necrosis surrounded by epitheliod macrophages and multinucleated, Langhans-type giant cells admixed with few lymphocytes were detected in the lung and pulmonary lymph nodes (Figure 1). Some granulomas were located within the walls of bronchioli. Multiple granulomas showed marked fibrosis admixed with few multinucleated giant cells. Ziehl-Neelsen and Fite-Faraco stains of multiple granulomas in the lung and lymph nodes revealed no acid fast bacilli. Based on these findings, a presumptive diagnosis of a pulmonary mycobacteriosis caused by organisms of the *M. tuberculosis* complex was made.

Using formalin-fixed and paraffin-embedded tissue samples of lungs and lymph nodes, *M. tuberculosis* was identified by PCR amplification of DNA extracts. Culture isolation was not attempted, as the specimens collected at necropsy were all processed for histology. Spoligotyping revealed a member of the *M. tuberculosis* complex exhibiting an orphan octal code (575247437763771).

Thus, results of postmortem examination of the horse revealed the presence of miliary TB with pleural effusion and necrotic lymph nodes, some of which had eroded into the bronchi.Earlier studies (reviewed in [9, 13]) indicate that most TB cases in horses are due to *M. bovis* and the most common site of infection is the gastro-intestinal tract, suggesting ingestion (oral route) as a major way of transmission in this host species. In contrast, we found disease due to *M. tuberculosis* predominantly localized in the thoracic organs. This feature combined with the observation that the infected horse was coughing for at least two days before death emphasizes the zoonotic concerns associated with this case.

3.3. Antibody Response. A serum sample collected from the index case at the time of euthanasia was tested by RT, MAPIA, and DPP assays with various antigens. The

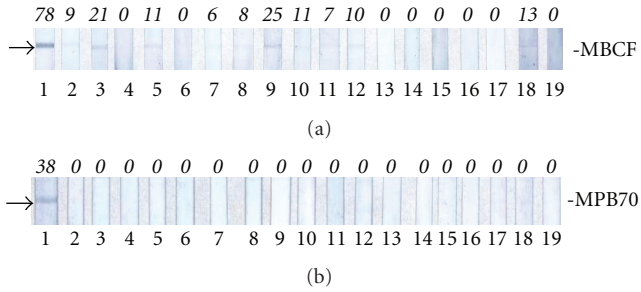


Figure 2: IgG (a) and IgM (b) antibody responses of the horse infected with *M. tuberculosis*. MAPIA was performed as described in the Materials and Methods Section. Strip images represent individual results obtained with serum samples from the infected horse (no. 1), and 18 presumed non-infected horses from the same farm (nos. 2–19). Arrows point at the bands indicating antibody reactivity found in the infected horse. Printed antigens are shown on the right. Figures in italic above the strips indicate MAPIA densitometry values (in arbitrary units) obtained for respective serum samples.

M. tuberculosis-infected horse was nonreactive by RT or DPP VetTB assay, thus suggesting the absence of detectable antibodies to ESAT-6, CFP10, and/or MPB83 in the serum. However, MAPIA revealed IgM antibody to MPB70 protein and strong IgG reactivity to *M. bovis* culture filtrate (MBCF), whereas no IgM against MPB70 and only borderline IgG binding to MBCF were found in sera from 18 potentially exposed but presumed noninfected horses from the farm (Figure 2) or among 5 negative control horses from the United States (data not shown). Other antigens used in MAPIA elicited no detectable antibody response in the infected animal or provided no discriminating power to distinguish the index case from the other horses. Further, using LAM of *M. tuberculosis* as a test antigen in DPP assay format allowed for detection of a strong serum IgG reactivity in the infected horse (DPP reader reflectance = 235 RLU) if compared to the control group (5 ± 12 RLU, $n = 5$) or to the potentially exposed but presumed non-infected horses (8 ± 14 RLU, $n = 18$). This observation is consistent with the MAPIA IgG reactivity to MBCF, presumably because this crude antigen preparation contains LAM which is known to elicit particularly robust antibody responses in humans and animals diagnosed with TB (our unpublished observations).

Thus, the infected horse developed a serologic response to certain *M. tuberculosis* antigens, such as LAM, MPB70, or MBCF. The antibody reactivity involved both IgM and IgG classes, as has also been shown for cattle experimentally infected with *M. bovis* [17]. Antigens recognized by the horse serum did not include the key specific targets identified in previous studies for other host species, such as ESAT-6, CFP10, or MPB83 protein [18]. As a result, the available animal-side assays employing the latter antigens and designed for those species failed to detect specific antibody in serum from the infected horse. The results illustrate the view that, when developing novel TB immunodiagnostics for various hosts, it is crucial to validate the choice of diagnostically important antigens being selected for each new species individually. Finding the antibody response in the M. tuberculosis-infected horse in the present study encourages further efforts to identify suitable antibody-reactive antigens for developing accurate TB serodiagnostics for this species.

3.4. Testing Exposed Animals. Fourteen horses lived in the same stable as the *M. tuberculosis*-infected horse. Other horses ($n = 4$), goats ($n = 29$), sheep ($n = 5$), donkeys ($n = 6$), and alpacas ($n = 14$) lived on a pasture adjacent to the horse stable and were taken care of by the same staff. Four dogs had free access to the stable and the pasture. These animals were considered exposed, as they had been in direct or indirect contact with the *M. tuberculosis*-infected horse. TST was negative in all goats and sheep. No TST was performed in horses, donkeys, alpacas, and dogs, because no validated protocols were available for these species. Serological testing with RT, DPP VetTB, and MAPIA of the alpacas, donkeys, and dogs showed no antibody reactors. To date, none of these animals has developed clinical symptoms that would be consistent with TB.

3.5. Testing Farm Personnel. Screening for LTBI was performed by the T-SPOT.TB assay, in accordance with the United States (Center for Disease Control and Prevention) and Swiss (Federal Bureau for Public Health) recommendations. The farm owner and the employees had to be tested for two objectives: (1) to search for a possible source of infection among the "care givers" of the infected horse (employees of the stable), since animal-to-animal transmission was highly unlikely, and (2) to search for subjects who could have been infected by the horse that had reportedly been coughing at least 2 days prior to death.

Among the 18 employees evaluated by chest X-ray, no indication for active TB was found (Table 1). One Swiss person showed a pulmonary nodule that was further investigated by the patient's general practitioner and was then presumed to be of neoplastic nature. For the 17 persons tested with the T-SPOT.TB assay, 12 results were negative, 4 were positive, and one was indeterminate (twice). Among the 4 IGRA-positive subjects, two originated from countries with moderate incidence of TB (Bosnia and Turkey). According to the history interview records, all 4 had definite or probable previous exposures to TB, as well as variable periods of recent direct or indirect contact with the infected horse. Treatment for LTBI was proposed to all patients with a positive IGRA result (one person refused treatment). To date, none of the tested employees has developed any signs of active TB.

3.6. Zoonotic Aspect. In contrast to *M. bovis* known to affect a wide range of natural hosts, susceptibility to *M. tuberculosis* remains not well defined for many mammal species [1, 2, 4] including horses and others (alpacas, donkeys, goats, sheep, and dogs) potentially exposed to the index case in the present study. Since no signs of infection were found in any of the remaining animals of the six species at the farm, it appeared unlikely that the equine TB case had resulted from animal-to-animal transmission of *M. tuberculosis*. Further, because the isolate was *M. tuberculosis*, not *M. bovis*, one could

Table 1: Human contact testing results.

Group	Gender[a]	Age, years	Country of origin	Chest X-ray	T-SPOT.TB
Owner, companion, veterinarian, and pathologists	M	93	Italy	Neg	ND[b]
	F	45	Switzerland	Neg	Neg
	M	61	Switzerland	Neg	Pos
	M	50	Switzerland	ND	Neg
	F	32	Italy	ND	Neg
Workers in stable	M	65	Switzerland	Neg	Indeterminate[c]
	F	65	Switzerland	Neg	ND
	M	48	Portugal	Neg	Neg
	M	79	Switzerland	Nodule, non-TB	ND
	M	57	Bosnia	Neg	Pos
	F	55	Bosnia	Neg	Neg
	M	58	Bosnia	Neg	Neg
Farriers	M	19	Switzerland	Neg	Neg
	M	55	Switzerland	Neg	Neg
	M	21	Switzerland	Neg	Neg
	F	23	Switzerland	Neg	Neg
	M	26	Switzerland	Neg	Neg
	M	44	France	Neg	Pos
	M	26	France	Neg	Neg
	M	51	Turkey	Neg	Pos

[a] M, male; F, female
[b] ND, not done
[c] Tested indeterminate repeatedly.

speculate that the horse infection had been acquired from a human case of active TB but not necessarily from any of the employees with a positive IGRA result. Moreover, an alternative scenario cannot be ruled out that LTBI detected in certain personnel during the follow-up investigation could have resulted from exposure to the *M. tuberculosis*-shedding horse. The IGRA-positive subjects have reportedly been exposed to TB in the past, but no earlier records on routine testing (TST or IGRA) were available to determine their pre-existing infection status. Thus, the origin of *M. tuberculosis* infection of the horse described herein remains unknown.

4. Conclusions

A rare case of TB due to *M. tuberculosis* was found in a horse. Based on the clinical evaluation results, TB was not suspected in this animal. However, postmortem examination by pathology and molecular methods led to diagnosis of pulmonary disease caused by *M. tuberculosis*. Antemortem testing of horses for TB remains challenging, because traditional methods, such as TST, have not been validated for this species. An antibody response to *M. tuberculosis* antigens was found in the infected horse, but not in the exposed animals that had no signs of disease. Follow-up testing of personnel involved in taking care of these animals revealed 4 IGRA-positive results indicating LTBI in those subjects. The findings suggest a possibility of interspecies transmission of *M. tuberculosis,* although the infection source remains unclear.

Acknowledgment

The authors thank Claudia Quinn for skillful assistance with serological testing.

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Hindawi Publishing Corporation
Veterinary Medicine International
Volume 2012, Article ID 798502, 13 pages
doi:10.1155/2012/798502

Research Article

Bovine Tuberculosis in Cattle in the Highlands of Cameroon: Seroprevalence Estimates and Rates of Tuberculin Skin Test Reactors at Modified Cut-Offs

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Received 19 October 2011; Revised 10 January 2012; Accepted 22 January 2012

Academic Editor: Jesse M. Hostetter

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The aim of this study was to obtain epidemiological estimates of bovine tuberculosis (TB) prevalence in cattle in the highlands of Cameroon using two population-based tuberculin skin test (TST) surveys in the years 2009 and 2010. However, prior to the TST survey in 2010, blood was collected from already chosen cattle for serological assay. Anti-bovine TB antibodies was detected in 37.17% of tested animals and bovine TB prevalence estimates were 3.59%–7.48%, 8.92%–13.25%, 11.77%–17.26% and 13.14%–18.35% for comparative TST at ≥4 mm, ≥3 mm and ≥2 mm cut-off points and single TST, respectively. The agreement between TST and lateral flow was generally higher in TST positive than in TST negative subjects. The K coefficients were 0.119, 0.234, 0.251 and 0.254 for comparative TST at ≥4 mm, ≥3 mm and ≥2 mm cut-off points and the single TST groups, respectively. Chi square statistics revealed that strong ($P < 0.05$; $\chi^2 > 48$) associations existed between seroprevalence rates and TST reactors. The study suggested that using lateral flow assay and TST at severe interpretations could improve the perception of bovine TB in Cameroon. The importance of defining TST at modified cut-offs and disease status by post-mortem detection and mycobacterial culture of TB lesions in local environments cannot be overemphasised.

1. Introduction

Bovine tuberculosis (TB) is a zoonotic disease with severe public health significance but it is neglected in Cameroon. The tuberculin skin tests (TSTs) are currently the best available techniques for international field diagnosis of bovine TB in live animals [1, 2] and it is based on delayed hypersensitivity reactions [3]. The single intradermal comparative cervical tuberculin (SICCT) test involving the intradermal injection of bovine tuberculin (BT) and avian tuberculin (AT) at separate sites in the skin of the neck gives more specific results than the single intradermal tuberculin (SIT) test which uses only BT [4, 5]. TST can effectively detect early stages of *M. bovis* infection in cattle and allows for rapid removal of infected animals, limited transmission, and fast eradication of bovine TB [6]. There are OIE-recommended cutoff points of the increase in skin thickness for SICCT-BT and SIT-BT to be positive [3]. However, the OIE-recommended cutoff values were established mainly in developed countries for *Bos taurus* cattle, and different cutoff

values are applied according to a particular country's disease status and objective of its disease control programme [4, 7–9].

The performance of TST could be affected by environmental factors, host factors (status of immunity, genetics), and nature of the tuberculin used [1, 4, 5, 9]. A perfect cutoff point in a specific geographic area may not be so useful in another environment [1, 4]. Also, the ability of the test to predict positive disease status depends on its sensitivity and specificity and prevalence of the disease in tested population [1]. Anergic animals, animals exhibiting reactions to both avian and mammalian tuberculins, animals in advanced stage of disease, periparturient cows, and animals with confined infection notably in the udder and with localised infection often in the lymphatic glands that has become inactive (latent) have been reported to be poor responders to TST [10]. However, severe interpretations are done in regions or herds where *M. bovis* infection has been confirmed, and SIT-BT reactors may also be subjected to an SICCT-BT test, based on the discretion of the veterinarian [4]. Veterinarians continue to play pivotal roles in inspections of animal (antemortem and postmortem) and animal products, diagnosis of *M. bovis* infected cattle, and impacting of cattle producers in bovine TB eradication programs [11]. Postmortem detection of TB lesions and other bovine TB diagnostic techniques (e.g., gamma-Interferon, ESAT-6 tests, serologic and fluorescence polarization assays) have been used to determine the ability of TST in the diagnosis of bovine TB in cattle in different environmental conditions around the world, including parts of Africa [1, 2, 6, 7, 9, 12–16]. However, TST-negative animals at slaughter with evidence of encapsulated lesions confirmed as caused by *M. bovis* have also been reported [10].

TST may demand physical exertion in the field but it is also simple and relatively inexpensive and offers reliable means of screening cattle populations in an entire region [4, 6]. Ancillary tests are being used and/or currently being validated to improve diagnosis and reduce the number of false positive results following TST [1, 2, 6, 7]. Also, rapid and simple immune-chromatographic assays for the serodiagnosis of bovine TB have been developed [17, 18] and proposed as additional tests to the TST for *antemortem* diagnosis [2, 19, 20]. These chromatographic immunoassays employ unique cocktails of selected *M. bovis* antigens as both qualitative captures and detectors of specific antibodies against *M. bovis* in plasma, serum, and whole blood [17, 21]. MPB83, ESAT-6, 14-kDa protein, CFP-10, MPB70, MPT63, MPT51, MPT32, MPB59, MPB64, Acr1, PstS-1, *M. bovis* purified protein derivatives, ESAT-6/CFP10 fusion protein, 16-kDa alpha-crystallin/MPB83 fusion protein, and *M. bovis* culture filtrate have been identified as the common seroreactive antigens in bovine TB [17, 18, 22]. The bound antibodies are visualized with the naked eye as colour band at the test device within some minutes of application [17, 21]. The assay requires no specific expertise or equipment, and the test kit may be kept without the need for refrigeration [17, 18, 21].

There are scanty reports of bovine TB prevalence in Cameroon, modifications of the OIE standards of TST applied elsewhere have been used to estimate the disease status in cattle in the country, and the findings have varied widely, even for the same sites [23–27]. This study was carried out to investigate bovine TB prevalence in cattle in the highlands of Cameroon through seroprevalence estimations, rates of TST reactors at modified cutoff points, and the epidemiological usefulness of the proposed screening algorithms. TST data of tested cattle in the years 2009 ($n = 2,853$) and 2010 ($n = 1,381$) were reanalyzed, and the epidemiological implication for applying TST at various cutoff points for a predominantly Zebu cattle population was discussed.

2. Materials and Methods

2.1. Study Area and Population. Cattle populations in the Western highlands (5°–7°N and 10°-11°E) and Adamawa plateaux (6-7°30′N and 12°30′–14°E) of Cameroon (Figure 1) were sampled in the years 2009 and 2010 as part of a bovine TB prevalence study. A SIT bovine TB prevalence rate of 26% recorded by Muchaal [25] in the Western highlands of Cameroon was used to estimate the number of cattle required to detect ≥1 positive reactor with a desired 95% confidence and precision of 5% as previously described [28]. The selection of cattle herds was done by the random-number generation method of cattle keeping communities, cattle owners, and locations of herds from records of annual livestock vaccination campaigns (contagious bovine pleuropneumonia, pasteurellosis, black quarter) at the Regional Delegations of MINEPIA (Ministère de l'Elevage, des Pêches et des Industries Animales (Ministry of Livestock, Fisheries and Animal Industries)). All animals within selected herds were tested except recently calved cows (within 2 months postpartum) and calves less than 6 months old because of immunosuppression in lactating cows and high maternal antibodies in calves that desensitizes them to tuberculin [29, 30].

During March to September 2009, a total of 2,853 cattle (84 herds) were tested in five administrative divisions in the Northwest regions of the Western highlands (Donga and Matung, Menchum, Bui, Mezam and Boyo) and one division in the Adamawa plateaux (Vina) of Cameroon (Figure 1). Similarly, 1,381 cattle (40 herds) were tested during May to September 2010 in Mezam and Bui divisions in the Western highlands which showed high bovine TB prevalence rates in the previous survey and also in the Vina division in the Adamawa plateaux. However, 30–60 minutes prior to the TST carried out in the year 2010, blood was collected from 807 cattle in 20 randomly selected herds of the 40 already chosen herds (1,381 cattle) to extract serum for lateral flow assay of antibovine TB antibodies (Antibovine TB Ab).

Risk assessments were done to avoid hazards to all persons and animals involved in the project. The project approval and ethical clearances were obtained from the required authorities in Cameroon including the National Ethics Committee, regional delegations of MINEPIA in the Northwest and Adamawa regions. The purpose of the study was explained to the targeted participants usually with

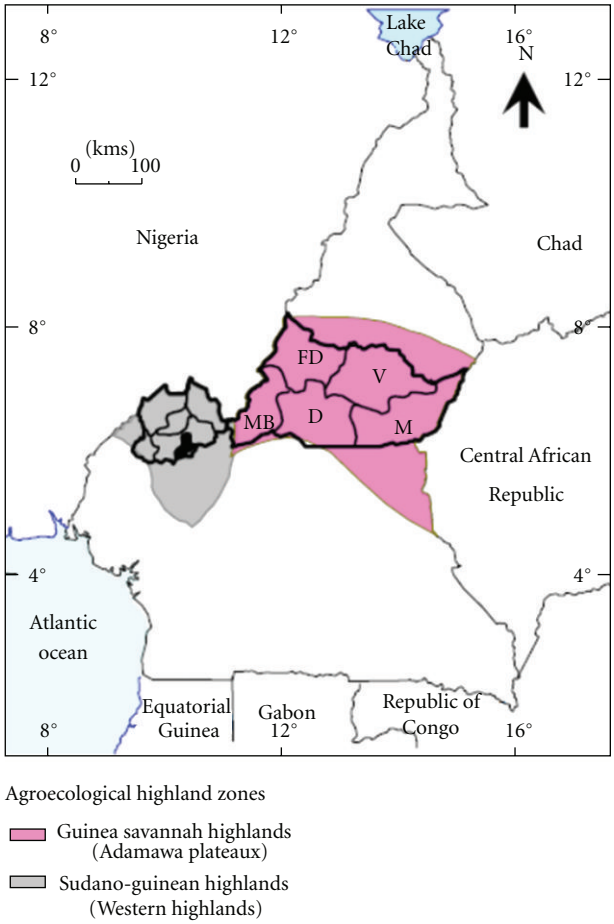

FIGURE 1: Map of Cameroon showing administrative regions within the Northwest and Adamawa Regions. Divisions in Northwest region are Donga and Matung, Menchum, Bui, Mezam, Boyo, and Ngo-Ketunja (shaded and not used in this study). Divisions in Adamawa region are V: Vina (study area); M: Mbere; D: Djerem; MB: Mayo-Banyo; FD: Faro et Deo.

the assistance of resident veterinarians, local community leaders, and trusted intermediaries. A herd was tested after an informed consent was given by the owner. Apart from minor jugular vein puncture for blood collection, intradermal injections of AT and BT, and procedural restraining manipulations for safety purposes, the animals were not subjected to suffering.

2.2. Antibovine Tuberculosis Antibody Assay. About 5 mL of blood was collected by jugular venipuncture of 807 cattle (20 herds) to extract serum for the detection of antibovine TB Ab against the *M. bovis* MPB70 antigen using the rapid lateral-flow test (Anigen Bovine Tb Ab, BioNote Inc., Republic of Korea), as described by the manufacturer. The immunochromatographic assay using recombinant MPB70 antigen as capture and detector in a direct sandwich method detected antibodies (IgM, IgG) against *M bovis*. Briefly, in the ready-to-use disposable test kit, 10 μL of test serum was poured into the sample well, and after 1 minute, 3 drops of developing buffer (provided as part of the kit) were placed in the buffer well. The result was interpreted after 20 minutes. The presence of two purple coloured bands within the result window, the test area and control line, indicated antibodies positive result whereas no band in the test area in addition to a visible control purple line was negative. An invalid test was one where no coloured band was visible within the result window. The appearance of a control colour band, for positive or negative assays, indicated that the test was working properly.

2.3. Tuberculin Skin Tests and Classification of Reactors. TSTs were carried out in the selected cattle (2,583 in the year 2009 and 1,381 in the year 2010 including the 807 blood donors but after blood collection) by intradermal injections of 0.1 mL each of AT (2500 IU/dose) and BT (3000 IU/dose) in two sites, at 12 cm apart in the right neck region. A correct intradermal injection was confirmed by palpating a small grain-like swelling at each injection site. The skin thickness was measured prior to and 72 hours after injecting the tuberculins using a digital calliper. The OIE-recommended ≥4 mm cutoff point of increase in skin fold thickness [3] as well as ≥3 mm and ≥2 mm cutoff points was assessed for SICCT-BT reactor status. The corresponding ranges ≥1 mm to <4 mm, ≥1 mm to <3 mm, and ≥1 mm to <2 mm were classified as doubtful responses, respectively. SICCT-BT was noted as negative if the skin response was <1 mm. SIT-BT interpretations were done using skin fold thickness of ≥4 mm, ≥2 mm to <4 mm, and <2 mm for positive, doubtful, and negative responses, respectively [3]. These cutoff points were assessed against the demonstrated circulating antibovine Tb antibodies status and classified as adapted from Martrenchar et al. [23] to determine the cutoff zone and risk group of TST reactors for consideration (Figure 2).

2.4. Data Management and Statistical Analysis. The lateral flow assay results and TST data at the ≥2 mm, ≥3 mm, and ≥4 mm cutoff points for individual cattle were entered into Microsoft Excel (Microsoft Corporation, USA) and also exported to SigmaPlot (Systat Software Inc, USA) for further analysis. The seroprevalence estimates, rates of TST reactors in the tested cattle population, and agreement between both methods at the predefined cutoff points were assessed [28].

The predictive values and diagnostic likelihood ratios of TST at the various cutoff points were compared against the antibovine TB Ab assay [28]. With sensitivity and specificity values obtained by Ameni et al. [9] and Pollock et al. [12], the observed prevalence rates were corrected using the Rogan-and-Gladen formula [28, 31]. The *kappa* statistics was used to estimate the degree of agreements between both tests while Chi-square techniques were applied to compare individual and herd prevalence of reactors in the different variables [28, 32].

The figure was adapted from Martrenchar et al. [23] where

(i) BT = (BT_{72}–BT_0) is the skin fold thickness at the injection site of bovine tuberculin at 72 hours;

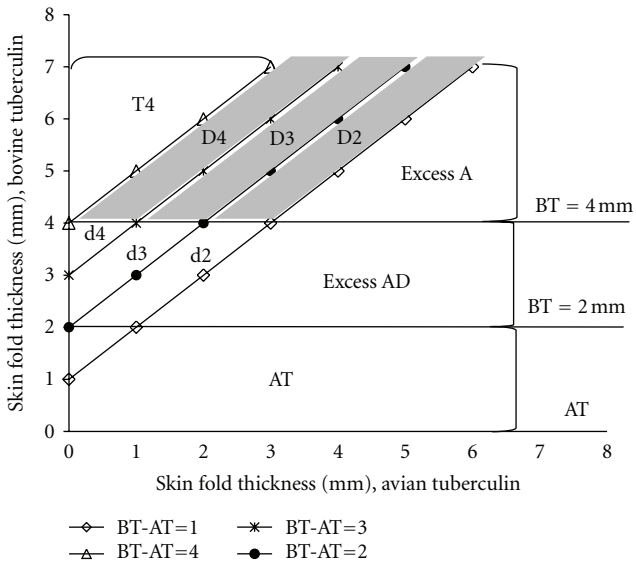

Figure 2: Classification of cattle according to their possible tuberculin skin tests response at ≥4 mm, ≥3 mm, and ≥2 mm cutoff points.

(ii) AT = (AT_{72}–AT_0) is the skin fold thickness at the injection site of avian tuberculin at 72 hours;

(iii) (D + d) is the SICCT-BT doubtful responses; the skin responses (D2 + d2), (D3 + D2 + d3 + d2), and (D4 + D3 + D2 + d4 + d3 + d2) are for ≥1 mm to <2 mm, ≥1 mm to <3 mm, and ≥1 mm to <4 mm cutoff ranges, respectively;

(iv) Excess d4 (Xd4) = d4 + d3 + d2 is the SICCT-BT doubtful responses (≥4 mm cutoff point) and classified as SIT-BT doubtful responses (when 1 mm ≤ (BT − AT) <4 mm and 2 mm ≤ BT < 4 mm);

(v) Excess d3 (Xd3) = d3 + d2 is the SICCT-BT doubtful responses (≥3 mm cutoff point) and classified as SIT-BT doubtful responses (when 1 mm ≤ (BT – AT) < 3 mm and 2 mm ≤ BT < 4 mm);

(vi) Excess d2 (Xd2) = d2 is the SICCT-BT doubtful responses (≥2 mm cutoff point) and classified as SIT-BT doubtful responses (when 1 mm ≤ (BT – AT) < 2 mm and 2 mm ≤ BT < 4 mm);

(vii) Excess D4 (XD4) = (D4 + D3 + D2) is the SICCT-BT doubtful responses at ≥4 mm cutoff point and classed as SIT-BT-positive animals (when 1 mm ≤ (BT – AT) < 4 mm and BT ≥ 4 mm);

(viii) Excess D3 (XD3) = (D3 + D2) is the SICCT-BT doubtful responses at ≥3 mm cutoff point and classed as SIT-BT-positive animals (when 1 mm ≤ (BT – AT) < 3 mm and BT ≥ 4 mm);

(ix) Excess D2 (XD2) = (D2) is the SICCT-BT doubtful responses at the ≥2 mm cutoff point and classed as SIT-BT-positive animals (when 1 mm ≤ (BT – AT) < 2 mm and BT ≥ 4 mm);

(x) T4 is the SICCT-BT-positive animals at ≥4 mm cutoff point (when (BT − AT) ≥ 4 mm);

(xi) T3 = (T4 + XD4 + Xd4) is the SICCT-BT-positive animals at ≥3 mm cutoff point (when (BT − AT) ≥ 3 mm);

(xii) T2 = (T3 + XD3 + Xd3) is the SICCT-BT-positive animals at ≥2 mm cutoff point (when (BT − AT) ≥ 2 mm);

(xiii) Excess A (XA) is the animals classed as SIT-BT-positive animals and infected with atypical mycobacteria according to SICCT-AT (when BT ≥ 4 mm and (BT – AT) < 1 mm);

(xiv) Excess AD (XAD) is the animals classed as SIT-BT doubtful responses and infected with atypical mycobacteria according to SICCT-AT (when 2 mm ≤ BT< 4 mm and (BT − AT) < 1 mm);

(xv) AT is the animals infected with atypical mycobacteria according to SICCT-AT and classed as SIT-BT negative animals (when BT < 2 mm and (AT − BT) > 0 mm).

3. Results

3.1. Observed Prevalence Rates and Agreements between Lateral Flow Assay and Tuberculin Skin Tests at ≥2 mm, ≥3 mm, and ≥4 mm Cutoff Points. The observed TST results at modified cutoff points and antibovine TB Ab assay in 807 cattle are summarized in Table 1. Of 807 tested cattle, antibovine TB Ab was detected in 37.17% (95% CI: 30.64–43.71) while 11.77% (95% CI: 9.55–14.00), 8.92% (95% CI: 6.96–10.88), and 3.59% (95% CI: 2.31–4.88) of them were SICCT-BT positive at ≥2 mm, ≥3 mm, and ≥4 mm cutoff points, respectively. The proportion of SICCT-BT/antibovine TB Ab reactors was highest ($P < 0.05$) at the ≥2 mm (9.42% (95% CI: 7.40%–11.43%)) followed by the ≥3 mm (7.93% (95% CI: 6.07–9.79)) and ≥4 mm (3.59% (95% CI: 2.31%–4.88%)) cutoff point groups.

However, analysis of all antibovine TB Ab reactors (300) revealed that 25.33%, 21.33%, 9.67%, and 27% of them were positive at the SICCT-BT ≥ 2 mm, ≥3 mm, and ≥4 mm cutoff points and SIT-BT, respectively. The proportion of SICCT-BT doubtful/antibovine TB Ab positive reacting cattle was highest ($P < 0.05$) at the SICCT-BT ≥ 4 mm (21%) followed by the ≥3 mm (5.67%) and ≥2 mm (1.67%) cutoff point groups. However, 0.62% (95% CI: 0.08%–1.16%), 3.47% (95% CI: 2.21%–4.73%), and 8.80% (95% CI: 6.84%–10.75%) of the 807 tested cattle showed SICCT-BT inconclusive results while 0.62% (95% CI: 0.08%–1.16%), 2.11% (95% CI: 1.12%–3.10%), and 7.81% (95% CI: 5.96%–9.66%) reactors were SICCT-BT doubtful and antibovine TB Ab positive at the o 2 mm, ≥3 mm, and ≥4 mm cutoff points, respectively. Over 27.14% (95% CI: 24.07%–30.21%) negative SICCT-BT reactors were also positive for antibovine TB Ab.

Furthermore, 13.14% (95% CI: 10.80%–15.47%) SIT-BT and 10.04% (95% CI: 7.96–12.11) SIT-BT positive/antibovine TB-Ab-positive animals were recorded.

Table 1: Distribution of reactors of tuberculin skin tests at various cutoff points and antibovine tuberculosis antibody assay (according to region, sex, age, and herd size) in cattle in Cameroon.

Variable/Label	Number of animals tested	SICCT-BT reactors (%)			SIT-BT reactors	SICCT BT doubtful/SIT-BT positive reactors (%)			SICCT BT doubtful/SIT BT doubtful responses (%)			Excess A* (%)	% of SICCT-BT doubtful and classed positive at inferior cutoff points (%)		Antibovine TB Ab reactors# (%±SE)
		T4	T3	T2		XD4	XD3	XD2	Xd4	Xd3	Xd2		XD4 at ≥3 mm	XD4 + XD3 at ≥2 mm	
All animals	807	3.59	8.92	11.77	13.14	3.72	0.87	0.50	5.08	2.60	0.12	4.46	2.97	3.84	37.17 ± 3.33
Agroecological Regions															
Adamawa plateaux	363	0.28	0.28	0.55	0.55	0.28	0.28	0.00	0.28	0.28	0.28	0.00	0.00	0.28	29.75 ± 4.70
Western Highlands	444	6.31	15.99	20.95	23.42	6.53	1.35	0.90	9.01	4.50	0.00	8.11	5.41	6.76	43.24 ± 4.61
Sex and Age															
Female	647	4.02	8.96	11.44	13.14	4.17	1.08	0.62	4.64	2.16	0.15	4.48	3.25	4.33	36.32 ± 3.71
Male	160	1.88	8.75	13.13	13.13	1.88	0.00	0.00	6.88	4.38	0.00	4.38	1.88	1.88	40.63 ± 7.61
Young (o 4 years)	481	3.33	8.32	11.02	11.85	3.33	0.42	0.21	4.99	2.49	0.00	3.53	2.91	3.33	38.46 ± 4.35
Adult (>4 years)	326	3.99	9.82	12.88	15.03	4.29	1.53	0.92	5.21	2.76	0.31	5.83	3.07	4.60	35.28 ± 5.19
Herd sizes															
Animals ≤ 40	169	5.92	10.06	12.43	13.02	6.51	1.18	0.59	2.96	1.78	0.00	4.14	5.92	7.10	28.99 ± 6.84
Animals > 40	638	2.98	8.62	11.60	13.17	2.98	0.78	0.47	5.64	2.82	0.16	4.55	2.19	2.98	39.34 ± 3.79

T4, T3, T2, XD4, XD3, XD2, Xd4, Xd3, Xd2, and Excess A are as defined in Figure 2.

SICCT-BT: Single Intradermal Comparative Cervical Tuberculin skin test for the detection of bovine tuberculosis.

SIT-BT: Single Intradermal Tuberculin skin test for the detection of bovine tuberculosis.

Antibovine TB Ab: Antibovine tuberculosis antibody assay.

#*Breed*: Upgraded/Exotic = 42.03 ± 6.72; Gudali = 31.30 ± 4.10; Namchi = 22.58 ± 14.72; Red Bororo = 67.53 ± 10.46.
Management and production system: Extensive = 31.76 ± 4.13; Intensive = 75.00 ± 30.01; Semi-intensive = 44.69 ± 5.53;
Beef herds = 38.07 ± 3.70; Dairy herds = 33.10 ± 7.66.

Table 2: Agreement between reactors of tuberculin skin tests and antibovine tuberculosis antibody assay according to various tuberculin skin response cutoff points.

	SICCT-BT cutoff points						SIT-BT	
	≥4 mm		≥3 mm		≥2 mm		≥4 mm	
	Number	%	Number	%	Number	%	Number	%
TST positive/Anti-BTB Ab positive	29	3.59	64	7.93	76	9.42	81	10.04
TST negative#/Anti-BTB Ab positive	271	34.82	236	29.24	224	27.76	219	27.14
TST positive/Anti-BTB Ab negative	0	0	8	0.99	19	2.35	25	3.10
TST negative#/Anti-BTB Ab negative	507	62.83	499	61.83	488	60.47	482	59.73
Total	807		807		807		807	
Agreement	29/807	3.59	64/807	7.93	76/807	9.42	81/807	10.04
Kappa statistics*	0.119		0.234		0.251		0.254	

TST: Tuberculin skin test.
Anti-BTB Ab: antibovine tuberculosis antibody assay.
#Not TST positive including TST doubtful reactors.
**Kappa* ranges from 1 (complete agreement beyond chance) to 0 (agreement is equal to that expected by chance), whereas negative values indicate that agreement less than that is expected by chance.

Among the SIT-BT reactors, 76.42% of them were antibovine TB Ab reactors and over 89.62%, 67.92%, and 27.36% were SICCT-BT reactors while 71.70%, 60.38%, and 27.36% were SICCT-BT-positive/antibovine TB-Ab-positive animals at the 2 mm; ≥3 mm, and ≥4 mm cutoff points, respectively. Overall, 31 (3.84%) SICCT-BT doubtful/SIT-BT-positive animals at superior cutoff points were classified as SICCT-BT reactors at ≥3 mm (2.97%) and ≥2 mm (3.84%) cutoff points (Table 1).

The agreement between TST at modified cutoff points and antibovine TB antibody assay was shown in Table 2. In all, the concordances (TST positive/antibovine TB Ab positive) were 100%, 88.89%, 80%, and 76.42% in positive subjects at SICCT-BT ≥4 mm, ≥3 mm, and ≥2 mm cut-offs and SIT-BT, respectively. The discordances (TST negative/antibovine TB Ab positive) were 34.83%, 32.11%, 31.46%, and 31.24%, at the SICCT-BT ≥4 mm, ≥3 mm, and ≥2 mm cutoff points and SIT-BT, respectively. However, the concordances (TST positive/antibovine TB Ab positive) in antibovine TB Ab positive subjects were 9.67%, 21.33%, 25.33%, and 27% while the discordances (TST negative/antibovine TB Ab positive) were 94%, 78.67%, 74.67%, and 73%, at the SICCT-BT ≥4 mm, ≥3 mm, and ≥2 mm cutoff points and SIT-BT, respectively. The bench marks (>0.80: very good agreement; 0.61–0.80: good agreement; 0.41–0.60: moderate agreement; 0.21–0.40 fair agreement and ≤0.20: poor agreement) for evaluating points estimates of *kappa* values [28] revealed a poor agreement between SICCT-BT test and antibovine TB Ab assay at the ≥4 mm skin response cutoff point and fair agreements at the other cutoff points (≥3 mm and ≥2 mm; and SIT-BT).

3.2. Comparison of Tuberculin Skin Tests at Modified Cutoff Points and Lateral Flow Assay in Cattle Reactors. The predictive values and likelihood ratios of SICCT-BT at various cutoff values and SIT-BT in cattle reactors against the antibovine TB Ab assay are shown in Table 3. Strong associations were noted between the seroprevalence estimates and rates of TST reactors irrespective of the TSTS cut-off value ($P < 0.05$; $\chi^2 > 48$) in this study. However, decreasing the cutoff points revealed inverse relationships with test predictive values and diagnostic likelihood ratios. The ability of SICCT-BT to produce no false negative result increased with increase in cutoff point (nonsignificant differences were noted between the ≥2 mm versus ≥3 mm and ≥3 mm versus ≥4 mm cutoff points). The findings also suggested that prediction of disease status improved with severe interpretation of TST (decreasing cutoff point). The study indicated that using antibovine TB Ab assays as ancillary diagnostic tests to SICCT-BT in cattle could significantly improve diagnosis of bovine TB cases. Statistically, the best all round SICCT-BT performance was realized at the ≥3 mm cutoff point. However, the ≥2 mm cut-off value showed the highest positive predictive value and a comparable positive diagnostic likelihood ratio to the others.

The detection of antibovine TB Ab positive cattle and proportions of SICCT-BT reactors and antibovine TB Ab/SICCT-BT reactors at the different cut-offs are shown in Figure 4. The SICCT-BT ≥ 2 mm cutoff value gave the highest ($P < 0.05$) rate (23.60%) followed by the ≥3 mm (15.15%) and ≥4 mm (4.98%) cutoff points. Overall, similar trends were observed for SICCT-BT and antibovine TB-Ab-positive/SICCT-BT-positive animals for the parameters considered. In all, 16.78% SIT-BT- and 12.73% SIT-BT-positive/antibovine TB-Ab-positive animals were detected (Figure 3). Among the SIT-BT reactors, over 98.59%, 61.23%, and 10.38% were SICCT-BT reactors and 78.88%, 60.19%, and 10.38% were SICCT-BT-positive/antibovine TB-Ab-positive animals at the ≥2 mm, ≥3 mm, and ≥4 mm cutoff points, respectively. Also, 84.07% SICCT-BT-positive/antibovine TB-Ab-positive animals were identified among the SIT-BT reactors, irrespective of the interpreting SICCT-BT cutoff point. SIT-AT positive reacting cattle was widespread in the study.

Table 3: Predictive values and likelihood ratios at the ≥2 mm, ≥3 mm, and ≥4 mm cutoff points for tuberculin skin tests and lateral flow assay of cattle reactors in Cameroon.

Cutoff point	Test predictive value; % (95% CI)		Diagnostic likelihood ratio; (95% CI)	
	Positive result	Negative result	LR+	LR−
(a) For SICCT-BT test against antibovine TB Ab assay				
≥2 mm	34.05 (29.16–38.50)	94.41 (91.66–96.41)	2.54 (2.03–3.08)	0.29 (0.45–0.18)
≥3 mm	29.55 (25.32–33.13)	97.58 (95.42–98.79)	2.77 (2.24–3.27)	0.16 (0.32–0.08)
≥4 mm	14.67 (12.15–15.94)	100 (98.88–100)	2.87 (2.31–3.17)	0* (0.19–0)
(b) For SIT-BT test against antibovine TB Ab assay				
≥4 mm	33.03 (28.13–37.61)	93.53 (90.87–95.58)	2.45 (1.94–2.99)	0.34 (0.50–0.23)

*The perfect diagnostic test would be expected to have an LR− equal to zero and an LR+ equal to infinity (producing no false negatives, but detecting all negatives and detecting all positives, and generating no false positives). The best test therefore for excluding a disease is the one with the lowest LR− and the test with the highest LR+ is the best for detecting disease [28].

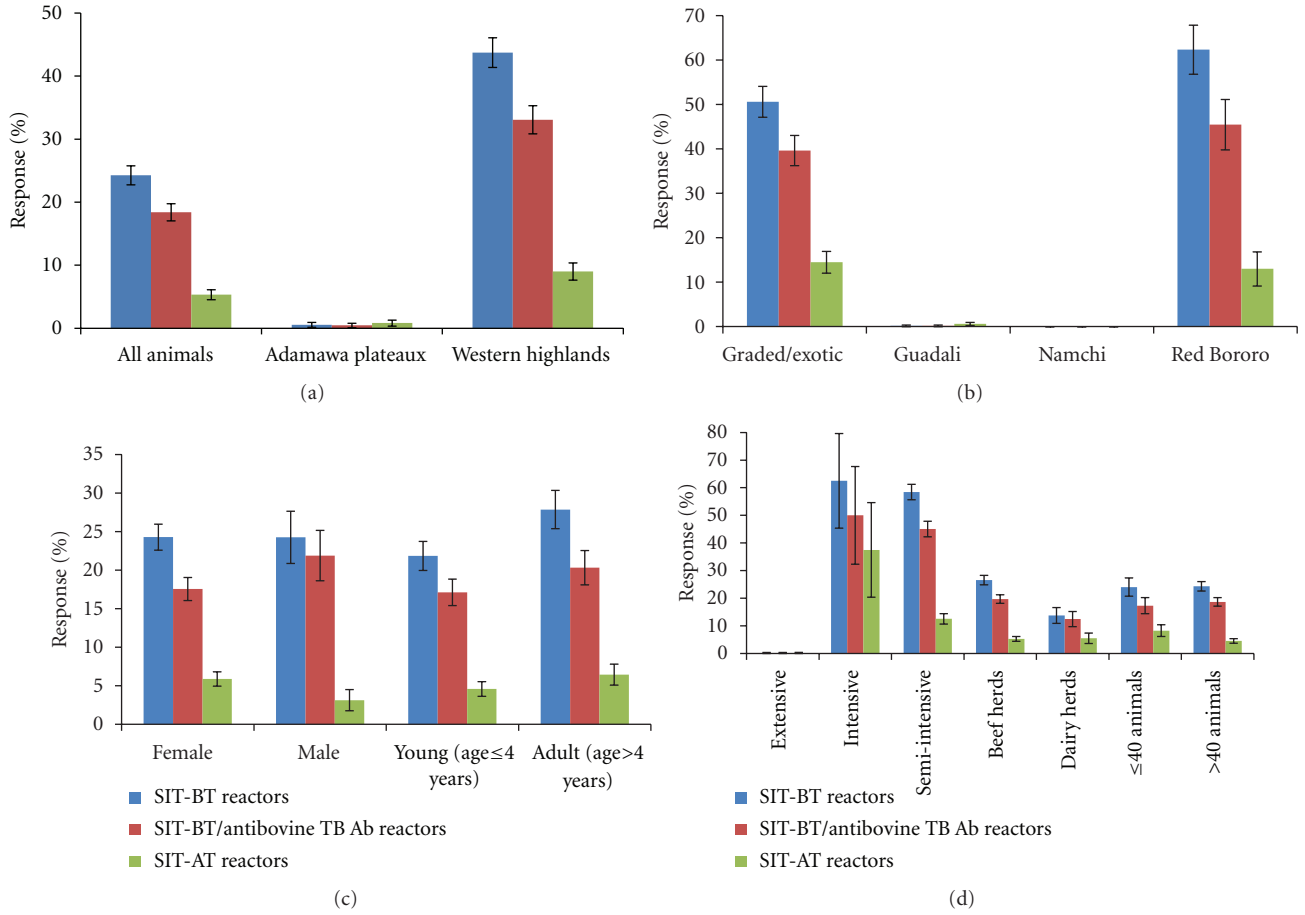

Figure 3: Detection of antibovine TB antibody and SIT-BT reactors in 807 tested cattle according to (a) study location, (b) breed, (c) sex and age group, and (d) management systems and herd sizes.

Furthermore, antibovine TB Ab assay revealed that over 95% (95% CI: 75.1%–99.9%) of the test herds had ≥1 antibovine TB-Ab-positive animal, while SIT-BT and SICCT-BT at ≥2 mm cutoff point gave nonsignificantly higher TST positive/antibovine TB Ab positive herds (36.84%, (95% CI: 16.3%–61.6%)) than SICCT-BT at ≥3 mm and ≥4 mm (30%, (95% CI: 12.6%–56.5%)) cutoff points. Indeed, the herd infection (i.e., ≥1 TST positive animal) rates were 35% (95% CI: 15.4%–59.2%) for SIT-BT and SICCT-BT ≥2 mm cutoff point and 30% (95% CI: 11.9%–54.3%) for the SICCT-BT at ≥3 mm and ≥4 mm cutoff points. Similarly, higher but comparable herd infection rates were obtained when severe interpretations were considered for complete TST screening of 1,381 cattle in 40 herds (i.e., for SICCT-BT: 40% (95% CI: 24.9%–56.7%) at ≥3 mm and ≥4 mm cut-offs; 45% (95% CI: 29.3%–61.5%) at ≥2 mm

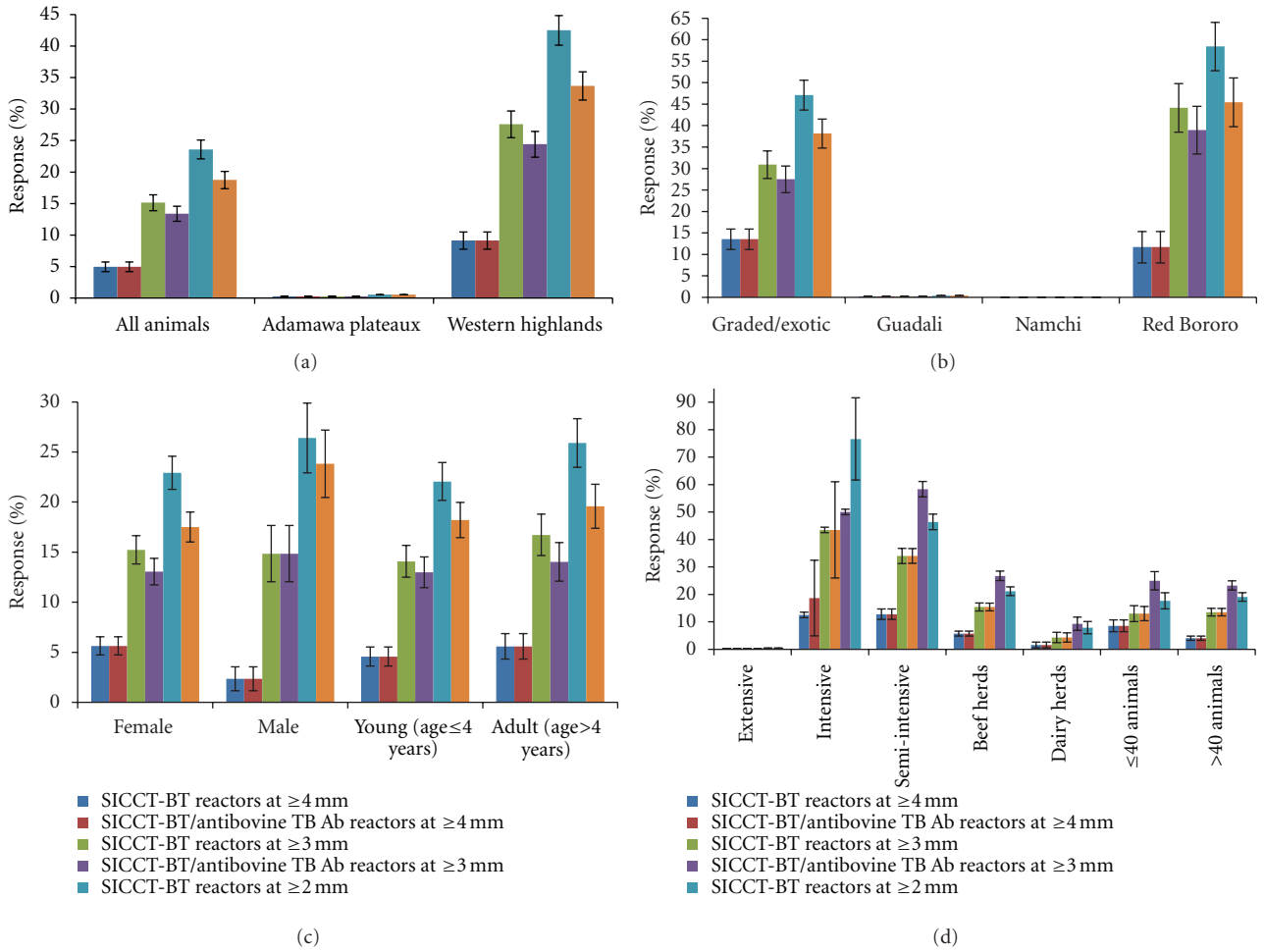

Figure 4: Detection of antibovine TB antibody and SICCT-BT reactors in 807 tested cattle at the ≥4 mm, ≥3 mm, and ≥2 mm cutoff points according to (a) study location, (b) breed, (c) sex and age group, and (d) management systems and herd sizes.

cut-off and also 47.5% (95% CI: 33.8%–66.2%) for SIT-BT). Also, significantly higher ($P < 0.05$) SICCT-BT- and SIT-BT-infected herds were recorded in the Western highlands (48.39% (95% CI: 30.2%–66.9%) at the SICCT-BT ≥4 mm and ≥3 mm cutoff points; 51.61% (95% CI: 33.1%–69.8%) at the SICCT-BT ≥2 mm cutoff point and 54.84% (95% CI: 36%–72.7%) for SIT-BT) than in the Adamawa plateaux (11.11% (95% CI: 24.9%–56.7%) for the SICCT-BT ≥4 mm and ≥3 mm cutoff groups and 22.22% (95% CI: 2.8%–60%) for the SICCT-BT ≥2 mm cut-off and SIT-BT groups).

3.3. Prevalence Rates of Bovine Tuberculosis in Previously Tested Cattle at the Modified Cutoff Points. The TST survey in the year 2009 (2,853 cattle) and complete data of 2010 (1,381 cattle) were reanalysed using the predefined cutoff points (Tables 4 and 5). Overall, the prevalence rates and trends of bovine TB in both surveys were very similar. The differences in the prevalence of SICCT-BT reactors were significantly higher between the cutoff points (≥4 mm versus ≥3 mm: $\chi^2 = 46.021$; $P \leq 0.001$; ≥4 mm versus ≥2 mm: $\chi^2 = 64.015$; $P \leq 0.001$; ≥3 mm versus ≥2 mm: $\chi^2 = 16.056$; $P \leq 0.001$). Age, sex, breed, animal site, and husbandry systems were significant ($P < 0.05$) risk factors to the epidemiological status of bovine TB in the regions.

4. Discussion

There is gross inadequacy in the implementation of the existing bovine TB control policy in Cameroon. Culling of TST reactors as part of a national animal disease control policy is not a routine practice due to political, economic, and social limitations. However, veterinarians continue to identify bovine TB lesions in slaughtered cattle across the country [33–35]. TB lesions have been detected in TST reactors at cutoff points less than the OIE-recommended optimal 4 mm cut-off [8, 9, 15] and TST negative reactors [10]. TB lesions were also observed in TST doubtful and negative reactors in Mezam Division in the present study. Lack of knowledge on the actual magnitude and distribution of the disease, inadequate laboratories and field expertise, and politicoeconomic deficiencies are common factors that limit bovine TB control in most of Africa [36]. The current control approach in Cameroon is based on controlling animal movements, culling suspected bovine TB cases and

Table 4: Prevalence of SICCT reactors in 1,381 cattle tested in the year 2010 at modified cutoff points and SIT reactors in the highlands of Cameroon.

Variable	No animals tested	SICCT-BT reactors % (95% CI)			SICCT-AT reactors*; %(SE)	SIT-BT reactors; %(95% CI)	SIT-AT reactors*; %(SE)
		≥4 mm	≥3 mm	≥2 mm	≥4 mm	≥4 mm	≥4 mm
All animals	1,381	7.41 (6.02–8.79)	13.25 (11.47–15.04)	17.26 (15.36–19.25)	0.65 ± 0.42	18.35 (14.35–22.35)	7.46 ± 1.39
Agroecological location							
ADP	363	0.43[a] (0–1.11)	0.40[a] (0–1.04)	0.79[a] (0–1.70)	0.55 ± 0.76	0.59[a] (0–2.13)	0.83 ± 0.93
WHC	1,018	9.89[b] (8.06–11.72)	17.84[b] (15.49–20.19)	23.13[b] (20.54–25.72)	0.69 ± 0.51	24.68[b] (19.49–29.88)	9.82 ± 1.83
Breed							
Upgraded/Exotic	764	10.87[a] (8.66–13.08)	16.12[a] (13.52–18.73)	21.07[a] (18.18–23.96)	0.79 ± 0.63	24.03[a] (18.09–29.96)	11.39 ± 2.25
Guadal	492	0.31 (0–0.79)	0.28 (0–0.75)	0.57 (0–1.24)	0.41 ± 0.56	0.40 (0–1.50)	0.61 ± 0.69
Namchi	31	0	0	0	0	0	0
Red Bororo	94	18.84[a] (10.94–26.75)	62.22[b] (52.42–72.02)	79.29[b] (71.10–87.49)	1.06 ± 2.07	72.28[b] (54.54–90.01)	13.83 ± 6.98
White Fulani							
Sex and Age							
Female	1,107	8.29[a] (6.66–9.91)	13.90[a] (11.87–15.94)	17.69 (15.44–19.94)	0.63 ± 0.47	19.73[a] (15.14–24.33)	8.49 ± 1.64
Male	274	3.83[b] (1.56–6.11)	10.62[a] (6.98–14.28)	15.51 (11.22–19.79)	0.73 ± 1.01	12.77[a] (5.02–20.51)	3.28 ± 2.11
Age ≤ 4 years	716	4.41[c] (2.91–5.91)	9.71[b] (7.55–11.88)	12.50[a] (10.07–14.92)	0.28 ± 0.39	11.93[b] (7.28–16.59)	4.05 ± 1.44
Age > 4 years	665	10.63[d] (8.29–12.97)	17.07[c] (14.21–19.92)	22.38[b] (19.22–25.55)	1.05 ± 0.78	25.26[c] (18.79–31.73)	11.13 ± 2.39
Management system							
Extensive	488	0.31 (0–0.80)	0.28 (0–0.75)	0.58 (0–1.25)	0	0.41 (0–1.51)	0.20 ± 0.40
Intensive/Zero grazing	552	8.95[a] (6.57–11.34)	12.03[a] (9.31–14.74)	16.50[a] (13.40–19.59)	1.09 ± 0.87	20.74[a] (14.11–27.36)	10.87 ± 2.60
Semi-intensive	341	15.05[b] (11.26–18.85)	33.89[b] (28.78–38.83)	42.36[b] (37.11–47.60)	0.88 ± 0.99	40.17[b] (29.97–50.37)	12.32 ± 3.49
Beef herds	692	7.39[c] (5.44–9.34)	16.40[c] (13.64–19.16)	20.63[c] (17.61–23.64)	0.43 ± 0.49	18.97[c] (13.25–24.70)	5.49 ± 1.70
Dairy herds	689	15.15[d] (12.48–17.83)	10.10[d] (7.85–12.35)	13.87[d] (11.29–16.45)	0.87 ± 0.69	17.73[c] (12.14–23.32)	9.43 ± 2.18
Herd size (No animals per herd)							
≤40 animals	713	9.41[a] (7.27–11.55)	12.72[a] (10.27–15.17)	16.38[a] (13.67–19.11)	1.12 ± 0.77	19.14[a] (13.48–24.81)	9.96 ± 2.20
>40 animals	668	5.26[b] (3.57–6.96)	13.82[a] (11.21–16.44)	18.18[a] (15.26–21.11)	0.15 ± 0.29	17.50[a] (11.86–23.15)	4.79 ± 1.62

[a,b,c,d] Label in a category with the different letters in a column are significantly different ($P < 0.05$).

*Observed prevalence.

ADP: Adamawa plateaux of Cameroon.

WHC: Western highlands of Cameroon.

SICCT-BT: Single Intradermal Comparative Cervical Tuberculin skin test for the diagnosis of bovine TB.

SIT-BT: Single Intradermal Tuberculin skin test for the diagnosis of bovine TB.

Table 5: Prevalence of SICCT-BT reactors in 2,853 cattle tested in the year 2009 at modified cutoff points in the highlands of Cameroon.

Variable	Animals tested	SICCT-BT reactors % (95% CI)		
		≥4 mm	≥3 mm	≥2 mm
All animals	2,853	7.48 (6.51–8.44)	11.52 (10.35–12.69)	12.92 (11.69–11.15)
Agroecological location				
ADP	727	4.10[b] (2.66–5.54)	5.32[b] (3.69–6.95)	7.07[a] (5.21–8.93)
WHC	2,126	8.63[a] (6.51–8.44)	13.64[a] (12.18–15.10)	14.92[b] (13.40–16.43)
Breed				
Upgraded/Exotic	368	12.49[a] (9.12–15.87)	19.39[a] (15.35–23.43)	21.05[a] (16.88–25.21)
Guadali	1,317	6.01[b] (4.73–7.30)	10.32[b] (8.68–11.96)	12.32[b] (10.54–14.09)
Namchi	33	3.03	3.03	3.03
Red Bororo	487	11.62[a] (8.77–14.46)	15.64[a] (12.42–18.87)	16.52[a] (13.22–19.82)
White Fulani	648	4.60[b] (2.99–6.22)	6.72[b] (4.80–8.65)	7.23[b] (5.24–9.23)
Sex and Age				
Female	2,212	7.73[a] (6.62–8.85)	12.30[a] (10.93–13.67)	13.92[a] (12.48–15.36)
Male	641	6.60[a] (4.67–8.52)	8.83[b] (6.63–11.02)	9.45[b] (7.19–11.72)
Age ≤ 4 years	1,481	5.82[b] (4.63–7.01)	8.40[c] (6.99–9.82)	9.72[c] (8.21–11.22)
Age > 4 years	1,372	9.27[c] (7.73–10.80)	14.88[d] (13.00–16.77)	16.37[d] (14.41–18.33)
Management system				
Extensive	1510	6.77[a] (5.50–8.03)	9.32[a] (7.85–10.78)	9.93[a] (8.42–11.44)
Intensive	138	6.38[a] (2.03–10.46)	17.62[b] (11.27–23.98)	19.81[b] (13.16–26.46)
Semi-intensive	1205	8.49[a] (6.92–10.07)	13.58[b] (11.64–15.51)	15.87[b] (13.81–17.93)
Beef herds	2,357	8.16[b] (7.05–9.26)	10.78[c] (9.53–12.03)	11.71[c] (10.41–13.00)
Dairy herds	496	4.24[c] (2.47–6.02)	15.03[d] (11.88–18.17)	18.67[d] (15.24–22.10)
Herd size (No animals per herd)				
≤40 animals	1,325	9.19[a] (7.64–10.75)	11.98[a] (10.23–13.72)	13.51[a] (11.67–15.35)
>40 animals	1,528	5.99[b] (4.80–7.18)	11.12[a] (9.55–12.70)	12.40[a] (10.75–14.06)

[a,b,c,d] label in a category with different letters in a column are significantly different ($P < 0.05$).
SICCT-BT: Single Intradermal Comparative Cervical Tuberculin skin test for the diagnosis of bovine tuberculosis.

carcass condemnation (partial or whole) at meat inspection [37]. Apparently, the strategies were designed to reduce the general prevalence and monitor spread of the disease in livestock. TST is presently a passive component of Cameroon's government strategy to control bovine TB which is of major concern to the veterinary and medical services.

Maximum detection of bovine TB in cattle populations in Cameroon is vital to understand its epidemiology and zoonotic potentials and also achieve significant reduction and control of the disease in livestock. Cell-mediated immune responses develop early after bovine TB infection in cattle while antibody responses may not become obvious until later and at advanced stages of the disease, when cell mediated reactions (TST reactions) are waning [38–40]. TST can boost antibody responses in *M. bovis* infected cattle and emphasizes the importance of timing of collection of blood samples on the interpretation the test [38]. In this study, the antibovine TB antibody detection (Anigen lateral-flow assay) that employed recombinant *M. bovis* MPB70 antigen as capture and detector was conducted prior to TST. This antibovine TB antibody test kit has a sensitivity of 90% against bovine TB confirmed by bacterial isolation and a sensitivity of 85.1% and specificity of 98.6% against TST [41]. Also using the Anigen lateral-flow assay, Whelan et al. [42] achieved a sensitivity of 84% and a specificity of 84.2% for serological diagnosis of *M. bovis* infection in cattle. Similar and relatively high sensitivity (86.5% and 84.6%) and specificity (83.8% and 91.4%) have been reported with other lateral flow techniques (CervidTB STAT-PAK and DPP VetTB assays, resp.) for the rapid diagnosis of bovine TB in farmed Red deers [43]. Furthermore, a sensitivity of 89.6% and specificity of 90.4% were achieved in the diagnosis of *M. bovis* infection in Eurasian wild boar using the DPP VetTB assay (based on combining two separate test antigens) [44]. However, the specificity of these test kits could be affected by cross-reacting members of the *M. avium* complex [43, 44], and high false positive results were observed when a commercial multiantigen lateral flow assay was performed in dairy cattle [45]. Nonetheless, significantly higher specificity of 98.4% and sensitivity of 93.1% in the diagnosis of bovine TB in cattle have been obtained for multiplex immunoassay based on a combination of antigens compared to those of assays based on a single antigen [22, 42]. The TST accuracy against postmortem detection of TB lesions revealed a sensitivity of 86% and specificity of 90% for SIT-BT [12], while sensitivity values of 69%, 65%, and 59% at SICCT-BT ≥2 mm, ≥3 mm, and ≥4 mm cutoff points and a specificity of 97% at these cutoff points have been reported [9]. The

lack of a well-established gold standard in this study was a key problem in calculating the sensitivity and specificity of the lateral flow assay and TST at the modified cutoff points.

The findings of this study suggest that TST at any cutoff point could be used to detect bovine TB in cattle and the test accuracy increased with increase in cut-off value. Cattle presenting differential SICCT-BT skin thickness of less than 4 mm in Cameroon should therefore not be excluded that they are negative for bovine TB. These animals may be infected but low reacting or not reacting at all if their immune systems were not stimulated enough for a positive response at the ≥4 mm cutoff point [46, 47] due to conditions such as stress that compromise immune function [48]. Also, the animals may have been sensitized to environmental mycobacteria [38]. Furthermore, delayed hypersensitivity to tuberculin may not develop for a period of 3–6 weeks following infection [3, 10]. Delaying TST of a herd/animal suspected to have been in contact very recently with infected animals in order to reduce the probability of false-negatives has been suggested [10] since it is unlikely that the control and eradication of TB from a herd will be achieved with only a single tuberculin test [3]. In this study, maximum positive prediction values and negative likelihood ratio were observed at the SICCT-BT ≥2 mm cutoff point and maximum negative prediction and positive likelihood ratio at the ≥4 mm cutoff point. The findings also revealed that 31 cattle (over 3.84%) considered as SICCT-BT doubtful reactors at the ≥4 mm cutoff point could be identified as positive bovine TB cases at the ≥3 mm and ≥2 mm cutoff points. The poor to fair agreements recorded suggested that severe interpretation of TST (i.e., decreasing skin response cut-off values) improved the agreement between TST and the lateral flow assay to detect TST positive reactors. The prevalence rates at the modified cutoff points could have influenced the estimated *Kappa* values. However, low *kappa* values have been obtained between good diagnostic and negatively correlated tests [28]. The poor correlation between comparative TST at the ≥4 mm cutoff point and antibovine TB antibody test results in the study was not unexpected. Therefore, the importance of determining appropriate localised TST cut-off values supported by validated methods in Cameroon cannot be overemphasized.

Though it is essential that tuberculin of sufficient potency to produce a reaction in the maximum number of infected animals is essential, a tuberculin of potency greater than that to which the majority of infected animals will respond has been proposed in TST [10]. However, Good and Duignan [10] had warned that highly potent tuberculin 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. Nonspecific responses in TST due to atypical or environmental mycobacteria have been widely reported [2, 3, 49–51]. Indeed, Lesslie et al. [52–54] recorded hypersensitivity responses to avian tuberculin that was equal or higher than responses to bovine tuberculin in cattle naturally infected with *M. bovis* and presenting visible lesions at slaughter. Therefore, severe interpretations of TST reactions should be employed when EU- and OIE-recommended tuberculin preparations are used in bovine TB endemic regions and environments where multiple mycobacteria are coexisting. The findings of this study agree with Martrenchar et al. [23] who reported high frequency of atypical mycobacteria which severely limited the reliability of SIT-BT and SICCT-BT results at the OIE-recommended 4 mm cutoff point in Northern Cameroon. Severe interpretations of TST results in the study revealed that many SIT-BT positive and SICCT-BT doubtful responses at ≥4 mm cutoff point could be appropriately identified as bovine TB cases at reduced cutoff points (some Excess D4 and Excess D3 reactors). The high detection of TST and antibovine TB antibody positive herds irrespective of TST cutoff point and findings of circulating antibovine TB antibody could suggest that the cattle were widely exposed to and affected bovine TB and other mycobacterial infections.

In this study, reducing the cutoff point from ≥4 mm improved the *ante mortem* detection of bovine TB in cattle using SICCT-BT and antibovine TB Ab tests. Overall, the maximum test ability was realized at ≥3 mm cutoff point and the best SICCT-BT positive predictive value was at ≥2 mm cutoff point. These findings revealed that interpreting SICCT-BT at the ≥2 mm cutoff point, and not at the ≥3 mm or ≥4 mm cutoff points, was beneficial from a public health perspective. However, there would be concrete risk of unnecessarily identifying more cattle at severe TST interpretations. This study cannot exclude that some SICCT-BT doubtful reactors at the ≥3 mm and the ≥4 mm cutoff points were infected cases detected at the ≥2 mm cutoff point. The application of the SICCT-BT ≥2 mm cutoff point should be considered in cattle in the agro-ecological highland environments of Cameroon for greater detection of bovine TB. Severe TST interpretation would be vital to effective control of the disease and reduction of its zoonotic risks to public health and food safety in the country.

5. Conclusion

The TST and antibovine TB antibody tests when used in parallel offered improved detection of bovine TB compared to individual tests. Bovine TB was detected at all the cutoff points and there were strong associations between both methods in the highlands of Cameroon. The best test performance was realized at the ≥3 mm cutoff point. However, interpreting SICCT-BT at ≥2 mm cutoff point was more strategic from a public health context since more affected cases would be predicted. The study revealed that the prevalence of bovine TB was high and atypical mycobacteria infection was widespread in the regions. Bovine TB-infected cattle which maybe anergic due to age, malnutrition, and/or suffering from concurrent diseases such as internal and external parasitosis (common scenarios in the study regions) could be detected at severe SICCT-BT interpretation. Their delayed hypersensitivity responses to tuberculin would be limited and cannot express the full OIE-recommended ≥4 mm cutoff point. However, it is important to investigate the performance of TST at modified cutoff points against

defined bovine TB status confirmed by postmortem examination and culture of TB lesions in reacting animals in the Cameroon environments.

Acknowledgments

This paper was supported by a Ph.D. grant of the Commonwealth Scholarship Commission in UK. The authors are also grateful to the Staff of MINEPIA and cattle professionals of the Northwest and Adamawa regions of Cameroon for their generous cooperation and for the support of IRAD-Wakwa.

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