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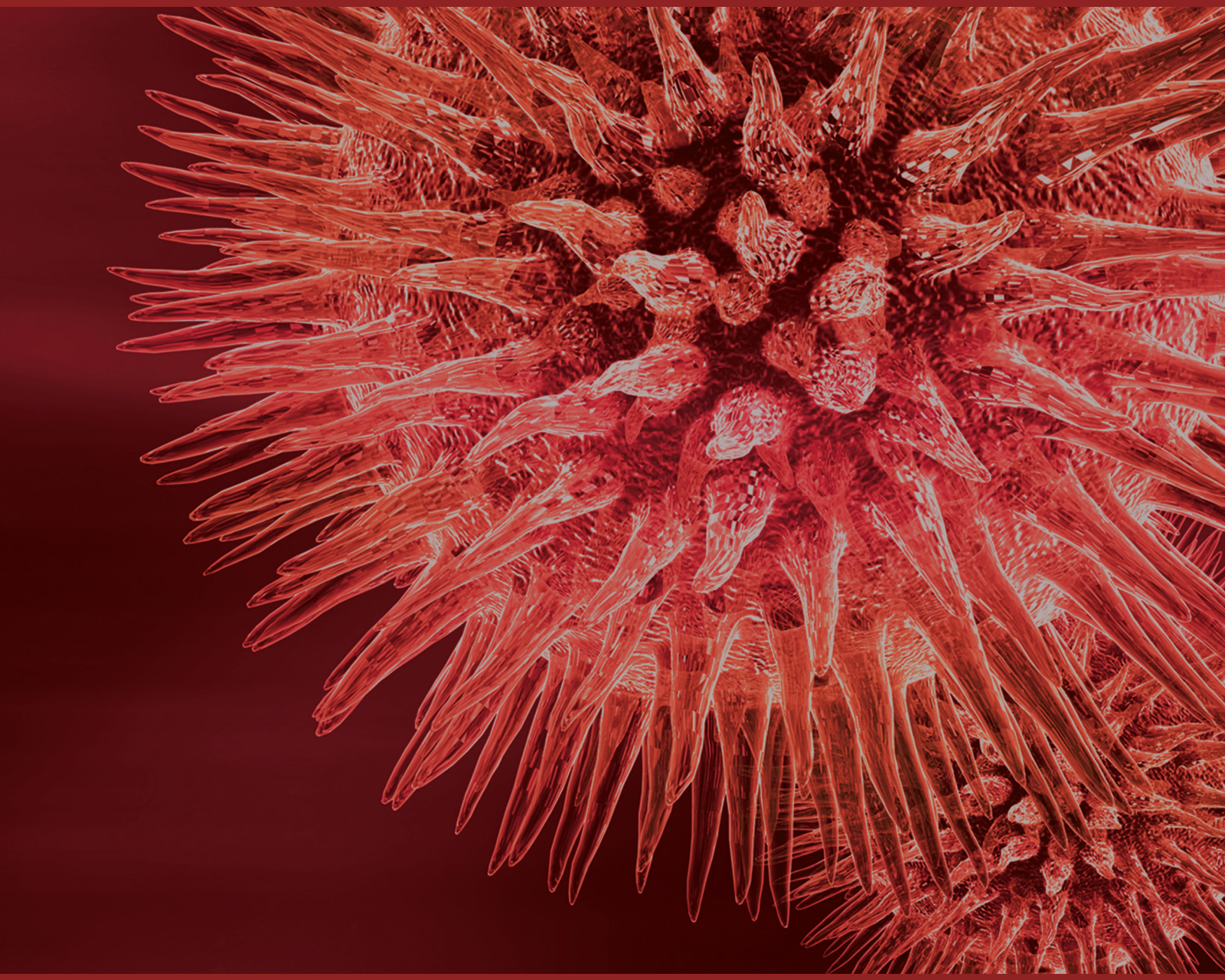
Guest Editors: Mehdi Mirsaeidi, Parissa Farnia, Ruxana Sadikot, Po-Ren Hsueh, and Stefano Aliberti



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Editorial

Nontuberculous Mycobacteria: Epidemiologic, Mycobacteriologic, and Clinical Aspects

Mehdi Mirsaeidi,[1] Parissa Farnia,[2] Ruxana Sadikot,[3] Po-Ren Hsueh,[4] and Stefano Aliberti[5]

[1]*Division of Pulmonary and Critical Care, University of Illinois, Chicago, USA*
[2]*Department of Mycobacteriology, National Research Center of Tuberculosis and Lung Disease, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[3]*Division of Pulmonary and Critical Care, University of Emory, Atlanta, GA, USA*
[4]*Divisions of Clinical Microbiology and Infectious Diseases, Departments of Laboratory Medicine and Internal Medicine, National Taiwan University College of Medicine, Taipei, Taiwan*
[5]*Department of Health Science, University of Milano-Bicocca, San Gerardo Hospital, Via Pergolesi 33, 20900 Monza, Italy*

Correspondence should be addressed to Mehdi Mirsaeidi; mmirsae@uic.edu

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Nontuberculous mycobacteria (NTM) are ubiquitous organisms that are highly prevalent in the environment. The evidence that these organisms originate in the environment is overwhelming; and there is no significant evidence for animal-to-human or human-to-human transmission. NTM were first recognized as human pathogens in 1950s [1] and since then more than 150 NTM species have been recognized [2]. Humans may be exposed to NTM on a regular basis through showering, bathing, and other activities using water, soil, or aerosols [3]. How, when, why, and in which population NTM infection might occur are largely unknown.

The incidence rate of NTM infections appears to have increased during the last decade globally resulting in a significant mortality and morbidity [4, 5]. Studies have shown a high incidence rate of NTM in elderly population and increasing susceptibility in very elderly people. It has been estimated that NTM incidence will continue to rise due to increasing elderly population by 2050 [6]. Little is known about the diseases caused by the NTM and only limited data are available showing its prevalence and associated factors [7, 8]. Bronchiectasis, chronic lung diseases, pulmonary fibrosis, and decreased lung immunity are some of known risk factors for pulmonary NTM diseases. NTM disease has also gained increasing attention in countries with high incidence of tuberculosis due to risk of misdiagnosis with multidrug resistant tuberculosis (MDR-TB) [9]. A recently published study showed the frequency of NTM misdiagnosis in up to 30% of patients with suspected MDR-TB [10].

The disease caused by NTM forms a broad clinical spectrum from asymptomatic infection to fatal disseminated disease in human. While many of NTM patients have underlying pulmonary diseases, NTM symptoms are undifferentiated from symptoms of patients with chronic pulmonary structural diseases such as bronchiectasis. Therefore, routine screening and having high suspicion for NTM disease are highly recommended.

Diagnosis of infection by NTM is another challenging issue. The diagnosis of NTM with conventional microbiologic methods may be misleading and molecular approaches are advised [11].

Increased knowledge and understanding of this type of mycobacterial disease could improve patient care of affected persons worldwide. We also highly recommend continuing translational studies to develop precision medicine in mycobacterial diseases [12].

In this special issue, a series of original articles as well as expert reviews on NTM are published to stimulate a greater understanding of NTM infections. Suggested areas of research are included: microbiologic and molecular diagnostic tools, drug susceptibility tests, human genetic susceptibility, prevalence and incidence studies, clinical presentations, and clinical trials for antibiotic therapy. We were particularly

interested in studies describing new laboratory diagnostic tools for NTM, modalities for clinical characterization of NTM diseases in patients with and without underlying diseases, and measuring outcomes of treatment trials.

In conclusion, the incidence of NTM disease is increasing and needs urgent attention by health policy makers and international societies. Better molecular tools for rapid diagnosis of NTM, understanding of immunopathogenesis, and host pathogen interactions are needed. In the last years, we have seen an explosion in NTM researches. Consequently, we hope to see more publications from basic and traditional research to answer our questions about NTM in coming years.

Acknowledgment

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Mehdi Mirsaeidi
Parissa Farnia
Ruxana Sadikot
Po-Ren Hsueh
Stefano Aliberti

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Research Article

Specific Proteins in Nontuberculous Mycobacteria: New Potential Tools

Patricia Orduña,[1] Antonia I. Castillo-Rodal,[1] Martha E. Mercado,[1] Samuel Ponce de León,[2] and Yolanda López-Vidal[2]

[1] *Programa de Inmunología Molecular Microbiana, Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 Mexico City, DF, Mexico*
[2] *División de Investigación Clínica, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 Mexico City, DF, Mexico*

Correspondence should be addressed to Yolanda López-Vidal; lvidal@unam.mx

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Nontuberculous mycobacteria (NTM) have been isolated from water, soil, air, food, protozoa, plants, animals, and humans. Although most NTM are saprophytes, approximately one-third of NTM have been associated with human diseases. In this study, we did a comparative proteomic analysis among five NTM strains isolated from several sources. There were different numbers of protein spots from *M. gordonae* (1,264), *M. nonchromogenicum* type I (894), *M. nonchromogenicum* type II (935), *M. peregrinum* (806), and *M. scrofulaceum/Mycobacterium mantenii* (1,486) strains, respectively. We identified 141 proteins common to all strains and specific proteins to each NTM strain. A total of 23 proteins were selected for its identification. Two of the common proteins identified (short-chain dehydrogenase/reductase SDR and diguanylate cyclase) did not align with *M. tuberculosis* complex protein sequences, which suggest that these proteins are found only in the NTM strains. Some of the proteins identified as common to all strains can be used as markers of NTM exposure and for the development of new diagnostic tools. Additionally, the specific proteins to NTM strains identified may represent potential candidates for the diagnosis of diseases caused by these mycobacteria.

1. Introduction

Mycobacteria that are not members of the *Mycobacterium tuberculosis* complex (*Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium canettii*, *Mycobacterium microti,* and *Mycobacterium tuberculosis*) or leprosy are classified as nontuberculous mycobacteria (NTM). The NTM group comprises more than 150 species that are widely distributed in many different environments (http://www.bacterio.cict.fr/m/mycobacterium.html). NTM have been isolated from water, soil, air, food, protozoa, plants, animals, and humans [1, 2]. Although most NTM are saprophytes, approximately one-third of NTM have been associated with human diseases [3, 4].

NTM infection is relatively uncommon and they are more frequently observed in immunocompromised individuals [5]. However, the rate of disease caused by NTM in individuals without abnormalities that would predispose them to infection appears to be increasing [6]. The development of new epidemiological tools, which are based on molecular techniques, has allowed an increased diagnosis of NTM disease and an increase in the identification of NTM species that are responsible for disease [4, 7].

Person to person transmission of NTM has not been reported, and it is generally accepted that NTM infections are acquired from environmental sources (water, soil) and that they are responsible for many nosocomial infections and occupational diseases. Some authors have indicated that NTM infections were directly related to exposure to contaminated water as they demonstrated to isolate the same clones from the water and patients [1, 8, 9]. Nevertheless, there is also a correlation between NTM-contaminated metallic fluids and aerosols with hypersensitive pneumonitis, asthma, and bronchitis observed in metallurgical workers [10]. The NTM species associated with human diseases have been isolated from the lungs, skin, and other soft tissues. Pulmonary infection is the most common disease manifestation and is associated with an increased age of the patient, while the skin

and soft tissue diseases have not been associated with age or gender [4, 7].

Previous exposure to NTM has been proposed as one of the main causes of reduced efficacy of BCG vaccination against pulmonary tuberculosis infection [11, 12]. Black et al. demonstrated that young adults living in the northern part of Malawi are immunologically reactive to NTM antigens prior to vaccination with BCG [13]. In addition, numerous animal model studies have provided evidence that exposure to NTM before the BCG vaccine application may modulate the immune response that is induced by the BCG vaccine [14, 15]. Mendoza-Coronel et al. demonstrated that *Mycobacterium avium* may be implicated in the induction of immune tolerance mechanisms, which could impact the T cell response that is induced by BCG vaccination [16].

Additionally, exposure to NTM is responsible for the low predictive value of the purified protein derivative (PPD) test. The PPD or the Mantoux reaction is the only available diagnostic tool to identify latent tuberculosis. Ideally, the PPD test could be used as a marker for tuberculosis infection, but unfortunately there is cross-reactivity between NTM infection and BCG vaccination [17–19]. Black et al. described that the IFN-gamma response to *M. tuberculosis* purified protein derivative (PPD) after vaccination was lower in individuals who reacted strongly to NTM antigens [13].

The increase of NTM infections, the variability of BCG vaccine protection, and the lack of a diagnostic tool for *Mycobacterium* species infection make it necessary to identify novel proteins that can potentially be used in the development of new vaccines and diagnostic tools against tuberculosis infection [20].

In this study, we did a comparative proteomic analysis of five different NTM strains: three were isolated from the pump water in Mexico City (*Mycobacterium gordonae*, *Mycobacterium nonchromogenicum* type II, and *Mycobacterium peregrinum*), one was isolated from human pulmonary infection (*Mycobacterium scrofulaceum/Mycobacterium mantenii*), and one was purchased (*M. nonchromogenicum* type I, ATCC 1953). We identified proteins common to all strains and specific proteins to each NTM strain. Some of the proteins that were common between the strains could be used as markers of NTM exposure.

2. Materials and Methods

2.1. Bacterial Strains. Five strains of NTM were used in this study: three were isolated from the pump water in Mexico City as previously described by Castillo-Rodal et al. (*M. gordonae*, *M. nonchromogenicum* type II, and *M. peregrinum*) [2], one was isolated from human pulmonary infection (*M. scrofulaceum/M. mantenii*), and one was purchased (*M. nonchromogenicum* type II, *ATCC 1953*) (Table 1). To ensure that all NTM strains were in the metabolically activated state, growth curves of each strain in Sauton medium were determined (Figure 1). All strains were grown to the mid-logarithmic phase (10 days for *M. nonchromogenicum* type I, 20 days for *M. nonchromogenicum* type II, 11 days for *M. gordonae*, 8 days for *M. peregrinum*, and 17 days for *M. scrofulaceum/M. mantenii*) at 37°C with shaking and then were harvested by centrifugation, washed three times, and suspended in sterile deionised water.

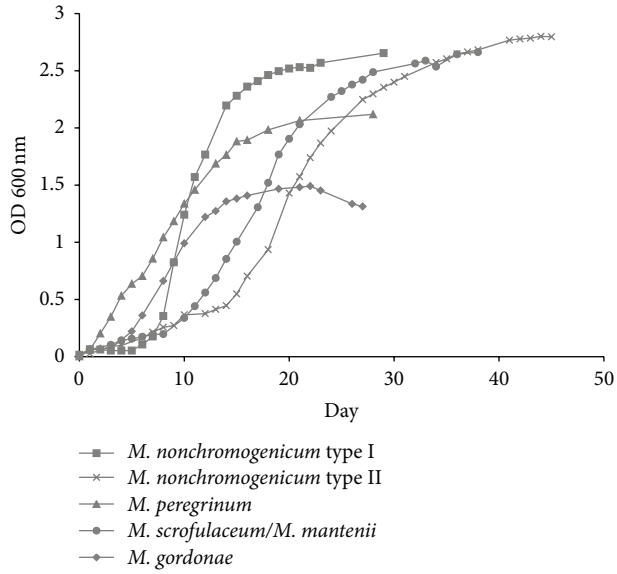

Figure 1: Representative growth curves of NTM strains included in this study. Growth curves were realised on Sauton medium at 37°C by duplicate.

2.2. Sample Preparation and 2D-PAGE. Cellular proteins were obtained by sonicating the bacteria (Ultrasonic Processor, Cole Parmer Corporation, USA) in the presence of protease inhibitors (10 mM PMSF, 1 mM EDTA; cycles: 1 min ON/1 min OFF) at 4°C. For 2D-PAGE, approximately 80 μg of protein for the analytical gels or 100–150 μg of protein for the preparative gels was solubilised, denatured, and reduced in sample buffer (4% CHAPS, 9 M urea, 70 mM l-dithiothreitol (DTT), 0.001% bromophenol blue, and 0.1% 3–10 ampholyte) and was used to rehydrate 11-cm, pH 4–7 IPG strips (ReadyStripTM, IPG strips, Bio-Rad, USA). IEF was carried out on a Multiphor II (Amersham Biosciences, UK) until 52,000 VH at 17°C. Prior to separation in the second dimension, IPG strips were equilibrated in a solution containing 6 M urea, 30% (v/v) glycerol, 50 mM Tris-base pH 8.8, and 2% (w/v) SDS. The strips were equilibrated first for 15 min with 70 mM DTT and then for 15 min with 120 mM iodoacetamide. The second-dimension electrophoresis was performed using a 12.5% polyacrylamide gel (Hoefer SE-600, Amersham Biosciences, UK) with a voltage gradient of 50–150 V for approximately four hours. Once fixed, the proteins were silver-stained and the gel images were then captured in a digital format for analysis (Molecular Imager GS-800TM Calibrated Densitometer, Bio-Rad, USA).

2.3. Gel Analysis and Spot Selection. 2D-PAGE was performed twice for each strain, and independent cultures were utilised to eliminate technical variation. Gel analysis was performed using PDQuest-Advanced 2D Analysis V8.0 (Bio-Rad, USA). A master image gel (MIG) was integrated with the two duplicate gels of each strain and was utilised for comparison. To estimate and overcome technical variations

Table 1: Description of the NTM strains used in this study.

Strain	Source	Growth
M. gordonae	Superficial water	Slow
M. nonchromogenicum type I (ATCC 19530)	Soil	Slow
M. nonchromogenicum type II	Superficial water	Slow
M. peregrinum	Water distribution system Mexico City	Fast
M. scrofulaceum/M. mantenii	Human pulmonary infection	Slow

Table 2: Spots identified in the 2D-PAGE gels of cellular proteins from various NTM strains.

Strain	Total spots in master image gel	CV (%)*
M. gordonae	1,264	0.2
M. nonchromogenicum type I	894	5.5
M. nonchromogenicum type II	935	0.07
M. peregrinum	806	0.02
M. scrofulaceum/M. mantenii	1,486	0.01

*Coefficient of variation: data were normalised according to the total density of the gel image.

between replicates, the spots were quantified for all of the gels. The variation in the coefficients was calculated using a previously described method [21, 22]. The spot intensity values were normalised to the total pixel count for each gel. Ten common spots for all strains, 7 specific spots for *M. gordonae* strain, and 2 specific spots for *M. arupense*, *M. nonchromogenicum,* and *M. peregrinum* strains were selected and identified by mass spectrometry. Spot selection criteria included the following: the spot was well defined, the spot had a high intensity, and the spot locations were diversely spaced throughout the gel.

2.4. Protein Identification. The selected spots were identified by a previously described protocol [21, 22]. Protein identification was performed using a 3200 QTRAP hybrid tandem mass spectrometer (3200 QTRAP, Applied Biosystems, USA) equipped with a nanoelectrospray ion source (NanoSpray II) and a MicroIonSpray II head. Proteins were identified based on their MS/MS spectra datasets using the MASCOT search algorithm (Version 1.6b9, Matrix Science, London, UK). A BLAST search was conducted comparing the sequences to the *M. tuberculosis* complex and Eubacteria kingdom sequences of the National Center for Biotechnology Information (NCBI) nonredundant database (NCBI nr20070623).

3. Results and Discussion

All of the NTM strains that were used in this study were slow growth strains, except for *M. peregrinum* that was a fast growth strain. We determined that the proteins did not have evidence of degradation by a polyacrylamide gel electrophoresis (data not shown).

The cell fractions from the five NTM strains in the mid-logarithmic phase were isolated and then analysed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). We identify different number of protein spots from *M. gordonae* (1,264), *M. nonchromogenicum* type I (894), *M. nonchromogenicum* type II (935), *M. peregrinum* (806), and *M. scrofulaceum/M. mantenii* (1,486) strains, respectively (Figure 2, Table 2). The distribution of the proteins by MM and pI was similar between the five NTM strains analysed (Figure 3).

Comparison of the protein profiles showed that 141 proteins were present in all NTM strains studied and approximately 80% of the proteins were shared between two or more strains (named common proteins). We also identified proteins present in only one NTM strain (named specific proteins). *M. gordonae* was observed to have the highest percentage of specific proteins, with 24% (Table 3).

A total of 23 proteins of the five NTM strains studied were selected for their identification by MS-based technologies. Spot selection criteria included the following: the spot was well defined, the spot had a high intensity, and the spot locations were diversely spaced throughout the gel.

We identify ten common proteins to all NTM strains studied (Table 4). Four of these proteins corresponded to informational pathways (RNA polymerase beta subunit, 50S ribosomal protein L7/L12, diguanylate cyclase, and DNA polymerase III), three were related to intermediary metabolism and respiration (adenylate kinase, probable aldehyde dehydrogenase, and enolase), two were identified as conserved hypothetical proteins (WAG31, Rv3075c), and one was related to lipid metabolism (short-chain dehydrogenase/reductase). The 50S ribosomal protein L7/L12, adenylate kinase, enolase, and two hypothetical proteins (WAG31 and Rv3075c) have been previously identified in the proteome of *M. tuberculosis* and *M. bovis* BCG showing that these proteins are shared with *M. tuberculosis* complex species [23–25]. Interestingly, the ribosomal protein L7/L12 and hypothetical protein WAG31 have been found in the proteome of PPD *M. tuberculosis* and/or PPD *M. bovis* [26]. Furthermore, the 50S ribosomal protein L7/L12 was described as an immunogenic protein in the BCG Mexico strain suggesting that this protein may be part of the cross-reaction observed between BCG vaccination and NTM exposure [27]. The five remaining proteins were identified for the first time in a mycobacterial proteome in this study.

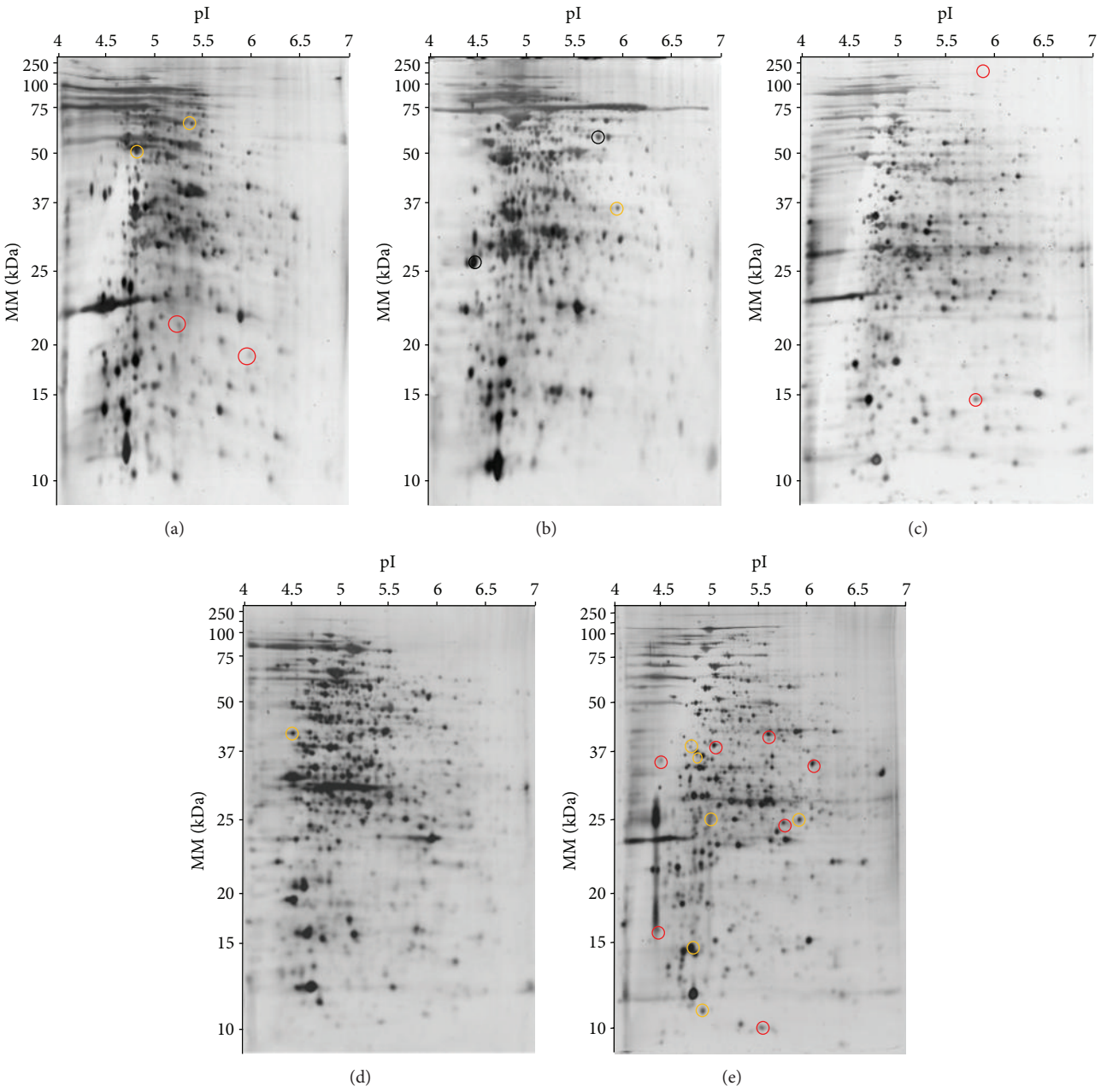

Figure 2: Representative 2D-PAGE of NTM cellular proteins of (a) *M. nonchromogenicum* type I, (b) *M. peregrinum*, (c) *M. nonchromogenicum* type II, (d) *M. scrofulaceum/M. mantenii*, and (e) *M. gordonae*. Eighty micrograms of cell proteins was isoelectrically focused in IPG strips (pH 4–7) and run on sodium dodecyl sulphate (SDS) 12.5% polyacrylamide gel. Gels were silver-stained and analysed with PDQuest 2D Analysis V8.0 (Bio-Rad, USA). The yellow and red circles were used to identify common proteins to all NTM strains and specific proteins to each NTM strain, respectively, by MS-based techniques.

The proteins of NTM that were identified in this study and have been previously described in the *M. bovis* BCG proteome can explain the cross-reactivity observed between BCG vaccination and NTM exposition. For example, the 50S ribosomal protein L7/L12, which we determined to be present in all proteomes of NTM strains studied, has been previously described as an immunogenic protein that upregulated the expression of the mannose receptor, CD80, CD86, and MHC class II molecules and it is associated with mycobacterial virulence [28, 29].

Moreover, the proteins that have previously been identified in the PPD *M. tuberculosis* proteome and that were identified in the NTM proteome, such as 50S ribosomal protein L7/L12, adenylate kinase, and hypothetical protein WAG31, may be the cause of the low predictive value of the PPD test to diagnose *M. tuberculosis* infection [26]. In fact,

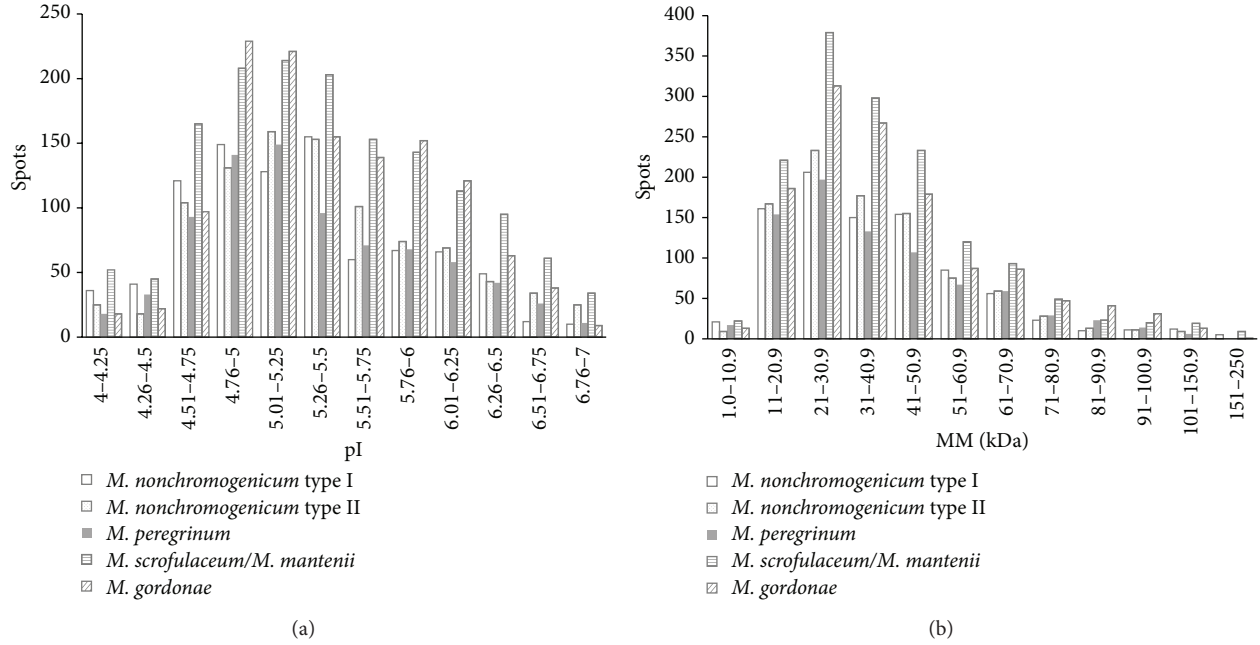

Figure 3: Distribution of proteins by isoelectric point (a) and molecular mass (b). Gel analysis was performed using PDQuest-Advanced 2D Analysis V8.0 (Bio-Rad, USA).

Table 3: Common and specific proteins in the NTM strains studied.

Strain	Common proteins* (%)	Specific proteins** (%)
M. gordonae	963 (76)	301 (24)
M. nonchromogenicum type I	701 (81)	166 (19)
M. nonchromogenicum type II	723 (81)	171 (19)
M. peregrinum	671 (83)	135 (17)
M. scrofulaceum/M. mantenii	1,231 (83)	255 (17)
Proteins common to all strains	**141**	

*Common proteins were defined as proteins that were present in at least two NTM strains.
**Specific proteins were defined as proteins present only in one NTM strain.

the 50S ribosomal protein L7/L12 is an immunogenic protein that induces a strong delayed-type hypersensitivity reaction [28], while the hypothetical protein WAG31 is involved in peptidoglycan synthesis and it has an important role in wall synthesis, cell growth, and cell division of mycobacteria [30].

Thirteen specific proteins from the NTM strains were identified by MS-based technologies (Table 4). We identified two specific proteins to *M. arupense* (deoxyuridine 5′-triphosphate nucleotidohydrolase and probable 3-hydroxylthioester dehydratase), *M. nonchromogenicum* (conserved hypothetical protein, catalase-peroxidase), and *M. peregrinum* (mannose-binding lectin, inositol-5-monophosphate dehydrogenase) and seven specific proteins to *M. gordonae* (probable cold shock protein A, putative mannose-specific lectin precursor, superoxide dismutase, malate dehydrogenase, F420-dependent glucose-6-phosphate dehydrogenase, luciferase-like protein, and hypothetical protein SKA58_12772). Four proteins identified as specific to *M. gordonae* (probable cold shock protein A, superoxide dismutase, malate dehydrogenase, and F420-dependent glucose-6-phosphate dehydrogenase) have also been identified in the proteomes of *M. tuberculosis* and/or *M. bovis* BCG [23–25]. Moreover, the F420-dependent glucose-6-phosphate dehydrogenase and superoxide dismutase proteins have been identified in PPD *M. avium* and *M. immunogenum* proteomes, respectively [26, 31]. Interestingly, Dong et al. described that the superoxide dismutase has one immunodominant epitope for cytotoxic T lymphocytes, which are implicated in protective immunity against tuberculosis [32]. These findings suggest that these proteins, which were identified as specific to *M. gordonae*, are shared with other NTM strains and these may be the cause of cross-reactivity against *M. tuberculosis*, *M. bovis* BCG, and/or other NTM.

On the other hand, we identified three specific proteins to *M. gordonae* (putative mannose-specific lectin precursor, luciferase-like protein, and hypothetical protein SKA58_12772) and one specific protein to *M. peregrinum* (mannose-binding lectin) that have not been previously described in any proteomic analysis of mycobacterial strains and did not align with any protein sequences in the *M. tuberculosis* complex database (data not shown). The function of these proteins is not well defined; however, we have known

Table 4: Proteins identified by sequencing in NTM strains.

Protein	Name	Function	Gene name	Peptides ID	Global score[a]	Sequence coverage %	Reference sequence	FC[b]
		Proteins identified in *M. nonchromogenicum* type I						
1	Deoxyuridine 5′-triphosphate nucleotidohydrolase	Involved in the biosynthesis of thymidylate. This enzyme is involved in nucleotide metabolism Catalytic activity: dUTP + H(2)O = dUMP + pyrophosphate	*dut*	2	109	11	*M. gilvum*	7
2	Probable 3-hydroxyl-thioester dehydratase	Unknown	*htdZ*	3	65	23	*M. tuberculosis* H37Rv	7
		Proteins identified in *M. nonchromogenicum* type II						
1	Conserved hypothetical protein	Unknown	—	4	33	27	*M. tuberculosis* complex	10
2	Catalase-peroxidase KATG	Multifunctional enzyme that exhibits a catalase, a broad-spectrum peroxidase, and peroxynitritase activities may play a role in the intracellular survival of mycobacteria within macrophages for the protection against reactive oxygen and nitrogen intermediates produced by phagocytic cells	*katG*	4	115	6	*M. avium* subsp. *paratuberculosis* K-10	0
		Proteins identified in *M. peregrinum*						
1	Mannose-binding lectin	Unknown	*MSMEG3662*	4	114	30	*M. smegmatis*	—
2	Inositol-5-monophosphate dehydrogenase	IMPDH catalyses the NAD-dependent oxidation of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP)	*PRK05567*	3	125	6	*M. vanbaalenii*	7
		Proteins identified in *M. gordonae*						
1	Probable cold shock protein A	Possibly involved in the acclimation to cold temperatures (the production of the protein is thought to be induced at low temperatures)	*cspA*	1	34	14	*M. tuberculosis* H3Rv	0
2	Putative mannose-specific lectin precursor	Lysine domain, found in a variety of enzymes involved in bacterial cell wall degradation. This domain may have a general peptidoglycan binding function	*MAB2373*	2	72	18	*M. abscessus*	10
3	Superoxide dismutase	Destroys toxic radicals that are normally produced within the cells Catalytic activity: 2 peroxide radicals + 2H(+) = O_2 + H_2O_2	*sodA*	2	81	24	*M. gordonae* and *M. assiaticum*	0
4	Malate dehydrogenase	Involved in the conversion of malate to oxaloacetate Catalytic activity: (S)-malate + NAD^+ = oxaloacetate + NADH	*mdh*	5	197	14	*M. marinum*	7
5	Luciferase-like protein	Energy production and conversion	*Mmcs 0532*	3	151	9	*Mycobacterium* sp.	—

Table 4: Continued.

Protein	Name	Function	Gene name	Peptides ID	Global score[a]	Sequence coverage %	Reference sequence	FC[b]
6	F420-dependent glucose-6-phosphate dehydrogenase	Catalyses oxidation of glucose-6-phosphate to 6-phosphogluconolactone using coenzyme F420 (hydroxy-5-deazaflavin derivative) as the electron acceptor	*fgd*	3	148	10	*M. avium* (*M. ulcerans, M. marinum, M. chelonae*)	7
7	Hypothetical protein SKA58_12772	Predicted phosphohydrolases	*SKA58 12772*	1	52	3	*Sphingomonas* sp. SKA58	—
		Common proteins to all NTM strains						
1	RNA polymerase beta subunit	Catalyses the transcription of DNA into RNA using the four ribonucleoside triphosphates Catalytic activity: N nucleoside triphosphate = N diphosphate + [1](N)	*rpoB*	2	30	72	*M. tuberculosis*	2
2	50S ribosomal protein L7/L12	Involved in translation mechanisms. Thought to be the binding site for several of the factors involved in protein synthesis and appears to be essential for accurate translation	*rplL*	4	115	35	*Mycobacterium* sp. (*M. smegmatis* and *M. vanbaalenii*)	2
3	Adenylate kinase	This enzyme is essential in intracellular nucleotide metabolism; in addition, it has been found to act as both a nucleoside mono- and diphosphate kinase suggesting it may have a role in RNA and DNA biosynthesis Catalytic activity: ATP + AMP = ADP + ADP	*adk*	1	53	6	*M. leprae*	7
4	Short-chain dehydrogenase/reductase SDR	Involved in the fatty acid biosynthesis pathway (first reduction step) (mycolic acid biosynthesis)	*fabG*	1	47	3	*Mycobacterium* sp.	1
5	Diguanylate cyclase	Catalytic activity: 2 GTP ↔ 2 diphosphate + cyclic di-GMP	*Pat1 0480*	1	52	2	*Pseudoalteromonas atlantica* T6c	2
6	Conserved hypothetical protein WAG 31	Unknown	*wag31*	3	112	12	*M. tuberculosis* H37Rv, *M. avium* subsp. *paratuberculosis* K-10	10
7	Conserved hypothetical protein	Unknown	*Rv3075c*	2	65	4	*M. tuberculosis* H37Rv	10
8	Probable aldehyde dehydrogenase	Interconversion aldehyde and acid. Catalytic activity: an aldehyde + NAD+ + H_2O = an acid + NADH	*Rv0458*	2	41	4	*M. tuberculosis, M. bovis, M. leprae*	7
9	Enolase	Catalysing the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate. It is essential for the degradation of carbohydrates via glycolysis Catalytic activity: 2-phospho-D-glycerate = phosphoenolpyruvate + H_2O	*eno*	4	77	10	*M. bovis* AF2122/97	7

Table 4: Continued.

Protein	Name	Function	Gene name	Peptides ID	Global score[a]	Sequence coverage %	Reference sequence	FC[b]
10	DNA polymerase III (beta chain) DNAN	DNA polymerase III is a complex, multichain enzyme responsible for most of the replicative synthesis in bacteria. This DNA polymerase also exhibits 3′ to 5′ exonuclease activity. The beta chain is required for initiation of replication once it is clamped onto DNA; it slides freely (bidirectional and ATP-independent) along duplex DNA Catalytic activity: N-deoxynucleoside triphosphate = N-diphosphate + (DNA)n	*dnaN*		44	11	*M. tuberculosis*	2

[a]According to Mascot search results, protein scores >25 are significant ($P < 0.05$).

[b]FC: functional category. 0: virulence, detoxification, and adaptation; 1: lipid metabolism; 2: information pathways; 3: cell wall and cell processes; 4: stable RNAs; 5: insertion sequences and phages; 6: Pe/PPE; 7: intermediary metabolism and respiration; 8: unknown; 9: regulatory protein; 10: conserved hypothetical (from BCGList World-Wide Web Server http://genolist.pasteur.fr/BCGList).

that the lectins can play a major role in the interaction with human cells [33, 34]. These proteins could be utilised to discriminate between NTM and *Mycobacterium tuberculosis* complex infections. However, the presence of these proteins in other NTM species that were not included in this study must be determined by specific assays.

4. Conclusions

NTM are a group of environmental bacteria that are considered to be potentially pathogenic both to immunocompetent and immunocompromised individuals. Exposure to these bacteria is a factor involved in the variability of the protective efficacy of the BCG vaccine because of cross-reactive antigens that are common between NTM and strains of the *M. tuberculosis* complex. Additionally, exposure to NTM is responsible for the low predictive value of the PPD test due to cross-reactivity with similar antigens.

In this study, we described the protein profiles of five NTM strains. Analysis of these profiles indicated the presence of proteins that were both common to and specific to each NTM strain. The common proteins can be utilised as markers of prior exposure to NTM. They can potentially provide a more specific diagnosis with a decreased number of false positives. Also, the proteins that were identified in the proteome of the NTM strains studied can be useful in the development of new diagnostic tools and may help explain the cross-reactivity between the PPD test and the BCG vaccination as described above.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Research Article

Beninese Medicinal Plants as a Source of Antimycobacterial Agents: Bioguided Fractionation and *In Vitro* Activity of Alkaloids Isolated from *Holarrhena floribunda* Used in Traditional Treatment of Buruli Ulcer

Achille Yemoa,[1] Joachim Gbenou,[2] Dissou Affolabi,[3] Mansourou Moudachirou,[2] André Bigot,[1] Séverin Anagonou,[3] Françoise Portaels,[4] Anandi Martin,[5] and Joëlle Quetin-Leclercq[6]

[1] *Unité de Formation et de Recherche en Pharmacie, Faculté des Sciences de la Santé (FSS), Université d'Abomey Calavi (UAC), 04 BP 494 Cotonou, Benin*
[2] *Laboratoire de Pharmacognosie et des Huiles Essentielles (LAPHE), Faculté des Sciences de la Santé (FSS) and Faculté des Sciences et Techniques (FAST), Université d'Abomey Calavi (UAC), 01 BP 188 Cotonou, Benin*
[3] *Laboratoire de Référence des Mycobactéries (LRM), Centre National Hospitalier de Pneumo-Phtisiologie (CNHPP), 01 BP 817 Cotonou, Benin*
[4] *Department of Biomedical Sciences, Institute of Tropical Medicine (IMT), Nationalestraat 155, 2000 Antwerpen, Belgium*
[5] *Laboratory of Microbiology, Department of Biochemistry and Microbiology, Faculty of Sciences, Ghent University, K.L. Ledeganckstraat 35, 9000 Gent, Belgium*
[6] *Pharmacognosy Research Group, Louvain Drug Research Institute (LDRI), Université Catholique de Louvain (UCL), B1 7203 Avenue E. Mounier 72, 1200 Bruxelles, Belgium*

Correspondence should be addressed to Achille Yemoa; ayemoa@yahoo.fr

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Buruli ulcer (BU) imposes a serious economic burden on affected households and on health systems that are involved in diagnosing the disease and treating patients. Research is needed to find cost-effective therapies for this costly disease. Plants have always been an important source of new pharmacologically active molecules. Consequently we decided to undertake the study of plants used in traditional treatment of BU in Benin and investigate their antimycobacterial activity as well as their chemical composition. Extracts from forty-four (44) plant species were selected on account of reported traditional uses for the treatment of BU in Benin and were assayed for antimycobacterial activities. Crude hydroethanolic extract from aerial parts of *Holarrhena floribunda* (G. Don) T. Durand and Schinz was found to have significant antimycobacterial activity against *M. ulcerans* (MIC = 125 μg/mL). We describe here the identification of four steroidal alkaloids from *Mycobacterium ulcerans* growth-inhibiting fractions of the alkaloidal extract of the aerial parts of *Holarrhena floribunda*. Holadysamine was purified in sufficient amount to allow the determination of its MCI (=50 μg/mL). These results give some support to the use of this plant in traditional medicine.

1. Introduction

Buruli ulcer (BU), caused by the environmental organism *Mycobacterium ulcerans* and characterized by necrotizing skin and bone lesions, poses important public health issues as the third most common mycobacterial infection in humans [1]. The disease has become substantially more frequent over the past decade, particularly around the Gulf of Guinea, and has been detected or suspected in at least 31 countries. Clinical diagnosis of BU disease should be confirmed by PCR, as recommended by the World Health Organization (WHO), and case patients should be treated with rifampin/streptomycin

daily for 8 weeks (therapy available since 2004), combined, if necessary, with surgery.

In Benin, sociocultural believes and practices strongly influence the health-seeking behaviours of people affected by BU. The first recourse is often traditional treatment. Most of the components in the traditional treatment belong to the plant kingdom [2]. Plants provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. Recently we carried out an ethnobotanical survey involving seventeen traditional practitioners within the Ouinhi community in the Zou Department (Benin). We noted that about forty-nine different plants were used for the traditional treatment of BU. Different parts of these plants were included in various pharmaceutical forms for internal or external use [2]. We realized a screening of 44 plant extracts used in traditional medicine to treat BU [2, 3]. Results showed that crude hydroethanolic extract of *Holarrhena floribunda* was effective in inhibiting the growth of *Mycobacterium ulcerans* (MIC 125 μg/mL) and worth further investigations.

Holarrhena floribunda (G. Don) T. Durand and Schinz grows as a shrub or tree up to 25 m tall, with a stem diameter of up to 30 cm. Its fragrant flowers feature a white corolla. Fruit is pale grey to dark brown with paired follicles, each up to 60 cm long. This species is known as four synonyms: *Rondeletia floribunda* G. Don, *Holarrhena africana* A. DC., *Holarrhena wulfsbergii* Stapf, *Holarrhena ovata*. In the Republic of Benin, the common names of this plant are: Fon: "kpakpatoun" [2]; Yoruba and Nago: "ako ire," "ire Ibedji;" Mina: "gaoti" [4]. Hoyer et al. [5] in 1978 isolated from the bark of the trunk a steroidal alkaloid called holarrhesine, but no activity was reported for this compound. They also isolated conessine from the bark of the root. A chemical study of the leaves of *H. floribunda* achieved by Janot team in 1959 led to the isolation of other new alkaloids including holaphylline and holaphyllamine, while other alkaloids were isolated later. Figure 1 shows the structures of the 9 chemical compounds isolated from *H. floribunda*: holaphylline, holaphyllamine, holamine, holaphyllinol, holaphyllidine, holadysamine, holarrhesine, conessine, and progesterone [6–9]. Phytochemical investigations on this plant have so far led to the identification of a crude *in vitro* active alkaloid extract on *M. tuberculosis* (MCI = 0.075 g/L) and other microorganisms [10, 11].

Here we describe compounds identified from bioactive fractions of the extract and evaluation of the inhibitory effect of one of them on the growth of *M. ulcerans*.

2. Materials and Methods

2.1. Experimental

2.1.1. Plant Materials. Plant species was collected and identified by a botanist from the National Herbarium of Benin and voucher specimens (Yemoa 06) are deposited at the same herbarium.

2.1.2. Preparation of Crude Hydroethanolic Extracts. Dried plants were ground to a powder with a pulverizator (National Mixer Grinder Mx-119N, Japan). 50 g of powder was then macerated 48 h (at room temperature) in 70% ethanol in a 1/10 (w/v) ratio. The material was filtered through a Millipore filter of 0.2 μm (Acrodisc, USA). The filtrate was concentrated under reduced pressure at less than 40°C using a rotary evaporator (Buchi Rotavapor R-200/205, Switzerland) to obtain a crude residue.

2.1.3. Fractionation of Crude Extracts and Isolation of Active Constituents. Crude hydroethanolic extract was fractionated on silica gel 60 (0,063–0,200 mm Merck, Germany) by atmospheric pressure liquid chromatography eluting with solvents of increasing polarity, namely, hexane, dichloromethane, ethyl acetate, and water, yielding 4 fractions (F1, F2, F3, and F4). Of these, fraction F2 (dichloromethane) was found to cause growth inhibition of *M. ulcerans* and was, as a result, selected for further work. This fraction was monitored using TLC plates (TLC silica gel $60F_{254S}$, Merck) and led to characterize the presence of alkaloids (toluene : ethyl acetate : diethylamine: [7 : 2 : 1], detection-Dragendorff's spray reagent). We then performed a more specific extraction to obtain an enriched alkaloid extract (Figure 2).

We therefore proceeded to fractionation of this enriched extract by Atmospheric Pressure Liquid Chromatography (APLC) and Medium Pressure Liquid Chromatography (MPLC) followed by gel filtration on Sephadex LH20 to purify this enriched alkaloid fraction.

Atmospheric Pressure Liquid Chromatography (APLC). Alkaloids enriched extract was repeatedly fractionated on silica gel 60 (0,063–0,200 mm, Merck, Germany) eluting with solvents of increasing polarity (mixture of CH_2Cl_2/MeOH 50 : 50 to 100% MeOH followed by MeOH : H_2O 90 : 10 and MeOH : acetic acid 90 : 10). Fractions were monitored by TLC (toluene : ethyl acetate : diethylamine : [7 : 2 : 1], detection-Dragendorff's spray reagent) and similar fractions were combined and concentrated in vacuo. Prepurified fractions obtained by APLC were rechromatographied by MPLC.

Medium Pressure Liquid Chromatography (MPLC). MPLC was performed on glass columns packed with LiChroprep Si 60 (15–25 μm) from Merck with a mobile phase composed of CH_2Cl_2/MeOH 50 : 50 to 100% MeOH. Prepurified fractions obtained were monitored by TLC (toluene : ethyl acetate : diethylamine: [7 : 2 : 1], detection-Dragendorff's spray reagent) and similar fractions were combined and concentrated in vacuo followed by gel filtration on Sephadex LH20 (MeOH). Five different purified fractions were obtained and analyzed by high pressure liquid chromatography coupled to mass spectroscopy (HPLC-MS or LC-MS) (Thermo Scientific Accela LC Systems, orbitrap).

LC-MS. High pressure liquid chromatography coupled to a diode array detection and mass spectrometry with positive electrospray ionization (HPLC-ESI-MS^n) was employed to rapidly separate and identify the constituents in these five purified fractions. The LC MS/MS system consisted in a Thermo Accela pump, autosampler, photodiode array detector, and Thermo Scientific LTQ orbitrap XL mass spectrometer.

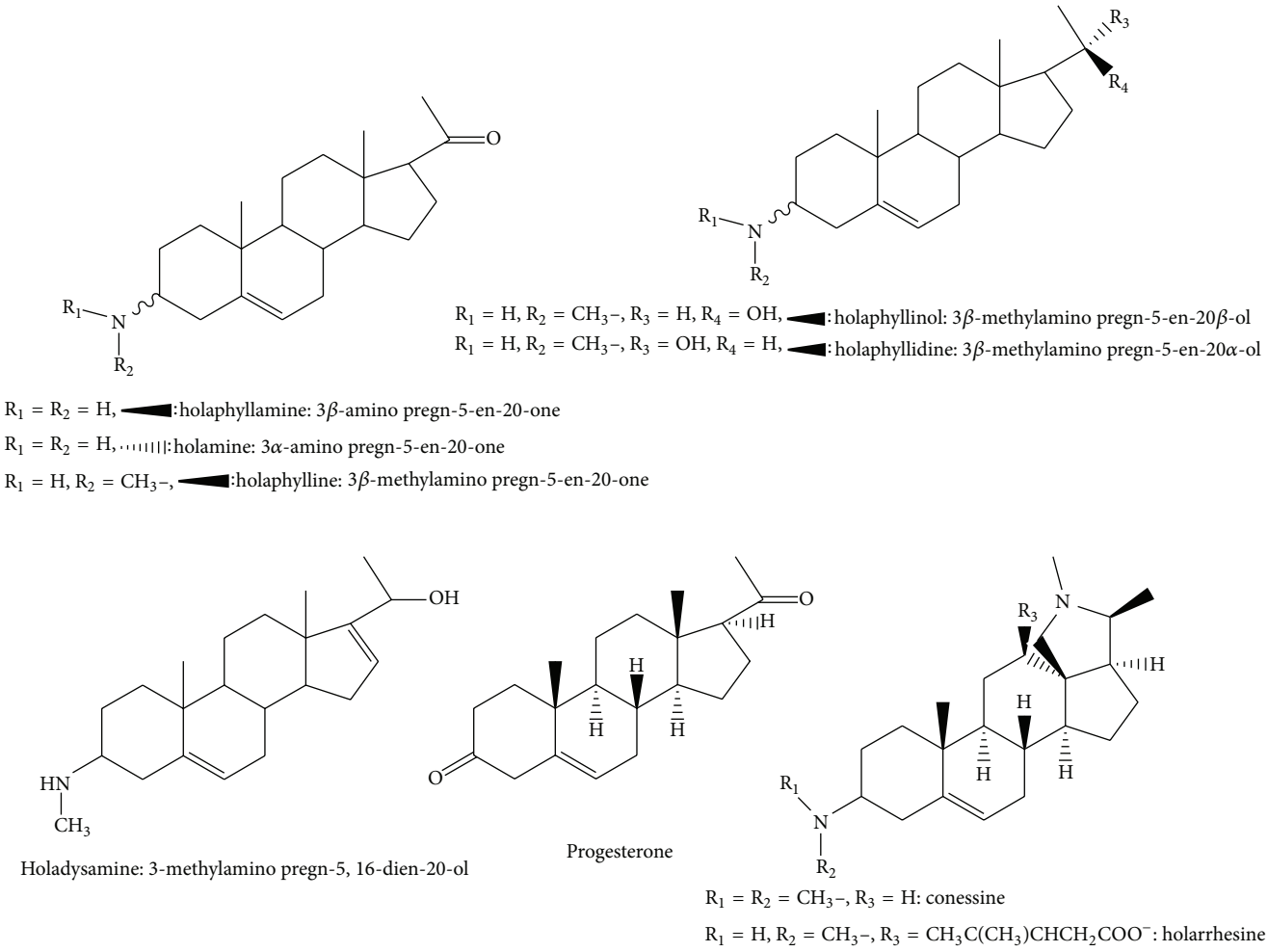

Figure 1: Structures of chemical compounds isolated from *H. floribunda*.

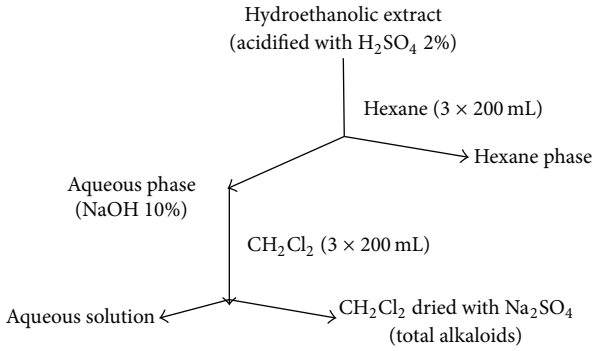

Figure 2: Scheme for preparation of alkaloids enriched extract.

Separation was performed using an analytical RP-C18 Lichrospher-100 column (250 × 4 mm, particle size 5 μm) with a gradient using acetonitrile and 0, 05% trifluoroacetic acid (TFA) aqueous solution as the mobile phase. The gradient used starts at 90% aqueous solution and 10% acetonitrile, going to a plateau of 100% acetonitrile in 25 min. These conditions are held for 15 min before returning to the initial conditions. The gradient was linear and the flow rate was 0.4 mL/min. The injection volume was 10 μL; the column temperature was 30°C.

High-resolution MS was measured with ESI source in the positive mode. The following inlet conditions were applied: capillary temperature 275°C, capillary voltage 35 V, tube lens 110 V, sheath gas flow 8 u.a, auxiliary gas flow 0 u.a, and sweep gas flow 0 u.a. Data acquisition and processing were performed with Xcalibur software version 2.0.7.

NMR. NMR spectra (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, and ^{1}H-^{13}C HSQC) were recorded using a Bruker-300, 300 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shifts were expressed in ppm (δ) using TMS (tetramethylsilane) (Aldrich Sigma, Germany) as reference. For NMR analysis, the sample was dissolved in 400 μL of $CDCl_3$-MeOD (2 : 1) and transferred to a NMR tube.

3. Results and Discussion

Our previous investigations allowed determining that the hydroethanolic extract of *H. floribunda* inhibited growth

of *M. ulcerans* with a MIC value of 125 μg/mL using the resazurin microtiter assay (REMA) [3]. The CH_2Cl_2 fraction of this extract also inhibited growth of *M. ulcerans* with a MIC value of 125 μg/mL and contained alkaloids. Fractionation of an enriched alkaloid extract (MIC = 62.5 μg/mL) was undertaken as described in the Experimental Section, allowing the identification of four different alkaloids in five different fractions and isolation of one of them with high purity. The structure of the major compound was identified as holadysamine (3-methyl amino pregna5,16-dien-20-ol) by MS and NMR [8]. LC-MS was used to identify alkaloids in the other fractions, named compounds A, B, and C, and gave molecular formula of, respectively, $C_{22}H_{37}ON$ (MW: 331.29), $C_{21}H_{33}ON$ (MW: 315.26), and $C_{23}H_{37}ON$ (MW: 343.29). According to bibliographic data on compounds isolated from *H. floribunda* [7, 8], they could correspond to holaphyllinol (MW: 331.29), holamine, or holaphyllamine (MW: 315.26), N,N-dimethyl holamine, N-dimethyl holaphyllamine or methyl 6 holaphylline (MW: 343.29).

HPLC-ESI-MS/MS allowed us to show that compound A could correspond to holaphyllinol and B to holamine or holaphyllamine while C seems to be a new compound. On the basis of the analysis of spectroscopic data, we observed the presence of a fragment at *m/z* 326 ($[M\text{-}H_2O+H]^+$) and the loss of a water molecule in the MS/MS fragmentations of C allowing us to propose an alcoholic and not a ketonic function on C20 (Figure 3).

Further studies must be performed to confirm these chemical structures. It could not be done because of the lack of compound.

Fraction containing pure holadysamine was found to be more active (MIC = 50 μg/mL) than fractions containing A, B, or C or the crude hydroethanolic extract (125 μg/mL) but less active than the reference rifampicin (MIC = 2 μg/mL). This low activity could not totally explain the use of this plant in traditional treatment but as this plant is used in mixtures with other plants; the various compounds present in these plant extracts can act synergistically as it is the case with the antibiotics ethambutol, clarithromycin, and rifampicin [12]. Rastogi and Labrousse in their study showed that the use of these antibiotics in combination results in increased bactericidal effects compared to drugs used alone or in combination with two of them.

4. Conclusions

Our results show that extracts of aerial parts of *H. floribunda*, used by traditional healers to treat BU, exhibited significant *in vitro* antimycobacterial activities. Four active alkaloids have been identified. Holadysamine the major compound was found to be the most active one (MIC = 50 μg/mL), but this activity is lower than that of rifampicine. As synergy may be found between different compounds, it would be interesting to analyze the efficacy of the other alkaloids and their combinations. Furthermore, as this plant is used in association with other ones, it is also interesting to test plant associations as used by traditional practitioners. Remedies that would prove effective activity should be applied for toxicological and pharmacological studies. There is also an urgent need of standardization of traditional remedies based on plants.

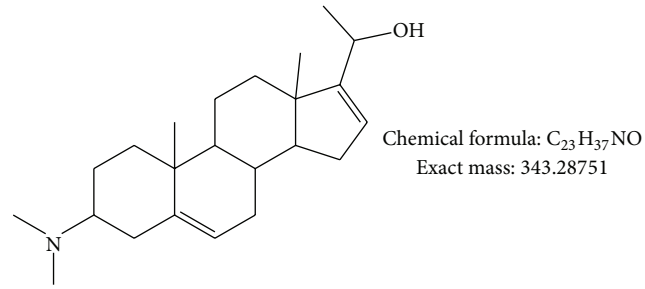

Figure 3: Proposed chemical structure for compound C.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Sliding Motility, Biofilm Formation, and Glycopeptidolipid Production in *Mycobacterium colombiense* Strains

Milena Maya-Hoyos,[1] John Leguizamón,[1] Leonardo Mariño-Ramírez,[2,3] and Carlos Y. Soto[1]

[1]*Chemistry Department, Faculty of Sciences, Universidad Nacional de Colombia, Carrera 30, No. 45-03, Ciudad Universitaria, Bogotá, Colombia*
[2]*Computational Biology Branch, NCBI, NLM, NIH, Bethesda, MD 20894-6075, USA*
[3]*Pan American Bioinformatics Institute, Santa Marta, Magdalena, Colombia*

Correspondence should be addressed to Carlos Y. Soto; cysotoo@unal.edu.co

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Mycobacterium colombiense is a novel member of the *Mycobacterium avium* complex, which produces respiratory and disseminated infections in immunosuppressed patients. Currently, the morphological and genetic bases underlying the phenotypic features of *M. colombiense* strains remain unknown. In the present study, we demonstrated that *M. colombiense* strains displaying smooth morphology show increased biofilm formation on hydrophobic surfaces and sliding on motility plates. Thin-layer chromatography experiments showed that *M. colombiense* strains displaying smooth colonies produce large amounts of glycolipids with a chromatographic behaviour similar to that of the glycopeptidolipids (GPLs) of *M. avium*. Conversely, we observed a natural rough variant of *M. colombiense* (57B strain) lacking pigmentation and exhibiting impaired sliding, biofilm formation, and GPL production. Bioinformatics analyses revealed a gene cluster that is likely involved in GPL biosynthesis in *M. colombiense* CECT 3035. RT-qPCR experiments showed that motile culture conditions activate the transcription of genes possibly involved in key enzymatic activities of GPL biosynthesis.

1. Introduction

The *Mycobacterium avium* complex (MAC) is widely distributed in soil and water [1], and this complex has been frequently identified as an infectious agent in animals and humans [2, 3]. The MAC comprises the species *M. avium*, *M. intracellulare* [3], *M. colombiense* [4], *M. chimaera* [5], *M. marseillense*, *M. timonense*, *M. boucherdurhonense* [6], *M. vulneris* [7], *M. arosiense* [8], 4 subspecies of *M. avium* [9], and "MAC-other" species [10]. The opportunistic infections generated from mycobacteria in HIV-infected and immunosuppressed patients have recently highlighted the clinical relevance of MAC [11, 12]. *M. colombiense* was originally isolated from HIV-positive patients in Bogotá, Colombia [4]. This species is responsible for lymphadenopathy in immunocompetent children in Spain and France [13, 14] and has recently been associated with pulmonary infections that complicate cases of cystic fibrosis [15] and disseminated coinfections with cytomegalovirus [16].

Urease-positive tests and the mycolic acid pattern by thin-layer chromatography (TLC) demonstrate the phenotypic characteristics that distinguish *M. colombiense* from other MAC species [4]. We recently used TLC to show that the mycolate profile of *M. colombiense* is characterised by the presence of mycolates I (α-mycolate), IV (ketomycolate), and VI (carboxymycolate) and two additional spots with chromatographic behaviours similar to those of mycolates III (hydroxyl-mycolate) and VI [17]. A unique *16S* rDNA and the internal transcribed spacer (ITS), MAC-X, facilitated the classification of *M. colombiense* as a novel sequevar [4]. We also identified a 450-bp exclusive genomic region suitable for *M. colombiense* identification through PCR [17].

The physiological and molecular bases for MAC virulence have not been entirely established. However, the virulence of MAC strains has been associated with variations in colony morphology [18, 19], genetic markers, and glycolipid composition [20]. MAC strains display three different morphologies: smooth transparent, smooth opaque, and rough [18, 19], with

the smooth variants being the most virulent morphology [18, 19]. In addition, MAC strains spread on solid hydrophilic surfaces through sliding motility mechanisms that are independent of extracellular structures [21, 22]. Bacterial motility plays a significant role in the colonisation of environmental surfaces and cells [21], which in turn has been correlated *in vitro* with the capacity to form biofilms on hydrophobic surfaces [23]. In *M. avium* strains, motility and biofilm formation have been correlated with colony morphology and the presence of glycopeptidolipids (GPLs) in the cell envelope [24, 25]. Specifically, smooth transparent *M. avium* variants show higher motility on hydrophilic surfaces and increased GPL production; conversely, rough variants show diminished motility and impaired GPL production [22].

GPLs are glycolipids attached to the outermost portion of some nontuberculous mycobacteria, including *M. avium*, *M. smegmatis*, *M. abscessus*, and *M. fortuitum* [25]. This type of glycolipid comprises a mixture of 3-hydroxy or 3-methoxy C_{26}–C_{34} fatty acids amidated to a tripeptide-amino-alcohol (D-phenylalanine-D-*allo*-threonine-D-alanine-L-alaninol) [24, 25]. The lipopeptide core is subsequently glycosylated through 6-deoxytalose (linked to the *allo*-threonine) and α-L-rhamnose (linked to L-alaninol), and the resulting oligosaccharide residues are methylated to form the nonserovar-specific GPLs (nsGPLs) present in all GPL-producing mycobacteria. However, MAC strains also synthesise polar GPLs, in which some oligosaccharides residues are attached to the 6-deoxytalose producing serovar-specific GPLs (ssGPLs) [24, 25]. In *M. avium*, the consecutive actions of a peptide synthetase (PstA/PstB), a fatty acid synthase system (FAS) and polyketide synthetase (Pks), polyketide synthetase associated protein (PapA), glycosyl and methyl transferases (GtfA/GtfB/RtfA and MtfA/MtfB/MtfC/MtfD), and glycolipid transporters (TmtP and Gap) are responsible for the biosynthesis and subsequent translocation of GPLs to the cell wall surface [24–26].

Currently, the existence of GPLs in *M. colombiense* strains is completely unknown. In the present study, we showed that *M. colombiense* contains glycolipids with chromatographic behaviours similar to GPLs. In addition, this novel species forms biofilms on the hydrophobic surfaces of polystyrene, and motility is increased in strains displaying smooth colony morphology. Moreover, we examined the genes likely involved in GPL biosynthesis in the CECT 3035 strain.

2. Material and Methods

2.1. Bacterial Strains, Culture Conditions, and Genomic DNA Isolation. The *M. colombiense* clinical isolates 19B, 57B, the *M. colombiense* genome sequence strain CECT 3035, *M. avium* 104 [27], and *M. smegmatis* mc^2155 [28] were used in this study (Table 1). Planktonic mycobacteria were cultured at 37°C with agitation (76 rpm) in Middlebrook 7H9 media supplemented with ADC (0.5% (w/v) bovine serum albumin, 0.2% (w/v) dextrose, 0.085% (w/v) NaCl, and 0.0003% (w/v) beef catalase) and 0.05% (v/v) glycerol, until an OD_{600} of 0.5 was obtained (planktonic conditions). For the cell motility assay, mycobacteria were cultured in motility medium containing 7H9 supplemented with ADC and 0.35% agarose. *Pseudomonas aeruginosa* ATCC27853 [29] cultured in motility medium was used as a positive control in the drop-collapsing test.

For DNA extraction, the mycobacteria were grown in 7H9-ADC broth to an OD_{600} of 0.5, centrifuged and resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8). Subsequently, the bacilli were inactivated through incubation at 80°C for 20 minutes. Genomic DNA was extracted using lysozyme, SDS/proteinase K, and CTAB/NaCl for cell disruption and chloroform : isoamyl alcohol (24 : 1, v/v) for getting rid of proteins [30, 31]. The DNA pellets were treated with DNAse-free RNAse resuspended in 0.1X TE, followed by quantification using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, MA, USA). The DNA quality was assessed using agarose gel electrophoresis and spectrophotometry (OD_{260}/OD_{280}).

2.2. Cell Motility Assay and Congo Red Staining. For the cell motility assay, plates containing 7H9 media supplemented with ADC and 0.35% agarose were incubated at room temperature overnight prior to cell inoculation [21]. Subsequently, 3 μL of mycobacterial culture at an OD_{600} of 0.6 (~2.7×10^5 CFU) was carefully spotted onto the centre of the plates to minimise the spread of cells from the inoculation point. The plates were dried under sterile conditions for 30 min, sealed with Parafilm, and incubated at 37°C for 4-5 weeks under humidified conditions. The motility rate was recorded as the colony growth halo. The motility assays were performed in triplicate from three independent experiments using *M. avium* 104 as a control. Differences among the experimental data were compared using Student's t-test, and $P < 0.05$ was considered significantly different.

For the characterisation of colony morphology, plates containing 7H10-OADC (ADC + 0.05% oleic acid) media supplemented with 100 μg/mL Congo Red (Sigma-Aldrich, MO, USA) [32] were inoculated as in the cell motility assay, and the resulting cultures were incubated for 4-5 weeks at 37°C under humidified conditions. The morphology was defined as rough or smooth, opaque or transparent, and yellow or beige.

2.3. Biofilm Formation Assay. Biofilm formation was assessed as previously described [22], with some modifications. Briefly, the wells of polystyrene microtiter plates (TPP, Switzerland) were filled with a mix of 75 μL of planktonic mycobacterial culture (OD_{600} = 0.6) and 125 μL of 7H9-ADC broth. The plates were incubated at 37°C for 6 weeks. Subsequently, the plates were washed three times with 1X PBS (0.14 M NaCl, 5 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4), 200 μL of 0.1% crystal violet solution was added, and the cells were incubated at 37°C for an additional 30 min. The residual crystal violet solution was removed, and the wells were washed three times with 1X PBS buffer. A total of 200 μL of absolute ethanol was added to each well, and the plates were incubated for 1 hour at room temperature. Colouration was quantified at 570 nm using a iMark Microplate Absorbance Reader 168-1135 (BioRad, PA, USA). The biofilm assays were performed in triplicate from three independent experiments using *M. avium* 104 as a

Table 1: Bacterial strains and primers used in this study.

Strain and primers	Description	Source
M. colombiense (CECT 3035)	Sequence genome strain	Clinical isolate
M. colombiense 19B and 57B	Clinical isolates	Clinical isolate
M. smegmatis mc^{2}155	Reference strain	[28]
M. avium 104	Reference strain	[27]
P. aeruginosa ATCC27853	Reference strain	[29]
Primers	Sequence (5′-3′)	
pstA dir	ACAGGGCACGAGGAATTCTA	This study
pstA rev	TAGTCCTCGGAGGCTTCGTA	This study
gftA dir	ATGTGTGCTGGCCAGTTATG	This study
gftA rev	GGAAGAACGACGTCCAGAAG	This study
rtfA dir	GACTTTTGGAGCGACGAGTT	This study
rtfA rev	GCCAAATCCTGGTAAAGCTG	This study
mtfB dir	GGACACCGAGCACTACGAG	This study
mtfB rev	TCATACAGATCGCCATCCAG	This study
mtfC dir	ACAAGGCGGATAAAGGGATT	This study
mtfC rev	CTCATACAGATCGCCATCCA	This study
mtfD dir	TACCTGCTCGACACCTTCG	This study
mtfD rev	TCGACCTGCTCGAGTGTCT	This study
tmtpC dir	TTCATTCGGGATACCAGGAG	This study
tmtpC rev	TTGATCCTGACCCGAAGTTT	This study
tmtpA dir	CTCTCGGCTTTGACGACAC	This study
tmtpA rev	ATGGCCGACATCAGCTACTT	This study
tmtpB dir	GAGTGCCCTTGAGTGATTCC	This study
tmtpB rev	CCTCCAAGAATGACGATTCC	This study
16 sRNA dir	GAGATAGGCGTTCCCTTGTG	This study
16 sRNA rev	CTGGACATAAGGGGCATGAT	This study

positive control. Differences among experimental data were compared using Student's t-test, and $P < 0.05$ was considered significantly different.

2.4. Drop-Collapsing Test. Mycobacteria grown on motility medium were scraped and resuspended in 5 mL 1X PBS, gently vortexed, and centrifuged at 8000 rpm for 1 h. Subsequently, 20 μL of supernatant was carefully spotted onto the 15.4 mm wells of polystyrene plates (TPP, Switzerland) previously coated with 100 μL of mineral oil and incubated for 24 h at room temperature [33, 34]. The aqueous drops were visually examined, incubated for 1 min at room temperature, and subsequently photographed. The tests were performed in triplicate from three independent experiments using deionised water and 1% SDS, *M. smegmatis* mc^{2}155, and *P. aeruginosa* as controls.

2.5. Lipid Extraction and TLC Analysis. Crude mycobacterial lipid extracts were obtained as previously described [35], with some modifications. Briefly, mycobacteria cultured under planktonic and motility conditions were extracted using chloroform-methanol (1 : 2, v/v) through vigorous stirring for 48 h at room temperature, followed by chloroform-methanol (2 : 1, v/v) extraction under the same experimental conditions. Pooled and dried organic extracts were partitioned using a chloroform-methanol-water mixture (8 : 4 : 2, v/v/v). The organic phase was separated and evaporated to dryness, and the free glycolipids extracts were weighed. For GPL detection, 1 mg of the lipid extracts was dissolved in chloroform-methanol (2 : 1, v/v) at 20 mg/mL and analysed on 20 × 20 Silica Gel 60 TLC plates (Merck, NJ, USA) using chloroform-methanol-water (65 : 25 : 4, v/v/v) [35]. The carbohydrate-containing compounds were visualised after spraying the plates with 1% anthrone (Sigma-Aldrich, MO, USA) in sulphuric acid, followed by heating at 120°C. Crude *M. avium* 104 lipid extract was used as a control, and the glycolipid profile was obtained from three independent experiments performed in duplicate.

2.6. Bioinformatics Analysis. The genes implicated in the biosynthesis of GPLs in the *M. avium* 104 strain (http://www.ncbi.nlm.nih.gov/nuccore/CP000479.1) were used as model for the bioinformatics analysis. The gene sequences for *pstA*/*pstB* (peptide synthetases), *pks10* (polyketide synthetase), *gtfA*, *gtfB*, and *rtfA* (glycosyl transferases), *mtfA*, *mtfB*, *mtfC*, and *mtfD* (methyl transferases), *tmtpC*, *tmtpA*, and *tmtpB* (glycolipid transporters), and *papA2* (acyl transferase) (Table 1) were identified in *M. colombiense* CECT 3035 using the Genomic BLAST tool (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). The orthologous sequences identified in the *M. colombiense* CECT 3035 genome [36] were subsequently used as targets for primer design in the RT-qPCR experiments.

2.7. RNA Isolation and RT-qPCR Analysis. RNA of *M. colombiense* 19B, CECT 3035, and 57B cultured on both motility medium and planktonic conditions was isolated for RT-qPCR experiments. Mycobacteria grown on motility medium

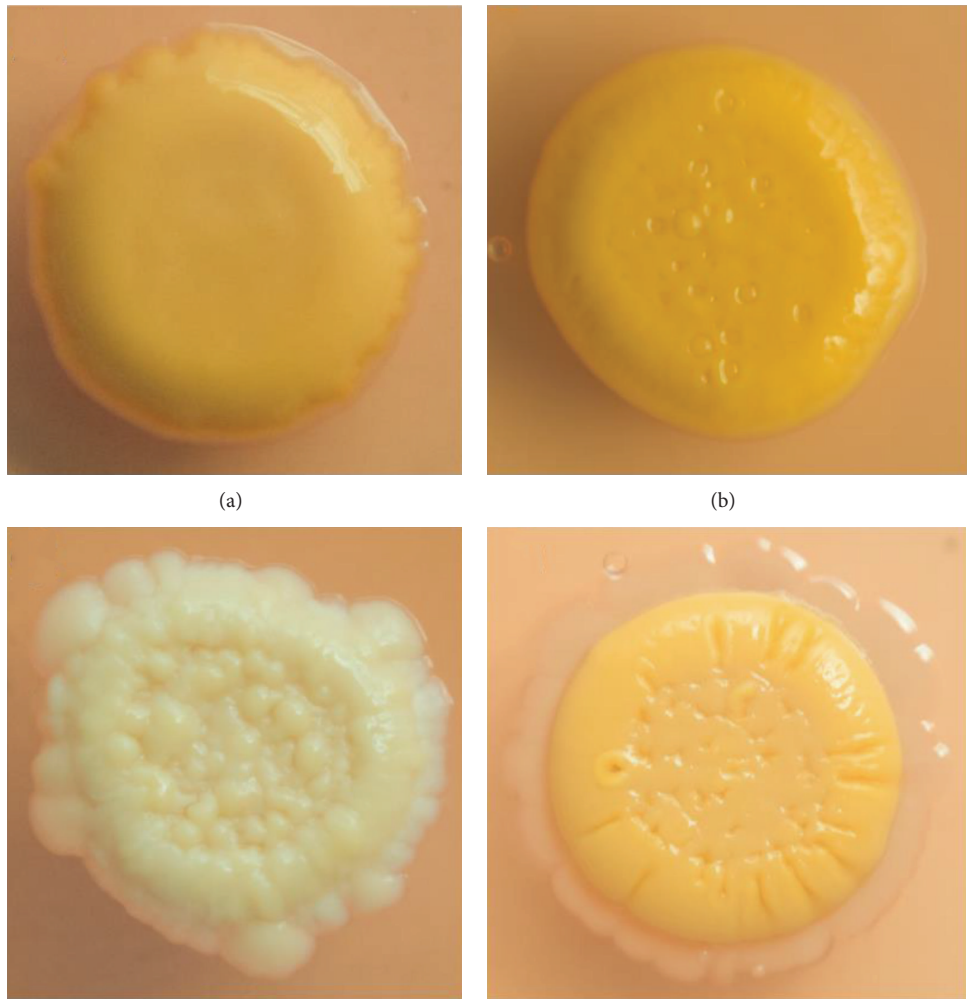

Figure 1: Colony morphology of *Mycobacterium colombiense* strains. Mycobacterial strains were grown on 7H10-OADC plates supplemented with Congo Red and incubated at 37°C for 4-5 weeks. (a) *M. colombiense* 19B, (b) CECT 3035, (c) 57B, and (d) *M. avium* 104.

were scraped and resuspended in diethylpyrocarbonate-(DEPC-) treated water. Motile and planktonic mycobacteria were centrifuged, washed with DEPC-treated water, and resuspended in TRIzol (Invitrogen, USA). The cells were lysed using a Mini-Bead Beater 16 (BioSpec, OK, USA) and glass beads (0.1 mm). Total RNA was isolated as previously described [37], and quantified using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, MA, USA). After DNase I treatment, the RNA quality was evaluated through agarose gel electrophoresis and spectrophotometry (OD_{260}/OD_{280}). cDNA libraries were constructed from 1 μg of RNA using the RevertAid First cDNA Synthesis Kit (Fermentas, Lithuania). For reverse transcription, the samples were incubated at 37°C for 30 min, 42°C for 5 h, 72°C for 1 min, and 4°C for 10 min.

The primers used for the RT-qPCR analyses are listed in Table 1. The RT-qPCR reactions were set up in triplicate using the Express SYBR GreenER Universal qPCR SuperMix Kit (Invitrogen, NY, USA) and a CFX-96 thermocycler (Biorad, PA, USA) under conditions including a denaturation step for 5 min at 95°C, followed by 39 cycles of 95°C for 10 sec, 58°C for 10 sec, and 72°C for 15 sec. Primers or cDNA were omitted in the negative controls. The gene transcription was normalised to the mean value of 16S rRNA (*rrs*) gene expression. The transcription profile was obtained from three independent experiments performed in duplicate. For each experiment, the differences among experimental data were compared using Student's *t*-test, and $P < 0.05$ was considered significantly different.

3. Results

3.1. M. colombiense Is Motile and Forms Biofilms on Hydrophobic Polystyrene Surfaces. The *M. colombiense* strains used in this study, including the genome sequence strain CECT 3035, presented smooth opaque and brilliant colonies on 7H10-OADC plates supplemented with Congo Red, except strain 57B, which displayed a rough and wrinkled phenotype (Figure 1). Similar results were obtained using medium lacking oleic acid (7H9-ADC-0.35% agarose, data not shown). Other *M. colombiense* strains not included in the present

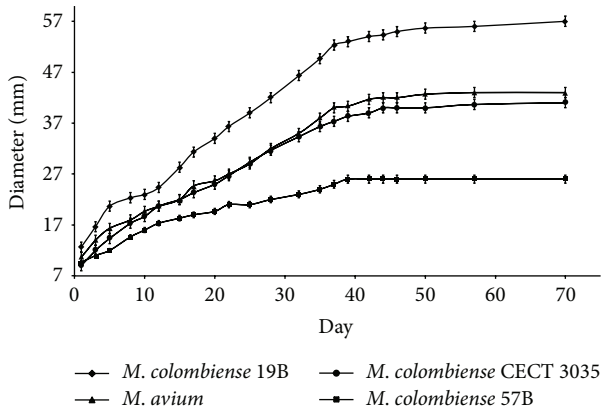

Figure 2: Motility rates for *Mycobacterium colombiense* strains. Mycobacterial strains were cultivated on 7H9-ADC-0.35% agarose and incubated at 37°C for 4-5 weeks. The colony growth halos were measured daily to determine the motility rate. Each reported value represents the mean of the colony growth halos from three independent experiments. The presented data have statistically significant differences compared with the values obtained from *M. avium* 104 ($P < 0.05$).

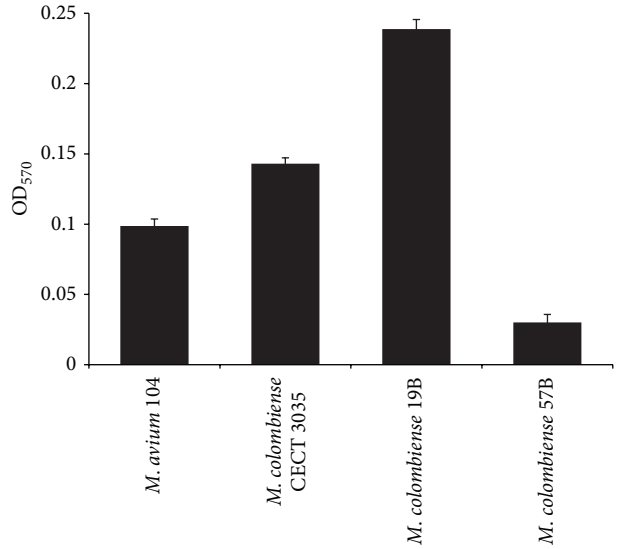

Figure 3: Capacity of *Mycobacterium colombiense* strains to form biofilms. The OD_{570} nm represents the proportion of mycobacteria added to the microtiter wells that reacted with the crystal violet solution. The bars represent the SD calculated from two independent experiments, each performed in triplicate.

study (6B, 7B, 9B, and 16B) also displayed a smooth colony morphology (data not shown). Both smooth and rough bacterial phenotypes were stable after repetitive culture on solid media for the duration of the study (3 years). The smooth *M. colombiense* strains developed yellow pigmentation with age, specifically at the stationary phase, in all culture media; however, the rough variant (57B) remained beige in colour under the same experimental conditions (Figure 1).

Because alterations in the phenotypic characteristics associated with colony morphology have been observed in other MAC species, motility and biofilm formation were compared between smooth and rough *M. colombiense* strains. We observed that the *M. colombiense* 19B and CECT3035 strains (smooth colony morphology) showed increased spreading ($40.94 \pm 0.05\,\mu$m/day and $29.72 \pm 0.05\,\mu$m/day, resp.) on hydrophilic agarose compared with the natural rough variant 57B, which showed impaired spreading (Figure 2).

Crystal violet staining was used to quantify the degree of *M. colombiense* biofilm formation on polystyrene. The highest OD_{570} values for all *M. colombiense* strains were obtained using an inoculum of $\sim 2.7 \times 10^5$ CFU/mL. In general, the strains displaying smooth morphology showed increased biofilm formation on hydrophobic surfaces, while the rough variant 57B showed reduced adhesion to polystyrene (Figure 3).

As biosurfactants could potentially influence cell motility, the "drop-collapsing" method was used to detect biosurfactant secretion in *M. colombiense* strains [22]. As shown in Figure 4, drops of 1% SDS and *P. aeruginosa* aqueous extract (positive controls) spread over the oily surface after the samples were incubated, indicating the presence of biosurfactant substances. In contrast, drops of distilled water, *M. smegmatis* (negative control), and *M. colombiense* aqueous extracts did not collapse on the oily surface, but rather appeared as firm drops, suggesting the absence of biosurfactants in the culture supernatants.

3.2. M. colombiense, Displaying a Smooth Colony Morphology, Contains Glycolipids with Thin-Layer Chromatographic Behaviour Similar to That of the GPLs of M. avium. TLC analysis of noncovalently attached lipids extracted from mycobacteria cultured under planktonic and motile conditions showed that *M. colombiense* strains contain glycolipids with chromatographic behaviour similar to that of the GPLsof *M. avium* [23]. Regarding planktonic cells (Figure 5(a)), the strains with smooth colony morphology (19B and CECT 3035) showed multiple lipid spots migrating in the region of control GPLs (*M. avium* 104); however, the spots observed in the CECT 3035 lipid extract were more intense than those in the 19B strain. The natural rough variant (57B) showed only one spot that migrated in the same GPLs region.

Under motile conditions (Figure 5(b)), the smooth colony morphology strains 19B and CECT 3035 showed a GPL profile similar to that obtained for cells cultured under planktonic conditions; nevertheless, the counterpart spots were more intense than those observed in planktonic cells, particularly for the 19B strain. Nevertheless, the unique spot observed for motile 57B cells (rough variant) were more intense than that observed for planktonic 57B cells. Interestingly, all motile *M. colombiense* cells exhibited reduced production of polar glycolipids, such as phosphatidyl-inositol mannosides (PIMs) and phosphatidylglycerol (PG), compared with planktonic cells (Figure 5(b)).

3.3. In Silico Identification of GPL Biosynthesis Genes in M. colombiense CECT 3035. The genes involved in GPL biosynthesis in *M. colombiense* are currently unknown.

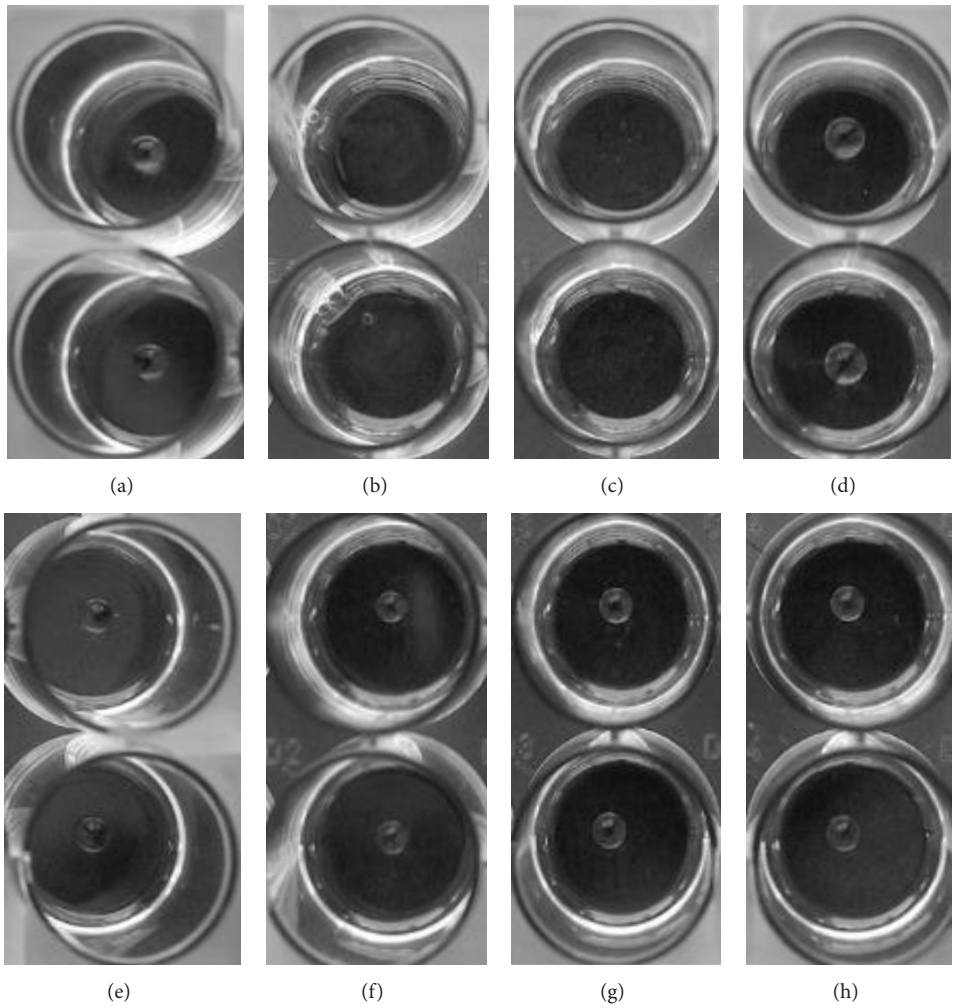

Figure 4: Drop-collapsing test. (a) Water (no surfactant), (b) 1% SDS (surfactant), (c) *P. aeruginosa*, (d) *M. smegmatis* mc^2155, (e) *M. colombiense* 19B, (f) *M. colombiense* CECT 3035, (g) *M. colombiense* 57B, and (h) *M. avium* 104. Drop spreading over the oily surface after samples incubation indicates the presence of biosurfactant substances in the aqueous sample. This assay was conducted in three independent experiments, each performed in duplicate.

Based on the GPL biosynthetic pathway reported for *M. avium* 104 [25], 30 open reading frames (ORF), encoding peptide synthetase, fatty acid synthases (FAS), polyketide synthases (PKs), acyltransferase (PapA), glycosyltransferases, carbohydrate synthetases, methyl and acyl transferases, glycolipid transporters, potential biosynthesis regulators, and proteins with unknown functions [25, 26], were searched within the recently reported nucleotide sequence of *M. colombiense* CECT 3035 [36]. This *in silico* analysis (see Table S1 of the Supplementary Material available online at http://dx.doi.org/10.1155/2015/419549) showed that all enzymatic activities searched were encoded by different ORFs in *M. colombiense* CECT 3035 with an identity ranging between 77% and 96%. The ORFs in the genome of the *M. colombiense* counterpart were located in the contigs 00001, 00002A, 00003A, and 00007 of the genome sequence of the CECT 3035 strain (Figure 6). Among the 30 genes of the GPL synthetic pathway in *M. avium* 104, 7 genes, (*mtfA, gtfB, gtfD, dghA, fadD23, pe*, and *gap-like*) did not have orthologous sequences in *M. colombiense* CECT 3035 (Table S1).

3.4. Differential Transcription of the GPL Biosynthesis Genes in M. colombiense Displaying Smooth and Rough Colony Morphologies. RNA was isolated from *M. colombiense* CECT 3035, 19B, and 57B strains cultured under planktonic and motile conditions to quantify the transcription levels of the *pstA, gtfA, rtfA, mtfB, mtfC, mtfD, tmtpC, tmtpA,* and *tmtpB* genes, predicted to encode key enzymes of the GPL biosynthetic pathway (Table S1). For relative quantification, *M. colombiense* CECT 3035 was grown to the exponential phase under planktonic conditions and used as the reference pattern for two reasons: (1) CECT3035 is the *M. colombiense* genome sequence strain [36] and, consistent with the TLC analysis, (2) planktonic cells displayed the lowest GPL production.

With regard to planktonic cells, the rate of *M. colombiense* 19B gene transcription was consistently higher than that for the *M. colombiense* 57B genes. Conversely, the rough variant (57B) displayed a dramatic decrease in the rate of transcription for the 9 selected genes, particularly those encoding peptide synthetase *pst*A, glycosyltransferase *gtf* A,

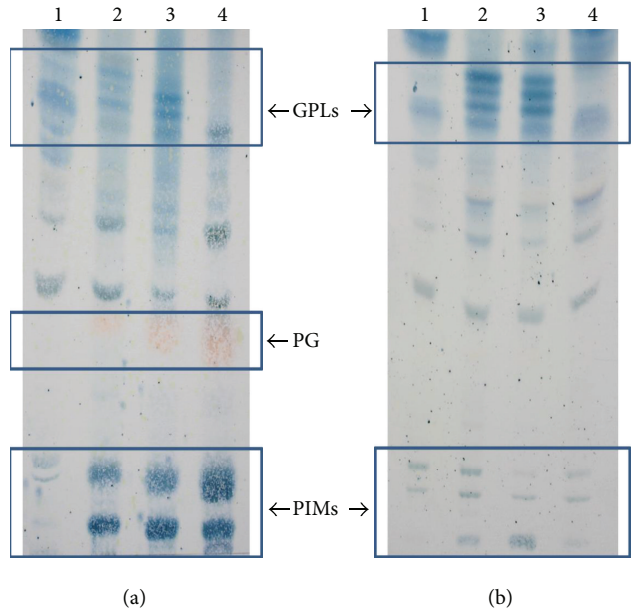

Figure 5: TLC profile of the GPLs in *Mycobacterium colombiense* strains. Crude lipid extracts of (1) *M. avium* 104, (2) *M. colombiense* 19B, (3) *M. colombiense* CECT 3035, and (4) *M. colombiense* 57B. Cells cultivated (a) under planktonic and (b) motile conditions were developed using chloroform-methanol-water (65 : 25 : 4, v/v/v). GPLs: glycopeptidolipids, PIMs: phosphatidyl-inositol mannosides, and PG: phosphatidylglycerol.

the methyltransferases *mtf*B and *mtf*C, and the lipid transporters *tmtp*A, *tmtp*B, and *tmtp*C (Figure 7(a)). Interestingly, *rtfA* (rhamnosyltransferase) in the 19B strain (smooth colony morphology) was the only gene among the strains cultured under planktonic conditions that showed an increased transcription rate (2.21-fold higher) compared with the control cells (planktonic *M. colombiense* CECT 3035 at the exponential phase of growth).

Under motile growth conditions, *M. colombiense* 19B showed the highest transcription rate for all selected genes compared with the CECT 3035 and 57B strains (Figure 7(b)). In contrast, the transcription rates for all genes in the 57B strain (rough variant), except *tmtp*A and *tmtp*C (lipid transporters), were reduced. In motile CECT 3035 cells, whereas *rtfA, mtfB* (methyl transfers), and *tmtpA* showed increased transcription, the *pstA, gftA, mtfC, mtfD, tmtpB,* and *tmtpC* genes exhibited reduced transcription compared with control cells.

4. Discussion

In the present study, TLC experiments were able to show that *M. colombiense* contains glycolipids with chromatographic behaviour similar to the GPLs of *M. avium* 104, which have been previously identified and completely characterised [23]; in addition, the colony morphology was associated with the GPL profile of *M. colombiense* strains. Thus, the TLC analysis revealed that (1) strains displaying smooth colony morphology (19B and CECT3035) produce a higher amount of variable GPLs compared with the rough natural variant (57B) strain and (2) the GPL production was augmented in *M. colombiense* cells cultivated under the motile growth conditions. As expected, the TLC analyses did not provide detailed information about GPL structure; however structural determination was not the objective of the present study.

The media composition influences the glycolipid content of the mycobacterial cell wall. The MAC strains growing on solid medium exhibit a significant rate of transition from smooth transparent to smooth opaque and from smooth to rough morphologies [18, 19, 38]. We compared planktonic and motile *M. colombiense* cells grown in the same culture medium (7H9-ADC or 7H9-ADC-agarose) to avoid differences in lipid content produced through alterations in the media composition. The results showed that the low GPL content observed in the rough 57B variant is less frequent among *M. colombiense* strains; therefore, *M. colombiense* shows a preference for augmented GPL production and smooth colony morphology. Interestingly, planktonic *M. colombiense* cells displayed increased PIM and PG production and diminished GPL content compared with cells cultivated under motile conditions, suggesting a potential compensatory mechanism in cell wall lipid synthesis that compensates for the low GPL content.

The abundance of GPLs in the outermost portion of the cell envelope could be associated with the motility of *M. colombiense* strains on hydrophilic surfaces. Recht and others [22] proposed that GPLs are linked through the hydrophilic head to the cellular capsule and the hydrophobic fatty acid chain is exposed to the bacterial surface, thereby reducing interactions with hydrophilic agarose surfaces and facilitating the spread of cells on solid medium [21, 22]. Thus, the augmented GPL production of motile 19B and CECT 3035 cells compared with that of the rough variant 57B is consistent with the sliding motility on the hydrophilic agarose surface observed for these strains. It has also been suggested that bacterial sliding motility might be favoured

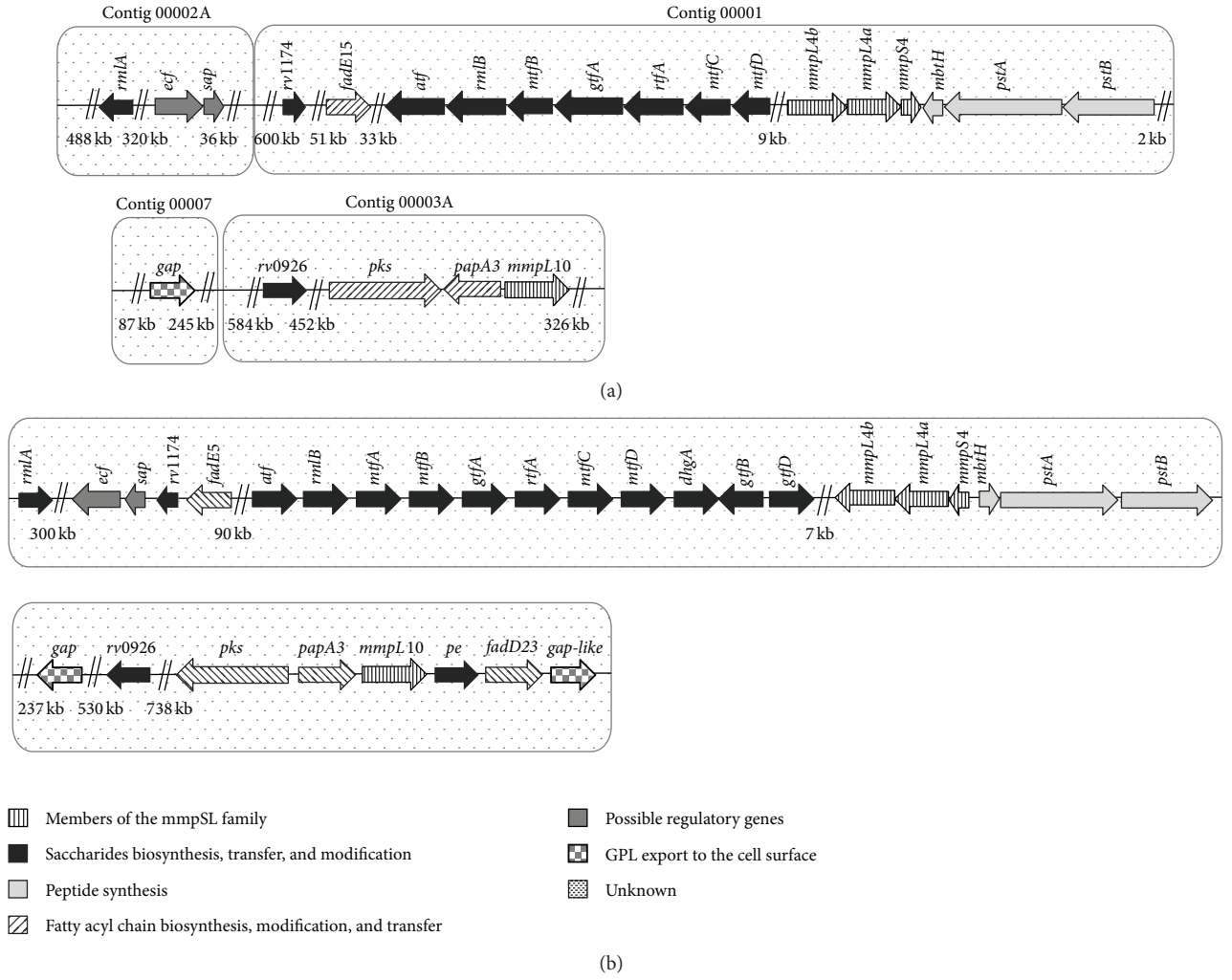

Figure 6: Model showing the gene cluster proposed for the GPL biosynthesis of *Mycobacterium colombiense* CECT 3035 (a). The location and identity of the *M. colombiense* genes were established using the previously described biosynthetic pathway for GPL biosynthesis annotated in the *M. avium* 104 genomic sequence (b).

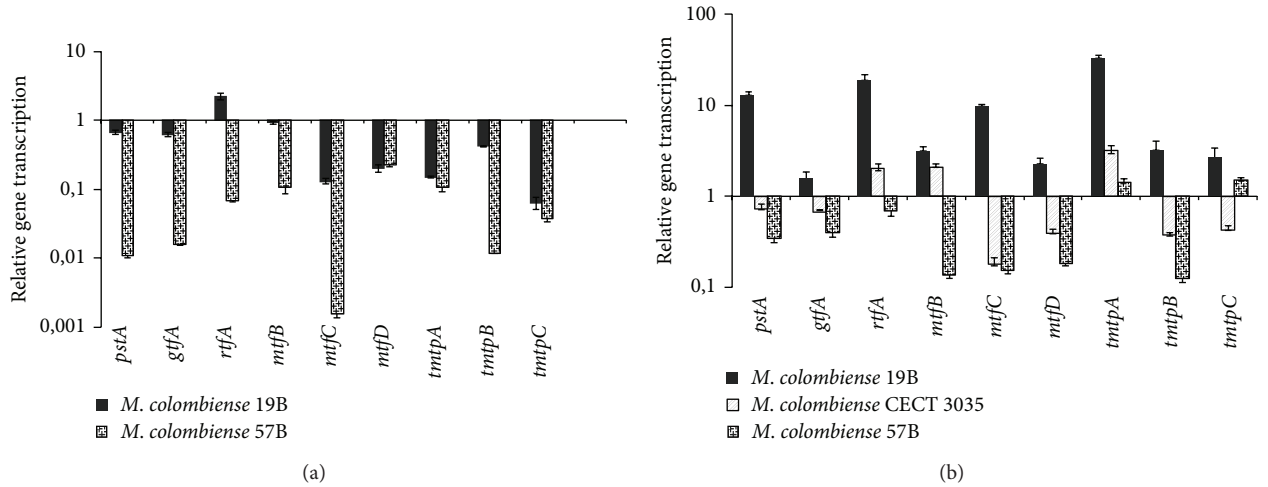

Figure 7: *In vitro* transcription of *Mycobacterium colombiense* genes involved in GPL biosynthesis. Relative quantification expressed as the ratio of gene transcription in (a) *M. colombiense* cells grown under planktonic conditions/transcription of *M. colombiense* CECT 3035 cells grown under planktonic conditions and (b) *M. colombiense* cells grown on the motility medium/transcription of *M. colombiense* CECT 3035 grown under planktonic conditions. The presented data have statistically significant differences compared with the values obtained from *M. colombiense* CECT 3035 cells ($P < 0.05$).

through the secretion of surfactant substances from cells [21, 22]. The results of the drop-collapsing test showed that biosurfactants were likely not secreted from *M. colombiense* cells, suggesting that the motility of *M. colombiense* could be favoured through hydrophobic molecules, such as GPLs, bound to the outermost portion of cells; however, we cannot completely rule out the possibility that some of the surfactant substances secreted from *M. colombiense* remained adhered to the mycobacterial cell surface, thereby influencing cell motility.

GPLs on the outermost portions of the *M. colombiense* cell envelope generate a more hydrophobic cell surface that facilitates initial interactions with hydrophobic surfaces and increase biofilm formation on polystyrene wells. In addition, the increased GPL production in 19B and CECT 3035 cells is consistent with the augmented biofilm formation observed for *M. colombiense* strains displaying smooth colony morphology. Moreover, increased biofilm formation on hydrophobic surfaces and enhanced sliding over motility plates for 19B cells compared with the CECT 3035 strain, which also displays smooth colony morphology, likely suggest possible differences in GPL structure between *M. colombiense* strains. Differences in GPL production between strains could influence the *M. colombiense* ability to form biofilms on polyvinylchloride (PVC) pipes, which would facilitate the dissemination of these bacteria in natural environments, such as in-hospital spaces, thereby increasing the chance of infection in immunosuppressed patients [23, 39, 40].

Based on the GPL biosynthetic pathway previously described for the *M. avium* 104 strain [25], we are currently constructing a potential gene cluster for GPL biosynthesis in *M. colombiense*, which will be finished when the nucleotide sequence of the CECT 3035 is completely assembled. RT-qPCR experiments showed that the planktonic and motile cells of the rough 57B strain showed decreased transcription of the 9 selected genes, leading to the low production of GPLs in this rough variant compared with the *M. colombiense* strains displaying smooth colony morphology. Interestingly, planktonic 19B cells exhibited lower expression of most of the selected genes compared with planktonic CECT 3035 cells. This behaviour results in the reduced production of GPLs in planktonic 19B cells compared with the CECT 3035 strain, consistent with the results of the TLC experiments.

It has been previously shown that an *rtfA* mutation in the *M. avium* serovar-2-specific strain resulted in the loss of serovar-specific GPLs, thereby diminishing the variability of these glycolipids in the cell envelope [41, 42]. Thus, it is possible that the enzymatic activity of the RtfA protein could be relevant for the increased glycosylation and/or augmented production of ssGPLs in strains displaying smooth colony morphology compared with the rough variant 57B. In strains displaying smooth colony morphology, the genes encoding rhamnosyl and methyl transferases (*rtfA* and *mtfB*) are overtranscribed, suggesting the increased production of nsGPLs, precursors for ssGPL biosynthesis [24–26], thereby increasing the production of GPLs. The diminished transcription of *pstA, gftA, mtfC, mtfD, tmtpB*,and *tmtpC* in motile CECT 3035 compared with control cells (planktonic CECT 3035) is intriguing; however, this behaviour also suggests the relevance of rhamnosyl transferases, particularly RtfA, in GPL biosynthesis in *M. colombiense*. Nevertheless, the actual role for RtfA in *M. colombiense* strains should be confirmed using *rtfA*-null mutants.

We did not identify 7 of the 30 known GPL cluster genes of *M. avium* 104 in the genome sequence of *M. colombiense* CECT 3035. This interesting observation suggests that the GPLs between these two closely related MAC species could have different structural characteristics. However, further experiments using mass spectrometry and NMR are necessary to evaluate potential differences in the GPL structure among *M. colombiense* strains.

5. Conclusions

In conclusion, the results of the present study show that *M. colombiense* strains displaying smooth morphology exhibit increased biofilm formation on hydrophobic polystyrene surfaces and enhanced sliding over motility plates. Bioinformatics analyses indicate that the gene cluster established for GPL formation, modification, and translocation is intact, strongly suggesting that GPLs are putatively present in *M. colombiense*. In addition, motile culture conditions activate the transcription of the genes implicated in the key enzymatic activities of GPL biosynthesis.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Clinical Relevance of Nontuberculous Mycobacteria Isolated from Sputum in a Gold Mining Workforce in South Africa: An Observational, Clinical Study

Clare L. van Halsema,[1] Violet N. Chihota,[2] Nicolaas C. Gey van Pittius,[3] Katherine L. Fielding,[1] James J. Lewis,[1] Paul D. van Helden,[3] Gavin J. Churchyard,[1,2,4] and Alison D. Grant[1]

[1]*London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK*
[2]*The Aurum Institute, Postnet Suite No. 300, Private Bag X30500, Houghton, Johannesburg 2041, South Africa*
[3]*DST/NRF Centre of Excellence for Biomedical Tuberculosis Research, MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Private Bag X1, Matieland, Cape Town 7602, South Africa*
[4]*School of Public Health, University of Witwatersrand, Private Bag 3, Wits 2050, South Africa*

Correspondence should be addressed to Clare L. van Halsema; claretaylor@doctors.org.uk

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Background. The clinical relevance of nontuberculous mycobacteria (NTM), detected by liquid more than solid culture in sputum specimens from a South African mining workforce, is uncertain. We aimed to describe the current spectrum and relevance of NTM in this population. *Methods*. An observational study including individuals with sputum NTM isolates, recruited at workforce tuberculosis screening and routine clinics. Symptom questionnaires were administered at the time of sputum collection and clinical records and chest radiographs reviewed retrospectively. *Results*. Of 232 individuals included (228 (98%) male, median age 44 years), *M. gordonae* (60 individuals), *M. kansasii* (50), and *M. avium* complex (MAC: 38) were the commonest species. Of 38 MAC isolates, only 2 (5.3%) were from smear-positive sputum specimens and 30/38 grew in liquid but not solid culture. MAC was especially prevalent among symptomatic, HIV-positive individuals. HIV prevalence was high: 57/74 (77%) among those tested. No differences were found in probability of death or medical separation by NTM species. *Conclusions*. *M. gordonae, M. kansasii*, and MAC were the commonest NTM among miners with suspected tuberculosis, with most MAC from smear-negative specimens in liquid culture only. HIV testing and identification of key pathogenic NTM in this setting are essential to ensure optimal treatment.

1. Introduction

The nontuberculous mycobacteria (NTM) form a group of organisms diverse in many characteristics, including pathogenicity and clinical disease. They have environmental reservoirs and are associated with a broad spectrum of clinical presentations from cutaneous to lung or disseminated disease. There is uncertainty regarding clinical relevance of many of the species, particularly in settings where facility to isolate and identify them is relatively recent.

Liquid mycobacterial culture is recommended for investigation of suspected tuberculosis in resource-limited settings [1]. Compared with solid culture, time to positive culture is decreased and yield increased [2–4]. NTM accounted for 77% of the additional yield from liquid versus solid culture of smear-negative sputa in a gold mining population [2]. It is therefore important to understand the clinical relevance of NTM isolates, particularly in settings of high HIV and tuberculosis prevalence, where NTM may be isolated from large numbers of individuals in tuberculosis programmes.

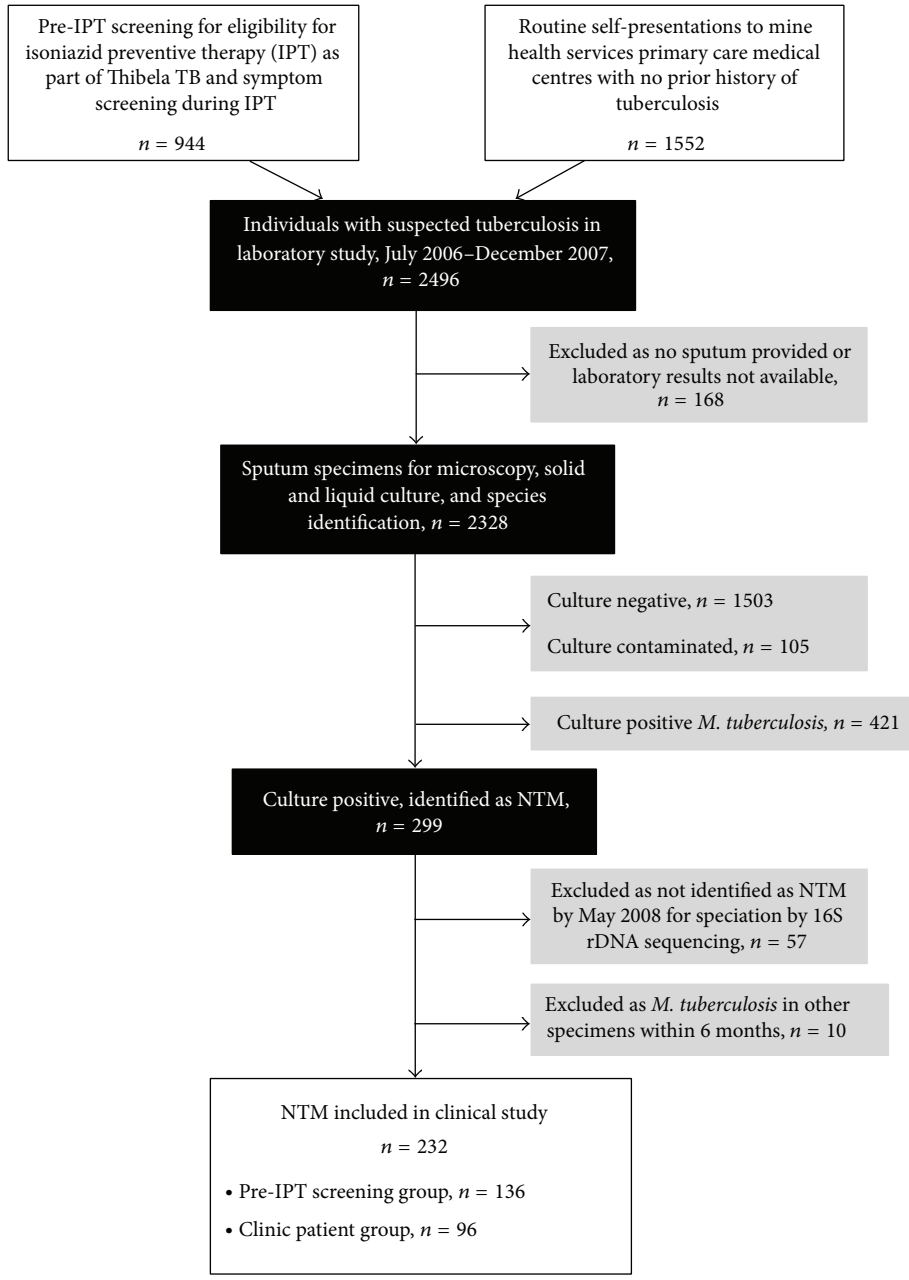

Figure 1: Flow chart showing sources of study participants.

To date, there is limited information from such settings, as culture and speciation facilities have not been widely available or generally applied [5].

Gold miners in South Africa have a very high incidence of tuberculosis [6] and high prevalence of HIV (estimated at 29% in 2000-2001 [7]) and silicosis [8, 9]. Previous studies in this population demonstrated disease due to NTM, particularly *M. kansasii*, *M. scrofulaceum*, and *M. avium* complex [8, 10–13]. We have previously reported on NTM species identification in this population [14] and now describe the clinical features of those with NTM isolated from sputum, to inform laboratory and clinical practice. Previous work on molecular characteristics of *M. gordonae* isolates [14] led to the specific objective of reevaluating the clinical relevance of *M. gordonae* in this setting. We used a cross-sectional, observational study design, in the context of "Thibela TB," a cluster randomised trial of community-wide isoniazid preventive therapy (IPT) [15, 16].

2. Methods

For a laboratory study evaluating mycobacterial culture media [2], participants were recruited from two sources (Figure 1).

(1) Screening for active tuberculosis at enrolment to Thibela TB (pre-IPT screening group): enrolment was open to the whole workforce. Screening was by symptom questionnaire and chest radiograph; nine months' IPT was given to those with negative screen. If tuberculosis was suspected at screening, sputum was taken for mycobacterial culture. All those providing sputum, regardless of tuberculosis history, were eligible for this laboratory study.

(2) Clinics run by mine health services (clinic patient group): individuals self-presented with symptoms or were referred from occupational health services, which conducted radiological screening of all employees every 6–12 months. Recruitment was restricted to individuals with no prior history of tuberculosis, aiming to strengthen diagnostic services for this group who would not routinely have been investigated with sputum culture.

Participants, recruited from July 2006 to January 2009, gave one sputum specimen, after nebulisation if necessary, and were interviewed using a standardised symptom questionnaire, which was more detailed for the clinic patient group. For this study, we included all those with NTM from sputum collected up to the end of December 2007 and with species identification using standard biochemical testing (SBT) completed by May 2008. Those with *M. tuberculosis* isolated within six months of the index NTM were excluded as clinical disease was likely to be due to tuberculosis. The pre-IPT screening and clinic patient groups are described separately, as characteristics of the two groups were expected to differ in terms of symptoms and stage of disease. Study participants with suspected tuberculosis were referred to the mine health service for subsequent investigation and treatment; no additional management of patients was done by investigators and this study was entirely observational, reporting routine practice in this setting.

2.1. Laboratory Methods. As described previously [2, 14], sputum smears were examined for acid fast bacilli by fluorescence microscopy; 0.5 mL sediment was cultured in BACTEC MGIT 960 system (BD Diagnostic Systems, Sparks, MD, USA) and a further 0.5 mL on Löwenstein-Jensen slants, which were incubated for up to eight weeks at 37° Celsius. Mycobacteria were identified by SBT as previously described [14], anti-MPB64 assay (TAUNS, Numazu, Japan) and microscopic cording. Subsequently, NTM underwent DNA sequence amplification (using 45 amplification cycles) and analysis of the hypervariable regions of the 16S rRNA gene by PCR, with referral to the RIDOM and GenBank databases for identification [17, 18]. For specimens in which chromatograms indicated mixed NTM species, the main species is reported; numbers with mixed species are given in Table 1. A new species was defined as an isolate whose sequence did not match a known sequence in the reference databases.

2.2. Clinical Record Review. Medical and laboratory records, accessed at primary health care centres and mining hospitals, were reviewed retrospectively, using a standardised case report form in order to report routine clinical practice in this setting. Mine health services policy is to offer HIV tests to those diagnosed with tuberculosis: tests were offered to individuals in this study according to medical staff practice, with no testing done for research purposes.

2.3. Chest Radiographs. The chest radiograph closest to sputum specimen collection was read using a standardised reporting form, recording features consistent with tuberculosis, along with a silicosis score according to the International Labour Office guidelines [20]. Signs were classified as consistent with definite, probable, possible, or no disease for both active and previous tuberculosis, according to the judgment of the reader, following guidance of the chest radiograph reading and recording system [21]. The reader was masked to clinical details. For the pre-IPT group, an additional 10% of radiographs were added to further mask the reader, from those who did not have NTM isolated (no isolates or *M. tuberculosis*).

2.4. Follow-Up. Individual records were linked to mine company human resources records to establish employment and vital status of participants. Survival analysis was used to examine proportions remaining in the workforce by NTM species isolated, for majority species, from the date of sputum specimen collection. Participants were followed up from the date of sputum specimen collection until the earliest of death, medical separation (individuals leaving the workforce for medical reasons), leaving employment, or censoring date (31 December 2010).

2.5. Definitions. Individuals were considered to be HIV-positive if a positive HIV test was recorded before or up to one year following enrolment and HIV-negative if a negative HIV test (and no subsequent positive test) was recorded after or up to one year preceding enrolment. Treatment outcomes for those treated with standard tuberculosis regimens were recorded according to WHO definitions [22]. Members of the *M. avium* complex were grouped together for the most of the descriptive work presented here.

2.6. Statistical Methods. Categorical variables were compared using chi-squared or Fisher's exact test and continuous variables using the Kruskal-Wallis test. The log rank test and Kaplan-Meier curves were used to compare proportions remaining in the workforce by NTM species.

2.7. Ethical Approval. This study was approved by the Research Ethics Committees of the University of KwaZulu-Natal and the London School of Hygiene and Tropical Medicine. All participants gave written or witnessed verbal consent.

Table 1: Species identified using 16S sequencing, with smear status, HIV status, and CD4 counts ($n = 232$).

Species	Number isolated (% of group)	Number (%) smear positive	Number with mixed species[4]	HIV prevalence[1] (%)	Median CD4[2] (cells/μL) (range (number included))
Clinic patient group					
M. avium complex	25 (26)	2 (8)	5	8/10	87 (14, 827 [5])
M. kansasii	21 (22)	6 (29)	3	5/8	85 (39, 763 [5])
M. parascrofulaceum	9 (9)	1 (11)	0	3/3	102 (66, 138 [2])
M. fortuitum	9 (9)	2 (22)	1	1/1	[0]
M. gordonae	7 (7)	0	1	1/1	195 [1]
New species	7 (7)	1 (14)	0	3/3	475 (236, 512 [3])
Other species	18 (19)	0	2	4/5	291.5 (154, 492 [4])
Total	96	12 (12.5)	12 (12.5% of 96 isolates)	25/31 (80.6)	215.5 (14, 827 [20])
Pre-IPT[3] screening group					
M. gordonae	53 (39)	8 (15)	5	10/12	298 (66, 396 [8])
M. kansasii	29 (21)	11 (38)	1	9/14	183.5 (69, 544 [8])
M. avium complex	13 (10)	0	3	4/5	211 (92, 534 [4])
M. parascrofulaceum	12 (9)	2 (17)	1	1/2	223 [1]
M. fortuitum	11 (8)	0	1	3/5	358 (245, 471 [2])
M. szulgai	5 (4)	0	2	2/2	132.5 (123, 142 [2])
New species	3 (2)	0	0	0/0	[0]
Other species	10 (7)	0	2	3/3	311 (252, 370 [2])
Total	136	21 (15.4)	15 (11.0% of 136 isolates)	32/43 (74.4)	245 (66, 544 [27])

[1]Fisher's exact test for difference in HIV prevalence by species, both groups combined, $P = 0.79$.
[2]Median CD4 count by species for those known to be HIV-positive, combining both groups (clinic patients and IPT screening group) and including 8 species compared using Kruskal-Wallis test, which showed no evidence for difference by species, $P = 0.42$.
[3]IPT: isoniazid preventive therapy.
[4]For specimens containing mixed NTM species on PCR sequencing, the main species is reported; minority species were not identified, but the numbers with mixed species are given here. This does not apply to those with concurrent *M. tuberculosis*, who were excluded from this study.

3. Results

3.1. Study Population. From July 2006 to December 2007, 2496 individuals were recruited and provided sputa. 720 specimens (28.8%) yielded mycobacterial growth, as described previously [2]. Of these, 421/720 (58.5%) grew only *M. tuberculosis* and 299/720 (41.5%) grew NTM. Fifty-seven were excluded because species identification by SBT was not completed by May 2008; 10 were excluded because of concurrent *M. tuberculosis*. Of 232 individuals included (Figure 1), 136 were enrolled through pre-IPT screening and 96 were clinic patients.

Two hundred and twenty-eight (98.3%) of study participants were male, with median age of 44 years (interquartile range (IQR) 36, 49 years; median age 44 years [IQR 36–48] for pre-IPT group and 45 years [IQR 36–50] for clinic patient group), compared with 43 years (IQR 37, 49) in the parent laboratory study [2] and 40 (IQR 31, 46) among 23299 individuals enrolling into Thibela TB at intervention clusters [23]. Median time worked in mining was 21 years (IQR 10, 28). In the pre-IPT screening group, 63/136 (46.3%) had a history of prior tuberculosis treatment, compared with 10.7% of the 23299 enrolling at intervention clusters [23].

3.2. Mycobacterial Species and Sputum Smear Status. Species isolated are shown in Table 1, by recruitment route. In the clinic patient group, 25 individuals, two of whom were sputum smear positive, had *M. avium* complex (12 *M. colombiense*; six *M. vulneris*; five *M. intracellulare*;one each of *M. avium* and *M. chimaera*). *M. kansasii* was isolated from 21 individuals, six of whom were sputum smear positive, and *M. fortuitum* from nine. Seven had new mycobacterial species. In the pre-IPT screening group, *M. gordonae* (53 individuals) was the commonest species, followed by *M. kansasii* (29 individuals) and then members of the *M. avium* complex (13 participants), principally *M. colombiense* (seven of 13). Eight of 53 *M. gordonae*, 11 of 29 *M. kansasii*, and none of the *M. avium* complex were isolated from smear-positive sputum specimens.

3.3. HIV Prevalence. Combining groups, HIV status was documented for 74/232 (31.9%) individuals (Table 1). HIV prevalence among those with *M. avium* complex was 12/15 (80.0%), for *M. kansasii* 14/22 (63.6%), and for *M. gordonae* 11/13 (84.6%). Median CD4 count among HIV-positive individuals with *M. avium complex* was 135 cells/μL (range 14, 827; $n = 9$), *M. kansasii* 169 cells/μL (range 39, 763; $n = 13$),

Table 2: Species distribution by HIV status, among 74 individuals with known status.

Species	HIV-positive (total = 57) *n* (%)	HIV-negative (total = 17) *n* (%)
M. kansasii	14 (25)	8 (47)
M. avium complex	12 (21)	3 (18)
M. colombiense	5	1
M. intracellulare	4	1
M. vulneris	3	1
M. gordonae	11 (19)	2 (12)
M. fortuitum	4 (7)	2 (12)
M. parascrofulaceum	4 (7)	1 (6)
M. szulgai	2 (4)	0
New species	3 (5)	0
Unknown	7 (12)	1 (6)

and *M. gordonae* 291 cells/μL (range 66, 396; $n = 9$). Species distribution by HIV status is shown in Table 2.

Of 57 HIV-positive individuals, 43 (75.4%) were attending HIV care and 8/43 (18.6%) had evidence of taking antiretroviral therapy.

3.4. Clinical and Radiological Features. Symptoms reported at the time of sputum specimen collection are shown in Table 3. In the clinic patient group, cough (17/25; 68.0%) and night sweats (13/25; 52.0%) were prevalent among those with *M. avium* complex. Cough was common for all NTM species and fever was reported most frequently by those with *M. parascrofulaceum* (6/9) and *M. gordonae* (4/7).

In the pre-IPT screening group, cough was less common than in the clinic patient group reported by 59/136 (43.4%) versus 66/96 (68.8%) individuals. Cough was reported by 8/13 (61.5%) of those with *M. avium* complex and 26/53 (49.1%) of those with *M. gordonae*.

Combining pre-IPT screening and clinic patient groups, of 60 individuals with *M. gordonae*, 21 (35.0%) reported weight loss and 14 (23.3%) night sweats, while, of 38 with *M. avium* complex, 18 (47.4%) reported night sweats and 14 (36.8%) weight loss.

Chest radiographs were available for 171/232 (73.7%) individuals. Prevalence of silicosis, grade 1/0 or above, was 38/171 (22.2%) and grade 1/1 or above 13/171 (7.6%). *M. kansasii* was most frequently associated with chest radiograph appearance suggestive of definite or probable active tuberculosis (17/34, 50.0%); corresponding figures for *M. avium* complex were 3/26 (11.5%) and for *M. gordonae* 14/49 (28.6%). Lung cavitation was present in 47/171 (27.5%) radiographs including 22/34 (64.7%) of those with *M. kansasii*, 11/49 (22.5%) with *M. gordonae*, 2/15 (13.3%) with *M. fortuitum*, and 2/26 (7.7%) with *M. avium* complex (chi^2 $P < 0.001$ for comparison of four species). Among those with cavitation, this was considered to be due to active disease in 6/11 (54.5%) with *M. gordonae* and 8/22 (36.4%) with *M. kansasii*.

M. gordonae isolates with discrepant identification on SBT were further identified by strain, as described previously, with no differences seen between clinical features of those few isolates identified as strain D versus other *M. gordonae* strains [14].

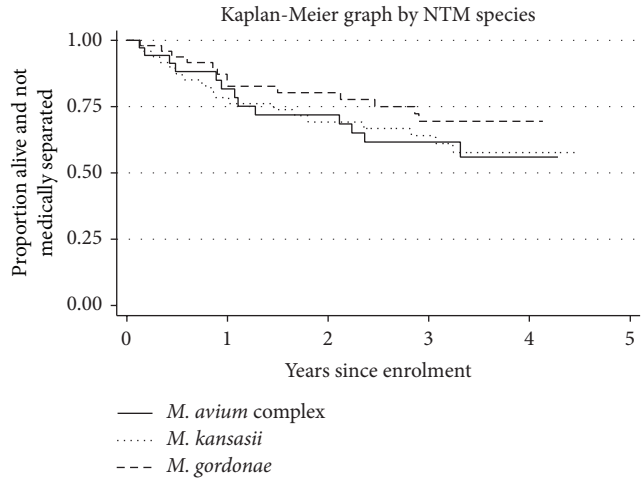

Figure 2: Kaplan-Meier graph showing death or medical separation by NTM species isolated for the three most prevalent species (n = 138: 51 *M. gordonae*; 49 *M. kansasii*; and 38 *M. avium* complex).

3.5. Species Isolated by Culture Method. Of 38 *M. avium* complex isolates, 30 (78.9%) were cultured on liquid but not solid culture medium. Of those 30, 9/12 individuals with known status were HIV-positive and none were sputum smear positive. For other species, 6/20 (30.0%) *M. fortuitum*, 38/60 (63.3%) *M. gordonae*, 12/50 (63.3%) *M. kansasii*, and 15/21 (71.4%) *M. parascrofulaceum* were isolated only on liquid medium (chi^2 test, $P < 0.001$). Combining all species, no significant differences in reported symptoms were observed between those cultured on solid medium and those cultured only on liquid medium (data not shown).

3.6. Outcomes and Loss to Workforce. Linking to human resources data was successful for 218/232 (94.0%) individuals. Median follow-up time was 31.9 months (range 0.07 to 54.7 months), during which time there were 10 deaths and 63 medical separations, as shown in Table 4. At 24 months, 80.3% with *M. gordonae*, 69.2% with *M. kansasii*, and 72.9% with *M. avium* complex remained in the workforce (Table 5). Comparing the three commonest species (*M. gordonae*; *M. kansasii; M. avium* complex), log rank test for differences in proportions remaining in the workforce gave $P = 0.47$ (Figure 2).

4. Discussion

In this mining population with high HIV prevalence, NTM were common in sputum culture of those with suspected tuberculosis. *M. gordonae, M. kansasii*, and *M. avium* complex were the commonest species, with *M. avium* complex

Table 3: Symptoms reported by route of recruitment to study and species of nontuberculous mycobacterium isolated (n = 232).

Species	Number isolated	Number reporting cough n (%)	Number reporting night sweats[1] n (%)	Number reporting haemoptysis n (%)	Number reporting weight loss[2] n (%)	Number reporting fever n (%)	Number reporting any symptom n (%)	Number with chest radiograph classified as definite or possible active tuberculosis n/number with chest radiograph available
Clinic patient group								
M. avium complex	25	17 (68)	13 (52)	4 (16)	11 (44)	11 (44)	19 (76)	2/14
M. kansasii	21	11 (52)	9 (43)	1 (5)	10 (48)	9 (43)	14 (67)	4/9
M. parascrofulaceum	9	8 (89)	7 (78)	0	8 (89)	6 (67)	9 (100)	1/7
M. fortuitum	9	7 (78)	5 (56)	0	5 (56)	3 (33)	7 (78)	1/5
M. gordonae	7	5 (71)	2 (29)	0	4 (57)	4 (57)	6 (86)	1/3
New species	7	5 (71)	3 (43)	1 (14)	2 (29)	4 (57)	5 (71)	0/5
Other species	18	13 (72)	11 (61)	3 (17)	7 (39)	10 (56)	15 (83)	1/7
Total	96	66 (69)	50 (52.1)	9 (9.4)	47 (49.0)	47 (49.0)	75 (78.1)	10/50
Pre-IPT[3] screening group								
M. gordonae	53	26 (49)	12 (23)	§	17 (32)	§	34 (64)	13/46
M. kansasii	29	8 (28)	2 (7.0)	§	6 (21)	§	12 (41)	13/25
M. avium complex	13	8 (62)	5 (39)	§	3 (23)	§	9 (69)	1/12
M. parascrofulaceum	12	2 (17)	2 (17)	§	4 (33)	§	6 (50)	4/11
M. fortuitum	11	5 (46)	3 (27)	§	1 (9)	§	5 (46)	5/10
M. szulgai	5	4 (80)	1 (20)	§	2 (40)	§	4 (80)	2/5
New species	3	1 (33)	1 (33)	§	1 (33)	§	1 (33)	1/2
Other species	10	5 (50)	1 (10)	§	5 (50)	§	6 (60)	1/10
Total	136	59 (43.4)	27 (19.9)	§	39 (28.7)	§	77 (56.6)	40/121

[1]Night sweats were defined by the wording of the question put to study participants: "Do you have drenching night sweats? (Sweat so much at night that clothes/pillows are soaking wet?)."
[2]Weight loss was defined by the wording of the question put to study participants: "Do you have unintentional weight loss? (In the last 6 months have your clothes become looser?)."
[3]IPT: isoniazid preventive therapy.
[§]Data not available as questionnaire used in pre-IPT screening for tuberculosis did not include this question.

Table 4: Deaths and medical separations, from recruitment until end of 2010, median 32 months (n = 218).

NTM species	Death n (row %)	Medical separation n (row %)	Total number of individuals
M. gordonae	0	13 (26)	51
M. kansasii	2 (4)	16 (33)	49
M. avium complex	1 (3)	12 (32)	38
M. parascrofulaceum	2 (11)	2 (11)	19
M. fortuitum	1 (5)	11 (58)	19
M. szulgai	0	1 (20)	5
New species	1 (10)	1 (10)	10
Other species	3 (11)	7 (26)	27
Total	10 (4.6)	63 (28.9)	218

particularly prevalent among symptomatic individuals presenting to routine health services and *M. gordonae* most common in the pre-IPT screening group.

Table 5: Probability of remaining in the workforce during follow-up for individuals with the three predominant NTM species.

Species	6 months (%, 95% CI)	12 months (%, 95% CI)	24 months (%, 95% CI)
M. gordonae	93.8 (82.0–98.0)	85.0 (91.0–92.5)	80.3 (65.5–89.3)
M. kansasii	87.3 (73.9–94.1)	78.4 (63.5–87.8)	69.2 (53.6–80.5)
M. avium complex	88.3 (71.7–95.4)	81.7 (63.7–91.4)	72.9 (52.9–94.4)

HIV prevalence was 77.0% where known. Importantly, though this study population comprised individuals with suspected tuberculosis, a minority had HIV status documented, suggesting that testing coverage is poor and that those tested may not be representative of all those with NTM. It is possible that those tested have higher prevalence of HIV than those not tested, as there may have been clinical reasons for offering tests. However, routine testing in this setting is essential to improve care and it is very likely that diagnoses of HIV are being missed. Silicosis grade 1/1 or above (definite silicosis)

was found in 7.6% of individuals, compared with 2.6% in the parent study Thibela TB [16], which would be expected in a subpopulation with NTM, as silicosis is a known risk factor [8].

Symptoms varied by recruitment method, as expected, with cough more prevalent among those self-presenting to routine health services than those undergoing screening. Lung cavitation was more prevalent among those with *M. kansasii* than those with *M. avium* complex, which would be expected, as *M. avium* complex more typically causes disseminated disease in the context of advanced HIV-related immunosuppression and sputum isolates may represent disseminated, rather than exclusively pulmonary, disease. It is not possible to definitively diagnose disseminated disease without further sampling, but, with low CD4 counts, systemic symptoms, and chest radiographs without cavitation, it is likely that some of this group had disseminated *M. avium* complex, with the organism isolated from respiratory specimens here. Similarly, radiographs were reported as consistent with active tuberculosis less frequently in those with *M. avium* complex. There was a higher than expected proportion with cavitation on chest radiograph among those with *M. gordonae*. This is difficult to interpret with limited clinical data available and cavitation may have been a result of previous or other diseases. We did not detect differences in proportions remaining in the workforce between groups of individuals with the three commonest NTM species, although there is a suggestion of lower retention in the workforce among those with *M. avium* complex and *M. kansasii*. The study design did not include a comparator group without NTM and from these data alone we can only conclude that loss to the workforce for health reasons among those with NTM isolated from sputum is substantial. However, from the mining population participating in Thibela TB, mortality was 0.91/100 person-years and combined mortality and medical separation were 4.34/100 person-years [16], suggesting higher combined mortality and medical separation among those with NTM than the workforce overall, which may be due to mycobacterial disease or untreated HIV. Differences by species may have been detected with larger numbers.

The distribution of NTM species differs from that found in previous studies in this population. In the 1990s, *M. kansasii* and *M. scrofulaceum* were reported to be the commonest species [8, 11], whereas this study found higher proportions of *M. avium* complex and *M. gordonae*, with notable absence of *M. scrofulaceum* using 16S sequencing. This is likely to be largely due to newer speciation methods: organisms identified by SBT as *M. scrofulaceum* in this study were almost all subsequently identified as *M. gordonae* by 16S sequencing [14]. The higher proportion of *M. avium* complex may be due to the use of liquid culture, as suggested by laboratory studies in this population [2] and elsewhere [3, 4], and may also be due to increased HIV prevalence in this population: from 1.4% among those with sexually transmitted infections in 1991 [24] to 19.0% among those with NTM in 1993-6 [11, 25] and 29% in a representative sample of the workforce in 2000-1 [7]. The median age of the population does not appear to have increased, estimated at 41 years in 2001 [7] and 40 years among those enrolling at Thibela TB intervention clusters [23], although the median age among those with NTM was higher.

M. gordonae was more prevalent in the pre-IPT screening group than in clinic patients, perhaps because, in the context of active case finding, not all isolates will be genuine pathogens. *M. gordonae* is well documented to be a laboratory and tap-water contaminant [19]. However, in this group of individuals with suspected tuberculosis, *M. gordonae* was associated with systemic symptoms (night sweats, weight loss) in some, with radiological abnormalities in 14/51 (27.5%), and with HIV infection in 11/15 (73.3%). It has previously been reported to cause disseminated disease in advanced HIV [26–28] and may be pathogenic in some individuals here, with isolates from sputum indicating pulmonary or more widespread disease. Laboratory and clinical records would be expected to have detected concurrent *M. tuberculosis* or bacterial infections if present. Longer term, close follow-up of these individuals was not incorporated into the study design. Without more clinical data and follow-up, firm conclusions on *M. gordonae* in this population should not be drawn, but those with *M. gordonae* and clinical symptoms or abnormal radiology warrant further investigation for other pathologies, particularly HIV infection, and repeat culture of sputum and other relevant samples.

M. avium complex was particularly common among symptomatic individuals in the clinic patient group, who had high prevalence of HIV infection. *M. avium* complex is known to be common where it is sought and appropriate diagnostics used in HIV-positive populations and disease is associated with low CD4 cell counts [29–32]. These individuals in particular require further investigation and specific treatment, with early antiretroviral therapy and cotrimoxazole prophylaxis. It is noteworthy that 30/38 *M. avium* complex isolates grew in liquid but not solid culture media and none of these 30 were smear positive, suggesting that *M. avium* complex infection may be underdiagnosed in settings of high HIV prevalence where liquid culture medium is not used. Pulmonary isolates may reflect disseminated disease and liquid culture will be needed to diagnose this infection in settings where the Xpert MTB/RIF assay is used as first line diagnostic test for tuberculosis. There was a notable absence of *M. avium*, with other *M. avium* complex subspecies found (*M. colombiense*, *M. vulneris*, and *M. intracellulare*). This may reflect immunosuppression, as *M. colombiense* is closely related to *M. avium* and has been isolated from HIV-positive individuals previously [33] or may reflect geographical variation in NTM species [34].

Mining populations have long been known to be at risk of NTM infection and disease [12] and to some extent results presented here are relevant primarily to this population. However, comparisons of species distribution, HIV prevalence, and clinical features are relevant to other settings in which HIV and tuberculosis are prevalent and liquid mycobacterial culture media are used. Similar results have been seen in Southeast Asia in a study showing high prevalence of NTM isolated by liquid culture of sputum among HIV-positive individuals, with *M. kansasii* most frequent in

Table 6: American Thoracic Society criteria for lung disease due to NTM (adapted from [19]).

Criteria for diagnosis of lung disease due to NTM	
Clinical	(1) Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules And (2) Appropriate exclusion of other diagnoses
Microbiological	(1) Positive culture results from at least two separate expectorated sputum samples Or (2) Positive culture results from at least one bronchial wash or lavage Or (3) Transbronchial o other lung biopsy with mycobacterial histopathological features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathological features and one or more sputum or bronchial washings that are culture positive for NTM

those with pulmonary disease and *M. avium* complex among those with disseminated disease [35].

The clinic patient group was restricted to individuals with no prior tuberculosis treatment, in order to maximise the impact of the parent laboratory study on routine health services. In addition, the clinic patient group was more symptomatic than the pre-IPT screening group and there are likely to have been differences in disease spectrum between these groups. For this reason, characteristics of the two groups are presented separately. In addition, despite excluding individuals with *M. tuberculosis* isolated from other specimens within six months of the NTM, we cannot definitively exclude culture negative tuberculosis.

ATS criteria for lung disease due to NTM [19] include, amongst other criteria shown in Table 6, a requirement for NTM to be isolated from expectorated sputum samples on more than one occasion. We were not able to apply these criteria in this study because of its retrospective design. This is a limitation of this study. However, authors of the ATS criteria, primarily intended to guide diagnosis of lung disease, are clear that they were written with the United States setting in mind and are not validated in other settings. Among miners in South Africa, these criteria have been shown to be difficult to apply even in prospective studies, because of the requirement for repeated sampling and detailed imaging and frequent use of presumptive antimycobacterial treatment [10] in a programme designed for tuberculosis detection and treatment. Such repeat intensive laboratory diagnostics are not part of the national guidelines for tuberculosis control in South Africa [36].

5. Conclusions

In conclusion, *M. avium* complex, largely found among symptomatic, smear-negative individuals, will be underdiagnosed where liquid culture is not used. Newer culture techniques are advisable wherever feasible, although there are of course cost and infrastructure obstacles to widespread use. Key pathogenic organisms should be identified where possible, so that specific treatment can be given if required. HIV testing coverage was poor and prevalence among those with known HIV status was high. Improved HIV testing strategies are required for those being investigated for tuberculosis in this and other settings of high HIV prevalence.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Clare L. van Halsema contributed to study design and implementation, data analysis, and paper writing; Violet N. Chihota contributed to study design, parent study implementation, laboratory work, and paper writing; Nicolaas C. Gey van Pittius carried out laboratory work, contributed microbiology advice, and reviewed the paper; Katherine L. Fielding contributed to study design, parent study design and implementation, epidemiological input, and paper review; James J. Lewis contributed to data management, parent study implementation, statistical and epidemiological advice, and paper review; Paul D. van Helden contributed to laboratory and study design advice and reviewed the paper; Gavin J. Churchyard contributed to study design of this and parent studies, epidemiological advice, and paper review; Alison D. Grant contributed to study concept and design, epidemiological advice, parent study design and implementation, and paper writing.

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Clinical Study

Lung and Nodal Involvement in Nontuberculous Mycobacterial Disease: PET/CT Role

Ginevra Del Giudice,[1] Andrea Bianco,[2] Antonio Cennamo,[1] Giulia Santoro,[3] Marco Bifulco,[4] Carlo Marzo,[1] and Gennaro Mazzarella[1]

[1]*Department of Cardiothoracic and Respiratory Sciences, Second University of Naples, "dei Colli" Hospital, Via Leonardo Bianchi, 80131 Naples, Italy*
[2]*Department of Medicine and Health Sciences, University of Molise, Via Giovanni Paolo II, Loc. Tappino, 86100 Campobasso, Italy*
[3]*Department of Laboratory Pathology and Microbiology, Section of Mycobacteriology, "dei Colli" Hospital, Via Leonardo Bianchi, 80131 Naples, Italy*
[4]*Department of Radiology, Section of Nuclear Medicine, "dei Colli" Hospital, Via Leonardo Bianchi, 80131 Naples, Italy*

Correspondence should be addressed to Ginevra Del Giudice; ginevradelgiudice@gmail.com

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Introduction. Systematic use of ^{18}F-FDG PET/CT has the potential to simultaneously assess both pulmonary and lymph node involvement in nontuberculous mycobacterial (NTM) lung infection. *Objective*. The aim of the study was to evaluate the role of ^{18}F-FDG PET/CT in the assessment of both mediastinal lymph nodes and lung involvement in NTM patients compared with active tuberculosis (TB) patients. *Methods*. 26 patients with pulmonary NTM disease were selected; six consecutive patients had undergone ^{18}F-FDG PET/CT and data was compared with 6 active TB patients. *Results*. NTM exhibited different radiological lung patterns with an average SUV max value at PET/CT scan of 3,59 ± 2,32 (range 1,14 to 9,01) on pulmonary lesions and a mean value of SUV max 1,21 ± 0,29 (range 0,90 to 1,70) on mediastinal lymph nodes. Pulmonary lesions in TB showed an average SUV max value of 10,07 ± 6,45 (range 1,20 to 22,75) whilst involved mediastinal lymph nodes exhibited a mean SUV max value of 7,23 ± 3,03 (range 1,78 to 15,72). *Conclusions*. The differences in PET uptake in a broad range of lung lesions and lymph nodes between NTM and *M. tuberculosis* patients suggest a potential role for PET/CT scan in the diagnosis and management of pulmonary mycobacterial disease.

1. Introduction

Epidemiological observations indicate an increase in the prevalence of NTM disease worldwide.

NTM infection is caused by a group of versatile opportunistic bacterial pathogens and remains underdiagnosed due to difficulties in pathogen isolation and nonspecificity of clinical signs. Isolation of NTM from respiratory specimens does not always indicate disease; clinical, radiographic, and microbiologic criteria must all be met to make a diagnosis of NTM lung disease according to ATS guidelines [1, 2].

Although ATS criteria represent the cornerstone of NTM diagnosis, some limitations need to be addressed: the uncertain significance of isolation of NTM; the experience and training required in using specific microbiological diagnostic kits; and use of X-ray investigations for NTM identification.

Radiological patterns of lung NTM disease include nodular lesions, cavitary pattern, bronchiectasis, lobar consolidation, and consolidation associated with fibrothorax, as well as possible mix patterns (e.g., nodular-bronchiectasis) [3]. Lymphadenitis is the main clinical manifestation of NTM in children aged 1–5 years [4, 5], while skin and soft tissue involvement is not uncommon.

Lymph node involvement in NTM is not easily interpreted at thoracic CT scan unlike *M. tuberculosis* lung infections; NTM patients do not have a primary complex, colliquation of nodal stations is not detectable, and lymph node repair in calcification or fibrocalcification is absent.

^{18}F-FDG positron emission tomography-computed tomography (PET/CT) is a functional imaging technique which detects metabolically active areas through the accumulation of the radiotracer 18-fluorodeoxyglucose. PET/CT is widely used in cancer management [6, 7] and is already employed in the assessment of inflammatory/infectious respiratory diseases with lymph node involvement such as sarcoidosis, TB, and intracellular infections [8–11].

The purpose of the study was to assess and compare, through the use of ^{18}F-FDG PET/CT, lung and mediastinal lymph node involvement in patients with NTM and patients with active TB. The data were collected at "Vincenzo Monaldi" Hospital, Naples, center for Phthisiology and clinical laboratory investigation in mycobacteriology.

2. Materials and Methods

Between 2008 and 2011 79 biological culture samples positive for NTM were evaluated in our mycobacteriology center.

In order to make an accurate disease diagnosis, according to ATS 2007 guidelines for NTM lung pathology, clinical, microbiological, and radiological criteria were all considered. 26 NTM patients were enrolled.

During the same period (2008–2011) 486 samples positive for *Mycobacterium tuberculosis* were evaluated. Only 6 patients affected by active TB according to medical history, clinical, radiology, and acute-phase reactants underwent ^{18}F-FDG PET/CT.

The clinical records and radiological examinations of patients with positive cultures for NTM species and TB by sputum or bronchial wash were reviewed retrospectively, including information about age, sex, respiratory symptoms, preexistent pulmonary and nonpulmonary illnesses, results of anti-HIV antibody, number of positive isolates, and available lung biopsy results (Table 1).

Six patients with NTM infection and 6 patients with diagnosis of TB who underwent an ^{18}F-FDG PET/CT were considered eligible for the study.

2.1. NTM Patients. All were male, Caucasian, with mean age of 61 (between 38 and 77 years), HIV negative presenting with nonspecific symptoms such as cough with sputum, evening fever, and dyspnea. Comorbidities such as COPD, diabetes mellitus, and bronchial and allergic asthma were present, and one patient had received an aortic valve replacement. Four NTM patients had a positive microbiological diagnosis on sputum samples (*M. kansasii* $n = 2$, *MAC* $n = 1$, and *M. xenopi* $n = 1$) and two on bronchial aspirate (*M. kansasii* $n = 1$, *MAC* $n = 1$).

2.2. Active Tuberculosis Patients. Three females and three males, Caucasian, were selected, with mean age of 52 (between 34 and 78 years). Symptoms included fever, dry tickly cough with or without sputum, weight loss, and episodes of hemoptysis; C reactive protein (PCR) and sedimentation rate were mostly raised. Four patients presented with comorbidities such as diabetes mellitus, sinus tachycardia, HBV+, atrial fibrillation, arterial hypertension, hemorrhoids, rheumatoid arthritis, and ischemic heart disease. *M. tuberculosis* isolation in five patients was made on bronchial aspirate and in one on sputum.

2.3. Microbiological Technique. Microscopic examinations were made with Ziehl-Neelsen coloration and confirmed on samples of sputum or bronchial aspirate using trough techniques of gene amplification (PCR), cultures on solid media (Lowenstein-Jensen) and liquid, and radiometric technique BACTEC MGIT 960. DNA probes were performed for molecular speciation: the technique of gene amplification PCR, and the technique INNO-LiPA mycobacterium V2.

2.4. ^{18}F-FDG PET/CT Imaging. Patients underwent ^{18}F-FDG PET/CT using a whole-body scanner (Siemens Biograph 16 PET-CT scanner). Patients were fasted prior to scanning for at least 4–6 hours, well hydrated, with the recommendation not to undergo intense physical activity the day before and blood glucose levels were assessed to ensure values <150 mg/dL. For diabetic patients insulin was administered three hours before to normalize blood glucose level. After establishment of venous access, the dose of radiotracer (^{18}F-FDG) was administered according to the weight of the patient (5.18 MBq/kg). Expected average dose is 370–555 MBq. The administration of the radiotracer was followed by oral hydration (to promote an appropriate distribution of the tracer in the tissues and its urinary excretion) and by a rest period in which the patient was informed not to walk, speak, or make any kind of effort to avoid the physiological uptake by muscle overactivity especially at the level of the eye muscles and vocal cords. 50 minutes after the injection of ^{18}F-FDG the patient was positioned supine in the PET scanner. The PET/CT was composed of a dedicated PET scanner with a detector LSO with crystal dimensions of $4.0 \times 4.0 \times 20$ mm, transaxial field of view of 585 mm, an axial field of view of 162 mm, intrinsic axial and transaxial resolution that was between 4.6 and 5.8 mm, and a multislice CT. The parameters used for the acquisition included CT 120 kV, 80 mA, 16 slices helical, and 0.5 s for rotation. Initial TC without contrast was carried out followed by a PET scan performed on the body of the patient in the same position from the head to knee level. In order to better assess the involvement of the lung and lymph node.

Initial CT without contrast was carried out followed by a PET scan performed on the body of the patient in the same position from the head to knee level.

After reconstruction of the coronal, sagittal, and transverse planes, the images were interpreted qualitatively and semiquantitatively with the standardized uptake value (SUV).

An experienced radiologist in nuclear medicine evaluated radiotracer uptake in mediastinal lymph nodes.

2.5. Statistical Analysis. The values were expressed as mean ± SD and were compared using the Mann-Whitney U test for two unpaired samples. Differences were considered statistically significant when $P < 0,05$. The results have been obtained through the use of MATLAB program.

3. Results

NTM patients CT scans showed nodular or pseudonodular involvement ($n = 3$), parenchymal consolidation ($n = 2$),

Table 1: The clinical records and radiological examination of patients with positive cultures for NTM species and *M. tuberculosis*.

Patient	Age	Sex	Agent isolated	Sample	Anti-HIV antibody	ESR	Clinic	Comorbidity	Lymph node	Lymph node uptake PET (SUV max)	Radiological lung patterns	Lung lesion uptake PET (SUV max)
1	40	M	*M. kansasii*	BAS	—	18	Evening fever	Bronchial and allergic asthma	4 R 3 4 L	1,03 0,92 1,04	Small consolidation LUL	No uptake
2	77	M	*M. xenopi*	Sputum	—	24	Fever, cough	Diabetes mellitus, arterial hypertension	7 3 4 R	1,68 1,50 1,19	Pseudonodular consolidation RLL Right basal pleural effusion	9,01 6,06
3	38	M	*M. kansasii*	Sputum	—	31	Fever, cough with sputum	No	3 7	1,70 1,20	Cavitary lesion RUL Cavity apical segment RLL Cavity LUL	2,60 3,15 5,36
4	70	M	*MAC*	Sputum	—	21	Evening fever, cough with mucopurulent sputum	COPD, diabetes mellitus	7	0,9	Right posterior basal pleural thickening Left posterior basal pleural thickening Calcification of the anterior segment of RUL	1,80 1,14 No uptake
5	67	M	*M. kansasii*	Sputum	—	27	Cough, dyspnea	COPD, arterial hypertension	4 L	0,96	Apical nodular consolidation RUL Pleural thickening anterior basal lateral right	1,64 1,39
6	76	M	*MAC*	BAS	—	23	Dyspnea on exertion, evening fever	Aortic valve replacement	7	1,16	Subpleural nodular consolidation anterior segment RUL Subpleural nodular consolidations LUL Mild bilateral parenchymal consolidation Mantle calcified sequelae of UL and of left hilum	4,50 2,70 3,70 No uptake
7	34	M	*M. tuberculosis*	BAS	—	94	Fever, cough with sputum	No	2 R 4 R 10 R 7	15,72 6,93 7,55 8,20	Consolidation + small cavitary lesions apical segment RUL Basal consolidation segments RLL GGO + micronodules of apical segment LUL	15,10 22,75 3,60
8	49	M	*M. tuberculosis*	BAS	—	63	Fever, cough with sputum	Arterial hypertension	2 R 2 L 3 4 R 4 L 5 7 8 10 R 10 L	8,55 8,55 8,55 8,55 8,55 8,55 8,55 8,55 8,55 8,55	Diffuse subpleural consolidation of right lung Diffuse subpleural consolidation of left lung Consolidation of posterior segment RUL Consolidation of apical-dorsal segment LUL Diffuse micronodules in both lungs (miliary)	11,58 12,27 12,24 17,06 8,95

Table 1: Continued.

Patient	Age	Sex	Agent isolated	Sample	Anti-HIV antibody	ESR	Clinic	Comorbidity	Lymph node	Lymph node uptake PET (SUV max)	Radiological lung patterns	Lung lesion uptake PET (SUV max)
9	61	F	*M. tuberculosis*	BAS	—	27	Weight loss, cough with sputum	Diabetes mellitus, sinus tachycardia, HBV+	2 R 3 4 R 8 10 R Right axillary Retromandibular left	3,30 8,10 5,90 7,70 7,56 12,94 7,60	Consolidation with excavation and various contextual calcifications of posterior segment RUL	4,19
10	78	F	*M. tuberculosis*	BAS	—	79	Fever, dry cough	Atrial fibrillation, arterial hypertension, and hemorrhoids	No linphonodes		Multiple focal bilateral consolidations; the greater is the left hilar Bilateral pleural effusions	8,00 No uptake
11	39	F	*M. tuberculosis*	Sputum	—	61	Fever, dyspnea on exertion	No	2 R 4 R	2,77 3,50	Consolidation of posterior and lateral basal segments RUL Consolidation RUL	19,55 3,54
12	51	M	*M. tuberculosis*	BAS	—	32	Hemoptysis, fever	Rheumatoid arthritis, ischemic heart disease	3 5 Left axillary	1,78 1,78 4,17	Cavity apical segment LUL Intrascissural left consolidation Lung consolidation RUL	6,55 4,53 1,20

GGO: ground glass opacity. RUL: right upper lobe. LUL: left upper lobe. BAS: bronchoaspirate. ESR: erythrocyte sedimentation rate.

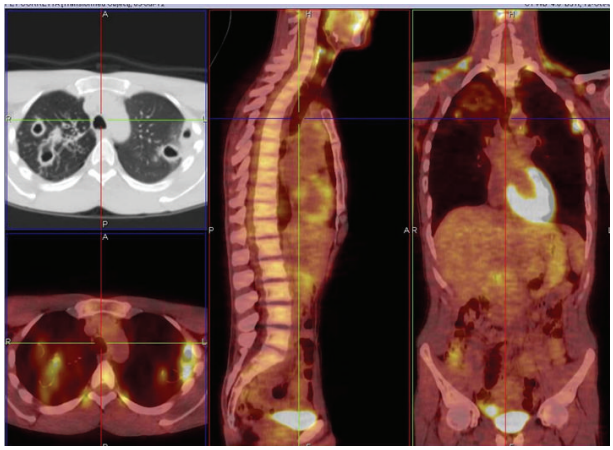

Figure 1: *M. kansasii* infection, cavitary lesions in the right upper lobe and in the apical segment of the right lower lobe and left upper lobe, SUV max 5.36. Related to patient 3 (PET/CT. Courtesy "V. Monaldi" Hospital).

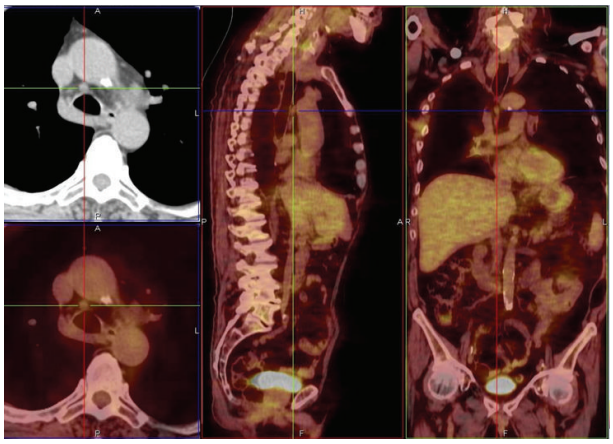

Figure 2: *M. xenopi* infection, lower right paratracheal lymph node (4R), SUV max 1.19. Related to patient 2 (PET/CT. Courtesy "V. Monaldi" Hospital).

pleural thickening ($n = 2$), calcified sequelae ($n = 2$), cavitary lesions ($n = 2$), and pleural effusion ($n = 1$). More than one radiological pattern was simultaneously found in five NTM patients. PET/CT scan in NTM patients showed areas of consolidation or parenchymal thickening with SUV max between 1,64 and 9,01 ($n = 3$), pleural thickenings with SUV max that ranged from 1,14 to 1,80 ($n = 2$), subpleural nodular lesions with SUV max 2,7–4,5 ($n = 1$), and multiple cavitary lesions with SUV max between 2,60 and 5,36 ($n = 1$) (Figure 1), and in one patient a baseline monolateral pleural effusion with maximum uptake (SUV max of 6,06) was observed.

One patient had no lesions with significant uptake.

The mean value of the SUV max of these metabolically active areas was 3,59 ± 2,32 (range 1,14 to 9,01).

Carinal, precarinal, paratracheal, and prevascular lymph nodes exhibited an average value of SUV max 1,21 ± 0,29 (range 0,96 to 1,70) (Figures 2 and 3).

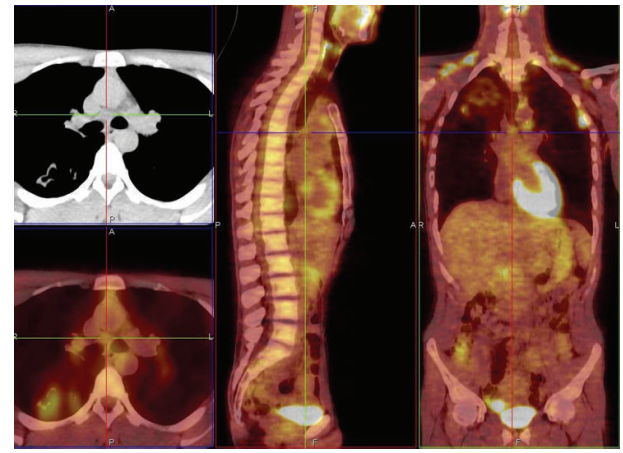

Figure 3: *M. kansasii* infection, subcarinal lymph node (7), SUV max 1.20. Related to patient 3 (PET/CT. Courtesy "V. Monaldi" Hospital).

Table 2: SUV max of mediastinal lymph nodes detected by PET/CT in patients with lung NTM disease.

Patient	Mediastinal lymph nodes	SUV max
1	Station 4 R	1,03
	Station 4 L	1,04
	Station 3	0,92
2	Station 7	1,68
	Station 4 R	1,19
	Station 3	1,50
3	Station 3	1,70
	Station 7	1,20
4	Station 7	0,90
5	Station 4 L	0,96
6	Station 7	1,16

Table 2 reports the details of the mediastinal lymph node involvement in NTM patients according to the classification revisited by Mountain.

At CT scan more than one radiological pattern was simultaneously found in active tuberculosis patients. CT findings included parenchymal consolidation or ground glass opacity (GGO) ($n = 6$) with SUV max between 1,20 and 22,75, excavation ($n = 3$) SUV max 4,19–15,10, and miliary ($n = 1$) SUV max 8,25, with lymph node involvement; in one case a combination of nodular consolidation PET-positive and bilateral pleural parietobasal effusion PET-negative was identified. PET/CT showed the presence (for all of 6 patients) of extensive areas of high metabolic activity (SUV max range 1,20–22,75 (Figure 4) and average values of SUV max of 10,07 ± 6,45).

In addition, 5 patients showed increased metabolic activity in paratracheal lymph nodes, Barety space (Figure 5), prevascular, carinal, paraesophageal, hilar, and aortopulmonary window and axillary with mean values of SUV max of 7,23 ± 3,03 (range 1,78–15,72).

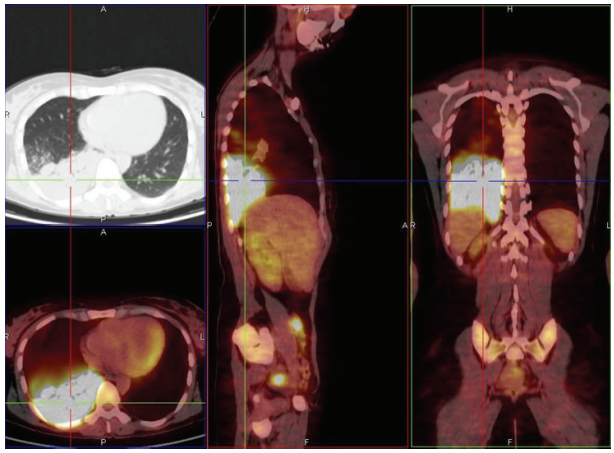

Figure 4: *M. tuberculosis* infection. Massive area of high metabolic activity, SUV max 19.55, lower right lobe. Related to patient 11 (PET/CT. Courtesy "V. Monaldi" Hospital).

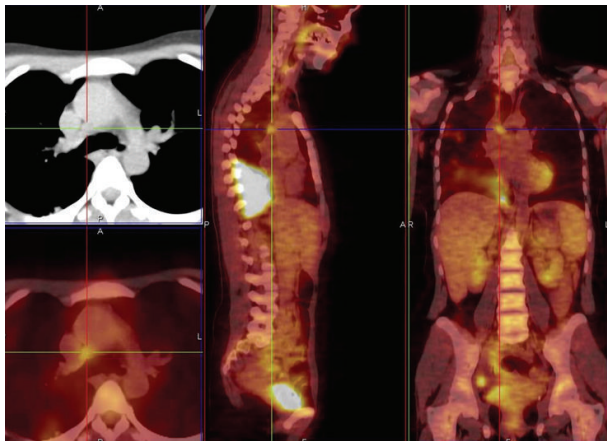

Figure 5: *M. tuberculosis* infection with lymph node of Barety space (4R), SUV max 3.50. Related to patient 11 (PET/CT. Courtesy "V. Monaldi" Hospital).

Table 3 reports the details of the mediastinal lymph nodes in active TB.

The data obtained with PET/CT (Table 4) shows that patients with active TB have high metabolic activity in lung lesions and in mediastinal lymph nodes compared to NTM. Furthermore NTM patients, while presenting with lung lesions exhibiting high uptake, showed little or no lymph node involvement.

Patients with active TB showed a mean value of SUV max of mediastinal lymph nodes equal to 7,23 ± 3,03 (range 1,78 to 15,72), significantly higher than the average value of SUV max 1,21 ± 0,29 (range 0,90 to 1,70); $P = 2{,}62 \times 10^{-6}$ detected in patients with NTM (Figure 6). The average SUV max of lung lesions in patients with active TB was equal to 10,07 ± 6,45 (range 1,20 to 22,75) and significantly higher than the average SUV max of lung lesions in NTM patients 3,59 ± 2,32 (range 1,14 to 9,01); $P = 0,0043$ (Figure 7).

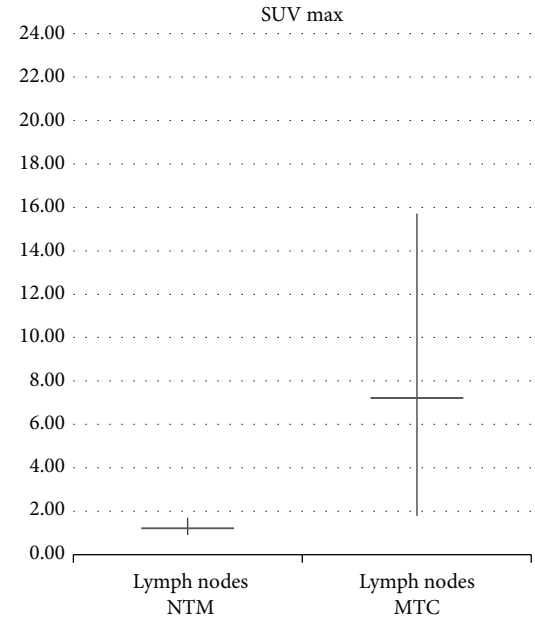

Figure 6: Comparison between average SUV max of mediastinal lymph nodes in NTM and *M. tuberculosis* patients.

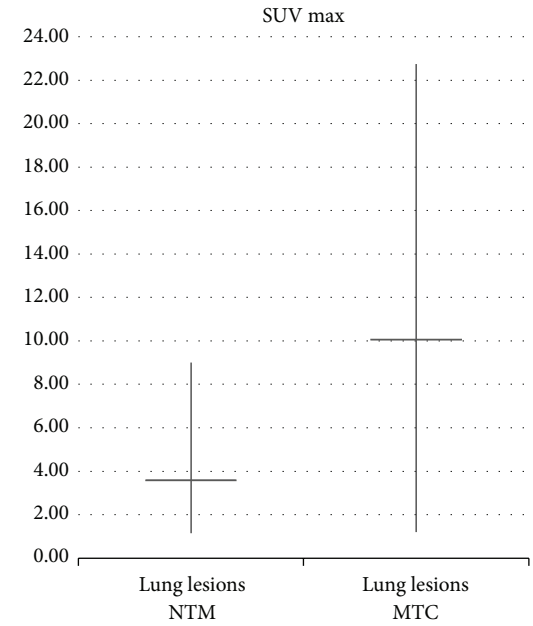

Figure 7: Comparison between average SUV max of lung lesions in NTM and *M. tuberculosis* patients.

4. Discussion

Our study suggests that ^{18}F-FDG PET/CT is a useful diagnostic technique which may be helpful in the management of pulmonary NTM and *M. tuberculosis* infections.

Indeed we have demonstrated that both NTM and *M. tuberculosis* patients exhibit an increase of SUV in lung lesions although average values were less in NTM than in *M.*

Table 3: SUV max of mediastinal lymph nodes detected by PET/CT in patients with TB.

Patient	Mediastinal lymphonodes	SUV max
7	Station 2 R	15,72
	Station 4 R	6,93
	Station 10 R	7,55
	Station 7	8,20
8	Station 2 R	8,55
	Station 2 L	8,55
	Station 3	8,55
	Station 4 R	8,55
	Station 4 L	8,55
	Station 5	8,55
	Station 7	8,55
	Station 8	8,55
	Station 10 R	8,55
	Station 10 L	8,55
	Station 8	8,55
	Station 10 R	8,55
	Station 10 L	8,55
9	Station 2 R	3,30
	Station 3	8,10
	Station 4 R	5,90
	Station 8	7,70
	Station 10 R	7,56
10	No lymph nodes	
11	Station 2 R	2,77
	Station 4 R	3,50
12	Station 3	1,78
	Station 5	1,78

Table 4: SUV max of the mediastinal lymph nodes and lung hypermetabolic areas in patients with NTM and TB.

	Mean value SUV max	Range
Mediastinal lymph nodes NTM patients	1,21 ± 0,29	0,90–1,70
Hypermetabolic lung areas NTM patients	3,59 ± 2,32	1,14–9,01
Mediastinal lymph nodes TB patients	7,23 ± 3,03	1,78–15,72
Hypermetabolic lung areas TB patients	10,07 ± 6,45	1,20–22,75

tuberculosis; lymph nodes SUV value was low or zero in NMT whilst results were high in active tuberculosis patients.

Despite well-established diagnostic criteria, NTM lung disease remains challenging for clinicians and new approaches are required for improving disease management.

The literature provides only a few studies regarding the potential usefulness of PET/CT as a diagnostic tool in nontuberculous mycobacteriosis.

Currently the ^{18}F-FDG PET/CT is widely used in cancer management for diagnosis, staging, and response to therapy [7, 12]. There is now a growing interest in the potential role of ^{18}F-FDG PET in the functional assessment of inflammatory and infectious pulmonary diseases including mycobacteria.

A study conducted by Goo et al. showed that the pulmonary tuberculoma commonly causes an increase in uptake of ^{18}F-FDG suggesting caution in differentiating benign from malignant pulmonary abnormalities [13].

Similarly, the literature reports various cases of pulmonary nodular lesions with PET/CT high uptake caused by infection with NTM (especially MAC) whose differential diagnosis with malignant nodule was performed through histological typing [14, 15].

Another study by Demura et al. evaluated the use of ^{18}F-FDG PET/CT in the diagnosis and monitoring of therapy for mycobacteriosis. The average uptake was higher in patients with MAC than those with TB. The study also confirms that, following one or two years of treatment for MAC, despite the persistence of nodules on HRCT, the ^{18}F-FDG PET uptake was more likely to correlate with disease state [16].

Our data differ from that reported by Demura, which may be due to differing patient selection criteria including the lung lesion characteristics and subtype of NTM species in patients enrolled.

Davis et al. have shown the utility of PET/CT to detect tuberculous lesions and to monitor response to chemotherapy in animal models hypothesizing the possible application for humans [17].

In our study we have demonstrated the usefulness of ^{18}F-FDG PET/CT in reflecting the activity and extent of disease by monitoring metabolic activity not only in nodular lesions but also within a broad range of radiologically visible lung lesions in NTM and *M. tuberculosis*. This is the first study in which PET/CT characteristics of lesions different from tuberculoma or single nodular lesion have been evaluated.

The broad range of radiological patterns seen in NTM and *M. tuberculosis* includes nodular or pseudonodular lesions, parenchymal consolidation, cavitary lesions, pleural thickenings, and pleural effusions and may reflect differences in cellular immune response at the level of the diseased lung [18–20].

One of the most important findings from this study is the role of ^{18}F-FDG PET/CT in the comparative evaluation of both pulmonary lesions and mediastinal lymph nodes in patients with NTM and those with active TB.

Our results have shown that, in both NTM and *M. tuberculosis*, increased uptake of the radioisotope at PET/CT scan is representative of active inflammatory areas within lung lesions which appears to reflect the real extent of disease.

PET/CT scan allows simultaneous assessment of both parenchymal and lymph node lesions which is of great value in tuberculous disease where lung involvement is almost always accompanied by lymphadenopathy.

Although performed on a small number of patients, our observations suggest a potential role for PET/CT in assessing extent of disease, evolution, and follow-up in both NTM and *M. tuberculosis* patients. Further investigations are required to verify whether there is the possibility to identify specific SUV cutoffs which in turn may define the

potential indications and limitations of PET/CT scan in both pulmonary NTM and *M. tuberculosis* infections.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Research Article

Use of MALDI-TOF MS for Identification of Nontuberculous *Mycobacterium* Species Isolated from Clinical Specimens

María Concepción Mediavilla-Gradolph,[1] Inmaculada De Toro-Peinado,[1] María Pilar Bermúdez-Ruiz,[1] María de los Ángeles García-Martínez,[1] María Ortega-Torres,[1] Natalia Montiel Quezel-Guerraz,[2] and Begoña Palop-Borrás[1]

[1] *Infectious Diseases and Microbiology Service, Regional University and Virgen de la Victoria University Hospitals, IBIMA, 29010 Malaga, Spain*
[2] *Microbiology Unit, Hospital Costa del Sol, 29603 Marbella, Spain*

Correspondence should be addressed to María Concepción Mediavilla-Gradolph; gradolphilla@hotmail.com

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The aim of this study was to compare the results obtained for identification by MALDI-TOF of nontuberculous mycobacteria (NTM) isolated in clinical samples with those obtained by GenoType *Mycobacterium* CM/AS (common mycobacteria/additional species). A total of 66 *Mycobacterium* isolates from various clinical specimens (mainly respiratory) were tested in this study. They were identified using MALDI-TOF Bruker from strains isolated in Lowenstein, following the recommended protocol of heat inactivation and extraction, and were simultaneously analyzed through hybridization by GenoType *Mycobacterium* from liquid culture MGIT. Our results showed that identification by MALDI-TOF was correct in 98.4% (65/66) of NTM isolated in our clinical practice (*M. avium, M. intracellulare, M. abscessus, M. chelonae, M. fortuitum, M. mucogenicum, M. kansasii, and M. scrofulaceum*). MALDI-TOF was found to be an accurate, rapid, and cost-effective system for identification of mycobacteria species.

1. Introduction

Nontuberculous mycobacteria (NTM) are environmental organisms found in soil and water throughout the world. The large majority of NTM are not pathogenic for humans, but almost all can behave as opportunists and thus be responsible for disease in the presence of predisposing conditions. Presentation is typically pulmonary, skin/soft tissue, lymphatic, or disseminated [1, 2]. The incidence of diseases caused by this group of pathogens is on the rise, due among other reasons to an increase in immunocompromised patients [3, 4]. The distribution of NTM in our environment and the high variability in the pathogenicity of some species means that it is important to differentiate them clinically depending on whether they arise in an asymptomatic setting, due to environmental contamination, or are real infections, in order to apply appropriate antimicrobial therapies. Rapid and accurate diagnosis of mycobacterial infection is of utmost importance as inappropriate treatment may lead to drug resistance or unnecessary exposure to drug toxicity. The American Thoracic Society and Infectious Disease Society of America (ATS/IDSA) recommended that clinically significant NTM isolates be identified to the species level whenever possible [5].

Historically, species-level identification of NTM was a long and complicated process. Growth characteristics in culture (development of color and grow rate) and substrate utilization were for decades the only methods available and sometimes no accurate identification was possible. High-performance liquid chromatography (HPLC) analysis of mycolic acid has been used, but this method is labor-intensive and requires initial culture of isolates on solid

medium [6–8]. In the last decades new strategies have been developed using molecular techniques. Currently, nucleic acid sequencing is the most rapid and accurate method for identifying *Mycobacterium* species; however, species-level discrimination may require analysis of several genes [6, 9] and, as with HPLC, requires a pure isolate obtained from solid medium, which delays the turnaround time. Thus, these methods remain limited to specialized laboratories. Tests based on hybridization directly from liquid culture medium are available in many laboratories, although the number of species that can be identified is limited. GenoType *Mycobacterium* CM/AS allows the detection of 36 species of NTM. It is a technology that can be performed in solid and liquid cultures, using the same PCR product [10, 11].

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is recognized as a powerful tool for the identification of bacteria and yeasts in the clinical laboratory [12, 13]. This technique allows identification of organisms on the basis of unique spectral fingerprints produced by extracted proteins. The method is relatively simple, rapid, and associated with significantly lower consumable costs than traditional microbiological identification methods. Although the MALDI-TOF MicroFlex LT mass spectrometer and associated software are expensive initially (approximately $200,000), the continuing consumable costs are inexpensive (less than $1 per isolate). Some authors have used MALDI-TOF for rapid identification of *Mycobacterium* species [6, 14–16], but few have undertaken prospective studies using clinical isolates [16].

We undertook a prospective study with NTM recovered from clinical samples in the routine practice of a microbiological laboratory and demonstrated the usefulness of MALDI-TOF for the daily identification of mycobacteria in our laboratory. We here compare the results of MALDI-TOF and GenoType *Mycobacterium* CM/AS for the identification of NTM from clinical specimens.

2. Materials and Methods

Between July 2013 and July 2014 we studied NTM isolates in the microbiology laboratories of the Regional University Hospital and the Virgen de la Victoria University Hospital, both in Malaga, Spain.

2.1. Mycobacterium Isolates. A total of 66 isolates (from 66 patients) from different types of clinical specimens (mainly respiratory) were analyzed. Samples from nonsterile sites were treated with N-acetyl cysteine and NaOH and cultured on Lowenstein-Jensen medium (LJ) and in a mycobacterium growth indicator tube (MGIT) (Becton Dickinson Microbiology Systems, Cockeysville, MD) and incubated in Bactec MGIT 960. Blood and bone marrow were inoculated into a vial of Myco/F Lytic (Becton-Dickinson, Sparks, MD). In positive cultures, Ziehl-Neelsen staining was performed to confirm the presence of acid-fast bacilli. GenoType was performed using liquid culture medium MGIT and identification using MALDI-TOF was made from colonies isolated on Lowenstein solid culture medium.

In the case of nonidentification of mycobacteria by either of the methods, the strains were sent to the *Mycobacterium* Reference Laboratory at the National Center of Microbiology (CNM) in Majadahonda (Madrid), where identification of the strains was performed by genotypic methods.

2.2. Mycobacteria Extraction Protocol for MALDI-TOF MS. We used a modification of the Inactivated Mycobacteria bead preparation method (inMBpm) of Bruker Daltonics [17], described below.

We use a fresh culture from a Lowenstein medium with enough biomass to undertake the process. In a biological safety cabinet we transfer enough colonies to obtain a 5 μL pellet and, with the help of a sterile swab, transfer it to an Eppendorf tube with 300 μL of water (HPLC grade). This is then inactivated for 30 minutes at 95°C in a thermoblock. At this point, we centrifuge at the maximum speed (13.000–15.000 rpm) for 2 minutes, after which the supernatant is removed. We add 300 μL of water (HPLC grade) and mix the sediment carefully. We then add 900 μL of 100% ethanol and mix it again using vortex. We centrifuge it once more at the maximum speed (13,000–15,000 rpm) for 2 minutes after which we remove the supernatant. Afterwards, we resuspend the pellet in 500 μL of water (HPLC grade), centrifuge it at maximum speed (13,000–15,000 rpm) for 2 minutes, and remove the supernatant. We resuspend the pellet in 50 μL of water (HPLC grade) and heat it for 10 minutes at 95°C in a thermoblock. When the sample has cooled, we add 1200 μL of absolute alcohol previously stored in a freezer (−18°C/−20°C) and the mixture is centrifuged at the maximum speed (13,000–15,000 rpm) for 2 minutes. Subsequently we remove all the supernatant and leave the pellet to dry for 5 minutes with the tube open to remove the ethanol completely.

Using the tip of a small spatula we add silica beads (0.5 mm zirconia/silica beads) and 20 μL of pure acetonitrile and mix well for one minute in vortex. We then add 20 μL of 70% formic acid and centrifuge this at the maximum speed (13,000–15,000 rpm) for 2 minutes. From each sample, 1 μL of supernatant is placed in three of the 96 spots of the steel target plate (Bruker) and is allowed this to dry at room temperature. Finally, we add 1 μL of HCCA matrix solution (α-cyano-4-hydroxycinnamic acid) and leave this to dry before further analysis by MALDI-TOF MS. Each sample is analyzed in triplicate, using the highest score for further analysis.

2.3. MALDI-TOF MS Analysis. Spectra are acquired in a linear positive ion mode at a laser frequency of 60 Hz across a mass/charge ratio (m/z) of 2,000 to 20,000 Da using the Microflex LT MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany). The protein profile is obtained by the software FlexControl 3.3 (Bruker Daltonik GmbH, Bremen, Germany) and analyzed by the program FlexAnalysis 3.3 (Bruker Daltonik GmbH, Bremen, Germany). The Mycobacteria Library v 1.0 is used, containing 173 mycobacterial protein profiles, representing 94 species. We consider a range of between 2.0 and 3.0 as acceptable, and scores between 1.6 and 2.0 are considered consistent when the same identification is repeated in most of the 10 possibilities provided

Table 1: Table of results.

	GenoType	MALDI-TOF	Score		GenoType	MALDI-TOF	Score	Sequencing
1	*M. avium*	*M. avium*	2.078	34	*M. fortuitum*	*M. fortuitum*	2.157	
2	*M. avium*	*M. avium*	1.657	35	*M. fortuitum*	*M. fortuitum*	1.877	
3	*M. avium*	*M. avium*	1.932	36	*M. fortuitum*	*M. porcinum*	2.222	
4	*M. avium*	*M. avium*	2.041	37	*M. chelonae*	*M. chelonae*	2.307	
5	*M. avium*	*M. avium*	1.626	38	*M. chelonae*	*M. chelonae*	1.667	
6	*M. avium*	*M. avium*	2.101	39	*M. chelonae*	*M. chelonae*	1.857	
7	*M. avium*	*M. avium*	2.100	40	*M. chelonae*	*M. chelonae*	2.064	
8	*M. avium*	*M. avium*	2.038	41	*M. chelonae*	*M. chelonae*	1.715	
9	*M. avium*	*M. avium*	2.009	42	*M. abscessus*	*M. abscessus*	2.107	
10	*M. avium*	*M. avium*	2.038	43	*M. abscessus*	*M. abscessus*	2.060	
11	*M. avium*	*M. avium*	1.858	44	*M. abscessus*	*M. abscessus*	2.320	
12	*M. avium*	*M. avium*	2.191	45	*M. abscessus*	*M. abscessus*	2.198	
13	*M. avium*	*M. avium*	2.100	46	*M. mucogenicum*	*M. phocaicum*	2.264	
14	*M. avium*	*M. avium*	2.180	47	*M. mucogenicum*	*M. phocaicum*	2.027	
15	*M. avium*	*M. avium*	2.003	48	*M. mucogenicum*	*M. phocaicum*	2.150	
16	*M. avium*	*M. avium*	2.009	49	*M. kansasii*	*M. kansasii*	1.600	
17	*M. intracellulare*	*M. intracellulare*	2.168	50	*M. kansasii*	*M. kansasii*	1.891	
18	*M. intracellulare*	*M. intracellulare*	2.217	51	*M. kansasii*	*M. kansasii*	2.199	
19	*M. intracellulare*	*M. intracellulare*	1.600	52	*M. kansasii*	*M. kansasii*	2.257	
20	*M. intracellulare*	*M. intracellulare*	2.005	53	*M. gordonae*	*M. gordonae*	1.914	
21	*M. intracellulare*	*M. intracellulare*	2.182	54	*M. gordonae*	*M. gordonae*	2.232	
22	*M. intracellulare*	*M. intracellulare*	2.148	55	*M. gordonae*	*M. gordonae*	2.096	
23	*M. intracellulare*	*M. intracellulare*	2.275	56	*M. gordonae*	*M. gordonae*	2.274	
24	*M. intracellulare*	*M. intracellulare*	2.272	57	*M. lentiflavum*	*M. lentiflavum*	2.171	
25	*M. intracellulare*	*M. intracellulare/Ch*[1]	2.016	58	*M. lentiflavum*	*M. lentiflavum*	2.205	
26	*M. intracellulare*	*M. intracellulare*	2.369	59	*M. lentiflavum*	*M. lentiflavum*	2.278	
27	*M. intracellulare*	*M. intracellulare*	2.057	60	*M. scrofulaceum*	*M. scrofulaceum/para*[2]	**1.985**	
28	*M. intracellulare*	*M. intracellulare/Ch*[1]	1.730	61	*M. scrofulaceum*	*M. scrofulaceum/para*[2]	**1.900**	
29	*M. intracellulare*	*M. intracellulare*	2.050	62	*M. peregrinum*	*M. peregrinum*	2.056	
30	*M. fortuitum*	*M. fortuitum*	2.376	63	*M. marinum*	*M. marinum*	2.046	
31	*M. fortuitum*	*M. fortuitum*	2.169	64	*M. gastri*	*M. gastri*	**2.270**	
32	*M. fortuitum*	*M. fortuitum*	2.643	65	No mycobacteria	*M. elephantis*	1.744	*elephantis*
33	*M. fortuitum*	*M. fortuitum*	2.223	66	*Mycobacterium* sp.	Unidentified		*duvalii*

[1] *M. intracellulare/M. chimaera*.
[2] *M. scrofulaceum/parascrofulaceum*.

by the project. Lower scores (1.7-1.6) are reported to give a correct identification [18–20].

2.4. GenoType Mycobacterium CM/AS. GenoType *Mycobacterium* CM/AS is a commercial DNA strip assay used for the identification of mycobacteria. These two strips, CM ("Common Mycobacteria") and GenoType AS ("Additional Species"), are designed to identify different patterns, of which 23 patterns can be assigned to single species and 8 patterns are allocated to two or more *Mycobacterium* species.

2.5. Molecular Identification in Mycobacterium Reference Laboratory. Strains sent to the *Mycobacterium* Reference Laboratory were identified by typing by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis with BstEII and HaeIII restriction enzymes of gene hsp-65 [21], using the updated band patterns that can be found on the web page (http://app.chuv.ch/prasite/index.html), and sequencing and analysis of gene 16S rRNA.

2.6. Statistical Analysis. To study the degree of concordance between the two methods we used the Spearman correlation and the Kappa coefficient. A $P < 0.05$ was considered statistically significant. The analyses were done with PSPP (0.8.3) for Mac.

We have considered the identifications of species closely related inside some complexes to be concordant: *M. phocaicum-mucogenicum, M. porcinum-fortuitum, M. intracellulare-chimaera*, and *M. parascrofulaceum-scrofulaceum*.

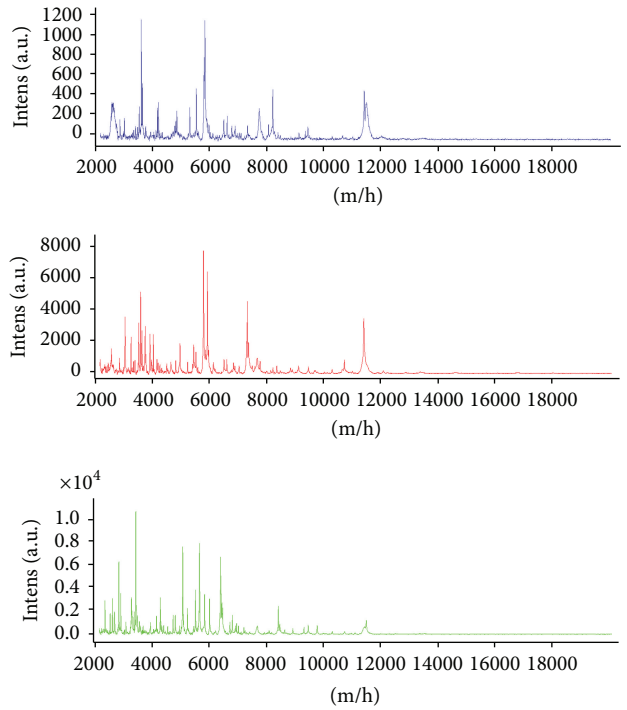

Figure 1: Protein profile of the most common species isolated (*M. intracellulare, M. avium,* and *M. fortuitum*).

3. Results

We obtained result with MALDI-TOF in 65 and with Genotype in 64 of the 66 MNT which were studied.

MALDI-TOF MS generated acceptable confidence scores (score > 2.000 or >1.600 if the same species was repeated in the 10 possibilities given by the project) for 65 (98.5%) isolates: 16 *M. avium,* 13 *M. intracellulare,* 6 *M. fortuitum,* 5 *M. chelonae,* 4 *M. abscessus*, 3 *M. phocaicum*, 4 *M. kansasii,* 4 *M. gordonae,* 3 *M. lentiflavum,* 2 *M. parascrofulaceum,* 1 *M. peregrinum,* 1 *M. porcinum,* 1 *M. marinum,* 1 *M. gastri*, and 1 *M. elephantis*. This included 49 (74.2%) identified with a score > 2.000 and 17 (25.7%) with a score between 1.600 and 2.000.

Scores obtained for the more frequently isolated species were *M. avium* (75% > 2.000, 87.5% > 1.800), *M. intracellulare* (84.6% > 2.000), *M. fortuitum* (85.7% > 2.000, 100% > 1.800), *M. chelonae* (40% > 2.000, 60% > 1.800), *M. abscessus* (100% > 2.000), and *M. kansasii* (50% > 2.000, 75% > 1.800).

Protein profile of the most common species isolated is represented in Figure 1.

The results obtained by GenoType were 16 *M. avium*, 13 *M. intracellulare*, 7 *M. fortuitum*, 5 *M. chelonae*, 4 *M. abscessus*, 3 *M. phocaicum*, 4 *M. kansasii*, 4 *M. gordonae* 3 *M. lentiflavum*, 2 *M. scrofulaceum*, 1 *M. peregrinum*, 1 *M. marinum*, 1 *M. gastri*, and 1 *Mycobacterium* genus; in one no *Mycobacterium* genus was found. This strain was identified as *M. elephantis* by MALDI-TOF (score 1.744) and also by sequencing at the *Mycobacterium* Reference Laboratory. One strain identified as *Mycobacterium* genus by GenoType was not recognized by MALDI-TOF and was identified as *M. duvalii* by the sequencing method at the *Mycobacterium* Reference Laboratory.

The strains identified using MALDI-TOF as *M. phocaicum* were identified as *M. mucogenicum* by GenoType. A strain identified with MALDI-TOF as *M. porcinum* was identified as *M. fortuitum* by GenoType. Two strains were identified by MALDI-TOF as *M. intracellulare/M. chimaera* (both identifications alternating in all the results [10] of each project) and recognized by GenoType as *M. intracellulare*. Two strains were identified by MALDI-TOF as *M. scrofulaceum/parascrofulaceum* (both identifications alternating in all the results [10] of each project) and identified by GenoType as *M. scrofulaceum*. The results are shown in Table 1.

The concordance analysis between the two methods (MALDI-TOF and GenoType) showed agreement in 64 of the 66 cases (96.9%). In one case no identification was made with either of the two methods and the other was identified as *M. elephantis* by MALDI-TOF but was not identified by GenoType. The Spearman correlation was 0.997 ($P < 0.001$) and the Kappa value was 0.965 ($P < 0.001$).

4. Discussion

The usefulness of MALDI-TOF for the identification of common bacteria has already been demonstrated [12, 13, 22, 23]. However, its main aim is to improve the identification of fungi (yeast and filamentous fungi) and mycobacteria, which have traditionally been the most difficult microorganisms to identify.

Molecular methods for the study of mycobacteria have resulted in great improvement in species identification and have permitted the description of new species. However, these techniques are not available in all laboratories because of their cost and complexity. Whilst commercial hybridization methods are available in most laboratories, their capacity to identify different NTM species is limited (fewer than 40 species), though there are more than 150 recognized species of NTM. Infections produced by mycobacteria are still a great problem, particularly tuberculosis, as are those caused by NTM, which mainly affect children, immunosuppressed persons, and patients with other pathologies like cystic fibrosis. The MALDI-TOF can provide laboratories with a new technique for the identification of mycobacteria, especially given its simplicity and the large number of species included in its database.

Although the number of strains in our study was not large (66) and the number of species was small (15), our results show that identification using MALDI-TOF is correct in the majority of clinically relevant strains of NTM (*M. avium, M. intracellulare, M. abscessus, M. chelonae, M. fortuitum, M. mucogenicum, M. kansasii,* and *M. scrofulaceum*), showing a good agreement with GenoType (96, 9%).

In the three isolates that were identified by GenoType as *M. mucogenicum*, the result obtained by MALDI-TOF was *M. phocaicum*, which cannot be identified by GenoType. *M. phocaicum* belongs to the *Mycobacterium mucogenicum* group (*M. mucogenicum*, *M. phocaicum*, *M. aubagnense*, and *M. llatzerense*) [14, 24, 25], which has such genetic

similitude as to make it difficult to distinguish one from the other, even using sequencing techniques [26]. One of the strains identified by GenoType as *M. fortuitum* was identified by MALDI-TOF as *M. porcinum*, a species that GenoType is unable to identify. This species belongs to the group known as *Mycobacterium fortuitum* (*M. fortuitum, M. peregrinum, M. senegalese, M. mageritense, M. septicum, M. alvei, M. houstonense, M. boenickei, M. conceptionense, M. porcinum, M. neworleansense*, and *M. brisbanense*) [25]. Some studies have shown almost complete phenotypic and molecular identity between clinical isolates of the *M. fortuitum* group and strains of *M. porcinum* [27]. Two strains were identified by MALDI-TOF as *M. intracellulare/M. chimaera* (both identifications alternating in all the results [10] of each project), being identified by GenoType as *M. intracellulare*. This finding has been described previously because they are two closely related organisms, differing by only 1 base pair in their 16S RNA gene regions [28, 29]. Thus, the ambiguity encountered when these organisms are identified by MALDI-TOF is not surprising. Moreover, it is not clear whether differences exist in the pathogenicity of these two species [18]. Two strains were identified by MALDI-TOF as *M. scrofulaceum/parascrofulaceum* (both identifications alternating in all the results [10] of each project), being identified by GenoType as *M. scrofulaceum.* Bruker's mycobacteria Library v 1.0 shows difficulties in the distinction between *M. scrofulaceum* and *M. parascrofulaceum*, likely due to the similarity in the protean profile. These mycobacteria show a similar antibiotic sensitivity profile, so differentiation could be irrelevant in a clinical context [14, 24]. In one strain identified as *M. elephantis* by MALDI-TOF the score was low (1.744), but the species identification was repeated in all the trials and was confirmed in the Reference Laboratory by sequencing. The only strain not identified by MALDI-TOF was identified in the Reference Laboratory as *M. duvalii,* probably because this species is not included in the mycobacteria Library v 1.0.

In most cases identification with MALDI-TOF generated a good score (74.5% > 2.000), especially in species with greater clinic relevance: *M. avium* (75% > 2.000, 87.5% > 1.800), *M. intracellulare* (84.6% > 2.000), *M. abscessus* (100% > 2.000), and *M. kansasii* (50% > 2.000, 75% > 1.800). In our opinion, it is important to evaluate both the score and the repetition of the result.

Previous studies have demonstrated the potential of MALDI-TOF for accurate *Mycobacterium* identification, both *M. tuberculosis* complex and NTM [6, 8, 15, 16, 18, 30]. Some authors found differences in the correct identification percentage according to the culture media from which the strains were obtained [6, 8, 30], the culture growth time [14], the extraction protocol applied to the mycobacteria [6, 15, 30], or the library used [6]. Our results were obtained from isolates on Lowenstein media, most of them recent cultures, though late growth cultures were used in some cases, which could explain the worse quality spectra.

There is no doubt that the appropriate extraction protocols will be optimized, especially in liquid culture media, in order to reduce the time to diagnosis. There is now a new Mycobacteria Library (v 2.0), but it is still necessary to optimize and update it with new species, and it should be able to discriminate the different species of the *M. Tuberculosis Complex*, which is not possible at present.

To date, NTM species level identification has been limited to specialized laboratories. The MALDI-TOF offers quick results, is easy to perform, and involves a low cost for reagents. Also, the procedure involves little handling and few working hours, which reduces the potential risk of acquiring infections caused by mycobacteria in the laboratory.

Our study supports the idea that MALDI-TOF is a suitable, reliable, and fast technique for identification of NTM. However, it is important to standardize the procedure in liquid media inoculated with clinical specimens to reduce the time to diagnosis. Accordingly, further study is required to validate these results in clinical practice.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Species Identification and Clarithromycin Susceptibility Testing of 278 Clinical Nontuberculosis Mycobacteria Isolates

Wenjuan Nie,[1] Hongfei Duan,[1] Hairong Huang,[2] Yu Lu,[3] and Naihui Chu[1]

[1]*Department of Tuberculosis, Beijing Chest Hospital, Capital Medical University, Tongzhou District, Beijing 101149, China*
[2]*Reference Lab, Beijing Chest Hospital, Capital Medical University, Tongzhou District, Beijing 101149, China*
[3]*Department of Pharmacology, Beijing Chest Hospital, Capital Medical University, Tongzhou District, Beijing 101149, China*

Correspondence should be addressed to Hairong Huang; hairong.huangcn@gmail.com and Naihui Chu; chunaihui1994@sina.com

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Purpose of this paper is to analyze different species' proportion of nontuberculosis mycobacteria (NTM) and susceptibility to clarithromycin of different species. 278 clinical NTM isolates were identified into species by using 16S rRNA, *rpoB* and *hsp65*. Then clarithromycin susceptibility testing against different species was done separately, using microplate Alamar Blue assay. Finally, resistance isolates' *erm(41)* of *M. abscessus* were sequenced in order to analyze mechanisms for clarithromycin resistant. In this test, 131 isolates (47%) belonged to *M. avium* complex (MAC), and 70 isolates (25%) belonged to *M. abscessus*. Nearly all the *M. abscessus* subsp. *abscessus* resistant to clarithromycin had T28 in *erm(41)*. However, all the *M. abscessus* subsp. *abscessus* susceptible to clarithromycin had C28 in *erm(41)*. In this study, we find that MAC was the most common pathogens of NTM, and the second one was *M. abscessus*. However, *M. chelonei, M. fuerth*, and *M. gordon* were rare. Clarithromycin had a good inhibition activity against all the NTM species except *M. abscessus* subsp. *abscessus*. The *erm(41)* genotype is of high relevance to clarithromycin resistance.

1. Introduction

Nontuberculosis mycobacteria (NTM) are globally recognized as pathogens especially in immunocompromised population including HIV/AIDS (Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome) patients. In addition to causing infections in immune-compromised patients, this group of organisms has been reported to be increasingly recognized as pathogens from immunocompetent individuals as well [1]. Different species have different susceptibility to antibiotics, so it is important to identify NTM into different species in order to make the respective therapy plan. The American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA) recommended treatment for NTM pulmonary and disseminated disease is a macrolide-containing, multidrug regimen. And clarithromycin, a macrolide drug, is described as the key therapy for NTM diseases [2]. Clarithromycin forms the cornerstone of NTM treatment and is the only drug recommended by the Clinical and Laboratory Standards Institute (CLSI) for drug susceptibility testing (DST) [3]. However, antibiotic treatment of pulmonary *M. abscessus* infections remains problematic. Infections due to the *M. abscessus* group are difficult to treat because these mycobacteria are intrinsically resistant not only to the classical antituberculous drugs but also to most of the antibiotics that are currently available. Clarithromycin had been the drug of choice for *M. abscessus* infections for the past 20 years before a resistance gene, *erm(41)*, was described in *M. abscessus*. The discovery of a novel inducible *erm* gene, *erm(41)*, provides an explanation for the lack of efficacy for macrolides against *M. abscessus* infections [4]. A polymorphism in *erm(41)* (T or C) was described at position 28 and its association with the susceptibility was examined. Our objective was to identify NTM isolates into different species and to do the DST separately and then to seek the presence of *erm(41)* in a large collection of clinical strains precisely identified as *M. abscessus* and finally to correlate these results with clarithromycin susceptibility.

2. Materials and Methods

2.1. Species Identification. All of the 278 clinical strains in Reference Lab of Beijing Chest Hospital were isolated from sputum samples of patients, which were previously identified as NTM strain using growth experiments of p-nitrobenzoic acid (PNB) by Reference Lab of Beijing Chest Hospital. All the strains were subcultured on the Lowenstein-Jensen medium at 37°C to observe colony morphology. The incubation time ranged from 4 to 28 days. Then colonies were used for species identification based on 16S rRNA sequence analysis. Preparation of the PCR reaction mixture and amplification were done as described previously [5, 6]. PCR products were purified and sequenced by the BGI corporation, using forward and reverse primers. Both strands were sequenced as a crosscheck. Species identification of these strains was accomplished by the sequencing of 16S rRNA, using BLAST search to measure the similarities.

2.2. Drug Susceptibility Testing. We performed DST of NTM isolates by broth microdilution using 96-well plates, which is a reference method as recommended by CLSI [3]. DST of these NTM isolates was done as described previously [6]. To *M. abscessus*, plates were submitted to an extended incubation of 14 days' incubation at 30°C. However, to other NTM isolates, the incubation time ranged from 3 to 10 days. A color change from blue to pink indicates bacterial growth. MIC was defined as the lowest concentration of the drug that showed no color change, which was the lowest concentration of drug capable of inhibiting the visible growth of tested isolates. MIC_{50} and MIC_{90} values were defined as drug concentrations, which inhibit 50% and 90% of isolates, respectively. Susceptibility was evaluated according to CLSI breakpoint recommendations [3]. The type strains of American Type Culture Collection (ATCC) were used for comparison.

2.3. Clarithromycin Resistant Mechanism. Crude DNA preparations suitable for PCR were obtained by heating suspensions of mycobacteria in Tris-EDTA buffer at 100°C for 15 to 20 min. Debris was removed from the preparations by centrifugation at 16,000 rpm for 3 min. The basic 40 μL PCR mixture comprised 2 μL DNA, 2 μL DNA polymerase, and 1 μL each primer and finally was complete to 40 μL by double distilled water. Reactions were run for 3 min at 94°C, followed by 30 cycles at 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min. For cloning of *erm(41)*, gene segments were amplified with primers 5-ACG TTG GAT CCG AGC GCC GTC ACA AGA TGC ACA-3 and 5-GCG AGA AGC TTG ACT TCC CCG CAC CGA TTC CAC-3. The resulting amplification products were purified and sequenced by the BGI corporation, using forward and reverse primers. Both strands were sequenced as a crosscheck. The sequence results were compared with *M. abscessus* standard strains.

3. Result

3.1. Species Identification. Of all the 278 NTM clinical strains (Table 1), 100 (36%) isolates belonged to rapidly growing *Mycobacterium* (RGM), and 178 (64%) isolates belonged to slowly growing *Mycobacterium* (SGM). Among these species, 70 isolates (25%) belonged to *M. abscessus*, 23 isolates (8%) belonged to *M. fortuitum*, 5 isolates (2%) belonged to *M. chelonei*, 2 isolates (1%) belonged to *M. fuerth*, 131 isolates (47%) belonged to *M. avium* complex (MAC), 34 isolates (12%) belonged to *M. kansasii*, and 13 isolates (5%) belonged to *M. gordon*. Of the 70 *M. abscessus* strains, according to *rpoB* and *hsp65*, 45 strains (64%) belonged to *M. abscessus* subsp. *abscessus*, and 25 strains (36%) belonged to *M. abscessus* subsp. *massiliense*.

Table 1: The result of NTM species identification.

	Species	Number (%) of strains
RGM $n = 100$ (36%)	*M. abscessus*	70 (25%)
	M. fortuitum	23 (8%)
	M. chelonei	5 (2%)
	M. fuerth	2 (1%)
SGM $n = 178$ (64%)	MAC	131 (47%)
	M. kansasii	34 (12%)
	M. gordon	13 (5%)
Total $n = 278$ (100%)	NTM	278 (100%)

All the 70 *M. abscessus* isolates were divided into two subspecies, *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* (Table 2). There was no *M. abscessus* subsp. *bolletii* strain observed. There were two ways to identify subspecies: sequencing *rpoB* PCR-restriction fragments and sequencing *hsp65* PCR-restriction fragments. Results of these two methods were the same (*M. abscessus* subsp. *abscessus*, 64%; *M. abscessus* subsp. *massiliense*, 36%), which means that all the *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* strains divided by *rpoB* sequence were all verified by *hsp65* sequence. There was no discordant result between *rpoB* and *hsp65* sequences analysis. The *rpoB* similarity of *M. abscessus* subsp. *abscessus* to the reference sequences was 98% to 100%, and the *hsp65* similarity was 98% to 100%. The *rpoB* similarity of *M. abscessus* subsp. *massiliense* to the reference sequences was relatively low, 97% to 100%, and the *hsp65* similarity was 98% to 100%.

3.2. Drug Susceptibility Testing. The breakpoint, MIC range, MIC_{50}, MIC_{90}, and proportions of susceptible, moderately susceptible, and resistant strains of each drug against NTM are shown in Table 3, respectively. As RGM, *M. fortuitum* should be incubated for 3 days (CLSI recommends), and then the MIC value is read. In Table 3, it shows that most of *M. fortuitum* (92%) strains were susceptible to clarithromycin, and the resistant proportion was low (4%). Although *M. abscessus* belong to RGM, the new CLSI guideline extended the incubation time to 14 days, which is the shortest time CLSI recommends for final reading. In this test, *M. abscessus* was divided into different subspecies, *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense*, and the DST was done separately. It appeared that most of the *M. abscessus* subsp. *massiliense* strains are susceptible to clarithromycin, whereas susceptibility was less clear for *M. abscessus* subsp. *abscessus*, with most strains being resistant

Table 2: Each subspecies' proportion identified by using *rpoB* and *hsp65*.

Sequence analysis	Number (%) of *M. abscessus* subsp. *abscessus*	Number (%) of *M. abscessus* subsp. *massiliense*	Total number (%)
rpoB	45 (64%)	25 (36%)	70 (100%)
hsp65	45 (64%)	25 (36%)	70 (100%)

Table 3: Clarithromycin susceptibility testing result of different species.

Subspecies	Incubation days	Susceptible breakpoint	Moderately susceptible breakpoint	Resistant breakpoint	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Number (%) of susceptible strains	Number (%) of moderately susceptible strains	Number (%) of resistant strains
M. abscessus subsp. *abscessus* ($n = 45$)	14	≤2	4	≥8	1–>32	>32	>32	5 (10%)	3 (7%)	37 (83%)
M. abscessus subsp. *massiliense* ($n = 25$)	14	≤2	4	≥8	≤0.0625–>32	0.25	0.25	24 (96%)	0 (0%)	1 (4%)
M. fortuitum ($n = 23$)	3	≤2	4	≥8	≤0.0625–>32	0.5	4	21 (92%)	1 (4%)	1 (4%)
MAC ($n = 131$)	7	≤8	16	≥32	≤0.0625–>32	0.5	8	124 (95%)	1 (1%)	6 (4%)
M. kansasii ($n = 34$)	7	≤16	—	>16	≤0.0625–16	≤0.0625	1	34 (100%)	—	0 (0%)

Table 4: Clarithromycin susceptibility testing result of *M. chelonei* and *M. fuerth*.

Species	Incubation days	Isolate's number	MIC value	DST result
M. chelonei ($n = 5$)	3	2	≤0.0625	Susceptible
		3	≤0.0625	Susceptible
		13	≤0.0625	Susceptible
		14	0.125	Susceptible
		17	≤0.0625	Susceptible
M. fuerth ($n = 2$)	3	12	0.25	Susceptible
		25	≤0.0625	Susceptible

and others susceptible. In Table 3, it shows that most *M. abscessus* subsp. *massiliense* (96%) strains were susceptible to clarithromycin, and the resistant proportion was low (4%). However, the other subspecies, *M. abscessus* subsp. *abscessus*, had an entirely different result. Most of *M. abscessus* subsp. *abscessus* (83%) strains were resistant to clarithromycin, and there were only 10% susceptible to it. As SGM, MAC had the biggest proportion of isolates, and the number was 131 isolates. Similarly, most MAC (95%) were susceptible to clarithromycin, and the resistant proportion was low (4%). *M. Kansasii* had the highest susceptible proportion. All of them (100%) were susceptible to clarithromycin. The sample size of *M. chelonei* and *M. fuerth* was small, so it is inappropriate to calculate the MIC_{50}, MIC_{90}, and proportions of susceptible, moderately susceptible, and resistant strains. Table 4 displays each isolate's MIC value of *M. chelonei* and *M. fuerth*. All of them were susceptible to clarithromycin.

3.3. Clarithromycin Resistant Mechanism. erm(41) genes in subspecies of *M. abscessus* showed characteristic differences. Compared with its homologues in *M. abscessus* subsp. *abscessus*, the *erm(41)* in *M. abscessus* subsp. *massiliense* maybe dysfunctional due to deletion of nucleotides. For 49 isolates, the *erm(41)* gene segment was sequenced successfully. Nine isolates belonged to *M. abscessus* subsp. *massiliense*, and all the *erm(41)* of which were incomplete. The rest 40 isolates belonged to *M. abscessus* subsp. *abscessus*, and all the *erm(41)* of which were complete. After analyzing these *erm(41)* gene segments, it showed that (Table 5) except 1 *M. abscessus* subsp. *abscessus* isolate which had C28 in *erm(41)*, nearly all the *M. abscessus* subsp. *abscessus* strains resistant to clarithromycin had T28 in *erm(41)*. However, all the *M. abscessus* subsp. *abscessus* strains susceptible to clarithromycin had C28 in *erm(41)*. It shows that *erm(41)* sequence differentiated these two subspecies, and its specific features were predictive for clarithromycin susceptibility or resistance.

4. Discussion

4.1. Species Identification. The proportions of different species are variable, according to geographical distribution. Geographical variation occurs in the pattern of NTM isolation across different countries. *M. fortuitum* was the commonest species reported from Iran and Turkey whereas MAC predominated most of the European countries and Brazil. In Korea, *M. abscessus* is the second most common pathogen responsible for lung disease caused by NTM, following the MAC [7]. This is the first study to focus primarily on the clinical relevance of the species

Table 5: The relationship between clarithromycin's resistance and *erm(41)*.

Subspecies	MIC value of day 14 (μg/mL)	Number	DST result	The base at size 28 of *erm(41)*
M. abscessus subsp. *abscessus* ($n = 40$)	>32	$n = 34$	R	T
	8	$n = 1$	R	C
	1–4	$n = 5$	S	C

differentiation among NTM in Beijing, and the largest study of its kind, with almost 278 patients' sputum samples included. In this test, SGM had the larger proportion of NTM than RGM. Among SGM, MAC had the largest proportion in NTM. Nearly half of NTM belonged to MAC. And the second-largest proportion of NTM was *M. abscessus*. Nearly a quarter of NTM belonged to *M. abscessus*. This result is similar to Korea's [8]. *M. chelonei*, *M. fuerth,* and *M. gordon*'s proportion was small. There were lots of special species, including *M. chelonei*, *M. fuerth,* and *M. gordon*, which had only few amounts, so it is difficult to calculate and analyze the characteristics of these rare species *in vitro*. In the future we should collect more samples to assess these special species in order to guide clinical treatment.

Two new *M. abscessus*-related species, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii*, were identified, which were previously grouped with *M. abscessus* [5]. Among 40 patients monitored at the National Institutes of Health (Bethesda, MD), the prevalence of *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* was 28% and 5%, respectively [9]. In the Netherlands, 21% of 39 clinical isolates of *M. abscessus* were identified as *M. abscessus* subsp. *massiliense* and 15% were *M. abscessus* subsp. *bolletii* [10]. In France, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* accounted for 22 and 18% of 50 patients with cystic fibrosis infected with *M. abscessus*, respectively [11]. In Korea, *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* are isolated in almost equal numbers among *M. abscessus* infections, whereas *M. abscessus* subsp. *bolletii* is rare. A previous study showed that nearly half (47%) of all *M. abscessus* clinical isolates were identified as *M. abscessus* subsp. *massiliense*, although the prevalence of *M. abscessus* subsp. *bolletii* was low (2%) [5]. Our test has the same result as Korea's. In this test, more than half *M. abscessus* belonged to *M. abscessus* subsp. *abscessus* and a little less than a half belonged to *M. abscessus* subsp. *massiliense*. There was no one *M. abscessus* subsp. *bolletii* observed in our test, which means that *M. abscessus* subsp. *bolletii* accounts for a very small proportion of *M. abscessus*. Differentiation among *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* is challenging for clinical microbiology laboratories. Recent work proved the inaccuracy of single-target sequencing for separating these three taxa within the *M. abscessus* group. In some strains, additional housekeeping genes were analyzed because of the discordant results between *rpoB* and *hsp65* genes analysis [5, 12]. Sequencing of more other targets (such as *secA1*, *soda,* and ITS), combined with phylogenomic analysis, has been shown to increase identification accuracy among these different taxa [8]. More recently, a multilocus sequence analysis targeting 8 housekeeping genes and a multispacer sequence analysis were reported [13, 14]. However, all of these methods require genomic sequencing, which is relatively costly and time consuming and may not be available in all clinical microbiology laboratories.

4.2. Drug Susceptibility Testing. NTM infection is a refractory disease that, unlike tuberculosis, does not respond well to conventional antituberculosis drug. With the progress of mycobacterial taxonomy and speciation, it appeared that antibiotic susceptibility of NTM is tightly related to mycobacterial species and, conversely, that a homogenous intrinsic susceptibility pattern may reflect the homogeneity of the species [2, 7]. Since many new species have been described recently with the assistance of molecular biology and genomics, we need to assess their intrinsic susceptibility patterns species by species. The macrolide clarithromycin is considered a cornerstone in antimicrobial chemotherapy of NTM infections. Clarithromycin is a 14-membered ring macrolide that binds to the large ribosomal subunit in the vicinity of the peptidyl transferase center and inhibits protein synthesis, which results in the arrest of bacterial growth. Clarithromycin is given orally and is highly active against many species of NTM. It is the only drug of demonstrated efficacy that can be administered orally. In all of the studies so far, most variations were reported in the clarithromycin susceptibility results within different NTM species even with strains precisely identified and even with reference strains [5, 15, 16]. For example, *M. abscessus* subsp. *bolletii* was shown to be clarithromycin resistant by Adékambi and Drancourt [15] and susceptible by Leao et al. [16]. NTM species show a characteristically different response to clarithromycin from others, and precise identification of these species would be important for the treatment of infected patients. This is why we would like to propose that clarithromycin susceptibility be assessed on a molecular basis for all new cases of *M. abscessus* group infections and that the outcomes of clarithromycin therapy be observed as follows. In this study, we evaluated clarithromycin's activity against different NTM species, since this is the single drug CLSI recommended in *in vitro* testing. The CLSI recommends the use of the radiometric BACTEC 460, the broth-based macrodilution, or the broth microdilution method for the testing of NTM susceptibility to clarithromycin, and it requires weeks to complete from the time of sample collection because of the growing rate of organism.

As the most common NTM species, the treatment outcome of MAC pulmonary disease was disappointing before the use of newer macrolide antibiotics such as clarithromycin and azithromycin. Since 1990 introduction of

newer macrolide antibiotics, treatment of pulmonary MAC disease has improved patient outcome. The ATS and IDSA recommend that a three-drug regimen be utilized for the treatment of MAC infection, including a macrolide (clarithromycin or azithromycin). The macrolides are the only class of drugs for which a correlation has been demonstrated between *in vitro* susceptibility results and *in vivo* clinical response in MAC disease (Griffith, 2007). For this reason, the ATS only recommends routine susceptibility testing for clarithromycin, the macrolide most commonly used for treating MAC disease (Griffith, 2007). In this test clarithromycin showed an excellent inhibition activity against MAC. 95% MAC were susceptible to it. And the resistance proportion was low. Considering the big sample size of 131 isolates, this result again proves clarithromycin's good inhibition activity against MAC. However, ATS guidelines also reported that, in contrast to *Mycobacterium tuberculosis*, DST maybe of little use for the treatment of MAC infection, although clarithromycin is the only drug for which the results of susceptibility testing should be taken into consideration in the treatment of MAC infection [2]. We need to conduct a prospective clinical study in order to compare efficacy *in vivo* and susceptibility data *in vitro*.

As the second-largest NTM species and one of the most notorious causative agents of disease, drug therapy of *M. abscessus* disease is long, costly, resistant to many antibiotics, and often associated with drug-related toxicities, leading to unsatisfactory treatment results. In the 1990s, clarithromycin became the drug of choice for *M. abscessus* infections, and therapeutic successes were reported. Combination therapy of intravenous amikacin with cefoxitin or imipenem and an oral macrolide has been recommended by the ATS/IDSA and many other experts [2]. However, treatment response rates are not satisfactory and optimal therapeutic regimens and treatment durations are not well established. Two new *M. abscessus*-related species, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii*, were identified, which were previously grouped with *M. abscessus*, so the previous *M. abscessus* now includes *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense,* and *M. abscessus* subsp. *bolletii*. But the data regarding *in vitro* DST results of these new subspecies are limited. It is reported that *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* have different clinical characteristics and treatment responses *in vivo* and have different DST result *in vitro* [12, 17]. For instance, an experiment in Japan shows that with *M. abscessus* subsp. *abscessus* bronchiectasia may occur more frequently than with *M. abscessus* subsp. *bolletii in vitro*, and the resistant proportion of imipenem against *M. abscessus* subsp. *abscessus* is lower than *M. abscessus* subsp. *bolletii* [17]. An experiment in Korea shows that *M. abscessus* subsp. *abscessus* may have a worse radiographic response on HRCT and microbiologic response than *M. abscessus* subsp. *bolletii in vitro*, and similarly, the resistant proportion of imipenem against *M. abscessus* subsp. *abscessus* is lower than *M. abscessus* subsp. *bolletii* [12]. In our test, it shows that *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* displayed different susceptibility to clarithromycin. Most of *M. abscessus* subsp. *abscessus* strains were resistant to clarithromycin. However, *M. abscessus* subsp. *massiliense*'s MIC to clarithromycin is small, and nearly all of the isolates were susceptible to it. From a clinical standpoint, our study has some important therapeutic implications. The inducible resistance to clarithromycin of *M. abscessus* subsp. *abscessus* isolates means that it can be much more difficult to treat *M. abscessus* subsp. *abscessus* lung disease. A much longer duration of intravenous antibiotic therapy and more effective oral antibiotics may be needed to improve treatment outcomes in *M. abscessus* subsp. *abscessus* lung disease. The low MIC for clarithromycin and the absence of inducible resistance to clarithromycin suggest that *M. abscessus* subsp. *massiliense* lung disease may be more effectively treated with a clarithromycin-based antibiotic regimen [12].

M. kansasii is the second most common cause of NTM disease in the United States. In our test it is the third-largest species, but the sample size is small. In this test, *M. kansasii* showed an excellent susceptibility to clarithromycin. All the *M. kansasii* isolates were susceptible to it. However, according to ATS and IDSA, routine susceptibility testing of *M. kansasii* isolates is recommended for rifampin only, and the recommended treatment of *M. kansasii* pulmonary disease does not include clarithromycin. But the excellent *in vitro* activity of clarithromycin against *M. kansasii* suggests that these agents may be useful in retreatment regimens. *M. fortuitum* infections are rare, and they are usually susceptible to multiple oral antimicrobial agents. In this test, *M. fortuitum* showed a good susceptibility to clarithromycin. All of the *M. fortuitum* isolates were susceptible to clarithromycin, except 1 isolate resistant to it. *In vitro* susceptibility data are limited because of the extreme fastidiousness of the organism [2]. Optimal therapy is not determined, but ATS and IDSA also recommend that multidrug therapies including clarithromycin appear to be more effective than those without clarithromycin. *M. chelonei* and *M. fuerth* are extremely rare species. In our test, isolates of these two species are very few, so Table 4 only shows every isolate's MIC value. In Table 4, it shows that the MIC value is very low and all the *M. chelone* and *M. fuerth* strains were susceptible to clarithromycin. Although the sample size is small, we may still suppose that clarithromycin may have a good inhibition activity against these two species.

4.3. Clarithromycin Resistant Mechanism. For *M. abscessus*, one of the most notorious causative agents of disease, the macrolide clarithromycin is considered as cornerstone in antimicrobial chemotherapy of pulmonary *M. abscessus* infection. Paradoxically, however, *M. abscessus* infections of the lung are often intractable to clarithromycin chemotherapy, although high doses of clarithromycin may lead to clinical improvement in some patients [2, 4]. The lack of efficacy of clarithromycin is puzzling, as pretreatment isolates are usually reported as susceptible to clarithromycin when CLSI procedures are used [3, 4]. Intriguingly, Brown et al. reported that the clarithromycin MIC increased for some *M. abscessus* isolates if the incubations for the susceptibility assays were extended, although these isolates would still be deemed clarithromycin susceptible if an incubation period based on the previous CLSI guidelines was used [4, 18]. Similarly, Nash and colleagues showed that although a strain

might appear susceptible after 3 days of *in vitro* incubation [3], due to induction of the synthesis of a methyltransferase [4], the strain was clarithromycin resistant if incubation was extended to 14 days or if the strain was preincubated with clarithromycin. So in 2012, CLSI guideline extends *M. abscessus* DST incubation time from 3 days to 14 days. Although *M. abscessus* subsp. *massiliense* remained a high susceptible proportion after this adjustment, the resistant proportion of *M. abscessus* subsp. *abscessus* increased to a high level. An explanation for the instability of clarithromycin MIC is that *M. abscessus* might have inducible resistance to clarithromycin. Inducible resistance to clarithromycin has been suggested as an explanation for the lack of efficacy of clarithromycin-based treatments against *M. abscessus* infection [12]. An erythromycin ribosomal methylase gene, *erm(41)*, has been identified in several isolates of *M. abscessus* and the presence of the gene was associated with inducible resistance to clarithromycin. The induction of resistance was shown to result from increased expression of *erm(41)* as demonstrated by an increase in *erm(41)* RNA levels after exposure to clarithromycin. The functionality of the methylase is dependent on the nucleotide at position 28 of the *erm(41)* gene. Wild type T28 sequevars show inducible clarithromycin resistance, while C28 sequevars do not [4]. In our test, of all the 40 *M. abscessus* subsp. *abscessus* isolates which were sequenced successfully, 35 isolates were resistant to clarithromycin, and 34 isolates in them had T28 in *erm(41)*. However, all the 5 *M. abscessus* subsp. *abscessus* isolates which were susceptible to clarithromycin had C28 in *erm(41)*. This result also highlights the resistant mechanism above, which means that *erm(41)* may be of relevance to clarithromycin resistance. There is also 1 isolate which has C28 in *erm(41)* and still resistant to clarithromycin. Maybe there are some other resistant mechanisms needed to be analyzed. Although the therapeutic implications of *erm(41)* require further study, it is anticipated that expression of this gene does confer clinically significant resistance to clarithromycin. And still, the clinical impact of inducible resistance remains to be determined.

5. Conclusions

In this study, we find that MAC was the most common pathogen of NTM, and the second one was *M. abscessus*. However, *M. chelonei*, *M. fuerth,* and *M. gordon* were rare. Clarithromycin had a good inhibition activity against all the NTM species except *M. abscessus* subsp. *abscessus. erm(41)* genotype which is likely to be a major determinant of clarithromycin resistance of *M. abscessus*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Wenjuan Nie and Hongfei Duan contributed equally to this work.

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Review Article

Nontuberculous Mycobacteria Isolation from Clinical and Environmental Samples in Iran: Twenty Years of Surveillance

Ali Akbar Velayati,[1] Parissa Farnia,[1] Mohadese Mozafari,[1] and Mehdi Mirsaeidi[2]

[1] *Mycobacteriology Research Centre, National Research Institute of Tuberculosis and Lung Disease (NRITLD), Shahid Beheshti University of Medical Sciences, P.O. Box 19575-154, Tehran 19569-44413, Iran*
[2] *Section of Pulmonary, Critical Care, Sleep and Allergy Department of Medicine M/C 719, University of Illinois at Chicago, 840 S. Wood Street, Chicago, IL 60612-7323, USA*

Correspondence should be addressed to Parissa Farnia; pfarnia@hotmail.com

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Nontuberculous mycobacteria (NTM) are opportunistic pathogens that are widely distributed in the environment. There is a lack of data on species distribution of these organisms from Iran. This study consists of a review of NTM articles published in Iran between the years 1992 and 2014. In this review, 20 articles and 14 case reports were identified. Among the 20 articles, 13 (65%) studies focused on NTM isolates from clinical specimens, 6 (30%) studies examined NTM isolates from environmental samples, and one (5%) article included both clinical and environmental isolates. *M. fortuitum* (229/997; 23%) was recorded as the most prevalent and rapid growing mycobacteria (RGM) species in both clinical (28%) and environmental (19%) isolated samples ($P < 0.05$). Among slow growing mycobacteria (SGM), *M. simiae* (103/494; 21%) demonstrated a higher frequency in clinical samples whereas in environmental samples it was *M. flavescens* (44/503; 9%). These data represent information from 14 provinces out of 31 provinces of Iran. No information is available in current published data on clinical or environmental NTM from the remaining 17 provinces in Iran. These results emphasize the potential importance of NTM as well as the underestimation of NTM frequency in Iran. NTM is an important clinical problem associated with significant morbidity and mortality in Iran. Continued research is needed from both clinical and environmental sources to help clinicians and researchers better understand and address NTM treatment and prevention.

1. Introduction

In 1996, the Working Group of the Bacteriology and Immunology Section of the International Union against Tuberculosis and Lung Disease contacted 50 laboratories in several countries, including Iran, in order to collect and analyze epidemiological data for nontuberculous mycobacteria (NTM) or mycobacteria other than tuberculosis (MOTT). At this time, the Iranian reference laboratory provided data from 98 patients (1980–1983), of which *M. fortuitum* and *M. kansasii* were identified as the most dominant NTM in clinical samples [1]. In the following years, many researchers attempted to determine the prevalence of NTM and its importance in Iran [2–4]. Unfortunately, these studies failed to capture a comprehensive measure of NTM in Iran. The majority of NTM in Iran consist of small samples or data confined to small geographical areas that cannot be generalized. As a result, no clear data on the epidemiology of NTM is available on the national scale.

Iran is an intermediate tuberculosis- (TB-) burden country where TB remains a major public health problem. The incidence of TB in Iran is 21 per 100,000 people.

The significant number of multi-drug resistant (MDR), extensive drug resistant (XDR), and totally drug resistant (TDR) tuberculosis underline the possibility of NTM infection among tuberculosis suspected cases [5, 6]. In most cases, patients with positive sputum smear microscopy are treated with first line pulmonary tuberculosis therapy. Clinical failures prompt the transfer of TB samples to central laboratories for further identification of isolates and in case

of NTM infection. Therefore, the diagnosis and treatment of resistant TB begin with considerable delay [7]. So far, limited investigation on NTM infections is reported from TB endemic countries with limited laboratory resources. Instead, NTM infection is more documented in developed countries including geographical variability [8–10]. For example, *Mycobacterium avium* complex (MAC) followed by *M. gordonae* and *M. xenopi* is considered the most predominant NTM in the United States and Europe [11, 12]. Given the complex treatment challenges particularly in low resource countries, understanding geographical diversity of NTM within the country is particularly important. With this background, we aim to retrospectively analyze and compare the NTM data published in the last 20 years. In addition, we evaluated the long-term trends of NTM isolation from clinical and environmental specimens.

2. Methods

A literature search was performed in PubMed, Scopus, SID and Google Scholar, Embase, and the Cochrane Library on nontuberculous mycobacteria in Iran. The search keywords were "atypical Mycobacteria," "nontuberculous mycobacteria," and "Iran," Original articles, case reports, and reviews published on nontuberculous mycobacteria in Iran in peer-reviewed journals including Persian and English journals were considered [2–4, 13–40]. Congress abstracts were excluded. The following data were abstracted for the purpose of review: the name of the city, research methods, and individual NTM species as well as sample source. The statistical significance of observed trends of NTM in the last 20 years was tested using Poisson log-linear regression. All analyses were performed using the statistical software packages SPSS version 21 (IBM SPSS, Inc., Chicago, IL).

3. Results

Twenty original articles about NTM isolates were identified. The selected articles were published from 1992 to 2014. The majority of these articles (13/20; 65%) included data from clinical samples, six studies outlined the frequency of NTM in the environment, and a single (5%) article studied both clinical and environmental NTM (Figure 1). The geographical setting of these studies was Tehran in 6 articles (30%), Isfahan in 6 (30%), Khuzestan in 2 (10%), and Golestan in 2 (10%). The remaining articles included provinces such as Sistan and Baluchestan (1/20; 5%), Kerman (1/20; 5%), West Azerbaijan (1/20; 5%), and Gilan (1/20; 5%) (Table 1).

Among 14 case report articles from different cities of Iran, 4 were reported from Tehran (28.5%), 3 from Isfahan (21.4%), 2 from Sari (14.2%), and one from other cities including Shiraz (7.1%), Khomein (7.1%), Babol (7.1%), Ilam (7.1%), and Karaj (7.1%) (Table 2).

The majority of NTM species with known sources were isolated from respiratory specimens including sputum (134/494; 27.1%), bronchoalveolar lavage (51/494; 10.3%), bronchial washing (7/494; 1.4%), pleural samples (6/494; 1.2%), and lung tissue biopsy (5/494; 1%). Extrapulmonary samples were collected from urine (9/494; 1.8%), abscess (6/494; 1.2%), lymph node biopsy (4/494; 0.8%), gastric lavage (2/494; 0.4%), vaginal discharge (2/494; 0.4%), CSF (1/494; 0.2%), dermal lesion (3/494; 0.6%), subcutaneous nodule in hand or finger (4/494; 0.8%), and corneal biopsy (1/494; 0.2%). In a considerable number of reports (259/494; 52.4%), the sources of isolation were not documented.

As shown in Table 1, the primary method of NTM detection was based on culture using Löwenstein-Jensen media. Identification was performed by conventional methods in 38% (8/20) and molecular methods in 15% (3/20) of articles. In 9 (45%) studies, both molecular and conventional methods were applied.

3.1. NTM in Clinical Samples. The geographic locations of samples were mainly Tehran (261/480; 54.3%), Isfahan (153/480; 31.8%), Khuzestan (34/480; 7.1%), Golestan (19/480; 3.9%), Kermanshah (7/480; 1.4%), Kerman (3/480; 0.6%), and Sistan-Baluchestan (3/480; 0.6%). From 13 studies using clinical samples, 480 NTM species were isolated. Of these isolates, 269 (56%) were grouped as SGM and 211 (43.9%) as RGM. The most prevalent RGM in clinical samples was *M. fortuitum* (136/480; 28.3%) in all locations (Isfahan 105/153, 68.6%, Khuzestan 9/34, 26.4%, and Golestan 4/19, 21%) except for Tehran. The prevalence of RGM was *M. chelonae* (29/261; 11.1%) in Tehran. Among SGM species, *M. simiae* (103/480; 21.4%) showed the highest rate. Geographical distribution of SGM in clinical samples was *M. simiae* in Tehran (88/261; 33.7%) and Golestan (6/19; 31.5%), *M. gordonae* (16/153; 10.4%) in Isfahan, and *M. intracellulare* (6/34; 17.6%) in Khuzestan (Table 3).

In the case report articles, *M. marinum* (4/14; 28.5%) had higher detection rate, most frequently isolated from nodules or lesions of the hand (Table 2).

3.2. NTM in Environmental Samples. Data regarding environmental distribution of NTM were primarily from Tehran (193/503; 38.3%), Isfahan (51/503; 10.1%), Golestan (161/503; 32%), West Azerbaijan (65/503; 12.9%), and Gilan (33/503; 6.5%). In total, 503 NTM from environmental samples were isolated, which included 221 (43.9%) SGM and 282 (56%) RGM. Among RGM species, *M. fortuitum* (93/503; 18.4%) showed higher frequency in environmental samples in different locations including Golestan (35/161; 21.7%), West Azerbaijan (21/65; 32.3%), Isfahan (20/51; 39.2%), Tehran (10/193; 5.1%), and Gilan (7/33; 21.2%) (Table 4). Regardless of geographical locations, the frequency of *M. fortuitum* was high in both water (195/503; 38.7%) and soil (308/503; 61.2%) samples. SGM frequencies varied in different locations; in Gilan *M. terrae* was 11/33, 33.3%, in Golestan *M. triviale* was 10/161, 6.2%, and in Isfahan *M. gordonae* was 7/51, 13.7.

3.3. Trends of NTM. As shown in Figure 2, the frequency of NTM among pulmonary TB cases was studied in only 8 studies. In 1995, 18 (8%) of 225 respiratory samples were recorded as NTM as compared to 2013 when 55 (18%) were recorded as NTM from 291 samples. This trend shows a significant increase in NTM detection rates during the study period ($P < 0.05$).

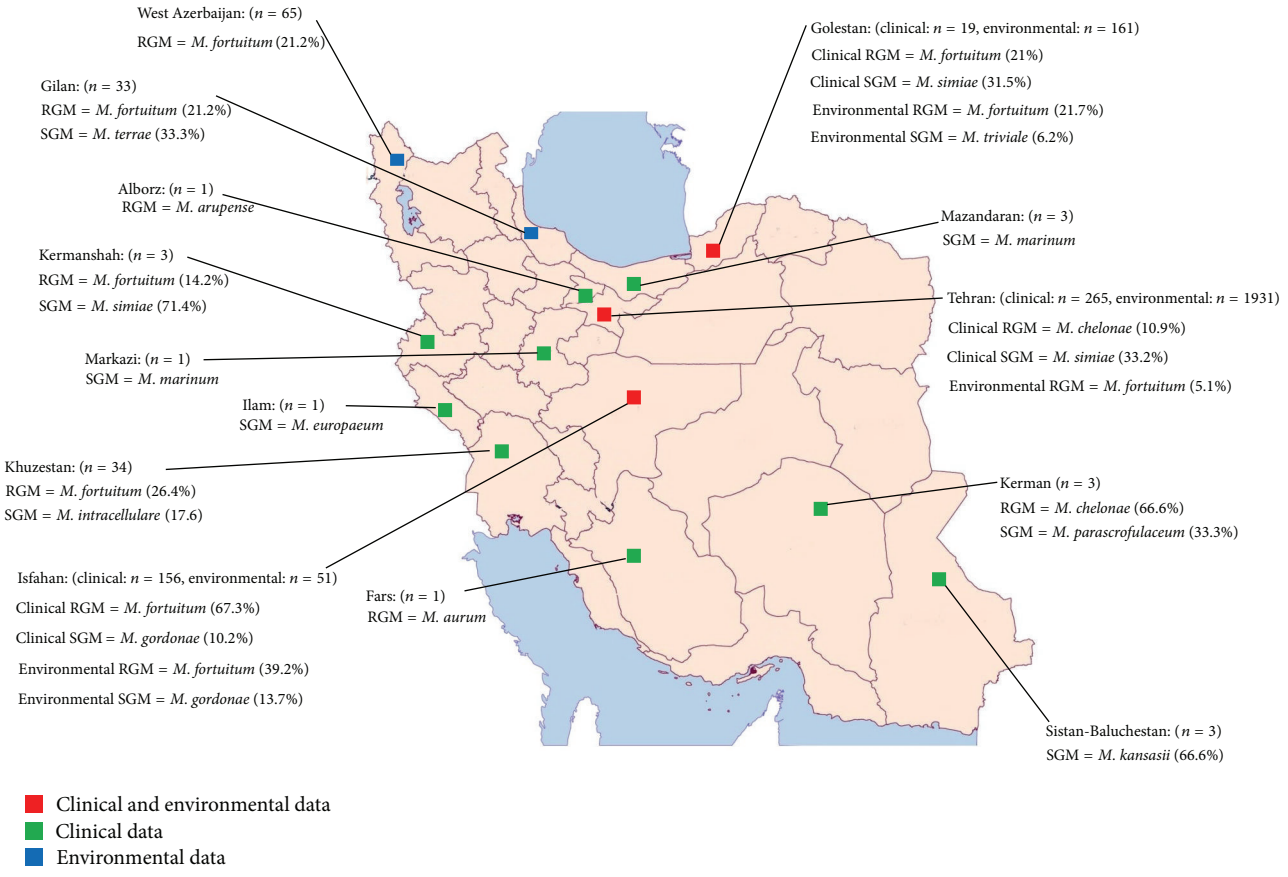

Figure 1: The provinces of Iran which work on NTM in clinical or environmental samples. The most prevalent SGM and RGM were noted.

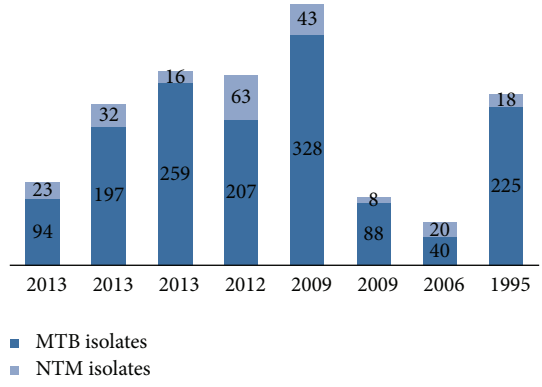

Figure 2: Number of NTM and MTB isolates in 8 studies.

4. Discussion

To the best of our knowledge this is the first study in which trends in clinical and environmental NTM species have been investigated over the past twenty years. Overall, from 34 published reports (original and case reports), 997 NTM strains were identified (494 isolated from clinical samples and 503 from environmental samples). The majority of clinical (86.2%) and environmental (65.2%) NTM species were isolated from Tehran ($n = 261$) and Isfahan ($n = 53$), respectively. These data are incomplete considering that Iran consists of 31 provinces. Here, we showed that NTM was isolated only from 14 (46%) provinces during the past years (Figure 1). In the remaining 17 provinces, there is no data available on prevalence of environmental and clinical NTM. These numbers suggest that NTM is a neglected disease in Iran, which is likely true for other neighboring countries in the region, where the incidence of TB is higher, such as Afghanistan, Iraq, and Pakistan [41–48]. In Pakistan, three clinical NTM reports were published in 1984, 2011, and 2013 with total sample size of 4, 62, and 104 subjects, respectively [43, 44]. *M. fortuitum* was identified as the most prevalent NTM in Pakistan (13.5%). In Iraq, few studies recently reported the frequency of NTM in dairy products and environmental samples such as milk powder and fresh milk, drinking water, and fecal samples from horses [46–48]. The most prevalent NTM species in Iraq was *M. chelonae* (18.2%) [46, 48].

In this study we also aimed to identify NTM distribution and trends within Iran. Variable techniques were used in different laboratories. From 1992 to 2006, most laboratories used traditional methods for identification of NTM in both clinical and environmental samples. However, from 2009 to 2014, advances in laboratory technique allowed combinations

Table 1: Detection and identification procedures for nontuberculous mycobacteria (NTM) in different laboratories.

Region	Laboratory/year of publication	Number of isolated NTM	Detection	Identification
Tehran	Mycobacteriology Research Center (MRC)-NRIT LD-2014 [51]	Clinical isolates: 124	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Amplification of *IS6110* and PCR-RFLP for *hsp65*
	Mycobacteriology Research Center (MRC)-NRIT LD-2013 [52]	Environmental isolates (RGM): 36; total: 193	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Amplification of *IS6110* and PCR RFLP of *hsp65* and *16 s–23 s rRNA*
	Department of Mycobacteriology, Pasteur Institute of Iran-2013 [2]	Clinical isolates: 32	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation, biochemical properties, and PCR based on *hsp65*
	Masoud Laboratory, TB Reference Centre of Ahvaz and Kermanshah, 2013 [54]	Clinical isolates: 23	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation, biochemical properties, amplification, sequencing of *16S rRNA*, *rpoB*, *hsp65*, and *ITS* (Internal Transcribed Spacer)
	Masoud Laboratory-2012 [13]	Clinical isolates: 63	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation, biochemical properties, amplification of *IS6110*, and PCR based on *hsp65*
	Mycobacteriology Research Center (MRC)-NRIT LD-2009 [14]	Clinical isolates: 43	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation, biochemical properties, and PCR-RFLP for hsp65
Isfahan	Dept. of Microbiology, Isfahan University of Medical Sciences-2014 [15]	Clinical isolates: 34	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Amplification and sequencing of *16SrRNA* and RFLP-PCR for *hsp65*
	Dept. of Microbiology, Isfahan University of Medical Sciences-2013 [16]	Clinical isolates: 21	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation and biochemical properties
	Dept. of Microbiology, Isfahan University of Medical Sciences-2012 [17]	Environmental isolates: 21	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation and biochemical properties
	Dept. of Microbiology, Isfahan University of Medical Sciences-2012 [18]	Environmental isolates: 22	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation and biochemical properties
	Isfahan University of Medical Sciences-2011 [4]	Clinical isolates: 67	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation, biochemical properties, amplification, sequencing of *16SrRNA*, and RFLP-PCR for *hsp65*
	Isfahan University of Medical Sciences-2010 [19]	Clinical isolates: RGM:25	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation, biochemical properties, genus and species specific PCR, and PCR based on *hsp65*
Golestan	Health care centers of Golestan province-2013 [20]	Clinical isolates: 19	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation, biochemical properties, amplification, and sequencing of *16S rRNA*
	Microbiology Laboratory of Urmia University of Medical sciences-2006 [21]	Environmental isolates: 161	Culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation and biochemical properties

Table 1: Continued.

Region	Laboratory/year of publication	Number of isolated NTM	Detection	Identification
Khuzestan	Tuberculosis reference laboratory, PHLS of Khuzestan province-2009 [22]	Clinical isolates: 8	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation, biochemical properties, and PCR based on *hsp65*
	Ahwaz University of Medical Sciences-1995 [23]	Clinical isolates: 18	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation and biochemical properties
West Azerbaijan	Microbiology Laboratory of Urmia University of Medical Sciences-2010 [3]	Environmental isolates: 65	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation and biochemical properties
Gilan	1992 [24]	Environmental isolates: 33	Culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation and biochemical properties
Sistan-Baluchestan	2006 [25]	Clinical: 3 NTM identified	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation and biochemical properties
Kerman	2007 [26]	Clinical NTM: 3 NTM identified	Smear microscopy and culture on solid media (Löwenstein-Jensen)	Growth characteristics and pigmentation and biochemical properties

Table 2: Case report studies of Iran.

Species	Year	City	Infected organ
Mycobacteriumbranderi [27]	2014	Tehran	Bone marrow
Mycobacterium marinum [28]	2014	Sari	Papule-hand
Mycobacterium iranicum [29]	2013	Isfahan	Bronchoalveolar lavage/hand wound
Mycobacterium arupense [30]	2013	Karaj	Respiratory system and blood
Mycobacterium chelonae [31]	2013	Tehran	Sputum and cervical lymph node
Mycobacterium aurum [32]	2012	Shiraz	Corneal biopsy
Mycobacterium europaeum [33]	2012	Ilam	Sputum
Mycobacterium marinum [34]	2011	Khomein	Nodule on the dorsum of fourth finger
Mycobacterium marinum [35]	2011	Babol	Lesions and pustules of the right forearm
Mycobacterium monacence [36]	2012	Isfahan	Sputum
Mycobacterium parascrofulaceum [37]	2011	Isfahan	Vaginal discharge
Mycobacterium lentiflavum [38]	2010	Tehran	Sputum
Mycobacterium marinum [39]	2008	Sari	Nodules and bulls on the back of the right hand
Mycobacterium thermoresistibile [40]	2006	Tehran	Cervical lymph node

of traditional and molecular methods to be used (Table 1) resulting in the detection of more species of NTM in clinical and environmental samples. Reports from high-tech laboratories proposed the use of commercial line probe assay supplemented with sequencing for identification [49, 50]. Ideally, the use of the commercial method may support standardization and it facilitates the comparison of results within different settings. Our data demonstrate PRA (PCR restriction analysis) methods with either *hsp65* or *16s-23s rRNA*, *rpoB* genes as the optional molecular test [18, 51, 52]. This highlights the need for standardized methods and guidelines for NTM identification in Iran.

We also showed that the majority of NTM were collected from respiratory samples. The results underline the importance of identifying NTM from suspected pulmonary TB patients. Molecular and phenotypic identification revealed a geographical distribution of NTM in Iran. From 494 clinical NTM isolates, 28.3% and 21.4% were recorded to be *M. fortuitum and M. simiae*, respectively. Analyzing the previous studies [15–19] showed geographical differences for *M. fortuitum* distribution, where Isfahan had the highest prevalence of *M. fortuitum* (105/153; 68.6%), while in Tehran the prevalence rate was less than 10% (17/261; 6.5%) ($P > 0.05$). These results make the analysis a bit difficult, as we are not sure if the report is a laboratory cross contamination or if it is *M. fortuitum* endemicity in some parts of Iran.

The results from environmental samples also showed the high frequency of *M. fortuitum* (93/282; 32.4%) followed by *M. chelonae* (38/282; 13.4%) in water and soil samples. This suggests the possible risk of *M. fortuitum* transmission from nature to human. Among SGM species, *M. simiae* isidentified as the dominant NTM in Tehran (88/261; 33.7%) and Golestan provinces (6/19; 31.5%) [20, 53, 54]. In three other regional settings (Isfahan, Sistan-Baluchestan, and Kerman), *M. simiae* was not isolated. The clinical importance of *M. simiae* in various geographical regions of Asia, including Turkey and Japan, has been already documented [55, 56]. The frequency of *M. simiae* wasreported to be from 1.5% to 10% across studies [53, 54]. For environmental SGM, the frequency of *M. flavescens* (44/503; 9%), *M. thermoresistibile* (24/503; 5%), and *M. terrae* (21/503; 5%) was higher than other species (Table 4). In contrast to RGM group, the distribution and frequency of slow growing mycobacteria in clinical and environmental samples were different.

The current study found a considerable number of environmental NTM (157/503; 31.2%) that remained unidentifiable (Table 3). This highlights the importance of the implementation of new techniques in order to improve NTM identification. At present, 8 regional and one national reference TB laboratories are functioning in Iran. Recently, due to global fund, they have been equipped with molecular diagnostic testing capabilities. As a result, it is our expectation that NTM detection will increase within the next few years.

Drug susceptibility tests (DST) for NTM were not performed in the majority of published studies in Iran. In developed countries, a variety of susceptibility testing methods such as the *E*-test, TREK, and microbroth dilution are used to carry out DST [57]. Given the well-described resistance patterns emerging in developed countries with low incidence of NTM, susceptibility testing is a particularly important clinical tool for countries such as Iran.

In conclusions, the trends of isolation and identification of NTM have been increased in Iran in the last 20 years. This increasing trend is attributable to the implementation of enhanced molecular techniques that have improved the detection coupled with the enhanced awareness of NTM in the clinical setting. However, further research is needed to address this important public health threat including enhancing the epidemiology of NTM throughout Iran, standardizing laboratory techniques for detection and drug susceptibility testing, and improving clinicians knowledge on NTM diagnosis and treatment in Iran.

Table 3: Species distribution of clinical nontuberculous mycobacteria isolated in articles reported from Iran.

Species	Kermanshah (2013)	Kerman (2007)	Isfahan (2010–2014)	Khuzestan (1995–2009)	Tehran (2009–2014)	Sistan-Baluchestan (2006)	Golestan (2013)	Total
M. fortuitum	1		105	9	17		4	136
M. simiae	5			4	88		6	103
M. kansasii	1		14	2	38	2		57
M. gordonae			16	5	19		1	41
M. chelonae		2			29		1	32
M. intracellulare			2	6	11			19
M. abscessus					17			17
M. scrofulaceum				1	7			8
M. avium			1	3	1	1		6
M. conceptionence			3		2			5
M. marinum					1		3	4
M. lentiflavum			1		2		1	4
M. thermoresistibile			1		3			4
M. szulgai				2	1			3
M. branderi					3			3
M. parascrofulaceum		1	1		1			3
M. gastri					2		1	3
M. malmoense					3			3
M. porcinum			3					3
M. phlei			2		1			3
M. massiliense					3			3
M. monacense			1		1			2
M. nonchromogenicum					1		1	2
M. senegalense					2			2
M. genavense					1			1
M. triviale				1				1
M. sherrissii					1			1
M. xenopi				1				1
M. montefiorense					1			1
M. triplex					1			1
M. arupense					1			1
M. nebraskense					1			1
M. flavescens							1	1
M. smegmatis			1					1
M. austroafricanum			1					1
M. elephantis			1					1
M. novocastrencse					1			1
M. aurum					1			1

Table 4: Species distribution of environmental nontuberculous mycobacteria isolated in articles reported from Iran.

Species	Isfahan (2012-2013)	West Azerbaijan (2010)	Golestan (2006)	Gilan (1992)	Tehran (2013-2014)	Total
M. fortuitum	20	21	35	7	10	93
M. flavescens	1	10	33			44
M. chelonae	5	6	27			38
M. thermoresistable		4	20			24
M. terrae	2		8	11		21
M. phlei	1		14			15
M. pregrinum		11			3	14
M. mucogenicum	5	6			2	13
M. gordonae	7		4			11
M. triviale			10			10
M. senegalense					9	9
M. xenopi	1			7		8
M. avium	1			6		7
M. abscessus	2	3			1	6
M. smegmatis	4	2				6
M. parafortuitum					5	5
M. fallax	1		4			5
M. conceptionence	1				3	4
M. gastri			3			3
M. kansasii			1	2		3
M. neoaurum		2				2
M. marinum			2			2
M. aurum					1	1
M. poriferae					1	1
M. obuense					1	1
Unidentified SGM					157	157

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Nontuberculous Mycobacteria in Noncystic Fibrosis Bronchiectasis

Giulia Bonaiti,[1] Alberto Pesci,[1] Almerico Marruchella,[1] Giuseppe Lapadula,[2] Andrea Gori,[2] and Stefano Aliberti[1]

[1]*Health Science Department, University of Milan Bicocca, AO San Gerardo, Via Pergolesi 33, 20052 Monza, Italy*
[2]*Department of Internal Medicine, Division of Infectious Diseases, AO San Gerardo, Via Pergolesi 33, 20052 Monza, Italy*

Correspondence should be addressed to Stefano Aliberti; stefano.aliberti@unimib.it

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During the past decades, a growing interest has been raised in evaluating nontuberculous mycobacteria (NTM) in patients with noncystic fibrosis bronchiectasis (NCFBE). This paper reviews several aspects of the correlations between NTM and NCFBE, including pathogenesis, radiological features, diagnosis, and management. Bronchiectasis and NTM lung disease are connected, but which one comes first is still an unresolved question. The rate of NTM lung disease in NCFBE varies through the studies, from 5% to 30%. The most frequent species isolated is MAC. NCFBE patients affected by NTM infection frequently present coinfections, including both other different NTM species and microorganisms, such as *P. aeruginosa*. Once a diagnosis of NTM disease has been reached, the initiation of therapy is not always mandatory. NTM species isolated, patients' conditions, and disease severity and its evolution should be considered. Risk factors for disease progression in NCFBE patients with NTM are low body mass index, cavitary disease, consolidations, and macrolide resistance at presentation.

1. Introduction

Nontuberculous mycobacteria (NTM) comprise mycobacteria other than *Mycobacterium tuberculosis* complex and *M. leprae*. Until now, more than 160 species have been isolated, many of which may be pathogen for humans. In 1959, Runyon proposed a classification of NTM into four main categories: group I of slow-growing photochromogens (e.g., *M. kansasii* and *M. simiae*), group II of slow-growing scotochromogens (e.g., *M. szulgai* and *M. xenopi*), group III of slow-growing nonphotochromogens (e.g., *M. avium* complex and *M. malmoense*), and group IV of the rapid growers (e.g., *M. fortuitum, M. chelonae,* and *M. abscessus*) [1].

A simplified classification of NTM is based on the rate of growth: according to this criterion, they have been divided into slowly and rapidly growing species. The first group takes more than 7 days to complete the growing in culture (e.g., *Mycobacterium avium* complex—MAC—and *M. kansasii*), while rapidly growing take less than 7 days (e.g., *M. abscessus*).

NTM are ubiquitous in the environment and have been isolated from soil and water, which are the presumed sources of infection. Until now, there is no evidence of animal-to-human or human-to-human transmission. Recent data seem to reveal the possibility of indirect human-to-human transmission of *M. abscessus* subspecies *massiliense* in patients affected by cystic fibrosis (CF). Bryant and colleagues have performed a genetic analysis of *M. massiliense* isolates in CF patients and have found near-identical isolates in different patients attending their centre; genetic data have revealed frequent transmission of multidrug resistant *M. massiliense* [2]. Although the authors cannot demonstrate the exact way of transmission, they postulate that it could be indirect. A single recent case report from United Kingdom suggests the possibility of human-to-human transmission of *M. kansasii*. A woman and her husband in London were infected by identical strains of *M. kansasii*, while no common source of infection could be found [3].

The exact prevalence of NTM disease is unknown because reporting is not mandatory in many countries and discrimination between colonization and active disease can

be challenging. Certainly, the rates of isolation of NTM are increasing in the last years because of many factors, including the progress in diagnostic techniques and the increasing attention to this topic. The prevalence of NTM infection in the United States of America (USA) from 2004 to 2006 raised from 1.4 to 6.6 per 100,000 persons [4]. In England, Wales, and Northern Ireland, the rate of all NTM reports increased from 0.9 per 100,000 population in 1995 to 2.9 per 100,000 in 2006 [5].

NTM disease usually affects patients with chronic lung disease (e.g., chronic obstructive pulmonary disease—COPD—or CF) but it has been also described in healthy individuals [6]. During the past decades, a growing interest has been raised in evaluating NTM in patients with non-CF bronchiectasis (NCFBE). Reports of NTM lung infection in NCFBE patients are increasing in the last years, although the exact pathogenesis of NTM infection in this population is not well known, along with its impact on clinical outcomes [7].

The aim of this paper is to review several aspects of the correlations between NTM and NCFBE, including pathogenesis, radiological findings, diagnosis, and management.

2. Pathogenesis of NTM in Non-CF Bronchiectasis

The three major factors involved in the pathogenesis of NTM infection are the exposure, a damage substrate, and a possible immune defect.

2.1. Exposure. NTM are ubiquitous in the environment, so, in theory, all people can be exposed to them. The way of contact with NTM is probably inhalation, most likely via aerosols from natural surface water or hot water systems. Some conditions may increase the risk of lung exposure. Microaspiration of ingested contaminated water can be a way of access for NTM to the lung. Patients affected by gastroesophageal reflux (GORD) or by lung disease associated with GORD—such as bronchiectasis—might be more predisposed to NTM lung infection [8]. However, in a group of patients affected by NCFBE, no difference in the presence or absence of gastroesophageal reflux has been found between patients affected or not affected by NTM disease [9].

2.2. Substrate. NTM lung disease is common in structural lung disease, such as CF, bronchiectasis, and COPD. A structural damage, as an injured epithelium or a deficit in mucociliary clearance, is a predisposing factor to NTM lung infection. MAC has the ability to adhere to the extracellular matrix that is exposed in areas of epithelial damage and in areas where fibrous mucus is poorly cleared, due to impaired mucociliary clearance [10].

2.3. Defective Immunity. Despite the fact that NTM are ubiquitous in the environment, relatively few individuals develop NTM lung disease, suggesting a possible intrinsic predisposition, such as a deficit in immunity response.

The pathogenesis of NTM lung disease involves many components of both innate and adaptive immunity. The innate immune system is the first line of defence against mycobacteria. It involved many pattern recognition receptors, such as toll-like receptors (TLR) to help identification, phagocytosis, and activation of defense mechanisms against mycobacteria. TLRs help begin rapid defense mechanism, such as phagocytosis and activation of antimicrobial activity and modulating adaptive immune responses [11].

Macrophages ingest mycobacteria and deliver them to degradative compartments where they are eliminated [12]. Macrophages stimulate cytokines, such as interleukin-12, which in turn upregulates interferon- (IFN-) γ and tumor necrosis factor-alpha (TNF-α) [13]. Cytokines recruit and stimulate T-lymphocytes and Natural Killer cells to help kill mycobacteria [14]. Some data about association with specific human leukocyte antigen (HLA) alleles exist. Kubo et al. founded an association between HLA-DR6/HLA-DQ4 and MAC pulmonary disease and associations between HLA-A26 and deterioration of nodular-bronchiectatic MAC infection [15]. The importance of these factors in immune response is confirmed by the increased number of NTM diseases in case of defect in one of them. Genetic defects in IFN-γ signaling are rare disorders associated with high risk of developing disseminating NTM infection [16]. Some evidences exist about a high risk of developing active tuberculosis (TB) in patients with latent TB infection undergoing anti-TNF-α therapy as well as the development of NTM disease during this therapy [17, 18]. In a recent study performed on mice, Renna et al. reported a possible association between long-term azithromycin treatment and development of NTM infection in CF patients [19]. They supposed that azithromycin impaired lysosomal degradation of both autophagosomes and phagosomes and can lead to failure of intracellular killing of mycobacteria and development of chronic infection with *M. abscessus* in mouse models. In HIV-positive patients, disseminated NTM infection usually takes place if $CD4^+$ T-cell count is very low, suggesting the importance of cell-mediated immunity in antimycobacterial defense [20]. Furthermore, the initiation of antiretroviral therapy in case of NTM lung disease may be followed by development of the "immune reconstitution inflammatory syndrome," due to restoration of pathogen-specific immune responses. In the majority of cases, this syndrome associated with MAC presents with fever and lymphadenopathy or painful lymphadenitis [21]. NTM pulmonary disease is frequent in women with a similar body habitus, characterized by elderly age, low body mass index, bronchiectasis, and centrilobular nodules, a condition noted with the acronym of Lady Windermere syndrome [22]. Other conditions frequently observed in patients affected by NTM pulmonary disease are scoliosis, *pectus excavatum*, mitral valve prolapse, and cystic fibrosis transmembrane conductance regulator (CFTR) mutations. Despite this association, no common immune defect has been found in these patients, in terms of neither cytokine production nor cell-mediated immunity [23].

Mutations of CFTR gene have been found in many patients with bronchiectasis and MAC lung disease; although these patients have not a diagnosis of CF, it seems that they have some defects in bronchial mucosal ion and in

water transport. These factors might be associated with development of bronchiectasis [23, 24].

Other evidences about possible genetic factors contributing to host susceptibility to NTM lung infection come from a retrospective review of six familial clusters of pulmonary NTM infection with at least two members affected by NTM lung infection [25]. The majority of patients were nonsmokers women, scoliosis was present in 31% of patients, and CFTR mutation without CF diagnosis was found in 42% of affected individuals. Furthermore, another common condition associated with bronchiectasis in patients with NTM infection is alpha-1 antitrypsin deficiency [26]. Systemic compromised host defenses, such as diabetes mellitus, malignancy, or transplant recipients, have been shown to be predisposing factors in the development of NTM lung disease, although a clear and definitive mechanism has not been yet identified [27–29].

3. NTM and Non-CF Bronchiectasis: Who Comes First?

Undoubtedly, bronchiectasis and NTM lung disease are connected, but which one comes first is an unresolved question so far. In some diseases, such as CF or posttuberculosis bronchiectasis, it seems reasonable that anatomic alterations of bronchi precede NTM infection [30]. On the other hand, few experiences have reported a possible role of NTM infection in causing bronchiectasis. Okumura et al. reported the case of a woman in which pulmonary MAC lesions seemed to precede the central bronchial lesion with later development of bronchiectasis [31]. Fujita and colleagues retrospectively studied pathological abnormalities in patients undergoing surgical resection for MAC lung disease and bronchiectasis [32]. Destruction of bronchial cartilage and smooth muscle layer, airways' granulomas, and ulcerated bronchial mucosa were found. The authors assumed that cartilage and smooth muscle destruction, caused by MAC, could result in bronchiectasis and that granulomas constitute the evidence that bronchiectasis is not antecedent but a consequence of chronic MAC infection.

4. Prevalence and Radiological Manifestations of NTM Species

The rate of NTM lung disease, according to American Thoracic Society (ATS) criteria, in patients with bronchiectasis varies through the studies from 5% to 10% and to 30% [9, 33, 34].

In a recent meta-analysis, the overall prevalence of NTM in patient with bronchiectasis was 9.3% [5]. In CF-bronchiectatic patients, prevalence of NTM varies from 5% to 20% through different studies [35, 36]. In a multicentre study performed in US in CF patients, the prevalence was 13% with range by centres from 7 to 24% [37].

The most frequent species isolated in bronchiectatic patients is MAC, with a percentage up to 50% and also 80% of the total of NTM [9, 39, 40]. The rate of isolation of different species varies from study to study. In an observational prospective study performed in Korea analysing 105 bronchiectatic patients, MAC constituted 50% of NTM isolated [39]. Other species isolated *were M. abscessus* (39%), *M. kansasii* (3%), and *M. fortuitum* (3%). In another observational prospective study performed in London, apart from MAC (isolated in 53% of 30 patients), other NTM isolated were *M. kansasii* (28%), *M. chelonae* (1%), *M. malmoense* (1%), *M. fortuitum* (1%), and *M. simiae* (only one patient) [41]. NTM lung infection can present itself with different radiological patterns. Two major radiological patterns have been described: the fibrocavitary form and the nodular/bronchiectatic one. The fibrocavitary form is characterized by areas of increased opacity and cavitations, usually localized in the upper lobes, with or without calcification; see Figure 1. Apical pleural thickening and fibrosis with volume loss and traction bronchiectasis are frequent [39]. Lower lobe involvement, adenopathies, and pleural effusion are uncommon. The radiologic presentation is similar to postprimary tuberculosis; however, NTM infection usually progresses more slowly than active tuberculosis [39, 42]. Wallace et al. reported some differences between NTM disease and tuberculosis. NTM tend to cause thin-walled cavities with less surrounding parenchymal infiltrate, have less bronchogenic but more contiguous spread of disease, and produce more marked involvement of pleura over the involved areas of the lungs [43]. Patients affected by these forms of NTM infection are typically elderly men with underlying lung disease [39].

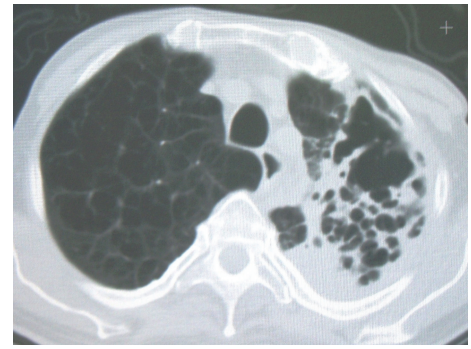
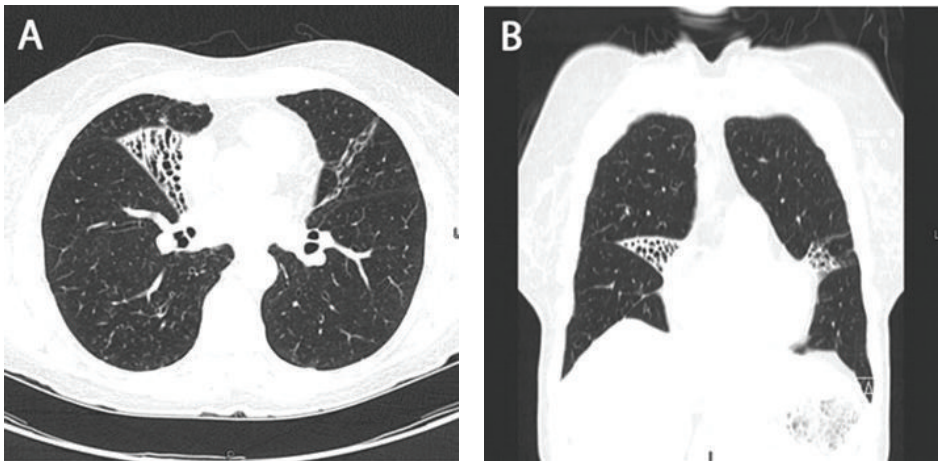


Figure 1: Fibrocavitary form of *Mycobacterium avium* lung disease.

The second radiologic pattern consists in cylindrical bronchiectasis and multiple small centrilobular nodules, localized especially in middle lobe and lingual; see Figure 2 [39, 44]. Reich and Johnson first used the term "Lady Windermere syndrome" to describe this pattern of NTM lung disease in elderly white woman without underlying lung disease symptomatic for chronic cough [22]. One study compared different radiological presentations of NTM lung disease: MAC was the most common species isolated in bronchiectatic (42%) and consolidative (43%) forms and *M. chelonae/M. abscessus* the most common in cavitary form (37%) [45]. Furthermore, in the same study, *M. kansasii* seems more common in cavitary (15%) and consolidative (13%) pattern than in bronchiectatic pattern (9%).



(a) (b)

Figure 2: Nodular/bronchiectatic form of nontuberculous mycobacterial lung disease. Axial (a) and coronal (b) computed tomography images demonstrating focal bronchiectasis in both the right middle lobe and lingula, characteristic of Lady Windermere syndrome [38].

5. Coinfection of NTM and Other Pathogens in Non-CF Bronchiectasis

Patients affected by NTM infection frequently present some coinfections, including both other different NTM species and microorganisms. *P. aeruginosa* is the most common copathogen isolated, with a percentage ranging from 27% to 52% [40, 46]. Infection with *P. aeruginosa* has been reported to worsen lung functions in bronchiectasis, but, in a recent study, no differences were reported concerning this aspect between patients infected and those not infected with this pathogen [46, 47]. Wickremasinghe and colleagues, in a retrospective analysis of 100 bronchiectatic patients performed in Brompton, found that the first pathogen isolated together with NTM was *P. aeruginosa* (52% of patients with multiple isolations), but only a quarter was chronic isolation [40]. In the same study, the second copathogen was *S. aureus* with a percentage of 28%, followed by *H. influenzae* (12%), *A. fumigatus* (4%), *C. albicans* (8%), and *S. maltophilia* (4%). Wallace and colleagues, in a prospective study of 26 patients affected by MAC infection, reported also *Nocardia* spp. (12% of patients) and *M. fortuitum* [43]. Also *M. abscessus* is described in these patients [30]. Another interesting point is that patients with nodular bronchiectasis pattern seem to frequently have multiple and/or repeated MAC infections with multiple isolated genotypes. On the other hand, patients with cavitary patterns are usually infected with one single strain [48]. Coinfection of NTM and *M. tuberculosis* is described also in immunocompetent patients, but no data are available in NCFBE [49, 50].

6. Challenges in NTM Diagnosis in Patients with Non-CF Bronchiectasis

NTM lung disease diagnosis is often difficult to establish, because of a frequent possibility of sample contamination. Furthermore, the respiratory tract can be infected with NTM without clear symptoms/signs of active disease, a condition that has been named colonization; however, there are no data proving that colonization is not a slowly progressive infection [30]. According to ATS guidelines, the diagnosis of NTM lung disease is based on specific criteria: two clinical criteria (pulmonary symptoms with compatible radiologic pattern and exclusion of other diagnoses) and one among the microbiological findings [30]. In light of the high probability of sample contamination, ATS criteria require more than one positive sample for diagnosis. One exception is patients with classic symptoms and radiologic pattern of nodular/bronchiectatic alterations without sputum production. According to ATS guidelines, the identification of NTM in one bronchoscopic specimen, especially MAC, is considered adequate in this specific type of patients for the diagnosis of NTM lung disease [30]. One more consideration is that NTM isolation in patients with CF is reported to be particularly difficult to culture if *P. aeruginosa* colonization is present [51]. So far, no studies are available in non-CF bronchiectasis patients evaluating difficulties in NTM isolation in case of a *P. aeruginosa* coinfection.

Differently from TB, in which transbronchial needle aspiration (TBNA) has a possible role in diagnosis, only few data exist about NTM infection. One study assessed the utility of endobronchial ultrasound-guided- (EBUS-) TBNA for the diagnosis of suspected granulomatous mediastinal lymphadenopathy [52]. Low et al. retrospectively reviewed 13 cases of suspected granulomatous mediastinal lymphadenopathy undergoing EBUS-TBNA, which was diagnostic in 9 of them (69%) with a final diagnosis of TB/NTM.

In specialized laboratories, molecular tests are available for rapid identifications of most common NTM species. Sequencing of genomic targets (such as 16S rRNA) allows accurate and rapid identification, even if some technical limitations exist, such as in case of samples with polymicrobial patterns and the deficiencies in public sequence databases [53].

Table 1: Treatment recommendations for nontuberculous mycobacteria, according to 2007 American Thoracic Society Guidelines.

	Initial therapy for nodular/bronchiectatic disease	Initial therapy for cavitary disease	Severe disease or previously treated disease
Mycobacterium avium complex	Clarithromycin 1,000 mg TIW or azithromycin 500–600 mg TIW Ethambutol 25 mg/kg TIW Rifampin 600 mg TIW	Clarithromycin 500–1,000 mg/d or azithromycin 250–300 mg/d Ethambutol 15 mg/kg/d Rifampin 450–600 mg/d ±Streptomycin or amikacin or none	Clarithromycin 500–1,000 mg/d or azithromycin 250–300 mg/d Ethambutol 15 mg/kg/d Rifabutin 150–300 mg/d or rifampin 450–600 mg/d ±Streptomycin or amikacin
Mycobacterium kansasii	Rifampin 10 mg/kg/d (maximum 600 mg/day) Ethambutol 15 mg/kg/d Isoniazid 5 mg/kg/d (maximum 300 mg) Pyridoxine 50 mg/d		

TIW: three times in a week; d: day.

7. Treatment of NTM in Non-CF Bronchiectasis

Once a diagnosis of NTM disease has been reached, the initiation of therapy is not always mandatory. NTM therapy is usually based on a prolonged treatment with at least two drugs leading to several side effects. In view of this, a risk-benefit evaluation should be carefully considered in each patient before deciding whether to treat or not an NTM infection. First of all, it should be considered which NTM species has been isolated, while clinicians should know which NTM species are more likely to be pathogen for humans. *M. kansasii* is considered one of the most virulent species and *M. fortuitum* is one of the less virulent ones, while species as *M. gordonae* and *M. terrae* are usually considered contaminants [30]. After that, clinicians should consider patients' conditions, evaluating disease severity, its evolution, and tolerability of drugs. Finally, the diagnosis of NTM lung infection in patients affected by bronchiectasis is crucial in the management of these patients because previous data have shown the role of these pathogens in worsening preexisting bronchiectasis [54, 55].

Radiological and clinical presentations are crucial to determine schemes and duration of treatment. Patients with more cavities, consolidations, and more severe and widespread bronchiectasis are more likely to require treatment [40]. Lee et al. evaluated retrospectively computed tomography (CT) scans of 399 patients with nodular-bronchiectatic form of MAC disease [7]. The presence of cavity and consolidation at initial CT was independent factors associated with disease progression and treatment requirements. They suggest that, in patients who initially were not candidates for treatment, a radiological progression of the disease later on could be a criterion for therapy.

Because of the fact that the majority of studies are focused on MAC, several guidelines recommendations concerning other NTM species tend to be based on MAC findings.

In terms of drug choice, ATS guidelines provide therapeutic schemes specific for some pathogen and general indications for others, such as rapidly growing mycobacteria; see Table 1.

Because of the known discrepancy between in vitro and in vivo drug susceptibility, the only drugs for which susceptibility of MAC should be evaluated are macrolides (azithromycin or clarithromycin) [56]. Rifampin should also be tested for *M. kansasii*. In case of drug-resistant NTM, the choice of drugs is based on in vitro susceptibility and expert opinion. One general rule is that macrolide monotherapy should be absolutely avoided in order to prevent the emergence of resistances.

For most patients with nodular-bronchiectatic MAC disease, intermittent, three-time weekly therapy is recommended [30]. In case of severe nodular/bronchiectatic disease or fibrocavitary presentation, a more aggressive regimen is recommended. According to ATS guidelines, two-drug regimen (macrolide and ethambutol) is acceptable only in case of nodular/bronchiectatic MAC disease if drug intolerance or mild disease is present.

In CF patients who are candidates for macrolide monotherapy, it is recommended to have sputum cultured for NTM before and during therapy. Furthermore, patients with repeated isolation of NTM should not receive macrolide monotherapy [30]. No specific recommendations exist for NCFBE, but it seems reasonable to have a similar behavior, in particular for subjects with a past history of NTM isolation [57]. Differently from *M. tuberculosis*, therapy with fluoroquinolones for bronchiectatic exacerbations does not seem to be a risk factor for delayed diagnosis of NTM or fluoroquinolones NTM resistance, although there are no clear data about it [57].

8. Risk Factors for Treatment Failure and Follow-Up

In patients with nodular/bronchiectatic MAC disease undergoing therapy, sputum conversion is frequently achieved, without the development of resistances. In a retrospective review evaluating the efficacy of macrolide/azalide-containing regimens for nodular/bronchiectatic MAC lung disease, sputum conversion to culture negative occurred in 86% of patients and nobody developed macrolide resistance during treatment [58]. In the same study, microbiologic recurrences occurred in 48% of patients who completed treatment, of which 75% were reinfected with the isolation of new MAC genotypes and 25% showed true relapse with

recurrence of the pretreatment MAC genotype. True relapses isolates occurred significantly earlier after completion of therapy than reinfection isolates: 6.2 months versus 17.5 months. In a prospective observational study about MAC lung disease in patients with nodular bronchiectasis, relapse was rare in patients who are culture negative for more than ten months of appropriate treatment and most infections at this time are caused by new strains (85% of subsequent infection). On the other hand, most of the infections in patients who were culture negative for less than ten months were a relapse (86% of infections), rather than new infection [59]. No late isolates were macrolide resistant in both studies. Noncompliance to therapy has to be carefully evaluated in case of treatment failure. Some studies evaluated risk factors for disease progression in nodular/bronchiectatic MAC. Kitada et al. performed an observational retrospective study in 72 patients with nodular/bronchiectatic MAC lung disease and showed that risk factors for disease progression were low body mass index, cavitary disease, consolidations, and macrolide resistance at presentation [60]. In another recent observational retrospective study, Zoumot et al. showed that chronic pulmonary aspergillosis, cavitation within nodules, and emphysema at presentation are associated with increased mortality in NCFBE affected by MAC infection [46].

MAC disease in nodular/bronchiectatic patients is a slow but progressive long-term infection [60].

Considering that relapse or new infections are possible after treatment, follow-up of these patients is mandatory during and after therapy. Patients under antibiotics therapy for NTM should be closely monitored, with sputum exam and visit, to assess response to therapy and possible side effects. Also patients who do not receive treatment should be monitored to evaluate eventual disease progression.

Some reports exist about the role of fluorine-18 fluorodeoxyglucose positron-emission tomography/computed tomography (F-18 FDG PET/CT) in evaluation of treatment response. Sato et al. reported a case of disseminated MAC infection and a FDG PET/CT performed after 4 and 9 months of antimycobacterial therapy that showed a decreased FDG accumulation [61]. Drijkoningen et al. reported another case of regression of PET avidity in disseminated MAC disease after 2 months of specific therapy [62]. No specific recommendations exist about the use of PET/CT in the follow-up of NTM disease, but these reports may indicate a possible role in both evaluation of successful treatment and follow-up.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Nontuberculous Mycobacterial Ocular Infections: A Systematic Review of the Literature

Wajiha J. Kheir, Huda Sheheitli, Maamoun Abdul Fattah, and Rola N. Hamam

Department of Ophthalmology, Ocular Immunology and Uveitis Service, American University of Beirut, Riad El Solh, Beirut 1107 2020, Lebanon

Correspondence should be addressed to Rola N. Hamam; rh46@aub.edu.lb

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Nontuberculous or atypical mycobacterial ocular infections have been increasing in prevalence over the past few decades. They are known to cause periocular, adnexal, ocular surface and intraocular infections and are often recalcitrant to medical therapy. These infections can potentially cause detrimental outcomes, in part due to a delay in diagnosis. We review 174 case reports and series on nontuberculous mycobacterial (NTM) ocular infections and discuss etiology, microbiology, risk factors, diagnosis, clinical presentation, and treatment of these infections. History of interventions, trauma, foreign bodies, implants, contact lenses, and steroids are linked to NTM ocular infections. Steroid use may prolong the duration of the infection and cause poorer visual outcomes. Early diagnosis and initiation of treatment with multiple antibiotics are necessary to achieve the best visual outcome.

1. Introduction

Nontuberculous mycobacteria (NTM) are defined as mycobacteria other than *Mycobacterium tuberculosis*. NTM infections are found ubiquitously in the environment in soil, dust, and water [1, 2]. Human infection is thought to be acquired from environmental exposures [1].

Nontuberculous or atypical mycobacterial ocular infections were first reported in a case of *Mycobacteria fortuitum* keratitis by Turner and Stinson in 1965 [3]. Reports of these infections increased in frequency and variety over the years, with cases reported of choroiditis [4] in 1969, orbital infections [5] in 1969, and endophthalmitis [6] in 1970, to list a few. With the advent of laser-assisted in situ keratomileusis (LASIK), nontuberculous mycobacteria began to be further implicated in cases of keratitis [7]. In more recent years, these infections have become subject to much study considering their potentially detrimental outcomes.

NTM infections are difficult to identify, with a significant delay in diagnosis or initial misdiagnosis causing a delay in treatment [8]. Their course is indolent, additionally prolonged with the use of topical corticosteroids [9], and often refractory to multiple medical therapies and surgical interventions. Timely diagnosis and proper treatment of these infections are paramount. In this paper, we systematically review 174 case reports and case series of 420 eyes from 379 patients infected with NTM. Etiology, microbiology, risk factors, diagnosis, clinical presentation, and treatment of the different types of ocular infections are discussed.

2. Methodology

Pubmed, Medline, and Scopus databases were accessed in November 2014 and a thorough search of the literature was conducted. The keywords *mycobacter*, atypical or nontuberculous or non-tuberculous, or avium or intracellulare or avium-intracellulare complex or avium complex or leprae or malmoense or marinarum or scrofulaceum or simiae or szulgae or ulcerans or xenopi or abscessus or chelonae or fortuitum or gordonae or smegmatis or ulcerans or massiliense, ophthalmic or eye or ocular or ophthalmological or eye disease* and Medical subject headings (MeSh) *nontuberculous mycobacteria* and *eye infections* were used. Non-English language case reports/series were excluded from the review. No other exclusion criteria were employed. 174 eligible case reports and series on NTM ocular infections were identified from August 1965 to September 2014. The distribution of these papers over

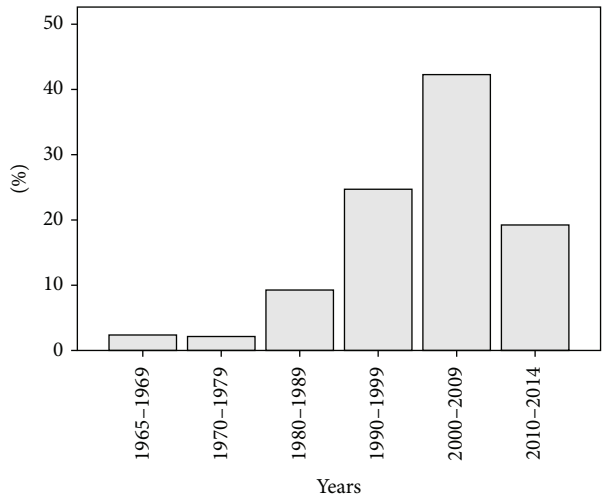

Figure 1: Distribution of reports of NTM infections over time.

time can be found in Figure 1. Additional three non-English reports were excluded.

From each paper, information on patient characteristics and course of infection was collected. Patient characteristics included age, gender, past medical history, past ocular history, and immune status. Information related to the course of infection was laterality, location, onset, initial and final visual acuity, clinical manifestations, type of samples taken, need for additional samples, tests determining diagnosis, delay in diagnosis, pathology, result of acid-fact bacillus (AFB) stain, type of organism, coinfection, possible source of infection, preceding interventions, associated trauma or foreign body, use of steroids, implants and contact lenses, medical and surgical treatment, prolonged course of infection, type and mode of delivery of antibiotics, duration of treatment, and outcome.

3. General Results

3.1. Location of Infection. NTM has been reported to cause periocular and adnexal infections, ocular surface infections, intraocular infections, and uveitis, as summarized in Table 1.

There exist several case reports of NTM infections involving more than one structure in the eye. Clare and Mitchell reported a case of iris root abscess and necrotizing scleroker-atitis in an immunocompetent woman with no apparent risk factors [177]. Sclerokeratitis was seen after cataract surgery in 4 eyes [40, 123], and keratitis leading to endophthalmitis was also seen in 6 eyes after cataract surgery [157, 158, 161] and 2 eyes after penetrating keratoplasty (PKP) [133, 156]. In one case of an elderly lady with dry eyes, punctal plug insertion caused secondary upper lid canaliculitis and keratitis [29]. Furthermore, endophthalmitis with a preseptal abscess was reported after cosmetic contact lens use in an eye with a failed corneal graft [164].

3.2. Patient Demographics. A total of 379 patients were reviewed. There was no gender predominance in NTM infections with a female to male ratio of 0.96 ($n = 368$). The average age of patients was 46 years (median 44, range 5–89) ($n = 369$). If we look at the distribution of infections across age groups, 19 patients (5.1%) were below age 20, 244 patients (66.1%) were between ages of 20 and 59, and 106 patients (28.7%) were above age of 60.

Table 1: Distribution of the types of ocular NTM infections.

Type of infection	Number of eyes ($n = 420$)
Periocular and adnexal infections	
Orbital [5, 10–16]	11 (2.6%)
Eyelid and periocular skin [14, 17–25]	28 (6.7%)
Lacrimal system [14, 18, 26–34]	17 (4.0%)
Total	**56 (13.3%)**
Ocular surface infections	
Keratitis [3, 4, 8, 27, 35–133]	290 (69.0%)
Scleritis [134–143]	18 (4.3%)
Conjunctivitis [144–146]	3 (0.7%)
Total	**311 (74.0%)**
Intraocular infections and uveitis	
Endophthalmitis [6, 74, 92, 106, 116, 133, 138, 147–172]	44 (10.4%)
Choroiditis [4, 173–176]	6 (1.5%)
Iridocyclitis [177]	1 (0.2%)
Panuveitis [178]	2 (0.5%)
Total	**53 (12.6%)**

3.3. Microbiology. In our review, the culprit organism was found to be *M. chelonae* in 179 eyes (42.7%), *M. abscessus* in 46 eyes (11.1%), and *M. fortuitum* in 62 eyes (14.7%). These 3 species in addition to other NTM species we encountered are listed in Table 2 with their frequencies.

4. Results Specific to Location of Infection

A summary of the results specific to location of infection can be found in Table 3.

4.1. Orbital Infections

4.1.1. Etiology. NTM orbital infections are quite rare; only 11 cases have been reported in the literature [5, 10–16]. Approximately half the cases were preceded by an intervention (5/11 eyes, 45.5%); orbital reconstruction and fracture repair after trauma in 4 eyes (36.4%) and blepharoplasty in 1 eye (9.1%). Orbital implants were implicated in 5/11 eyes (45.5%).

4.1.2. Clinical Presentation. Patients presented with a variable course ranging from days to months. Time to presentation was on average 8 weeks (range: 3 days–44 weeks). Patients presented with an intraconal mass gradually causing restricted motility and proptosis in 2/11 eyes (18.2%). Periorbital cellulitis with secondary orbital abscesses/lesions was

Table 2: NTM species in ocular infections.

Mycobacterial species	Number of Eyes $n = 420$
M. chelonae	179 (42.6%)
M. fortuitum	62 (14.8%)
M. abscessus	46 (11.0%)
M. avium complex	8 (1.9%)
M. szulgai	8 (1.9%)
M. avium	4 (1.0%)
M. gordonae	4 (1.0%)
M. immunogenum	4 (1.0%)
M. haemofilis	3 (0.7%)
M. kansasii	3 (0.7%)
M. massiliense	3 (0.7%)
M. chelonae-fortuitum complex	2 (0.5%)
M. mucogenicum	2 (0.5%)
M. aurum	1 (0.2%)
M. flavescens	1 (0.2%)
M. goodii	1 (0.2%)
M. houstonense	1 (0.2%)
M. intracellulare	1 (0.2%)
M. marinarum	1 (0.2%)
M. phlei	1 (0.2%)
M. smegmatis	1 (0.2%)
Unknown NTM species	84 (20.0%)

seen in 2 eyes (18.2%). Associated osteomyelitis of the frontal bone with bone erosion was also seen in 2 eyes (18.2%), while infections that occurred after enucleation with Teflon ball implantation led to Teflon ball implant exposure or extrusion in 2 eyes (18.2%).

4.1.3. Diagnosis. Diagnosis of orbital NTM infections was achieved through culture of specimens obtained from the site of infection in 9/10 eyes (90%). DNA sequence analysis had to be done to confirm the diagnosis in 1/10 eyes (10%). The types of specimens used to make the final diagnosis were purulent material collected through simple swab in 1/7 eyes (14.2%) or drainage of abscesses in 2/7 eyes (28.5%) and orbital tissue biopsy of associated lesions in 4/7 eyes (57.1%). Pathology specimens revealed a chronic inflammatory lesion with granulomatous noncaseating features. Acid fast bacilli were revealed in the area of necrosis and were also found in lipid vacuoles in cases where there was orbital fat involvement.

Authors referred to status of delay in diagnosis of NTM infection in only 4 eyes. The reported delay was due either to not sending samples for culture initially or to the fact that no growth on culture once sample was sent or to misdiagnosis. For example, an orbital infection was confused for pseudotumor causing a 26-week delay in proper diagnosis and therefore effective treatment [11].

4.1.4. Treatment. Orbital NTM infections were treated with systemic antibiotics alone in 3/11 eyes (27.3%) or in combination with surgery in 8/11 eyes (72.7%). Most infections required the use of more than two types of antibiotics (6/11 eyes, 54.6%), most commonly combinations of macrolides, fluoroquinolones, and amikacin. Of the 8 eyes that underwent surgery, 3 had excision of the infectious lesion, 1 had a simple incision and drainage, and 1 had to undergo removal of an orbital implant. In fact, out of the 4 eyes that did not respond to initial medical therapy, 3 had to undergo a surgical intervention.

4.1.5. Outcome. Orbital NTM infections had a variable prognosis. Treatment led to complete resolution of the infection with no loss of vision in 7/10 eyes (70%). However, 3 eyes had a final visual acuity of 20/200 or worse, thereby rendering them legally blind. In our review, a prolonged course was determined as initial failure of medical therapy or more than one required surgical intervention. Four eyes with orbital NTM infections had a prolonged course (4/10 eyes, 40%), but the infection eventually resolved with no loss of vision in these eyes. There were no reported cases requiring enucleation, evisceration, or exenteration.

4.2. Eyelid/Periocular Skin Infections

4.2.1. Etiology. Twenty-eight cases of eyelid and periocular skin infections have been reported [14, 17–25]. All with the exception of 1 eye had a preceding intervention (27/28 eyes, 96.4%). The most common interventions were ptosis repair and/or blepharoplasty in 22 eyes (78.6%) and dacryocystorhinostomy (DCR) with or without stent placement in 4 eyes (14.3%). There were isolated cases of infections in eyes preceded by reconstruction/fracture repair after trauma and chalazion excision. Implants were implicated in 9/28 eyes (32.1%), and these included orbital implants, fat injections, lacrimal plug or stents, and silicone rods depending on the type of preceding intervention.

The one case that was not related to an intervention was that of a young man who got *M. marinum* preseptal cellulitis of his lower and upper lids after self-manipulating a hordeolum. The authors attributed his infection to exposure from his work place, a tropical fish shop [17].

4.2.2. Clinical Features. Eyelid and periocular skin NTM infections typically had a subacute presentation. However, reported symptoms ranged from immediate postoperative to month after any prior intervention (1 week–12 weeks). Presenting symptoms were mainly firm erythematous single and multiple nodular lesions along surgical wounds in 17/24 eyes (70.8%) and were associated with progressive swelling and surrounding periorbital cellulitis. Frequently, the red nodules drained purulent discharge. Elevated infectious nodules also presented without erythema or other inflammatory signs in 7/24 eyes (29.1%). There were no associated intraocular manifestations.

4.2.3. Diagnosis. The diagnosis of atypical mycobacterial infection was made by culture in 23/26 eyes (88.5%) or on histopathologic examination in 3 eyes (11.5%). Histopathologic findings included chronic granulomatous inflammation and necrotizing granulomata. The specimen used to make

Table 3: Summary of the results specific to location of infection.

Location of NTM infections	Total cases	Etiology	Medical treatment	Surgical treatment	Outcomes
Orbital infections	11	(i) Orbital reconstruction/Fracture repair 4/11 (36.4%) (ii) Orbital Implant 5/11 (45.5%)	Systemic ATB combination (>2 ATB) 6/11 (54.6%)	Excision of lesion 3/11 (37.5%)	(i) Resolution 7/10 (70%) (ii) VA of 20/200 or worse 3/10 (30%) (iii) Prolonged course 4/10 (40%)
Eyelid/periocular skin infections	28	(i) Ptosis Repair and/or Blepharoplasty 22/28 (78.6%) (ii) Implants 9/28 (32.1%)	Systemic ATB combination (>2 ATB) 14/28 (50%)	(i) Excision/incision and drainage/debridement 13/28 (46.4%) (ii) Removal of implant 7/28 (25%)	(i) Resolution 22/28 (78.5%) (ii) Prolonged course 6/28 (21.4%)
Lacrimal system infections	17	(i) Implants 13/17 (76.5%) (ii) Punctal Plug or Lacrimal Tube Insertions 9/17 (52.9%) (iii) DCR +/− stent placement 5/17 (29.4%)	Topical + systemic 7/17 (41.2%)	Removal of implant 10/17 (58.8%)	(i) Resolution 13/17 (76.5%) (ii) Prolonged course 3/17 (17.6%)
Keratitis	290	(i) LASIK 130/273 (47.6%) (ii) Trauma 43/264 (14.8%) (iii) Foreign body 51/211 (17.6%) (iv) Contact lenses 19/276 (6.4%) (v) Implants 44/254 (17.3%)	(i) Topical 108/203 (53.2%)* (ii) Topical + systemic 85/203 (41.9%)*	(i) Removal of corneal flap 49/283 (17.3%) (ii) PKP 40/283 (14.1%)	(i) Resolution 190/235 (80.9%) (ii) VA 20/40 or better 112/204 (54.9%) (iii) VA of 20/200 or worse 40/204 (19.6%)
Scleritis	18	Scleral buckling 14/18 (77.8%)	Topical 16/17 (94.1%)	Explanation of buckle 13/17 (76.5%)	(i) Resolution 16/17 (94.1%) (ii) VA of 20/200 or worse 10/14 (71.4%)
Endophthalmitis	44	Cataract surgery (IOL insertion) 18/37 (48.6%)	(i) Intraocular + topical/systemic/periocular 14/38 (36.8%) (ii) Intraocular 5/38 (13.2%)	PPV 13/37 (35.1%)	(i) Resolution 12/36 (33.3%) (ii) Loss of eye 12/36 (33.3%) (iii) Loss of vision 12/36 (33.3%)
Uveitis	9	HIV/AIDS 5/9 (55.6%)	Systemic/topical + systemic 6/9 (66.7%)	(i) PPV 1/8 (12.5%) (ii) Evisceration 1/8 (12.5%) (iii) Enucleation 1/8 (12.5%)	(i) Resolution 4/9 (44.4%) (ii) Loss of eye 3/9 (33.3%) (iii) VA 20/40 or better 2/4 (50%)

Denominator differs from total number of cases due to missing data.
* Analysis was done based on infections that led to eventual resolution without loss of vision.

the diagnosis was the purulent discharge collected by swab in 5/15 eyes (33.3%). Other cases required incision and drainage in 4/15 eyes (26.7%) as well as biopsy of nodular lesions in 3/15 (20%) to achieve a diagnosis. In two cases diagnosis was only achieved after intraoperative tissue, taken from debrided nodules and lesions that were excised as part of the treatment, was sent for culture and histopathology. When AFB stain was performed, it was positive in 9/10 eyes (90%).

Delay in diagnosis of eyelid and periocular skin NTM infections was encountered in 6/12 eyes (50%). Reasons for the delay included no growth or slow growth on culture and not sending samples for cultures initially.

4.2.4. Treatment. Treatment of eyelid and periocular skin infections consisted of either medical therapy in 8/28 eyes (28.6%) or surgical therapy in 2/28 eyes (7.1%) or

a combination of both modalities in 18/28 eyes (64.3%). Regarding medical therapy, systemic antibiotics were used in all 26 eyes treated medically, with 3 eyes additionally treated with topical antibiotics. More than half the infections required more than 2 antibiotics in the regimen (14/28 eyes, 50%). These were usually a combination of amikacin, macrolides, and fluoroquinolones. Antibiotics that were used alone were fluoroquinolones in 4/28 eyes (14.3%) and macrolides in 8/28 eyes (28.6%).

Of the infections that failed to respond to initial medical therapy (14/23 eyes, 60.9%), 11 (78.6%) had to undergo surgical treatment. In fact, excision of infectious lesions alone was sufficient to clear the infection in 2/28 eyes (7.1%). Examples of the types of surgical treatment that had to be performed are excision of lesions, incision and drainage, and debridement in 13/28 eyes (46.4%). Of the 9 infections that implicated an implant, removal of that implant was necessary for the resolution of the infection in 7 eyes.

4.2.5. Outcome. Patients with eyelid NTM infections had a relatively good prognosis; infection generally resolved with no major sequel affecting the eyelid function in 22/28 eyes (78.5%). There were no reported cases of loss of eye due to NTM eyelid infection. Six cases had a prolonged course but eventually recovered with no change in vision or eyelid dysfunction. There were no reported cases leading to loss of vision or enucleation, evisceration, or exenteration.

4.3. Lacrimal System Infections

4.3.1. Etiology. There are seventeen cases of lacrimal system infections, dacryocystitis, and canaliculitis, reported to be due to NTM [14, 18, 26–34]. Most cases (14/17 eyes, 82.3%) were preceded by one of two types of interventions, punctal plug or lacrimal tube insertions in 9/17 eyes (52.9%) and DCR with or without stent placement in 5/17 eyes (29.4%). Implants were implicated in 13/17 eyes (76.5%). Previous ocular history of epiphora and nasolacrimal duct obstruction was found in the 2 eyes that had not had prior intervention. One of these eyes belonged to a patient who was HIV-positive.

4.3.2. Clinical Presentation. Presentation was subacute, with onset of symptoms ranging from 2 to 26 weeks after intervention. Patients presented with epiphora and purulent discharge from the puncta, along with swelling and erythema at the medial canthal area or at the site of the DCR incision, with or without associated nodular lesions in 12/14 eyes (85.7%). Less frequently, they had blood tinged purulent discharge in 2/14 eyes (14.2%).

4.3.3. Diagnosis. Diagnosis was mainly made through swab cultures taken from draining purulent material expressed from canaliculi 8/11 (72.7%). In certain situations where the draining material was insufficient for diagnosis, biopsy of the associated nodular lesion confirmed diagnosis (2/11 eyes, 18.2%). Chronic granulomatous reaction was frequently seen in the affected material and acid fast staining revealed bacilli within the area of necrosis.

Delay in diagnosis as reported by authors was seen in 6/14 eyes (42.9%). Causes of delay included delay in sending samples for culture, no growth on initial samples taken, and misdiagnosis.

4.3.4. Treatment. Like eyelid and periocular skin infections, treatment of lacrimal system infections consisted of medical therapy in 2/17 eyes (11.8%), surgical therapy in 2/17 eyes (11.8%), or a combination of both in 13/17 eyes (76.5%). Surgery alone was enough to clear the infection in 2/17 eyes (11.8%). All 8/13 eyes (61.5%) that did not respond to initial medical therapy had to undergo surgery. The most common type of surgery was removal of the implant with or without debridement in 10/17 eyes (58.8%). Other surgical therapies included excision of lesions, incision and drainage, and canaliculotomy.

The most common mode of antibiotic administration was a combination of both topical and systemic (7/17, 41.2%). Topical antibiotics alone were used in 3/17 eyes and systemic antibiotics alone were used in 2/17 eyes. More than 2 types of antibiotics were used in 8/17 eyes (47%), and these included a combination of mainly amikacin, fluoroquinolones, and macrolides. When a single antimicrobial was used, choices were amikacin in 3/17 eyes (17.6%), fluoroquinolones in 2/17 (11.8%) eyes, and macrolides in 2/17 eyes (11.8%).

4.3.5. Outcome. Dacryocystitis and canaliculitis also had a good prognosis. The majority of infections (13/17 eyes, 76.5%) had complete resolution of the infection. Three cases had a prolonged course with eventual resolution. One patient was lost to follow-up. There were no reported cases leading to loss of vision or enucleation/evisceration.

4.4. Keratitis

4.4.1. Etiology. Keratitis is the most common type of ocular NTM infection, with 290/420 eyes (69%) reported so far in the literature [3, 4, 8, 27, 35–133]. The vast majority of keratitis infections are preceded by an intervention (190/273 eyes, 69.3%), most commonly LASIK in 130/273 eyes (47.6%).

Other interventions include cataract surgery in 24/273 eyes (8.8%), penetrating keratoplasty (PKP) in 26/273 eyes (9.5%), and pterygium/pinguecula excision in 4/273 eyes (1.5%). NTM keratitis has also been seen following radial keratotomy, cataract surgery with PKP, laser epithelial keratomileusis, deep anterior lamellar keratoplasty, and endokeratoplasty.

Other possible risk factors for the development of NTM keratitis are trauma (43/264 eyes, 14.8%) and presence of a foreign body (51/211 eyes, 17.6%). The most common type of foreign body implicated was metallic in 31/51 eyes (60.8%), with wood, glass, plant debris, shale, and clay accounting for a few cases. Not all authors outlined the mechanism by which the foreign body got in the eye.

Keratitis was found to be a serious complication of contact lenses, whether soft or hard. In our review, contact lenses were used in 19/276 eyes (6.4%), including one case involving a bandage contact lens. Steroids were used in more than half of the cases of keratitis (101/176 eyes, 57.4%). As for

implants, they were found in 44/254 eyes (17.3%) and were mainly intraocular lenses (22/254 eyes, 8.7%) and corneal grafts/tissues (24 eyes, 9.4%).

Patients with keratitis and no other obvious risk factors were found to have certain medical problems including miliary tuberculosis, rheumatoid arthritis, bullous pemphigoid, and a history of malignancy. Relevant ocular history was determined to be ocular surface disease in 2 eyes and exposure keratopathy in 1 eye.

4.4.2. Clinical Presentation. With NTM keratitis, time to presentation varied from 1 day to 1 year, with an average of 5.6 weeks ($n = 158$). On examination, patients typically exhibited a "cracked windshield" appearance of the cornea around the edge of the central area of the infiltrate. Infiltrates at times had irregular margins or satellite lesions, mimicking fungal keratitis [38, 39, 44, 64, 80, 81, 92]. Dendritic epithelial defects with minimal stromal infiltration were also seen in NTM infections, prompting authors to falsely diagnose herpes keratitis [50, 88].

4.4.3. Diagnosis. The diagnosis of NTM keratitis was done mostly through culture of samples from the eye. Occasionally, polymerase chain reaction (PCR), PCR probes/hybridization, PCR restriction endonuclease analysis, and gene sequencing were needed to establish or confirm the diagnosis. AFB stain tested positive in samples from 101/115 eyes (87.8%). Regarding samples collected, isolation of the causative organism in NTM keratitis often required only a superficial corneal scraping (127/198 eyes, 64.1%). When scrapings did not reveal the organism, corneal biopsy was needed to reach a diagnosis (19/198 eyes, 9.5%). When NTM keratitis occurred after LASIK, infiltrates appeared within the lamellar flap or at the flap interface. Making a swift diagnosis required the lifting of the flap to obtain scrapings for microbiological evaluation in 27/198 eyes (13.6%). In eyes that necessitated corneal transplant, the corneal button was often used to determine diagnosis (11/198 eyes, 5.5%). In isolated cases, surgical instruments and a lens care system were used when more traditional methods failed to offer a causative organism.

Delay in making the diagnosis was reported in 61/110 eyes (55.5%). Reasons provided for this delay were misidentification of the causative organism, delay in taking cultures, no growth or slow growth of the organism, and misdiagnosis. Organisms misidentified as the causative agent were *Nocardia* species [35, 36, 70, 71, 128] and *Corynebacterium* species [74]. Relevant misdiagnoses made were herpes keratitis [46, 50, 53, 55, 66] and fungal keratitis [39, 46, 48, 54, 94]. The duration of delay ranged from 1 week to 30 weeks, with an average of 8 weeks ($n = 24$).

4.4.4. Treatment. Most cases of NTM keratitis were treated with medical therapy alone in 127/283 eyes (44.9%) or a combination of medical and surgical therapy in 156/283 eyes (55.1%). Surgical treatment was required in 156/283 eyes (55.1%). Of the infections that had an initial lack of response to medical therapy (141/193 eyes, 73.1%), 111/140 eyes (79.3%) had to undergo a surgical intervention. The most common types of surgeries were removal the corneal flap in 49/283 eyes (17.3%), PKP in 40/283 eyes (14.1%), extirpative keratectomy in 15/283 eyes (5.3%), and removal of implant, whether a corneal graft or an IOL, in 9/283 eyes (3.6%). In fact, removal of the flap for resolution of infection was needed in 49/61 eyes after LASIK (80.3%).

Table 4: Medical treatment of NTM keratitis.

Mode of delivery	Number of eyes $n = 203$
Topical	108 (53.2%)
Topical and systemic	85 (41.9%)
Systemic	5 (2.5%)
Topical, systemic, and periocular	2 (1%)
Topical and intraocular	1 (0.5%)
Topical and periocular	1 (0.5%)
Systemic and periocular	1 (0.5%)
Antibiotic (ATB) regimen	**Number of eyes $n = 192$**
Amikacin alone	56 (29.2%)
Amikacin + macrolide	27 (14.1%)
Amikacin + fluoroquinolone	24 (12.5%)
Amikacin + fluoroquinolone + macrolide	18 (9.4%)
Fluoroquinolone + macrolide	16 (8.3%)
Other*	14 (7.3%)
Fluoroquinolone alone	13 (6.8%)
Amikacin + 1 or more ATB*	11 (5.7%)
Macrolide + 1 or more ATB*	7 (3.6%)
Macrolide alone	5 (2.6%)
Fluoroquinolone + 1 more ATB*	1 (0.5%)

*Antibiotic not including amikacin/fluoroquinolone/macrolide.

Results related to medical therapy were centered on treatment of infections that led to eventual resolution without severe loss of vision (final visual acuity better than 20/200). The mode of delivery and antibiotics used are summarized in Table 4. The most common modes of delivery of antibiotics were topical in 108/203 eyes (53.2%) and a combination of both topical and systemic in 85/203 eyes (41.9%). More than two antibiotics had to be used in 112/203 eyes (55.2%), with the majority of the combinations including amikacin (80/203, 39.4%). In fact, amikacin constituted sole therapy in 56/203 eyes (27.6%). Other commonly used antibiotics were fluoroquinolones and macrolides, alone or in combination with other antibiotics.

4.4.5. Outcome. The majority of cases of NTM keratitis resolved without severe loss of vision (190/235 eyes, 80.9%). Among these, 48 (25.8%) had a prolonged course that necessitated either multiple medical therapies or more than 1 surgical intervention before resolution was reached. With respect to final visual acuity, more than half of the cases had a good outcome of 20/40 or better (112/204 eyes, 54.9%). Nonetheless, NTM keratitis was a potentially debilitating infection, with 40/204 eyes (19.6%) ending up with loss of vision or legal blindness. More so, 3 cases ended up with

loss of the eye (3/235 eyes, 1.3%). Patients who underwent a surgical intervention were more likely to end up with visual impairment (RR = 2.7, P value 0.001).

4.5. Scleritis

4.5.1. Etiology. There are eighteen cases of NTM scleritis reported in the literature [134–143]. Almost all cases were directly preceded by an intervention (17/18 eyes, 94.4%). These included scleral buckling procedure in 14/18 eyes (77.8%) and isolated cases of a pterygium excision, an intravitreal injection, and a pars plana vitrectomy (PPV). The one case of NTM scleritis not following a procedure occurred in an immunocompromised man with severe medullar hypoplasia on interleukin-2 (IL-2) treatment. He had a disseminated *M. chelonae* infection with spondylodiscitis and spinal epidural abscess in addition to the scleritis [137].

4.5.2. Clinical Presentation. In eyes that had undergone scleral buckling, NTM infections occurred weeks to months after the surgery (1.5 weeks–40 weeks). Manifestations included nonspecific symptoms of chronic redness, pain, and discharge. Infection was shown to lead to marked scleral thinning along with scleral buckle erosion and exposure, along with scleral abscess/subconjunctival nodules with mucopurulent discharge mainly late in the disease course. Scleral abscesses were seen after pterygium excision, and focal lesions were seen around the sutures at the scleral ports after vitrectomy. The immunocompromised patient presented with a nodular necrotizing scleritis. Although only conjunctival inflammation was seen after intravitreal injection, a hypoechoic excavation of the sclera at the site of the injection was evident on anterior segment ultrasound.

4.5.3. Diagnosis. Diagnosis of NTM scleritis was made through samples obtained from scleral biopsies of abscesses and nodules sent for culture. In 1 case, the etiological agent was confirmed by 116S rRNA sequencing [134]. Explanted scleral buckles were also used to isolate NTM. Conjunctival biopsy specimens from vitrectomy port sites revealed the diagnosis after PPV. None of the authors reporting on time to diagnosis found a significant delay (6 eyes).

4.5.4. Treatment. Eyes with NTM scleritis were treated with a combination of medical and surgical therapy in 15/17 eyes (88.2%) and medically alone in 2/17 eyes (11.8%). All 14 eyes associated with scleral buckles required surgery, 13/17 eyes had explantation of the buckle with debridement of necrotic tissue. One eye needed debridement after the removal of an exposed scleral buckle [141]. Other types of surgical interventions were debridement procedures with or without the use of scleral patch grafts for scleral thinning.

Medical therapy consisted of topical antibiotics (16/17 eyes, 94.1%), in combination with systemic antibiotics (5/17 eyes, 29.4%), periocular antibiotics (1/17 eyes, 5.8%), or both (4/17 eyes, 23.5%). Most eyes (12/17 eyes, 70.6%) were treated with 2 or more types of antibiotics. Amikacin and other aminoglycosides such as gentamicin and kanamycin were the most common antibiotics used (12/17 eyes, 70.6%), and these were usually combined with macrolides and/or fluoroquinolones.

4.5.5. Outcome. Although NTM scleritis resolved on proper treatment in the majority of the cases (16/17 eyes, 94.1%), the visual outcome was poor; 10/14 eyes (71.4%) had a final visual outcome of 20/200 or worse. Of the cases that resolved, 2/16 eyes (12.5%) had a prolonged course requiring a change in the medical treatment regimen in both and an additional surgery for debridement in one. Only 1 case (1/17 eyes, 5.9%) ended up with loss of the eye.

4.6. Conjunctivitis. There were only 3 cases of isolated NTM conjunctival involvement reported in the literature [144–146]. One occurred in an AIDS patient with bacillary angiomatosis of the palpebral conjunctiva with coinfection by NTM and cytomegalovirus. He presented with a large mass protruding from his eye, which underwent debulking. Cultures of intraoperative specimens revealed the diagnosis. On pathology, acid fast bacilli (AFB) were concentrated in areas of microabscesses. He was treated with topical erythromycin and systemic azithromycin, ethambutol, isoniazid, rifampicin, pyrazinamide, and acyclovir. The mass resolved with no effect on visual acuity [144].

A second case occurred in a healthy middle-aged woman who raised parrots and wore soft contact lenses but did not have any risk factors otherwise. She presented with a large, fleshy, elevated conjunctival mass that was treated with excision and topical ciprofloxacin and amikacin. After conventional staining techniques failed, diagnosis of *M. fortuitum* was established by PCR. Pathology revealed suppurative granulomatous inflammation. A recurrence was successfully treated with repeat excision and oral clarithromycin and moxifloxacin [145].

The last case occurred in an elderly man who developed nodular bulbar conjunctivitis 6 weeks after cataract surgery with IOL placement. He had no apparent risk factors other than steroid treatment postoperatively. An incisional biopsy, revealing suppurative granulomas with AFB, suggested mycobacterial infection. A swab culture later confirmed infection with *M. abscessus*. After failure of oral antimicrobial therapy, the patient was successfully treated with topical ciprofloxacin and lubrication [146].

4.7. Endophthalmitis

4.7.1. Etiology. There were 44 cases of endophthalmitis reported in the literature [6, 74, 92, 106, 116, 133, 138, 147–172]. Most were preceded by an intervention (28/37 eyes, 75.7%), mainly cataract surgery with IOL insertion (18/37 eyes, 48.6%). Other predisposing procedures were penetrating keratoplasty, intravitreal injection, scleral buckling, filtering surgery, and Descemet's stripping automated endothelial keratoplasty or DSAEK (1 eye, 2.7%). Implants were implicated in 26/37 eyes (70.3%) and included IOLs, glaucoma filtering tubes, corneal grafts/tissues, and a scleral buckle.

Possible etiological factors for eyes that did not have a direct intervention were identified. NTM endophthalmitis occurred in 6/10 eyes (60%) of immunocompromised

patients, as well as in 3/10 eyes (30%) of patients with disseminated NTM infections. Other factors were steroid use in 4/10 eyes (40%), contact lens use in 1/10 eyes (10%), and an old glaucoma filtering tube implant in 1/10 eyes (10%).

4.7.2. Clinical Presentation. For eyes that were preceded by a procedure, patients presented within days and up to 35 weeks after intervention, with an average of 11.5 weeks ($n = 13$). On examination, anterior chamber reaction with hypopyon was seen in 19/32 eyes (59.3%). Granulomatous keratic precipitates were also observed in 2/32 eyes (6.3%). 10/32 eyes (31.2%) had an associated vitreous inflammatory reaction. Postcataract NTM endophthalmitis revealed whitish fluffy plaque like material on the intraocular lens in 1 eye. Corneal infiltrates and/or abscesses formed in the needle-knife tract of the cataract wound in 3/32 eyes (9.2%).

4.7.3. Diagnosis. In patients with NTM endophthalmitis, diagnosis was achieved mostly through culture of aqueous samples from anterior chamber tap in 11/36 eyes (30.5%) and vitreous samples from tap in 13/36 eyes (36.1%) and vitrectomy in 6/36 eyes (16.7%). Samples that underwent AFB staining tested positive in 19/20 eyes (95%). In six eyes with endophthalmitis associated with keratitis, corneal specimens were sufficient to establish the diagnosis.

On histopathology of the eyes requiring enucleation or evisceration, eye contents revealed extensive infiltration of the anterior chamber and vitreous cavity with a dense granulomatous reaction.

Delay in the diagnosis of NTM endophthalmitis was reported in 12/25 eyes (48%). Reasons identified for this delay were misidentification of the causative organism, no or slow growth on culture, and misdiagnosis. For example, chronic endophthalmitis has been mistaken for granulomatous iridocyclitis [151]. The duration of delay varied from 3 to 9 weeks and was 5.6 weeks on average ($n = 5$).

4.7.4. Treatment. All eyes with endophthalmitis were treated medically, and 32/37 eyes (86.5%) were also treated with surgery. In fact, out of the 24 eyes that did not respond to initial medical therapy, 22 (91.7%) required surgical intervention. Types of surgeries were PPV in 13/37 eyes (35.1%), removal of ocular implant in 8/37 eyes (21.6%), enucleation of 5/37 eyes (153.5%), and evisceration of 3/37 eyes (8.1%).

The most common route of antibiotic administration was intraocular combined with topical/systemic/periocular antibiotics in 14/38 eyes (36.8%), topical in 8/38 eyes (21.1%), topical combined with systemic/periocular in 7/38 eyes (18.4%), and intraocular in 5/38 eyes (13.2%). Regarding the types of antibiotics, amikacin alone (5/38 eyes, 13.2%) or in combination with other antibiotics (19/38 eyes, 50%) was the most used. Combinations of antibiotics included fluoroquinolones in 7/38 eyes (18.4%) and macrolides in 11/38 eyes (28.9%). At least 2 types of antibiotics were used in 29/38 eyes (76.3%).

4.7.5. Outcome. The prognosis of NTM endophthalmitis is poor. Loss of eye occurred in 12/36 eyes (33.3%) and loss of vision in 12/36 eyes (33.3%). Resolution of the infection took place in 12/36 eyes (33.3%) and, of these, 5 had a prolonged course. With respect to final visual acuity, only 5/22 eyes (22.7%) had a visual acuity of 20/40 and better. One patient with AIDS and a disseminated *M. avium* infection passed away [150]. We found no significant correlation between PPV and visual outcome.

4.8. Uveitis

4.8.1. Etiology. There are 9 reported cases of uveitis caused by NTM in the literature: choroiditis in 6 eyes [4, 173–176], iridocyclitis in 1 eye [177], and granulomatous panuveitis in 2 eyes [178]. Predisposing factors included an intervention of cataract with PPV in 1/9 eyes (10%) and treatment with steroids in 3/7 eyes (42.9%). Uveitis due to NTM occurred in 5/9 eyes (55.6%) of immunocompromised patients, all of whom had HIV/AIDS. 3/9 eyes (33.3%) were patients with a disseminated NTM infection or localized infection elsewhere in the body. Regarding ocular history, 3/4 eyes (75%) had history of previous CMV retinitis.

4.8.2. Clinical Presentation. Cases with systemic NTM infections led to the development of choroidal and other intraocular lesions in 3/9 eyes (33.3%). Choroiditis presented as multiple yellowish, round, subretinal pigment epithelial (sub-RPE) lesions. Along with the multifocal choroiditis, eyes exhibited associated panuveitis with anterior chamber reaction, iris nodules, and vitritis in 2/9 eyes (22.2%). In a patient with AIDS, the presentation was subclinical in nature prior to initiation of HAART therapy, with minimal evidence of inflammation. After initiation of therapy, massive granulomatous inflammation and panuveitis resulted bilaterally [176].

In one case of hemorrhagic anterior uveitis, a subconjunctival mass extending to the cornea turned out to be a nodular iris root abscess extending from the anterior chamber to the iris and ciliary body on ultrasound [177].

4.8.3. Diagnosis. Culture of ocular samples from eyes with uveitis recovered NTM in 3/8 eyes (37.5%). Molecular techniques such as PCR confirmed the diagnosis in 3/8 eyes (37.5%). NTM were not retrieved in 2/8 eyes (37.5%), and the diagnosis was made because the patients had systemic NTM infections. Microbial analysis was carried out on vitreous samples in 5/6 eyes (83.3%) and on a corneal biopsy in 1 eye with the iris root abscess. The result of the AFB stain was mentioned in 4 eyes, all of which had a positive result. On histopathology of enucleated eyes, choroidal lesions had a suppurative center surrounded by granulomatous inflammation.

Delay in diagnosis was encountered in 5/6 eyes (83.3%). Reasons reported for the delay were misdiagnosis, misidentification of the organism, and delay in taking cultures. In one eye, *Nocardia* species was initially thought to be the causative organism [177]. In another, choroiditis was mistaken for ocular lymphoma [175].

4.8.4. Treatment. NTM uveitis was treated medically, with 3/8 eyes (37.5%) requiring surgical treatment. Types of surgeries were PPV, evisceration, and enucleation. Lack of initial resolution on medical therapy occurred in 3/7 eyes (42.9%), all of which had to undergo surgical intervention. Regarding medical therapy, mode of administration of antibiotics was systemic in 3/9 eyes (33.3%), topical and systemic in 3/9 eyes (33.3%), topical, systemic and intraocular in 2/9 eyes (22.2%), and systemic, intraocular, and periocular in 1/9 eyes (11.1%). All eyes were treated with 2 or more types of antibiotics. A common treatment regimen consisted of antituberculous medications in 4/9 eyes (44.4%), while others were combinations of macrolides, amikacin, and fluoroquinolones in 5 eyes (55.6%).

4.8.5. Outcome. Treatment of NTM uveitis resulted in resolution in 4/9 eyes (44.4%), with a prolonged course in 1 eye. Loss of eye occurred in 3 cases and patient passed away in 2 cases. One eye had stable choroidal lesions at the time of reporting. Final visual acuity was reported as 20/40 or better in 2/4 eyes (50%) and 20/50 in 2/4 eyes.

5. Discussion

Despite the increasing reports on NTM ocular infections, they remain relatively uncommon. The most frequently reported type of infection caused by NTM is keratitis and accounts for 69% of all eyes reviewed [3, 4, 8, 27, 35–133]. On the other hand, Fong et al. analyzed the clinical and mycobacterial characteristics of 476 cases of microbial keratitis at the National Taiwan University Hospital over a 10-year period (January 1992 to December 2001). NTM accounted for only 7.9% of culture positive isolates [179].

Incidence of NTM keratitis may have increased with the advent of LASIK. The American Society of Cataract and Refractive Surgery (ASCRS) Cornea Clinical Committee published data on the incidence, microbiology, treatment, and outcomes collected from a post-LASIK infectious keratitis survey. NTM accounted for 33/69 (48%), the majority of culture positive cases [180]. A study from Spain on 204,589 LASIK procedures conducted by Llovet et al. did not find any mycobacterial infections among the 72 eyes that developed infectious keratitis. The authors postulated, however, that the 33 samples with negative cultures had a late presentation and may likely be due to atypical organisms like NTM [181].

While most cases of NTM keratitis were sporadic, few have been due to outbreaks. Winthrop et al. investigated NTM keratitis outbreaks after LASIK from August 2000 to June 2001 and reported on 3 clusters of infections [182]. One outbreak was linked to the use of corneal masks from contact lens fragments [43], while another was linked to *M. szulgai* contaminated ice used to chill syringes utilized in flap lavage [54, 183]. Another cluster of 5 patients presenting with *M. chelonae* keratitis after PKP has been reported. Although the source of the outbreak was not traced, all donor corneas were harvested from the same collection center [113].

In our review, we identified several potential risk factors for ocular NTM infections, namely, any history of interventions, trauma, foreign body, implant, contact lens wear, and steroid use. When these factors were excluded from the analysis, observations on relevant past medical and ocular history were made in order to determine any added risks. Five patients had HIV/AIDS [18, 144, 154, 176, 178], and four patients had an NTM infection, either disseminated or at another site in the body [147, 150, 167, 175]. Individual cases of miliary tuberculosis [6], rheumatoid arthritis [38], bullous pemphigoid [66], and history of cancer [34] were also reported. Regarding relevant ocular history, two patients had nasolacrimal duct obstruction [18, 34], two had ocular surface disease [38, 66], and one had exposure keratopathy [66].

Of the diverse clinical presentations of ocular infections, certain ones would be suggestive of an NTM infection. An indolent infection with a delayed onset after intervention should raise the suspicion for NTM as the causative organisms. Clinical clues were especially evident in NTM keratitis; a "cracked windshield" appearance around the edge of the central area of infiltrates is practically pathognomonic. Other features such as irregular margins or satellite lesions mimicking fungal keratitis or dendritic epithelial defects mimicking herpes keratitis were also reported. If these are encountered in patients not responding to the corresponding antifungal or antiherpetic therapies, then NTM should be on the differential and therefore be investigated.

NTM have been divided into 4 Runyon groups based on their pigment and photoreactivity (Group I, photochromogens; Group II, scotochromogens; Groups III and IV, nonchromogens). Groups I, II, and III NTM are slow growing mycobacteria with a growth rate of 2 to 4 weeks. Group IV NTM are rapidly growing with a growth rate of 7–10 days [1, 2, 40, 95]. The rapidly growing *M. chelonae*, *M. abscessus*, and *M. fortuitum* were the organisms isolated from most ocular infections we reviewed. This is consistent with previous reports about the prevalence of ocular infections with these clinically important species [184, 185].

NTM species have different antimicrobial susceptibilities which makes species identification of NTM clinically important [1]. Identification of NTM is conventionally based primarily upon growth rate, pigmentation, colonial morphology, and results of biochemical tests such as arylsulfatase, catalase, and niacin tests. More recently, molecular and genetic techniques such as polymerase chain reaction (PCR), gene sequencing, and molecular probes have been used to characterize these organisms [2].

Regarding the staining of different specimens for AFB, the fluorochrome technique, the Ziehl-Neelsen, and the Kinyoun stain are used. NTM may appear pleomorphic, showing as long filaments or coccoid forms, with uniform staining properties. It is important to note that nonmycobacterial organisms, including *Rhodococcus* species, *Nocardia* species, and *Legionella micdadei*, as well as *Microsporidium* spores and the cysts of *Cryptosporidium, Isospora,* and *Cyclospora* may show various degrees of acid fastness. In this review, we came across 8 isolates of NTM ocular infections that were initially misidentified as *Nocardia* species [35, 36, 70, 71, 128, 177]. However, considering the high rate of positive AFB stains we found, reaching up to 95% in cases of NTM endophthalmitis, we recommend AFB stain as an initial diagnostic test pending microbial culture results.

When sending isolates for culture with the suspicion of NTM, both solid and broth (liquid) media should be included. The recommended broth system is the mycobacteria growth indicator tube (MGIT), and the recommended solid media include Löwenstein-Jensen and Middlebrook (7H10 and 7H11) media [1].

Isolates grown on culture media can be speciated by genotypic and molecular techniques as they allow for a rapid identification of NTM. In our review it is evident that there is increasing utilization of PCR and PCR probes/hybridization to accurately and rapidly identify specific NTM species [54, 61, 87, 121, 130, 144, 145, 151, 154–156, 178]. Another genotypic method that was frequently employed is the PCR restriction endonuclease analysis [35, 45, 165]. DNA sequencing and identification of the signature sequences of the 16S rRNA gene was also used for the initial diagnosis and confirmation of culture results [11, 81, 103, 136, 168, 169, 177]. Furthermore, the restriction fragment length polymorphism (RFLP) technique using the IS*6110* repetitive sequence as a probe [1, 2] was utilized by Palani et al. [161].

NTM can lead to a wide range of ocular infections requiring various sampling techniques for obtaining isolates for organism identification. Getting the appropriate sample for microbial analysis is critical. When swab cultures of infection sites are negative, incision and drainage or biopsy of lesions should be undertaken. In keratitis after LASIK, lifting the flap for scrapings of infiltrates at the interface is often necessary.

With all that is known about sampling and tests needed for diagnosis, it is important to determine how often delay in diagnosis was encountered in reviewed cases as well as the reasons for that delay. A delay, ranging from 1 week to 30 weeks, was seen in approximately half the cases of eyelid and periocular infections, lacrimal infections, keratitis, and endophthalmitis.

Reasons determined to play a role in the delay were no growth or slow growth of NTM, misidentification of organism, misdiagnosis, and delay in sending samples for microbiological evaluation. Some eyes had more than one reason for the delay. Examples of organisms wrongly determined to have caused the infection are *Nocardia* species [35, 36, 70, 71, 128, 177] as well as *Corynebacterium* species [74]. Some examples of the incorrect diagnoses made were herpes keratitis [46, 50, 53, 55, 66] and fungal keratitis [39, 46, 48, 54, 94]. An orbital infection was confused for pseudotumor [11]. As for intraocular infections, chronic endophthalmitis was mistaken for granulomatous iridocyclitis [151], and choroiditis was mistaken for ocular lymphoma [175].

Considering all the different causes of delay, we recommend swiftly obtaining samples for microbial analysis when infection is on the differential. Any lack of growth or slow growth encountered should prompt resorting to molecular techniques for diagnosis. Clinicians should also keep in mind the different diagnoses that NTM infections are often mistaken for while evaluating their cases.

Many studies have investigated the proper medical treatment regimen of NTM, and recently more of these have been concerned with ocular infections. Girgis et al., the largest retrospective study on ocular NTM infections to date (143 eyes), found that most isolates from NTM infected eyes were sensitive to clarithromycin (93.2%) and amikacin (81.3%), followed by linezolid (36.4%), gatifloxacin (30.9%), moxifloxacin (21.4%), and ciprofloxacin (10.3%) [184]. Another retrospective study by Brown-Elliott et al. found *M. abscessus* to be most susceptible to amikacin and clarithromycin, *M. chelonae* to amikacin, clarithromycin, and tobramycin, and *M. fortuitum* to amikacin and imipenem [186].

In vivo rabbit studies have been conducted to compare effectiveness of treatment with different fluoroquinolones; gatifloxacin was found to be synergistic with clarithromycin and amikacin [187] and found to be the most potent among fluoroquinolones [188]. In fact, the combination of amikacin and clarithromycin was not effective unless combined with gatifloxacin. In a review by Abschire et al., however, *M. chelonae* was found to be more susceptible to moxifloxacin than gatifloxacin, keeping in mind that *M. chelonae* is responsible for most NTM ocular infections [189].

Dolz-Marco et al. reported a case of *M. chelonae* resistant to amikacin, clarithromycin, and moxifloxacin, which led them to resort to linezolid to clear the infection [49]. With the threat of resistance looming demonstrated by Dolz-Marco et al., an effective treatment strategy ought to be devised. Hose et al. found no effectiveness in the combination of clarithromycin and amikacin as compared to a basic salt solution in vivo, but a combination of gatifloxacin, clarithromycin, and amikacin was found to be effective, as was treating with gatifloxacin alone [187]. Monotherapy does, however, increase the risk of resistance. We recommend treating ocular NTM infections with a combination of amikacin, a fluoroquinolone, and a macrolide pending antimicrobial susceptibilities.

Regarding surgical therapeutic interventions, we found that eyes that experienced a lack of initial response to medical therapy were 10 times more likely to undergo a surgery ($P < 0.05$). NTM infections are potentially detrimental, so any surgery deemed necessary by clinicians to clear the infection ought not be delayed.

In fact, surgery should be a prime consideration whenever an ocular implant is involved. Infections occuring after an implant was placed in the eye were more likely to necessitate surgery (OR = 3.5, P value < 0.001). Several therapeutic surgical interventions involved removing implants from infected eyes. Eyes with implants were almost 6 times more likely to end up with loss of vision (P value < 0.05), in contrast to eyes with a history of a foreign body, which were less likely to develop loss of vision (OR = 0.3, P value = 0.03).

Steroid use has been linked to the initial failure of medical therapy of keratitis [190]. In a rabbit model of *M. fortuitum* keratitis, eyes treated with topical corticosteroids had larger infiltrates and lesions [9]. We found that eyes which received steroids prior to diagnosis of the infection being made were almost three times more likely to have lack of initial resolution to medical therapy (OR = 2.8, P value = 0.001), more likely to develop a prolonged course of infection (OR = 2.7, P value = 0.001), and less likely to reach resolution (OR = 0.5, P value = 0.006). Because steroid use may lead to a prolonged course with a potentially worse outcome, we

recommend its discontinuation whenever NTM infection is suspected.

Statistical analysis relating certain locations of the ocular infection with the outcome was performed. Intraocular infections were less likely to resolve (OR = 0.2, P value < 0.001), with a higher likelihood of undergoing a therapeutic surgical intervention (OR = 2.7, P value = 0.012). They were also more likely to result in loss of vision (OR = 5.3, P value < 0.001) and extremely more likely to result in loss of the eye (OR = 34.4, P value < 0.001). Predictably, ocular surface infections were less likely to result in loss of vision (OR = 0.2, P value < 0.001) or in loss of the eye (OR = 0.1, P value < 0.001). They were also less likely to require more than one therapeutic surgical intervention (OR = 0.4, P value < 0.001). Intraocular NTM infections, therefore, should be treated aggressively due to significant potential morbidity.

6. Conclusion

Nontuberculous mycobacterial infections of the eye are uncommon but are potentially detrimental. NTM can cause periocular infections, adnexal infections, ocular surface infections, intraocular infections, and uveitis, with ocular surface infections, specifically keratitis, making up the majority of cases. NTM keratitis is especially noted after LASIK procedures. The most common species causing ocular NTM infections are *M. chelonae*, *M. abscessus*, and *M. fortuitum*. Ocular NTM infections are frequently indolent and unresponsive to initial medical therapy, especially when preceded by an intervention. NTM infections are also encountered after trauma, foreign bodies, implants, and contact lens wear. Immunocompromised patients are more likely to develop intraocular NTM infections, which are associated with a greater risk of loss of vision or even loss of the eye.

Considering the potential detrimental outcomes associated with these infections, clinicians should have a high index of suspicion of NTM when faced with a challenging case. If suspecting NTM, diagnosis is made by taking appropriate samples to be sent for microbiological evaluation which includes acid fast staining and culture in liquid broth mycobacteria growth indicator tube (MGIT), or on Löwenstein-Jensen and Middlebrook (7H10 and 7H11) solid media. Molecular and genetic techniques can also be used to hasten species identification. In treatment, we recommend a combination of antibiotics based on culture sensitivities or NTM species found in order to decrease the likelihood of developing resistance. Steroid use should be avoided in suspected cases as it is associated with prolonged infections and worse visual outcomes. Therapeutic surgical intervention may be needed in order to control the infection. Timely diagnosis and initiation of therapy are key factors in achieving resolution of the infection as well as a good visual outcome.

Conflict of Interests

The authors have no proprietary interests in the subject matter of the review.

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Research Article

Mycobacterium avium subsp. *hominissuis* Infection in Swine Associated with Peat Used for Bedding

Tone Bjordal Johansen,[1] Angelika Agdestein,[1] Bjørn Lium,[1] Anne Jørgensen,[2] and Berit Djønne[1]

[1] *Norwegian Veterinary Institute, P.O. Box 750, Sentrum, 0106 Oslo, Norway*
[2] *Animalia, P.O. Box 396, Økern, 0513 Oslo, Norway*

Correspondence should be addressed to Tone Bjordal Johansen; tone.johansen@vetinst.no

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Mycobacterium avium subsp. *hominissuis* is an environmental bacterium causing opportunistic infections in swine, resulting in economic losses. Additionally, the zoonotic aspect of such infections is of concern. In the southeastern region of Norway in 2009 and 2010, an increase in condemnation of pig carcasses with tuberculous lesions was seen at the meat inspection. The use of peat as bedding in the herds was suspected to be a common factor, and a project examining pigs and environmental samples from the herds was initiated. Lesions detected at meat inspection in pigs originating from 15 herds were sampled. Environmental samples including peat from six of the herds and from three peat production facilities were additionally collected. Samples were analysed by culture and isolates genotyped by MLVA analysis. *Mycobacterium avium* subsp. *hominissuis* was detected in 35 out of 46 pigs, in 16 out of 20 samples of peat, and in one sample of sawdust. MLVA analysis demonstrated identical isolates from peat and pigs within the same farms. Polyclonal infection was demonstrated by analysis of multiple isolates from the same pig. To conclude, the increase in condemnation of porcine carcasses at slaughter due to mycobacteriosis seemed to be related to untreated peat used as bedding.

1. Background

Mycobacterium avium subsp. *hominissuis*, a member of the *M. avium* complex, is regarded as an opportunistic pathogen for pigs and humans [1]. Infection in pigs is typically characterised by granulomatous lesions in lymph nodes associated with the digestive system, but lesions in internal organs like the liver, lungs, and kidneys may also occur. The lesions are usually discovered at meat inspection and can imply serious economic losses for the producer if detected in several pigs and in multiple organs [2, 3]. Occasionally, clinical symptoms like wasting and abortion are seen [4]. The gross pathological presentation of lesions is not possible to distinguish from those caused by *M. bovis*, causing issues of proper management of carcasses and the herds of origin before the diagnosis is confirmed. In humans, *M. avium* subsp. *hominissuis* is a known cause of systematic infections in immunocompromised patients, lung infections in patients with underlying pulmonary disorders, and lymphadenitis in the head and neck region of children. A zoonotic aspect of *M. avium* infections has not been ruled out [3].

Nontuberculous mycobacteria, like *M. avium* subsp. *hominissuis*, are known to be ubiquitous in the environment, where they are able to survive and multiply [5, 6]. They have been isolated from a variety of environmental samples, like water, food, soil, sawdust, and peat [7–15]. In the Norwegian pig production, sawdust, wood shavings, and peat are materials commonly used for bedding. Peat has become more popular as bedding material, due to the higher costs and limited accessibility of sawdust and wood shavings. Additionally, peat is used as a feed supplement for piglets, both as iron enrichment for suckling piglets and for regulation of intestinal function in newly weaned piglets [16]. Contaminated peat and sawdust have been associated

with outbreaks of *M. avium* subsp. *hominissuis* infections in swine as confirmed by molecular fingerprinting methods [7, 9, 11, 14, 15].

In the southeastern region of Norway, starting in December 2009 and lasting through the beginning of the year 2010, there was an increase in the number of condemnations of swine carcasses due to tuberculous lesions in lymph nodes, liver, and lungs. Several herds were involved and some had involvement of multiple carcasses. A common factor for many of the herds was the use of peat as bedding material. It was, therefore, hypothesised that peat might be the cause of mycobacterial infection in these herds, and a project examining lesions detected at meat inspection as well as environmental samples, including peat, from the herds and from peat production facilities was initiated.

Table 1: Samples examined for mycobacteria.

Sampled material	Number examined	Number positive for *M. avium* subsp. *hominissuis*
Pigs (organ samples)	46 (91)	35 (72)
Peat intended for bedding[a]	4	4
Peat intended for bedding[b]	16	12
Peat intended for feed supplement[b]	2	0
Sawdust[a]	4	1
Hay/straw[a]	5	0
Water[a]	10	0

[a]Sampled at farms.
[b]Sampled at peat production facilities.

2. Materials and Methods

The majority of pigs included in the study were slaughtered at Furuseth AS located in the county Akershus. Additionally, some of the pigs were slaughtered at Nortura Sarpsborg in Østfold. The animals originated from seven counties in the southeastern part of Norway: Østfold, Vestfold, Buskerud, Telemark, Akershus, Hedmark, and Oppland. This was a descriptive study conducted in order to clarify the infection status of the herds, and sampling was, therefore, not randomized and systematically performed, but based on inclusion of the samples sent to the laboratory. Forty-six pigs with gross lesions indicating mycobacterial infection and originating from 15 herds (A–I and K–P) were sampled, and tissue samples were sent to the Norwegian Veterinary Institute for analysis. Only carcasses showing visible lesions at regular meat inspection were sampled. From each pig, lymph nodes, liver, and/or lungs were sampled. Twenty-three environmental samples, including peat intended for bedding, sawdust, hay/straw, and water, were collected from six of the herds (A, B, I, J, O, and P). Additionally, 16 samples of peat intended for bedding were retrieved from three different production facilities (facilities I, II, and III), of which peat producer II delivered peat to farm B and producer III to farm A. Two samples drawn from peat intended as feed supplement for piglets were also examined (facility IV) (Table 1).

Isolation of mycobacteria from organ and environmental samples was performed as described earlier on slants of Middlebrook 7H10 (BD Diagnostics, Sparks, MD) w/10% oleic acid (BD Diagnostics) with and without antibiotics and fungicides (final concentrations of 100 μg/mL carbenicillin, 200 U/mL polymyxin B sulphate, 19.5 μg/mL trimethoprim lactate, and 10 μg/mL amphotericin B), Dubos P, and Stonebrink's medium [7, 15]. Slants were incubated for eight weeks at 37°C, and colonies resembling mycobacteria were subcultured on Middlebrook 7H10 and the medium they were initially observed to grow on. On primary isolation, attempt was made to pick one colony of each morphotype, when more than one was present. When more than one organ/lymph node from the same pig was positive, one isolate from each organ was included for further analysis. Also from some environmental samples, more than one isolate was examined further. Isolates shown to be acid-fast rods by the Ziehl-Neelsen (ZN) staining method were identified as *M. avium* by Accu Probe (GenProbe Inc., San Diego, CA), and further determination of subspecies was based on the presence or absence of IS*901* and IS*1245*, analysed by PCR using 1U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). Primers 901a and 901c were used for the amplification of IS*901* and primers P40 and P41 for IS*1245* [18, 19]. PCR conditions were set as described earlier [20]. The reference strain *M. avium* subsp. *avium* ATCC 25291 was included as a positive control and MQ water as negative control. Acid fast bacteria not identified as *M. avium* were analysed by 16S rDNA sequencing as described previously [21].

Isolates identified as *M. avium* subsp. *hominissuis* were analysed by multiple locus variable number of tandem repeat analysis (MLVA), also referred to as MIRU-VNTR typing, using the eight loci as described by Thibault et al. [17]. Product size of PCR fragments was analysed by capillary electrophoresis using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) as described [7]. *Mycobacterium avium* subsp. *avium* ATTC 25291 was used as a control in each run. Sizes of the PCR products were converted to a corresponding tandem repeat number for each locus as described by Thibault et al. [17]. The data was entered into BioNumerics version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium), and cluster analysis was performed using the categorical method and the unrooted UPMGA tree. Only isolates of 100% similarity, that is, isolates having the same number of tandem repeats in each locus, were assigned to the same cluster. The HG index/diversity index was calculated as described [22, 23], using the Discriminatory Power Calculator (http://insilico.ehu.es/mini_tools/discriminatory_power/index.php).

When more than one organ from the same pig was positive for *M. avium* subsp. *hominissuis*, the MLVA profile of the isolates was subjected to minimum spanning tree (MST) analysis in BioNumerics 6.1, illustrating the relationship and possible mutation pathways within the clusters based on single locus variations (SLV). *Mycobacterium avium* subsp. *avium* ATCC 25291 was used as a reference strain. MST is a tool that takes a unidirectional graph and extracts

the subgraphs with the smallest weights [24, 25]. The MST was created based on the MLVA data used for the cluster analysis of the complete dataset. The nodes (circles) consist of identical genotypes and the edges (lines) of weights based on number of mutations (steps) taken from the loci used. Long weights (steps) indicate multiple mutations, while short weights indicate few mutations. The MST algorithm was then applied to this graph to extract all subgraphs with the minimal overall weight sum. Hence, the most similar strains are clustered closely together with short and thick edges, while increasing genomic variation leads to thin and longer edges.

3. Results

Thirty-five out of the 46 slaughtered pigs sent for analysis showed positive growth of mycobacteria. These 35 pigs originated from 12 herds. All together 72 isolates were obtained from different organs from the 35 pigs. All isolates from pigs were verified as *M. avium* subsp. *hominissuis* based on identification with Accu Probe, absence of IS*901* and presence of IS*1245*. *Mycobacterium avium* subsp. *hominissuis* was also detected from 16 of 20 samples of peat intended for bedding (herds A, B, I, and J and peat producers I, II, and III) and in one out of four samples of sawdust (herd A) (Table 1). From three of the herds *M. avium* subsp. *hominissuis* was not detected, neither from peat (herds O and P) nor from pigs (herds N, O, and P). None of the samples of hay/straw or water were positive for mycobacteria. The two samples of peat intended as feed supplements for piglets were also negative for mycobacteria. Additionally, seven samples of peat intended for bedding were positive for *M. bohemicum*, one sample of peat showed growth of *M. palustre,* and two peat samples showed growth of *Mycobacterium* sp. that could not be further identified with the methods used in the current study.

All isolates from pigs ($n = 71$), peat ($n = 22$), and sawdust ($n = 1$) underwent MLVA analysis. Two isolates, one from peat and one porcine isolate, were excluded from analysis due to double amplification product in one locus. MLVA analysis identified 16 different profiles among the 92 analysed isolates, distributed on eight clusters and eight singletons (Figure 1). Clusters were recognised when containing ≥2 isolates with identical profile. All tandem repeats were present in the isolates analysed, except for TR10 which was lacking in one isolate. The range and mode for the different tandem repeats were as follows: TR292 (range 0–2, mode 2), TRX3 (1–5, 4), TR25 (2-3, 2), TR47 (2-3, 2), TR3 (1-1, 1), TR7 (1-1, 1), TR10 (2-2, 2), and TR32 (8-8, 8). The discriminatory index for MLVA was calculated to 0,819.

Four of the VNTR loci were monomorphic markers (TR3, TR7, TR10, and TR32). Of these, TR7 had an amplicon size of between 180 and 200 bp, which is between one and two copies as described by Thibault et al. [17], but as it has been experienced that the size of these amplicons differs between *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis*, the experienced amplicon size corresponds to one copy of TR7 as described [26, 27].

For illustration, each MLVA profile was labelled from A to P (Figure 1). Not only identical porcine isolates from the same farm but also identical isolates from pigs from different farms were detected. On several occasions, environmental isolates and porcine isolates were found to be identical. From farm A, one isolate from peat (number 2007) and one from sawdust (number 2008) were identical to five porcine isolates originating from three pigs (numbers 2013, 2014, 2023, 2024, and 2025) (MLVA profile M). Additionally, another isolate from peat from the same farm (number 2006) was identical to seven porcine isolates from four pigs (numbers 1997, 2000, 2004, 2005, 2017, 2016, and 2018) (profile K). This farm received peat from peat producer III, where isolates with the same two profiles were detected (number 2095 with profile M and number 2096 with profile K). Farm B used peat from peat producer II, and identical isolates from pigs and from this peat producer were detected. Four porcine isolates from two pigs (numbers 2019, 2020, 2030, and 2031) showed identical MLVA profiles with isolates from peat producer II (number 2086 and number 2091) (profile J).

Isolates originating from different organs from the same pig did on several occasions show differences in MLVA profiles. In all, 52 isolates from 20 pigs were compared by MST (Figure 2). Some isolates originating from the same pig showed difference in only one locus, exemplified by isolate number 2022 (profile J) and number 2023 (profile M), where there was a difference in the number of repeats in locus X3 (Figure 1). Other isolates from one pig, like number 2021 (profile A) and number 2022 (profile J), as well as number 2044 (profile P) and number 2045 (profile E), differed in multiple loci (Figure 2).

4. Discussion

Peat is used both as bedding material for piglets, grower pigs, and finisher pigs and as feed additive providing iron supplement and intestinal regulation for piglets. However, even when used as bedding material, pigs will often ingest some of the peat. If the peat is contaminated with mycobacteria, risk of infection is increased, especially if ingested by young animals. The presence of *M. avium* subsp. *hominissuis* in the majority of samples of peat intended for bedding, together with the detection of identical isolates from swine and peat in some of the herds, confirmed that peat is a product capable of introducing the infectious agent into the pig herds. Such massive infection pressure might cause condemnation of carcasses as slaughter, which is an economic concern for the farmer. It has additionally been proven that pig herds with *M. avium* infections can have unapparent animals at slaughter that still harbour *M. avium* subsp. *hominissuis* in lymph nodes [7, 15]. As long as the zoonotic aspect of *M. avium* infections is not ruled out, this might be of concern for the pig industry.

This study has certain limitations, being mainly descriptive and, therefore, lacking randomized and systematic sampling. Environmental samples were only collected from six herds, while pigs were sampled from fifteen herds. Additionally, information about management was not obtained from all herds. It is, therefore, difficult to draw firm conclusions about the source of *M. avium* subsp. *hominissuis* for all

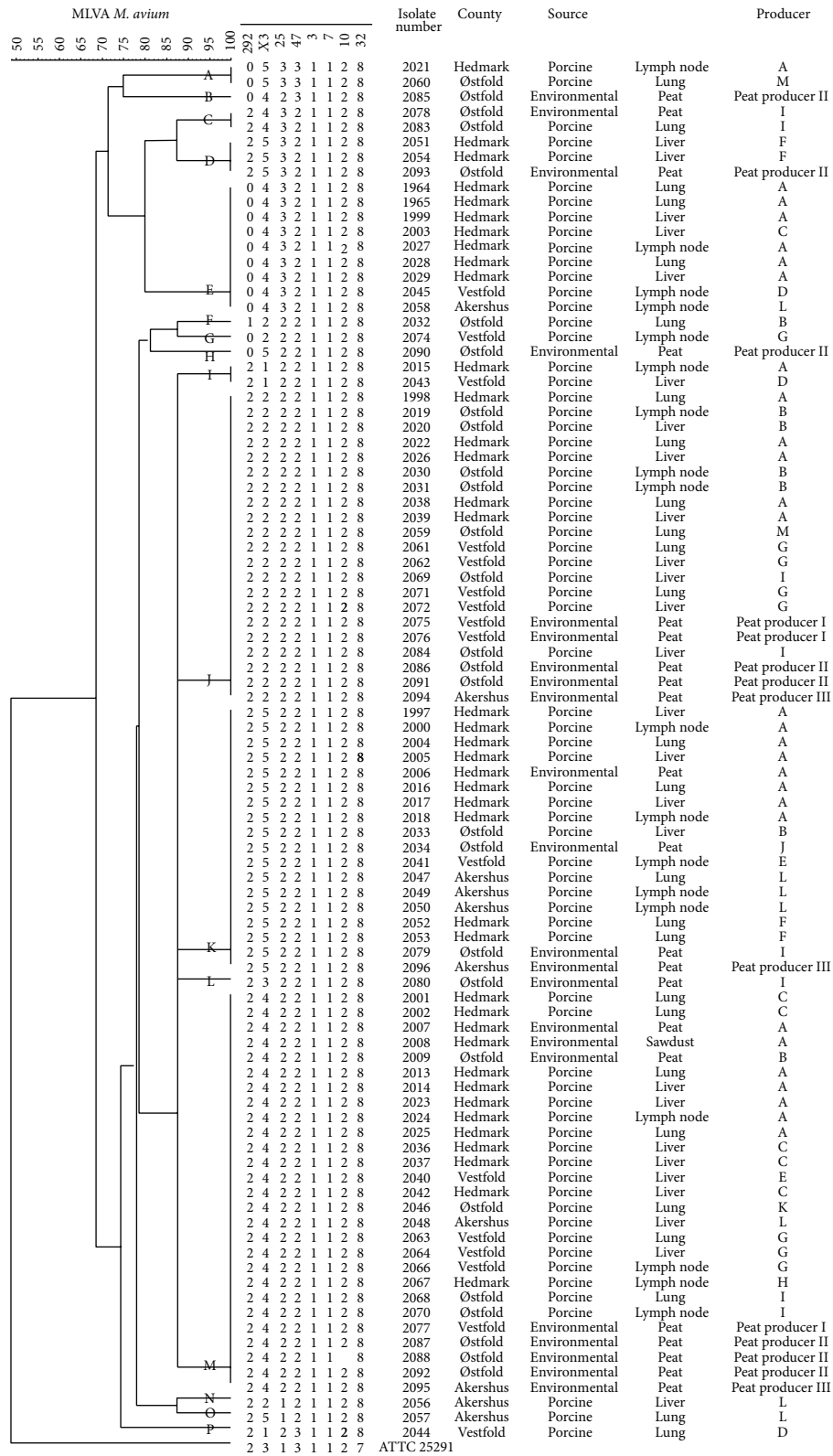

Figure 1: An unrooted tree showing genetic relationship between isolates of *Mycobacterium avium* subsp. *hominissuis* originating from peat, sawdust, and lymph nodes from slaughtered pigs in Norwegian herds. The dendrogram is based on eight-locus MLVA analysis [17]. The tree was created in BioNumerics 6.1, using categorical data and the unweighted pair group method with arithmetic mean (UPGMA). *Mycobacterium avium* subsp. *avium* ATCC 25291 was used as a reference strain. The different MLVA profiles are named A–P.

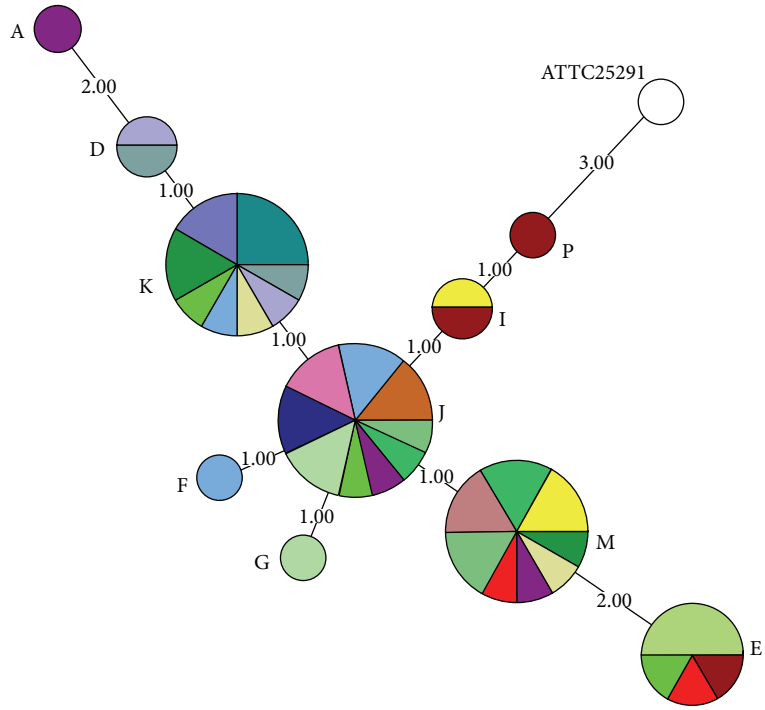

Figure 2: Minimum spanning tree (MST) analysis made in BioNumerics 6.1 illustrating the relatedness of isolates when more than one organ from the same pig was positive on culture for *Mycobacterium avium* subsp. *hominissuis*. All isolates ($N = 52$) originating from 20 pigs were subjected for analysis. The figure illustrates the relationship and possible mutation pathways within the clusters based on single locus variations (SLV). The MST was created based on the MLVA data used for the cluster analysis of the complete dataset. The nodes (circles) consist of identical genotypes and the edges (lines) of weights based on number of mutations (steps) taken from the loci used. Long weights (steps) indicate multiple mutations, while short weights indicate few mutations. Isolates originating from the same pig are illustrated in the same color. The size of the nodes represents the number of isolates showing the same genotype, and the size of the colored fields represents the number of isolates from the same pig within the nodes. Each node is labelled with the letter describing the MLVA profile as shown in Figure 1. *Mycobacterium avium* subsp. *avium* ATCC 25291 was used as a reference strain.

the herds in the study. In two of the involved farms, however, isolates from peat sampled at the factory supplying the farm, from the peat intended for bedding, and from slaughtered pigs were of the same genotype, which is yet another indication of peat being the probable source of infection for these farms. The results are in concordance with other publications that document the presence of *M. avium* subsp. *hominissuis* in peat [7, 9, 15].

Other species of mycobacteria were also detected in peat samples. Both *M. bohemicum* and *M. palustre* have previously been detected in both peat and lymph nodes from swine and can cause lesions similar to those caused by *M. avium* [7, 28]. No mycobacteria were detected in the peat intended as a feed supplement. Such peat is treated with acetic acid and formic acid to control the microbial flora but not heat treated. Mycobacteria would probably survive such treatment. The production site for this peat was different from the factories producing peat for bedding included in the study. However, as only two samples of this type of peat were analysed, no firm conclusions can be made regarding the risk factor of this feed additive when it comes to mycobacteriosis in pigs.

Peat seems to be a habitat where mycobacteria, including *M. avium,* thrive. Low pH, low oxygen content, and high organic matter are factors that have been correlated with increased levels of mycobacteria in soil samples, suggesting that peat might provide excellent conditions for *M. avium* [5, 29, 30]. Peat has many positive qualities in the pig production like the ability to bind ammonium, water, and urine, thereby improving the animals' environment and reducing the risk of diseases like joint infections and diarrhoea [31]. The cost for the farmer is also low. On the downside is the risk of infectious agents that may be introduced by peat like mycobacteria and pathogenic fungi [16, 31], which makes increased knowledge about the frequency of mycobacteria in peat essential for an adequate risk-benefit analysis of the use of peat in the pig production. Also, the age of the pigs at the time of peat introduction might be of importance, as young animals have a weak immune system and are more at risk of infections.

One sample of sawdust showed growth of *M. avium* subsp. *hominissuis*, which is in concordance with findings from other studies [11, 15]. The other environmental samples analysed were negative for mycobacteria, although one could assume that a higher sample volume would allow detection of mycobacteria in such samples. Water, in particular, has previously been described as a source of *M. avium* subsp.

hominissuis for both humans and pigs [6, 14]. However, the detection frequency of mycobacteria in the other environmental samples, when compared to peat, suggests that these types of bedding materials might be a safer choice for the farmer.

The study demonstrated a large proportion of pigs infected with *M. avium* subsp. *hominissuis*, and in multiple cases isolates with different MLVA profile were detected from the same animal. Such findings have been described by other authors analysing isolates from both pigs and humans [27, 32]. Also for other mycobacteria, as *M. avium* subsp. *paratuberculosis* and *M. tuberculosis*, the same phenomenon has been described [24, 25, 33]. The finding of genetic different isolates based on MLVA from the same animal could be a result of mutation of the strain during the course of infection or of coinfection with multiple isolates. When the MLVA profiles of the isolates differ only by one locus, mutation during infection could explain the observed difference. However, when isolates differ on more than one locus, polyclonal infection is a more likely explanation, as the alternative would have to be multiple mutations occurring in the same strain during infection. These findings could indicate a large infection pressure in the herd, probably caused by contaminated peat.

The eight-locus MLVA method used in this study is a rapid PCR based typing method well suited for discrimination of bacterial isolates. The discriminatory power experienced in the present study is slightly reduced compared to what has been described by others [27, 32, 34]. This could be explained by the epidemiologic link between the isolates, as multiple isolates were retrieved from the same farms and production sites and also from the same pigs. Not all loci are equally suited for discrimination. Four monomorphic markers were described in this study (TR3, TR7, TR10, and TR32). Of these, three have showed a low allelic diversity for isolates of *M. avium* subsp. *hominissuis* in other studies, while TR3 has been demonstrated as monomorphic also in other studies [17, 26, 32, 35]. The employment of these markers in this MLVA analysis is, therefore, not adding as much information as the more diverse loci, and the tandem repeats could be excluded or replaced with other targets, such as one or more of the tandem repeats used in the MATR-VNTR described by Inagaki et al. [26].

To conclude, the increase of condemnation of porcine carcasses at slaughter due to *M. avium* subsp. *hominissuis* experienced by the Norwegian pig industry in 2009 to 2010 seemed to be related to contaminated peat used as bedding in the herds. As a result of the findings, the use of peat was reduced in most herds and the situation stabilized. Pig farmers that consider use of peat in their herds must be aware of the risk for mycobacteriosis.

Conflict of Interests

The authors state that there are no competing interests related to the present study.

Authors' Contribution

Tone Bjordal Johansen was responsible for conception and design of the experiment, laboratory work, and data analysis and drafted the paper. Angelika Agdestein contributed to conception and design of the experiment, data analysis, and critical revision of the paper. Bjørn Lium was involved in conception and design of the experiment and critical revision of the paper. Anne Jørgensen was involved in conception and design of the experiment, sampling, and critical revision of the paper. Berit Djønne participated in conception and design of the experiment, laboratory work, and critical revision of the paper. All authors have read and approved the final paper.

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Review Article

Highlight on Advances in Nontuberculous Mycobacterial Disease in North America

Mehdi Mirsaeidi,[1] Maham Farshidpour,[2] Mary Beth Allen,[3] Golnaz Ebrahimi,[1] and Joseph O. Falkinham[4]

[1] *Section of Pulmonary, Critical Care, Sleep and Allergy, Department of Medicine M/C 719, University of Illinois at Chicago, 840 S. Wood Street, Chicago, IL 60612-7323, USA*
[2] *Institute of Human Virology, University of Maryland School of Medicine, Baltimore, MD 21201, USA*
[3] *Department of Health, University of Louisville, Louisville, KY 40202, USA*
[4] *Department of Biological Science, University of Virginia Tech, Blacksburg, VA 24061, USA*

Correspondence should be addressed to Mehdi Mirsaeidi; mmirsae@uic.edu

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Nontuberculous mycobacteria (NTM) are ubiquitous in the environment and exist as an important cause of pulmonary infections in humans. Pulmonary involvement is the most common disease manifestation of NTM and the incidence of NTM is growing in North America. Susceptibility to NTM infection is incompletely understood; therefore preventative tools are not well defined. Treatment of pulmonary nontuberculous mycobacterial (NTM) infection is difficult and entails multiple antibiotics and an extended treatment course. Also, there is a considerable variation in treatment management that should be considered before initiating treatment. We highlight the new findings in the epidemiology diagnosis and treatment of mycobacterial infections. We debate new advances regarding NTM infection in cystic fibrosis patients and solid organ transplant recipients. Finally, we introduce a new epidemiologic model for NTM disease based on virulence-exposure-host factors.

1. Introduction

Nontuberculous mycobacteria (NTM) are an important cause of morbidity in the United States. A few available prevalence studies show that NTM disease is increasing in the elderly population and suggesting NTM disease causes higher morbidity than TB in the US [1]. Patients with pulmonary NTM disease have significantly impaired health-related quality of life (HRQL) due to impaired lung function [2, 3]. The genus *Mycobacterium* includes over 150 species, many of which may cause disease [4]. Approximately 80% of pulmonary NTM (PNTM) infections in the United States are caused by members of the *Mycobacterium avium* complex (MAC) [5–7]. Molecular sequence data show that MAC includes 10 different subspecies such as *M. avium*, *M. hominissuis*, *M. silvaticum*, and *M. paratuberculosis, M. intracellulare*, *M. colombiense*, *M. bouchedurhonense*, *M. timonense*, *M. arosiense,* and *M. marseillense* [8].

Current published studies report that the prevalence of pulmonary NTM disease is rising throughout the United States, particularly among older adults [3, 9]. As the baby boomer cohort continues to age thus increasing the proportion of older Americans in the general population, it is expected that the incidence and prevalence of pulmonary NTM disease will likewise increase. Also, patients with NTM disease require frequent and intense healthcare resources such as hospitalizations and frequent office visits as well as complicated therapy and associated treatment challenges. These challenges are confounded when multiple comorbidities are also present, which are common in this population.

Many of the potential challenges with treating NTM infection in the US are offset by the improvement of medical knowledge over the last decade. This paper reviews important new developments in the prevalence, pathogenesis, diagnosis, and management of mainly pulmonary NTM disease in North America.

2. Methods

A literature search was conducted using search keywords "nontuberculous mycobacteria," "MAC," "*M. abscessus,*" "epidemiology," "treatment," "North America," "mortality," "cystic fibrosis," "transplantation," "prevention," and "diagnosis" from studies that have been published between the years 2009 and 2014. PubMed, Cinahl, Scopus, Embase, and the Cochrane Library were searched. A total of 382 articles were reviewed from which 65 papers were selected that met our selection criteria. Titles of interest were further reviewed by all authors. Reference lists of relevant studies were hand-searched in order to identify other potentially relevant articles. Studies included in this review met the following criteria:

(i) study populations included patients with NTM;

(ii) articles were full reports, case reports or reviews;

(iii) articles were in English and published from the US based institutes;

(iv) articles were published in peer-reviewed journals.

3. Epidemiology

Nontuberculous mycobacteria (NTM) are an important cause of morbidity and mortality, often in the form of progressive lung disease [5, 10, 11]. Few reports are accessible on NTM disease prevalence in the United States; however based on the recent data the incidence of pulmonary NTM has been reported to be rising in North America [3]. Winthrop et al. described the pulmonary NTM disease prevalence in the state of Oregon, USA [12]. The total age-adjusted prevalence of NTM was reported 8.6 per 100,000 population in the 2005-2006. However, 50 years of age and older had a higher rate of 20.4 per 100,000. The median age was 66 years and 59% were females [12]. In a combined report of four other regions in 2010, the mean annual prevalence was 5.5/100,000, ranging from 1.7/100,000 in Southern Colorado to 6.7/100,000 in Southern California [5]. Moreover, according to the national Medicare claims data by Adjemian et al., the annual prevalence of NTM in the population older than 65 years old significantly increased from 20 cases/100,000 persons in 1997 to 47 cases/100,000 persons in 2007, in which Caucasians account for 90% of cases followed by Asians/Pacific Islanders and Blacks [3, 13]. The prevalence of pulmonary nontuberculous mycobacterial disease differs by geographic region since specific environmental factors linked to water and soil exposure seem to increase the risk of PNTM infection. Adjemian et al. reported the 55 counties in 8 states with a particularly high risk of infection, including parts of California, New York, Florida, Hawaii, Louisiana, Oklahoma, Pennsylvania, and Wisconsin [14].

According to another study, NTM were found in 30% of patients with noncystic fibrosis bronchiectasis [15]. The frequency of NTM in the bronchiectasis population was 37%, 30% of which met the ATS criteria for NTM disease. MAC was the most common isolate (88%) found in this particular patient population [15].

In Ontario, Canada, the population cohort study showed that the NTM isolation prevalence raised from 9.1/100,000 in 1997 to 14.1/100,000 in 2003 [16]. Furthermore, Damaraju et al. found 10.8% patients with culture-proven pulmonary tuberculosis (PTB) in Ontario had NTM coisolated, including *Mycobacterium avium* complex (55%), *M. xenopi*, (18%), and *M. gordonae* (15%) [17].

4. Extrapulmonary NTM

Although the incidence of extrapulmonary NTM in the US remained largely unknown, it has been reported that up to 10% of NTM disease manifests as extrapulmonary [18]. The incidence of extrapulmonary NTM may be higher than our current estimation. NTM have potential to involve any human body organ and are commonly isolated from skin and soft tissue, lymphadenitis, septic arthritis, bone, and as disseminated infection [19–21]. A high index of clinical suspicion of disease and isolation of NTM from sterile site or any NTM growth from biopsy or compatible histopathology with mycobacterial disease are main keys to diagnose extrapulmonary NTM. A recently published study on 42 patients with confirmed NTM infection in upper extremity showed that there was a significant diagnosis delay due to its indolent presentation and lack of physician suspicion [22]. Table 2 shows nontuberculous mycobacteria strains associated with osteoarticular infections and skin diseases.

5. NTM in Elderly

According to a review conducted by Mirsaeidi et al., older people are at an increased risk for developing NTM infections and are most likely to use significant health care resources including long-term care services to manage NTM infections [23]. Given the aging of the US people and the incidence and severity of NTM disease in the elderly population, an increasing focus on research in the area of NTM including highly valid studies in the elderly should be considered. Another important factor when treating this population is therapy considerations given comorbidities and associated concomitant therapies. For this reason, drug-drug interaction is an important issue in elderly population. This is especially true regarding macrolides, rifamycins, and fluoroquinolones that are commonly used for NTM treatment [24, 25]. These treatment regimens usually cause interaction with the metabolisms of other drugs via interacting with cytochrome P-450 [25].

6. Mortality

United States population-based data demonstrate that the number of deaths from nontuberculous mycobacterial disease is growing. During the years 1999 through 2010, NTM disease was reported as an immediate cause of death in 2,990 people in the United States with a combined overall mean age-adjusted mortality rate of 0.1 per 100,000 person-years. Persons aged 55 years and older, women, those living in Hawaii and Louisiana, and those of non-Hispanic, white

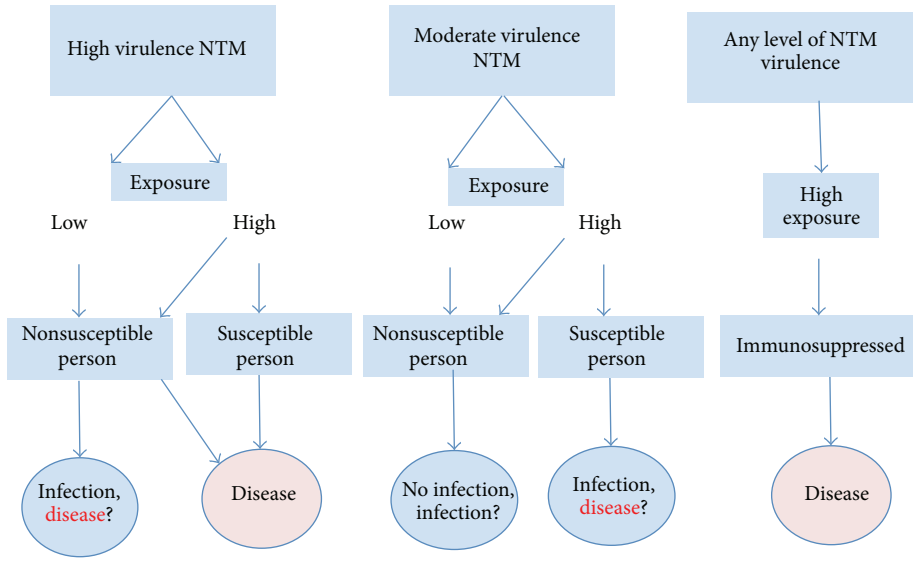

Figure 1: Illustrates our proposed virulence-exposure-host model for NTM disease. Virulence: High virulence NTM carries virulence antigens, although those antigens are largely unknown. Susceptible patient is defined as a person with chest wall abnormality, anatomical lung abnormalities, such as bronchiectasis, COPD, and asthma, and minor immune system abnormalities such as Mendelian susceptibility to mycobacterial disease. Infection: infection is defined as epithelial colonization by NTM without any evidence of tissue invasion including clinical and radiological evidence. Immunosuppressed patient is defined as a person with active malignancy except skin basal cell carcinoma on chemotherapy medication(s) and radiotherapy and HIV/AIDS, significant primary immunodeficiency, and corticosteroids therapy.

ethnicity had higher mortality rates. The majority of NTM deaths were reported in the hospital setting [34]. Additionally, there is a strong association between age and NTM mortality, which was found to be significantly higher in patients older than 65 years. In addition to the presence of comorbidities common in this population, advanced age itself was determined to be a strong predictor of mortality [34, 35].

7. Pathogenesis and Risk Factors

Everyone is virtually exposed to NTM, although most do not develop clinical signs of infection. The factors predisposing one to infection are not well described, but likely result from interaction between host defense mechanisms and the load of exposure [13]. Figure 1 illustrates our proposed epidemiologic model for NTM disease based on virulence-exposure-host factors. The infectious dose for NTM infection is largely unknown. It has been estimated that 10-10^2 *M. bovis* organisms can cause pulmonary disease [36]. In mouse model for *M. ulcerans* infections an infectious dose of 10^3-10^4 colony-forming units are sufficient to induce swelling [37]. However, this data have never been extrapolated to other NTM species and also for humans.

Although for this reason NTM are considered opportunistic pathogens, they frequently cause infection in patients with no known underlying diseases. Even in seemingly normal hosts, some level of immunodeficiency or preexistent pulmonary disease probably exists [38]. Four categories of susceptible persons for NTM infection have been identified [23]. First, structural or preexisting pulmonary diseases such as cystic fibrosis, chronic obstructive pulmonary disease (COPD), and bronchiectasis have been strongly associated with the risk of developing several infectious lung diseases including NTM. Second, patients with autoimmune disorders who are being treated with antitumor necrosis factor-α (TNF-α) drugs are at risk for developing NTM as well as many other opportunistic infections. Third, HIV infected persons with AIDS are also at an increased risk for developing NTM along with many other opportunistic infections. In fact, a CD4+ T cell count of less than 50 cells/μL is associated with increased risk of disseminated NTM disease. Fourth, patients with genetic syndromes involving mutations in the interleukin-12 or interferon γ pathways are also at risk for developing opportunistic infections including NTM. Mutations in these pathways are associated with both autoimmune disorders as well as immune suppression [39–42]. Additionally, nonsmoker elderly females with a slender body and some with characteristic features such as scoliosis, pectus defects, or mitral valve prolapsed are more prone to pulmonary NTM compared to the normal population [15, 43, 44]. The last group forms the majority of patients that are seen in our practice in Chicago.

There are limited data on the genetic susceptibility to NTM infection. The familial clustering of pulmonary NTM infections has only been rarely reported [45]. There is some evidence for association between NTM disease and natural resistance-associated macrophage protein 1 gene (NRAMP1) [46]. NRAMP1 regulates intramacrophage iron concentrations to limit the availability of iron for intracellular bacteria [47], as demonstrated in *Mycobacterium bovis* residing within the phagolysosome [47, 48].

8. NTM and Organ Transplantation

Solid organ transplant recipients could also have increased risk of NTM disease for several reasons. Posttransplantation immunosuppressive therapy may increase the likelihood of clinical disease from environmental exposures [49]. Also, underlying lung disease in lung transplant patients could place patients at a higher risk for NTM infection during the pretransplant period. Possible risk factors for reinfection or new disease with NTM after lung transplantation are immunosuppression and the development of structural lung disease over time secondary to bronchiolitis obliterans syndrome [50, 51]. Longworth et al. reported 34 cases of solid organ patients with NTM, which were predominantly males with a median age of 55 years with disease incidence following a median of 8 months after transplantation. *Mycobacterium abscessus* and *Mycobacterium avium complex* were the most common pathogens, and the lung (including pleura) was the most common site of disease. In this adjusted case-control analysis, lung transplant recipients had the highest risk of NTM disease [52]. According to Knoll et al., NTM were isolated from 53 of 237 patients (22.4%) following lung transplantation over a median of 25.2 months follow-up. The incidence rate of NTM isolation was 9.0/100 person-years, and the incidence rate of NTM disease was 1.1/100 person-years. The most common NTM isolated was MAC (69.8%), followed by *M. abscessus* (9.4%) and *M. gordonae* (7.5%) [51]. Huang et al. found out NTM infection notably increased the risk of death after lung transplantation (HR = 2.61, $P = 0.001$) following an assessment of 201 primary lung transplant recipients transplanted between January 2000 and August 2006. The increased risk was observed for both NTM colonization and NTM disease [53].

9. NTM and Cystic Fibrosis

Cystic fibrosis (CF) possesses a strong association with NTM for a number of reasons. First, the underlying lung problems characteristic of CF put patients with this disease at a unique risk for developing NTM following exposure. Also, the increasing lifespan of CF patients secondary to improvements in management places CF patients at a longer lifetime risk for developing NTM infection as compared to the general population [42]. NTM could be present intermittently in low quantities in the airways of CF patients. NTM have been isolated from up to 32% of CF patients [54]. Although the effect of chronic and recurrent NTM infection in the CF course is not clear, it is quite possible that progressive respiratory decline because NTM disease may also affect CF disease outcomes. Identifying NTM in CF patient is rather difficult for clinicians given the common symptoms exhibited by CF patients without NTM [55]. Although NTM are usually not believed to be a transmissible disease, current evidence by Aitken et al. documented an outbreak of *M. abscessus* subspecies *massiliense* with similar genome sequencing in five CF patients at the University of Washington. This report has brought to light the possibility that *M.* abscessus can indeed be transmitted among CF populations [56]. In 2010, Esther et al. reported microbiological data from 1216 CF patients demonstrating that chronic *M. abscessus* infection was associated with clinical deterioration as measured by an increased rate of decline in FEV1 [57].

10. NTM and TNF-α

The therapeutic use of TNF-α receptor antagonist drugs, particularly in rheumatoid arthritis and other connective tissue disorders patients, is a risk factor for NTM infection. In a review of 8418 anti-TNF-α users, Winthrop et al. reported that 18 cases developed NTM and 16 individuals were diagnosed with tuberculosis after drug initiation. The rates (per 100,000 person-years) for NTM, respectively, for etanercept were 35 (95% CI: 1 to 69), infliximab were 116 (95% CI: 30 to 203), and adalimumab were 122 (95% CI: 3 to 241) [40]. Most cases of NTM infections were pulmonary (67%), but there were considerable (22%) extrapulmonary sites of involvement as well. *M. avium* was accountable for half of the cases and in a review of 8,000 users of anti-TNF-α medications the rate of NTM was 74/100,000 person years [40, 58]. The same group reported that *M. avium* (49%) following rapidly growing mycobacteria (19%) were the most common etiologic microorganism in anti-TNF-α receivers [40].

11. Diagnosis

The diagnosis of NTM infection can be quite challenging. First, culturing NTM can be tricky because the bacteria are ubiquitous in the environment and may contaminate clinical samples from nonsterile sites. Contamination may occur before, during, and even after sampling. For example, collected sputum samples may be contaminated if rinsed in the mouth with tap water before expectoration [59]. Fibrotic bronchoscope suction channel contamination with *Mycobacterium chelonae* has also been reported as a cause of pseudoepidemic [60]. In order to distinguish between contamination and infection, a diagnosis of NTM pulmonary disease should be established in a combination of clinical, radiological, bacteriological, and histological criteria [39, 59, 61]. A clinical and radiological diagnostic criteria overview is outside of scope of this review and could be found elsewhere [23].

12. Methods Used for the Detection of NTM

12.1. Staining and Culture. Smear staining is routinely performed in a two-step procedure. First, samples are screened by fluorochrome (auramine) staining due to the high sensitivity and positives are confirmed by classical Ziehl-Neelsen staining [62]. Once preparing specimens for isolation, decontamination by N-acetyl-L-cysteine-sodium hydroxide (NALC/NaOH) is needed to prevent the growth of other bacteria; however, samples from patients with cystic fibrosis should be treated with an additional decontamination step with oxalic acid to diminish the Gram-negative overgrowth and increase the frequency of detection of NTM by culture [63]. In general, liquid media are more sensitive rather than

solid media such as Lowenstein-Jensen [64]. The highest frequency of recovery of NTM is expected to be obtained if both solid and liquid media are applied and incubated at both 37 and 30°C if M. marinum is suspected [62]. Most NTM strains grow within 2 to 3 weeks with the exception of rapidly growing mycobacteria types like *M. abscessus, M. fortuitum, Mycobacterium chelonae,* and *M. massiliense,* which may grow within 7 days [65].

12.2. Molecular Methods. The methods for the identification of mycobacteria in clinical laboratories have improved considerably over the last 2 decades. Also, species identification offers an opportunity to further expand the clinical and epidemiologic database regarding NTM which may ultimately produce treatment trials and accurate outcome studies [66]. Current rapid techniques for the identification of NTM consist of probes, high-performance liquid chromatography (HPLC), and other molecular techniques [67]. HPLC recognizes mycobacteria according to variations in mycolic acids, the long-chain fatty acids resided in the cell wall of mycobacteria [68]. Molecular DNA probes have now been applied for identifying MAC, *M. gordonae*, and *M. kansasii*; however, this process is costly and probes are not provided for all species of mycobacteria [69]. Polymerase chain reaction (PCR) restriction fragment length polymorphism analysis is another molecular technique for identifying mycobacteria on account of differences in restriction fragments of the 65 kD heat-shock protein. Sequence analysis of the rpoB gene and 16S ribosomal RNA has been expanded recently as another method for speciation of NTM [70, 71]. A recent study proposed serodiagnosis of pulmonary NTM infection as a possible diagnostic method in order to identify antibodies specific to lipid antigen in NTM [72].

Reverse hybridization is a commonly used method in clinical laboratories to identify those NTM species uncovered by the Accuprobe assay [73]. Most species can be identified by using Genotype and Inno-Lipa diagnostic kits which are mainly used in Europe [74].

13. Drug Susceptibility Test

Most NTM infections are managed with antimicrobial agents. Consequently, the role of drug susceptibility testing (DST) on NTM isolates is critical in the determination of drug therapy regimens for NTM disease [75]. The current ATS/IDSA guidelines recommend drug susceptibility tests for MAC (macrolides), *M. kansasii* (rifampin), and rapid growing mycobacteria [59]. There are not enough data available regarding the role of DST in other species of NTM [76].

Most NTM strains are resistant to conventional antituberculous agents, leaving fewer options for treatment than many other diseases. Also, clarithromycin is along the most preferred agent in many cases if the isolate is susceptible, which further emphasizes the need for DST [59]. Recently, Babady et al. [77] discovered the clarithromycin susceptibility testing of MAC by the SLOMYCO panel and the JustOne strip methods are simple to set up and easy to interpret. BACTEC 460 system is a well-established assay for clarithromycin susceptibility testing of MAC isolates. The concordance between the SLOMYCO panel or the JustOne strip and the BACTEC 460 method was 90%, with the kappa score indicating sound agreement between the methods.

The JustOne strip and the SLOMYCO panel are both broth microdilution methods, and they exhibit ≥90% correlation with both the radiometric method and a broth microdilution reference method. Additionally, the SLOMYCO panel and the JustOne strip have the advantage of being commercially accessible and simple to set up and read and the susceptibility results are frequently available within 7 days. This is much quicker than the BACTEC 460 method, which also avoids the use of costly instrumentation and allows therapy to be initiated sooner [77, 78].

Table 1: The most common NTM species isolated from patients in North America.

Slow-growing mycobacteria (SGM)	Rapid-growing mycobacteria (RGM)
M. avium complex	*M. abscessus*
M. kansasii	*M. chelonae*
M. xenopi	*M. fortuitum*
M. simiae	*M. marinum*
M. malmoense	
M. szulgai	

14. Treatment

The management of NTM infection is mainly by drug therapy. However, drug used to treat NTM disease is often expensive; the course is lengthy, and treatment is often correlated with drug-related toxicities [76, 79]. The treatment regimens vary by species with the most important distinction being that between slow *versus* rapid growing NTM [6] (Table 1). For most slow growing strains, the optional regimen includes rifampicin (Rifapentine or rifabutin) and ethambutol and a macrolide is administrated for 18–24 months; amikacin or streptomycin should be added in the initial 3–6 months in cases of severe disease. For the rapid growing strains, regimens are based on *in vitro* DST results. For *Mycobacterium abscessus*, these regimens usually consist of a macrolide, amikacin and either cefoxitin, imipenem, or tigecycline [75, 80]. Jarand et al. reported the management results for *M. abscessus* pulmonary disease patients who received antibiotic treatment that was individualized according to patient tolerance and drug susceptibility outcomes. Sixteen different antibiotics were administrated with forty-two different combinations for an average of 4.6 drugs per patient over the course of a median of 6 months. Forty-nine patients converted sputum cultures to negative, but 16 (23%) experienced relapse later [80]. Additionally, Safdar showed that aerosolized amikacin with a range of 7,600 to 95,400 mg was effective in the treatment of eight PNTM patients who previously failed combination oral drug therapy [81]. Patients with anti-IFN-γ autoantibodies (a rare underlying disease

Table 2: Some of extrapulmonary NTM diseases reported from skin, soft tissue, bone, and joints.

Clinical presentation	Mycobacterium species	Comorbidities	References
Arthritis	*M. Chelonae* *MAC* *M. fortuitum* *M. marinum*	Rheumatoid arthritis	[26]
Tenosynovitis	*MAC* *M. chelonae*	Bone fracture, penetrating injury	[27]
Osteomyelitis	*M. szulgai* *M. abscessus* *M. fortuitum* *M. chelonae*	Inherited STAT1 deficiency, hepatitis C, former intravenous drug user, none	[28–31]
Skin and soft tissue	*M. chelonae* *M. marinum* *M. avium*	Tattoos None None	[32, 33]

for NTM) have impaired IFN-γ signaling which may lead to severe disseminated infections with intracellular pathogens including primarily NTM [82, 83]. Rituximab has no role in the treatment of NTM disease except this rare condition. Browne et al. used rituximab (anti CD-20) in 4 patients with disseminated infection with *Mycobacterium abscessus*, *M. avium*, and *M. intracellulare* due to high-titer anti-IFN-γ autoantibodies. All subjects had received ≥3 antimycobacterial agents before rituximab treatment. Rituximab was given at 375 mg/m^2 weekly for ≥4 doses and then at wider intervals. All patients received between 8 and 12 doses over the first year with subsequent additional doses determined by the recurrence of infection. Within 2–6 months after initiation of the rituximab treatment, all patients had marked clinical, radiologic, and laboratory improvement [84]. Moreover, one more case of anti-IFN-γ autoantibody syndrome with disseminated infection by *M. abscessus* was successfully treated with rituximab at a dose of 375 mg/m^2 by Czaja et al. [85].

Cure rates of pulmonary NTM disease is different by species, ranging from 30–50% in *M. abscessus* disease to 50–70% in *Mycobacterium avium* complex and 80–90% in *Mycobacterium malmoense* and *Mycobacterium kansasii* disease [80, 86]. According an *in vitro* study by Van Ingen et al., clofazimine and amikacin illustrated significant synergistic activity against a variety of NTM, including both slow and rapid growing strains. This *in vitro* study consisted mostly of MAC, *M. abscessus*, and *M. simiae*, which are all well-known causative agents of human disease with challenging drug treatment options and inferior clinical outcomes [87]. Regarding MAC management, Wallace et al. recently demonstrated that among 180 cases with nodular/bronchiectatic (NB) MAC lung disease, treatment with macrolide/azalide-containing regimens such as clarithromycin or azithromycin may lead to 84% successful sputum conversion without true microbiologic relapse. Interestingly, no patient developed macrolide resistance during treatment and intermittent therapy was effective and considerably better tolerated than daily therapy [88]. On the other hand, long-term monotherapy with azithromycin in 191 persons with CF appeared to be associated with a lower frequency of incident NTM infections. However, since macrolide monotherapy could lead to macrolide resistance, routine screening for NTM should be considered for persons with CF [89].

A new report has documented that tigecycline as part of a multidrug regimen resulted in improvement in >60% of 52 patients with *M. abscessus* and *M. chelonae* infections, including those with underlying cystic fibrosis despite having failed prior antibiotic therapy. However, adverse events with tigecycline were reported in >90% of cases, the most common being nausea and vomiting [90].

Indications for surgery have not been uniformly accepted although would be considered in the events of medication intolerance, drug resistance, and/or localized cavitation. Other indications include recurrent or massive hemoptysis and the presence of a destroyed lung. In experienced hands and careful patient selection, the safety of lung resection for NTM lung disease, particularly thoracoscopic right middle lobe lobectomy and lingulectomy, seems good [79]. Data have shown that surgery provides improved microbiologic response for refractory organisms such as *M. abscessus* as compared to medication regimens alone [79, 80, 91].

15. Cost of Treatment

The treatment of pulmonary nontuberculous mycobacterial (NTM) infection is difficult and entails multiple antibiotics and an extended treatment course. However, limited data are available regarding the cost of NTM treatment in the United States. Leber and Marras determined the monthly mean cost of treating 91 patients with pulmonary NTM infections in a tertiary care facility in Toronto, Ontario, Canada was equal to 292 US dollars (USD). The median total duration and cost per treated patient were 14 months (interquartile range (IQR) 9–23 months) and 4,484 USD, correspondingly. The most costly oral regiment includes rifampin in addition to fluroquinolone and macrolide [92]. Collier et al. reported that the direct cost

of inpatient treatment for NTM was $21,041 per each episode of admission [93].

16. Conclusions

The incidence of NTM infection is growing in North America. In addition to population distribution factors resulting in more elderly Americans coupled with the higher incidence of disease occurring in this population placing more people at risk for disease following exposure. Also, more immunosuppressed patients of all ages are likewise susceptible to disease often due to medical advances in treating autoimmune disorder, HIV/AIDS, and the availability of solid organ transplantation as an option for treating a myriad of diseases. In addition to disease susceptibility, diagnostics of NTM have demonstrated remarkable improvement allowing cases to be better identified. These improvements include liquid culture techniques and advance molecular methods. Despite these significant advances over the last few years, susceptibility to disease is incompletely recognized. This is an undermined effort to identify a complete understanding of at risk populations and determine preventative tools. Additionally, given the difficulty of eradicating NTM and its considerable reoccurrence, identifying appropriate candidates for treatment and the timing of initiation of therapy are likewise challenging. There is a considerable variation in treatment management that should be deliberated before initiation. While the US populations are aging and NTM diseases are rising in elderly population, we would hope to see an increasing focus on research in NTM infection and multicenter trials. It is critical that this condition is recognized as an important public health issue with potentially significant consequences for affected patients. Finally, the applicability of the virulence-exposure-host model in NTM disease should be investigated.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Conception, review literature, design, and modeling for review writing of the paper were done by Mehdi Mirsaeidi. The review literature, design, and modeling for review writing of the paper were by Mehdi Mirsaeidi and Maham Farshidpour. Writing the paper or substantial involvement in its revision before submission was by Mehdi Mirsaeidi, Maham Farshidpour, Mary Beth Allen, Golnaz Ebrahimi, and Joseph O. Falkinham.

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