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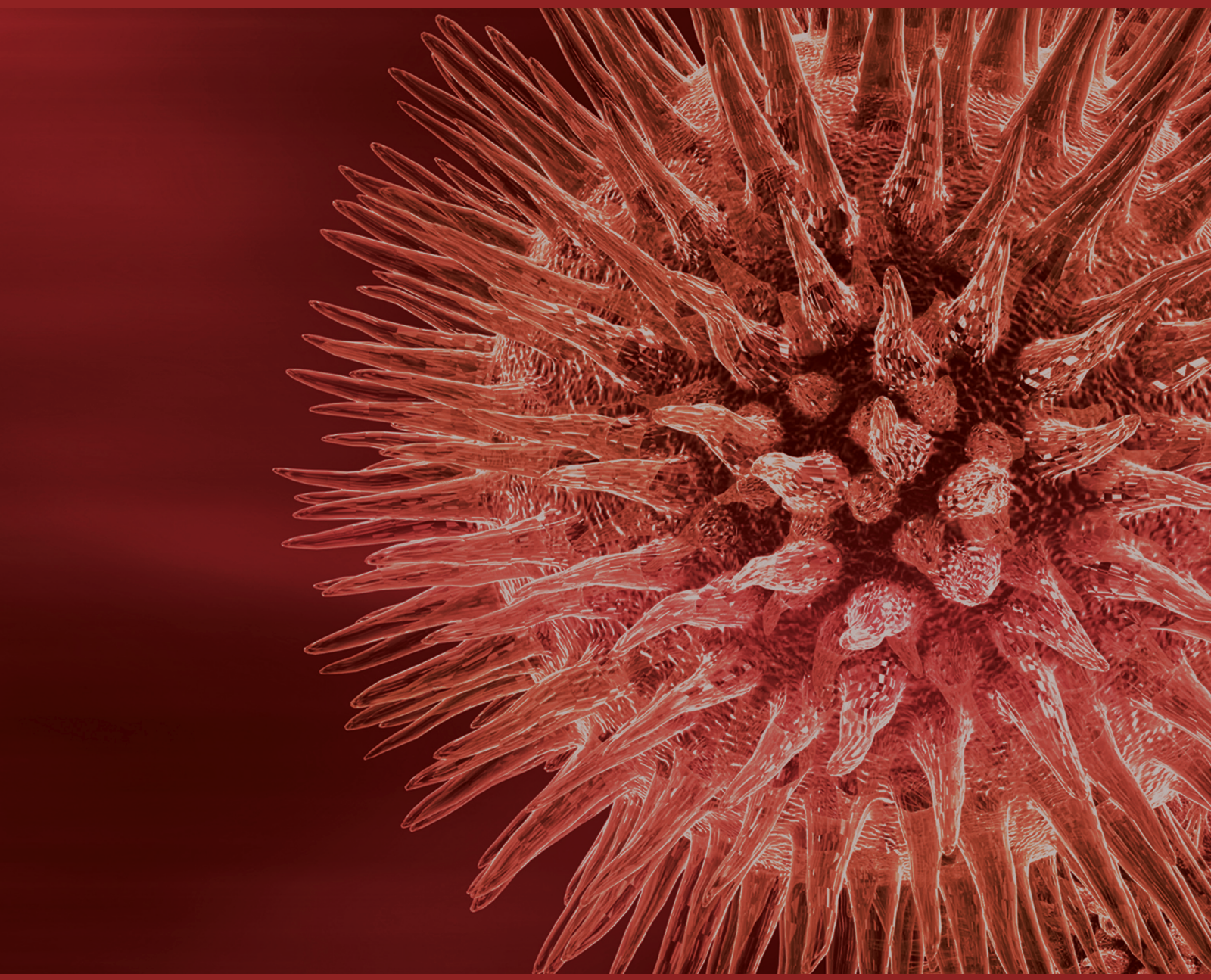
Guest Editors: Tomasz Jagielski, Jakko van Ingen, Nalin Rastogi, and Jarosław Dziadek



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Contents

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Editorial

Recent Developments in Mycobacteriology: A Clinical and Diagnostic Perspective

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Mycobacteria comprise a diverse group of bacteria that are widely distributed in nature, some of which cause significant diseases in humans. The most prominent representative of this group is the *Mycobacterium tuberculosis* complex, which includes the etiological agents of tuberculosis (TB), which, with over 8 million new cases and nearly 2 million deaths annually, continues to cause one of the major health burdens for humans. This complex also includes the causative agents of TB in animals, of which *Mycobacterium bovis*, the causative agent of bovine TB, is the most prominent. Whereas the prevalence of TB has been closely monitored in most parts of the world, the epidemiology of nontuberculous mycobacterial (NTM) infections remains poorly defined, in spite of the fact that the importance of NTMs as a cause of opportunistic infections of humans has been increasingly recognized over the last two decades, particularly in areas where the incidence of TB is in decline.

The purpose of this special issue is to provide the reader with some recent achievements in mycobacteriology, with particular emphasis on the developments which have direct relevance to the clinical practice and diagnostic performance.

The issue opens with a comprehensive state-of-the-art review on the molecular typing methods for *M. tuberculosis* and some NTM species, most commonly associated with human disease. For the various methods, technical practicalities as well as discriminatory power and accomplishments are discussed.

The next two studies focus further on genotyping methods in the molecular epidemiology of TB. Both describe the principle, technical advantages, and discriminatory power of the ligation-mediated PCR systems, namely, fast ligation amplification polymorphism (FLAP) method and fast ligation-mediated PCR (FLiP) (A. Żaczek et al.).

Genotyping methods allow for delineating phylogenetic relationships between the strains. To improve the utility of genotyping and to increase international consensus in the interpretation of genotyping results, M. Aminian et al. propose a new algorithm, publicly available online, useful for classification of *M. tuberculosis* complex isolates into phylogenetic clades, based on the spoligotyping profiles.

Molecular interrogation has entered both the epidemiological and the clinical field today. For clinical practice, molecular tools can help in detecting mycobacteria in clinical samples as well as detecting markers for drug resistance. In this context, Z. Bakuła et al. examine the mutational "hot spots" in the *embB* gene potentially related with resistance of *M. tuberculosis* to ethambutol (EMB). Similarly, but from a different angle, N. Alvarez et al. investigate the molecular mechanisms behind resistance of tubercle bacilli to fluoroquinolones and the binding of levofloxacin to the mycobacterial DNA gyrase in particular.

Identification of *M. tuberculosis* complex isolates by nonmolecular means is described in the contribution by D.

Machado et al. Here, the usefulness of the MGIT TBc Identification Test (Becton Dickinson), an immunochromatographic assay which detects the MPB64 protein, is evaluated for routine identification of *M. tuberculosis* complex in a network of hospital mycobacteriology laboratories in Portugal and other Portuguese-speaking countries.

Despite all advances in clinical diagnostics and molecular epidemiology, there still is no truly effective vaccine against TB. In a broad stream of research on new anti-TB vaccines is a study of M. V. Bianco et al., who investigate the efficacy of a new vaccine candidate against TB based on a *M. bovis* mutant in *p27–p55* operon.

Pharmacokinetic analyses are gaining foothold in the treatment of TB, where they are available. An interesting issue is addressed by A. Zabost et al., who explore the correlation between the N-acetyltransferase 2 (NAT2) genotype with isoniazid (INH) acetylation in TB patients and demonstrate NAT2 genotyping as an important tool for the adjustment of INH dosing regimens.

Four studies deal with NTM. The first one focuses on *Mycobacterium kansasii* which is among the most frequently isolated NTM species from human clinical cases worldwide. Since only certain genotypes of *M. kansasii* are clinically important, a paper by Z. Bakuła et al. gives a snapshot of the distribution of *M. kansasii* subtypes among patients in Poland suspected of having pulmonary NTM disease. In the second paper, M. Slany et al. report on a distinct NTM skin disease in humans, known as fish tank granuloma and caused by *Mycobacterium marinum*. The remaining two NTM papers come from the veterinary field. C. Goepfert et al. report cases of *Mycobacterium avium* subsp. *avium* infection in veal calves, highlighting the diagnostic problems surrounding these infections. A. Ledwoń et al. report the results of the experimental infection with *M. avium* subsp. *avium* in budgerigars positive for the beak and feather disease circovirus (BFDV) in order to determine how mycobacteriosis affects the course of the viral disease.

The articles within this issue differ considerably from each other with respect to their research scopes and methodologies, thus reflecting the multidirectional character of the research in the ever-expanding field of mycobacteriology. We face an exciting era in mycobacteriology, where greater understanding of the mycobacteria leads to technical improvements that change clinical practice and these techniques in turn help to curb the epidemic of mycobacterial diseases of humans and animals.

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Research Article

The Structural Modeling of the Interaction between Levofloxacin and the *Mycobacterium tuberculosis* Gyrase Catalytic Site Sheds Light on the Mechanisms of Fluoroquinolones Resistant Tuberculosis in Colombian Clinical Isolates

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We compared the prevalence of levofloxacin (LVX) resistance with that of ofloxacin (OFX) and moxifloxacin (MFX) among multidrug resistant (MDR) *MTB* clinical isolates collected in Medellin, Colombia, between 2004 and 2009 and aimed at unraveling the underlying molecular mechanisms that explain the correlation between QRDR-A mutations and LVX resistance phenotype. We tested 104 MDR isolates for their susceptibility to OFX, MFX, and LVX. Resistance to OFX was encountered in 10 (9.6%) of the isolates among which 8 (7.7%) were also resistant to LVX and 6 (5.7%) to MFX. Four isolates resistant to the 3 FQ were harboring the Asp94Gly substitution, whilst 2 other isolates resistant to OFX and LVX presented the Ala90Val mutation. No mutations were found in the QRDR-B region. The molecular modeling of the interaction between LVX and the DNA-DNA gyrase complex indicates that the loss of an acetyl group in the Asp94Gly mutation removes the acid base interaction with LVX necessary for the quinolone activity. The Ala90Val mutation that substitutes a methyl for an isopropyl group induces a steric modification that blocks the LVX access to the gyrase catalytic site.

1. Introduction

According to reports from the World Health Organization (WHO), one-third of the world population is infected with *MTB* and about 10% develop the disease during their life. It is estimated that in 2011 there were 8.7 million new tuberculosis (TB) and 1.4 million deaths [1]. Even though effective drugs to treat TB have been available for more than 50 years, the absolute number of cases has continued to increase every year, as slow reductions in incidence rates continue to be outbalanced by increases in population [2]. Since 1993, the WHO has insisted on the practice of directly observed therapy (DOT) in which the patient is administered the medication by a health worker and observed taking it [3]. This measure was designed to not only increase treatment success rates but also to prevent further development of MDR-TB after the emergence of strains resistant to at least rifampin and isoniazid [4, 5]. However, insufficient government commitment, inadequate patient management, and public health policies as well as poor adherence to treatments and misuse of antibiotics have rendered MDR-TB a significant public health issue that poses a serious threat to global TB control [6].

As a result, the need for novel classes of anti-TB drugs has increased, with fluoroquinolones (FQ) becoming the drug of choice for second line use in MDR-TB treatment [7, 8] or in patients with intolerance to one of the first-line drugs [9]. Today the alarming current TB situation resides not only in its overall incidence but also in the emergence in 2006 of cases of extensively drug resistant (XDR)-TB [10] caused by *MTB* that, in addition of being MDR, are resistant to any FQ and to at least 1 of 3 injectable second line drugs amikacin, kanamycin, or capreomycin [1].

FQ to which OFX, MFX, and LVX belong are an important group of synthetic antibiotics that inhibit the bacterial DNA gyrase, thus inhibiting the DNA replication and transcription processes by preventing the ATP-dependent introduction of negative supercoils into closed circular DNA, as well as ATP-independent relaxation of supercoiled DNA [7, 11]. DNA gyrase is formed by GyrA and GyrB subunits which form a heterotetrameric A_2B_2 complex, the A and B subunits being encoded by the gyrase A (*gyrA*) and gyrase B (*gyrB*) genes, respectively [11]. The A subunit carries the active site for double-stranded DNA breakage and reunion, whereas the B subunit promotes ATP hydrolysis [12–14].

Studies have shown that amino acid substitutions occurring in the quinolone resistance, determining region of GyrA (QRDR-A) mainly clustered in codons 88, 90, 91, and 94, account for up to 96% of the mechanisms that confer FQ resistance in *MTB* [11, 15, 16]. In contrast, the substitution at position 95 in GyrA, which encodes a serine or threonine, has been shown to have no influence on FQ resistance [15].

Amino acid substitutions occurring in the quinolone resistance-determining region of GyrB (QRDR-B) are less related to FQ resistance and its implication in FQ resistance remains largely unclear. Among the 21 GyrB substitutions described in the literature, only two have been demonstrated to be implicated in resistance to FQ (N538D and E540V) [17].

It is known that the new FQ MFX and Gatifloxacin (GFX) have higher activity against *MTB* than LFX and their pharmacokinetic and pharmacodynamics properties make them an excellent alternative for treating MDR-TB cases [18]. However some studies have suggested that gatifloxacin may have more side effects that MFX and LVX such as glucose metabolism alterations [18].

LVX is an optical isomer of OFX and is characterized by its broad spectrum against gram positive, gram-negative bacteria, and other pathogens such as *Mycoplasma*, *Chlamydia*, *Legionella,* and *Mycobacteria* spp. Studies have reported that LVX is active against OFX resistant organisms including *MTB* and reaches high levels in the CSF as it can pass the blood brain barrier making it an excellent choice in cases of tuberculous meningitis [19].

The use of LVX in antituberculosis therapy presents certain advantages. The bioavailability of oral LVX is very rapid and complete, approaching 100%, and it is not affected when it is ingested after meals. LVX pharmacokinetics are similar during multiple-dose and single doses regimens and the pharmacokinetics are not appreciably affected by age, gender, or race when differences in renal function and body mass and composition are taken into account [19].

Colombia reported an overall incidence of 25–49 TB cases per 100,000 population for 2011 [1] and a proportion of MDR-TB among new TB cases of 2.4% between 2004 and 2005 [20]. However, the rate of MDR-TB among previously treated patients can reach more than 30%. Colombia is also one of the 84 countries until 2011 to have reported at least one case of XDR-TB [1]. Even though Colombia is not considered a high burden country for TB, current epidemiological data show that first line drugs as well as FQ resistance is a growing problem that needs to be urgently addressed. It even becomes alarming in the city of Medellin, an area of 2.4 million habitants out of which 681,000 (28.3%) live with less than 104 USD per month [21] and that has reported for 2011 an incidence of 78.9 TB cases per 100,000 population [22] with 175 MDR-TB cases since 2009 [23].

Our study first aimed to compare the prevalence of LVX resistance compared with resistance to OFL and MFX among MDR-TB clinical isolates collected between 2004 and 2009 in the metropolitan area of Medellin, Colombia, and establish a correlation with QRDR-A and QRDR-B mutations. Additionally, the study examined the structural interactions between the mutated GyrA enzyme and the LVX molecule using a homology modeling approach.

2. Materials and Methods

2.1. Isolates Selection and Mycobacterial Culture. A total of 104 MDR-*MTB* clinical isolates collected in Medellin, Colombia, between 2004 and 2009 and stored at −70°C at the Corporacion para Investigaciones Biológicas were used. These isolates had previously been tested for susceptibility to rifampin and isoniazid using a screening test in thin layer agar Middlebrook 7H11 (TLA 7H11) (Becton Dickinson) [24] and confirmed as MDR-TB using the Multiple Proportion Method in agar [25].

The MDR-*MTB* clinical isolates were grown in TLA 7H11 and incubated for 3 weeks at 37°C in a 5% CO_2 atmosphere until luxuriant growth became apparent.

2.2. Fluoroquinolone Susceptibility Testing. Each of the 104 MDR-*MTB* isolates was tested for FQ susceptibility using the Multiple Proportion Method in agar. *MTB* colonies were first inoculated in liquid Middlebrook 7H9 medium and incubated for 2 to 3 weeks until MacFarland standard 1.0 was reached. Subsequently, serial 10^{-2} and 10^{-4} dilutions were prepared for inoculation into TLA 7H11 with antibiotics [24, 25]. OFX (Sigma-Aldrich), MFX (Kemprotec Ltd.), and LVX (Sigma-Aldrich) stock solutions were prepared at 1 mg/mL in water and stored at −70°C. The final concentrations that were used were OFX (2 μg/mL), MFX (0.5 μg/mL), and LVX (2 μg/mL). Readings were performed weekly for 4 weeks and an isolate was considered resistant when the number of colonies on medium with the antibiotics was equal or greater than 1% of the number of colonies on the control media without antibiotics. The reference strain H37Rv *MTB* was used as a control.

Table 1: Primers used to amplify and sequence QRDR-A and QRDR-B regions.

Target region	Primer	Nucleotide sequence	Method
QRDR-A	gyrA15F	5′-GATGACAGACACGACGTTGC-3′	PCR
	gyrA19R	5′-GCCAGCTCACGCAGGTTG-3′	
	gyrA17F	5′-ATCGACTATGCGATGAGCGTG-3′	Sequencing
	gyrA18R	5′-ATGCCGCCTGACCCGTTG-3′	
QRDR-B	gyrB5F	5′-ACCTTCGCCAACACCATCAACACC-3′	PCR and sequencing
	gyrB10R	5′-CGAACCGAGGGATCCATGGTG-3′	
	gyrB7F	5′-CGGTTCTGCAAAAAGCGGTCGC-3′	Sequencing
	gyrB8R	5′-CGGAAGTATCGCCTGGAACATCG-3′	

2.3. QRDR-A and QRDR-B PCR Amplification. *MTB* isolates were cultured on TLA 7H11 for 3 weeks. Total DNA was isolated using the CTAB/NaCl method [26] and quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific) by measuring the absorption at 260 nm. Ten nanograms of DNA were used for PCR amplification using 2.5 U of the Fermentas Recombinant Taq DNA Polymerase (Thermo Scientific) and 60 pmol of each forward and reverse primer described in Table 1. The amplification was performed under the following conditions: 4 min at 94°C (1 cycle) followed by 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C (40 cycles) and a final extension of 5 min at 72°C on a DNA Engine Thermal Cycler (Bio-Rad). DNA fragments corresponding to the QRDR of *gyrB* and *gyrA* were amplified under PCR conditions previously described in this methodology (Table 1).

2.4. Nucleotidic Sequences Analysis. The sequencing of the amplicons was outsourced and performed by Macrogen Korea (Seoul, Republic of Korea) with each strand being sequenced using a forward and a reverse primer. Two primers for *gyrA* and four for *gyrB* were used (Table 1). The chromatograms were analyzed and edited using FinchTV v 1.4 (http://www.geospiza.com/Products/finchtv.shtml) and the consensus sequences were established with ClonManager V.9.0 (Sdi-Ed Software). Nucleotidic sequence alignments were performed with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and predictions of related aminoacid sequences were carried out with the ExPASy program (http://expasy.org/tools/dna.html).

2.5. Minimum Inhibitory Concentration (MIC). OFX, MFL, and LVX minimum inhibitory concentrations (MIC) were determined for all isolates, and *MTB* H37Rv was used as a control. MIC were determined according to procedures previously described [25]. Briefly, *MTB* H37Rv and isolates were grown in Middlebrook 7H9 liquid medium until MacFarland standard 0.5 was reached. Cultures were homogenized on a minishaker using 4 mm glass beads. The FQ MIC assay was performed in Middlebrook 7H9 liquid medium in BACTEC MGIT 960. For each FQ, the concentrations tested were 0.25, 0.50, 1.00, 2.00, 4.00, 8.00, and 16.0 μg/mL.

MGIT tubes were supplemented with 0.8 mL of supplement (BACTEC MGIT Growth Supplement; Becton Dickinson) and were inoculated with 0.1 mL of the drug solution and 0.5 mL of the test strain suspension. For preparation of the drug-free growth control tube, the organism suspension was diluted 100-fold in sterile saline solution, and then 0.5 mL was subsequently inoculated into the tube (susceptibility testing). The susceptibility testing sets were placed in the MGIT 960 instrument and results were interpreted as follows. At the time when the growth units (GU) of the drug-free control tube was >400, if the GU of the drug-containing tube to be compared was >100, the strain was Resistant. If the GU of the drug-containing tube was <100, the strain was susceptible [27].

2.6. Homology Modeling Analysis for QRDR-A. A homology modeling analysis was performed on *MTB* H37Rv and isolates TBR-67 and TBR-49 to determine the structural interactions between LVX and the gyrase A QRDR region. A search for identification of a three-dimensional structure of a protein known to serve as a model for determining the structure of the target protein was performed using the RCSB Protein Data Bank (PDB) accessible at http://www.pdb.org/pdb/home/home.do. A comparison was carried out using the *E. coli* DNA gyrase sequence (PDB access number 1AB4) and visualized with DeepView/Swiss-PdbViewer v3.7 (http://www.expasy.org/spdbv/). Additionally, the three-dimensional coordinates were read by Molecular RasWin Graphis Windows v2.6 (http://mc2.cchem.berkeley.edu/Rasmol/v2.6/).

The molecular characteristics of the GyrA catalytic site as well as the LVX chemical structure were determined using the semiempirical Austin Model 1 method which uses parameters derived from results of experimental quantum calculations to simplify and reduce computational costs [28]. Also, Austin Model 1 focuses on external electrons only considering that they are responsible for the reactivity. Models were optimized using global minimum geometry and energy parameters using PC Spartan Pro 1.0.5 (Wave function). The affinity values between the drug and the enzyme catalytic site were calculated for both the H37Rv reference strain and the two resistant isolates (TBR-49 and TBR-67) and the hydrogen bond length between the Asp94 and Arg98 residues and

Table 2: QRDR-A and QRDR-B mutations spectrum for the 10 FQ resistant *MTB* isolates.

Isolates	SNP	Amino acid change	Codon	Susceptibility to FQ		
				OFX	LVX	MFX
TBR-67*	GAC/GGC	Asp/Gly	94	R	R	R
TBR102*(XDR)	GAC/GGC	Asp/Gly	94	R	R	R
TBR-49*	GCG/GTG	Ala/Val	90	R	R	S
TBR-111*(XDR)	GAC/GGC	Asp/Gly	94	R	R	R
TBR-31*	GCG/GTG	Ala/Val	90	R	R	S
TBR-176*(XDR)	GAC/GGC	Asp/Gly	94	R	R	R
TBR-73*	No mutation			R	R	R
TBR-18*(XDR)	No mutation			R	S	S
TBR-107*(XDR)	No mutation			R	S	S
TBR-103*(XDR)	No mutation			R	R	R

*Isolates with Ser95Thr mutation.
R: resistant isolate; S: susceptible isolate.

the LVX ionizable groups were also calculated using PC Spartan Pro 1.0.5 (Wave function).

3. Results

3.1. MDR Isolates FQ Susceptibility. Of the 104 MDR-TB isolates that were analyzed, 94 (90.4%) were sensitive to the 3 FQ tested. The remaining 10 (9.6%) were resistant to OFL, among which 8 (7.7%) were also resistant to LVX and 6 (5.7%) to MVX (Table 2).

3.2. QRDR-A and QRDR-B Mutations. Of the 10 (9.6%) isolates that were resistant to OFL, all were harboring the QRDR-A Ser95Thr substitution, a mutation also encountered in 88 (84.6%) isolates sensitive to the 3FQ (not shown). Four isolates (TBRs 67, 102, 111, and 176) which were all resistant to the 3 FQ were carrying the Asp94Gly mutation, while 2 (TBR-49 and TBR-31) which were resistant to only OFX and LVX had the Ala90Val substitution (Table 2). The 4 remaining isolates (TBR-73, 18, 103, and 107) did not harbor any mutation in positions 90 and 94 but carried the Ser95Thr substitution (Table 3). No mutations were found in the QRDR region of the *gyrB* gene.

3.3. Minimum Inhibitory Concentration (MIC) of FQ Resistant Isolates. The MIC for OFL, MFX, and LVX were determined for the 10 FQ resistant isolates (Table 3). The highest MIC for OFL (16 mg/L) were encountered in 2 isolates harboring the Asp94Gly substitution (TBR-67 and TBR-111); however TBR-102 and TBR-176 also harboring the same mutation showed a MIC of 8 mg/L. The 2 isolates bearing the Ala90Val mutation both had a MIC of 4 mg/L and the MIC for the isolates with no QRDR-A mutation were equally distributed between 4 mg/L and 8 mg/L. For LVX, the MIC were spread between 2 mg/L and 8 mg/L with no particular trend in the distribution. For MFX, TBR-49 and TBR-31 both had a MIC of 0.5 mg/L. The highest MIC (2 mg/L) was measured in isolates with either no QRDR-A mutation (TBR-73) or the Asp94Gly substitution. Within 10 FQ resistant *MTB* isolates were found 6 XDR (Table 2).

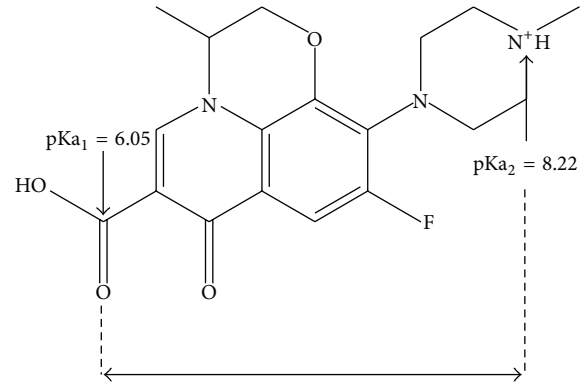

Figure 1: Structural representation of the LVX molecule with its ionizable groups.

3.4. Modeling of the Interaction between LVX and GyrA. We hypothesized an acid-base interaction between LVX and the GyrA catalytic site in which the amino acids 90, 94, and 98 have been previously shown to be directly involved in the antibiotic fixation.

We established a first model using the H37Rv reference strain in which we calculated that the interatomic distance between the Asp94 and Arg98 residues was 10.72 Å. This distance between both amino acids correlates with the distance between LVX ionizable groups (10.83 Å) and is therefore thought to allow enough space for LVX to interact (Figures 1 and 2).

Taking the interatomic distances into consideration a structural representation of the interaction between LVX and the H37Rv GyrA catalytic site was performed (Figure 2), showing the nonimplication of the Thr in position 95. The Ser95Thr substitution in TBR-49 and TBR-67 did not affect the interaction between LVX and GyrA catalytic site (Figures 2 and 3).

The Ala90Val substitution in TBR-49 resulted in an increase of the amino acid electron density volume from 83.88 $Å^3$ to 119.62 $Å^3$ due to the substitution of a methyl group by an isopropyl group (Figure 3(b)). This generated a steric

Table 3: OFL, LVX, and MFX MIC for ten isolates fluoroquinolones resistant X: last dilution with growth.

Isolate	Mutation in GyrA	Mutation in GyrB	OFL MIC mg/L							Cut-off MIC for resistance	LVX MIC mg/L							Cut-off MIC for resistance	MFX MIC mg/L							Cut-off MIC for resistance
			0.25	0.5	1	2	4	8	16		0.25	0.5	1	2	4	8	16		0.25	0.5	1	2	4	8	16	
TBR-49	Ala90Val	NM					X									X				X						
TBR-67	Asp94Gly	NM							X							X					X					
TBR-73	NM	NM						X								X						X				
TBR-31	Ala90Val	NM					X								X					X						
TBR-103	NM	NM						X							X						X					
TBR-18	NM	NM					X			>2.0				X				>2.0		X						>0.5
TBR-107	NM	NM					X							X					<0.25							
TBR-111	Asp94Gly	NM							X							X						X				
TBR-102	Asp94Gly	NM						X								X						X				
TBR-176	Asp94Gly	NM						X							X						X					

Table 4: Interaction energy and hydrogen bond length between GyrA and LVX.

Complex	Interaction energy	Hydrogen bonds (Arg98-LVX)	Hydrogen bonds (Asp94-LVX)
H37Rv-LVX	−970.83 kcal/mol	4.778 Å	2.599 Å
TBR-49-LVX	−955.83 kcal/mol	5.143 Å	2.673 Å
TBR-67-LVX	−866.48 kcal/mol	4.500 Å	—

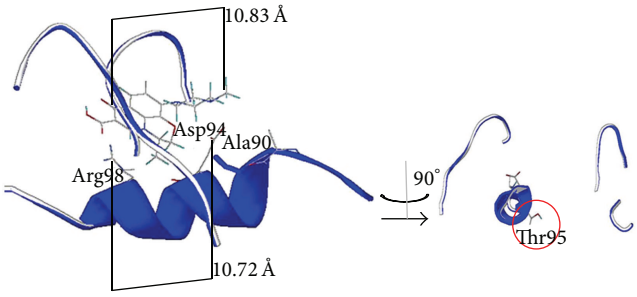

Figure 2: Model representation of the interaction between LVX and H37Rv *MTB* GyrA catalytic site featuring interatomic distances.

hindrance impairing LVX interaction with GyrA catalytic site.

The TBR-67 Asp94Gly generated the loss of an acid (acetyl) group, thus preventing the acid-base interaction between the LVX amino group and the Asp acid group at the catalytic site (Figure 3(c)).

In addition the affinity values between the drug and the enzyme catalytic site were calculated for both the H37Rv reference strain and the two resistant isolates (TBR-49 and TBR-67), and we found that the more stable configuration was given for the H37Rv-LVX complex with an interaction energy of −970.83 kcal/mol. For both strains with *gyr*A mutations we found that the interaction energies were −955.83 kcal/mol and −866.48 kcal/mol for the TBR-49 (Ala90Val)-LVX and the TBR-67 (Asp94Gly)-LVX complexes, respectively. The more negative the affinity value (kcal/mol) between the drug and the enzyme is, the more stable the interaction complex is. Also, we calculated the hydrogen bond length between the Asp94 and Arg98 residues and the LVX ionizable groups (Table 4). Interestingly, the distance between the Arg98 residue and LVX is greater for the TBR-49 (Ala90Val) isolate (5,143 Å) due to the steric effect generated by increasing the volume density when an isopropyl group is added to the catalytic site. For the TBR −67 isolate (Asp94Gly) the loss of the acetyl group prevents the interaction with the LVX amino group, consequently nonhydrogen bond could be calculated.

4. Discussion

MDR-TB is an increasing global problem as it has now been identified in almost every country and continues to climb in many parts of the world with alarmingly high levels as in some areas, one in five (19%) TB patient is infected by an MDR-TB strain [1]. The situation is even more dramatic in retreatment cases for which the prevalence is up to 60% [29, 30].

FQ broad spectrum of antibacterial activities and convenient use have made them a drug of choice for empiric therapy of a variety of common infections, such as urinary tract, upper and lower respiratory tract, enteric and gonococcal infection, even sometimes when a causative organism has not been identified. With the expanded use of these broad-spectrum agents for many infections, the selective pressure applied onto microbial pathogens has resulted in the emergence of FQ resistant strains in a diversity of organisms, including *MTB* [31].

Since the 1990s, the incidence of FQ-resistant *MTB* has been gradually increasing first with ciprofloxacin and OFX, and today with MFX and LVX [32]. The impact of FQ resistant TB as well as the critical need to control it and understand its mechanisms is underscored by the rapid emergence and spread of XDR-TB [33]. High MDR-TB burden countries located in Eastern Europe and Central Asia reported XDR-TB in more than 10% of MDR-TB cases. To date, a cumulative total of 84 countries (Colombia reported its first XDR-TB case in 2009) have confirmed at least one case of XDR-TB.

In this study, we endeavored to examine the resistance to MFX, OFX, and LVX of 104 clinical isolates of MDR *MTB* that had been collected in Medellin, Colombia, between 2004 and 2009. Those isolates had been phenotypically characterized and stored at the Mycobacterial Unit of the Corporación para Investigaciones Biológicas. However, we had no data indicating whether they were new or retreatment cases isolates. We encountered an FQ resistance rate of 9.6% which is comparable with the one described by other recent studies in Latin America [34] but far below the prevalence reported in Russia [35]. FQ resistance in Colombia is scarcely documented and in fact, to our knowledge, this is the 1st study reporting numerical data on this particular issue. The 10 isolates that displayed FQ resistance were further tested for the injectable amikacin and capreomycin as well as for ethionamide and para-aminosalicylic acid (PAS) and 6 of them (5.8%) (TBR-102, 111, 176, 18, 107, and 103) were found to be XDR (data not shown). This XDR prevalence among MDR strains is comparable to what has been reported in other regions of Latin America [36].

It has been widely reported that the majority of FQ-resistant *MTB* isolates carry mutations in the QRDR part of the *gyrA* gene [35, 37, 38]. However, the mutation we encountered the most frequently is known to be a natural polymorphism unrelated to resistance [39]. The Ser95Thr substitution is carried by 88 susceptible and the 10 resistant isolates.

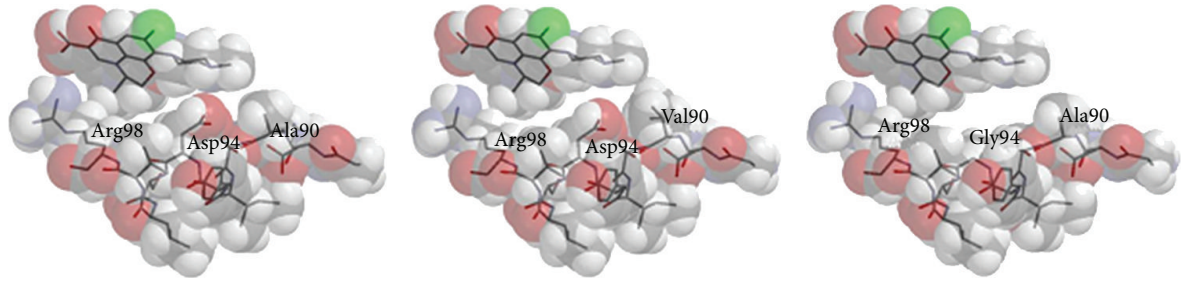

Figure 3: Comparative structural three-dimensional model representation of the interaction between LVX and *MTB* GyrA catalytic site in H37Rv (3(a)), TBR-49 (3(b)), and TBR-67 (3(c)).

Substitution in GyrA codons 90 and 94 have often been linked to FQ resistance [37, 40]. Of the 8 isolates resistant to the 3 FQ, 4 carried the Asp94Gly substitution (TBR-67, 102, 111, and 176) and 2 carried the Ala90Val mutation (TBR-49 and 31) (Table 2). None of the resistant isolates harbored both mutations unlike what was reported in several studies [15, 41]. Recently Nosova and colleagues showed that isolates carrying the double mutations exhibit higher MICs, strongly suggesting a synergistic contribution of both substitutions. Two isolates (TBR-73 and TBR-107) did not harbor any mutation in positions 90 and 94, indicating that additional GyrA independent mutations or mechanisms could also confer FQ resistance. Mutations in GyrB such as Asn538Asp, Asp500His, and Asp495Asn have been shown to be associated with *MTB* cross-resistance to FQ [35, 42], but in this study no mutations in QRDR-B region were found. On the other hand, TBR-18 that resist to OFL and MXF but not to LVX do not carry any mutation in position 90 or 94 indicating that if OFX and MFX resistance can be expressed without those substitutions, susceptibility never occurs in their presence. Additionally this shows that OFX and MFX resistance can be expressed independently of that of LVX.

It is important to clarify that all the isolates that were sensitive to LVX or MFX had a MIC below the cutoff (2.0 μg/mL), except for the TBR-107 isolate, with a MIC in the cutoff value for LVX (2.0 μg/mL), but was truly sensitive to MFX (<0.25 μg/mL). Other resistance mechanisms such as those involving the action of efflux pumps may explain the phenotypic FQ resistance in *MTB* without mutations in *gyrA* or *gyrB* genes. There are studies that show that the overexpression of efflux pumps from the ABC family can be correlated with expulsion of fluoroquinolones and a concomitant MIC increase. Further studies are necessary to assess the role efflux pumps in phenotypic resistance to FQ of *MTB* clinical isolates [43].

We determined MICs for the 10 FQ tested (Table 3). Even though there was no proper correlation between MIC levels and any of the mutations, the isolates harboring the Asp94Gly substitution tend to resist higher FQ concentrations. The stronger effect of Asp94Gly corresponds to a trend previously suggested by several studies [35, 44].

In order to better understand the mechanisms of LVX resistance conferred by substitutions in positions 90 and 94 and study them at the atomic level, we established a three-dimensional molecular model of the interaction between the hypothesized structure of the GyrA QRDR region and LVX. We used the same homology modeling technique that had allowed Cunha and colleagues to propose the first *MTB* GyrA hypothetical structure three-dimensional model [13]. Calculating the distance between Asp94 and Arg98 facilitated the visualization of a pocket that is believed to accommodate LVX (Figures 1 and 2), thus underscoring the critical role played by both residues in the FQ binding. LVX is a chiral fluorinated carboxyquinolone that possesses 2 ionizable functional groups, a carboxylic moiety (pKa_1 = 6.05) and a basic piperazinyl group (pKa_2 = 8.22) [45] that are critical to explain the acid-base interaction with GyrA catalytic site (Figure 1).

The Ala90Val change generated the addition of an isopropyl group at the catalytic site inducing an electron density volume increase (Figure 3(b)) and a steric effect that reduces the interaction between the LVX amino group and GyrA. Studies in *E. coli* suggest that a DNA gyrase that possesses such mutations is not affected in its function of DNA breakage [46] and maintains its functionality.

In the case of the Asp94Gly substitution, that acid-base interaction is hampered by the loss of an acetyl group (Figure 3(c)) thus rendering LVX binding less efficient and providing resistance to the drug by target modification. Modifying the enzyme does prevent LVX action; however it has been shown to have no effect on DNA binding, therefore maintaining a fully active gyrase [46].

Different studies have demonstrated the presence of individuals within the same *MTB* population causing infection in the same patient and expressing different susceptibility to drugs. This phenomenon is known as heteroresistance and has been described as present in 25% of isolates resistant to OFX [47]. Although the presence of heteroresistance was not assessed in this study, we cannot totally exclude this phenomenon without further experiments.

FQ are actually the more active drugs in the treatment of MDR-TB; nevertheless, it is still unknown many aspects related to resistance mechanisms including those nonassociated to mutations. As FQ resistant TB continues to develop

into more alarming and threatening public health issue, gaining structural information on the resistance mechanisms can facilitate the rational engineering for improving the actual drugs. The homology modeling approach has become a promising tool that can provide a deeper insight of antibiotic-target interaction essential to the design of much needed new molecules.

5. Ethical Considerations

This project is classified in the category "research without risk" according to the Helsinki Declaration. For this study applies only experimental techniques of laboratory to isolates of MTB and at no time were biological, physiological, psychological, and social interventions in humans performed.

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

Predicting *Mycobacterium tuberculosis* Complex Clades Using Knowledge-Based Bayesian Networks

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We develop a novel approach for incorporating expert rules into Bayesian networks for classification of *Mycobacterium tuberculosis* complex (MTBC) clades. The proposed knowledge-based Bayesian network (KBBN) treats sets of expert rules as prior distributions on the classes. Unlike prior knowledge-based support vector machine approaches which require rules expressed as polyhedral sets, KBBN directly incorporates the rules without any modification. KBBN uses data to refine rule-based classifiers when the rule set is incomplete or ambiguous. We develop a predictive KBBN model for 69 MTBC clades found in the SITVIT international collection. We validate the approach using two testbeds that model knowledge of the MTBC obtained from two different experts and large DNA fingerprint databases to predict MTBC genetic clades and sublineages. These models represent strains of MTBC using high-throughput biomarkers called spacer oligonucleotide types (spoligotypes), since these are routinely gathered from MTBC isolates of tuberculosis (TB) patients. Results show that incorporating rules into problems can drastically increase classification accuracy if data alone are insufficient. The SITVIT KBBN is publicly available for use on the World Wide Web.

1. Introduction

Tuberculosis (TB) represents a reemerging serious health threat worldwide. TB is caused by the *Mycobacterium tuberculosis* complex (MTBC) bacterium. One-third of the world population is latently or actively infected with TB. Molecular epidemiology now plays a crucial role in the tracking and control of TB [1]. DNA fingerprinting methods have made it possible to distinguish between cases of recent transmission of TB and reactivation of latent infections. This has enabled the tracking of transmission routes and the timely identification of outbreaks. Thus, knowledge about the genotypes of prevailing strains has revolutionized traditional approaches to the epidemiology of TB. Moreover, the predominance of certain strains or groups of strains in certain host populations has been clearly observed [2]. Studies of the genetic and biogeographic diversity of the MTBC have revealed differences in the virulence, immunogenicity, and drug resistance of strains [2]. This has consequences for the development of control measures for TB.

The classification of MTBC strains into genetic groups or clades is important to help track transmission patterns and develop a better understanding of pathologic specificities in TB. Phylogeographic clades have been defined based on genetic similarities between strains and observed associations between groups of similar MTBC genotypes with host populations [3]. A variety of molecular techniques including the analysis of phylogenetically informative single nucleotide polymorphisms (SNPs) and long sequence polymorphisms (LSPs) are used to genotype MTBC strains [4]. Classification based on SNPs and LSPs is considered to be the gold standard. However, studies of such variations in DNA sequences

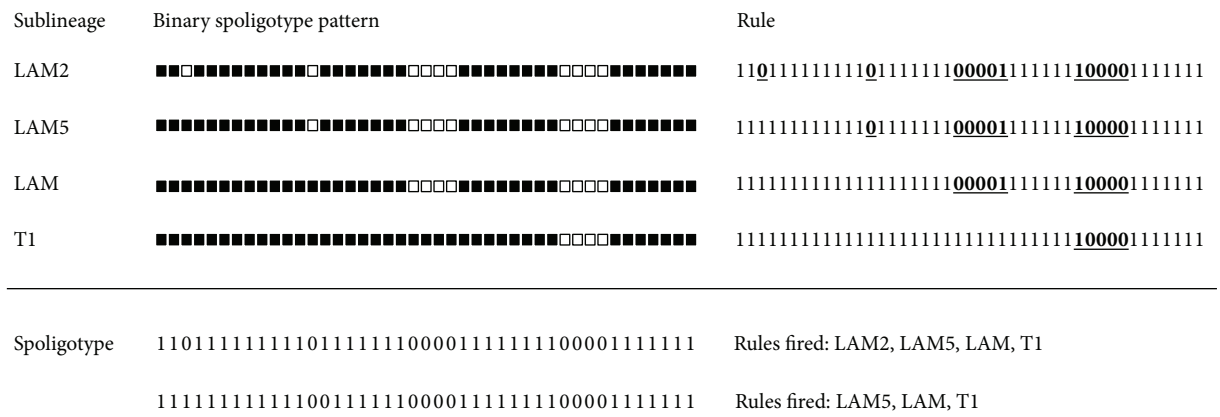


Figure 1: Example rules from SpolDB4. The rule column represents characteristic patterns specified by the visual rules as underlined subsequences in the spoligotype patterns. Each line corresponds to a rule. The underlined portions of the spoligotype must match exactly while the portions not underlined can take any value. All of these rules fire for the spoligotype 11011111111101111111000011111111100001111111, while three of the rules fire for 11111111111100111111000011111111100001111111.

of MTBC strains are not performed frequently for public health purposes. Spacer oligonucleotide typing (spoligotyping) and mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTRs) typing are two polymerase-chain-reaction- (PCR-) based DNA fingerprinting methods routinely used in the United States for genotyping all identified culture-positive TB cases. Large databases of spoligotypes have been collected. Each spoligotype for a strain is determined by the presence or absence of 43 specific spacers in the DR region, producing a 43-bit number. Each spacer separates two direct repeats. These strains have been assigned sublineage labels using mixed expert-based and bioinformatics approaches derived from visual rules applied to spoligotypes as shown in Figure 1.

These visual rules are based on the identification of characteristic deletions of one or more adjacent spacers in spoligotypes. Certain inferred mutations (deletions of blocks of adjacent spacers) in progenitor strains are considered to be lineage defining [5]. These deletions are conserved in all descendent strains since studies have shown that the mechanism of mutation observed in the direct repeat (DR) region involves loss of spacers, and spacers are rarely gained [6]. Additionally, the existence of these sublineages has been independently verified by clustering based on spoligotype and MIRU types of strains [7, 8]. Therefore, while it has been established that strains of TB belong to distinct sublineages, the definitions of these sublineages based on spoligotypes are not clear. The visual rules for a sublineage are generalizations of spoligotype patterns that belong to the sublineage. However, directly applying visual rules to spoligotype patterns can lead to multiple assignments of sublineage labels since spoligotype patterns may match patterns prescribed by more than one rule, and sometimes spoligotype patterns do not exactly match the patterns specified by any rule. This is an inherent limitation of a rule-based system, wherein rules need to be broad enough to capture general patterns but narrow enough to delineate classes. Additionally, spoligotyping is based on polymorphisms in a single locus, the DR region, and therefore has the potential for convergent evolution. Relying on specific subsequences within the spoligotypes for the study of genetic diversity is hence error prone.

This paper presents a hierarchical probabilistic graphical model, the knowledge-based Bayesian network (KBBN), that encodes rules of thumb and large training databases to classify data into given classes. Expert knowledge is modeled in the top level of variables in the BN representing the rules. The middle level variables represent the class and the lower level represents various features of interest. KBBN uses the strategy of not directly modeling the dependency of the rules on the features. This greatly reduces the model parameter space which helps reduce the amount of data required for training while capturing the knowledge in the rules.

The overall goal of this paper is to construct a model for predicting clades based on spoligotypes as determined by SITVIT using published rules from SPOLDB4 and other sources and to make this model available via the World Wide Web. For MTBC clade classification in this model, the visual rules of thumb are the top-level variables, the clades are the classes, and the 43 spacers that constitute the spoligotypes are the features. The KBBN for MTBC sublineages builds on the conformal Bayesian network previously designed for that domain. The structure of the KBBN encodes the knowledge base captured in the rules of thumb helping to improve overall accuracy while overcoming any potential problems such as ambiguous, inaccurate, or incomplete rules. A secondary goal is to assess the effectiveness of the KBBN in the MTBC lineage classification task. Thus we do extensive experiments on SITVIT as well as an additional testbed: CDC. The CDC test set consists of data and rules from the United States Centers for Disease Control and Prevention.

This paper is organized as follows. We first examine prior Bayesian networks for MTBC classification and then introduce the KBBN approach to incorporate rules. We then examine the rules of thumb and data associated with MTBC clades. We present results for the KBBN-SITVIT model and assess its accuracy. Finally, computational studies examine how KBBN can improve accuracy over Bayesian networks on the two KBBN testbeds (SITVIT and CDC). The results

show that KBBN is quite resilient to incomplete, inaccurate, or ambiguous rules and can obtain better performance than BN using less data.

Previously in the MTBC domain, other approaches to incorporate advice in the form of rules have been shown to improve discriminative learning models of MTBC major lineages and other problems [9]. However, those methods are limited to rules expressed in less-intuitive polyhedral form that requires preprocessing of data and rules.

The proposed KBBN model allows the existing rules of thumb to be incorporated with no modification resulting in improved classification over the predictions made with the rules or Bayesian networks alone. Also, unlike visual rules, the flexibility offered by the KBBN enables it to handle these common problems with the following rules of thumb.

(i) Incompleteness: rules only exist for some of the classes or only partially cover a class.

(ii) Ambiguity: multiple rules of thumb for different classes apply to the same exemplar. This frequently occurs if there is no precedence associated with the rules.

(iii) Inaccuracy: rules may incorrectly classify some exemplars.

Visual rules with precedence have been established for six major MTBC lineages [10]. A prior online knowledge-based support vector machine (SVM) approach combined these visual rules and precedence into a set of rules expressed in polyhedral form [9]. The method produced a high-accuracy SVM using much less data. However, as discussed in Section 5, this elegant work has several practical limitations that we sought to overcome in this study. First, expressing rules and precedence as polyhedral rules [9] can be challenging for a large number of rules. Second, the method works best with linear SVMs, but linear SVMs do not capture the underlying complexity of the biomarkers and their mechanism of evolution. This can be overcome by using nonlinear SVMs (SVMs using 3-degree polynomial kernels work very well), but then incorporating the polyhedral rules becomes even more challenging. Third, the complexity of training increases with the introduction of rules. Thus, the proposed design of the KBBN has the following salient features:

(i) incorporates rules easily without modification and without imposing precedence,

(ii) models known properties of the domain such as biomarkers and their mutation mechanisms,

(iii) provides an efficient training method for classes with and without rules,

(iv) achieves high prediction accuracy,

(v) overcomes ambiguity, incompleteness, and inaccuracy of the rules,

(vi) provides additional information about the effectiveness of each rule.

The overall approach produces a high quality model for predicting SITVIT clades which has been made available for use by other researchers.

2. Bayesian Network Background

A Bayesian network (BN) is a graphical representation of a probability distribution. Formally speaking, a BN is a directed acyclic graph $G(N, E)$ consisting of a set of nodes $X = \{x_i \mid x_i \in N\}$ to represent the variables and a set of directed links to connect pairs of nodes [11]. Each node has a conditional probability distribution that quantifies the probabilistic relation between the node and its parents such that for a network of k nodes

$$P(x_1, x_2, \ldots, x_k) = \prod_{i=1}^{k} P(x_i \mid \text{parents}(x_i)). \quad (1)$$

Therefore, one can compute the full joint probability distribution from the information in the network. In other words, a well-represented Bayesian network can capture the complete nature of the relationship among a set of variables.

The SPOTCLUST Bayesian network was the first generative model used for analysis of MTBC sublineages [7]. SPOTCLUST uses mixture models based on spoligotypes to identify strain families of MTBC. SPOTCLUST models the asymmetric evolution of spacers using a Bayesian network with "hidden parents" [7]. The hidden parents of a lineage generate the members of the lineage. They capture the evolution of spoligotypes without generating the full phylogeny. A spacer in the hidden parent may be lost with small probability. A spacer that is absent in the parent is almost never gained. The design models the evolution mechanism of the DR region, allowing the Bayesian network to capture the deletions that are known to characterize spoligotype lineages. The hidden parent technique of SPOTCLUST is used for the spoligotype-associated parts of the KBBN model.

The conformal Bayesian network (CBN) is another generative model for analysis of both spoligotype and MIRU type data for MTBC strains [9, 12] (spoligotype CBN is shown in Figure 2(a)) originally designed for predicting major MTBC lineages. CBN captures the domain knowledge about the properties of spoligotypes and MIRU and uses this information to classify MTBC strain genotyping data into major lineages. CBN reflects the known mutation mechanisms of the spoligotypes and MIRU. With rare exceptions, ancestral strains have 2 or more repeats at MIRU24. Thus the top-level variable, M_{24}, indicates whether MIRU24 is less than two (indicating one of the modern lineages with high probability) or at least two (indicating one of the ancestral lineages with high probability).

One can think of the MIRU CBN model "generating" the data as follows. The value of locus MIRU24 generates the lineage, which in turn determines the number of repeats in the remaining MIRU loci. Thus, patterns in the occurrences of repeats at each locus for each lineage are captured. The lineage also generates the hidden parents of the lineage which in turn generate the spoligotype spacers. The MIRU24 determines the lineage priors.

We tried using the CBN model as designed for major lineages to classify MTBC genotyping data into sublineages. But using the single rule, *if MIRU*24 $\geq$ 2*, then lineage is ancestral*, as in the original CBN was not enough to generate a good

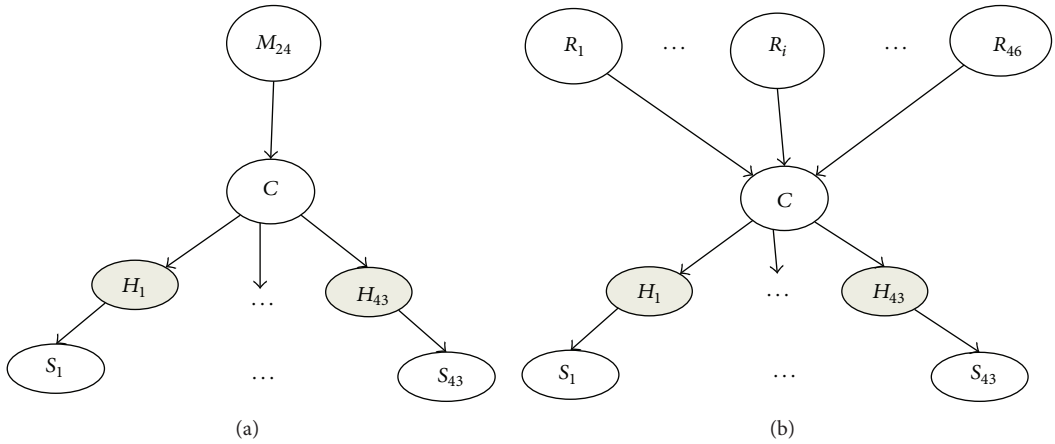


Figure 2: (a) The spoligotype conformal Bayesian network uses a single rule based on the number of repeats at the MIRU24 locus as the first level of a hierarchical Bayesian network. It uses the 43 spacers as features. CBN predicts the major lineage with high accuracy. (b) The KBBN uses multiple rules based on the presence of characteristic deletions as the first level of a hierarchical Bayesian network. As with the CBN, it uses the 43 spoligotype spacers.

model. KBBN grew out of the effort to incorporate all of the visual rules available from SpolDB4 [3] into CBN.

2.1. Knowledge-Based Bayesian Network. The knowledge-based Bayesian network (KBBN) is a hierarchical probabilistic graphical model which encodes the knowledge obtained from expert-defined rules derived from true observations with large databases to solve classification problems. KBBN incorporates the rules of thumb as high level variables or class priors in the Bayesian network and therefore combines the information obtained from rules of thumb with the information provided by a BN model specifically designed for the domain. The method is designed and tested on widely used simple BNs such as naïve Bayes and polytrees with polynomial time learning and inference algorithms. The KBBN for the tuberculosis domain uses CBN as its base and is used for both CDC and SITVIT testbeds.

KBBN, represented in Figure 2(b), is a novel hierarchical Bayesian network probability model for sublineage classification of MTBC. KBBN captures domain knowledge about the properties of spoligotypes and incorporates additional information provided by SpolDB4 or CDC rules to predict the class with high accuracy. The corresponding probability density function for the naïve KBBN model, shown in Figure 2(b), is

$$P\left(C, S_\Omega, R_\Psi\right) = \prod_{j\in\Omega}\left(\sum_{H_{j\in\{0,1\}}} P\left(S_j \mid H_j\right) P\left(H_j \mid C\right)\right) \times P\left(C \mid R_\Psi\right) P\left(R_\Psi\right), \quad (2)$$

where the random variable C represents the sublineage class, the random variable $S_\Omega = \{S_j \mid j \in \Omega\}$ with $\Omega = \{1, \ldots, 43\}$ represents the spoligotype spacers, and $R_\Psi = \{R_k \mid k \in \Psi\}$ represents the set of binary rules indicating whether each specific rule is fired. The spacer variables S and class variable C are assumed to follow binomial and multinomial distributions, respectively. The conditional probabilities of R given C are represented as a table which maps the set of possible combinations of rules fired in the data to the probability of each class. Laplacian smoothing is used.

For spoligotypes, we followed the SPOTCLUST model [7]. It captures the fact that spacers are lost but almost never gained, by introducing a variable for the unobserved hidden parent (H_j) and for each spacer S_j, both of which follow a binomial distribution. Given a 43-dimensional spoligotype S and its spacer position j, $S_j = 1$ if the spacer is present, and $S_j = 0$ if the spacer is absent. The probabilities of the spacer given the parent $P(S_j \mid H_j)$ are assumed to be known. As in Vitol et al., 2006 [7], we considered the probability of losing a spacer as 10^{-1} and the probability of gaining a spacer as 10^{-7}.

The KBBN assumes that the spoligotype hidden parents are conditionally independent given the sublineage. The conditional independence assumption of spacers is a model simplification previously made in the SPOTCLUST BN model. This conditional independence of the biomarkers in the BN model enables KBBN to conform to the set of available biomarkers without any expensive missing value computations. None of the genotyping variables in the BN are treated as unobserved except for the hidden parent spacers, which are always unobserved.

Using Bayes' rule, one can predict the sublineage for new data by determining the sublineage with maximum probability:

$$P\left(C \mid S_\Omega, R_\Psi\right) \propto \prod_{j\in\Omega}\left(\sum_{H_{j\in\{0,1\}}} P\left(S_j \mid H_j\right) P\left(H_j \mid C\right)\right) \times P\left(C \mid R_\Psi\right) P\left(R_\Psi\right). \quad (3)$$

3. Data Domains and Biology Rules

This study focused on creating a predictive model for clades that emulated SITVITWEB, a publicly available international

Table 1: SITVIT and CDC MTBC testbeds.

Testbed	Dataset	Size	Number of classes	Max class size	Min class size	Number of rules
SITVIT	Train	2714	69	390	1	69
	Test	7949	69	1107	1	69
	CV	2593	45	390	11	45
CDC	CV	1286	8	356	39	8

multimarker database for tuberculosis molecular epidemiology [13]. Different datasets were created for training, testing, and cross validation. To validate the approach we also used a dataset of isolates collected by the CDC for cross validation studies. The following two sections describe the datasets in detail. Table 1 summarizes them.

3.1. SITVIT Testbed. SITVIT-Train and SITVIT-Test are based on the SITVIT, a MTBC genotyping markers database provided by the Institut Pasteur de la Guadeloupe, and on the SpolDB4 rules that are published in Brudey et al., 2006 [3], plus one rule recently developed for the URAL1 clade. KBBN was trained on the SITVIT-Train dataset of 2714 records, each corresponding to a spoligotype and clade. There were 69 classes, the minimum sublineage size was 1, and the maximum sublineage size was 390. To test this model while keeping all classes we used SITVIT-Test, a large dataset based on SITVIT with 7949 records, each record corresponding to a spoligotype and clade. This dataset contained the same 69 classes as SITVIT-Train with different class distributions and again with the minimum class size of 1. SITVIT-Train and SITVIT-Test do not overlap so the total SITVIT dataset consists of 10633 distinct spoligotypes. To enable 10-fold cross validation (CV) with at least one spoligotype per class, the SITVIT-CV dataset was created which consists of the SITVIT-Train data restricted to the 45 classes with at least 11 spoligotypes each.

Note that some lineages have been reclassified while the KBBN model was under development. Two LAM sublineages were recently raised to lineage level: LAM10-CAM as the Cameroon lineage [14] and LAM7-TUR as the Turkey lineage [15, 16]. Some spoligotype patterns previously classified as H3 and H4 sublineages were relabeled "Ural" [17]. The latter include patterns belonging to H4 sublineage that were relabeled "Ural-2" and some patterns previously classified as H3 sublineage but with an additional specific signature (presence of spacer 2, absence of spacers 29 to 31 and 33 to 36), which are now relabeled "Ural-1." With their definitive reclassification pending, we hereby refer to these as H4-Ural-2, H3, and H3-Ural-1. Spoligotype patterns labeled as EAI and EAI5 were merged into a single group called EAI since one rule covers both patterns.

A sample of SpolDB4 rules is presented in Figure 1. Each line corresponds to a rule. The underlined portions of the spoligotype must match exactly while the portions not underlined can take any value. Note that in Brudey et al. [3] the rules are expressed using the octal coding of spoligotypes; here we express them in binary for simplicity. While these rules establish characteristic patterns for sublineages of MTBC, they are not exclusive and in some cases overlap. Up to 4 rules fired per example. The mode of the number of rules fired per record was 2. In practice, a precedence or order is introduced over the rules using expert knowledge so that unambiguous sublineage predictions are generated. However, this precedence has not been published for sublineages and is up to the individual user of the rules. The SpolDB4 rules have continued to evolve as new lineages such as H3-URAL-1 which are added and refined, and thus the exact rules that we used are provided in Supplement 1 in the Supplementary Materials available online at http://dx.doi.org/10.1155/2014/398484.

3.2. CDC-Sublineage and CDC Rules. The second dataset, CDC-Sublineage, examines 1286 MTBC isolates genotyped by spoligotyping and labeled with 8 sublineages. Dr. Lauren Cowan of the CDC was interviewed to obtain 8 rules of thumb. The data is a subset of 31,482 MTBC isolates genotyped by spoligotyping and 12-locus mycobacterial interspersed repetitive units (MIRU) typing with known lineages from a set collected by the CDC as part of routine TB surveillance in the United States from 2004 to 2009. Since only spoligotypes are used in the rules, the data for training were restricted to spoligotypes with labeled sublineages.

There are 8 rules expressed as numeric formulas based on the 43 spacers in the spoligotypes. For example, the rule for the Indo-Oceanic sublineage Manila (or EAI2-Manila in SpolDB4 rules) is

> If absence of spacers 3, 20, 21, 29–32, 34, sum(spacers33–36) > 0 and presence of spacers 2, 4, 19, 22, then Indo-Oceanic Manila.

There is one rule per sublineage. This dataset was preprocessed by adding an array of 8 bits, one bit per rule. The value of a bit was set to 1 if the rule was fired and zero otherwise. Note that the sublineage sizes are unequal. Overall, the minimum sublineage size was 39, the maximum sublineage size was 356, and the median was 138 records. The rules were ambiguous and no precedence was imposed. In some cases no rules fire for a record. A maximum of 2 rules is fired for each record. The mode of the number of rules fired per record was also 2. If multiple rules fire for a record and the sublineages determined conflict or if no rules fire, the record is considered to be misclassified. Details of the CDC rules can be found in Supplement 2.

4. SITVIT Experimental Results

In this section, we examine the effectiveness of the KBBN model for prediction of SITVIT classification results. Our experiments consist of two parts: (1) in-sample accuracy

Table 2: Training F-measure for KBBN trained on all 10633 SITVIT isolates.

Clade	F-measure	Clade	F-measure	Clade	F-measure
AFRI	0.800	H	0.736	PINI	0.750
AFRI_1	0.944	H1	0.924	PINI1	1.000
AFRI_2	0.908	H2	0.875	PINI2	0.667
AFRI_3	0.966	H3	0.915	S	0.976
Beijing	1.000	H3-Ural-1	0.873	T	0.926
BOV	0.948	H37Rv	0.958	T1-RUS2	0.778
BOV_1	0.993	H4-Ural-2	0.933	T2	0.953
BOV_2	1.000	LAM	0.947	T2-Uganda	0.991
BOV_3	0.644	LAM1	0.977	T3	0.964
BOV_4-Caprae	0.891	LAM11-ZWE	0.954	T3-ETH	0.65
Cameroon	0.929	LAM12-Madrid1	0.947	T3-OSA	0.626
CANETTI	1.000	LAM2	0.991	T4	0.988
CAS	0.937	LAM3	0.988	T4-CEU1	1.000
CAS1-Delhi	0.961	LAM4	0.970	T5	0.984
CAS1-Kili	0.973	LAM5	0.978	T5-Madrid2	1.000
CAS2	0.921	LAM6	0.856	T5-RUS1	0.949
EAI	0.982	LAM8	1.000	T-Tuscany	1.000
EAI1-SOM	0.986	Manu_ancestor	1.000	Turkey	0.928
EAI2-Manila	0.984	Manu1	0.991	X1	0.989
EAI2-Nonthaburi	1.000	Manu2	1.000	X2	0.963
EAI3-IND	0.963	Manu3	1.000	X3	0.995
EAI4-VNM	1.000	Microti	0.750	ZERO	0.800
EAI6-BGD1	0.989				
EAI7-BGD2	1.000			AVERAGE	**0.930**
EAI8-MDG	1.000				

of the SITVIT KBBN model trained using all available data and (2) out-of-sample accuracy of the SITVIT KBBN model trained on the SITVIT-Train and tested on the much larger SITVIT-Test set.The accuracy of the results was measured using the F-measure on the testing data (harmonic mean of precision and recall) averaged over the classes. The F-measure was selected since it effectively captures performance on the unbalanced multiclass data sets studied here. Reporting class accuracies/errors can be misleading for unbalanced classes such as those in the TB data. The minimum and maximum class sizes are reported in Table 1. The F-value was computed as

$$F = 2 \times \frac{\text{precision} \times \text{recall}}{\text{precision} + \text{recall}}, \quad (4)$$

where recall is the percentage of the isolates in a given clade correctly identified as being in that clade and precision is the percentage of isolates predicted to be in a clade that are actually in the clade.

4.1. SITVIT KBBN Model Accuracy. The SITVIT KBBN model was trained to predict 69 sublineages using the combined SITVIT-Train and SITVIT-Test data extracted from SITVITWEB along with the SPOLDB4 rules. Overall the model is very accurate; it correctly classifies 94.3% of all of the spoligotypes, achieving an average F-value of 0.93 across the 69 clades. Table 2 presents the in-sample results of SITVIT-KBBN for each clade. The errors that do occur primarily come from lack of specificity not sensitivity. The model achieves a sensitivity of greater than 82% on all of the clades, but the specificity is below 82% on 13 clades. The T clade, which is known to be ill defined, contributes errors leading to reduced specificity in a wide variety of clades including LAM6, T1-RUS, T3-ETH, T3-OSA, and AFRI. Within the *M. africanum* clades, AFRI is primarily confused with other *M. africanum* clades (AFRI_1, AFRI_2, and AFRI_3) which is an acceptable error. A few Cameroons, H3, and T isolates are mistakenly identified as AFRI. Many BOV isolates are assigned by the model as belonging to BOV_3 indicating that a more expansive definition of BOV_3 may be warranted. There are some minor confusions within the Haarlem sublineages H1, H2, and H3 combined with the new H4-URAL-2 and H3-URAL-1 sublineages. About 16% of H3 is assigned to other classes. This suggests that further refinement of the definition of these sublineages will be ongoing. Microti, PINI, and PINI2 have lower F-values, but this is partially due to the fact that these sublineages have only a few exemplars. More data is needed for these rare lineages to improve the model. The F-value of ZERO is reduced by 6 CAS misclassified as ZERO. The overall specificity averaged over the clades is 0.909 and the sensitivity is 0.965.

Table 3: Results of the F-measures of KBBN based on out of-sample test. The KBBN model was trained on SITVIT-Train (with 2714 records) and tested on SITVIT-Test with 7949 records. Overall average F-measure is 0.939.

Clade	F-measure	Clade	F-measure	Clade	F-measure
AFRI	0.889	H	0.942	PINI	0.667
AFRI_1	0.975	H1	0.977	PINI1	0.923
AFRI_2	0.926	H2	0.625	PINI2	0.522
AFRI_3	1.000	H3	0.944	S	0.956
Beijing	0.980	H3-Ural-1	0.887	T	0.969
BOV	0.981	H37Rv	1.000	T1-RUS2	0.956
BOV_1	0.996	H4-Ural-2	0.960	T2	0.991
BOV_2	1.000	LAM	0.949	T2-Uganda	1.000
BOV_3	1.000	LAM1	0.986	T3	0.969
BOV_4-Caprae	0.914	LAM11-ZWE	0.976	T3-ETH	0.977
Cameroon	0.967	LAM12-Madrid1	1.000	T3-OSA	0.978
Canetti	0.500	LAM2	0.993	T4	0.984
CAS	0.990	LAM3	0.973	T4-CEU1	1.000
CAS1-Delhi	0.990	LAM4	0.967	T5	1.000
CAS1-Kili	0.846	LAM5	0.985	T5-Madrid2	1.000
CAS2	1.000	LAM6	0.889	T5-RUS1	0.883
EAI	0.989	LAM8	0.970	T-Tuscany	0.889
EAI1-SOM	1.000	Manu_ancestor	1.000	Turkey	0.941
EAI2-Manila	1.000	Manu1	0.995	X1	0.963
EAI2-Nonthaburi	0.933	Manu2	0.997	X2	0.944
EAI3-IND	1.000	Manu3	1.000	X3	0.971
EAI4-VNM	1.000	Microti	0.667	ZERO	0.800
EAI6-BGD1	1.000				
EAI7-BGD2	0.993			Average	**0.939**
EAI8-MDG	1.000				

4.2. Predictive Accuracy Results. To assess the out-of-sample predictive accuracy of the KBBN SITVIT model we trained the model on SITVIT-Train and tested it on SITVIT-Test. The model was very accurate overall achieving an average out-of-sample test F-value of 0.939, almost identical to the in-sample estimate of above 0.930. The average recall (percentage of the isolates in a given clade correctly identified as being in that clade) between all lineages is 97.5%, and the average precision (the percentage of isolates predicted to be in a clade that are actually in the clade) among all lineages is 91.9%. As shown in Table 3, the results for each clade are very similar to those reported in Table 2. The T clade and small rarer clades such as PINI variants and Microti account for much of the decrease in precision.

4.3. Model Validation. The next set of experiments evaluates the effectiveness of the KBBN approach with respect to other techniques and the effectiveness of incorporating rules. All experiments were done on both the SITVIT and CDC datasets to ensure that the results are not an artifact of a single dataset. For each dataset, first we used 10-fold stratified cross validation. Each training set was divided into 10 parts with 9 parts available as the training data for creation of models and 1 part held out as an independent test set. For all experiments the same test sets were employed, but the training dataset or the set of rules used may be varied. The accuracy of the results was measured using the F-measure on the testing data (harmonic mean of precision and recall) averaged over the classes. To facilitate a fair comparison, the data were constructed so that there are at least 10 records per class. In the SITVIT domain, this required removing clades that do not commonly infect human beings (e.g., PINI1 and PINI2). We refer to this subset of the SITVIT-Train dataset as SITVIT-CV. SITVIT-CV had 45 classes and 2593 records. The minimum sublineage size was 11, and the maximum sublineage size was 390 with a mode of 21 records.

4.3.1. Comparison with Other Techniques. We designed several sets of experiments on the two datasets SITVIT-CV and CDC-Sublineage to determine the following: if incorporating rules improved the performance of the Bayesian network over the performance of the BN or rules alone. The results were gathered for KBBN, BN, and the rules used alone. In addition, linear and nonlinear SVM results were provided as a baseline for comparison. The SVM implementation in WEKA (http://www.cs.waikato.ac.nz/ml/weka/) was used. The SVM kernels and parameters were selected using a grid search of 9-fold cross validated accuracy of the training set. The degree-three polynomial kernel and radial basis function kernels were found to work best. All SVM data was normalized

Table 4: Average F-measure of KBBN, BN, Rules-only, and SVM (nonlinear and linear) on two testbeds. While using Rules-only provides poor results, KBBN is able to provide results that are significantly better or at least not worse than BN and SVM on both domains. Results significantly different from KBBN at 5% significance level are shown in bold.

Dataset	Model				
	KBBN	BN	Rules-only	SVM nonlinear	SVM linear
SITVIT-CV	0.945	**0.771**	**0.345**	**0.903**	**0.914**
CDC-Sublineage	0.981	**0.934**	**0.312**	0.994	0.993

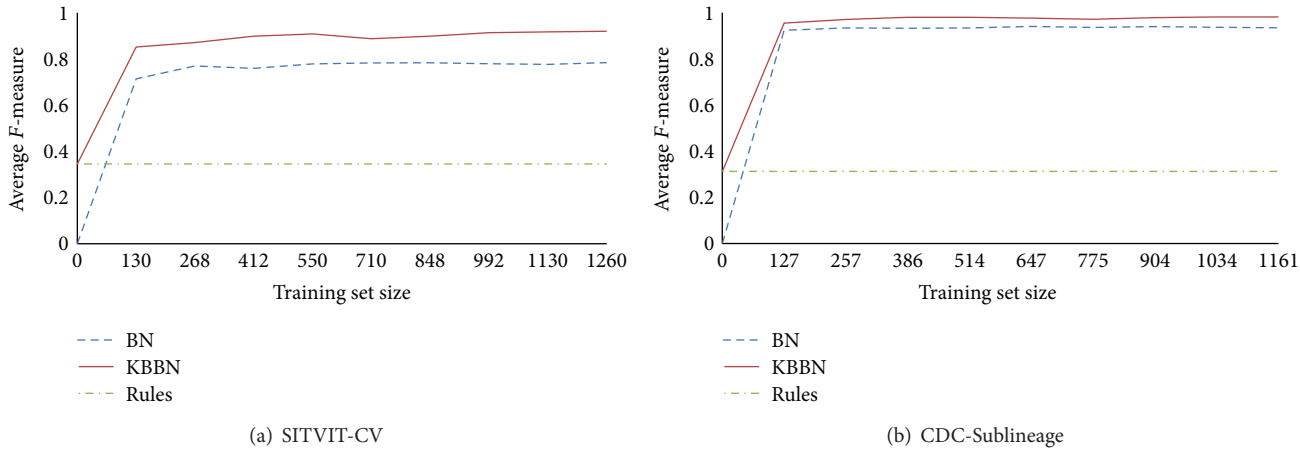


Figure 3: The result of adding rules to different training set sizes for the (a) SITVIT-CV and (b) CDC-Sublineage testbeds.

before training. Also, we are interested in the nature of the misclassification because it tells us about the potential inaccuracies in the definition of the lineages.

Table 4 compares the results of KBBN, BN, Rules-only, and SVM (nonlinear and linear) on the two testbeds. The rules themselves have very poor overall accuracy, but they led to improvements over the baseline BN accuracy on both datasets with statistically significant improvements on CDC-Sublineage and SITVIT-CV. The SVM results indicate that KBBN's accuracy is competitive with state-of-the-art nonlinear and linear classification methods. But note that KBBN, being a generative method, has many advantages over SVM such as availability of posterior probabilities of each class given the observation that can be interpreted as the confidence of the prediction, easier interpretation, and ease of incorporation of domain knowledge.

4.3.2. Effectiveness of Rules in Bayesian Network. Next, we designed several sets of experiments to determine the following: how the quality and quantity of rules and data affected the performance of KBBN. The basic underlying experimental design was the same for experiments across the two testbeds.

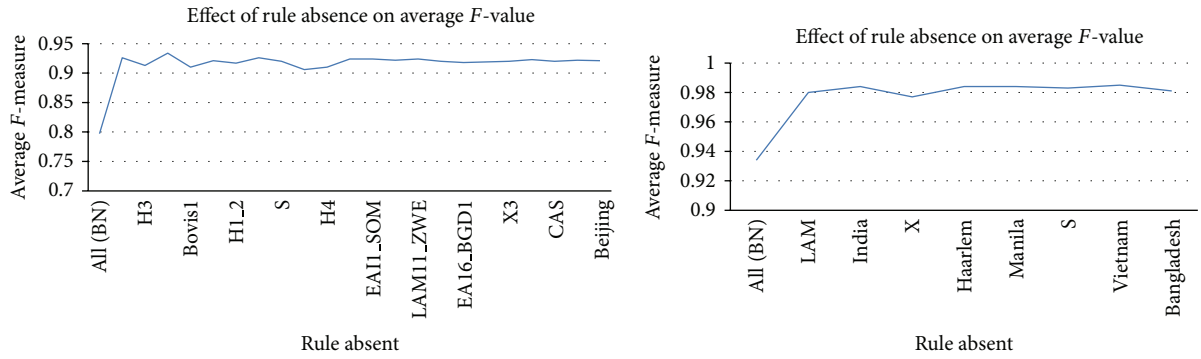
Our hypothesis was that KBBN can learn the concept faster with less data by adding rules. We wanted to show that rules can improve learning especially where you have less data. For each dataset, first we used 10-fold stratified cross validation. Next each training set was divided into 9 parts providing models using 1/9, 2/9,... or 9/9 of the training set and tested on the corresponding test set. The test sets were kept the same for different training set sizes. We measured the amount of F-value for different training set sizes with or without the rules and compared the result with the case of using no data at all (i.e., BN case) or only rules. The results are presented in Figure 3. Similar smaller testing set studies on CDC-Sublineage and SITVIT-CV found that KBBN always performs better than or as good as BN for all training set sizes.

To further examine the effect of incorporating rule sets and using incomplete rules, we performed two sets of experiments described in the following section: (1) using increasing percentages of the available rules and (2) using subsets of rules, removing rules for a given class at a time.

4.3.3. Removal of Rule Sets for a Class . In these experiments, we examined the effect of removing all the rules associated with a given class. We examined the KBBN accuracy and recorded the amount of average F-value between all classes after all the rules corresponding to a single class are removed. Again, 10-fold stratified cross validation was performed. The results are presented in Figure 4. "All (BN)" is when no rules are used in KBBN, which is equivalent to BN performance. Clearly, KBBN can lead to significant improvements compared to when no rules exist for entire classes of MTBC. We leave a more comprehensive study of when rules are most helpful for problems in other domains to future work.

5. Quality of Rules

KBBN can provide us with information about the quality of each rule. We studied posterior probabilities of rules given the class to provide insight into the utility and accuracy of each



(a) SITVIT-CV (b) CDC-Sublineage

Figure 4: Effect of removing rules for each class on the average F-value for (a) SITVIT-CV and (b) CDC-Sublineage.

Table 5: Posterior probability of each rule given class for CDC-Sublineage dataset. Blanks indicate 0.

	Class						
	Haarlem	LAM	S	X	India	Manila	Vietnam
Rule							
Haarlem	0.707				0.015		
LAM		1.000					
S			1.000		0.015	0.005	0.033
X				1.000	0.015		0.017
India					0.970	0.022	0.017
Manila						0.735	
Vietnam							0.283
No rule	0.297				0.015	0.243	0.700

rule. The $P(r \mid c)$ is of great interest because it tells us how good rule r is for a given class c. The posterior probability of the rules given the classes for the CDC-Sublineage data is presented in Table 5. The table includes a row for "No rule" indicating the probability of no rule getting fired. When no rule is fired a regular BN is used instead of KBBN. Note that the probabilities within columns may sum to more than 1 since rules are not mutually exclusive.

For CDC, the rule for LAM exactly corresponds to the class LAM on this data, since $P(\text{Rule} = \text{LAM} \mid \text{Class} = \text{LAM}) = 1$ and all other probabilities in the LAM row or column are 0. The rules for S and X correctly fire for their respective classes, but they also fire incorrectly for other lineages as indicated by the other entries in the S and X rows. The rules for Haarlem and Manila correctly predict their corresponding sublineages, but the fact that "No Rule" occurs 29.6% and 24.3% of the time, respectively, indicates that these rules fail to cover all members of their class. For the India class, the India rule is quite accurate, but the rules can be ambiguous as indicated by the multiple entries in the India column. Most Vietnams are not covered by any rules and for those that are covered the rules may be ambiguous.

We provide the posterior probability distribution of each rule given the sublineage for the SITVIT-CV dataset as a heat map in Figure 5. Good rules only have red on the diagonal. A rule fires for multiple classes if it has multiple red entries in a row. The rule set is ambiguous for a class if there are multiple red entries within a given class column. Notice that the rules that are fired for many classes with high probability

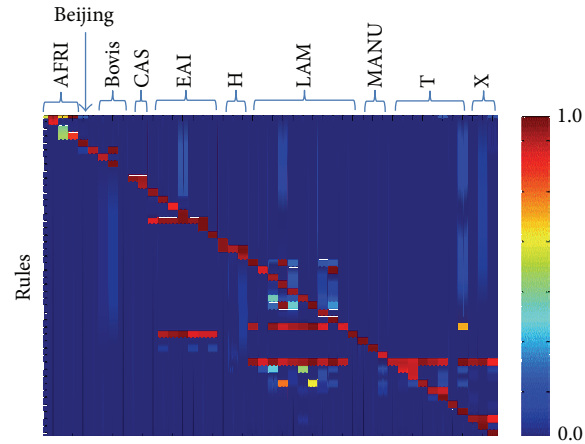


Figure 5: The heat map represents the posterior probability of each rule given the sublineage for the SITVIT dataset. A strong association of a rule in predicting a sublineage is shown with a red square while a blue square represents no relation. Here H includes URAL-1 and URAL-2 and LAM includes Turkey and Cameroon sublineages.

(e.g. T) are not very effective in indicating the associated class as opposed to Beijing which is an effective rule.

6. Discussion of Alternative Knowledge-Based Approaches

The KBBN has a great appeal over alternative knowledge-based approaches such as knowledge-based SVM (KBSVM) and knowledge-based neural networks (KBANN) [8, 9, 18]. The first advantage is that no special encodings of the rules are required. In KBANN, the rules are mapped into a neural network by converting the data to numeric form and designing appropriate nodes, links, and weights in the neural network. KBSVM requires each rule to be encoded as a polyhedral rule, such as *if* $x \in R^n$ *satisfies* $Bx \leq d$ *then class* $= 1$. In KBSVM, the process of converting rules to probabilities can greatly increase the number of rules. For example, for the task of predicting major lineages of MTBC, the 13 original logical rules published in a prior study [8] were mapped into 29 polyhedral rules. The added rules help capture the precedence of the original rules which made them mutually exclusive. There is no easy way to capture rule precedence in KBSVM or KBANN. KBSVM must add rules of the form "*if x satisfies condition A then x is not in Class y*." In KBBN, the data can be numeric or symbolic and each rule may be any arbitrary function of the observations to the classes. As reported in the preliminary study [9], KBBN works effectively on rules with and without precedence.

A second advantage of KBBN is the computational complexity of training. For the polytree type KBBN studied here, both training and inference can be done very efficiently in polynomial time. The MAP estimation of KBBN parameters has a closed form solution enabling globally optimal solutions to be found by simple counting algorithms. The only additional computation required over BN is representation of the probabilities of the classes given the rules. Unlike KBSVM and KBANN, no special purpose software is required for mapping and training KBBN beyond knowing which rules are fired for which example. KBSVM requires the solution of challenging nonconvex programs with many constraints and variables introduced for each rule over the original SVM. Similarly, KBANN also requires the solution of a nonconvex program of considerably greater complexity than the original ANN network due to the addition of weights and nodes to capture the rules. Special purpose software is needed to create the structure of the KBANN network, but any neural network training algorithm can be used to train it.

Additional benefits of KBBN over KBSVM and KBANN include that KBBN can be easily used for multiclass problems, it provides estimates of the posterior probabilities of the classes, and the resulting classification function is more transparent and explainable. KBSVM classification results published to date are limited to two-class problems and how to do efficient multiclass KBSVM remains an open research question. In both KBSVM and KBANN, the rules are used only to bias the construction of the prediction function and the prediction is typically a black box. In contrast, the KBBN probability density functions are readily interpretable as soft relaxations of the visual rules already used in TB. The posterior probabilities of the rules can be used to explain the effectiveness of these rules.

7. Conclusions

We have developed an effective classifier to predict SITVIT MTBC clades with high accuracy. The result is a publicly available web-based tool for SITVIT clade classification to support TB control and research efforts available for use at TB-INSIGHT (http://tbinsight.cs.rpi.edu/run_tb_lineage.html) and later on SITVIT2. We established that the clade estimates are robust by performing two out-of-sample testing experiments. Furthermore, the results on the two testbeds show that KBBN is a highly accurate classifier that can outperform methods based on rules or Bayesian networks trained on data alone and that meets or beats the performance of nonlinear and linear SVM models. KBBN proved to be robust to ambiguity, incompleteness, and inaccuracy of the rule set. The results here are limited to simple commonly used BN that are polytrees using MAP estimation, but future work is needed to examine the KBBN approach on more general BN models and algorithms.

As a general approach, KBBN has many attractive properties. It allows any type of rules to be incorporated into a Bayesian network with little increase in the model and training complexity. Prior knowledge-based SVM required manipulation of the rules, models, data, and/or kernel [8, 9]. There is no need to introduce precedence or resolve inconsistency of the rules for KBBN. The KBBN model can provide posterior class probabilities as well as information on how the rules were used and how classification decisions were made. We studied the posterior probability of rules given the class to provide insight into the utility of each rule. This underlines another advantage of KBBN as a generative model over its discriminative competitor models, like KBSVM. Thus KBBN offers a promising research direction for combining rule and data-driven predictive methods that may be applicable in many domains.

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

Experimental Inoculation of BFDV-Positive Budgerigars (*Melopsittacus undulatus*) with Two *Mycobacterium avium* subsp. *avium* Isolates

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Beak and feather disease virus- (BFDV-) positive (naturally infected) but clinically healthy budgerigars (*Melopsittacus undulatus*) were inoculated with two isolates of *Mycobacterium avium* subsp. *avium* isolated from naturally infected golden pheasant (*Chrysolophus pictus*) and peafowl (*Pavo cristatus*). During a period of more than two months after inoculation, samples of cloacal and crop swabs, faeces, and blood were obtained for BFDV and *Mycobacterium avium* testing with PCR. Birds were euthanized nine weeks after inoculation. All infected budgerigars developed signs typical of mycobacteriosis, but more advanced clinical and pathological changes were visible in the group infected with the pheasant isolate. Only a few cloacal and crop swab samples were positive for *Mycobacterium avium* subsp. *avium* despite advanced pathological changes in the internal organs. In the groups infected with mycobacterium isolates the frequency of BFDV-positive samples was higher than in the control group. In the infected groups the frequency of BFDV was substantially higher in the cloacal swabs of birds inoculated with the pheasant isolate than in the peafowl-isolate-infected group.

1. Introduction

Mycobacterioses in pet birds occur with constant prevalence which can be even more than 1% of total necropsy submissions [1]. They are mainly caused by *Mycobacterium avium* subsp. *avium* [2, 3] and *Mycobacterium genavense* [4, 5].

Mycobacterioses are often correlated with immunosuppression, which can be caused by viral agents [6]. In psittacine birds the most important immunosuppressive viral disease is psittacine beak and feather disease—PBFD—which is caused by the beak and feather disease circovirus (BFDV) [7–10]. Circovirus will selectively attack the thymus and bursa of Fabricius preventing lymphocyte production and severely impairing the bird's immune system. The younger the bird is infected the more severe the immunosuppression is. Normally birds develop the antibody diversity in the bursa Fabricius during first 3-6 weeks of their lives provided no infection had taken place before this period, otherwise an adequate immune system will be never established. These birds with a suppressed immune system due to PBFD will commonly suffer from a range of secondary infections [11].

A psittacine circoviral infection shows a relatively high degree of spread throughout parrot colonies. The virus was also detected in birds free of clinical signs [7–9].

We used BFDV-positive budgerigars for the experimental infection with *Mycobacterium avium* subsp. *avium* to check

relations as to the presence of these viral and mycobacterial pathogens in the crop and cloacal swabs as well as blood and faeces.

2. Material and Methods

2.1. Course of an Experiment. The authors obtained a positive opinion from the Local Ethics Commission (nr 39/2008), prior to using budgerigars in the experiment. A total of 18 of about-six-month-old budgerigars were used. Birds with no signs of illness were randomly assigned to experimental groups, each consisting of six individuals. The budgerigars were fed a commercial seed mix, Prestige Premium (Versele Laga, Belgium), supplemented with vitamin mixture Vinka (Beaphar, The Netherlands) and cuttlefish bone (Vadigran, Belgium). Parakeets were fed *ad libitum*.

Budgerigars were inoculated with *M. avium* subsp. *avium*. Two experimental groups were created. GroupPh was inoculated withmycobacteria isolated from a necropsied golden pheasant (*Chrysolophus pictus*) with advanced mycobacteriosis. The second group P was infected with *Mycobacterium avium* subsp. *avium* isolated from Indian peafowl (*Pavo cristatus*) with respiratory mycobacteriosis. Both *Mycobacterium avium* subsp. *avium* isolates were cultured on BBL Lowenstein-Jensen Medium + PACT (Becton Dickinson, USA) for 4 weeks in 37°C. The inoculum was administered into the pectoral muscles at a dose of about 5×10^5 colony-forming units/kg body weight [12]. Another six birds comprised the negative control group.

During the experimental period, body weight control and cloacal and crop swabs were obtained weekly and blood samples from the right jugular vein were obtained every second week. Swabs and blood samples were tested by QPCR. Before the start of experiment feather samples were tested for BFDV by PCR.

The birds were submitted to euthanasia 10 weeks after inoculation. Euthanasia was performed using pentobarbital sodium (Morbital Biowet Pulawy, Poland) intravenously. During necropsy, samples of the proventriculus, gizzard, intestine, pancreas, heart, lung, pectoral muscle, brain, kidney, and gonads were collected for histopathological examinations. Tissue samples were fixed in 10% buffered formalin. The fixed tissue samples were stained with haematoxylin and eosin stain or according to the Ziehl-Neelsen method.

2.2. DNA Extraction. DNA was extracted from crop and cloacal swab samples using the Swab 100 DNA extraction kit (A&A Biotechnology, Poland) according to the manufacturer's instructions with the exception of time of incubation, which was prolonged to two hours.DNA extraction from the feathers and blood was performed with 5% Chelex (Bio-Rad Laboratories, Canada) [13].

2.3. Primers. BFDV PCR was performed according to Katoh et al. [14]. A selection of specific *Mycobacterium avium* subsp. *avium* primers was supported by Bacon Designer software 7 (PREMIER Biosoft International, Canada) on *Mycobacterium avium* subsp. *avium* ATCC 25291 NZ_ACFI01000238 [15]. The chosen forward primer MAA-s (ACACCGTCAGCATCAAGG, Tm°C: 53.7) was located at nucleotides 489 to 506 *Mycobacterium avium* subsp. *avium*, and MAA-as (GAAGTTAGCGGAAATTCAAGC, Tm°C: 53.2) corresponded to nucleotides 588 to 608. BFDV in feather samples was detected with PCR according to Ypelaar et al. [16].

2.4. Positive Controls. The positive control for BFDV was DNA isolated from the feathers of parrots with clinical signs of PBFD. This test was considered only as qualitative. *M. avium* subsp. *avium* DNA was obtained from a pure culture of *Mycobacterium avium* subsp. *avium* using the Sherlock AX kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. The quantity of DNA was measured with NanoDrop (NanoDrop Technologies, USA) to ng/μL and recounted to the number of DNA copies with Stratagene Mx3005, MxPro QPCR Software, 2007.

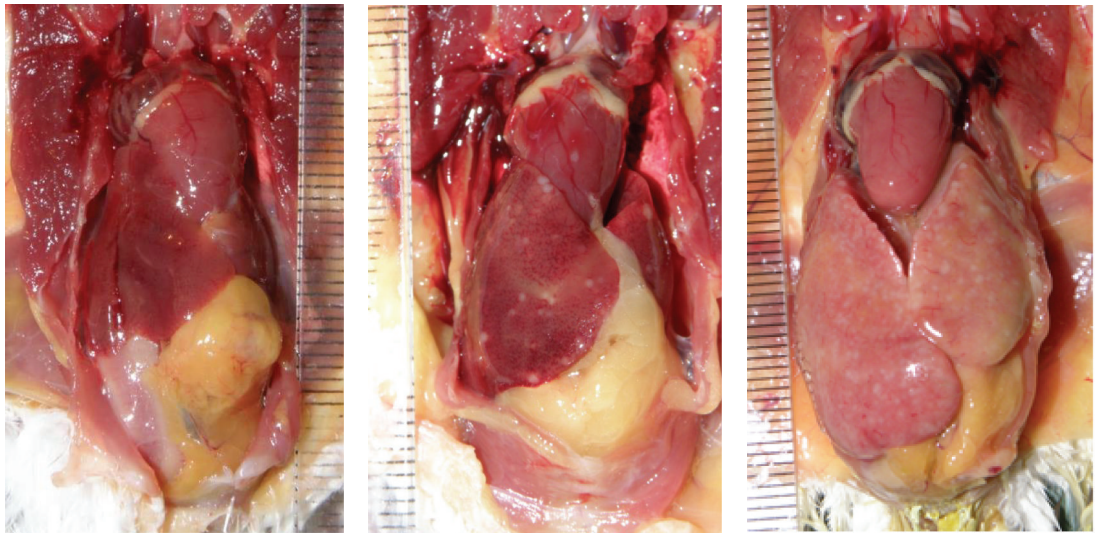
2.5. Real-Time PCR Assay. Real-time PCR amplification was carried out in a total volume of 25 μL using Brilliant SYBR Green QPCR Master Mix (Stratagene, Canada) containing 0.5 μL of each primer and 3 μL of the DNA template. The PCR was performed in the Stratagene Mx3005PTM cycler (Stratagene, Canada). BFDV reactions were performed according to published data [14]. *Mycobacterium avium* subsp. *avium* QPCR was performedwith the following protocol: initial denaturation for 10 min at 95°C followed by 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 60 s, and elongation at 72°C for 60 s. Fluorescence data were collected during the elongation step. After termination of the reaction by a final extension step at 72°C for 5 min, a DNA-melting curve was generated to verify the correct product by its specific melting temperature. Melting-curve analysis was performed by heating at 95°C for 1 min, followed by cooling to 55°C for 30 s and subsequent heating to 95°C for 30 s. For each real-time PCR reaction, software associated with the Stratagene Mx3005PTM system determined a threshold of the cycle number (C_t). The specific melting temperature value of the real-time product was about 76.8°C.

To determine the sensitivity of the real-time PCR assay, 11-fold serial dilutions of positive control DNA ranging from 2.86×10^{10} to 2.86×10^0 of DNA copies were tested. C_t value range was 19.7 (2.86×10^{10}) to 40 (2.86×10^1).

2.6. Statistics. General linear models with a binomial link function [17] were used to compare the three groups in terms of BFDV and *M. avium*. For the analysis, the repeated measures character of the data was ignored, and what was of interest to us was how many samples were positive out of all the samples for a particular bird. Multiple comparisons for generalized linear models [18] were used when the general hypothesis of the lack of a difference among the groups was rejected.

3. Results

3.1. Clinical Observations. Despite the partial lack of remiges in a few birds there was an absence of the clinical signs



(a) (b) (c)

Figure 1: Liver necropsy (groups: (a) control; (b) infected with *M. avium* subsp. *avium:* peafowl isolate; (c) infected with *M. avium* subsp. *avium:* pheasant isolate).

Table 1: Body mass analysis.

Group	Values mean
Pa	44.0[a1]
K	40.5[a]
Ph	40.3[a]

[1]The same letters in a column mean that birds within the three groups studied did not significantly differ in body mass ($P = 0.606$).

of PBFD disease. The first symptoms of mycobacteriosis appeared in group Ph (infected with the pheasant isolate of *M. avium*) 3 weeks after infection as moderate polyuria. Five weeks after infection a yellow discolouration of the ureates and in week 8 excessive polyuria with green ureates was observed. In week 9 diarrhoea appeared in one budgerigar and one week later this bird died. In group Pa the first clinical signs of yellow urine discolouration appeared in one budgerigar in week 9; polyuria and biliverdinuria in all Pa parakeets were present in week 10. Body mass in the three groups was compared by means of linear mixed-effects models [19] in which observations of body mass were nested within particular birds (Table 1).

3.2. Pathology. Typical advanced changes were observed in the liver (Figure 1) and spleen just as in the place of inoculation in all of the necropsied birds from the Ph group. Miliary abscesses were observed in the liver and marked hepatomegaly was observed in five birds.In birds infected with the peafowl originating isolate (Pagroup) the lesions were less prominent but typical of avian mycobacteriosis.

Prevalence and severity of typical of mycobacterioses histopathological changes are shown in Table 2. Other abnormalities also found in the control group were splenic white pulp proliferation and microvesicular steatosis. Splenic white pulp proliferation was observed in 4/6 of birds in the control group, 2/6 in the Pa group, and 2/6 in the Ph group; microvesicular steatosis was observed in 3/6 of the control group, 3/6 of the Pagroup, and 1/6 of the Phgroup.

Table 2: Prevalence of typical mycobacteriosis histopathological changes: g, granulomas; i, infiltration of granulocytes; if, inflammation; n, necrosis.

Organ	Group Ph	Group P	Control
Liver	6 (g)	4 (g) 1 (if)	—
Spleen	1 (g) 3 (i)	1 (i)	—
Proventriculus	1 (i)	—	—
Gizzard	—	—	—
Intestine	—	1 (if)	—
Pancreas	—	—	—
Heart	2 (i)	—	—
Lung	1 (if, n)	—	—
Pectoral muscle	5 (g) 1 (i)	5 (g)	—
Brain	—	—	—
Kidney	2 (i)	—	—
Gonads	—	—	—

All of the infected budgerigars developed changes typical of mycobacteriosis, but more advanced pathological changes were visible in the group infected with the pheasant isolate. Five out of 18 samples were BFDV negative, during the experiment, despite the fact that all of the birds were in the same room and the spread of the virus undoubtedly took place. One parakeet with negative feather samples was only once a blood-positive bird, in the Ph group; another bird from

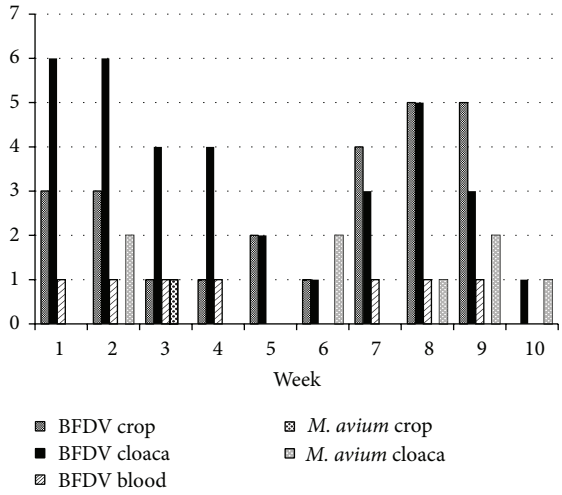


Figure 2: Frequency of mycobacteria and BFDV-positive samples in budgerigars infected with the peafowl isolate of *M. avium* subsp. *avium*.

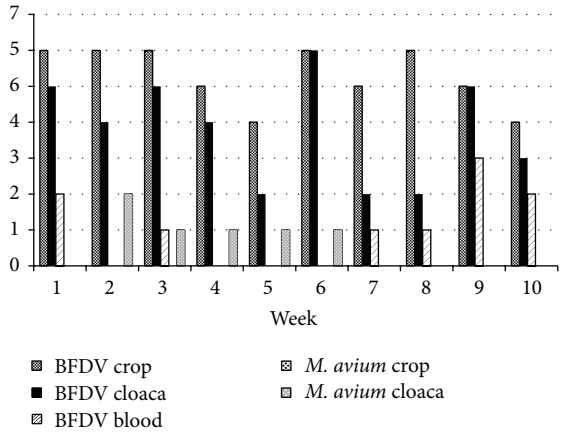


Figure 3: Frequency of mycobacteria and BFDV-positive samples in budgerigars infected with the pheasant isolate of *M. avium* subsp. *avium*.

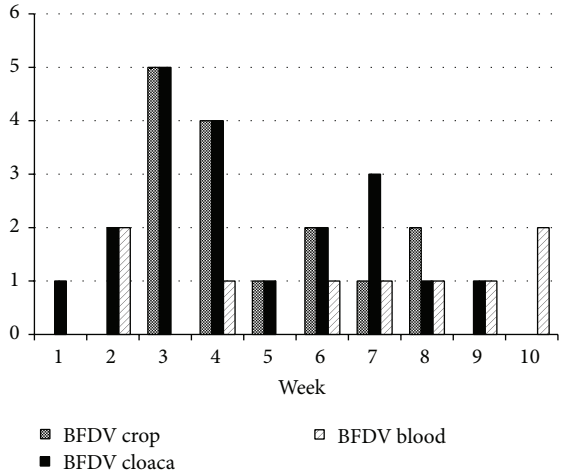


Figure 4: Frequency of mycobacteria and BFDV-positive samples in the control group of budgerigars.

Table 3: Occurrence of BFDV-positive samples.

Group	Crop swab	Cloacal swab	Blood
P	0.491[b1]	0.660[b]	0.333
Ph	0.900[c]	0.600[b]	0.267[a]
K	0.267[a]	0.333[a]	0.217[a]

[1]The different letters in the column represent a different mean share of the positive samples for birds within the two corresponding groups.

Table 4: Occurrence of *M. avium* positive samples.

Group	Crop swab	Cloacal swab
P	0.019[1]	0.154[b2]
Ph	0.000	0.136[b]
K	0.000	0.000[a]

[1]Crop swabs for *M. avium* were not analyzed because of too few positive samples in the experiment (for the two groups, K and Ph, *M. avium* was not detected at all, and for C it was detected in only a few samples).
[2]The different letters in the column represent a different mean share of the positive samples for birds within the two corresponding groups.

the control group was blood negative during the course of the experiment (Table 3).

For the two groups, K and Ph, M. *avium* was not detected at all, and for Pa it was detected in only a few samples (Table 4).

4. Discussion

In our previous experiment, published in 2008, budgerigars were infected with *Mycobacterium avium* subsp. *avium*, which caused no clinical or pathological changes typical of mycobacteriosis [12]. In the present study when we used the same amount of bacteria in the same environmental conditions, advanced mycobacteriosis was endangered. The most important factor was probably bacteria pathogenicity. Isolates used in the current study were isolated from lethal cases of spontaneous mycobacteriosis in gallinaceous birds and were used shortly after isolation from the tissues. Strain and isolate used in the previous study originated from the collection of the National Tuberculosis and Lung Diseases Research Institute in Warsaw or were isolated from the faeces of healthy birds. Another important reason was the subclinical viral infections in the budgerigars which affected their immunological system. However, the histopathology was not typical of circoviral infections. Microscopy of the spleen in some of the control as well as infected birds revealed the proliferation of white pulp, which can be consistent with the presence of a chronic inflammation. By contrast, circoviruses commonly cause lymphocyte depletion and spleen atrophy [20].

Yet circovirus shedding was correlated with mycobacterial infection (Figures 2, 3, and 4). Budgerigars inoculated with pheasant isolate of *Mycobacterium avium* subsp. *avium* were more frequently BFDV positive (Table 2) than with peafowl isolate (Table 3) and control group, respectively. Therefore an important finding is that a chronic bacterial infection depending on it severity can cause excess of viral

particles shedding. The research can also be used to evaluate QPCR for diagnostics of mycobacterioses in live birds. In human patients tuberculosis sputum samples are the most commonly examined [21], whereas in our research cloacal and crop swabs were tested. Only a few samples were positive despite the advanced pathological changes in the internal organs. Our previous study involving other species of mycobacteria cultures of faeces proved also unsatisfactory in terms of the anticipated number of positive samples [12]. Thus, cloacal and crop swabs do not constitute valuable material for diagnostics of *Mycobacterium avium* subsp. *avium* in budgerigars.

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

Comparison of Ligation-Mediated PCR Methods in Differentiation of *Mycobacterium tuberculosis* Strains

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Fast and inexpensive identification of epidemiological links between limited number of *Mycobacterium tuberculosis* strains is required to initially evaluate hospital outbreaks, laboratory crosscontaminations, and family or small community transmissions. The ligation-mediated PCR methods (LM-PCR) appear sufficiently discriminative and reproducible to be considered as a good candidate for such initial, epidemiological analysis. Here, we compared the discriminative power of the recently developed in our laboratory fast ligation amplification polymorphism (FLAP) method with fast ligation-mediated PCR (FLiP). Verification of the results was based on analyzing a set of reference strains and RFLP-IS*6110* typing. The HGDI value was very similar for both LM-PCR methods and RFLP-IS*6110* typing. However, only 52% of strains were correspondingly grouped by both FLiP and FLAP methods. Differentiation by FLAP method demonstrated a limited similarity to IS*6110*-RFLP (37,7%). As much as 78,7% of strains were grouped identically when differentiated by FLiP and IS*6110*-RFLP methods. The analysis differentiated 31, 35, and 36 groups when using FLAP, FLiP, and RFLP-IS*6110* methods, respectively.

1. Introduction

Recent development of molecular methods has substantially improved the identification of many bacterial pathogens, both at the species and strain levels. *M. tuberculosis*, the causative agent of tuberculosis, is still one of the most dangerous human pathogens causing high morbidity and mortality worldwide. The genetic typing of mycobacteria has greatly improved knowledge about tuberculosis epidemiology and enabled a molecular-guided control of the disease. Various genetic markers are used in molecular epidemiology of tuberculosis. In particular, identification of repeated sequences in mycobacterial genome and their analysis at molecular level allowed to develop the intraspecies discrimination methods for mycobacteria [1].

The current international standard for epidemiological typing of *M. tuberculosis* is restriction fragment length polymorphism (RFLP) based on the detection of variability in the number of copies and chromosomal locations of IS*6110* insertion sequences [2–5]. The second widely used method is mycobacterial interspersed repetitive unit-variable number of tandem repeats typing (MIRU-VNTR) based on variable number of tandem repeats [6]. Finally spoligotyping (spacer oligonucleotide typing) based on polymorphism in the chromosomal direct repeat (DR) locus is often used as a fast screening method [7]. Interesting alternative for the methods mentioned above are those based on ligation-mediated PCR (LM-PCR), which have proven useful in epidemiological analysis of a number of bacterial species [8–10]. Such methods can be adapted to mycobacterial typing when they are based on variability in IS*6110* flanking regions [11–15].

Here, we assess the usefulness of a recently described in our group LM-PCR method, termed fast ligation amplification polymorphism (FLAP), for differentiation of *M. tuberculosis* strains [15]. We present the results of its application in context of published results of reference set [14, 16] and compare its discriminatory power to that of IS*6110*-RFLP and FLiP (fast ligation-mediated PCR) methods.

2. Materials and Methods

2.1. Bacterial Strains. The 61 strains used in this analysis were obtained in 2006-2007 from patients hospitalized in the Center for Lung Diseases Treatment and Rehabilitation in Lodz, Poland. All strains were tested for susceptibility to isoniazid, rifampicin, pyrazinamide, streptomycin, and ethambutol using the Bactec 460 TB system (BD Diagnostic Systems, Sparks, MD, USA), as described previously [16]. This set of strains was previously characterized by IS*6110*-RFLP analysis, 15 locus MIRU-VNTR typing, and spoligotyping [16].

2.2. DNA Preparation. Genomic DNA was extracted and purified from all the isolates using the protocol by van Embden et al. [2]. The concentration of DNA was measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA).

2.3. The FLiP Method. The FLiP analysis was performed as originally described by Reisig et al. [13]. Briefly, the method is based on the ligation of oligonucleotide adaptors. Following restriction digestion, genomic DNA is ligated with an adapter composed of two oligonucleotides, one of which is complementary to the end created by restrictase, while the other contains uracil instead of thymine. A pair of starters is used for amplification; one of them is specific to the IS*6110* sequence and the other is complementary to the oligonucleotide ligated with restricted genomic DNA fragments. Amplification products are analyzed using electrophoresis; the obtained band patterns are strain specific.

2.4. The FLAP Method. The FLAP method was performed as we previously described [15]. Briefly, genomic DNA of *M. tuberculosis* strains was digested with *Pvu*II and *Sal*I restriction enzymes. The *Pvu*II endonuclease recognizes a single nucleotide sequence within IS*6110* and generates blunt ends. After the digestion step, oligonucleotide adaptors (36 and 40 nucleotides in length) are ligated to *Sal*I cohesive ends. All restriction fragments are used as templates for PCR amplification, with one primer complementary to adaptor sequence and the second primer complementary to the inner fragment of IS*6110*. The PCR products were separated on acrylamide gels and visualized by UV light illumination to generate the FLAP patterns.

2.5. Clustering and Computer Analysis. The fingerprint patterns obtained by both methods were analysed by using BioNumerics software, version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms were generated based on the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) algorithm for clustering and Dice similarity coefficient. The Hunter-Gaston discriminatory index (HGDI) was calculated as previously described and used to evaluate the discriminatory power of the typing methods [17].

3. Results and Discussion

The "gold standard" method for epidemiological typing of *M. tuberculosis* is IS*6110*-RFLP analysis providing the best resolution at the population level [2–5]. It seems obvious that appropriate epidemiological analyses of *M. tuberculosis* clinical strains should be based on more than one molecular method. Recently developed PCR-based genotyping methods are rapid, do not require a large quantity of purified DNA, and provide reproducible digital results. In particular, the relatively novel ligation-mediated PCR FLiP and FLAP typing methods seem to be promising alternatives for genotyping of *M. tuberculosis*, as well as for the detection of genotypic heterogeneity, mixed infection, and crosscontamination of mycobacterial samples [11–15].

In this study, we compared the results obtained by FLAP for 61 strains of *M. tuberculosis*, isolated from TB patients in 2006-2007, with both FLiP and IS*6110* RFLP methods, and estimated their discriminatory power by HGDI. All the methods are based on detection of insertion sequence IS*6110*.

The FLAP analysis subdivided the 61 analyzed strains into 31 clusters; 13 of which demonstrated unique patterns. The remaining 48 strains were grouped into two clusters of 5 strains each, three clusters of 4 strains each, and thirteen clusters of 2 strains each (Table 1).

The total of 35 FLiP patterns were detected and distributed in 14 clusters within 40 strains (65,6%) and 21 unique patterns (34,4%). One cluster consisted of 8 strains with identical FLiP pattern, three clusters contained 5, 4, and 3 strains, respectively, while eleven clusters comprised 2 strains each (Table 1).

The discriminatory power of the FLAP typing for the 61 *M. tuberculosis* isolates, calculated as HGDI, was 0.9757 compared to 0.9713 for FLiP method.

Previously performed analysis by reference methods, IS*6110*-RFLP and MIRU-VNTR typing, grouped this set of strains into 36, for IS*6110*-RFLP, and 27 patterns, for MIRU-VNTR, and gave the resolving power, 0.9743 and 0.9697, respectively [16].

We observed differences in the number of copies of IS*6110* when determined by reference method IS*6110* RFLP and LM-PCR methods (FLiP and FLAP). Therefore minimal and maximal number of bands in particular patterns were calculated. The copy number of IS*6110*, as determined by the reference method in each of 61 strains ranged from 6 to 14. The majority, 48 (78,7%) of the strains, contained 8–12 copies. FLAP patterns obtained for the same set of strains varied from 4 to 12, with 51 (83,6%) of strains containing 7–9 bands in pattern. In contrast, the number of IS*6110* in FLiP patterns varied from 3 to 8, and 49 (80,3%) of strains possessed 6–8 bands in pattern.

We found that FLAP patterns of 46 strains (75,4%) possessed more bands in particular than when analyzed by FLiP, 5 strains (8,2%) consisted of less bands, and 10 (16,4%) of strains contained the same number of bands in both methods. Next, we compared band patterns between FLAP and IS*6110* RFLP methods. 41 strains (67,2%) possessed less bands than when analyzed by IS*6110* RFLP typing; in eight patterns (13,1%) we observed more bands while 12 (19,7%) strains contained identical number of band patterns. Comparison of

Table 1: The comparison of 61 clustered strains by FLAP, IS*6110*-RFLP, and FLiP analysis.

	Strain	FLAP	IS*6110*-RFLP	FLiP
1	50/8	**F.1**	R.1	**1**
2	674/7		R.2	
3	149/8	**F.2**	R.3	**2**
4	146/7	**F.3**	R.4	**3**
5	319/7	F.4	R.5	4
6	19/7			
7	118/7		R.6	5
8	126/7			
9	147/8	F.5	R.7	6
10	54/8		R.8	7
11	102/7	**F.6**	R.9	
12	176/7			**8**
13	171/8	**F.7**	R.10	
14	412/7			**9**
15	129/7	**F.8**	R.11	
16	41/7			**10**
17	216/8	**F.9**	R.12	
18	307/7			**11**
19	165	**F.10**	R.13	**12**
20	218/8	**F.11**	R.14	**13**
21	230	**F.12**	R.15	
22	9/7			**14**
23	84	**F.13**	R.16	**15**
24	321	F.14	R.17	16
25	65/7	F.15		
26	632			
27	179/8	F.16		
28	386/7			
29	391/7			
30	611			
31	564			
32	152/7	F.17	R.18	17
33	690/7		R.19	18
34	34/7			
35	549/7		R.20	19
36	550			
37	567/7	F.18		
38	696			
39	232/8	**F.19**	R.21	**20**
40	571/7			
41	565	**F.20**	R.22	**21**
42	80/7	**F.21**	R.23	**22**
43	490/7	**F.22**	R.24	**23**
44	704	F.23		24
45	222		R.25	25
46	237/8	**F.24**	R.26	**26**
47	253			
48	1/7	**F.25**	R.27	**27**
49	10/7			
50	601			
51	120/7		R.28	
52	723	F.26	R.29	28
53	91/8		R.30	29
54	459	**F.27**	R.31	**30**
55	306/7	F.28	R.32	31
56	725			
57	724		R.33	
58	108/8		R.34	32
59	671	**F.29**	R.35	**33**
60	305/7	**F.30**	R.36	**34**
61	37/7	**F.31**		**35**

The identical clusters determined in both FLiP and FLAP methods are marked in bold.

patterns obtained by FLiP and IS*6110* RFLP typing revealed lower number of bands in 56 strains (91,8%) analyzed by FLIP when compared to IS*6110* RFLP, and 2 strains (3,3%) possessed higher number of bands, and 3 (4,9%) strains presented identical number of bands in these two methods. The intrinsically lower band numbers of FLiP patterns might contribute to slightly lower discriminatory power of this method in comparison to IS*6110* RFLP. The results indicate that the different number of bands in FLiP and FLAP DNA fingerprints may not necessarily reflect the number of IS*6110* copies in the strains tested. Moreover, the observed differences could be caused by technical difficulties, including mistakes in interpretation of visualized bands and insufficient separation of PCR fragments in poliacrylamide gel, or by difficulties of the PCR amplifying large products itself. It has been shown that PCR-based DNA fingerprinting patterns could result from nonspecific amplification of some of the products [13]. However, the analysis of identically clustered strains clearly showed the highest concordance for the FLiP and IS*6110* RFLP methods (48 strains, 78,7%). Both FLAP and FLiP methods allowed to group 32 strains (52,4%), whereas the lowest clustering identity was observed for FLAP and IS*6110* RFLP methods for 23 strains (37,7%).

4. Conclusions

In summary, the LM-PCR methods proved to be effective and reproducible for the differentiation of *M. tuberculosis* strains, showing high discriminatory power comparable to that of the IS*6110* RFLP. Based on the previous and present results, the LM-PCR methods seem to be a valuable alternative (highly discriminating, inexpensive, and very fast) to the widely applied and standardized IS*6110* RFLP method. However, the main limitation of the PCR-based methods, including LM-PCR, is the incapability in construction of reference database, especially containing strain patterns of isolates from different laboratories. Therefore, these methods might be rather used as a second-line test for verification of epidemiological links and could be valuable molecular epidemiology tools for analyzing collections with a limited number of strains. Nevertheless, based on our results, it is clear that it is necessary to apply more than one PCR-based method simultaneously.

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

Mycobacterium avium Subsp. *avium* Infection in Four Veal Calves: Differentiation from Intestinal Tuberculosis

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Mycobacterium avium subsp. *avium* (Maa) is an intracellular pathogen belonging to the *Mycobacterium avium-intracellulare* complex (MAC). Reservoirs of MAC are the natural environment, wildlife and domestic animals. In adult bovine, MAC infections are typically caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map). Maa infections in bovine are rarely reported but may cause clinical disease and pathological lesions similar to those observed in paratuberculosis or those induced by members of the *Mycobacterium tuberculosis* complex (MTBC). Therefore, differentiation of MAC from MTBC infection should be attempted, especially if unusual mycobacterial lesions are encountered. Four veal calves from a fattening farm dying with clinical signs of otitis media, fever, and weight loss were submitted for necropsy. Samples from affected organs were taken for histologic investigation, bacteriologic culture, and bacterial specification using PCR. Macroscopic thickening of the intestinal mucosa was induced by granulomatous enteritis and colitis. Intracytoplasmic acid-fast bacteria were detected by Ziehl-Neelsen stains and PCR revealed positive results for *Mycobacterium avium* subsp. *avium*. Clinical and pathological changes of Maa infection in veal calves had features of *Mycobacterium avium* subsp. *paratuberculosis* and the MTBC. Therefore, *Mycobacterium tuberculosis* complex infection should be considered in cases of granulomatous enteritis in calves.

1. Introduction

Mycobacterium (*M.*) *tuberculosis* and *M. bovis* are two important pathogens belonging to the *M. tuberculosis* complex (MTBC) causing human and bovine tuberculosis. Tuberculosis occurs spontaneously in cattle in developed countries and is endemic in Africa, Asia, and Central and South America with a herd infection rate of 10–35% [1]. Horses, small ruminants, swine, dogs, and cats can also be affected [1, 2]. Typical lesions are caseous granulomas mainly affecting the respiratory tract. In addition, enteric forms of tuberculosis with granulomatous changes and thickening of the intestinal mucosa also occur [1]. To prevent spread via the food chain, MTBC infections in food producing animals are classified as reportable diseases in many countries, and suspicious lesions in food animals must be investigated for the etiologic agent.

Nontuberculous bacteria of the *Mycobacterium avium-intracellulare* complex (MAC) most commonly cause enteric disease in animals [3]. Important members of this group are *M. avium* subsp. *paratuberculosis* (Map), the pathogenic agent of Johne's disease in ruminants that has also been isolated from human Crohn's disease patients [4], and *M. avium* subsp. *avium* (Maa). Maa is a primary pathogen in wild and domestic birds; mammals are however sporadically affected [5]. In humans, bacteria have been isolated from healthy individuals [6], but clinical disease may develop with immunosuppression [7]. MAC are widely distributed in the environment and have been isolated from soil, wastewater, water tanks, municipal water, aerosols, protozoa, deep litter, fresh tropical vegetation, animals, and humans [8]. Typically, MAC infections are characterized by granulomatous enteric lesions and intestinal lymphadenitis, but systemic disease

can also occur [1]. Granulomatous lesions in the intestine of adult bovine are typically caused by Map (paratuberculosis), but this disease is usually not encountered in calves [9]. Maa induced lesions in calves have mainly been reported in experimental infections and are an unusual differential diagnosis of enteritis in veal calves [10]. Because of the similarity of enteric lesions induced by MTBC, differentiation of unusual mycobacterial enteric lesions in animals requires identification of the causative agent.

In the present case report we describe pathological and microbiological findings in four severely affected calves with granulomatous lesions in the intestine and mesenteric lymph nodes similar to those seen in Map. Due to the young age of the animals and the paucity of bacteria in the lesion, an infection with Map was however unlikely and MTBC as an infectious agent could not be excluded. Bacterial specification using PCR revealed positive results for Maa, which so far has only rarely been reported as enteric pathogen in calves.

2. Materials and Methods

2.1. Animals and Antemortem Evaluation. Calves bought at 7 to 34 days of age from different farms in western and central Switzerland were fattened for approximately five months. During the first five to six weeks of the fattening period, they were initially housed in groups of five and later on in groups of 45 calves on deep litter with open-air area. Within the last 9 weeks before death, three calves were housed on deep litter without open-air area in groups of 26, and one calf was housed in a group of 44 calves in a pen with open-air area. Medical attendance has been taking place regularly by a private veterinary clinic (Kaelberpraxis, Rickenbach, Switzerland).

2.2. Clinical Signs. Upon arrival to the fattening farm, calves weighed between 43 and 69 kg. On day one every calf was vaccinated against bovine respiratory syncytial virus (BRSV) and parainfluenza-3 virus (PI-3) and received Vitamin E, selenium, and iron per os or by injection. The prophylactic antibiotic applications were either SK60 (chlortetracycline and spiramycin) or Amoxan 70 (amoxicillin) 10 days per os combined with an injection of Draxxin 10% (tulathromycin). Shortly after arrival one calf showed colic signs, which was treated with Buscopan (butylscopolamine) and ColoSan (Sterculiae gummi) per os. For reasons of otitis media, respiratory symptoms, and fever, the affected fattening groups including each of the presented calves were treated once or several times with antibiotics, depending on the severity of the symptoms (CAS 45K, Primadox 50, SK60, or Amoxan 70). Over time three of the four calves were treated individually with antibiotics and anti-inflammatory drugs because of clinical signs of otitis media and elevated temperature. After six weeks of fattening, calves weighed between 74 and 103 kg, and after two to four months one calf was euthanized due to poor health (otitis media, apathy, and fever) and three calves died without apparent clinical signs. All four animals were submitted for necropsy.

2.3. Postmortem Examination. Dead calves were transported to the Institute of Animal Pathology, Vetsuisse Faculty, University of Bern, where a complete necropsy was performed. Samples from major organs were fixed in 4% neutral buffered formalin, routinely processed for paraffin embedding, sectioned (4 μm), and stained with hematoxylin and eosin (H&E). Additionally, sections of mesenteric lymph nodes and intestine were stained with Ziehl-Neelsen (ZN) acid-fast stain.

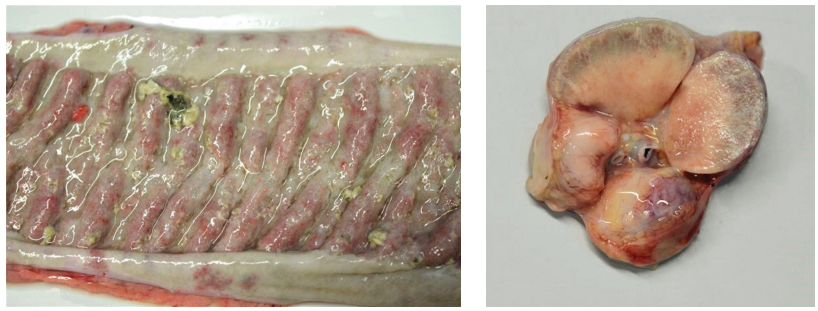
Microscopically, lesions were graded as nonaffected (−), mildly (+), moderately (++), or severely (+++) affected as previously described [11]. Numbers of acid-fast bacilli were scored − (no acid-fast organisms), + (1–5 acid-fast bacilli/10 400x fields), ++ (6–50 acid-fast bacilli/10 400x fields), and +++ (>50 bacilli/10 400x fields).

2.4. PCR. DNA from fresh tissue (small and large intestine) of two calves was extracted using "QIAamp cador Pathogen Mini Kit" (Qiagen, Hilden, Germany) and subjected to specific real-time PCRs for detection of mycobacteria of the *Mycobacterium tuberculosis* complex (artus *M. tuberculosis* TM PCR Kit, Qiagen) and *Mycobacterium avium* subsp. *paratuberculosis* (TaqVet*Mycobacterium paratuberculosis* Advanced Real-Time PCR Kit, LSI, Lissieu, France). Additionally, sequencing of the 16 S rDNA was performed as previously described [12].

3. Results

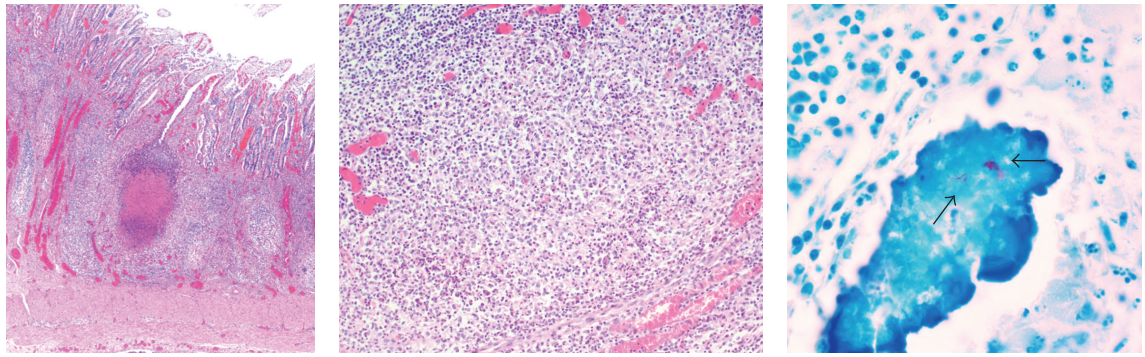
3.1. Macroscopical Findings. Macroscopic changes were present within the small (jejunum and ileum) and large intestine (caecum and colon) and adjacent mesenteric lymph nodes. There was mild to severe segmental thickening of the intestinal mucosa, in severe cases with prominent horizontal folds (Figure 1(a)). The thickened mucosa was hyperaemic, and in three calves it contained multiple nodules of about 3 mm in diameter, which were ulcerated and filled with necrotic material. The intestinal contents were green and slurry and became pasty in the colon and rectum. Jejunal and ileocaecal lymph nodes were enlarged and measured up to 10 cm in diameter. External and cut surfaces were evenly pale and contained multiple caseous or mineralized areas of few millimetres in one calf (Figure 1(b)). Cranioventral areas of both lungs of two calves were firm and dark red and on the cut surfaces dry, white, and sometimes filled with pus. The pleura was multifocally roughened and covered with fibrin. Secretion residue was present on the fur of the ears in one calf.

3.2. Microscopical Lesions. The main histological findings of the small and large intestine are summarized in Table 1. There were mild to severe, multifocal infiltrates of macrophages and neutrophils in mucosa and submucosa. Macrophages were often loosely arranged in aggregates. Villi in the affected areas were shortened or fused, and there were numerous ulcers and necrotic foci (Figure 2(a)). Additional small aggregates of lymphocytes, plasma cells, and eosinophilic granulocytes were present in mucosa and submucosa. In



(a) (b)

Figure 1: Macroscopic changes of intestine and lymph nodes. (a) Diffuse thickening of the ileal mucosa. (b) Lymphadenomegaly of ileocaecal lymph node.



(a) (b) (c)

Figure 2: Histologic changes of intestine and lymph nodes: (a) pyogranulomatous enteritis (H&E stain, magnification 40x). (b) Higher magnification of A (H&E, magnification 200x). (c) Mesenteric lymph node. Granuloma with central calcification and intralesional, acid-fast bacilli (arrows, Ziehl-Neelsen stain, magnification 1000x).

the cortex of affected lymph nodes multiple areas of granulomatous inflammation containing multinucleated giant cells, macrophages, and central necrosis or mineralization were evident. Ziehl-Neelsen stains revealed intracytoplasmic acid-fast bacteria in macrophages and multinucleated giant cells (Figure 2(c)), which were only rarely observed in some affected areas of two calves (+). A low to moderate amount (+/++) of acid-fast bacilli was detected in affected tissues of the other two calves (Table 1).

In two animals the liver showed random necrotic foci, which were infiltrated by macrophages and neutrophils. One calf showed similar lesions in the spleen. The lung of two calves affected by bronchopneumonia showed severe, acute to chronic, necrosuppurative bronchitis/bronchiolitis and a fibrinous pleuritis. No acid-fast organisms were found from liver, spleen, or lung.

3.3. Bacteriology. Bacterial cultures of the intestine were negative in three calves, and in one calf high (small intestine)/moderate (large intestine) amounts of *Escherichia coli* type F41 were isolated. *Mycoplasma bovis* and *Bibersteinia trehalosi* were isolated from the lung of one calf and *Pasteurella multocida* subsp. *multocida/septica* from the second calf affected by bronchopneumonia.

3.4. PCR. Real-time PCR for detection of DNA of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* subsp. *paratuberculosis* in intestinal and lymph node samples of two calves was negative.

Forward and reverse sequencing of the 16 S rDNA [12] followed by sequence comparison to the BLAST database revealed a sequence similarity of 99% for *Mycobacterium avium* subsp. *avium*.

Final diagnoses for the four calves were multifocal to coalescing, severe, and granulomatous enteritis and colitis. Additionally, two calves presented severe, acute/chronic active, necrosuppurative bronchopneumonia and fibrinous pleuritis, and one of those showed bilateral, acute, moderate to severe, purulent otitis media.

4. Discussion

Here, we report on four veal calves suffering from an unusual form of intestinal mycobacteriosis due to infection with Maa. Clinical signs were symptoms of otitis media, fever, and chronic weight loss, which represent common clinical complaints in veal calves. Bovines are prone to be infected by Map. Infection can take place under 30 days of age, but clinical disease does not develop until 2–5 years of age [9]. In contrast,

Table 1

Calf number	Granulomatous inflammation*					Necrosis*					Acid-fast organisms**				
	Jejunum	Ileum	Caecum	Colon	Lnn.	Jejunum	Ileum	Caecum	Colon	Lnn.	Jejunum	Ileum	Caecum	Colon	Lnn.
1	−	++	+	++	−	−	+	+	++	−	−	++	−	++	−
2	+	+++	−	−	+	−	++	−	−	+++	−	+	−	−	+
3	+	−	−	+	++	++	−	+	+	+	−	−	−	−	+
4	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+	++	++	+	+

*+: mild change; ++: moderate change; +++: severe change; −: negative.
**+: low number of acid-fast organisms; ++: moderate number of acid-fast organisms; +++: high number of acid-fast organisms.
Lnn.: Ileocaecal lymph node.

disease in these calves already developed at 1.5 to 2 months of age. Macroscopic changes in paratuberculosis are segmental thickening of the ileum, caecum, and proximal colon with multifocal ulceration due to granulomatous inflammation and usually numerous intracytoplasmic acid-fast bacteria in macrophages [13]. In the reported cases, similar lesions were present in the intestinal tract; however, the macroscopic and histopathological appearance of ulcerative and necrotizing lesions as well as the various amounts (moderate to small amounts) of acid-fast bacilli warranted differentiation of other mycobacteria. In particular the observation of small numbers of intralesional acid-fast bacilli can be suspicious for MTBC infection [14]. MTBC in bovine is a reportable disease, and entrance of zoonotic bacteria into the food chain has to be avoided. Therefore, microbiological investigation at the National Reference Laboratory for mycobacteria was initiated. Final diagnosis of Maa as the etiologic agent was achieved by PCR and 16 S rDNA sequencing, thereby excluding *Mycobacterium tuberculosis* complex infection in these veal calves. Because Maa infections in animals are not reportable in Switzerland, no further action was mandatory at the veal calf operation. Nevertheless, remaining animals in the affected groups were closely monitored for signs of diarrhoea or weight loss. After slaughter of the remaining calves 5 months later and at approximately 230 kg no intestinal lesions or enlarged lymph nodes were reported at meat inspection. The source of infection of these calves remained unknown. Maa are widely distributed throughout the environment and it is likely that most calves housed in this goup were exposed. The affected calves additionally had other diseases during the fattening period, such as bacterial bronchopneumonia or otitis, and thus might have been predisposed to develop additional enteric mycobacteriosis. In humans it is well documented that Maa infection mainly occurs in immunocompromised individuals [15]. However, mycobacteriosis in these calves may also have been predisposed to secondary infections.

In conclusion, Maa infection in young calves can mimic clinical and pathological signs of paratuberculosis and intestinal tuberculosis. Because the disease is rarely reported as a cause of diarrhoea in calves, enteric mycobacterioses in this age group might be underdiagnosed. In addition, affected animals are expected to shed high numbers of Maa within the feces. In order to differentiate Maa lesions from those of MTBC and to reduce the distribution of bacteria in food animals such as calves, it is important to identify suspicious animals and initiate molecular testing of affected tissues.

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

Assessment of the BD MGIT TBc Identification Test for the Detection of *Mycobacterium tuberculosis* Complex in a Network of Mycobacteriology Laboratories

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We evaluate the performance of the TBcID assay in a panel of 100 acid-fast bacilli cultures. Sixty-four isolates were TBcID positive for *Mycobacterium tuberculosis* complex (MTBC), whereas 36 gave negative results. These included 28 nontuberculous mycobacteria, one nonmycobacterial isolate, one *M. tuberculosis*, and six *M. bovis* BCG strains. This corresponds to a sensitivity of 90.14%, specificity of 100%, and positive and negative predictive values of 100% and 80.55%, respectively. The test is rapid, easy to perform and interpret, and does not require sample preparation or instrumentation. However, a negative result does not exclude the presence of a strain belonging to MTBC, especially when mutations in *mpb64* gene are present or some *M. bovis* BCG strains are isolated. The TBcID showed potential to assist in the identification of MTBC when the implementation and usage of molecular methods are often not possible, principally in resource-limited countries.

1. Introduction

The genus *Mycobacterium* comprises over 150 species of which more than 30 can cause disease in livestock, wildlife, and humans [1, 2]. Members of the *Mycobacterium tuberculosis* complex are the etiologic agents of tuberculosis and the responsible for about 8.6 million of new tuberculosis cases and 1.3 million deaths in 2012 [3]. Nontuberculous mycobacteria are a cause of opportunistic infections and are frequently encountered in clinical samples [1]. In some cases, clinical presentation of pulmonary disease caused by nontuberculous mycobacteria is very similar to that of tuberculosis [4]. Therefore, rapid diagnosis of patients with active tuberculosis is of major importance for the control of the disease demanding for reliable, easy to perform instrument-free identification assays, especially for low-income countries. Fast and accurate differentiation between *M. tuberculosis* complex and nontuberculous mycobacteria is essential, as it enables the implementation of appropriate measures to prevent the spread of the infection, allows the implementation of appropriate therapy, and prevents inappropriate drug susceptibility testing without species identification. Moreover, the differentiation of nontuberculous mycobacteria is important, since some of these species are resistant to most of the first line antibiotics used in tuberculosis therapy [4].

The conventional methods employed for the diagnosis of mycobacterial diseases rely on acid fast staining, culture, and phenotypic characterization. The development and implementation of liquid culture media allowed the reduction of the time for detection of positive cultures. From these, the BACTEC MGIT 960 is the most sensitive for recovery of mycobacteria from clinical samples [5]. After the positivity

of a culture, the verification of the presence or absence of acid fast bacilli in the culture can be achieved by means of microscopy. Nevertheless, it does not distinguish between *M. tuberculosis* and nontuberculous mycobacteria. Usually, most laboratories in resource-limited settings use the labor intensive standard biochemical tests to identify *M. tuberculosis* complex, which requires subculture of mycobacteria on solid media and delay the result by several weeks. This process increases the turnaround time for reporting positive results. Nucleic acid probe and amplification based methods have been used for the identification of mycobacteria from cultures or directly from clinical samples thus reducing the time for diagnosis [6, 7]. However, since molecular methods do not distinguish between live and death bacteria, culture confirmation is mandatory. Moreover, these techniques require specific equipment, expensive reagents requiring refrigeration, and highly trained personnel.

Lateral flow assays, also called immunochromatographic assays, have been developed for the discrimination between *M. tuberculosis* complex and nontuberculous mycobacteria. These include the SD Bioline Ag MPT64 Rapid assay (Standard Diagnostics, Kyonggi-do, Korea), Capilia TB (TAUNS, Numazu, Japan), and the MGIT TBc Identification Test (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD). These sandwich-type assays use a monoclonal antibody to detect the MPB64 protein (Rv1980c; also termed as MPT64), which is specifically secreted during growth by the *M. tuberculosis* complex [8]. The MPB64 is a 24 kDa protein, highly specific for the *M. tuberculosis* complex, except some variants of *Mycobacterium bovis* BCG [9, 10]. In this study we investigated the performance of the BD MGIT TBc identification test for the discrimination between *M. tuberculosis* complex and nontuberculous mycobacteria grown in both liquid and solid medium for the routine discrimination of *M. tuberculosis* complex in our setting and the reliability of the MPB64 protein for *M. tuberculosis* complex identification. This assay is intended to be used as a preliminary screening identification test when the hospital laboratories only perform acid-fast smear staining and culture and send their isolates to intermediate or reference laboratories for molecular identification at the species level and susceptibility testing of *M. tuberculosis*.

This study was carried out as part of the training programs in TB laboratory diagnosis for Mozambique and other Portuguese speaking countries, created and implemented to assess the usefulness of this assays for routine identification of *M. tuberculosis* in the network of hospital mycobacteriology laboratories in these countries as well as in Portugal.

2. Methods

2.1. Mycobacterial Strains and Culture. A total of 100 culture isolates received from seven hospitals of the Lisbon Health Region during the training period of three months were included in this study. These comprised 71 strains of the *M. tuberculosis* complex, 28 nontuberculous mycobacteria, and one acid-fast bacilli other than mycobacteria (Table 1). *M. tuberculosis* H37Rv and *M. bovis* BCG Pasteur strain were used as controls. As an intermediate level laboratory in the network of hospital mycobacteriology laboratories in Lisbon, the Mycobacteriology Laboratory of the IHMT/UNL only receives acid-fast smear positive cultures for molecular identification and drug susceptibility testing from the hospital laboratories. All isolates were processed for Ziehl-Neelsen staining and inoculated into MGIT culture tubes and Lowenstein-Jensen slants. Samples were digested and decontaminated, by the standard sodium hydroxide-N-acetyl-L-cysteine (NaLC-NaOH) method [11]. Once a positive signal was given by the BACTEC system, a Ziehl-Neelsen staining was performed and the presence or absence of serpentine cording morphology was observed. Only acid-fast bacilli positive cultures were included in the study. Presence of contamination was evaluated by inoculation of the cultures into blood agar plates and Ziehl-Neelsen staining.

Table 1: Identification of *M. tuberculosis* complex and differentiation from nontuberculous mycobacteria by the TBcID.

Mycobacterial species (n)	TBcID	
	Positive	Negative
M. tuberculosis complex (71)	64	7
Nontuberculous mycobacteria (28)	0	28
AFB high G+C bacteria (1)	0	1

AFB: acid-fast bacilli.

2.2. TBcID Assay. The TBcID assay (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD) consists of a nitrocellulose membrane on a test device with immobilized anti-MPB64 mouse monoclonal antibodies conjugated with gold colloidal for the detection of the MPB64 protein. In the presence of a sample, the antibody-colloidal gold conjugate binds to the MPB64 antigen and flows laterally through the membrane until the reaction zone. Here, the complex will be captured by a second antibody specific for MPB64. If the MPB64 protein is present, a purple band will be developed. Each TBcID device was inoculated with 100 μL of a positive MGIT culture. All cultures were tested with growth units above 100 (GU $\geq$ 100) and between day 1 and day 5 after positivity within the MGIT system. Selected *M. tuberculosis* isolates from the same collection were evaluated with cultures grown in Lowenstein-Jensen. For that, one loopful of colonies was suspended in 200 μL of extraction buffer (phosphate buffer with 0.05% Tween 20 and 0.02% sodium azide) and 100 μL of the suspension used in the assay. The results were interpreted 15 min after application of the sample. A positive result was indicated by the development of two purple bands, one in the control zone (C) and another in the test zone (T). The presence of the control band alone indicates a negative result.

2.3. Confirmatory Identification Tests. All the results obtained with TBcID were compared with Accuprobe MTBC culture identification test (GenProbe Inc., San Diego, CA) as the "gold standard" for the identification of the *M. tuberculosis* complex. Briefly, 1 mL of the each culture was centrifuged during 10 min at 13000 rpm and the pellet was used for hybridization according to the instructions of the manufacturer. The isolates that were negative for *M. tuberculosis*

Table 2: Comparison of the results obtained by the TBcID and the Accuprobe assay.

Mycobacterial species (*n*)	TBcID		Accuprobe	
	Positive (*n*)	Negative (*n*)	Positive (*n*)	Negative (*n*)
M. tuberculosis complex				
M. tuberculosis (64)	63	1	64	0
M. bovis BCG (6)	0	6	6	0
M. africanum (1)	1	0	1	0
Nontuberculous mycobacteria				
M. abscessus (2)	0	2	0	2
M. avium (4)	0	4	0	4
M. intracellulare (5)	0	5	0	5
M. chelonae (2)	0	2	0	2
M. fortuitum (1)	0	1	0	1
M. genavense (2)	0	2	0	2
M. gordonae (4)	0	4	0	4
M. kansasii (1)	0	1	0	1
M. marinum (2)	0	2	0	2
M. peregrinum (1)	0	1	0	1
M. scrofulaceum (1)	0	1	0	1
M. szulgai (1)	0	1	0	1
M. xenopi (1)	0	1	0	1
M. ulcerans (1)	0	1	0	1
Nonmycobacteria				
AFB high G+C bacteria (1)	0	1	0	1

BCG: Bacillus Calmette-Guérin. AFB: acid-fast bacilli.

complex by *Accu*probe were identified using the Genotype CM/AS (Hain, Nehren, Germany) according to manufacturer's instructions. Total genomic DNA for the Genotype CM/AS assays was extracted from the cultures using the QIAamp DNA mini kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions.

2.4. Assessment of Discordant Results. The isolates that were positive for *M. tuberculosis* complex with *Accu*probe but negative with the TBcID were tested using the Genotype MTBC (Hain) assay, according to manufacturer's instructions. Mutations in the *mpb64* gene of *M. tuberculosis* were analyzed by PCR amplification and DNA sequencing using the primers *mpb64*-F30 and *mpb64*-R433, *mpb64*-F404 and *mpb64*-R891, described elsewhere [12]. The reaction mixtures were prepared for a total reaction volume of 50 μL consisting of 1x Taq buffer (Fermentas, Ontario, Canada), 1.5 mM $MgCl_2$, 200 mM of each dNTP, 10 pmol of each primer, 1.5 U Taq DNA Polymerase (Fermentas), and 5 μL of chromosomal DNA. The PCR reactions were performed with the following amplification profile: initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min during 40 cycles. The final extension occurred at 72°C for 10 min. PCR products were sequenced with an ABI Prism 3130 capillary sequencer (Applied Biosystems, Foster City, CA) and the BigDye terminator kit (ABI Prism).

2.5. Performance Analysis. The sensitivity, specificity, and positive and negative predictive values of the TBcID assay were determined using the results of the Accuprobe MTBC culture identification test as the gold standard.

3. Results

The results obtained are summarized in Tables 1 and 2. Of the 100 mycobacterial cultures tested, 64 were correctly identified as *M. tuberculosis* complex by the TBcID. The assay failed to detect one *M. tuberculosis* isolate that carried a mutation in the *mpt64* gene and all *M. bovis* BCG tested ($n = 6$). We did not observe cross-reaction with any of the 28 nontuberculous mycobacteria and the one nonmycobacterial acid-fast bacilli culture tested. These correspond, in this study, to a sensitivity of 90.14%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 80.55% of the TBcID assay for the identification of the *M. tuberculosis* complex.

From the seven false negatives yielded by TBcID one corresponded to an *M. tuberculosis* strain as determined by Accuprobe. To evaluate if the negative result was due to a reduced amount of secreted MPB64 protein necessary for the detection, this isolate was subcultured and the test was repeated. The result was again negative. After this, the entire *mpb64* gene was sequenced and an insertion of two bases (CG) at position 335 of the gene was detected. This frame shift resulted in the generation of a premature stop codon at amino acid position 167, truncating the protein. The remaining six isolates were identified as *M. bovis* BCG by the Genotype MTBC assay and these isolates were later on found to belong to one child BCGitis and from patients that are being monitored for bladder cancer immunotherapy [13]. Testing by

(a) (b)

Figure 1: Identification of *M. tuberculosis* complex isolates by the MGIT TBc ID assay from (a) positive MGIT culture and (b) Lowenstein-Jensen slants. In the Figure it is shown the result of the TBcID for the same isolate when grown in different culture media. The positive result is indicated by the development of two purple bands, one in the control zone (C) and another in the test zone (T).

polymerase chain reaction with the four primers confirmed that the *mpb64* gene is absent in these strains.

The manufacturer's instructions of the TBcID system recommend its use with isolates grown in liquid media only. Nevertheless, we decided to test the capability of the test to detect MPB64 protein in strains grown on solid media. For that we selected 25 *M. tuberculosis* isolates from the panel of isolates grown in liquid media previously evaluated. The test demonstrates a good performance for detection of *M. tuberculosis* complex from solid cultures as all isolates were correctly identified as *M. tuberculosis* complex (Figure 1 and Table 3).

4. Discussion

In this work, the performance of the BD MGIT TBc identification test was evaluated for the identification of *M. tuberculosis* complex and differentiation from nontuberculous mycobacteria. We did not detect false positive results. However, the TBcID assay yielded seven false negative results. The assay demonstrates 100% of specificity which is similar to that published in other studies [14–16]. Nevertheless, the sensitivity was found to be 90.14%, which is lower than that reported by other authors (between 95.2 and 100%) [14–16]. This fact can be explained by the false negative results obtained.

The occurrence of false negatives can be due to the absence of the MPB64 protein or mutations in the coding gene, *mpb64*. Several studies have reported false negatives results for several BCG strains [14, 17, 18]. Among the *M. bovis* BCG variants, some of them do not produce the MPB64 antigen, whereas others are good secretors of this antigen [19]. This difference is due to the deletion of the *mpb64* gene together with the RD2 [20]. One of the strains included in this study was the BCG Pasteur, already described as a nonproducer of the MPB64 antigen [21, 22]. In Portugal, BCG SSI (strain 1331) is used for vaccination and BCG Medac is used for treatment of noninvasive urothelial bladder carcinoma and thus we assume that we have found both strains in our study, since one strain came from a child who developed osteomyelitis after BCG vaccination and five strains were isolated from patients undergoing cancer therapy [13]. Another possible explanation for the occurrence of negative results is the presence of mutations within the *mpb64* gene. Several mutations are reported in the literature for this gene [12, 15, 16, 18, 22–24]. In this study, we detected a GC insertion at nt 335, which resulted in a truncated protein and a TBcID negative result for this *M. tuberculosis* strain. As far as we know, this mutation has not been previously reported. Further, due to the existence of some strains of *M. tuberculosis* with delayed MPB64 secretion some cultures might lead to erroneous reporting of negative results. Vadwai et al. [25] propose that a culture must be tested with GU ≥ 300 to avoid false negative results. In our study, we did not detect false negative results using as cutoff MGIT cultures with GU ≥ 100.

Table 3: Results of the TBcID for a subset of 25 *M. tuberculosis* complex strains using different culture media.

Strain ID	TBcID result	
	MGIT	Lowenstein-Jensen
22	(+)	(+)
28	(+)	(+)
56	(+)	(+)
57	(+)	(+)
58	(+)	(+)
61	(+)	(+)
70	(+)	(+)
74	(+)	(+)
75	(+)	(+)
76	(+)	(+)
77	(+)	(+)
78	(+)	(+)
79	(+)	(+)
80	(+)	(+)
81	(+)	(+)
87	(+)	(+)
90	(+)	(+)
91	(+)	(+)
94	(+)	(+)
95	(+)	(+)
96	(+)	(+)
97	(+)	(+)
98	(+)	(+)
99	(+)	(+)
100	(+)	(+)
Total (25)	25	25

(+) positive result.

Differentiating *M. tuberculosis* complex from nontuberculous mycobacteria as soon as possible is important, mainly in situations in which nontuberculous mycobacteria strains represent a considerable portion of mycobacteria isolated [26]. The nontuberculous mycobacteria and one acid fast bacilli other than mycobacteria tested in this study correctly provided true negative results. Serpentine cord morphology can be used for rapid presumptive identification of *M. tuberculosis* in liquid culture and as a guide for the selection of auxiliary tests. Using this feature, we were able to improve the sensitivity of the test since the combination of the serpentine cording morphology with the TBcID comparing with AccuProbe assay as the gold standard corresponds to a sensitivity and specificity of 100% and identical positive and negative predictive values (data not shown). Similar results were reported by others [27]. The capacity of the TBcID was also evaluated for the identification of a subset of 25 *M. tuberculosis* strains grown on solid media, and all the 25 isolates were correctly identified as *M. tuberculosis* complex.

The main advantages of the test are the cost and rapidity. However, it has some disadvantages. The method (i) cannot be applied directly to clinical samples; (ii) does not allow the identification at species level; and (iii) requires further confirmatory tests for identification at species level. The MPB64 protein is the target of the test but it is also its weak point. All negative results suspected for *M. tuberculosis* complex had to be tested with other methodologies. Noteworthy, the isolation of *M. bovis* from human samples is uncommon, but sporadically, BCG strains are isolated from patients receiving BCG immunotherapy. The fact that all of our BCG strains are negative for MPTB64 can be useful to assist in the detection of BCG strains since it can be used as a screening tool in combination with molecular methods. Our results show that the TBcID is not an alternative to the Accuprobe system, at least in our work algorithm, due to the false negative results presented and this also precludes its use for confirmatory laboratory diagnosis of tuberculosis infections. Another limitation is related to the safety measures. The test necessitates biosafety level 3 conditions to be applied as it involves a considerable bacterial inoculum. When these conditions are not present, the test must be performed with heat-inactivated cultures [14].

The BD MGIT TBc identification test showed potential to assist in the identification of *M. tuberculosis* complex and differentiation from nontuberculous mycobacteria when the implementation and usage of molecular methods are often not possible, principally in resource-limited countries. The test is simple, rapid, easy to perform and interpret, and does not require sample preparation or instrumentation. In Mozambique, in 2012 the TB Reference Laboratories from Maputo and Beira have introduced the immunochromatographic assay, a very important step in a country for which very limited information regarding the occurrence of nontuberculous mycobacteria is available [28]. In 2012, among all positive cultures, 20.5% were from single or mixed infection from nontuberculous mycobacteria, a strong evidence of the high prevalence of nontuberculous mycobacteria in this country that are often misidentified and might be considered as multidrug resistant tuberculosis.

This study demonstrated the usefulness of the immunochromatographic assays for routine identification of *M. tuberculosis* in a network of mycobacteriology laboratories as preliminary screening identification test of cultures to be sent to the intermediate or reference laboratory as part of the network of TB laboratories of the national tuberculosis control programs.

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

Assessment of *Mycobacterium bovis* Deleted in *p27-p55* Virulence Operon as Candidate Vaccine against Tuberculosis in Animal Models

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A *Mycobacterium bovis* knockout in *p27-p55* operon was tested as an antituberculosis experimental vaccine in animal models. The mutant MbΔp27-p55 was significantly more attenuated in nude mice than its parental strain but more virulent than BCG Pasteur. Challenge experiments in mice and guinea pigs using *M. bovis* or *M. tuberculosis* strains showed similar protection conferred by MbΔp27-p55 mutant than BCG in terms of pathology and bacterial loads in spleen but lower protection than BCG in lungs. When tested in cattle, MbΔp27-p55 did not induce IL-2 expression and induced a very low production of IFNγ, suggesting that the lack of P27/P55 reduces the capacity of *M. bovis* of triggering an adequate Th1 response.

1. Introduction

Mycobacterium bovis (Mb), the causative agent of bovine tuberculosis (BTB), infects cattle and other animals, including humans [1]. Although vaccination of cattle may represent an intervention strategy to reduce the impact of BTB on livestock productivity and human health in the developing countries, to date there is no available vaccine against BTB.

The gene that encodes P27/LprG constitutes a virulence operon together with *p55* that encodes an efflux pump or transporter [2]. Although P27 induces Th1 immune response, in BALB/c mice, when administrated as vaccine with BCG produced an adverse effect [2]. Moreover, coadministration of P27 with *M. tuberculosis* aggravates the infection [2], suggesting that this protein plays a role in *M. tuberculosis* infection by inducing increased suppression of the immune response. In this study we investigated the capacity of a *M. bovis* strain knockout in *p27-p55* operon to induce protective immune response in cattle and to vaccinate mice and guinea pigs against infection with virulent *M. bovis* and *M. tuberculosis*, respectively.

2. Results and Discussion

2.1. Examination of MbΔp27-p55 Virulence in Nude Mice. In order to comply with the safety requirements for a live TB candidate vaccine we evaluated the virulence of the MbΔp27-p55 in immunodeficient mice. Nude mice (10 per group) were infected with 12,500 colony forming units (CFUs) of the wild type or MbΔp27-p55 strains, and survival was assessed. The median survival of wild type-infected mice (59 days) was statistically different ($P < 0.001$) to that of MbΔp27-p55-infected animals (109 days) (Figure 1). This result demonstrates that MbΔp27-p55 is attenuated in the absence of a T-cell adaptive immune response and therefore is a safe candidate to be tested as a TB vaccine.

2.2. Evaluation of MbΔp27-p55 as TB Vaccine Candidate in Animal Models. The experimental challenge model of progressive pulmonary tuberculosis was used in this study [3]. Groups of BALB/c mice (7 per group) were vaccinated subcutaneously in the base of the tail with 100,000 bacilli of either the MbΔp27-p55 mutant or BCG Pasteur. At 60 days

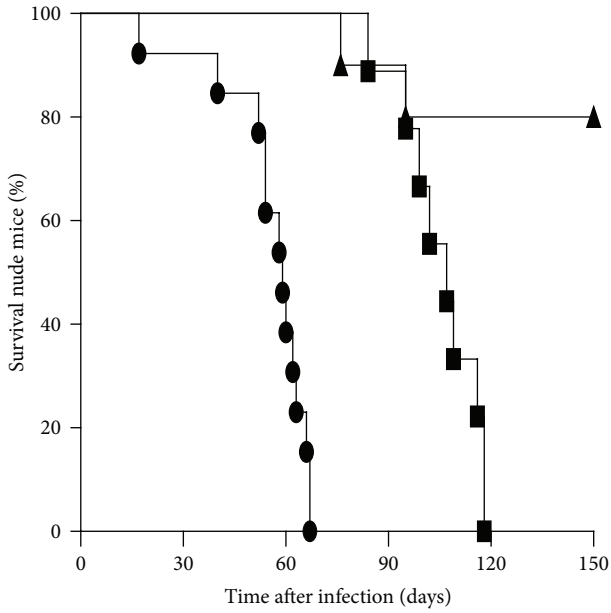


Figure 1: Survival of nude mice after intratracheal inoculation with 1.25×10^4 CFU of WT (circles), MbΔp27-p55 (squares), or BCG (triangles) bacteria. Statistical analysis for survival curves was performed using Mantel-Cox tests ($P < 0.001$).

after-vaccination, all mice were challenged intratracheally with 125,000 CFUs of a virulent *M. bovis* strain. Mice were then killed at 30 days after-challenge. Levels of protection were determined by evaluating the numbers of viable *M. bovis* strain bacilli recovered from lungs and spleen. The numbers of CFUs cultured from the organs of each group are shown in Figure 2(a). Mice vaccinated with either BCG or MbΔp27-p55 were protected compared to saline control ($P < 0.001$), in both lungs and spleen. However, in the lungs, the protection conferred by the mutant was statistically lower than that of BCG ($P < 0.05$).

Groups of 8 Dunkin-Hartley guinea pigs were used to evaluate the efficacy of MbΔp27-p55 compared with BCG Danish 1331 both delivered subcutaneously in a single dose at a concentration of 5×10^4 CFU. Twelve days after-immunization, animals were infected with a low aerosol dose of *M. tuberculosis* H37Rv. At 4 weeks after -challenge, guinea pigs were killed and organs were removed.

Protection was primarily assessed by measuring bacterial load in lungs and spleen and comparing the vaccinated groups of animals with the control group (saline). Guinea pigs vaccinated with either BCG or MbΔp27-p55 were protected compared to saline control ($P < 0.001$), in both lungs and spleen. However, in the lungs, the protection conferred by the mutant was statistically lower than that of BCG (Figure 2(b)).

A histopathological analysis of lungs and spleen lesions revealed that both vaccinated groups (MbΔp27-p55 and BCG) showed significantly reduced consolidation, foci of necrosis/caseation, and foci of calcification when compared with the unvaccinated group (Figure 2(c)). Again, guinea pigs vaccinated with BCG showed significantly reduced lung pathology when compared to animals vaccinated with the mutant strain.

2.3. Assessment of the Immune Responses Induced in Cattle after Inoculation of a M. bovis Strain Deleted in p27 and p55 Genes. In order to better understand the failure of MbΔp27-p55 to protect both mice and guinea pigs against tuberculosis, we used the cattle model to evaluate the immune response induced after infection with this mutant strain.

In peripheral blood monocyte cells (PBMCs) isolated 90 days after infection with the wild type strain, activation of CD4+ cells increased upon stimulation with PPDB ($P < 0.01$) (Figure 3(a)). In contrast, PBMCs isolated from animals infected with MbΔp27-p55 did not respond to specific stimulation with activation of CD4+ cells in any time point assayed.

We assessed the cytokine expression profile in PBMCs by measuring cytokine mRNAs after stimulating the cells with PPDB (Figure 3(b)). Values for sequential samples were normalized to values before inoculation for each animal. Given that there are available ELISA commercial assays to detect bovine IFNγ we used this methodology instead of quantification of IFNγ mRNA by RT-qPCR.

At 90 days after-infection (dpi), the expression of interleukin-2 (IL-2) in PBMCs was upregulated only in the group infected with the wild type strain, which is consistent with the CD4+ proliferative response, detected only in this animal group. Unexpectedly, only the group inoculated with the mutant responded to PPDB stimulation with production of IL-12 ($P < 0.05$), while the expression of TNFα was upregulated in both animal groups with no significant differences between them (Figure 3(b)).

The expression of IL-4, a Th2 cytokine, was downregulated in both groups (Figure 3(b)). This result is consistent with the low level of IFNγ detected at 90 dpi in both animal groups. It has been proposed that IL-4 is produced to compensate the inflammatory response induced by IFNγ.

At 120 dpi, the production of IFNγ in culture supernatant of PBMC stimulated with PPDB was significantly lower in the group inoculated with the MbΔp27-p55 mutant than in the group inoculated with the wild type strain. In fact, the group inoculated with the mutant strain produced very low quantities of IFNγ after PPDB stimulation throughout this study (Figure 3(c)).

Altogether, these results indicate that, although there is upregulation of IL-12 and TNFα, observed at 90 dpi, the lack of P27 and P25 in *M. bovis* reduces the capacity of the bacilli to induce a significant Th1 response when inoculated in cattle.

3. Conclusions

In this study we demonstrated that a *M. bovis* mutant in *p27-p55* operon did not confer better protection than BCG in both mice and guinea pigs. MbΔp27-p55 was more virulent than BCG in athymic mice, suggesting that its reduced protective capacity was not due to an inability to establish an infection. We found that the mutant induced in cattle the transcription of IL-12 and TNFα, two important Th1 cytokines. However, in contraposition, CD4+ cells from cattle inoculated with the

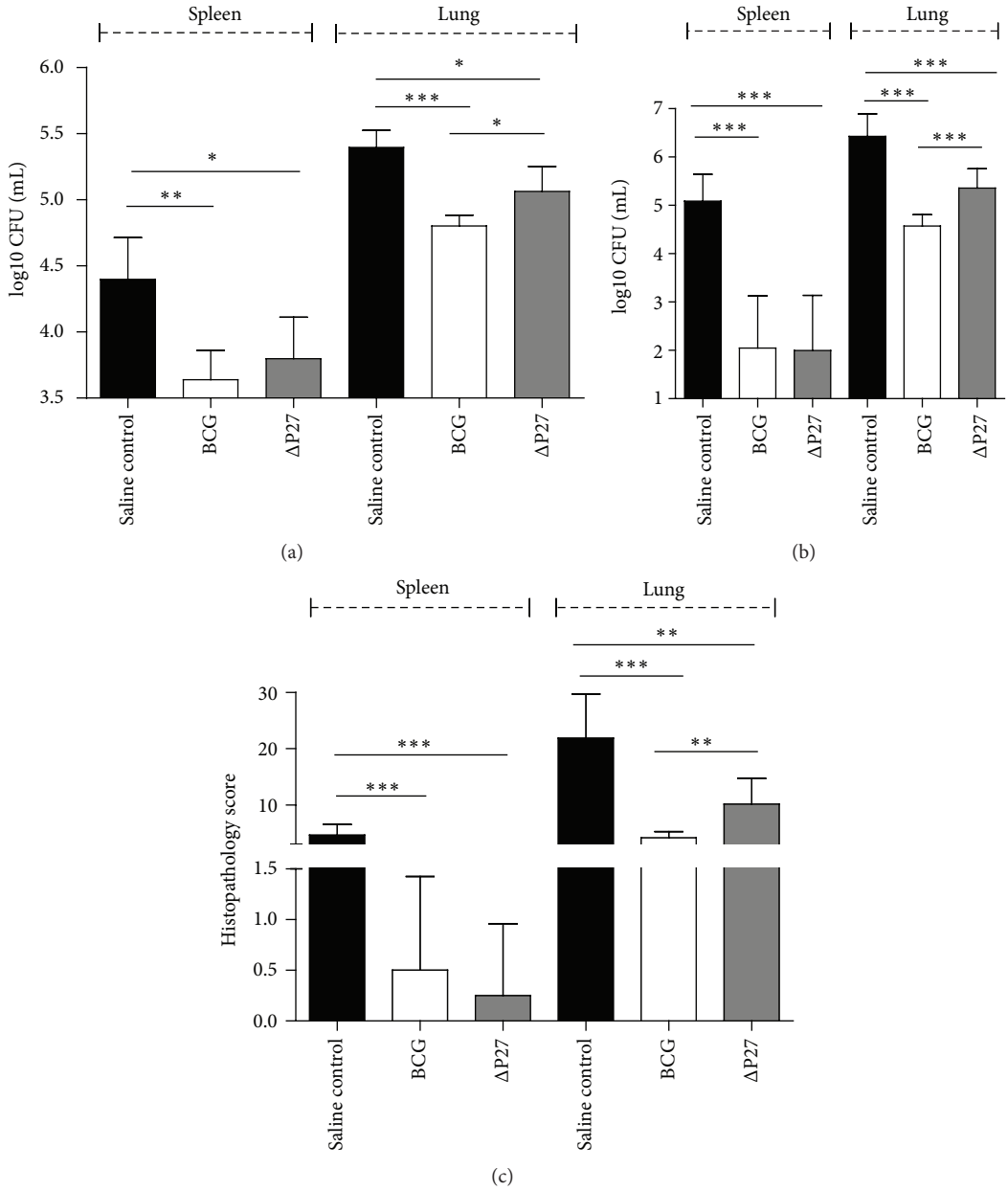


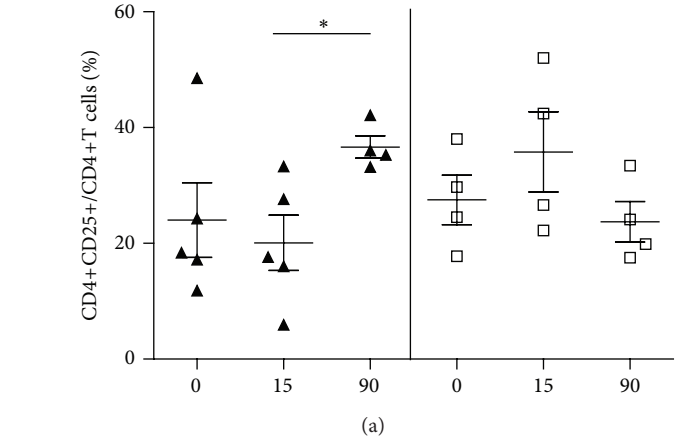
Figure 2: Protection assays in animal models. Organ bacillary loads after intratracheal challenge with *M. bovis* (a): BALB/c mice were vaccinated with the MbΔp27-p55 or BCG and compared with control nonvaccinated animals. CFUs were determined after 30 days of challenge. Organ bacillary loads (b) and histopathology (c) after aerosol challenge with *M. tuberculosis*: guinea pigs were vaccinated with the MbΔp27-p55 or BCG and compared with control nonvaccinated animals. Histopathology and CFUs were determined after 30 days of challenge. Data in (a) and (b) were analyzed using a two-tailed unpaired t-test, and data in (c) were analyzed using Mann-Whitney test ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

mutant did not proliferate in response to specific stimuli, and the production of IFNγ in blood was nearly undetectable in this animal group. Therefore, altogether these results suggest that the lack of *p27-p55* operon reduces the capacity of *M. bovis* to induce an adequate Th1 response, underlining the immunogenic properties of P27. In the light of the results of this study, a *M. bovis* deleted in *p55* virulence gene carrying an intact *p27* gene would be an attractive candidate to be tested as TB vaccine.

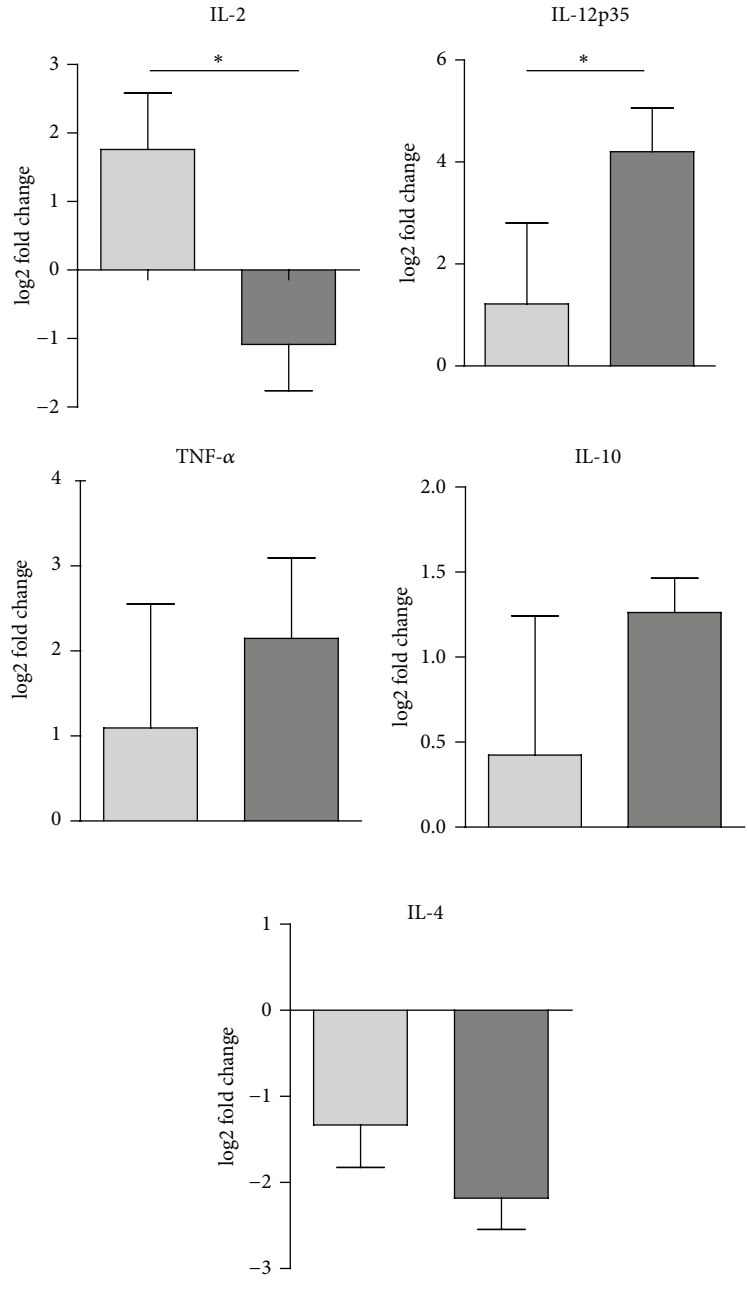
4. Materials and Methods

4.1. Mouse Vaccination and Infections. Groups of female nude (N:NIH (S)-*FoxnI*^{nu}) mice of 6–8 weeks old were used to assess the virulence of MbΔp27-p55 strain.

BALB/c mice aging 6–8 weeks old were used for vaccination and challenge experiments. *M. bovis* NCTC 10772 strain (the parental strain of mutant MbΔp27-p55) was used as challenge strain. This experiment was repeated twice.

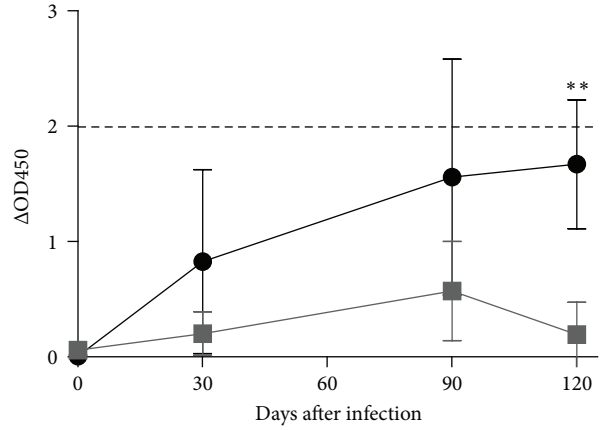


(a)



(b)

Figure 3: Continued.



(c)

Figure 3: Response of *M. bovis*-infected cattle to PPDB. (a) Percentages of the activated lymphocyte cell subsets CD4+ of PBMCs stimulated with PPDB from animals inoculated with MbΔp27-p55 ($N = 4$, white square) or NCTC 10772 ($N = 4$-5, black triangle) at 0, 15, and 90 days after infection. Data were analyzed using the Wilcoxon matched pair test for cells with and without PPDB stimulation (*statistically significant $P < 0.05$). The means ± SEM are indicated. (b) Relative cytokine gene expression. Gene expression was measured in PBMCs from animals infected with either MbΔp27-p55 ($N = 4$, gray bars) or NCTC 10772 ($N = 4$, dark gray bars) stimulated with PPDB at 90 dpi. Relative gene expression was calculated using the 2-ΔΔCt method with E correction, using *pol II* and *gadph* mRNA expression as reference genes and the preimmune condition as the calibrator. Data were analyzed using a two-tailed unpaired Student's *t*-test (*$P < 0.05$). The bars indicate the average ratios of infected animals/uninfected animals ± SEM. (c) IFN-γ release in response to *M. bovis* antigens PPDB in blood from animals inoculated with MbΔp27-p55 (gray squares), WT (black circles) at different time points (0, 30, 90, and 120 dpi). Results are expressed as ΔO.D. (OD450 PPDB − OD450 PBS). Significance was determined by Mann-Whitney test (**statistically significant $P < 0.01$).

Experiments with mice were performed in compliance with the regulations of Institutional Animal Care and Use Committee (CICUAE) of INTA. MbΔp27-p55 or BCG Pasteur were delivered subcutaneously in a single dose at a concentration of 1×10^5 CFU. 60 days after vaccination the animals were infected with $1,25 \times 10^5$ CFU of *M. bovis* by intratracheally instilation.

4.2. Guinea Pig Vaccination and Infection. Groups of 8 Dunkin-Hartley guinea pigs, weighing between 250 and 300 g (free of infection), obtained from a commercial supplier (Harlan, UK), were used to evaluate the efficacy of MbΔp27-p55 compared with BCG Danish 1331 (Statens Serum Institute, Copenhagen, Denmark), both delivered subcutaneously in a single dose at a concentration of 5×10^4 CFU, and a negative control unvaccinated group. Guinea pig experimental work was conducted according to UK Home Office legislation for animal experimentation and was approved by the local ethics committee.

Animals were infected with a low aerosol dose (10–50 CFU retained dose in the lung) of *M. tuberculosis* H37Rv [4] 12 weeks after vaccination. Nose only aerosol challenge was performed using a fully contained Henderson apparatus as previously described [5, 6] in conjunction with the AeroMP (Biaera) control unit [7]. The aerosol was generated from a water suspension containing 5×10^6 CFU/ml in order to obtain an estimated retained, inhaled dose of approximately 10–50 CFU/lung [7]. At 4 weeks after-challenge, guinea pigs were killed humanely by intraperitoneal injection of pentobarbital (Euthatal). Postmortem, lungs and spleens were taken and processed for bacteriology and histopathology analysis (subjective score), as described previously [8]. A significant reduction in CFU (bacterial load) and the nature and severity of the lesions (histopathology score) of vaccinated animals when compared with the control groups was considered a protective effect of the vaccine [9].

4.3. Cattle Infections and Immune Response Evaluations. Cattle infections were performed in compliance with the regulations of CICUAE and authorized by the National Service of Agricultural and Food Health and Quality (SENASA) and National Consultant Commission of Agricultural Biotechnology (CONABIA). Group of Holstein-Fresian calves (six months old) were inoculated intratracheally as described previously [10] with 10^4–10^5 CFUs of either *M. bovis* NCTC 10772 ($N = 4$-5) or MbΔp27-p55 ($N = 4$) and blood samples were taken at different points. After three months of infection, the calves were euthanized and then thin slices of lungs and lymph nodes of the head and pulmonary region were analyzed for granuloma formations. Only one of the animals inoculated with the wild type NCTC 10772 strain developed macroscopic lesions compatible with tuberculosis (data not shown). These lesions were located in retropharyngeal lymph nodes. No lesions were observed in animals inoculated with the mutant MbΔp27-p55. Flow cytometry determinations and cytokine expression analysis were performed as previously described [10]. IGRA Interferon Gamma (IFN-γ) release assays were performed on blood samples by using ELISA-based kit (Bovigam; Prionics) as previously described [10]. Duplicate samples for individual antigens were analyzed.

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

Current Methods in the Molecular Typing of *Mycobacterium tuberculosis* and Other Mycobacteria

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In the epidemiology of tuberculosis (TB) and nontuberculous mycobacterial (NTM) diseases, as in all infectious diseases, the key issue is to define the source of infection and to disclose its routes of transmission and dissemination in the environment. For this to be accomplished, the ability of discerning and tracking individual *Mycobacterium* strains is of critical importance. Molecular typing methods have greatly improved our understanding of the biology of mycobacteria and provide powerful tools to combat the diseases caused by these pathogens. The utility of various typing methods depends on the *Mycobacterium* species under investigation as well as on the research question. For tuberculosis, different methods have different roles in phylogenetic analyses and person-to-person transmission studies. In NTM diseases, most investigations involve the search for environmental sources or phylogenetic relationships. Here, too, the type of setting determines which methodology is most suitable. Within this review, we summarize currently available molecular methods for strain typing of *M. tuberculosis* and some NTM species, most commonly associated with human disease. For the various methods, technical practicalities as well as discriminatory power and accomplishments are reviewed.

1. Introduction

The genus *Mycobacterium* contains more than 140 species [1], which are separated in three major groups, that is, *M. tuberculosis* complex (MTBC), *M. leprae*, and mycobacteria other than MTBC and *M. leprae*, collectively referred to as nontuberculous mycobacteria (NTM). *Mycobacterium tuberculosis*, the most prominent member of the MTBC, is an obligate human pathogen and the causative agent of tuberculosis (TB), which remains one of the leading global public health problems. According to the World Health Organization (WHO), over 9 million new cases of TB occur each year, resulting in approximately 2 million deaths worldwide [2]. Conversely to *M. tuberculosis*, for which no environmental reservoir exists, NTM are ubiquitous organisms and are readily isolated from environmental sources, including soil and both natural and artificial water systems [3]. Despite reportedly low virulence of NTMs for immunocompetent human hosts, an increase in their isolation frequencies has been seen in the last decade, particularly in countries where TB incidence is on decrease [4, 5].

In the epidemiology of TB and other mycobacterioses, as in all infectious diseases, the key issue is to define the source of infection and to disclose its routes of transmission and

dissemination in the environment. For this to be accomplished, the ability of discerning and tracking individual *Mycobacterium* strains is of critical importance. The earliest discriminatory methods relied upon phenotypic characteristics, such as colony morphology, susceptibility to antituberculosis drugs, or mycobacterial phage typing. The usefulness of these methods is seriously limited by the phenotypic variability of mycobacteria. For instance, drug susceptibility patterns may change for the same isolate as it acquires resistance to specific drugs in the course of treatment. Otherwise, the limiting factor of phage typing is the low number of recognized mycobacteriophages [6–8]. Discrimination between the strains based on their biochemical and serological characteristics is even more unsuccessful [9, 10]. A turning point in this quest was the development of molecular biology tools in the mid 1980s. The DNA-based techniques have revolutionized the epidemiology of TB and other mycobacterial diseases, since they allow querying the whole genome that is unique and relatively stable for each strain.

In terms of genetic heterogeneity, the MTBC and NTM are vastly different and this has strong implications for the choice of typing methods and the achievable levels of discrimination.

Mycobacterium tuberculosis complex constitutes a remarkably genetically homogeneous group. This is perhaps best illustrated by the fact that, within the ribosomal DNA (rDNA) operon, not only genes encoding various types of rRNA but also regions between those genes, such as internal transcribed spacer (ITS) regions, which in many bacteria and fungi are highly polymorphic and thus are useful for identification and typing to subspecies or strain level, show complete conservation among members of the *M. tuberculosis* complex [11]. Likewise, many structural genes of *M. tuberculosis* complex show important sequence conservation, with an estimated rate of synonymous mutations of 0.01–0.03% [12–14]. Additionally, the lack of significant evidence for horizontal gene transfer between *M. tuberculosis* genomes speaks in favor of clonal evolution in *M. tuberculosis* complex [12–15]. This in turn renders strain-level discrimination of *M. tuberculosis* by means of molecular typing approaches challenging. However, recent studies have shown that the genetic diversity of the MTBC is much higher than previously assumed and that this genomic variance is attributed to the single nucleotide polymorphisms (SNPs), with potential impact on pathobiological phenotype [16, 17].

As for the NTM, the evolutionary time of divergence is believed to be much larger than that for the *M. tuberculosis* complex and this implies that less than a whole genome can be queried to obtain a highly discriminatory typing result [18].

Within this review, we summarize currently available molecular methods for strain typing of mycobacteria. For the various techniques, technical practicalities as well as discriminatory power and accomplishments are reviewed.

2. Restriction Site Analysis of Genomic DNA

First attempts of molecular typing of *M. tuberculosis* were based on restriction enzyme analysis of bacterial DNA (REA). In principle, chromosomal DNA of the analyzed strains is digested using various restriction enzymes and the resulting fragments are separated and visualized by gel electrophoresis. The obtained pattern of DNA fragments (*genetic fingerprint*) is characteristic for each strain. However, using the original procedure, the sensitivity of the method is rather limited due to technical difficulties in providing a high-resolution electrophoretic separation of fragments within a broad range of sizes. Also, when more restriction enzymes are used, the high number of DNA fragments makes a reliable analysis impossible [19]. Therefore, new methods have been proposed for a more accurate separation of DNA molecules, such as REA-PFGE (pulsed-field gel electrophoresis), which guarantees high resolution of the restriction patterns. More commonly used methods, which are modifications of the traditional REA, employ DNA hybridization assay in the process of specific pattern detection. Specifically, after electrophoresis, the separated DNA fragments are denatured in situ and transferred onto a membrane, which is then incubated with a radiolabeled probe (Southern blot) for the target sequence. Hybridization signals are visualized on autoradiography. The Southern blot technique was first applied to the analysis of restriction fragment length polymorphism (RFLP), which is hence a combination of REA and hybridization technology. Early studies utilizing RFLP methodology indicated that strains of *M. tuberculosis* display a considerably low degree of genetic diversity. However, such interpretation of the results was actually misleading since probes used in those studies targeted highly conserved regions and were of low specificity [20–22]. The resolution of the RFLP method increased substantially when insertion sequences (IS) were identified and used in probe construction [23, 24]. Different repetitive sequences found in the genomes of *M. tuberculosis* and NTM are an important source of genetic polymorphism and provide reliable markers allowing the determination of genetic relationships at both species and strain levels.

3. Pulsed-Field Gel Electrophoresis (PFGE)

A method related to REA, pulsed-field gel electrophoresis (PFGE), enables the separation of large DNA fragments, up to 10 Mb. In contrast, by using conventional electrophoresis, the threshold length exists at about 50 kb. The principle of the PFGE system is based on the application of an electric field that periodically changes its orientation across a gel matrix. This is achieved by varying the duration of the electrical pulse and shifting the direction of the current frequently. In general, the PFGE procedure involves digestion of chromosomal DNA with rare cutting restriction endonucleases, followed by agarose gel electrophoresis and analysis of the resolved electrophoretic patterns. A crucial step is the preparation of genomic DNA. Since large DNA molecules are prone to shearing and crushing, DNA is isolated in a gentle manner by first embedding a suspension of the organism in agarose plugs, lysing the cells in situ, and digesting the chromosomal DNA with restriction enzymes. The plugs are then loaded into the gel wells and sealed into place with agarose. After the electrophoresis, the resulting banding patterns are compared, using a predefined set of criteria for strain relatedness [25]. Although the PFGE patterns are well reproducible and the

overall discriminatory power of the method is high, a number of limitations are apparent. Firstly, the method is technically demanding and cost intensive. Secondly, it requires intact DNA for restriction enzyme treatment. Thirdly, the PFGE method has a long turn-over time, as the whole protocol usually takes not less than a week. Finally, no standardized procedure for performing PFGE has yet been recommended. Despite these disadvantages, the PFGE typing was successfully used to differentiate between strains of *M. tuberculosis* [26], *M. bovis* [27], and *M. bovis* BCG [28]. However, the PFGE typing is rarely used in *M. tuberculosis* complex due to technical, time, and cost considerations, as mentioned above. Moreover, PFGE analysis does not always generate sufficient discrimination between the strains [26, 29, 30].

Quite oppositely, PFGE often remains the most powerful typing system for nontuberculous mycobacteria. The method has been applied, with different degrees of success, to both slow-growing NTM species, including *M. kansasii* [31, 32], *M. avium-M. intracellulare* complex [33], *M. gordonae* [34], and *M. haemophilum* [35], and rapidly growing mycobacteria, such as *M. fortuitum* [36], *M. chelonae* [37], and *M. abscessus* [37, 38].

4. IS*6110*-RFLP Analysis

Study of the complete *M. tuberculosis* H_{37}Rv reference strain genome sequence revealed a relatively large amount of repetitive DNA elements [39]. Those elements vary in length, structure, and localization. Two main groups can be distinguished, that is, tandem repeats (TR) and interspersed repeats (IR). The first are short monomeric sequences (up to 100 bp) organized as head-to-tail arrays, whereas the latter are scattered as individual copies throughout the entire genome. An important class of IR sequences is insertion sequences (IS), which are mobile genetic elements.

The best known and investigated insertion sequence is IS*6110* first recognized by Thierry et al. in the early 1990s [40–43]. The IS*6110* sequence belongs to the IS*3* family and is a 1,355 bp long with unique 28 bp terminal inverted repeats (TIR). The region between those repeats includes two overlapping reading frames, *orfA* and *orfB*, encoding for a transposase, an enzyme responsible for transposition of the insertion sequence. The IS*6110* is found within the *M. tuberculosis* complex, and, in most members of the complex, the sequence is present at multiple copies, although *M. bovis* normally contains only one copy. In general, the copy number of IS*6110* ranges from 0 to 25 and depends on the frequency of transposition, which is largely conditioned by the nature of the genomic region at which transposition occurs [44]. Although the IS*6110* can be integrated into any place on the chromosome, there are regions with higher frequency of transposition. The so-called integration hot spots are usually located within coding regions of *M. tuberculosis* DNA [44, 45].

Differences in the copy number and locations within the genome, responsible for the high degree of IS*6110* polymorphism, have predisposed this sequence to be used as a specific molecular marker for genotyping of *M. tuberculosis* strains [46, 47].

IS*6110*-based typing is still among the most widely applied genotyping methods in molecular epidemiological studies of *M. tuberculosis*. The method includes digestion of genomic DNA with *Pvu*II restriction enzyme that cleaves the IS*6110* sequence only once, generating DNA fragments that are separated through gel electrophoresis, then transferred onto a membrane, and hybridized with a peroxidase-labelled probe complementary to part of the 3′-end of the IS*6110* sequence. As a result, every visualized fragment represents a single copy of IS*6110* surrounded by different in length flanking DNA (Figure 1(1)) [48]. Since the IS*6110*-RFLP methodology has been standardized and published, recommendations have been adopted by most of the research groups; the fingerprints generated in different laboratories can be compared and catalogued [49, 50]. The method is highly discriminatory and reproducible. An important characteristic of IS*6110*-RFLP typing is the stability of its profiles over time, allowing distinguishing epidemiologically related from unrelated isolates. Specifically, the half-time of change in IS*6110*-RFLP pattern was estimated to be *ca*. 3-4 years [51, 52]. Stability of the IS*6110*-RFLP patterns depends on the transposition process frequency; the more common is the transposition, the less stable is the number of the IS*6110* element in the genome. However, several important limitations exist for the IS*6110*-RFLP method. Firstly, there is a need for large amounts of high quality DNA (2 μg) for restriction enzyme digestion and therefore requires time consuming (up to several weeks) bacterial culturing. Secondly, the method is technically demanding and requires sophisticated and expensive computer software as well as experienced personnel of high technical expertise. Finally, the discriminatory power of IS*6110*-RFLP typing is insufficient for those strains whose copy number of IS*6110* is 6 or less (the so-called low-copy strains are seen among *M. bovis* isolates from cattle or *M. tuberculosis* isolates from Asia) [53–56]. In addition, some NTM have multiple copies of sequences that are homologous to IS*6110* and may thus hybridize with the IS*6110* probe [57].

Despite these limitations, the IS*6110*-RFLP method remains one of the most commonly used approaches for *M. tuberculosis* typing and was long considered the gold standard technique in the molecular epidemiological investigations of TB.

5. IS*6110*-Based PCR Fingerprinting

The IS*6110* is a target sequence in many methods currently used for molecular typing of *M. tuberculosis*. Among these, the most important is the mixed-linker PCR (ML-PCR) [58], ligation-mediated PCR (LM-PCR) [60], and fast ligation-mediated PCR (FliP) [59]. All these methods follow a similar four-step algorithm including genomic DNA fragmentation using restriction enzymes that generate protruding ends (i), ligation of those fragments with synthetic oligonucleotide linkers or adaptors (ii), amplification of the ligation products with one primer specific for the IS*6110* and a second primer complementary to a linker (iii), and analysis of the amplicons (iv).

In ML-PCR and FliP methods the products are 3′ fragments of IS*6110*, whereas in LM-PCR the IS*6110*-flanking

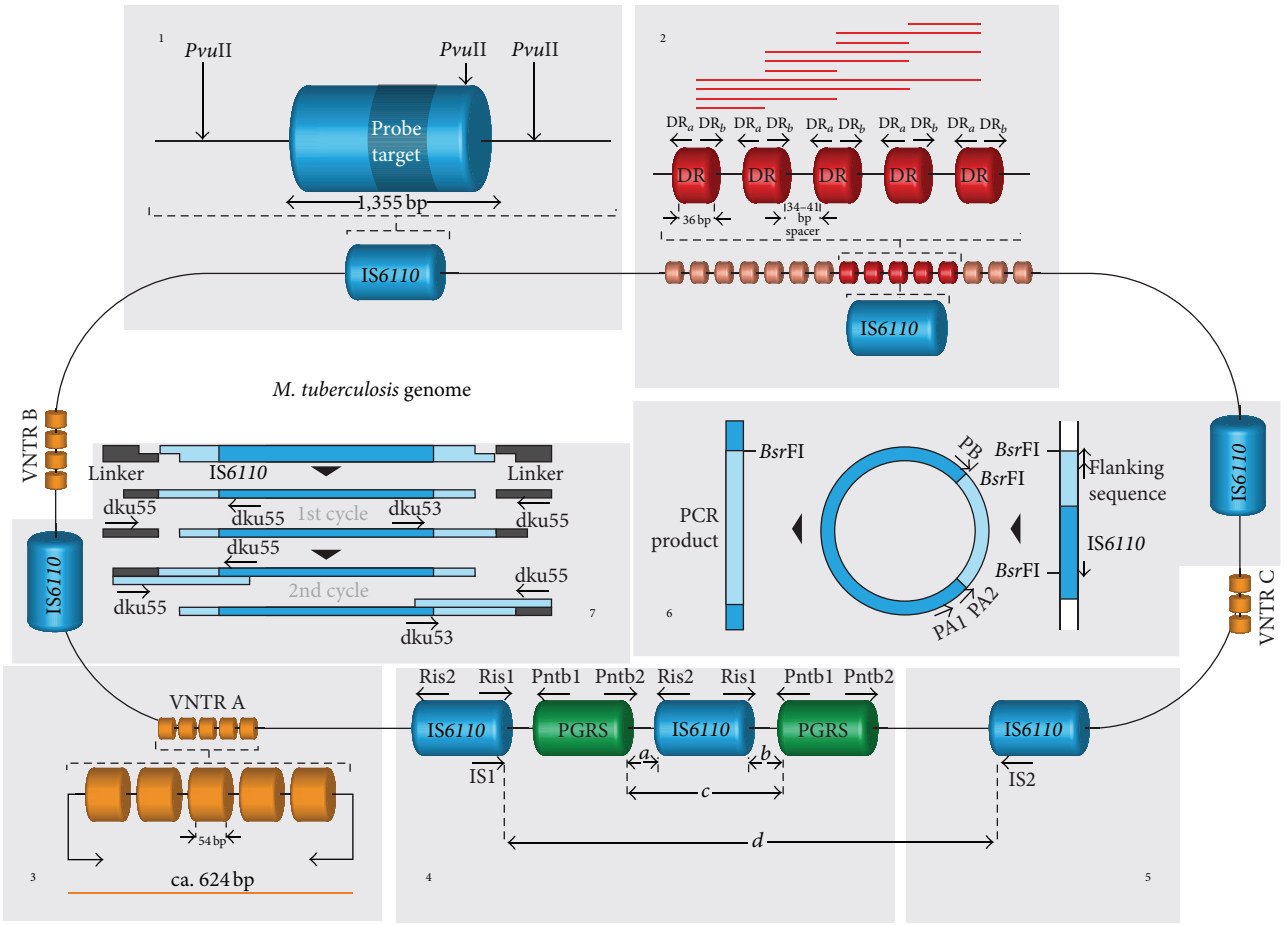

FIGURE 1: Schematic representation of the chromosome of a hypothetical *Mycobacterium tuberculosis* complex isolate with marked repetitive elements as targets for different typing methods. The principle of those methods is pictorially outlined. (1) In IS*6110*-RFLP typing, mycobacterial DNA is cleaved with the restriction endonuclease *Pvu*II, and the resulting fragments are separated electrophoretically on an agarose gel, transferred onto a nylon membrane by Southern blotting, and hybridized to a probe complementary to the 3′-end of the IS*6110* (probe target) yielding a characteristic banding pattern, in which every band represents a single IS*6110* element. (2) Spoligotyping relies upon PCR amplification of a single direct repeat (DR) locus which harbours 36 bp direct repeats interspersed with unique 34–41 bp spacer sequences. The PCR products (red horizontal lines) are hybridized to a membrane containing 43 oligonucleotides corresponding to the spacers from *M. tuberculosis* H_{37}Rv and *Mycobacterium bovis* BCG. The presence or absence of each of those 43 spacers in the DR region of the analysed isolate will be represented as the pattern of positive or negative hybridization signals. (3) The variable numbers of tandem repeat loci (VNTR) or mycobacterial interspersed repetitive units (MIRU) are PCR-amplified and the obtained products (yellow horizontal line) are sized on agarose gels to deduce the number of repeats in each individual locus. (4, 5) Two PCR-based typing methods, that is, double-repetitive-element PCR (DRE-PCR) and amplityping, are designed to amplify DNA between clusters of IS*6110* and polymorphic GC-rich sequences (PGRS) or between clusters of IS*6110* elements, respectively. Different distances between the repetitive elements and their different copy numbers result in variability of banding patterns, composed of DNA fragments amplified (*a*–*d*) and produced for individual isolates. (6) A heminested inverse PCR (HIP) depends on the amplification of the 5′-end of the IS*6110* sequence along with its upstream flanking sequence, bordered by the closest *Bsr*FI site. The size and number of PCR amplicons generated depend on the number of copies of IS*6110*. (7) Ligation-mediated PCR (LM-PCR) procedure allows, by introducing specifically designed linkers, amplifying the flanking sequences on both sides of the IS*6110* element. Names and positions of the PCR primers were excerpted from the original papers. For more details, read the text.

sequence on the 5′ side is amplified. (In the original LM-PCR procedure flanking sequences on both sides of the IS*6110* are amplified by using primers that are complementary to both termini of the IS*6110* sequence and one of the primers is also complementary to the shorter strand of the linker (Figure 1(7)) [68].)

All those methods are highly reliable and exhibit significant discriminatory potential, albeit slightly lower than that of IS*6110*-RFLP analysis [59, 69–71].

This potential can even be increased when using heminested inverse PCR (HIP). This method relies on amplification of a 5′ part of the IS*6110* and its flanking sequence up to the proximal *Bsr*FI restriction site [61]. Briefly, chromosomal DNA is cut with the restriction endonuclease *Bsr*FI and the restriction fragments are then self-ligated at low DNA concentration. The resulting circular molecules comprised of the 5′-end of the IS*6110* and its flanking region are subjected to PCR using primers that anneal to the IS at sites between its

5′-end and the closest *Bsr*FI site. Products of amplification vary in length depending on the length of the flanking sequence and are visualized by agarose gel electrophoresis (Figure 1(6)). The HIP method is highly reproducible and its discriminatory power is equivalent to that of standard IS*6110*-RFLP analysis but is much simpler and faster in performance [61, 72].

Another PCR-based IS*6110* typing method is amplityping. Here, outward-oriented primers hybridize with the ends of the IS*6110* sequence, so that DNA separating adjacent copies of this element on the genome is amplified (Figure 1(5)) [73–75]. A variant of the aforesaid method uses only a single primer, targeted to the terminal inverted repeat sequences of the IS*6110* [76, 77]. Differences in the length of the amplicons reflect the distance between the IS elements and are analysed by standard gel electrophoresis. An important limitation of this typing approach is that production of the PCR amplicons is dependent on the priming sites within the ISs being close enough for efficient PCR.

All PCR-based typing assays, targeting the IS*6110*, are easy-to-perform, time saving and require relatively small amounts of genomic DNA, which makes them applicable to nonviable organisms or directly to clinical specimens, without culture. The resolution of the IS*6110-PCR analysis may not be sufficient enough to differentiate among the isolates; however* when supplemented with an additional restriction digestion step, the differentiation capacity of the method is comparable to IS*6110*-RFLP [78]. *Apart from some technical difficulties, such as nonspecific priming, still* the major drawback of the methods discussed is the lack of discriminatory power for typing of the isolates with low copy numbers of IS*6110* [74–77].

6. IS-Based Typing of NTM

Since a number of different ISs have been described in various NTM species, these genetic mobile elements have widely been applied for strain-level typing.

Two closely related insertion sequences IS*1245* and IS*1311* were found very useful to differentiate within the *M. avium* complex.

Based on the IS*1245* or IS*1311*-RFLP patterns, various clades of *M. avium* and its subspecies could be distinguished, related to infections in birds, pigs, and humans [79–81].

Other insertion elements that are known and have been used for identification rather than typing purposes of *M. avium* complex bacilli include IS*900* present in *M. avium* subsp. *paratuberculosis* [82], IS*901* present in *M. avium* subsp. *avium* [83], IS*902* present in *M. avium* subsp. *silvaticum* [84], and IS*666*, IS*1110*, and IS*1626*, whose distribution among *M. avium* isolates has been ill-studied [85–87]. The insertion sequences: IS*900*, IS*901*, IS*902*, and IS*1245,* can be used for the identification of various *M. avium* subspecies as well as for differentiation within those subspecies.

One of the most important observations made upon IS*1245* RFLP typing was that birds are infected by a genetically highly conserved type of *M. avium* strains invariably revealing the same three-band pattern, while the banding patterns of *M. avium* isolates of porcine and human origin revealed highly variable and multibanded patterns. Consequently, it was proposed to reserve the naming *M. avium-avium* for the bird-type isolates and to introduce the designation *M. avium hominissuis* for typical isolates from humans and pigs [81].

Beyond the *M. avium* complex, RFLP typing has been pursued only sporadically. Yet potentially useful insertion sequences have been described in a variety of species. These include IS*1407* in *M. celatum* [88], IS*1395* in *M. xenopi* [89], IS*1511*/*1512* in *M. gordonae* [90], IS*2404* in *M. ulcerans*, IS*2606* in *M. ulcerans* and *M. lentiflavum* [91], IS*1652* in *M. kansasii* [92], and IS*6120* in *M. smegmatis* [93].

7. Spoligotyping

Spoligotyping is currently one of the most frequently used PCR-based approaches for studying the phylogeography of *M. tuberculosis* complex. The spoligotyping method is based on the polymorphism at one particular genomic region, the so-called direct repeat (DR) locus, initially identified by Hermans et al. in the vaccine strain *M. bovis* BCG P3 [94]. The DR locus comprises a series of well-conserved 36 bp direct repeats (DRs) interspersed with unique, nonrepetitive spacer sequences of 34–41 bp. The DR and the adjacent variable sequence form a direct variant repeat (DVR). The DR locus belongs to the clustered regularly interspaced short palindromic repeats (CRISPRs) family of repetitive DNA. It is postulated that these elements are reminiscent of centromere-like structures with a possible role in replication partitioning [95]. In the spoligotyping method, the entire DR locus is amplified by PCR, using two inversely oriented primers complementary to the sequence of short DRs. The PCR products, of different sizes, are hybridized to a membrane with 43 covalently bound synthetic oligonucleotides representing the polymorphic spacers identified in *M. tuberculosis* H_{37}Rv (spacers 1–19, 22–32, and 37–43) and *M. bovis* BCG (spacers 20-21 and 33–36). The hybridization signals are detected by chemiluminescence through biotin labeling of the PCR products (one of the primers is biotinylated) and a streptavidin-peroxidase conjugate system and then visualized by autoradiography (Figure 1(2)). Individual strains are differentiated by the number of the spacers that are missing from the complete 43-spacer set [62]. The lack of spacers is most probably the result of deletions mediated by various genetic mechanisms, such as homologous recombination or transposition (the DR region is a hot spot for IS*6110* integration) [96, 97].

Spoligotyping is a relatively simple, cost-effective, and high-throughput method, whose results are accurate and reproducible and are obtained in up to 2 days. The reliability of the results is linked to a high stability of the DR locus. The molecular clock of this genetic marker is believed to be very slow, since multiple *M. tuberculosis* isolates from the same patients corresponding to relapses or infections at different sites, even over time spans of several years, showed identical spoligotypes [98]. An important advantage of spoligotyping is its genuine sensitivity estimated at 10 fg of chromosomal DNA, equivalent to DNA from 2-3 bacterial cells [99], allowing the method to be applied directly in clinical samples, without the need for prior culture. Moreover, spoligotyping

has proven to be useful for typing on nonviable cultures, Ziehl-Neelsen smear slides, or paraffin-embedded tissue sections [100, 101].

Given the binary (present/absent) format of the data, the spoligotyping results can easily be interpreted, computerized, and compared between different laboratories [102].

In 2006, an international spoligotyping database (SpolDB4) was released. The database, which is accessible online (http://www.pasteur-guadeloupe.fr:8081/SITVIT-Demo/), describes 1,939 STs (shared types, i.e., spoligotype patterns shared by two or more isolates) and 3,370 orphan types (i.e., spoligotype patterns reported for only single isolates) from a total of 39,295 *M. tuberculosis* complex isolates, from 122 countries, classified temporarily into 62 clades/lineages [103]. In a recently erected publicly available multimarker database named SITVIT, a total of 7105 spoligotype patterns (corresponding to 58,180 clinical isolates)—grouped into 2740 shared types containing 53,816 clinical isolates and 4364 orphan patterns—were incorporated (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) [104].

Spoligotyping allows identification of *M. tuberculosis* complex isolates at the (sub)species level. For instance, the *M. tuberculosis* spoligotypes are characterized by the absence of spacers 33–36, whereas *M. bovis* spoligotypes usually lack spacers 39–43, and *M. bovis* BCG spoligotypes lack spacers 3, 9, and 16 [105]. Furthermore, spoligotyping allows identification of genotypes of significant, clinical, and epidemiological relevance. A clear example is the "Beijing" genotype, commonly encountered in the Beijing area, other regions of Asia, the former Soviet Union, and other geographical areas. Most of the "Beijing" genotype strains react only with the last 9 spacers (35–43) in the panel of 43 [105].

Spoligotyping has a lower level of discrimination than the IS*6110* RFLP typing, as evidenced in several studies [70, 106–110]. The introduction of 51 novel spacer sequences, mostly [45] from the DR region from the *M. canetti* genome only, slightly improved the resolution of the method [97]. The 68-spacer format, with 25 out of 51 new spacers, was shown to improve the discrimination for the *M. africanum* subspecies and for the East African-Indian (EAI) clade of *M. tuberculosis* [111, 112].

The reason for the limited discriminatory capacity of the spoligotyping method is due to the fact that it targets only a single genetic locus, covering less than 0.1% of the *M. tuberculosis* complex genome. Nevertheless, spoligotyping can be effectively used for the differentiation of *M. tuberculosis* strains with low IS*6110* copy numbers (≤5 bands in RFLP patterns) [54, 113]. As *M. tuberculosis* isolates with different spoligotypes invariably have distinct IS*6110* RFLP profiles, a genotyping strategy has been proposed, in which spoligotyping could be performed as a first-line, screening test, to be followed by another typing method of greater discriminatory power [108]. Spoligotyping, when used alone, is not sufficient for epidemiological linking studies. Furthermore, contaminated isolates and multistrain infections may not be detected by performing spoligotyping directly on clinical samples. However, this remark can be extended to any PCR-based technology, which is applied directly in clinical material.

Spoligotyping, when applied for nontuberculous mycobacteria, produced no signal, indicating the specificity of the method solely for *M. tuberculosis* complex [108].

Since the description of the spoligotyping method in its original form, another two formulations have been proposed. The first benefits from the Luminex technology, where the synthetic spacer oligonucleotide probes are immobilized on microspheres by means of covalent coupling, and detection is achieved via fluorochromes attached to the beads and hybridized PCR product. The Luminex platform, by eliminating the membrane step with the subjective manual data interpretation, provides, greater robustness and reproducibility. It is also well suited for high-throughput analysis, since it allows 96 isolates to be assayed simultaneously, as opposed to 45 isolates in a standard spoligotyping approach [112, 114].

A more recent alternative to a conventional spoligotyping scheme is a new multiplexed primer extension-based spoligotyping assay using automated matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Spoligotyping by MALDI-TOF MS improves the classical reverse line blot hybridization assay with respect to reproducibility, throughput, process flow, ease of use, and data analysis [115]. An important limitation of these innovative, technologically refined spoligotyping assays is that they require advanced and expensive equipment which many laboratories, especially those in resource-constrained settings, cannot afford.

8. Methods Based on Minisatellite Sequences

Mycobacterium tuberculosis was among the first bacterial species in which tandem repeat loci resembling minisatellite loci in eukaryotic genomes were identified. The mycobacterial tandemly repeated sequences were, per analogiam to those in humans and animals, called variable number of tandem repeat (VNTR) loci. However, as new VNTR-type loci have been discovered, they have been referred to under different names.

The first described VNTRs were major polymorphic tandem repeat (MPTR) and exact tandem repeat (ETR), found in 5 (A–E) and 6 (A–F) loci, respectively [63]. The MPTR consists of a 10 bp repeated sequence separated by unique 5 bp spacer sequences. These repetitive DNA elements were identified in *M. tuberculosis* complex as well as in other mycobacteria, including *M. gordonae*, *M. kansasii*, or *M. szulgai* [116]. The MPTR has been shown particularly useful in typing of *M. kansasii*. RFLP analysis with MPTR as a probe has revealed the existence of multiple subtypes within this species [92]. These subtypes have later been shown to have different degrees of pathogenicity in humans [117]. Interestingly, the MPTR sequences are part of the 3′-end of genes belonging to the PPE proteins (named after the conserved Pro-Pro-Glu (PPE) motifs near the N terminus of the molecule). The polymorphism of the PPE proteins in their C-terminal domains, linked to the presence of the MPTR motifs, is speculated to be the source of antigen variability in *M. tuberculosis* [118].

The ETR elements, found exclusively in *M. tuberculosis* complex strains, contain repeats ranging in size from 53 to

79 bp. Sequencing of the ETRs revealed that all of the ETR loci were variable. Contrastingly, among MPTR loci, only one (MPTR-A) showed some polymorphism, upon sequencing analysis [63]. Of eleven MPTR/ETR loci, only five (ETR-A–E) are routinely used for genotyping of *M. tuberculosis* strains. The principle of any typing system based on the polymorphism of VNTR loci is that each VNTR locus is PCR-amplified with specific primers complementary to the flanking regions, and the resulting PCR products are visualized by standard gel electrophoresis (Figure 1(3)). The number of tandem repeat units is determined by estimating the size of the amplicons, in relation to the known size of the repeat unit within the targeted VNTR locus. The results are expressed in a simple, digital format, in which each digit represents the number of copies at a particular locus [63]. Despite being fast, easy-to-perform, and highly reproducible, VNTR genotyping based on 5 ETR loci has a low discriminatory power, compared to IS*6110*-RFLP or spoligotyping [53, 70, 119, 120]. However, with the completion of the *M. tuberculosis* H_{37}Rv genome sequencing project, new VNTR-type loci have been identified. A specific class of these new VNTR elements is mycobacterial interspersed repetitive units (MIRUs). MIRUs were originally described by Supply et al. [121] as 46–101 bp tandem repeats scattered at 41 loci throughout the chromosome of *M. tuberculosis* H_{37}Rv. Based on the sequence analysis of each of those loci, 12 were found to display variations in tandem repeat copy numbers and were thus selected for genotyping of *M. tuberculosis* isolates [122, 123]. Among those 12 hypervariable loci, two (MIRU-4 and MIRU-31) are identical to formerly described ETR loci (i.e., ETR-D and ETR-E, resp.). MIRU-VNTR analysis, as every VNTR-based typing approach, involves PCR amplification of a specific MIRU locus, followed by determination of the sizes of the amplicons by gel electrophoresis or, after running multiplex PCRs, on an automated, fluorescence-based sequencer (Figure 1(3)). Since the length of the repeat units is known, the calculated sizes reflect the numbers of the amplified MIRU copies. The final result is a multidigit numerical code (the so-called MIRU-VNTR code), corresponding to the repeat number at each analyzed locus [64, 124]. This coding system allows the results to be readily compared across laboratories worldwide and facilitates the data to be deposited in the global databases via the Internet for large-scale epidemiological and population genetic studies [125, 126] (http://www.miru-vntrplus.org/). Recently, the biggest publicly available international database named SITVITWEB, which incorporates multimarker genotyping information (i.e., based on MIRU-VNTR typing and spoligotyping) on 62,582 *M. tuberculosis* complex clinical isolates from 153 countries of patient origin, has been released (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) [104]. Furthermore, a 12-locus MIRU scheme, based on the minimum spanning tress (MST) method, has been proposed for classification of *M. tuberculosis* complex genotypic lineages [127].

The MIRU-VNTR method is a reliable and efficient typing system, whose discriminatory capacity approximates or even exceeds that of IS*6110*-RFLP profiling. In general, the discriminatory power of MIRU-VNTR analysis increases with the number of loci evaluated. MIRU-VNTR typing based on 12 loci is slightly less discriminatory than IS*6110*-RFLP analysis for *M. tuberculosis* isolates with high copy number of IS*6110* [64, 123, 128, 129] but at the same time more discriminatory than the IS*6110*-RFLP if low-copy-number IS*6110* isolates are investigated [130, 131]. In principle, 12-locus MIRU-VNTR cannot be used as a sole typing method, as it may overestimate the number of true epidemiological links, especially in large, population-based studies [132, 133]. Consequently, it is suggested to use 12-locus MIRU-VNTR analysis in combination with other genotyping methods [128, 132]. An alternative way is to increase its resolution by expanding the investigation on other polymorphic VNTR loci.

The observed heterogeneity of the VNTR domains (they are still being discovered in the tubercle bacilli genome) provides great flexibility in designing new marker combinations that would enhance the discriminatory capacity of the genotyping method. In 2006, a new system employing 24 MIRU-VNTR loci (including 12 previously investigated) has been proposed [124]. Noteworthy, 15 (including 6 previously investigated) of those 24 loci account for 96% of all detected polymorphisms in *M. tuberculosis* strains. The discriminatory power of this new, 24-locus MIRU-VNTR typing system equals that of IS*6110*-RFLP profiling [125, 134, 135]. This has rendered 24-locus MIRU-VNTR typing the new gold standard in molecular typing of *M. tuberculosis* complex bacteria. Next to the proposed standardized MIRU-VNTR 15- or 24-loci sets, the use of another three loci, the so-called hypervariable loci (i.e., VNTRs 3232, 3820, and 4120), is recommended as a second-line typing step, particularly to differentiate Beijing genotype strains.

Overall, genotyping based on minisatellite sequences is a rapid, sensitive, and highly discriminating approach, which makes it well suited for large-scale investigations. A particular advantage of the VNTR genotyping, compared to the IS*6110*-RFLP typing, is its portability due to digitalization of the generated patterns and therefore simple intra- and interlaboratory comparability as well as the amenability to inclusion in web-based databases. Reproducibility of the method was expected to be good, due to the genetic stability of the targeted loci; yet, in the first worldwide proficiency study, both intra- and interlaboratory reproducibility proved to be suboptimal. Further harmonization of the laboratory methodology is still needed [136].

The occurrence of VNTR loci in the genomes of non-tuberculous mycobacteria remains largely obscure. Eight MIRU-VNTR-type loci have recently been described by Thibault et al. in *Mycobacterium avium* and *M. avium* subsp. *paratuberculosis* [137]. In Japan, a refined VNTR typing method using the *M. avium* tandem repeat (MATR) loci (MATR-VNTR) has been employed [138]. In *M. intracellulare* Dauchy et al. identified 45 potential MIRU-VNTR loci, 7 of which showed enough variability to be used as strain-level discriminatory markers [139]. The discriminatory power of the VNTR typing assays, based on the newly discovered loci in both species, was considered promising [80, 137, 139]. Recently, 13 VNTR loci have been described and applied to confirm clonal relationships between patient and environmental isolates of

M. ulcerans [140]. Twelve VNTR loci have been defined and tested against *M. marinum* isolates [141].

9. Methods Based on GC-Rich Sequences

The genome of *M. tuberculosis* has a particularly high GC content (>65.5%). The polymorphic GC-rich repetitive sequences (PGRS) are the most abundant type of repetitive DNA in the genome of *M. tuberculosis* complex. The PGRS elements occur at multiple loci and consist of several repeats of a 9 bp consensus sequence (5′-CGGCGGCAA-3′), tandemly arranged in up to 1.5 kb segments. Although the PGRS were initially identified in *M. tuberculosis* complex, they are now known to be present in other mycobacterial species, such as *M. kansasii*, *M. gastri*, and *M. szulgai* [142]. The PGRS bear important resemblance to the aforedescribed MPTRs. The similarities between those repetitive elements include host range, structure, genetic stability, and copy number across the mycobacterial genome [143]. Furthermore, PGRS, like MPTR sequences, are part of protein-coding regions. Multiple tandem repetitions of the PGRS-encoded motif AsnGlyGlyAlaGlyGlyAla are found in glycine-rich proteins with a characteristic proline-glutamate (PE) residue group at the N terminus of the peptide. Members of the PE-PGRS family of proteins, analogously to PPE-MPTR protein family, are suspected to play a role in antigenic variability [118].

Since the number of the PGRS element and its distribution vary in different strains, it has been applied as a genetic marker for typing of *M. tuberculosis*. The most extensively used method utilizing this marker is PGRS-RFLP, the procedure of which is quite the same as that for the IS*6110*-RFLP, except that the chromosomal DNA is cut with *Alu*I restriction endonuclease, instead of *Pvu*II, and that a 3.4 kb fragment of the PGRS sequence, cloned in a recombinant plasmid pTBN12, is used as a probe in a hybridization step [142]. The PGRS-RFLP analysis or pTBN12-RFLP fingerprinting has been shown to have a relatively high discriminatory power, especially for IS*6110* low-copy-number strains [56, 144, 145]. The pTBN12 fingerprinting method has similar limitations as the IS*6110*-RFLP typing, but additionally the hybridization patterns produced by PGRS typing are more complex and difficult in interpretation. A second method which benefits from the polymorphism of the PGRS sequence is double-repetitive-element PCR (DRE-PCR). This method relies upon amplification of the DNA segments located between IS*6110* sequences and the PGRS sequences, by using primers directed outwards from the ends of both these repetitive elements (Figure 1(4)). Based on the distance between IS*6110* and PGRS sequences and the copy number of these elements which differs between the strains, DRE-PCR yields strain-specific amplification patterns [65]. Although highly discriminative, the method suffers from poor reproducibility and a strong bias in the interpretation of the results [65, 146, 147].

A similar approach as that reported for DRE-PCR was used to design another typing method, called IS*6110*-ampliprinting [66]. In brief, the method measures the variability in the distances between IS*6110* elements and copies of MPTR sequences of *M. tuberculosis*, through unilateral-nested PCR with IS*6110* and MPTR targeted primers, followed by hybridization with an IS*6110*-specific probe. The method, however, has not been widely adopted, mainly due to the limited number and size of generated PCR products [70].

A recent method that harbors GC-rich sequences, the IS*6110*-Mtb1-Mtb2 PCR typing, is guided by a similar principle as that involved in DRE-PCR profiling. Two PCR assays are performed with primers of two kinds, that is, primers complementary to terminal inverted repeats (TIR) flanking the IS*6110* sequences and primers complementary to short (16 bp) GC-rich motifs, called Mtb1 (I PCR) and Mtb2 (II PCR). As a result, fragments of DNA located between Mtb1 and Mtb2 as well as between each of those elements and IS*6110* sequences are amplified [67]. Although the IS*6110*-Mtb1-Mtb2 PCR method has not been exploited sufficiently, the results from the insofar performed studies suggest its usefulness in the molecular epidemiological investigations of TB. Most notably the discriminatory power of this method has been demonstrated as almost equivalent to that of IS*6110*-RFLP typing [67, 148, 149].

Although PGRS is not commonly being applied for strain typing of NTM, RFLP analysis with PGRS isolated from *M. tuberculosis* as a probe was successfully used to differentiate isolates of the species of *M. kansasii* [150] and *M. ulcerans* [151].

10. Repetitive Sequence-Based- (rep-) PCR

Every genotyping method, whose principle is based on the PCR amplification of DNA sequences between repetitive DNA elements, is referred to as repetitive sequence-based-(rep-) PCR. By using primers designed as extended away from the repetitive sequence elements, multiple amplified fragments are generated, depending on the sequence length between the repetitive elements. The amplified fragments produce a fingerprint pattern upon gel electrophoresis. IS*6110* amplityping, DRE-PCR, IS*6110*-ampliprinting, and IS*6110*-Mtb1-Mtb2 PCR, all these methods are representatives of rep-PCR technology. A long list of various repetitive elements that have been applied for genotyping of *M. tuberculosis* and nontuberculous mycobacteria includes also DNA sequences that lack species or even genus specificity, such as enterobacterial repetitive intergenic consensus (ERIC) and the $(GTG)_5$ sequences [152–155].

Recently, a commercially available rep-PCR system (DiversiLab System, bioMérieux, France) has been adapted for use on mycobacteria. The DiversiLab System, which takes advantage of various repetitive elements interspersed throughout different bacterial genomes, was evaluated on a collection of *M. tuberculosis* and *M. avium* complex isolates [156, 157] as well as *M. abscessus* isolates [158]. For *M. tuberculosis* and *M. avium* subsp. *avium*, the discriminatory ability of the assay equaled or exceeded that of IS*6110*-RFLP and IS*1245*-RFLP [156, 157, 159]. In more recent reports, rep-PCR was successfully used to disclose the source of infection in a patient with hypersensitivity pneumonitis caused by *M. avium* [160].

11. Random Amplified Polymorphic DNA (RAPD) Analysis

Random amplified polymorphic DNA (RAPD) analysis, also referred to as arbitrary primer PCR, depends on amplification of random fragments of genomic DNA using arbitrarily designed primers (5 to 50 bp) under low stringency conditions. The interstrain polymorphism is assessed by comparing the electrophoretic pattern of the PCR products. Although the method provides high discriminatory power, a number of limitations exist for this technique, of which the apparent lack of reproducibility is the most important. This is because differences between the strain patterns generated by RAPD are due to technical and operating parameters of the method rather than true interstrain genetic polymorphism. Variations in RAPD patterns are chiefly attributed to variations in the priming efficiency during early rounds of amplification, and these in turn depend on template concentration and purity, primer/template ratio, or the ramp times of the cyclers used. Nonetheless, the RAPD typing has been used to differentiate among isolates of *M. tuberculosis* complex isolates [161–164] as well as among isolates of numerous NTM species, including *M. abscessus* [165], *M. phocaicum* [166], *M. gordonae* [167], *M. szulgai* [168], and *M. malmoense* [169].

The RAPD analysis is usually performed on the entire genomic DNA; however in some modifications of the method only a fragment of the bacterial chromosome is used as a substrate for PCR. Such a strategy was employed for genotyping of *M. tuberculosis* strains by Abed et al., who used the 16–23S rDNA internal transcribed spacer (ITS) region within the rDNA operon as a target for PCR. The subsequent RAPD analysis of the amplified product resulted in highly polymorphic and easily interpretable profiles [170]. Still, the reproducibility of this RAPD-based method was found to be poor [171, 172].

12. Amplified Fragment Length Polymorphism (AFLP) Analysis

Amplified fragment length polymorphism (AFLP) analysis is a PCR-based method in which genomic DNA undergoes digestion with two restriction enzymes, a rare cutter and a frequent cutter, typically having six- and four-nucleotide-long recognitions sites, respectively (e.g., *Eco*RI and *Mse*I, resp.), followed by ligation of two types of synthetic, double-stranded adaptors (10–30 bp) to the generated cohesive ends of DNA fragments. Amplification of a subset of restriction fragments is achieved by PCR with primers complementary to the adaptor sequence, the restriction site sequence, and a few nucleotides (1–3) extending beyond the restriction site. The amplicons are separated and visualized on denaturing polyacrylamide gels, usually through autoradiography, as the primers are radioactively labeled [173]. The conventional radioactive AFLP method has a lower discriminatory potential than IS*6110*-RFLP typing [174, 175]. However, the AFLP technology provides an extraordinary flexibility in designing the typing protocols of enhanced power of discrimination. Here, the choice of restriction endonucleases and the degree of primer selectivity may largely determine the final resolution of the method.

A refinement of the traditional AFLP assay is the fluorescent (f)AFLP, where the subsets of the DNA digestion fragments are PCR-amplified with five primers, a single, nonselective, unlabeled forward primer targeting the *Mse*I adaptor site and four reverse primers, targeting the *Eco*RI adaptor site, each containing the selective base A, G, C, or T, labeled with different fluorescent dyes. The amplified fragments are resolved by electrophoresis on an automated DNA sequencer and precisely sized using internal size standards [176]. Apart from the improved occupational safety, the discriminatory potential of fAFLP is higher than that of the radioactive AFLP and comparable to that achieved by IS*6110*-RFLP [176, 177].

The AFLP method has successfully been applied to various NTM species including the *M. avium* complex [178], *M. haemophilum* [179], and *M. marinum* and *M. ulcerans* [180], although its exact discriminatory power in larger sets of isolates has never been assessed.

Furthermore, AFLP analysis has clearly distinguished between *M. marinum* and *M. ulcerans*, conforming that these species which are difficult to distinguish by conventional methods, are genetically distinct [180, 181].

13. Multilocus Sequence Typing (MLST)

Genetic polymorphism between and within different mycobacterial species can also be investigated at the nucleotide sequence level. Such a concept gave rise to a new typing system, the so-called multilocus sequence typing (MLST), which evolved directly from the multilocus enzyme electrophoresis (MLEE or MEE) technique. Unlike MLEE, which targets the electrophoretic mobility of slowly evolving metabolic enzymes (usually 15 to 25), isolated from each bacterial isolate, MLST targets the sequences of a small number (up to 10) of housekeeping genes coding for vital enzymes and structural proteins. Although both MLEE and MLST schemes failed to reveal sufficient polymorphism among members of the *M. tuberculosis* complex [27, 182], these techniques were found useful for differentiation of various NTM species. For instance, by using MLEE, polymorphism was found among isolates of the *M. avium-M. intracellulare* complex [183–185]. On the other hand, the MLST analysis, based on 10 genetic loci, allowed the determination of the variability between subspecies and strains of *M. avium* and thus greatly improved our knowledge on genetic divergence and evolution of this group of NTM [186]. MLST has also proven to be valuable in investigations of NTM in laboratory outbreak settings [166].

Given the low degree of sequence polymorphisms in *M. tuberculosis* complex, standard MLST is poorly informative and inefficient. Its place has now been taken over by a new typing strategy based on analysis of single nucleotide polymorphisms (SNPs).

14. Single Nucleotide Polymorphism (SNP) Typing

Single nucleotide polymorphisms (SNPs) fall into two major groups: synonymous- (s-) SNPs and nonsynonymous- (ns-)

SNPs. The latter, if present in coding regions, introduce amino acid changes to the proteins. This in turn may influence the phenotype and be subjected to selection pressure. For instance, ns-SNPs are implicated in *M. tuberculosis* resistance to anti-TB drugs (drug resistance in *M. tuberculosis* is almost invariably associated with mutations (nonsynonymous point mutations, small deletions, and duplications) in specific, chromosomal loci) [187]. Screening for ns-SNPs in resistance-conferring genes provides important insights into the molecular mechanisms and dynamics of the development of drug resistance.

Contrariwise, s-SNPs do not alter the amino acid profiles and are thus phenotypically neutral. As s-SNPs are also believed to be evolutionary neutral, they are used for population genetics and for studying phylogenetic relationships among mycobacterial strains [188, 189]. A picture of the phylogenetic population structure of *M. tuberculosis* has recently been inferred by using a combination of s-SNP and non-SNP markers. Based on the SNPs at codon 463 of the *katG* gene and codon 95 of the *gyrA* gene, Sreevatsan et al. divided *M. tuberculosis* complex into 3 principal genetic groups (PGG1–PGG3) [14]. Those three PGG groups were further split into 9 major clusters (I–VIII and II.A) by analysis of additional 36 s-SNPs [189]. Recently, Dos Vultos et al. [190] have indicated SNPs in the 56 genes encoding 3R (DNA replication, recombination, and repair) system components as the key genetic markers to study the evolution of *M. tuberculosis*.

Although SNPs represent the most reliable markers for lineage classification of MTBC, their use is hampered by the need to test a large set of genes to achieve satisfactory resolution. Recently, Homolka et al. [16] have developed a SNP-based diagnostic algorithm allowing the identification of 17 MTBC phylogenetic lineages with high specificity. The algorithm involves sequence analysis of only five genes. The SNP typing approach is highly specific and sensitive, although SNP analysis has predominantly been used in genealogy, phylogenetic, and population genetics studies.

15. Deletion Mapping and Deligotyping

Comparative genomic studies of different strains of *M. tuberculosis* (e.g., H_{37}Rv, CDC1551) have proven that the loss of genetic material has stigmatized the evolutionary history of that species. Genomic deletions, also known as large-sequence polymorphisms (LSPs) or regions of difference (RD), have been detected across the *M. tuberculosis* genomes [191, 192]. For example, a total of 68 distinct deletions, ranging in size from 105 bp to approximately 12 kb, were found in 100 *M. tuberculosis* clinical isolates [192]. Deletions are not randomly distributed but rather appear in aggregations. They occur within both intra- and intergenic regions [191–194]. Noteworthy, almost half of the LSPs identified in *M. tuberculosis* H_{37}Rv and CDC1551 strains involved genes encoding PPE and PE family proteins [191]. Since LPSs have emerged as a significant source of interstrain genetic variability, they have been used as markers for genotyping. Analysis of chromosomal deletions has been shown as an extremely attractive approach for studying the phylogeny and evolution of *M. tuberculosis* complex [192, 195, 196]. Deletion analysis, also referred to as deligotyping, can be performed either by a simple PCR-based method or by automated microarray techniques. The resolution of the method can greatly be increased if specific sequences flanking each side of the deletion element are known. Recently, a high-throughput method for distinguishing LSPs was invented. Both the concept and procedure of this method were patterned upon the spoligotyping technique. Here, deletion events are detected in 43 genomic loci by amplifying them in a multiplex PCR assay and subjecting the amplicons to hybridization with a set of 43 probes, whose sequences directly correspond to the targeted loci [197]. Deligotyping is a very sensitive and efficacious approach for rapid screening of clinical isolates of *M. tuberculosis*. The method is also well suited for constructing robust phylogenetic relationships [198].

16. Concluding Remarks

As described above, there is a wide range of methods available for genotyping of *M. tuberculosis* complex and NTM species (Tables 1 and 2). Each method has its own benefits and shortfalls, and none of them have proven clearly superior to any of the others. The choice of the optimal typing system depends heavily on the sample under investigation, the setting in which typing is performed, and the expected outcome. For instance, spoligotyping is of particular value in population-based studies to define the phylogeographic specificity of circulating clades of tubercle bacilli. However, to assess the genetic relatedness and the epidemiological links among TB outbreak-related cases, the IS*6110*-RFLP or MIRU-VNTR typing is preferably chosen. Again, whereas spoligotyping is recommended as a preliminary screening test of a large number of *M. tuberculosis* isolates, disclosure of true genetic relationships between the isolates requires more discriminating methods, such as MIRU-VNTR typing, to be performed within the spoligotype-defined clusters.

An ideal molecular typing method should accommodate the requirements with respect to both performance and analytical criteria. The desired performance parameters include technical simplicity (easiness of performance), reproducibility, robustness, and time and cost effectiveness. An attractive feature of the method could be its applicability directly to clinical material. Another special advantage is a standardized and easily portable and interpretable format of the results (e.g., digital codes), facilitating databasing and interlaboratory comparative studies. As for the analytical parameters, the most important are the level of discrimination and stability of the genetic marker used. The general rule that the higher the discriminatory power of a given method, the more reliable the results obtained guides most of the molecular epidemiology investigations. However this may not always be the case. The validity of such assumption depends on several issues related to clustering, such as the characteristics of the study setting, the proportion of cases included (completeness of sampling), or the period of case recruitment (duration of the study). In other words, a number of important considerations have to be taken into account when choosing an appropriate typing methodology in terms of discrimination capacity.

Table 1: Selected typing methods for *Mycobacterium tuberculosis* complex and level of genetic polymorphism they reveal.

Typing method	DNA target	Polymorphism	References*
IS*6110*-RFLP	IS*6110*	High	van Embden et al., 1993 [48]
ML-PCR		High	Haas et al., 1993 [58]
FliP		High	Reisig et al., 2005 [59]
LM-PCR		High	Prod'hom et al., 1997 [60]
HIP		High	Kearns et al., 2000 [61]
Spoligotyping	DR locus	Low	Kamerbeek et al., 1997 [62]
VNTR typing	ETRs A–E	Low	Frothingham and Meeker-O'Connell, 1998 [63]
MIRU-VNTR typing	MIRUs	High	Supply et al., 2001 [64]
DRE-PCR	IS*6110*/PGRS	High	Friedman et al., 1995 [65]
IS*6110*-ampliprinting	IS*6110*/MPTR	High	Plikaytis et al., 1993 [66]
IS*6110*-Mtb1-Mtb2 PCR	IS*6110*/Mtb1/Mtb2	High	Kotlowski et al., 2004 [67]

*Papers with original description of a given method.
RFLP: restriction fragment length polymorphism; ML-PCR: mixed linker PCR; FliP: fast ligation-mediated PCR; LM-PCR: ligation-mediated PCR; HIP: heminested inverse PCR; VNTRs: variable numbers of tandem repeats; MIRU: mycobacterial interspersed repetitive units; DRE-PCR: double-repetitive-element PCR; DR: direct repeat; ETR: exact tandem repeat; PGRS: polymorphic GC-rich sequence; MPTR: major polymorphic tandem repeat.

Table 2: Discriminatory power of selected typing methods for *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria and level of genetic polymorphism they reveal.

Typing method	MTBC	NTM
RFLP	High	Insufficient data
RAPD	Medium	High
PFGE	Medium	High
AFLP	High	Insufficient data
Spoligotyping	Low/diverse	None
MIRU-VNTR typing	High/diverse	Insufficient data

MTBC: *Mycobacterium tuberculosis* complex; NTM: nontuberculous mycobacteria; RFLP: restriction fragment length polymorphism; RAPD: random amplified polymorphic DNA; PFGE: pulsed-field gel electrophoresis; AFLP: amplified fragment length polymorphism; MIRU-VNTR: mycobacterial interspersed repetitive unit-variable number of tandem repeats.

The discriminatory power of molecular markers is directly linked to the genetic stability of each marker. There have been observed minor changes in DNA fingerprint patterns of *M. tuberculosis* strains isolated not only from epidemiologically related TB cases, but also from the same patient at different points of time [52, 199–202]. The stability of the genotypic patterns over time reflects the evolutionary rate, also referred to as a "molecular clock," of each genetic marker. For example, a half-life of the IS*6110*-RFLP profiles has been shown to be much shorter than that of the spoligotype profiles [70, 98]. Likewise, the combined molecular clock of the MIRU-VNTR loci has been shown to be slower than that of IS*6110*-RFLP [203]. In general, the best molecular marker is the one, whose "molecular clock" is, on the one hand, fast enough to distinguish unrelated cases and, on the other hand, sufficiently slow to capture epidemiologically linked cases [204]. Markers evolving rapidly and those evolving at slow rates may either underestimate or overestimate the amount of recent transmission of the disease, respectively.

The choice of a genotyping method, with respect to its discriminatory ability, depends on the type of research in question. Whereas a highly discriminatory method, that is with a fast "molecular clock," would be required to determine whether an infection is a reactivation of an infection acquired in the past (latent infection) or rather a reinfection with a new strain, a method with a slow "molecular clock" would be needed for global strain tracking and evolutionary studies.

The resolution power of different typing methods, yielding very diversified genetic patterns, has had an impact on the definition of clustering. Indeed, there persists a controversy in the literature on whether or not isolates whose patterns show subtle differences, that is, of 1-2 bands, in the IS*6110*-RFLP patterns or single locus variations (SLVs) in the MIRU-VNTR patterns may be regarded as part of a genetic cluster and thus constitute an ongoing chain of transmission. Whereas some authors apply a strict cluster definition including only isolates with identical genotypes [134, 135, 205, 206], the others advocate the use of slightly relaxed criteria for defining clusters, with a tolerance of a single- or double-band difference in the RFLP profiles and/or SLVs or double locus variations (DLVs) in the MIRU-VNTR profiles [52, 200, 207, 208]. With the latter approach, another important question arises, that is, to what extent the fingerprint patterns of two isolates may differ before they are no longer considered to be clustered. Since no clear cut-offs exist for defining a cluster, the decision which isolates should be included or excluded from clusters is largely a matter of arbitrariness. In general, the more lenient the assumed criteria, the higher the chance of detecting clusters, but the lower the likelihood that a cluster represents epidemiologically related cases [209].

One has also to be mindful that the identity or high similarity of DNA fingerprints from two individuals, obtained by using even the most discriminating techniques, may not always mean a recently transmitted infection. Other explanations are possible, including the simultaneous reactivation of a previously acquired infection with the same organism (coincidence of time), the regional predominance of a particular strain, circulating over a long time, or a laboratory cross-contamination [210].

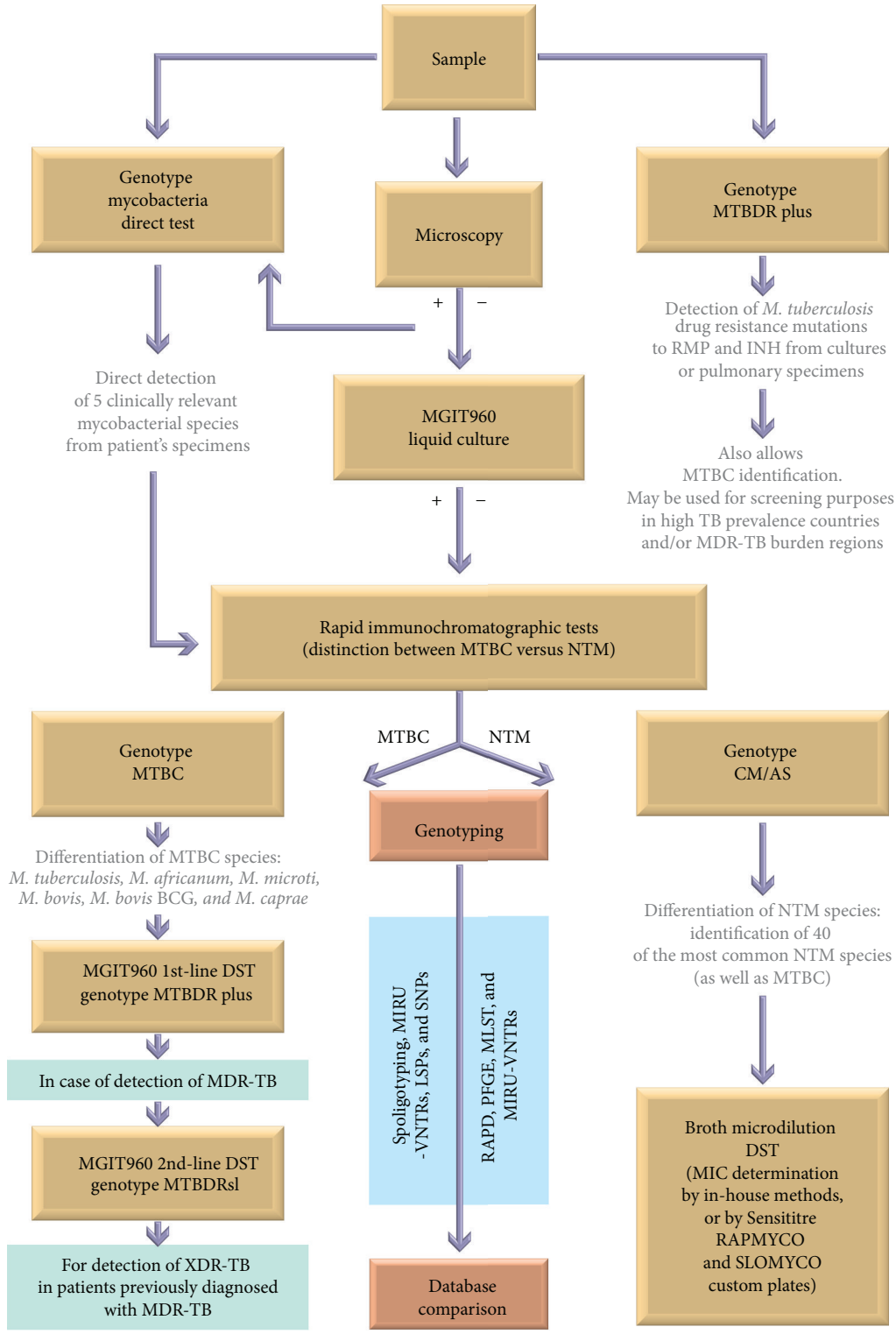

Figure 2: Schematic flow diagram illustrating processing of clinical samples for species identification, drug susceptibility testing (DST), and genotyping of mycobacteria belonging to *Mycobacterium tuberculosis* complex (MTBC) and nontuberculous mycobacteria (NTM). MIRU-VNTRs: mycobacterial interspersed repetitive units-variable number of tandem repeat loci; LSPs: large sequence polymorphisms; SNPs: single nucleotide polymorphisms; RAPD: random amplified polymorphic DNA; PFGE: pulsed-field gel electrophoresis; MLST: multilocus sequence typing.

Identical genotypic patterns may not indicate clonality, even when multiple genetic markers are employed. This was best evidenced by Niemann et al. who compared the complete genomes of two *M. tuberculosis* Beijing genotype isolates from a high-incidence region (Karakalpakstan, Uzbekistan), of which one was drug susceptible and the other was multidrug resistant. Both isolates shared the same IS*6110*-RFLP pattern and the same allele at 23 out of 24 MIRU-VNTR loci, yet they differed by 130 SNPs and one large deletion [211]. This finding implies some important messages. First, *M. tuberculosis* isolates exhibiting identical DNA fingerprinting profiles may still display substantial genomic diversity. This in turn may lead to misinterpretation of the extent of TB transmission in a community or invalid differentiation between disease relapse and exogenous reinfection, when using standard genotyping tools. It seems that the optimal option to fully explore the phylogenetic branching and variation on strain level and to justifiably draw epidemiological conclusions on the etiology, host-range, and transmission of TB disease would be the application of whole-genome sequencing (WGS) analysis. Although still an expensive solution, with the plummeting cost of DNA sequencing, the WGS may become an omnipotent approach to replace all previously known diagnostic tests for *M. tuberculosis*, including those for identification or drug susceptibility profiling [212]. As for the present, a status of the "gold standard" typing method for *M. tuberculosis* is still being held by the IS*6110*-RFLP. However, it is being increasingly replaced by the MIRU-VNTR typing, not only because its discriminatory power equals that of IS*6110*-RFLP but only because it has methodological and practical advantages over RFLP, most important of which is that the PCR-based typing method requires fewer bacteria and consequently shortens considerably delay in obtaining genotypes. Thus, the MIRU-VNTR typing can be regarded as the new standard for TB molecular epidemiology.

As for the NTM, a number of genotyping methods are available, most of which were first applied to *M. tuberculosis* complex and then turned out to work for specific NTM species, but the discriminatory power of those methods has not been fully examined. A method most commonly applied in various NTM species, with only slight species-specific modifications, has been PFGE typing. Due to its wide use, it could be considered the "gold standard" for all NTM species except for *M. avium*. For the latter, IS*1245* RFLP typing is the widely recognized reference method [79]. MLST has also the potential to become a reference method, although its wider use is hampered by limited access and high costs of the sequencing facilities. However, with the level of genetic diversity in NTM (sub)species being ill-defined and the discriminatory power of most of the markers not fully established, for a proposal of the "gold standard" typing system for NTM species to be delivered, further studies are required (Figure 2).

Although there is currently no genotyping method that would work in diverse settings and population groups or to be equally effective at answering particular epidemiological questions, the application of molecular typing methods has significantly advanced our knowledge of the transmission and pathogenesis of mycobacteria.

Molecular typing methods have permitted investigation of the outbreaks [36, 61, 100, 139, 165, 166, 213, 214], discrimination between exogenous reinfection and endogenous reactivation [209, 210, 215–217], identification of mixed infections [215, 218, 219], and cases of misdiagnosis due to laboratory contamination [220–223]. Molecular markers have also extensively been used for tracking transmission patterns within specific populations and/or defined geographical settings [106–108, 153, 206, 224–227]. Finally, genotyping methods have shed important light on the phylogeny and evolutionary history of the mycobacterial species [12, 14, 189, 194].

Methods of molecular typing constitute an integral element of virtually all epidemiological studies on mycobacterial infections. They continue to substantially improve our understanding of the biology of mycobacteria and are believed to provide novel and powerful tools to combat and/or protect against the diseases caused by these pathogens.

Conflict of Interests

The authors report no conflict of interests.

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Research Article

Short Communication: Subtyping of *Mycobacterium kansasii* by PCR-Restriction Enzyme Analysis of the *hsp65* Gene

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Mycobacterium kansasii is one of the most common causes of pulmonary disease resulting from nontuberculous mycobacteria (NTM). It is also the most frequently isolated NTM species from clinical specimens in Poland. The aim of this study was to investigate the distribution of *M. kansasii* subtypes among patients suspected of having pulmonary NTM disease. Fifty clinical isolates of *M. kansasii* recovered from as many patients with suspected mycobacterial lung disease between 2000 and 2010 in Poland were genotyped by PCR-restriction enzyme analysis (PCR-REA) of partial *hsp65* gene. *Mycobacterium kansasii* subtype I was the only genotype to be identified among the isolates, both disease-associated and non-disease-associated. Isolation of *M. kansasii* subtype I from clinical specimens may be indicative of infection but may also merely represent colonization.

1. Introduction

Mycobacterium kansasii, a non tuberculous mycobacterium (NTM), is an opportunistic pathogen that causes both pulmonary and extrapulmonary infections [1–3]. As with other NTM, *M. kansasii* infections are believed to be acquired from environmental exposure rather than by human-to-human transmission. The natural reservoir of *M. kansasii* remains largely unknown. Rarely have the bacteria been isolated from soil, natural water systems, or animals. Instead it has quite often been recovered from municipal tap water, which is considered to be its major environmental source [4]. *Mycobacterium kansasii* is one of the most frequent NTM pathogens isolated from clinical samples throughout the world [1, 5–7]. According to a recent study on the global prevalence of NTM species, *M. kansasii* was the sixth most frequently isolated NTM. When focused on Europe, Poland, Slovakia, and the United Kingdom had the highest *M. kansasii* isolations of 35%, 36%, and 11%, respectively, compared to a mean isolation of 5% in Europe [1]. In most places, *M. kansasii* ranks second, behind only *Mycobacterium avium* complex, as a cause of NTM lung disease [8]. The annual rates of infection due to *M. kansasii* reported in the general population fall within the range of 0.2 to 0.3 cases per 100 000 [4], yet significant geographical variability exists [9–12]. In Poland, among the cases of NTM disease, whose number has been increasing remarkably in recent years, those attributable to *M. kansasii* are in the majority [13]. One in three NTM species isolated from patients with pulmonary mycobacterial infections is *M. kansasii* [1, 13].

Several molecular analyses have demonstrated that *M. kansasii* is a heterogeneous species [14–19]. To date, seven *M. kansasii* subtypes (I–VII) have been identified by PCR-restriction enzyme analysis (PCR-REA) of the *hsp65* gene [20]. The heterogeneity within the *M. kansasii* species has important clinical and epidemiological implications. There

are reports that *M. kansasii* isolates that are involved in human disease belong almost exclusively to types I and II, with the former being the most commonly described [17, 20, 21].

The aim of this study was to determine the distribution of *M. kansasii* subtypes among 50 patients suspected of having pulmonary NTM disease.

2. Material and Methods

2.1. Strains. A total of 50 *M. kansasii* strains isolated from 50 patients with suspected *M. kansasii* infection (32 women and 18 men; median age: 64.6 ± 18.8 years; age range: 27–92 years), collected between 2000 and 2010 at the Department of Internal Medicine, Pneumonology, and Allergology of the Medical University of Warsaw, were included in the study. Patients were classified as having an infection in accordance with the criteria of the American Thoracic Society (ATS) [4]. The strains were cultured from sputa (28), bronchial washings (18), bronchoalveolar lavage fluids (3), and bronchial lavage fluid (1). The clinical samples were liquefied and decontaminated using soda lye with N-acetylcysteine and sodium citrate (final concentration: 2% NaOH, 0.5% NAC, and 1.3% $C_6H_5O_7Na_3$). The samples were then concentrated and cultured on Löwenstein-Jensen (L-J) medium. The isolates were identified as *M. kansasii* by using the high pressure liquid chromatography (HPLC) methodology, in accordance with the Centers for Disease Control and Prevention (CDC) guidelines [22].

2.2. DNA Extraction. Genomic DNA was extracted using Amplicor Respiratory Specimen Preparation Kit (Roche Diagnostics, Switzerland) as described elsewhere [23].

2.3. Amplification and Restriction Analysis. For the amplification of a 441 bp fragment of the *hsp65* gene Tb11 and Tb12 primers were used, as described by Telenti et al. [15]. The PCR mixtures were prepared with a TopTaq Master Mix kit (Qiagen) in a final volume of 50 μL containing ca. 10 ng of genomic DNA. After initial denaturation at 94°C for 3 min, the reaction mixture was run through 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 10 min. Amplified fragments were digested with HaeIII and Eco91I (BstEII) restriction enzymes (FastDigest), under conditions recommended by the manufacturer (ThermoScientific), separated by electrophoresis in 4% agarose gels, and visualized by staining with ethidium bromide (0.5 μg/mL) and exposure to UV light (λ = 320 nm).

Strains were classified into subtypes based on their PCR-REA patterns obtained in two separate PCR-REA assays.

3. Results and Discussion

Of the 50 patients under the study, 23 (46%; 15 women, 8 men aged 56.9 ± 20.3 years; age range: 27–87) met the ATS criteria for the definition of *M. kansasii* disease. For the remaining 27 (53%) patients, the NTM case definition criteria, either clinical or bacteriological, were not fulfilled.

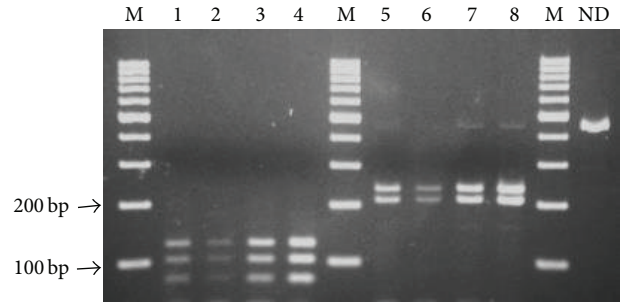

Figure 1: Differentiation of *M. kansasii* subtypes by PCR-REA of *hsp65*. Amplified *hsp65* fragments were digested with HaeIII (lanes 1–4) and BstEII (lanes 5–8). Lanes: M: GeneRuler 100 bp DNA Ladder (ThermoScienific), ND: nondigested fragment of *hsp65*.

All the *M. kansasii* isolates tested yielded, upon PCR amplification of partial *hsp65* gene, a single product of expected size (ca. 440 bp). When subjected to restriction endonuclease digestion with the enzyme HaeIII, the amplicons always produced three DNA fragments of 140, 105, and 80 bp in length. Likewise, digestion of the amplified *hsp65* fragment with BstEII yielded each time the same two-band pattern (fragments of 240 and 210 bp in length) (Figure 1). According to PCR-REA patterns obtained in two different PCR-REA assays, all the 50 *M. kansasii* isolates were categorized into type I.

Poland is the country with the highest *M. kansasii* isolation rate in Europe (35% of all NTM isolations in Poland compared to a mean isolation rate of 5% for Europe) [1]. This study is the first to document the distribution of *M. kansasii* genotypes among patients with pulmonary disease from Poland.

The reported results are consistent with those of previous studies. The investigations performed so far have suggested that *M. kansasii* type I is the most prevalent type from clinical isolates worldwide. The distribution of the genotypes (subtypes) among *M. kansasii* isolates was first studied by Picardeau et al. in the late 1990s [17]. Of the five (I–V) recognized genotypes, genotype I was the most common and included 25 (39.7%) of the 63 *M. kansasii* isolates, of both environmental and clinical origin. Among the latter group, all five genotypes were encountered with only 16 (42.1%) of the 38 isolates being differentiated into genotype I. The frequency of this genotype was found to be much higher in a study of Alcaide et al. [24]. *Mycobacterium kansasii* subtype I was present in 109 (66.9%) of the 163 clinical isolates from different European settings. A higher percentage of type I *M. kansasii* clinical isolates was found in three subsequent European studies. Taillard et al. reported 77.9% (60/77) of the isolates from Switzerland belonging to genotype I [20], whereas in a study by Gaafar et al., of the 252 *M. kansasii* isolates collected in Spain, only two belonged to genotype II, with all the remaining isolates being representatives of genotype I [19]. Another Spanish study revealed the absence of genotype II among *M. kansasii* clinical isolates, with 91 (97.8%) of

the 93 isolates tested representing genotype I and the remaining two isolates representing genotype VI [25]. An analysis of human *M. kansasii* isolates from the United States showed that all but three (78 of 81 isolates) belonged to subtype I. Of the remaining three isolates, two belonged to subtype III and one belonged to subtype II [26]. Similar results were obtained by Chimara et al. in Brazil, where out of 184 patient isolates of *M. kansasii* only two were other than type I isolates (one belonged to type II and the other to type III) [27].

Some authors suggest that in the absence of complete clinical information on patients from whom *M. kansasii* isolates are obtained the PCR-REA analysis of the *hsp65* gene may be useful in categorizing isolates as associated with mycobacterial disease (types I and II). However, as evidenced in our study, recovery of *M. kansasii* type I isolates from clinical samples does not necessarily correlate with clinical picture. This has also been observed by others [20, 28]. Isolation of *M. kansasii* subtype I from clinical samples may be indicative of infection but may also merely represent colonization.

4. Conclusions

To conclude, *M. kansasii* subtype I was the only subtype recognized among the 50 *M. kansasii* isolates, both disease-associated and non-disease-associated. High detection rate of *M. kansasii* subtype I in clinical samples may suggest that this genotype has a particular propensity for colonization, and thus a higher epidemiological potential for humans. More comprehensive studies, on large collections of *M. kansasii* isolates, are needed to provide a better understanding of the biology and pathogenicity of *M. kansasii* subtype I. An important consideration to be addressed in these studies is the possible high degree of heterogeneity of *M. kansasii* type I isolates.

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Research Article

Genotyping of Clinical *Mycobacterium tuberculosis* Isolates Based on IS*6110* and MIRU-VNTR Polymorphisms

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In this study, 155 clinical *Mycobacterium tuberculosis* isolates were subject to genotyping with fast ligation-mediated PCR (FLiP). This typing method is a modified mixed-linker PCR, a rapid approach based on the PCR amplification of *Hha*I restriction fragments of genomic DNA containing the 3′ end of IS*6110* and resolving the amplicons by polyacrylamide gel electrophoresis. The results were compared with previous data of the more commonly used methods, 15-locus mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing and, to verify combined FLiP/MIRU-VNTR clusters, the reference IS*6110* restriction fragment length polymorphism (RFLP). FLiP banding patterns were highly reproducible and polymorphic. This method differentiated 119 types among the study set compared to 108 distinct MIRU-VNTR profiles. The discriminatory power of FLiP was slightly higher than that of MIRU-VNTR analysis (Hunter-Gaston Discriminatory Index = 0.991 and 0.990, resp.). Detailed comparison of the clusters defined by each of the methods revealed, however, a more apparent difference in the discriminatory abilities that favored FLiP. Clustering of strains by using combined results of these two PCR-based methods correlated well with IS*6110* RFLP-defined clusters, further confirming high discriminatory potential of FLiP typing. These results indicate that FLiP could be an attractive and valuable secondary typing technique for verification of MIRU-VNTR clusters of *M. tuberculosis* strains.

1. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*, one of the most dangerous human pathogens. In 2009 almost two million people worldwide died of TB [1]. Recently, molecular typing of *M. tuberculosis* has greatly improved our knowledge of TB epidemiology and allowed for a better control of this disease [2]. Identification of epidemiologically linked *M. tuberculosis* strains helps to reveal the source of infection, to trace the transmission routes of various strains, and to determine the risk factors for TB transmission in a community. Molecular epidemiology enables distinguishing between exogenous reinfection and endogenous reactivation, thus helping in a more effective elimination of TB from the population. In a laboratory, molecular methods can be used to identify cross-contamination [3–5].

Various genetic markers are currently used in molecular epidemiology of TB. A high degree of DNA polymorphism in *M. tuberculosis* strains is associated with repetitive DNA elements such as insertion sequences (IS) and short repetitive DNA sequences [2]. The insertion sequence IS*6110* is especially useful in genotyping of *M. tuberculosis* [6]. IS*6110* restriction fragment length polymorphism (RFLP) is one of the methods based on the variability of this element and is the current international typing standard in the

epidemiology of TB [7]. The reference method relies on the analysis of the number of IS*6110* copies and their locations within genomes of *M. tuberculosis* strains and shows the highest discriminatory potential on the population level [2]. However, IS*6110* RFLP is labor-intensive and expensive and requires high quantities of purified genomic DNA (at least 2 μg). Moreover, it is not applicable to the analysis of *M. tuberculosis* strains with low copy numbers of IS*6110* or strains devoid of this element [8].

Due to those disadvantages of the IS*6110* RFLP, alternative PCR-based methods have been developed. They are easy to perform, require small amounts of genomic DNA, and can be performed even on nonviable organisms or directly from clinical specimens, thus reducing the time, cost and labor-intensity required for the analysis [8–10]. One of these genotyping methods exploits polymorphism in the variable number of tandem repeats (VNTRs) of mycobacterial interspersed repetitive units (MIRUs) in the genomes of *M. tuberculosis* strains [11]. Out of the 41 identified loci that contain MIRU repeats, 12, 15, or 24 the most variable sequences have been frequently used for differentiation of *M. tuberculosis* strains, based on the number of repeats in each locus investigated [12–14]. MIRU-VNTR typing has discriminatory potential close to that of the reference method or even higher in the case of isolates with low IS*6110* copy numbers [15].

Fast ligation-mediated PCR (FLiP) is another method based on IS*6110* polymorphism [16]. FLiP is a modified mixed-linker PCR, a rapid typing method based on the PCR amplification of RFLP fragments containing the 3′ end of IS*6110* and resolving the amplicons by polyacrylamide gel electrophoresis [17]. Compared with eight other typing methods for *M. tuberculosis* complex, the FLiP method shows similar high discriminatory power and reproducibility [18]. However, despite its ability to reliably differentiate between strains, FLiP has not been frequently used since its publication.

In this context, we extend our earlier, preliminary observations [19, 20] and present here the results on genotyping of 155 clinical *M. tuberculosis* isolates using FLiP in comparison with previously performed 15-locus MIRU-VNTR typing [21]. Clusters identified in both PCR-based methods were further analyzed by the reference IS*6110* RFLP.

2. Materials and Methods

2.1. Bacterial Isolates. The 155 *M. tuberculosis* isolates, of 234 isolates previously studied [21], were available for the present analysis. They were obtained in 2005–2008 from 153 TB patients diagnosed at the Center for Lung Diseases Treatment and Rehabilitation in Łódź, Poland. In two patients, a second isolate was obtained in a time interval and those two repetitive isolates were also included in the present study. The strains were cultured on Löwenstein-Jensen slants from sputum (n = 126), bronchial aspirate (n = 15), throat swab (n = 7), pleural fluid (n = 4), larynx swab (n = 1), gum pus (n = 1), and urine (n = 1). All the isolates were tested for their susceptibility to isoniazid (INH), streptomycin (SM), ethambutol (EMB), and rifampin (RMP) using the BACTEC 460-TB system (Becton-Dickinson, Sparks, MD, USA). Only six (3.9%) strains were resistant to at least one anti-TB drug: INH (n = 3); SM (n = 1); INH and RMP (n = 1); INH, RMP and SM (n = 1).

Genomic DNA was extracted and purified from all the isolates using the protocol by van Embden et al. [7], recommended for the standard IS*6110* RFLP typing. The concentration of DNA was measured with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA) prior it was used in molecular typing.

2.2. Fast Ligation-Mediated PCR. FLiP was performed as originally described by Reisig et al. [16]. Briefly, the linker was synthesized by annealing of the oligonucleotides NLO (5′-GCATTTGAATTCCACGTCAGCGACTGCACG-3′) and BA (5′-TGCAGUCGCUGACGUGGAA-3′). The oligonucleotides were added in equimolar amounts into 100 μL 1 × Gold Buffer (Invitrogen) and heated to 94°C for 15 min, followed by three cycles with 58°C for 10 min and 70°C for 5 min. Next, the DNA samples were digested with 0.5 U/μl *Hha*I (Fermentas Life Technologies) and the restriction fragments were ligated to the linker (0.67 mM) using 0.05 U/μL T4 DNA ligase in 20 μL 1 × ligation buffer (Fermentas Life Technologies). Both reactions were performed simultaneously for 2 h at 25°C. Five μL of the restriction-ligation mixture was used in amplification reaction. The PCR mix (50 μL) contained primers IS54 (5′-TCGACTGGTTCA-ACCATCGCCG-3′) and Flip1 (5′-TTTGAATTCCACGTC-AGCGACTGC-3′) (12.5 pM each), 1.25 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates, 0.5 U uracil DNA glycosylase (UDG, Invitrogen), and 2.5 U Ampli*Taq* Gold DNA polymerase (Invitrogen). The reaction began with an incubation at 50°C for 10 min to cut BA oligonucleotide of the linker with UDG. The cycling conditions consisted of an activation of Ampli*Taq* Gold DNA polymerase at 95°C for 10 min, followed by 30 cycles with 30 s at 69°C and 1 min at 72°C, and a final extension for 7 min at 72°C. Each experiment included negative (sterile ultrapure water) and positive (DNA of *M. tuberculosis* H37Rv) controls processed together with the test samples.

PCR products were resolved by 8% polyacrylamide gel electrophoresis. Banding patterns were visualized by ethidium bromide staining and photodocumented under UV light.

2.3. Genotype Analysis. The FLiP patterns were examined visually and then subject to computer-assisted analysis with BioNumerics 5.0 (Applied Maths, Sint-Martens-Latem, Belgium). The similarity (percentage) of patterns was calculated by the unweighted pair group method with arithmetic averages (UPGMA) and Dice similarity coefficient. A cluster was defined as a group of at least two isolates showing 100% identical DNA fingerprints.

The Hunter-Gaston Discriminatory Index (HGDI) [22] was used as a numerical index for the discriminatory power of the typing methods.

3. Results

Fast ligation-mediated PCR was applied in the present study to verify the usefulness of this method in differentiation of *M. tuberculosis* strains. The FLiP fingerprint patterns of 155 strains tested were highly variable. The number of bands in the FLiP patterns ranged from 3 to 11, with fragments between 100 and 1000 bp. The majority, 130 (84%) of the patterns, contained 5–8 bands, with an average of 6 bands. Figure 1 shows an example of fingerprinting results for *M. tuberculosis* strains obtained with FLiP method. FLiP typing proved to be highly reproducible. The reproducibility of FLiP was assessed by independent analysis of triplicate DNA samples of 16 randomly selected test strains. Also, DNA of *M. tuberculosis* H37Rv was included as a positive control in each experiment. The FLiP patterns obtained for the reference strain and test strains in independent reactions were identical, confirming the reproducibility of this method (data not shown). However, weak (Figure 1, lane 7) and/or smear-like bands (Figure 1, lanes 2, 4, and 6) were sometimes visualized. Therefore, the unweighted pair group method with arithmetic averages (UPGMA) and Dice similarity coefficient based on manual indication of bands was used for the comparative analysis of FLiP patterns instead of a densitometry-based algorithm.

FLiP typing identified 119 distinct patterns among 155 tested strains (Figure 2). One hundred and one (65%) strains showed unique band patterns, and remaining 54 (35%) strains clustered into 18 groups containing 2–12 identical genetic profiles representing tested strains. The largest cluster was composed of 12 strains. Also, three larger clusters of strains were detected, each containing six, five, and three strains, respectively. Majority of the clusters (14 out 18) were made up of two strains each. Patterns detected in two strains isolated from the same patient at different times were identical and clustered together, further confirming stability and reproducibility of the FLiP profiles (Figure 2). The overall degree of strain differentiation generated by FLiP was 0.768 and it was higher in comparison with 0.697 observed for the same set of strains when characterized by 15-locus MIRU-VNTR typing.

Previously performed, MIRU-VNTR analysis [21] identified 108 distinct profiles among the 155 strains tested. Eighty-one (52%) strains were unique, and 74 (48%) strains were grouped in 27 clusters including 2–11 isolates. Eleven strains were grouped into the largest cluster with identical MIRU-VNTR patterns. Three large clusters of strains were also identified, each containing seven, six, and four strains, respectively. The remaining 23 clusters were composed of two strains each. As in FLiP, the repetitive isolates from two patients were identical to the first isolate also in MIRU-VNTR typing.

Clustering results from both methods were compared to determine their discriminatory power. FLiP gave resolving power only slightly higher than the 15-locus MIRU-VNTR analysis (HGDI = 0.991 and 0.990, resp.). However, detailed comparison of the clusters defined by each of the methods revealed a more apparent difference in the discriminatory abilities that favored FLiP. As shown in Table 1, 13 (48%) of the 27 MIRU-VNTR clusters included strains with multiple FLiP patterns, whereas only 4 (22%) out of the 18 FLiP clusters could be subdivided by MIRU-VNTR analysis.

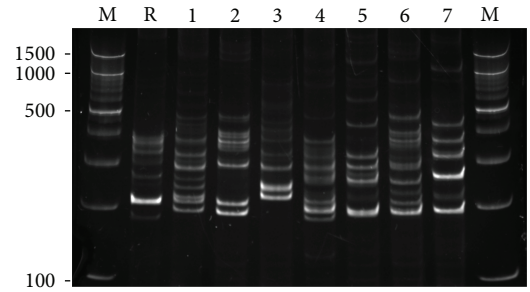

Figure 1: Polyacrylamide gel electrophoresis of PCR amplification products generated by FLiP analysis. Lanes 1–7: *M. tuberculosis* test strains; R: *M. tuberculosis* H37Rv; M: 100 bp DNA ladder (in base pairs).

The simultaneous use of both methods identified 42 isolates that clustered in 15 combined FLiP/MIRU-VNTR groups. Previously obtained IS*6110* RFLP data [21] revealed one unique pattern in each of three combined clusters consisting of seven, four, and three strains, respectively. The differences in IS*6110* RFLP patterns within the three subdivided FLiP/MIRU-VNTR clusters were, however, not significant and limited only to the presence of one or two additional IS*6110* hybridizing bands (data not shown).

4. Discussion and Conclusion

FLiP is a DNA typing method for *M. tuberculosis* complex strains described by Reisig et al. in 2005 [16]. It is based on the original mixed-linker approach that used one primer specific for IS*6110* and a second primer complementary to a linker ligated to the *Hha*I restriction fragments of genomic DNA in a PCR amplification. One strand of the linker molecule contained uracil instead of thymidine, and it was split by UDG assuring specificity of the reaction [17]. The mixed-linker method was successfully applied to outbreak investigations and population-based studies [17, 18, 23]. However, it requires reamplification that increases the risk of cross-contamination and multiple hands-on steps. To overcome those limitations, Reisig et al. constructed new linker oligonucleotides. That modification enabled specific amplification of RFLP fragments carrying IS*6110* after a single PCR step, following simultaneous restriction-ligation reactions, resulting in a simplified and faster typing of *M. tuberculosis* strains [16]. Kremer et al., in an extensive interlaboratory study comparing nine PCR-based assays, concluded that MIRU-VNTR and FLiP methods are both rapid, highly reliable, and discriminative epidemiological typing tools for *M. tuberculosis* [18]. However, in spite of its potential to differentiate between strains, FLiP has not been tested in molecular analyses since its publication.

To address this lack of data, we previously used FLiP to estimate molecular relationships among small sets of clinical *M. tuberculosis* isolates [19, 20]. In this study, we extend

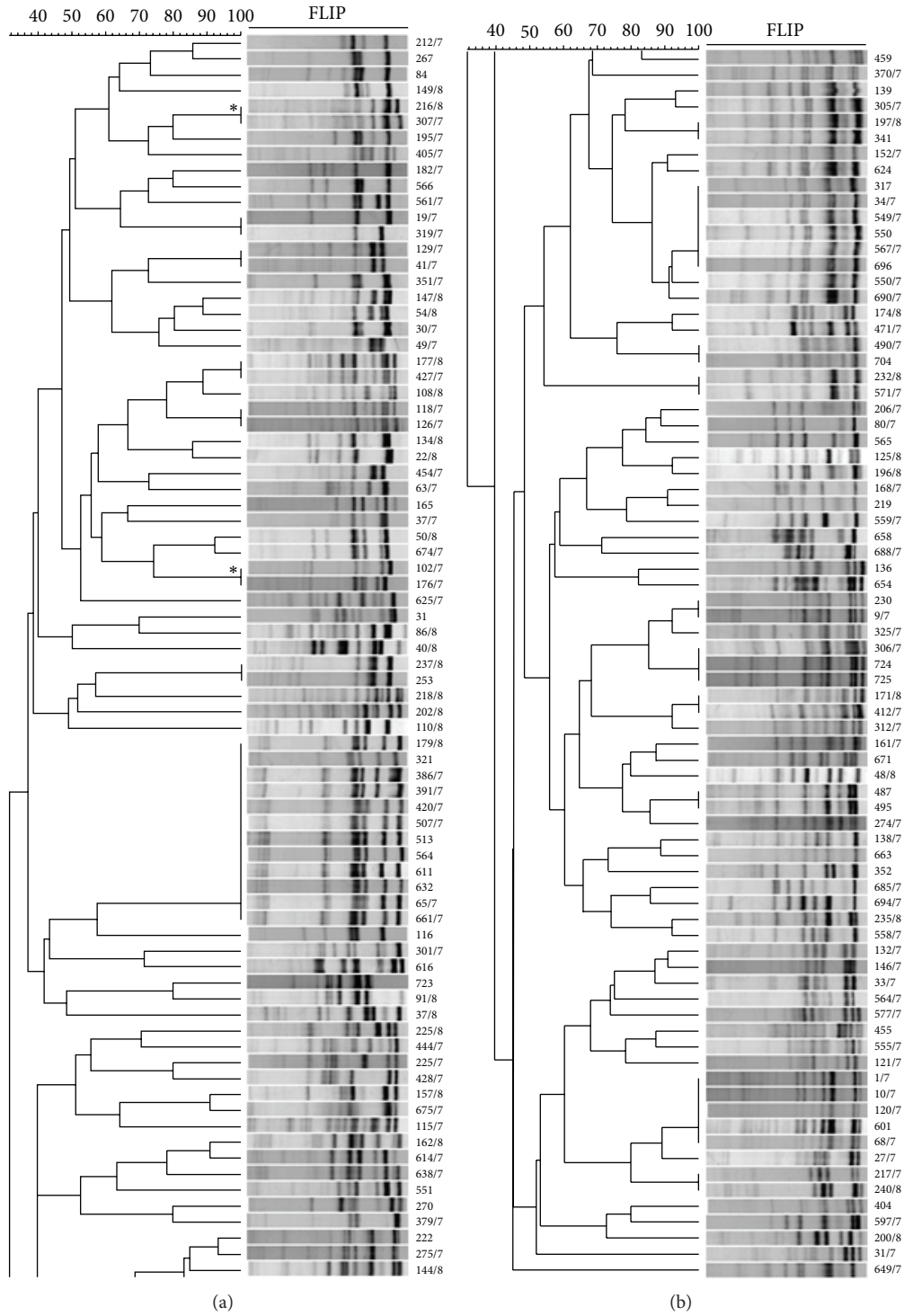

Figure 2: FLiP profiles of the 155 *M. tuberculosis* strains and the corresponding dendrogram. The similarity among the profiles is given as a percentage above the dendrogram. The numbers of strains are shown at the right side. Asterisks indicate two clusters of identical isolates from the same patients.

our preliminary observations and report a molecular characterization of 155 *M. tuberculosis* strains from Łódź, Poland, through genotyping by FLiP in comparison with previous results of 15-locus MIRU-VNTR analysis [21]. In accordance with our earlier reports [19, 20], FLiP patterns obtained in the present study consisted of 3–11 DNA fragments, with an average of 6 bands. This is slightly lower compared to 0–16 bands (8 average) described by Reisig et al. [16]. These authors did not comment on concordance between the numbers of IS*6110* copies detected by FLiP and IS*6110*

Table 1: Comparison of clustering in *M. tuberculosis* strains by the use of 15-locus MIRU-VNTR and FLiP typing methods.

MIRU-VNTR		FLiP pattern (no. of strains)	MIRU-VNTR		FLiP pattern (no. of strains)
Pattern (no. of strains)	Numerical code[a]		Pattern (no. of strains)	Numerical code[a]	
1 (11)	453531333243437	1 (6), 2 (1), 3 (1), 4 (2), 5 (1), 6 (1)	16 (2)	423533332242325	30 (1), 31 (1)
			17 (2)	432433343242325	32 (2)
2 (7)	482132544343228	7 (12)[b]	18 (2)	443433343242225	33 (2)
3 (6)	453533333443335	8 (3), 9 (1), 10 (1), 11 (1)	19 (2)	423434343242526	34 (1), 35 (1)
			20 (2)	433334343242626	36 (2)
4 (4)	343541333431446	12 (5)	21 (2)	432335343242525	37 (2)
5 (2)	443533333443437	13 (2)	22 (2)[d]	431535342232322	38 (2)[d]
6 (2)	453533333443337	14 (2)	23 (2)	443444333443545	39 (2)
7 (2)	432433333443437	15 (1), 16 (1)	24 (2)	3112132345443138	40 (1), 41 (1)
8 (2)	463634333443637	17 (1), 18 (1)	25 (2)	4102123454443226	42 (2)
9 (2)	433533333443637	19 (1), 20 (1)	26 (2)	492134544443138	43 (1), 44 (1)
10 (2)[c]	442431542132437	21 (2)[c]	27 (2)	482532544343228	7 (2)[b]
11 (2)	453531333243237	7 (1)[b], 22 (1)	28 (1), 29 (1)	453533333443345,	45 (2)
12 (2)	453531333443437	23 (2)		453533333443342	
13 (2)	463633333443235	24 (1), 25 (1)	30 (1), 31 (1)	453533333475347,	46 (2)
14 (2)	423432342242425	26 (1), 27 (1)		453543333463248	
15 (2)	432532342242425	28 (1), 29 (1)			

[a]Number of copies > 9 per locus is underlined.
[b]Members ($n = 12$) of the same cluster (FLiP pattern 7).
[c, d]Sequential isolates from two patients.

RFLP. However, we previously noticed 1–8 bands less in 90% of FLiP patterns in comparison with IS*6110* RFLP profiles of the respective strains [20]. Also, Prod'hom et al. reported that the number of bands generated in ligation-mediated PCR, a DNA typing technique related to mixed-linker amplification, was equal to or lower than IS*6110* copy number in the strains tested [24]. These results indicate that the number of bands in the FLiP DNA fingerprints may not necessarily reflect the number of IS*6110* copies in *M. tuberculosis* strains and rather should not be identified with them. Some limitation of FLiP, inherent to banding profiles-based methods, could be occasional appearance of weak and/or smear-like bands within a pattern. However, despite this potential difficulty, FLiP still proved to be fully reproducible confirming previous observations [18, 20].

FLiP typing revealed a high degree of polymorphism among the 155 strains investigated here confirming previous data of Kremer et al. [18], although its discriminatory power was somewhat lower in the present study (HGDI = 0.991 versus 0.994). The difference was most probably due to various geographical origins of the 90 *M. tuberculosis* strains tested earlier that resulted in lower degree of clustering compared to the present study on the strains originating from a single town only. Nevertheless, FLiP was still more discriminative than 15-locus MIRU-VNTR typing (HGDI = 0.990). On the other hand, Kremer et al. observed slightly lower discriminatory potential of FLiP compared to MIRU-VNTR analysis based on 12 loci (HGDI = 0.995) [18].

Strain resolution by MIRU-VNTR typing approaches that of the reference method but varies according to the loci analyzed and between strain families [18, 25–29]. Moreover, the exclusive use of this method leads to misinterpretations of epidemiological links among *M. tuberculosis* isolates [29, 30]. Methods based on IS*6110* and methods based on MIRU-VNTR polymorphisms detect changes in different regions of the chromosome (mobile versus core); therefore clustering generated by the two types of methods does not have to be identical. In the present study, only 7% (3/42) of the strains clustered both by MIRU-VNTR and FLiP methods could be subdivided by the reference IS*6110* RFLP. These results further confirm a high discriminatory ability of FLiP and its usefulness as a secondary typing technique for verification of MIRU-VNTR clusters.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Fish Tank Granuloma Caused by *Mycobacterium marinum* in Two Aquarists: Two Case Reports

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Mycobacterium marinum, the cause of chronic systemic infections in fish, occasionally causes granulomatous skin and soft tissue lesions in humans. Cutaneous mycobacterial infection in two patients owing to unusual circumstances is presented in this report. The first patient was infected through improper hygienic behavior, while infection in the second patient was previously misdiagnosed as rheumatoid arthritis and treated with methylprednisolone for a period of three months, which resulted in a rare systemic spread of *M. marinum* into the bones of the hand, testis, and epididymis. Simultaneously, screening for possible sources of *M. marinum* infection in patients' aquaria revealed positive fish harboring VNTR profiles identical to those obtained for clinical isolates from patients.

1. Introduction

Nontuberculous mycobacteria (NTM) are ubiquitous in the environment and are responsible for several diseases in animals and humans known as mycobacterioses [1–3]. A study published in 2013 has shown that *M. fortuitum, M. gordonae, M. kansasii,* and *M. peregrinum* are frequently present in the aquatic environment of surface waters in the Moravian region of the Czech Republic. In contrast, the most frequent NTM present in aquaria with ornamental fish were *M. marinum* [4].

Mycobacterium marinum is the cause of chronic systemic infections in fish and an occasional cause of granulomatous skin lesions in humans. Infections in humans result in skin and soft tissue infections characterized by their predilection for the upper extremities, often following minor trauma to hands with a history of typical exposure to aquarium tanks [5, 6]. Diagnosis is usually made after biopsy and culture of the lesion, but microbiologists should be alerted to the possibility of *M. marinum* if the injury originated from an aquarium or the water environment [7].

Here we report two cases of *M. marinum* infection in humans (Table 1). Previously described species-specific qPCR targeting the *erp* and IS*2404* genes together with a conventional culture method was used for the detection of *M. marinum* in clinical specimens of two infected humans (Table 2). Simultaneously, epidemiology screening for possible sources in patients' aquaria via VNTR fingerprinting of *M. marinum* isolates was carried out.

2. Materials and Methods

2.1. Sample Collection. A total of 49 samples were taken from two humans ($n = 9$), ornamental fish ($n = 20$), and the aquarium environment ($n = 20$; biofilms, water, and plants). Animal and environmental samples were examined within the framework of an epidemiological study carried out in the aquaria of infected aquarists.

2.2. Cultivation and Isolate Characterization. All fish and environmental samples were subjected to decontamination with N-acetyl-L-cysteine followed by cultivation at 25°C, 30°C, and 37°C for 8 weeks [8]. In case of clinical samples decontamination step was excluded. Mycobacterial isolates were grown on solid Löwenstein-Jensen and Ogawa media

Table 1: Cutaneous infection of *Mycobacterium marinum* in two patients.

Patient no.	Age/sex	Case background	Drug therapy		Treated
			Regimen	Course	
(1)	25/m	Hobby aquarist who cleaned injured knee with a brush usually used to clean the aquarium	Clarithromycin	Infiltrate with visible crusts together with two subcutaneous resistances on the left knee Extirpation of subcutaneous nodule because positivity for *M. marinum* in the sample of exploratorily excised cutaneous tissue was not detected	Yes
			Clarithromycin Ethambutol	Treatment changed due to the insufficient clinical response in the area of subcutaneous resistance	
(2)	60/m	Patient with a professional interest in fish breeding reported finger injury during aquaria cleaning	Methylprednisolone Methotrexate	Suspected rheumatoid arthritis due to swelling of the index finger joint Immunosuppression contributed to the hematogenous spread of infection	Yes
			Clarithromycin Ethambutol	Insufficient clinical response in the area of the right hand Severe right-hand oligoarthritis	
			Amikacin Ciprofloxacin Ethambutol Cycloserine Pyrazinamide	Osteomyelitis of the affected metacarpophalangeal joints Orchiectomy due to inflammation and necrosis in the epididymis with continuous spreading to the testis Surgical removal of the ring finger	

Table 2: The presence of *Mycobacterium marinum* in clinical specimens studied using the *erp* and IS*2404* qPCR assay.

Sample		*erp*/IS*2404* qPCR	ZN microscopy	Cultivation	Isolate identity
Matrice	Source				
		Patient 1			
Subcutaneous nodule	Left knee	1.6×10^7	AFB	+	*M. marinum*
Cutaneous tissue	Left knee	–	–	–	–
		Patient 2			
Sperm	Testis	–	–	–	
Puncture	Metacarpal joint	2.8×10^5	AFB	+	*M. marinum*
Pus	Ring finger	5.2×10^6	AFB	+	*M. marinum*
Blood	Vein	6.2×10^3	–	–	–
Subcutaneous nodule	Right forearm	4.1×10^5	–	+	*M. marinum*
Tissue	Epididymis	2.8×10^7	AFB	–	–
Tissue	Testis	1.2×10^5	AFB	–	–

Quantification of *M. marinum* is shown in genome equivalents per g of tissue or mL of liquid sample.
AFB: acid fast bacilli.

(Trios, Prague, Czech Republic) and in liquid Sula medium (Trios). Clinical specimens were subjected to microscopic examination after Ziehl-Neelsen (ZN) staining for the detection of acid fast bacilli (AFB).

Mycobacterial isolates were identified by their phenotypic properties (temperature preference, growth rate, and pigment production in the dark and upon exposure to light), microscopic examination after ZN staining, and sequence analysis of the *16S rRNA* gene [9]. Subsequently, VNTR analysis according to a previously published method was used to fingerprint isolates identified as *M. marinum* [10].

2.3. DNA Isolation and Molecular Detection of Mycobacterium marinum. DNA isolation from human, fish, and environmental samples was based on a protocol described previously [11]. The isolated DNA was analyzed according to a previously described *erp*/IS*2404* qPCR assay, which enables species-specific detection of *M. marinum* [12].

3. Patient Clinical Presentation

3.1. Patient 1. A Twenty-five-year-old male reported cleaning his injured left knee with a brush usually used to clean the

Table 3: VNTR analysis of *Mycobacterium marinum* isolates.

Isolate source	VNTR loci[a]								
	Locus 1	Locus 6	Locus 15	Locus 16	Locus 18	MIRU2	MIRU5	VNTR2067	VNTR3422
Patient 1									
Subcutaneous nodule	2	2	3	3	2	2	NA	3	4
Fish	2	2	3	3	2	2	NA	3	4
Patient 2									
Puncture	2	2	3	4	2	2	2	3	4
Pus	2	2	3	4	2	2	2	3	4
Subcutaneous nodule	2	2	3	4	2	2	2	3	4
Fish	2	2	3	4	2	2	2	3	4

[a]NA: no amplification. As Locus 9, Locus 14, and VNTR1132 failed in amplification from any isolates, they are not listed here.

aquarium, which resulted in infiltration and subsequently visible crusts together with two subcutaneous resistances caused by *M. marinum*. The patient was initially treated with clarithromycin. Subcutaneous resistance did not lessen after nine weeks; therefore, treatment was continued with ethambutol for an additional nine weeks.

3.2. Patient 2. The second case involved cutaneous infection of a 60-year-old male with a professional interest in fish breeding. He reported injury to the tip of his index finger, which occurred during aquarium clearing. He was misdiagnosed with rheumatoid arthritis and treated with methylprednisolone for a period of 3 months, which resulted in a rare systemic spread of *M. marinum* from the primary site into the joint and bones of the index and ring finger, forearm, testis, and epididymis. The patient was initially treated with a combination of clarithromycin and ethambutol. Because of the insufficient clinical response in the area of the right hand an eight-month-long treatment based on results of sensitivities to a combination of amikacin, ciprofloxacin, ethambutol, cycloserine, and pyrazinamide was administered. Due to the damage to the testis an orchiectomy was also carried out. Because of long-lasting pain and damage the distal part of the ring finger was surgically removed.

4. Results and Discussion

Generally, three main histopathological patterns of *M. marinum* infection are distinguished: granulomatous nodular or diffuse inflammation with mixed granulomas; abscesses with mild granulomatous reactions; and subcutaneous (patients 1) or deep dermal granulomatous inflammation [13]. Tenosynovitis, septic arthritis, bursitis, or osteomyelitis was also reported in the literature to follow from deep subcutaneous infection [14, 15]. *M. marinum* infection may have the potential for systemic dissemination in humans as has been reported earlier and was shown in the second patient [6].

Conventional microbiological methods used for *M. marinum* diagnostics are slow and rely solely on phenotypic characteristics, which can be very similar to different causative agents. Clinicians in general have to consider other causes besides *M. marinum* including deep fungal infection; therefore, a diagnostic ladder is needed in this kind of patients. Delayed diagnosis is the main cause of the patients' serious side effect. Rapid and accurate molecular diagnosis methods are essential. Rapid detection of mycobacteria using conventional broad range PCR assay was previously described in the literature [16]. The advantage of this approach is the ability to detect multiple mycobacterial species. Generally, conventional PCR could show weakness such as proneness to contamination or lack of sensitivity when used for detection in clinical specimens where pathogen is present in limited amount [17]. Sensitive and specific method for the direct detection of *M. marinum* in clinical specimens suitable to overcome this limitation would be beneficial. So far, there have been very few reports on the detection of *M. marinum* directly from infected tissue without previous culturing [12, 18].

The molecular based approach used in this study represents a fast, sensitive, and specific method for the detection of *M. marinum*, in comparison to more time-consuming conventional methods. The protocol used here enabled us to complete the analysis of a sample, including controls, in approximately 6 hrs. The applied qPCR assay enabled the detection of *M. marinum* in clinical specimens collected from both the infected humans prior to successful cultivation (Table 2). Rare systemic spread of *M. marinum* in the second patient was proven by qPCR, microscopy, and cultivation. Furthermore, direct qPCR detection was successful in the case of three culture-negative samples.

Screening for the presence of *M. marinum* in the aquarium environment of both patients was carried out. The qPCR analysis of the patients' ornamental fish and aquarium environment revealed positivity for *M. marinum* in eight fishes ($n = 3$, patient 1; $n = 5$, patient 2). The number of viable cells in qPCR-positive samples was possibly lower due to the applied decontamination procedure because only one fish from each patient was later culture-positive for *M. marinum*. As reported earlier, microbiological stains give positive results in less than 25% of cases and do not correlate with the severity of *M. marinum* infection [14]. Although *M. marinum* is described to be present in the water environment, it was detected only in fish.

From the epidemiological aspect it has been proven that the *M. marinum* strain isolated from fish had the same VNTR profile as the clinical isolate originating from the fish owner and thus it can be concluded that the aquaria were the source of infection in the case of both aquarists (Table 3).

5. Conclusion

With regard to health, it is important that many NTM species classified as potentially pathogenic survive not only in the water environment, but also in infected fish. Contact zoonosis may occur particularly in risk groups such as aquaculture and fishery professionals, fish processors, ornamental fish hobbyists, and also consumers. The diagnosis of infections is usually hampered by the unfamiliarity of clinicians with disease agents derived from aquatic species. Fish tank exposure is the source of most cases of cutaneous *M. marinum* infections. It should be considered as a possible threat to humans reporting close contact with this environment and may be prevented by the use of waterproof gloves by persons with acute or chronic open skin lesions.

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Research Article

Mutations in the *embB* Gene and Their Association with Ethambutol Resistance in Multidrug-Resistant *Mycobacterium tuberculosis* Clinical Isolates from Poland

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Ethambutol (EMB) continues to be used as part of a standard drug regimen for the treatment of tuberculosis (TB). Mutations in the *embB* gene and those within its conserved EMB resistance determining region (ERDR) in particular have repeatedly been associated with resistance to EMB in *Mycobacterium tuberculosis*. The aim of this study was to examine the mutational "hot spots" in the *embB* gene, including the ERDR, among multidrug-resistant (MDR) *M. tuberculosis* clinical isolates and to find a possible association between *embB* mutations and resistance to EMB. An 863-bp fragment of the *embB* gene was sequenced in 17 EMB-resistant and 33 EMB-susceptible MDR-TB isolates. In total, eight *embB* mutation types were detected in 6 distinct codons of 27 (54%) *M. tuberculosis* isolates. Mutations in codon 306 were most common, found in both EMB-resistant (9) and EMB-susceptible (11) isolates. Only mutations in codons 406 and 507 were found exclusively in four and one EMB-resistant isolates, respectively. Sequence analysis of the ERDR in the *embB* gene is not sufficient for rapid detection of EMB resistance, and the codon 306 mutations are not good predictive markers of resistance to EMB.

1. Introduction

One of the greatest challenges in the fight against tuberculosis (TB) has been the emergence and spread of drug-resistant (DR) and multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis*. The development of new molecular techniques targeting specific molecular mutations associated with drug resistance creates a valuable adjunct to conventional drug susceptibility testing (DST) for *M. tuberculosis*. These techniques can be performed directly on clinical samples without a culturing step and thus allowing a reliable diagnosis of drug-resistant TB to be achieved as fast as within a 24-hour period.

Ethambutol (EMB), an arabinose analogue, is a bacteriostatic, antimycobacterial drug, which has been used for the treatment of TB since the mid-1960s. The drug is routinely recommended for the intensive phase of TB therapy, as part of a four-drug regimen, including isoniazid (INH), rifampicin (RMP), and pyrazinamide (PZA) [1]. Disturbingly, almost 4% of all *M. tuberculosis* clinical isolates have been shown to display resistance to EMB [2].

Ethambutol appears to target the cell wall of tubercle bacilli through interfering with arabinosyl transferases, encoded by the *embCAB* operon, comprised of three homologous genes, designated *embC, embA*, and *embB*, and involved in the biosynthesis of arabinogalactan and lipoarabinomannan, the key structural components of the mycobacterial cell wall. The proposed scenario of EMB action on *M. tuberculosis* is that upon interaction with the EmbCAB proteins EMB inhibits the arabinan synthesis leading to a lack of arabinan receptors for mycolic acids and accumulation of mycolic acids results in cell death [3]. Resistance to EMB has repeatedly been associated with alterations in the *embB* gene, particularly in *embB* codon 306, referred to as EMB resistance

determining region (ERDR). Sequence analysis of the ERDR has been considered a rapid screening tool for detection of resistance to EMB [4–6]. Several allelic exchange studies have demonstrated that mutations in codons *embB*306, *embB*406, and *embB*497 are responsible for low and moderate levels of EMB resistance [7, 8]. However, this correlation is uncertain because all these codons have also been found mutated in isolates susceptible to EMB [9–12].

The aim of this study was to examine mutational "hot spots" in the *embB* gene, including the ERDR region, among MDR *M. tuberculosis* clinical isolates from Poland and to find a possible association between *embB* mutations and resistance to EMB.

Part of the results of this study was presented as a poster (A-527-0001-03736) at the 5th Congress of European Microbiologists (FEMS 2013), Leipzig, Germany, July 21–25, 2013.

2. Material and Methods

2.1. Strains and Drug Susceptibility Testing. A total of 50 *M. tuberculosis* strains isolated from 46 unrelated adult patients (40 men and 6 women; age range: 31 to 79 years; median age: 50.5 years) with pulmonary MDR-TB were included in this study. The isolates were collected at the National Tuberculosis and Lung Diseases Institute in Warsaw during the 3rd national survey on DR-TB throughout 2004 and represented all MDR-TB cases in Poland in that year [13]. Primary isolation, culturing, and species identification of the isolates were done according to standard mycobacteriological procedures, described elsewhere [14]. Resistance determination for first-line anti-TB drugs was performed by using the proportion method on Löwenstein-Jensen (LJ) medium [14]. The critical concentration used for EMB was 2 μg/mL. The *M. tuberculosis* H37Rv reference strain served as a quality control for EMB susceptibility testing.

2.2. DNA Extraction and Amplification. Genomic DNA was extracted from *M. tuberculosis* cultures on LJ slants by using the cetyl-trimethyl ammonium bromide (CTAB) method [15]. For EMB resistance, a 863-bp fragment of the *embB* gene was PCR-amplified, with the oligonucleotide primers embBF (5′-CGACGCCGTGGTGATATTCG-3′) and embBR (5′-CCACGCTGGGAATTCGCTTG-3′) and directly sequenced. Amplification reactions were performed in a final volume of 50 μL containing 1x TopTaq buffer PCR, 1.25 U of TopTaq DNA polymerase (Qiagen), 0.2 μM of each primer, 200 μM of each dNTP and 10 ng of DNA template. After initial denaturation at 94°C for 3 min, the reaction mixture was run through 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 50 s, followed by a final extension at 72°C for 5 min. Amplified fragments were separated by electrophoresis at 3.5 V/cm in 1% agarose gels in 0.5x TBE buffer and visualized by staining with ethidium bromide (0.5 μg/mL) and exposure to UV light (λ = 320 nm).

2.3. Amplicon Sequence Analysis. Purified PCR amplicons (Clean-Up, A&A Biotechnology) were sequenced by using the BigDye ver. 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems) in the ABI 3130xl Genetic Analyzer (Applied Biosystems). Sequencing was done in both directions using the same forward and reverse primers as those used in the PCR. Sequence data were assembled and analysed with the ChromasPro (ver. 1.7.1) software (Technelysium). The presence of mutations was determined by comparing the obtained sequences with the *M. tuberculosis* reference strain H37Rv sequence of *embB* from GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using the BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/).

2.4. Nucleotide Sequence Accession Numbers. The sequences with novel mutations were deposited in GenBank under the following accession numbers: KF694753 (Met306Ile, Arg507Gly), KF694754 (Leu413Pro), and KF694755 (Glu504Gln).

3. Results

Of the 50 MDR-TB isolates under the study, 17 (34%) were resistant to EMB, as measured by the proportion method.

In total, eight *embB* mutation types were detected in 6 distinct codons of 27 (54%) *M. tuberculosis* isolates tested. Thirteen (76.5%) EMB-resistant isolates and 14 (42.4%) EMB-susceptible isolates carried mutations in the analyzed *embB* region. An amino acid change at codon 306 was the most frequent and occurred in 20 (40%) isolates (i.e., in 9/17 EMB-resistant and in 11/33 EMB-susceptible isolates). The Met306Val substitution resulting from an A → G transition at nucleotide position 916 was detected in 4 EMB-resistant and 3 EMB-susceptible isolates, while the Met306Ile substitution, due to either a G → A transition or a G → C transversion at nucleotide position 918, was detected in 5 resistant and 8 susceptible isolates, respectively. The second most common amino acid change was Gly406Ala caused by a transition G → C at nucleotide position 1217. This alteration was found exclusively in 4 EMB-resistant isolates. Only one isolate (EMB-resistant) had a double mutation in the analysed region: G → A at position 918 (Met306Ile) and A → G at position 1519 (Arg507Gly). Other point mutations were identified only in EMB-susceptible isolates and were as follows: T → C at position 1238 (Leu413Pro), A → G at position 1490 (Gln497Arg), and G → C at position 1510 (Glu504Gln). A detailed summary of the sequencing results is provided in Table 1.

4. Discussion

Although EMB has been used for the treatment of TB for over 40 years, the molecular mechanisms of EMB resistance still remain poorly understood. Previous studies have correlated the EMB resistance phenotype with mutations in genes of the *embCAB* operon, most notably in the *embB* gene. Mutations at codon position 306 of the *embB* gene have been found to occur most frequently. The high detection rates of mutations at codon *embB*306 among EMB-resistant *M. tuberculosis* isolates were reported from Korea (47%) [16], China (55%)

Table 1: Mutations detected in 50 MDR *M. tuberculosis* isolates under the study.

Mutation[a]		No. (%) of isolates with detected mutation	
Nucleotide	Amino acid	EMB-resistant (n = 17)	EMB-susceptible (n = 33)
A → G (916)	Met → Val (306)	4 (23.5)	3 (9.1)
G → A (918)	Met → Ile (306)	3 (17.6)	5 (15.1)
G → C (918)	Met → Ile (306)	1 (5.9)	3 (9.1)
G → C (1217)	Gly → Ala (406)	4 (23.5)	—
T → C (1238)*	Leu → Pro (413)	—	1 (3)
A → G (1490)	Gln → Arg (497)	—	1 (3)
G → C (1510)*	Glu → Gln (504)	—	1 (3)
G → A (918) and A → G (1519)*	Met → Ile (306) and Arg → Gly (507)	1 (5.9)	—

[a]Numbers in brackets indicate nucleotide positions and amino acid residue positions; *novel mutation.

[17], Cuba and the Dominican Republic (70%) [12] and also from countries neighboring to Poland, such as Russia (48%) [18] and Germany (68%) [5]. The role of the *embB*306 alterations in creating resistance of tubercle bacilli to EMB was confirmed by allelic exchange mutagenesis [19]. Additionally, strains with the Met306Ile substitution were found to have lower MICs of EMB (20 μg/mL) than strains with Met306Val or Met306Leu replacements (40 μg/mL) [20]. However, a number of reports have indicated that only less than 35% of EMB-resistant *M. tuberculosis* isolates harbored mutations in the *embB* codon 306 [21, 22]. Moreover, mutations in codon *embB*306 have been found also in *M. tuberculosis* strains susceptible to EMB, and the frequencies of those mutations in EMB-susceptible strains approached those among EMB-resistant strains [9, 11].

The results of this study are in line with previous findings, showing mutations in *embB*306 codon to predominate (40% of all MDR-TB isolates) and to occur at higher frequency in EMB-resistant than EMB-susceptible isolates (53% versus 33%).

Studies on EMB resistance showed that mutations in the *embBAC* gene cluster, outside *embB* codon 306, do occur but are quite rare. Only two other substitutions, found in *embB* codons 406 and 497, have been consistently associated with EMB resistance. The percentage of *embB*406 mutations among EMB-resistant isolates is rather low, usually not exceeding 10%, whereas mutations in codon *embB*497 are twice as frequent [12, 17, 21].

Allelic exchange experiments performed at codons 406 and 497 of the *embB* gene have concluded that point mutations at these codons only slightly increase resistance to EMB [7]. Interestingly, mutations in codons 406 and 497 have—similarly to mutations in codon 306—been identified also in *M. tuberculosis* strains susceptible to EMB [17, 23]. In our study mutations at *embB*406 were found exclusively in 4 (23%) EMB-resistant strains, whereas a single mutation at *embB*497 was found only in an EMB-susceptible isolate. Mutations at codons other than 306, 406, and 497 were identified only in two EMB-susceptible and one EMB-resistant isolates.

Three novel mutations in the examined fragment of the *embB* gene were observed in this study. The sequence variations in codons 504 and 507 have already been described before in EMB-resistant isolates, yet the amino acid replacements were different from those observed here [24]. The substitution at codon 413 is reported for the first time. Of the three new mutations described in this work, only that in codon 507 may have an impact on EMB resistance, since it was found in an EMB-resistant isolate. Yet, the extent of this impact was masked by the cooccurrence of the *emb306* change in that isolate.

More than a half (54%) of MDR-TB clinical isolates tested had mutations in the examined region of the *embB* gene. These mutations occurred nearly twice as frequently in EMB-resistant than EMB-susceptible isolates (76.5% versus 42%).

The high frequency of *embB* mutations with no association between the presence of mutation and EMB-resistant phenotype can be explained by the fact that mutations in the *embB* gene occur significantly more frequently in MDR than EMB-monoresistant strains [9, 25, 26]. Several studies have demonstrated a strong association between *embB*306 mutations and resistance to INH or RMP, or MDR phenotype [19, 25, 26]. It has been suggested that *embB*306 mutations may have selective advantage upon treatment with multiple drugs. In other words, these mutations inhibit the synergistic effect of anti-TB drugs when used in combination [19]. The molecular mechanism behind this phenomenon can only be speculated and may involve changes in the cell wall permeability as a result of *embB*306 mutations [19].

Another possible explanation for the lack of correlation between the *embB* gene alterations and EMB resistance may relate to a cumulative effect of multiple mutations on the development of EMB resistance. Acquisition of resistance to EMB is thought to be a gradual process that may involve numerous genes [3, 27]. Strains bearing *embB* mutations are susceptible to EMB because these mutations alone are not sufficient to generate EMB resistance unless accompanied by alterations in other genetic loci. Recently, Safi et al. have shown that mutations in the *embB, embC*, *Rv3806c*, and *Rv3792* genes, involved in the decaprenylphosphoryl-β-D-arabinose (DPA) biosynthetic and utilization pathway, produce a wide range of ethambutol MICs by interacting in different ways and that the acquisition of EMB resistance does not occur in a single step but requires a multistep process [28].

Finally, conclusions concerning EMB resistance can be inaccurate because of the false-negative DST results, and thus importance of mutations in the *embB* gene can be underestimated. Quite often, the MIC values for EMB have varied depending upon culture medium, strain condition, or the DST method used [29]. The results of previous studies have

shown that EMB resistance can indeed be phenotypically missed by routine laboratory procedures [30, 31].

5. Conclusions

Despite the limitations of the study in terms of size and time frame of the sample, our results confirm previous observations that sequencing of the ERDR within the *embB* gene is not sufficient for rapid detection of EMB resistance and that the codon 306 mutations are not good markers for the prediction of resistance to EMB. Analysis of other genetic loci is needed for the identification of more specific mutations associated with EMB resistance.

Ethical Approval

The study was approved by the Ethics Committee at the National Tuberculosis and Lung Diseases Research Institute.

Acknowledgment

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Research Article

Correlation of N-Acetyltransferase 2 Genotype with Isoniazid Acetylation in Polish Tuberculosis Patients

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Isoniazid (INH), a key agent in the treatment of tuberculosis (TB), is metabolized primarily by the genetically polymorphic N-acetyltransferase 2 (NAT2) enzyme. Patients treated with INH can be classified as fast, intermediate, and slow acetylators. The objective of this study was to explore the relationship between NAT2 genotypes and the serum concentrations of INH. Blood samples from 130 patients were taken for the analysis, and plasma INH concentrations were determined by using the high-performance liquid chromatography (HPLC) technology. Acetylation genotype was determined on genomic DNA by using an allele-specific polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) assay. Once the NAT2 genotypes were established, patients were classified into three categories: fast, intermediate, and slow acetylators. Of the 130 patients studied, 84 (64.6%) were slow, 39 (30%) were intermediate, and 7 (5.4%) were fast acetylators. Analysis of INH concentrations in the blood of patients receiving the approximate doses of the drug revealed that, at the time intervals examined, the average concentration of INH was 2- to 7-fold higher among slow acetylators compared to fast and intermediate acetylators. *Conclusion*. Determining mutations in the NAT2 gene enabled the identification of the INH acetylation type in patients and the genotyping results were consistent with the phenotype determined by methods of measurement of drug bioavailability.

1. Introduction

Isoniazid (INH) is an essential component of all current chemotherapeutic regimens used for the treatment of tuberculosis (TB). INH is not only highly effective against *Mycobacterium tuberculosis*, but is also widely affordable, inexpensive, and well tolerated. The major pathway for metabolizing INH involves acetylation to acetylisoniazid by a noninducible hepatic and intestinal enzyme, N-acetyltransferase (NAT). The rate of acetylation is constant in every individual but varies between patients. The human population is divided into three different phenotypic groups according to acetylation rate, that is, slow, intermediate, and fast acetylators.

INH is an easily digestible and well-absorbed drug, and maximum plasma concentrations occur 2 hours after oral intake. INH is metabolized internally to acetylisoniazid by NAT, which is then hydrolyzed to acetylhydrazine. Acetylhydrazine can be transformed into diacetylhydrazine by a process of acetylation or oxidized by cytochrome P-4502E1 (CYP2E1) to form hepatotoxic compounds. Low NAT activity increases the risk of hepatic damage because the majority of acetylhydrazine is oxidized [1, 2]. Depending on its serum concentration, INH can be both an inducer or an inhibitor of CYP2E1. High concentrations of INH repress the CYP2E1 activity, whereas low INH concentrations induce the enzyme [1]. The most severe hepatocellular damage during INH treatment is linked to an excess of hydrazine to which acetylhydrazine is hydrolyzed by amidase. The hepatotoxic potential of INH is greatly influenced by the enzymatic isoform of NAT. Low NAT activity (free isoform) increases the risk of hepatocellular damage due to oxidation of acetylhydrazine by CYP2E1 (Figure 1).

N-Acetyltransferase 2 (NAT2) exhibits genetic polymorphisms. Different acetylation phenotypes within a population are the result of mutations in the NAT2 gene. These mutations influence the activity (leading to either high or low activity) of the NAT enzyme (slow and fast acetylators, resp.) [3]. The NAT2 gene, located on chromosome 8p22, is autosomal

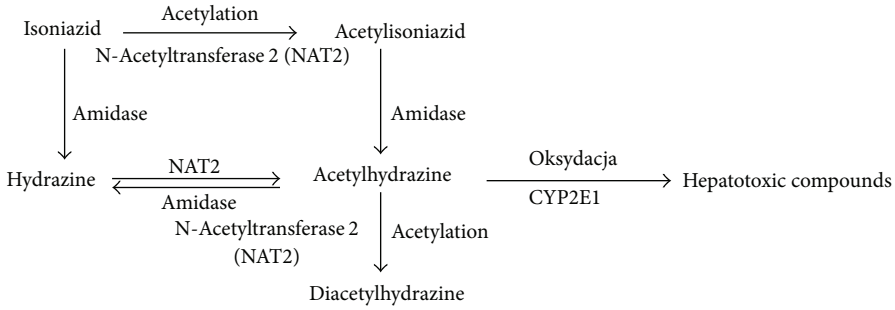

Figure 1: Schematic of the biotransformation of isoniazid [1].

dominant and intronless, with a single open reading frame of 870 bp. NAT2 enzyme detoxifies and inactivates drugs and xenobiotics in the liver. Polymorphisms of NAT2 confer slow, intermediate, or fast acetylator phenotypes with broad interethnic variations. There are currently known 53 NAT2 alleles, and each allelic variant reflects a combination of one, two, three, or four nucleotide substitutions. Within the coding region, there are seven missense mutations (G191A, T341C, A434C, G590A, A803G, A845C, and G857A) and four silent mutations (T111C, C282T, C481T, and C759A) [4]. The wild type NAT2*4 allele is associated with the fast acetylator phenotype and does not have any nucleotide substitutions. The acetylation phenotype can be predicted with 95% accuracy by genotyping [5].

The aim of this study was to explore the relationship between the NAT2 genotype and the INH acetylation phenotype.

2. Materials and Methods

The present study was conducted at the National Tuberculosis and Lung Diseases Research Institute on 130 adult TB patients treated at the Mazovian Center for Lung Diseases and Tuberculosis in Otwock, Poland, over a 2-year period (i.e., from 2006 to 2007). All patients received INH orally (200, 250, or 300 mg per day). Doses were calculated per kg of body weight. Patients were prohibited from ingesting food for 3 hours after INH intake.

Patient information, including name, age, and weight, was obtained, and blood samples were collected at 0, 1, 3, and 6 hours after administration of INH.

The study protocol was approved by the Internal Ethics Committee of the National Tuberculosis and Lung Diseases Research Institute. The aim of the study was fully explained to each patient, and the written informed consent was obtained from the participants.

2.1. Genotyping. Blood samples from 130 patients were drawn into sterile tubes (PAXgene Blood DNA, Qiagen), and total genomic DNA was extracted using the Blood DNA kit. To identify the three NAT2* mutations, C481T (NAT2*5), G590A (NAT2*6), and G857A (NAT2*7), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed following the method described by Spurr et al. [6], with some modifications. Briefly, after the initial amplification, PCR products were digested with KpnI, DdeI, TagI, and BamHI. The NAT2*5 mutant allele was identified by the loss of a KpnI restriction site and the gain of a DdeI site, NAT2*6, by the loss of a TagI site, and NAT2*7, by the loss of a BamHI site. Restriction digests were separated by electrophoresis on 8% polyacrylamide gels.

Patients were classified into three groups: fast acetylators (homozygous for wild type NAT2*4 allele), intermediate acetylators (heterozygous for NAT2*4 and a mutant allele), and slow acetylators (a combination of mutant alleles).

2.2. Measurement of INH Concentration

Chromatographic Method. Plasma concentrations of INH were measured by high-performance liquid chromatography (HPLC), as described by Seifart et al. [7]. Plasma samples were deproteinized with trichloroacetic acid. After centrifugation at 10,000 RPM for 10 min, 200 μL of the deproteinized sample was combined with 20 μL water and 40 μL 1% cinnamaldehyde. After 10 min at room temperature, the sample was eluted with solvent A (50 mM KH_2PO_4) and solvent B (acetonitrile-isopropanol, 4 : 1) on a C_8 250 mm column and detected at 340 nm. Two criteria were used to determine the INH acetylation phenotype: index of acetylation (I_3) (boundary value of 0.65) [8] and Armstrong's criteria, which is the plasma concentration of INH 6 hours after administration of the drug (C_6) (boundary value of 0.8 μg/mL) [9].

2.3. Pharmacokinetic and Statistical Analysis. Pharmacokinetic parameters were determined using a one-compartment open model with the Pharm/PCS program using the INH concentrations determined in this study [10, 11]. The AUC (AUC_{0-6}, the area below the concentration curve over time in μg/mL/h) was calculated as the sum of triangles and trapezoids. The K_e (fixed speed of elimination 1/h) was calculated by a computer program, and the $T_{0,5}$ (time of biological half-life in h) was determined by dividing 0.693 by K_e. For statistical analyses, the paired Student's t-test was used, with statistical significance defined as $P < 0.01$.

3. Results

There were 9 different NAT2 genotypes among the 130 TB patients examined. Seven (5.4%) patients with a NAT2*4/*4 genotype were classified as fast acetylators; 39 (30%) patients

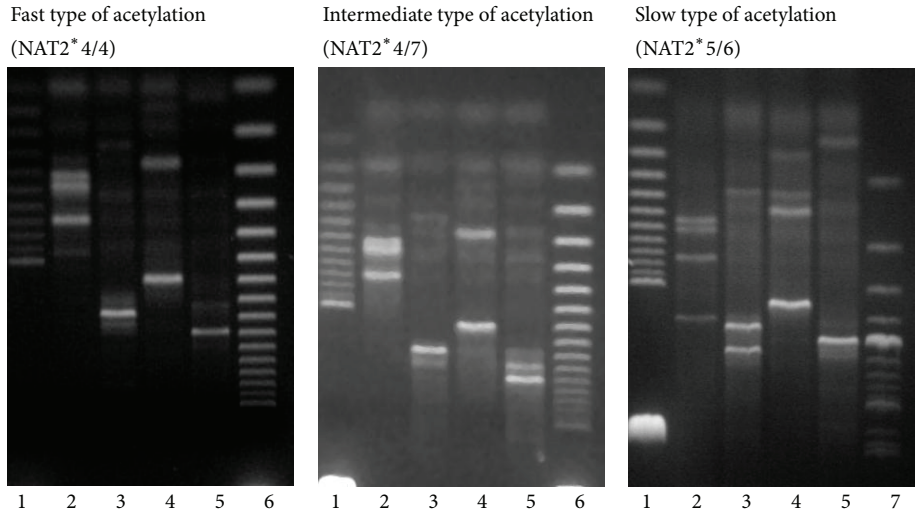

Figure 2: Identification of N-acetyltransferase 2 alleles. Restriction analysis of PCR product (lane 1: molecular size marker 25 bp, lane 2: Tag1, lane 3: Kpn1, lane 4: Dde1, lane 5: BamH1, lane 6: molecular size marker 50 bp, and lane 7: molecular size marker 100 bp).

Table 1: The INH concentration at 3 hours after drug administration among various NAT2 genotypes.

Genotype	Number	%	INH (μg/mL)	AUC_{0-6}
NAT2∗4/∗4	7	5,4	1,2 ± 0,6	8,55 ± 4,6
wt/wt	7		1,2 ± 0,6	8,55 ± 4,6
NAT2∗4/∗5	25	19,2	2,3 ± 1,2	15,7 ± 6,2
NAT2∗4/∗6	13	10	2,1 ± 1,4	14,7 ±6,3
NAT2∗4/∗7	1	0,8	1,2	15
wt/m	39	30	2,2 ± 1,3	15,4 ± 6,1
NAT2∗5/∗5	22	16,9	4,1 ± 1,2	23,5 ± 6,1
NAT2∗5/∗6	45	34,6	4,2 ± 1,5	24,5 ± 7,6
NAT2∗6/∗6	10	7,7	4,4 ± 1,0	25,9 ± 5,8
NAT2∗6/∗7	5	3,8	4,6 ± 1,7	25,9 ± 7,8
NAT2∗7/∗7	2	1,5	7,7 ± 1,1	44,1 ± 7,1
m/m	84	64,6	4,4 ± 1,5	25,0 ± 7,5

AUC_{0-6}: area under the concentration curve during the period of 0–6 hours

with a *4/*5, *4/*6, or *4/*7 genotype were classified as intermediate acetylators; and 84 (64.6%) patients with a NAT2*5/*5, *5/*6, *6/*6, *6/*7, or *7/*7 genotype were classified as slow acetylators (Table 1, Figure 2).

The mean plasma INH concentrations of homozygous wild type (wt/wt), heterozygous (wt/m), and mutant/mutant (m/m) groups were 1.2 ± 0.6, 2.2 ± 1.3, and 4.4 ± 1.5, respectively. The INH concentration in the m/m group was significantly higher than that in both the wt/m and wt/wt groups. Analysis of the correlation between genotype and plasma drug concentration revealed that the lowest concentrations of the drug were associated with the NAT2*4/4 genotype, whereas the NAT2*7/7 genotype, which is indicative of slow acetylation, was correlated with the highest INH concentrations (Table 1).

Examination of the acetylation type, as determined by genetic and chromatographic methods, demonstrated that the parameters that best differentiated between the three types of acetylation were AUC_{0-6}, AUC_{total}, and maximum concentration of INH (C_{max}).

For the pharmacokinetic parameters, I_3, C_6, K_e, and $T_{0,5}$, no statistically significant differences were observed between the fast ($I_3 = 0.36$) and intermediate ($I_3 = 0.55$) rates of acetylation as determined by genotyping. Significant differences for these indicators of bioavailability were, however, found between slow acetylation and the other two types of acetylation (fast and intermediate) (Table 2).

4. Discussion

The process of INH acetylation is well described, and its practical and theoretical aspects were systematically studied in the early years of INH use for the treatment of TB. In 1973, a report by the World Health Organization underscored the significance of determining a patient's acetylation phenotype during INH administration, as the drug is subject to biotransformation by NAT in the liver [12]. The way the INH is acetylated in the liver is polymorphic, and three types of individuals, namely, fast, intermediate, and slow acetylators, exhibit different plasma concentrations of the drug after the administration of the same dose. In addition, the rate of the elimination of INH is different between fast and slow acetylators [13].

The activity of the polymorphic enzyme NAT2 influences the metabolism of INH, the rate of its elimination from the body, and possibly the development of toxic side effects. Consequently, the rate of INH acetylation is a potential risk factor for liver failure. Acetylation polymorphisms are associated with interindividual variability in plasma concentration and half time of INH.

A fraction of INH is eliminated in its biologically active form, with the rest excreted as inactivated acetylated metabolites. Most of the INH (75–95%) is eliminated in the urine in a metabolized form within 24 hours of ingestion [14]. Depending on the acetylation type, 3–30% of INH is eliminated in an unaltered form [15]. These proportions also depend on

Table 2: Pharmacokinetic parameters of INH in plasma among fast, intermediate, and slow acetylators by the chromatographic method.

Genotype of acetylation	Pharmacokinetic parameters of INH						
	I_3	C_6	K_e	$T_{0,5}$	C_{max}	AUC_{0-6}	AUC_{total}
Fast	0,36[a]	0,27[a]	0,51[b]	1,59[a]	3,39[a]	8,6[a]	9,2[a]
Intermediate	0,55[a]	0,70[a]	0,41[b]	1,85[a]	5,80[b]	15,4[b]	17,5[b]
Slow	0,97[b]	2,20[b]	0,27[a]	3,14[b]	7,09[c]	24,5[c]	35,5[c]

The different letters in the columns indicate significant differences (at $P \leq 0.05$) between the respective types of acetylation.
I_3: index of acetylation.
C_6: concentration of INH 6 hours after drug administration.
K_e: fixed speed of elimination 1/h.
$T_{0,5}$: elimination half time.
AUC_{0-6}: area under concentration curve in the period 0–6 hours.
AUC_{total}: area under concentration curve.

the individual acetylation capacity. INH is partially eliminated in the feces, and some of the thus eliminated fraction is broken down into as yet unknown metabolites.

The results of this study demonstrate that differences in the dynamics of INH metabolism among slow, intermediate, and fast acetylators determine the concentration of the drug and its metabolites. Therefore, the bioavailability of the drug among fast and intermediate acetylators is much lower than it is among slow acetylators, and the anti-TB therapeutic range of INH is maintained for only a short period of time. Among slow acetylators, symptoms related to the accumulation and toxic effects of the drug occur more often. Consequently, slow acetylators should be monitored to avoid side effects [16–19].

Chromatographic methods of determining plasma concentrations of INH are precise and, compared to biological methods, involve a shorter waiting period (1 day). This method is useful for both scientific purposes and for monitoring TB patients undergoing treatment. This method allows to defining the type of acetylation and precisely monitor the changes in drug concentration. Published reports suggest that the therapeutic plasma concentration of INH should range from 1 to 2 μg/mL during the first 3 hours after oral administration [20]. Here, we observed that four patients (9% of fast and intermediate acetylators) did not exhibit the proper INH concentration.

We described the variability in the pharmacokinetics of INH in TB patients after standard dosing, its demographic characteristics, and its relationship with genetic variations in NAT2. In addition, we clearly showed that the overall INH exposure is indirectly proportional to the number of highly active NAT2 alleles, consistent with findings from recent clinical trials. Carriers of the NAT2*4/*4 genotype (two high-activity NAT2 alleles) have been reported to exhibit lower INH concentrations at 3 h after dose than those with other NAT2 genotypes [21]. Parkin et al. suggested a link between the NAT2 genotypes, consisting of the recently defined 14 mutant alleles, and the acetylator phenotype in TB patients. These findings could pave the way to individualized dosage regimens. In addition, further sequence analysis of an expanded population may discover other mutations in the NAT2 gene. The main purpose of the present study was to clarify the applicability of genotyping as an alternative to therapeutic drug monitoring in the clinical setting.

Mutations in the NAT2 gene have been shown to account for the majority of the slow acetylator phenotypes among humans (NAT2*5, NAT2*6, and NAT2*7) [22]. Previous investigation of the functional characteristics of NAT2 single nucleotide polymorphisms has revealed that different molecular mechanisms underlie the slow acetylator phenotype and variations in the enzymatic activity of NAT2 between different alleles [23].

Patients with the NAT2*4/4 genotype (two high-activity NAT2 alleles) have been reported to exhibit lower INH concentrations than those with other NAT2 genotypes. Slow acetylators had two- to sevenfold higher INH concentrations at 3 and 6 h after drug administration than did fast acetylators [24].

5. Conclusions

The method of NAT2 genotyping is simple and fast. Therefore, it is suggested to be used as an alternative to therapeutic drug monitoring in clinical practice.

The data from the present study and other published reports clearly suggest that the determination of the NAT2 genotype prior to INH administration is clinically relevant for the prediction of pharmacokinetic variability and the possible adjustment of INH dosing regimens.

It must also be noted that INH will be predominantly employed in TB control programs in the developing world. Here, large numbers of patients must be managed by programs with limited resources. Under these circumstances, an individualized approach to INH dosing may not be feasible, and, thus, a compromise must be adopted.

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