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Frequency of *Mycobacterium tuberculosis* among *Mycobacterium tuberculosis* Complex Strains Isolated from Clinical Specimen

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Abstract

Background: Rapid and accurate detection of *Mycobacterium tuberculosis* (Mtb) is of primary importance for infection control and selection of anti-tuberculosis drugs. However, most clinical laboratories report Mtb complex (MTC) without reporting Mtb because MTC comprising Mtb, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium caprae*, and *Mycobacterium pinnipedii* have 99.9% similarity at the nucleotide level and identical 16S rRNA sequences. This study was conducted to analyze the species frequency of MTC isolates obtained from clinical specimens. *Methods:* Of 310 MTC isolates obtained from clinical samples in a tertiary care hospital from February 2017 to August 2018, MolecuTech Real TB-Taq (YD Diagnostics, Korea) real-time polymerase chain reaction (PCR) was performed, specifically to detect Mtb. For DNA showing Mtb negative results by Mtb-specific real-time PCR or pyrazinamide-resistant strains, PCR-based MTC typing, spacer oligonucleotide typing (spoligotyping), and exact tandem repeat D gene sequencing were performed. *Results:* All the 310 MTC isolates were identified to be Mtb. Two Mtb strains of the East-African-Indian 4-Vietnam genotype, which have not been reported in Korea, were also found. *Conclusion:* There was no zoonotic tuberculosis in this study. Since we investigated only 310 MTC isolates detected in only one medical institution, a multi-center study is needed to accurately know the prevalence of zoonotic tuberculosis in Korea.

Keywords

Mycobacterium tuberculosis complex
Sequence analysis
Spoligotyping

1. Introduction

The pathogens that cause tuberculosis (TB) in humans and animals are the *Mycobacterium tuberculosis* complex (MTC), which are phylogenetically very similar but are characterized by the fact that certain animals are hosts or are found only in certain geographical regions. The MTC include *Mycobacterium tuberculosis* (Mtb), *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium caprae*, and *Mycobacterium pinnipedii* [1,2]. Although the clinical findings of TB caused by MTC are almost similar, they differ in pathogenicity, geographic range, resistance to anti-TB drugs, transmissibility, and host preference, making it necessary to identify MTC at the strain level; however, the 16S rRNA sequence of MTC is identical and the overall gene sequence homology is greater than 99%. Therefore, laboratories using commercialized molecular diagnostics for the identification of Mtb that cannot differentiate Mtb from MTC are forced to report the results as MTC, and clinicians consider MTC-positive patients to have TB caused by Mtb and treat them with anti-TB drugs.

With the increasing interest in zoonotic TB as part of TB control measures, the importance of accurate epidemiological data on zoonotic TB has been greatly emphasized, but there are few studies on the species-specific frequency of mycobacteria belonging to MTC in Korea. Therefore, in this study, we aimed to analyze the proportion of *M. bovis* and other species among MTC detected in clinical specimens and to identify diagnostic methods useful for differentiating the species of MTC.

2. Materials and methods

2.1. AdvanSure Mycobacterium GenoBlot Assay for MTC detection

2.1.1. Study subjects and DNA extraction

From February 2017 to August 2018, Mtb cultures were referred to a tertiary care hospital, and DNA was extracted from bacteria cultured in BACTEC MGIT liquid medium (Becton Dickinson Microbiology Systems, Sparks, MD, USA) or 3% Ogawa medium (Shinyang Chemical, Seoul, Korea). For DNA extraction, if grown in Ogawa medium, one loop was removed using an inoculation loop and swirled into a 1.5 mL microtube containing 200 μL of sterile distilled water, centrifuged at 13,000 rpm for 3 min, and the supernatant was removed. If grown in MGIT medium, 1 mL of liquid medium was added to a 1.5 mL microtube, centrifuged at 13,000 rpm for 3 min, and the supernatant was removed. To the precipitate remaining after the pretreatment, 1 mL of sterile distilled water was added, mixed vigorously, centrifuged at 13,000 rpm for 3 min, and the supernatant was removed. After adding 100 μL of extraction buffer solution to each tube, it was heated at 100°C for 20 min and centrifuged at 13,000 rpm for 3 min.

This study was conducted after review by the Institutional Review Board of Yonsei University Wonju Severance Christian Hospital (IRB No. CR318323).

2.1.2. AdvanSure Mycobacterium GenoBlot Assay

The extracted DNA was tested using the AdvanSure Mycobacterium GenoBlot Assay (LG Chemistry, Seoul, Korea), which can distinguish between MTC and 20 species of nontuberculous mycobacteria [3]. PCR was performed using a GeneAmp PCR System 9700 (Thermo Fisher Scientific, MA, USA) with denaturation at 50°C for 2 min and 95°C for 10 min, followed by 15 cycles of 94°C for 30 s, 65°C for 1 min, 72°C for 30 s, and 38 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 30 s, and then 72°C for 10 min. Reverse hybridization line blot assay was performed using AdvanSure hybridizer (LG Chemistry) according to the manufacturer's manual, and the strips after the chromogenic process were analyzed using AdvanSure GenoLine Scan (LG Chemistry), an instrument that automatically analyses the pattern of the bands.

2.2. Mtb-specific real-time polymerase chain reaction

The 310 strains identified as MTC in the AdvanSure Mycobacterium GenoBlot Assay (LG Chemistry) were subjected to a real-time polymerase chain reaction (PCR) using the MolecuTech Real TB-Taq (YD diagnostics, Yongin, Korea) product, which detects Mtb by targeting region of difference (RD) sites specific to Mtb. To summarize the test method, 5 μL of DNA extracted by preparing the master mix provided in the product was placed in a PCR tube and real-time PCR was performed with the SLAN Real-Time Detection System (LG chemistry). Real-time PCR was performed at 94°C for 3 min, followed by denaturation at 94°C for 20 s and 55°C for 40 s, and the fluorescence value was repeated 40 times. If the cycle threshold (Ct) value of the internal control (FAM) was detected as 30–35 and the Ct value for Mtb (HEX) was less than 35, it was judged as Mtb positive, and if the Ct value of the internal control (FAM) alone was detected as 30–35, it was judged as Mtb negative.

2.3. PCR-based MTC typing

To screen 310 MTCs for possible non-Mtb strains, commercially available PCR tests that specifically detect Mtb were used, and only limited Mtb-negative strains and pyrazinamide-resistant strains were subjected to PCR-based MTC typing, spacer oligonucleotide typing (spoligotyping), and sequencing of exact tandem repeats.

To identify the RD deletion sites among MTC chromosomal genes to distinguish MTC, seven pairs of primers specific to the Rv0577, IS1561 (Rv3349c), Rv1510 (RD4), Rv1970 (RD7), Rv3877/8 (RD1), Rv3120 (RD12), and Rv2073c (RD9) sites were selected [4], and hybridized to four types of reference DNA [Mtb H37Rv (ATCC 25619), *M. bovis* AN5, *M. bovis* BCG (ATCC 19274), and *M. africanum* (KCTC 9504)], *M. bovis* DNA isolated from one type of cattle, and DNas from seven pyrazinamide-resistant MTC strains which were then used for PCR (**Table 1**).

PCR was performed in AccuPower Taq PCR Premix (Bioneer, Daejeon, Korea) with 10 pmol each of primers and 10 ng of DNA to a total volume of 20 μL, followed by denaturation at 94°C for 5 min and 25 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and 72°C for 10 min using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). PCR amplification products were electrophoresed on a 2% agarose gel and

Table 1. Primers used in PCR-based Mtb complex typing

Primer type and the target locus	Primer name	The nucleotide sequence (5' → 3')	Size, base pair
Rv0577	Rv0577F	ATG CCC AAG AGA AGC GAA TAC AGG CAA	786
	Rv0577R	CTA TTG CTG CGG TGC GGG CTT CAA	
IS1561 (Rv3349c)	IS1561F	GCT GGG TGG GCC CTG GAA TAC GTG AAC TCT	943
	IS1561R	AAC TGC TCA CCC TGG CCA CCA CCA TTG ACT	
Rv1510 (RD4)	Rv1510F	GTG CGC TCC ACC CAA ATA GTT GC	1,033
	Rv1510R	TGT CGA CCT GGG GCA CAA ATC AGT C	
Rv1970 (RD7)	Rv1970F	GCG CAG CTG CCG GAT GTC AAC	1,116
	Rv1970R	CGC CGG CAG CCT CAC GAA ATG	
Rv3877/8 (RD1)	Rv3877/8F	CGA CGG GTC TGA CGG CCA AAC TCA TC	999
	Rv3877/8R	CTT GCT CGG TGG CCG GTT TTT CAG C	
Rv3120 (RD12)	Rv3120F	GTC GGC GAT AGA CCA TGA GTC CGT CTC CAT	404
	Rv3120R	GCG AAA AGT GGG CGG ATG CCA GAA TAG T	
Rv2073c (RD9)	Rv2073cF	TCG CCG CTG CCA GAT GAG TC	600
	Rv2073cR	TTT GGG AGC CGC CGG TGG TGA TGA	

Abbreviation: Mtb, *Mycobacterium tuberculosis*; IS, insertion sequence; RD, region of difference.

images were read with a Gel Doc system (Bio-Rad, Hercules, CA, USA).

2.4. Spoligotyping

Spoligotyping was performed using a spoligotyping strip (M&D Inc., Wonju, Korea) with four reference DNAs [Mtb H37Rv (ATCC 25619), *M. bovis* AN5, *M. bovis* BCG (ATCC 19274), *M. africanum* (KCTC9504)], DNA isolated from one bovine species, and DNAs from seven pyrazinamide-resistant MTC strains. The strips were labeled with biotin-labeled primers for the Mtb RD spacer region (**Table 2**). PCR was performed using the PCR premix provided in the kit, adding 3 μL of extracted DNA and 17 μL of distilled water, followed by denaturation at 94°C for 5 min and 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 7 min using the GeneAmpPCR System 9700 (Applied Biosystems). The amplified DNA products were subjected to reverse blot hybridization assay using spoligotyping strips I and II. The spoligotyping test process consisted of mixing the amplified DNA product with an equal amount of the denaturing solution provided in the kit and leaving it at room temperature for 5 minutes, then placing the spoligotyping strip I, II in the tray wells provided in the kit, mixing the DNA denaturing solution and 1 mL of the hybridization solution well, spraying the strip, and hybridizing at 60 rpm for 30 minutes in a 60℃-water bath. After removing all the solution in the wells, 1 mL of washing solution was dispensed and washed twice for 10 min at 60 rpm in a 60℃ constant temperature water bath, then 0.5 μL of conjugate (alkaline phosphatase) was diluted in 1 mL of conjugate dilution solution, dispensed, and reacted for 30 min at 60 rpm at room temperature. After two washes, a diluted NBT/BCIP solution (1:50) was added for 10 min of development. Finally, after two washes with distilled water, the strips were dried and read.

For the reading method, the direct repeat spacer region probes of 43 MTCs were applied along the lines of the data sheet, and the pattern was marked with 'n' for colored and 'o' for non-colored. For the analysis of the results, the data patterns were converted into binary code and octal code, and the shared international type (SIT) number and phylogeny were analyzed using the SITVIT Database website of Institut Pasteur de la Guadeloupe, SpolDB4, and http://www.mbois.org.

2.5. MTC exact tandem repeat D sequencing

The MTC-specific exact tandem repeat D (ETR-D) assay was sequenced to determine the diversity of tandem repeats in MTC [5,6]. PCR was performed using primers specific for ETR-D (**Table 2**) in AccuPower Taq PCR Premix (BIONEER) with 10 pmol of each primer, 10 ng of DNA in a total volume of 20 μL using a GeneAmp PCR System 9700 (Applied Biosystems) and denatured at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 60°C for 20 s, 72°C for 30 s, and 72°C for 3 min.

PCR amplification products were electrophoresed on a 2% agarose gel and images were read with a Gel Doc system (Bio-Rad). PCR for sequencing was

Table 2. Primers used in spoligotyping and exact tandem repeat D sequencing

Primer type and the target locus	Primer name	The nucleotide sequence (5' → 3')	Size, base pair
Spoligotyping			
DR	DRa	GGT TTT GGG TCT GAC GAC	Variable
	DRb	CCG AGA GGG GAC GGA AAC	
ETR-D sequencing			
ETR-R	ETRDF	GTT GAT CGA GGC CTA TCA CG	Variable
	ETRDR	GAA TAG GGC TTG GTC ACG TA	

Abbreviation: DR, direct repeat; ETRDF, exact tandem repeat D forward; ETRDR, exact tandem repeat D reverse.

performed by mixing 2 μL of Exo-SAP with 4 μL of PCR product and reacting at 37°C for 30 min and 85°C for 15 min. The purified PCR product was diluted and denatured at 95°C for 1 min, followed by 25 cycles of 95°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing PCR products were purified with ethanol and examined on a 3500 Genetic Analyzer (Applied Biosystems).

3. Results

3.1. Advansure Mycobacterium GenoBlot Assay for MTC Detection

If the AdvanSure Mycobacterium GenoBlot Assay (LG Chemistry) reported MTC during the study period and the same result was found in the same person, only the first result was analyzed. The median age of the 310 subjects was 71 years, with 51.6% aged 70 years or older. The gender was 58.7% male and 41.3% female, with 93.5% respiratory specimens (**Table 3**).

Table 3. Characteristics of 310 *Mycobacterium tuberculosis* complex cases in the clinical specimens

Variables	*M. tuberculosis* complex no. (%)
Age, years	
0–9	1 (0.3)
10–19	5 (1.6)
20–29	11 (3.6)
30–39	13 (4.2)
40–49	36 (11.6)
50–59	53 (17.1)
60–69	31 (10.0)
≥ 70	160 (51.6)
Sex	
Male	182 (58.7)
Female	128 (41.3)
Specimen	
Sputum	141 (45.5)
Bronchial washing	140 (45.2)
Pleural fluid	9 (2.9)
Urine	3 (1.0)
Cerebrospinal fluid	2 (0.6)
Colon	2 (0.6)
Lymph node	2 (0.6)
Others	11 (3.6)
Pyrazinamide susceptibility	
Resistant	7 (2.3)
Susceptible	303 (97.7)

3.2. Mtb-specific real-time PCR

Using the MolecuTech Real TB-Taq (YD diagnostics) test, 307 out of 310 cases of MTC were Mtb-positive and 3 cases were Mtb-negative with only Ct values of 30–35 for the internal control (FAM). When tested with standard DNA, Mtb H37Rv and *M. bovis* AN5 were Mtb positive, while *M. bovis* BCG and *M. africanum* were Mtb negative.

3.3. PCR-based MTC typing

In the PCR-based MTC typing test, Mtb H37Rv was confirmed positive at Rv0577, IS1561 (Rv3349c), Rv1510 (RD4), Rv1970 (RD7), Rv3877/8 (RD1), Rv3120 (RD12), and Rv2073c (RD9). *M. bovis* AN5 and *M. bovis* isolated from cattle were positive for Rv0577, IS1561, and RD1 and negative for RD4, RD7, RD12, and RD9. *M. bovis* BCG was positive for Rv0577 and IS1561 and negative for RD4, RD7, RD1, RD12 and RD9. *M. africanum* was positive for Rv0577, IS1561, RD4, RD1, and RD12, and negative for RD7 and RD9. All three isolates that were Mtb-negative by Mtb-specific real-time PCR and all seven isolates of pyrazinamide-resistant MTC were Mtb positive for Rv0577, IS1561, RD4, RD7, RD1, RD12, and RD9 (**Table 4**).

3.4. Spoligotyping

By spoligotyping pattern analysis, Mtb H37Rv among the standard DNA was read as T-H37Rv (SIT 451), *M. bovis* AN5 and *M.bovis* BCG were read as BOV_1 (SIT 482), *M. africanum* was read as AFRI_1 (SIT 181), and *M. bovis* isolated from cattle was identified as BON_2 (SIT 683). Of the three strains that were Mtb-negative

by Mtb-specific real-time PCR, one was T1 (SIT 520) and two were analyzed as East-African-Indian 4-Vietnam (EAI4-VNM, SIT 564), confirming that all were Mtb. The two patients with the EAI4-VNM sub-lineage were Vietnamese (**Table 5**).

3.5. MTC exact tandem repeat D sequencing

ETR-D analysis, performed to determine the tandem repeat diversity of MTC, identified three repeats of 77 base pairs in Mtb H37Rv. *M. bovis* AN5 and *M. bovis* isolated from cattle showed three repeats of 77 base pairs and one repeat of 53 base pairs for a total of four repeats, whereas *M. bovis* BCG showed two repeats of 77 base pairs for a total of two repeats, confirming that *M. bovis* and *M. bovis* BCG have different numbers of repeats. Among the three strains that were Mtb-negative by Mtb-specific real-time PCR, spoligotyping revealed EAI4-VNM (SIT 564) in two cases, one case

Table 4. PCR-based Mtb complex typing

Organisms (No.)	PCR-based Mtb complex typing						
	Rv0577	IS1561	Rv1510 (RD4)	Rv1970 (RD7)	Rv3877/8 (RD1)	Rv3129 (RD12)	Rv2073c (RD9)
Mtb H37Rv	+	+	+	+	+	+	+
M. bovis AN5	+	+	-	-	+	-	-
*M. bovis**	+	+	-	-	+	-	-
M. bovis BCG	+	+	-	-	-	-	-
M. africanum	+	+	+	-	+	+	-
Isolates (3)†	+	+	+	+	+	+	+
Isolates (7)‡	+	+	+	+	+	+	+

*Isolate from cattle. †Isolates were positive in AdvanSure Mycobacterium GenoBlot Assay but negative in *M. tuberculosis*-specific real-time PCR. ‡Pyrazinamide-resistant strains. Abbreviation: Mtb, Mycobacterium tuberculosis; IS, insertion sequence; RD, region of difference; BCG, Bacillus Calmette–Guérin.

Table 5. Spoligotyping-based Mtb complex differentiation

Species / Specimen No.	Octal value	SIT	Clade (strain)	Spoligotype pattern
Mtb H37Rv	777777477760771	451	T-H37Rv	■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■■■□□□□■■■■■■■
M. bovis AN5	676773777777600	482	BOV_1	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■□□□□□
M. bovis	664073777777600	683	BOV_2	■■□■■□■□□□□□■■■□■■■■■■■■■■■■■■■■■■■■■■□□□□□
M. bovis	676773777777600	482	BOV_1	■■□■■■■■□■■■■■■□■■■■■■■■■■■■■■■■■■■■■■□□□□□
M. africanum	770777777777671	181	AFRI_1	■■■■■■□□□■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■■
Y1*	777777777760571	520	T1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■
Y2*	777737774413771	564	EAI4-VNM	■■■■■■■■■■■■□■■■■■■■■■■■□□■□□□□■□■■■■■■■■■
Y3*	777737774413771	564	EAI4-VNM	■■■■■■■■■■■■□■■■■■■■■■■■□□■□□□□■□■■■■■■■■■
P1†	777737777760771	37	T3	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■
P2†	000000000003771	1	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■
P3†	000000000003771	1	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■
P4†	000000000003771	1	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■
P5†	000000000003771	1	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■
P6†	000000000003771	1	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■
P7†	000000000003771	1	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■
S1‡	777777777763771	54	Manu2	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■
S2‡	000000000003771	1	Beijing	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■

*Y1–3, *Mycobacterium tuberculosis*-negative strains for real-time PCR. †P1–7, pyrazinamide-resistant strains. ‡S1–2, *M. tuberculosis*-positive strains for real-time PCR. Abbreviation: Mtb, *M. tuberculosis*; SIT, shared international type; EAI4-VNM, East-African-Indian 14-Vietnam; BCG, Bacillus Calmette–Guérin.

each showed nine repeats of 77 base pairs and one repeat of 53 base pairs (10 repeats in total) and another case each showed seven repeats of 77 base pairs and one repeat of 53 base pairs (8 repeats in total), while one case analyzed by spoligotyping as T1 (SIT 520) showed the same repeats of two repeats of 77 base pairs and one repeat of 53 base pairs (3 repeats in total) (**Table 6**).

Table 6. Sequencing-based Mtb complex differentiation for exact tandem repeat D

Organisms	Total no. of ETR-D
Mtb H37Rv	3 (3 × 77 bp)
M. bovis AN5	4 (3 × 77 bp, 1 × 53 bp)
*M. bovis**	4 (3 × 77 bp, 1 × 53 bp)
M. bovis BCG	2 (2 × 77 bp)
M. africanum	4 (3 × 77 bp, 1 × 53 bp)
Isolate no. 1†	3 (2 × 77 bp, 1 × 53 bp)
Isolate no. 2†	10 (9 × 77 bp, 1 × 53 bp)
Isolate no. 3†	8 (7 × 77 bp, 1 × 53 bp)

*Isolate from cattle. †Isolate was positive in AdvanSure Mycobacterium GenoBlot Assay but negative in *Mycobacterium tuberculosis*-specific real-time PCR. Abbreviation: MTB, *Mycobacterium tuberculosis*; ETR-D, exact tandem repeat-D; bp, base pair; BCG, Bacillus Calmette–Guérin.

4. Discussion

The World Health Organization's (WHO) Global Tuberculosis Report 2018 included *M. bovis*-borne TB as an area of concern for the first time [7]. Before this, the Roadmap for *M. bovis* TB, published jointly by WHO and the United Nations in 2017, identified 10 priorities to continue the work in a phased manner, the first of which is to collect and report complete and accurate data, and the second is to improve diagnostic methods [8]. Based on a study by Müller *et al.* based on data from 61 countries [9], the estimated incidence of acquired TB is estimated to be less than 1.4% of all TB outside of Africa. In addition, among the countries in the Western Pacific region, of which Korea is a part, only three countries had WHO data: Australia, New Zealand, and parts of China, and the estimates of the incidence of MDR-TB in these countries were 0.2%, 2.7%, and 0.2%, respectively [9]. While the WHO data on *M. bovis* are limited to specific geographical areas or cover a specific time period, there are no data reporting the frequency of *M. bovis*-induced TB in Korea.

Human tuberculosis infection with *M. bovis* is mainly caused by the consumption of unpasteurized raw milk from infected livestock or eating undercooked meat or by-products or processed foods using them, so the frequency of extrapulmonary tuberculosis is higher than that of *M. tuberculosis*, and the frequency of occurrence by the site is in the order of lymph nodes, urinary system, bones and joints, intestines and abdominal cavity, and nervous system [10]. There has been one case of infection with *M. bovis* reported in Korea, which occurred in an empyema in a woman who migrated from Vietnam in 2011 [11].

M. bovis was not detected in the genotyping of MTC in this study, but it was thought that human TB infection with *M. bovis* was unlikely due to the introduction of pasteurization of milk in Korea, and the risk of contracting bovine TB among people engaged in livestock farming was also thought to be significantly lower due to the early detection and culling of TB-infected livestock through the implementation of bovine TB eradication programs. Among the differentiation methods for MTC in this study, Mtb-specific real-time PCR products were the easiest to test and read, but there were three false negatives (0.97%), one T1, and two EAI4-VNM in spoligotyping. These false-negative results are unknown due to the lack of disclosure of the Mtb-specific RD sites targeted by the real-time PCR products but may be due to the deletion of RD sites among Mtb. RD9 deletion has been reported in some Mtbs and is thought to be particularly relevant to the RD9 deletion observed in EAI lineage [12]. There are reports that *M. bovis* AN5, which has been identified as a false positive in Mtb-specific real-time PCR products, has a different RD9 deletion pattern than other *M. bovis* [13]. The PCR results using RD9 primers in **Table 4** showed that only Mtb H37Rv was RD9 positive, whereas *M. bovis* AN5, *M. bovis* BCG, *M. bovis* isolated from cattle, and *M. africanum*

were RD9 negative, confirming the importance of primer design for RD9. Spoligotyping is a method that distinguishes Mtb from *M. bovis* much more robustly than traditional methods, and by identifying the structure and polymorphism of the direct repeat locus of the MTC, it is possible to discriminate between species within the MTC and to genotype Mtb [14,15]. In this study, we identified two EAI4-VNM sub-lineages by spoligotyping, which have never been reported in Korea. EAI4-VNM is a genotype mainly found in Vietnam, and it has been reported that 25.3%–50.7% of Mtb isolates in Vietnam are EAI4-VNM [16], and the two EAI4-VNM sub-lineage genotypes identified in this study were also detected in Vietnamese living in Korea. This study demonstrated that spoligotyping is not only the most useful test for differentiating MTC strains but also provides Mtb genotypes, which can be useful for epidemiological investigations of the disease. However, there were cases in which strains identified as belonging to the same clade (EAI4-VNM) of the same SIT (564) in spoligotyping pattern analysis but showed different repeat counts in ETR-D analysis. Since these results indicate that strains of different genotypes were read as the same genotype in spoligotyping, it was thought that two or more different tests should be used in combination to identify the prevalent genotype of Mtb and to identify the route of transmission. In addition, spoligotyping has the disadvantage that the test method and the method of analyzing the results are somewhat cumbersome and time-consuming compared to the PCR method.

Since this study investigated 310 MTCs detected in a single medical institution, future multicenter studies are needed to accurately determine the prevalence of MTCs in Korea. In addition, the development of a multiplex PCR that can easily and accurately distinguish strains belonging to MTCs in clinical laboratories will help to determine the frequency of isolation of MTCs by strain in real-time.

Disclosure statement

The authors declare no conflict of interest.

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