Epidemiological analysis of human and animal originated Mycobacterium bovis subspecies by spoligotyping and mycobacterial interspersed Repetitive Unit-Variable Number of Tandem Repeat (MIRU-VNTR) methods

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ABSTRACT

This study aims to investigate the genotypic similarities between human and animal-originated isolates by spoligotyping and 24 loci MIRU-VNTR for molecular epidemiological analysis of *Mycobacterium bovis* isolates. In this study, isolates were obtained between 2019 and 2022 from 58 humans and 50 bovines. Initially identified with the GenoType MTBC kit, all isolates were genotyped using spoligotyping and 24 loci Mycobacterial Interspersed Repetitive Unit-Variable Number of Tandem Repeat (MIRU-VNTR) methods and their lineage relationships were illustrated in the dendrogram. When subjected to the spoligotyping method, the human and animal-originated isolates were revealed eight distinct clusters and 29 different genotypes. Notably the most prevalent genotypes were SIT1118/SB0989 (19.23%), SIT482/SB0120 (16.35%), SIT685/SB0288 (12.5%) detected in both human and animal-originated isolates. SB1593 (12.5%) was exclusively identified in animal-originated isolates. Additional genotypes included SIT3529/SB0920, SIT1185/SB0897, SIT3710/SB1595, SIT688/SB0129, SIT3687/SB1625, SB0419, SB2466, SB1231, and SB2510. MIRU-VNTR analysis resulted in nine distinct clusters and 55 different genotypes. ETR-C, QUB2163b, QUB26, and Mtub04 exhibited the highest allelic diversity, while MIRU02, MIRU20, MIRU24, MIRU27, and MIRU39 did not display allelic diversity. When the molecular typing results of the 95 isolates, tested with all three methods, 93.7 % agreement was observed between methods. In conclusion, it was determined that both tests could be safely employed. The presence of similar genotypes in humans and animals underscores the potential zoonotic transmission of *Mycobacterium bovis.*

Introduction

Tuberculosis (TB) is a chronic, contagious disease caused by members of the *Mycobacterium tuberculosis* complex (MTBC). It presents various clinical symptoms, high morbidity and mortality rates, and has a global impact on both humans and animals. The MTBC comprises 11 mycobacterial species including *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium caprae*, *Mycobacterium canettii*, and *Mycobacterium microti* (13, 25). World Health Organization (WHO) recognizes Bovine TB (BTB) as one of seven neglected zoonoses that poses a serious threat to public health. In 2019, an estimated 140,000 new cases of zoonotic TB occurred with *Mycobacterium bovis* being the most common causative agent. The actual zoonotic TB burden may be higher, considering other mycobacterial species causing such infections (4, 29). Bovine TB is a notifiable disease listed

by the World Organization for Animal Health (OIE) due to its socioeconomic impact and public health significance. The OIE advocates for the control and elimination of BTB (17), often achieved through the culling of potentially infected farm animals (1). BTB affects a broad range including domestic and wild animals as well as humans with domestic cattle being the primary source. Consequently BTB is challenging to control and eradicate, and zoonotic tuberculosis cases caused by *M. bovis* have increased globally in recent years (12). The main transmission sources to humans include the consumption of unpasteurized milk, contact with the body fluids of sick animals and aerosol transmission (8, 18). Although less pathogenic than *M. tuberculosis*, *M. bovis* can cause pulmonary tuberculosis transmission among humans, especially in immunocompromised individuals. However, detailed investigations into human TB cases caused by *M. bovis* lacking (10, 24). Diagnosis of bovine tuberculosis (BTB) is effective not only in preventing disease transmission disease between animal species but also in preventing transmission from animals to humans. Humans are a potential source of *M. bovis* transmission for Tuberculosis (TB) infection to cattle (28). For this reason, determining and applying methods that diagnose zoonotic TB is crucial in controlling the spread of the disease (3, 30). In programs implemented to eradicate TB worldwide, the strains obtained are identified using molecular methods and followed epidemiologically. Molecular characterization of circulating strains is essential for BTB control (29). The genotyping methods used for this purpose have resulted in significant progress in both diagnosis and determination of drug resistance in recent years.

Spoligotyping is a fast, simple, and highly reproducible Polymerase Chain Reaction (PCR) based on reverse dot blot hybridization. The MTBC genome contains a series of well-conserved 36 base pair (bp) direct repeat (DR) locus and nonrepetitive spacer sequences between the DR loci. The strains are differentiated based on variations in the number of DRs and the presence or absence of specific spacers (2, 4-6, 11, 15, 16, 19-21, 26).

The Mycobacterial Interspersed Repetitive Unit-Variable Number of Tandem Repeat (MIRU-VNTR) method is used to determine the repeat number and size of amplicons obtained by PCR using primers that recognize regions containing MIRU loci. Forty-one different VNTR regions in the MTBC genome, ranging from 50–100 bp, are considered MIRU loci. The MIRU-VNTR method has high specificity and reproducibility between laboratories (6, 7, 14, 16, 19, 23, 26).

This study investigates the genotypic similarities between human and animal-originated isolates using spoligotyping and 24 loci MIRU-VNTR for molecular epidemiological analysis of *M. bovis* isolates.

Materials and Methods

***Bacterial strains*:** In this study, 58 human-originated isolates (40 *M. bovis* subsp. *bovis*, 17 *M. bovis* BCG, and 1 *M. bovis* subsp. *caprae*) and 50 bovine-originated isolates (49 *M. bovis* subsp. *bovis* and 1 *M. bovis* subsp. *caprae*) obtained between 2019 and 2022 were used.

***Isolation and Identification*:** All isolation studies were performed at the *National Tuberculosis Reference Laboratory*. The human-originated isolates were obtained from various samples (tissue biopsy, sputum, abscess, starvation gastric lavage, urine, bronchoalveolar lavage) from individuals suspected of having TB. To obtain animal-originated isolates, 108 bovine tissue necropsy samples (lung lymph node) found positive for BTB using a tuberculin skin test resulting from a veterinary examination in *Tuberculosis, Paratuberculosis and Glanders Diagnostic Laboratory* were used. Isolation of MTBC members was done using conventional cultural methods. After decontamination of the samples using the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH), the isolates were incubated in Mycobacteria Growth Indicator Tube (MGIT) broth and incubated at 37 °C. The growth in the media was followed for 6 weeks. All isolates were identified as MTBC by immunochromatographic assay (BD MGIT TBc ID, Beckton Dickinson Diagnostic, Sparks, USA) made from positive liquid cultures, and drug susceptibility testing (DST) was performed. DST was performed with the proportion method using the first-line drug solutions in the MGIT 960 automated culture device (BD Diagnostic Systems, Sparks, MD, USA). All pyrazinamide-resistant isolates were first identified with the GenoType MTBC kit (HAIN Lifesciences, Germany). The remaining DNA extracts were frozen for genotyping studies (27).

***Spoligotyping*:** Spoligotyping was performed according to Kremer et al. (2004) (15). DRa (5'- GGT TTT GGG TCT GAC GAC-3') and DRb (5'- CCG AGA GGG GAC GGA AAC- 3') primer pairs targeting the DR area were synthesized for SpoligoPCR. The DRa primer was labeled with biotin at the 5' end and kept at +4 °C. During each process, positive (pure DNA from *M. bovis* BCG and *M. tuberculosis* H37Rv) and negative controls (ultrapure water) were used. The PCR master mix (50 µL) consisted of 6 µL dH2O, 1.0 µL DMSO, 25 µL 2X buffer mix, 4 µL DRa (20 pmol/µL), 4 µL DRb (20 pmol/µL), and 10 µL template DNA. The heat cycles were adjusted as follows: 10 min predenaturation at 95 °C, 25 cycles of 1 min denaturation at 96 °C, 1 min annealing at 55 °C, 30 s extension at 72 °C, and a 5 min final extension at 72 °C. Precisely 20 µL of the PCR product was added to 150 µL 2XSSPE 0.1% SDS. The PCR product was denatured by boiling for 10 min; it was then immediately transferred to

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the ice. The membrane (Isogen Bioscience BV, Maarssen, The Netherlands) and sponge pad (Immunetics Plastic Cushion PC200, Immunetics Inc., Boston, MA, USA) were placed in a mini-blotter (Mini-blotter-3024) perpendicular to each other. The denatured PCR product was slowly placed in the slots. The mini-blotter was hybridized horizontally at 60°C for 60 min in a hybridization oven. The products were then aspirated from the slots of the mini-blotter with an aspiration device. The membrane was taken from the mini-blotter and placed in the washing box. The membrane was washed with 250 mL of 2XSSPE 0.5% SDS twice at 60 °C for 10 min each time. The membrane was wrapped with a nylon membrane and put into the bottle. Exactly 3 µL of streptavidin-peroxidase and 15 mL of 2XSSPE 0.5% SDS at 42 °C were mixed in a bottle. The entire membrane was wetted with the mixture. The bottle attached to the rotor in the hybridization oven was rotated and incubated at 42 °C for 60 min. The membrane was removed from the bottle and washed twice with 250 mL of 2XSSPE 0.5% SDS at 42 °C for 10 min each time. Subsequently, the membrane was washed with 2XSSPE for 5 min at room temperature by shaking. Hybridized DNA was detected with a chemoluminescence imaging device (QUANTUM-ST4 3020–WL/BLUE/20M) after incubation with streptavidin-peroxidase. Hybrid regions were observed as black squares. The results were prepared in Excel format with '1' denoting the presence of black squares and '0' showing their absence. The results were converted to an octal code consisting of 15 characters between 0 and 7 using the below octal coding key.

□□□ = 0 □□■ = 1 □■□ = 2 □■■ = 3 ■ = 1 □ = 0

■□□ = 4 ■□■ = 5 ■■□ = 6 ■■■ = 7 Spacer 43

Using the Mbovis.org (http://www.mbovis.org) and SITVIT2 (http://www.pasteur-guadeloupe.fr:8081/SITVIT2/links.jsp) databases, groups, and clades were determined with the obtained data.

MIRU-VNTR genotyping: MIRU-VNTR was employed according to Supply et al. (23). The primers in Table 1 were obtained for the targeted MIRU loci of *M. bovis*. To determine the VNTR number of 24 loci MIRU for each isolate, 8 mixes with 3 primers were prepared. During each process, positive (pure DNA from *M. bovis* BCG and *M. tuberculosis* H37Rv) and negative controls (ultrapure water) were used. The storage conditions of the primers (–20°C) were strictly followed. The PCR master mix (25 µL) consisted of 12.5 µL 2X HS Prime Taq, 5 µL 5X Q, 3 µL forward primer, 1,5 µL reverse primer, 1 µL $MgCl_2$, and 2 µL template DNA. The heat cycles were adjusted as follows: 10-min predenaturation at 95 °C, 25 cycles of 45-s denaturation at 94 °C, 1-min annealing at 57 °C, 1-min extension at 72 °C, and a 5-min final extension at 72 °C. After PCR, electrophoresis was applied to amplicons formed at 120 volts for 60 min on a 1.5% agarose gel stained with GelRed. PCR products were observed by comparing them with a 1000-bp DNA ladder (Fermentas). After electrophoresis, the gel was visualized with an imaging device. The observed amplicon samples were analyzed in a capillary gel electrophoresis device. Two µL of diluted PCR sample, 1 µL of size marker, and 30 µL of sample-loading solution were added to the analysis plate. The separation plate was also prepared and loaded into the device. At the end of 24 loci MIRU-VNTR typing, the number of allelic repeats was determined for each MIRU locus according to fragment lengths. The fragment lengths taken from the device were prepared in Excel format. The codes obtained from spoligotyping and 24 loci MIRU-VNTR typing were analyzed with the computer program. Dendrograms were generated using an unweighted pair-grouping method analysis algorithm in BioNumerics Software 7.5 (Applied Maths, East Flanders, Belgium). The origin relationship was determined by calculating the similarity of coefficients between the isolates.

Results

***Isolation and identification*:** All growths were defined as MTBC using an immunochromatographic test performed with the liquid cultures of all isolates. With the GenoType MTBC kit, of the 58 human-originated isolates, 40 (69%) were identified as *M. bovis* subsp. *bovis*, 17 (29.3%) as *M. bovis* BCG, and one (1.7%) as *M. bovis* subsp. *caprae*. Of the 50 animal-originated isolates, 49 were identified as *M. bovis* subsp. *bovis* and one as *M. bovis* subsp. *caprae.*

***Spoligotyping*:** Of the 108 MTBC isolates, 102 were identified as *M. bovis* subsp. *bovis* by spoligotyping and two as *M. bovis* subsp. *caprae*. Among the 104 isolates, 13 different spoligotypes were identified (Table 2). The predominant spoligotypes found were SIT1118/SB0989 (19.23%, n = 20), SIT482/SB0120 (16.35%, n = 17), both belonging to the BOV_1 family, followed by SIT685/SB0288 (12.50%, n = 13), which belongs to the BOV family, and, finally, SB1593 (12.50%, n = 13) (Table 2). Only 5 spoligotypes were found in the *M. bovis* spoligotype database (www.mbovis.org). These spoligotypes are SB1231 (n = 1), SB2466 (n = 4), SB2510 (n = 1), SB0419 (n = 2), and SB1593 (n = 13). The other genotypes are SIT3529/SB0920 (n = 4), SIT1185/SB0897 (n = 2), SIT3710/SB1595 (n = 1), SIT688/SB129 (n = 1), all belonging to the BOV_1 family, and SIT3687/SB1625 (n = 1) from the BOV family. No codes were found in the databases for 24 isolates (23.08%), 10 of which were human-originated and 14 of which were animal-originated; these isolates were defined as 'new pattern.' They consisted of orphan strains not belonging to any group (Table 2).

Table 1. Primers and mixes used for the 24 locus MIRU-VNTR method.

MIRU primers	Primer sequences (5′ to 3′)	Primer Names	Mixes
MIRU 4; ETR D	GCGCGAGAGCCCGAACTGC (FAM)	MIRU4_ETR D-F	
	GCGCAGCAGAAACGCCAGC	MIRU4_ETR D-R	
MIRU 26	TAGGTCTACCGTCGAAATCTGTGAC	MIRU26-F	Mix1
	CATAGGCGACCAGGCGAATAG (VIC)	MIRU26-R	
MIRU 40	GGGTTGCTGGATGACAACGTGT (NED)	MIRU40-F	
	GGGTGATCTCGGCGAAATCAGATA	MIRU40-R	
MIRU 10	GTTCTTGACCAACTGCAGTCGTCC	MIRU10-F	
	GCCACCTTGGTGATCAGCTACCT (FAM)	MIRU10-R	
MIRU 16	TCGGTGATCGGGTCCAGTCCAAGTA	MIRU16-F	Mix2
	CCCGTCGTGCAGCCCTGGTAC (VIC)	MIRU16-R	
MIRU 31	ACTGATTGGCTTCATACGGCTTTA	MIRU31_ETR E-F	
	GTGCCGACGTGGTCTTGAT (NED)	MIRU31_ETR E-R	
Mtub04	CTTGGCCGGCATCAAGCGCATTATT	Mtub04-F	
	GGCAGCAGAGCCCGGGATTCTTC (FAM)	Mtub04-R	
ETR C	CGAGAGTGGCAGTGGCGGTTATCT (VIC)	ETR_C-F	Mix3
	AATGACTTGAACGCGCAAATTGTGA	ETR_C-R	
ETR A	AAATCGGTCCCATCACCTTCTTAT (NED)	ETR_A-F	
	CGAAGCCTGGGGTGCCCGCGATTT	ETR_A-R	
Mtub30	CTTGAAGCCCCGGTCTCATCTGT (FAM)	Mtub30-F	
	ACTTGAACCCCCACGCCCATTAGTA	Mtub30-R	
Mtub39	CGGTGGAGGCGATGAACGTCTTC (VIC)	Mtub39-F	Mix4
	TAGAGCGGCACGGGGGAAAGCTTAG	Mtub39-R	
QUB-4156	TGACCACGGATTGCTCTAGT	QUB-4156-F	
	GCCGGCGTCCATGTT (NED)	QUB-4156-R	
QUB-11b	CGTAAGGGGGATGCGGGAAATAGG	QUB-11b-F	
	CGAAGTGAATGGTGGCAT (FAM)	QUB-11b-R	
Mtub21	AGATCCCAGTTGTCGTCGTC (VIC)	Mtub21-F	Mix5
	CAACATCGCCTGGTTCTGTA	Mtub21-R	
QUB-26	AACGCTCAGCTGTCGGAT (NED)	QUB-26-F	
	CGGCCGTGCCGGCCAGGTCCTTCCCGAT	QUB-26-R	
MIRU 2	TGGACTTGCAGCAATGGACCAACT	MIRU2-F	
	TACTCGGACGCCGGCTCAAAAT (FAM)	MIRU2-R	
MIRU 23	CTGTCGATGGCCGCAACAAAACG (VIC)	MIRU23-F	Mix6
	AGCTCAACGGGTTCGCCCTTTTGTC	MIRU23-R	
MIRU 39	CGCATCGACAAACTGGAGCCAAAC	MIRU39-F	
	CGGAAACGTCTACGCCCCACACAT (NED)	MIRU39-R	
MIRU 20	TCGGAGAGATGCCCTTCGAGTTAG (FAM)	MIRU20-F	
	GGAGACCGCGACCAGGTACTTGTA	MIRU20-R	
MIRU 24	CGACCAAGATGTGCAGGAATACAT	MIRU24-F	Mix7
	GGGCGAGTTGAGCTCACAGAA (VIC)	MIRU24-R	
MIRU 27; QUB-5	TCGAAAGCCTCTGCGTGCCAGTAA	MIRU27_QUB-5-F	
	GCGATGTGAGCGTGCCACTCAA (NED	MIRU 27_QUB-5-R	
Mtub29	GCCAGCCGCCGTGCATAAACCT (FAM)	Mtub29-F	
	AGCCACCCGGTGTGCCTTGTATGAC	Mtub29-R	
ETR B	ATGGCCACCCGATACCGCTTCAGT (VIC)	ETR_B-F	Mix8
	CGACGGGCCATCTTGGATCAGCTAC	ETR_B-R	
Mtub34	GGTGCGCACCTGCTCCAGATAA (NED)	Mtub34-F	
	GGCTCTCATTGCTGGAGGGTTGTAC	Mtub34-R	

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Table 2. Genotypes detected by spoligotyping.

Number of strains	% rate	Genotype	Cluster	Number	Octal	Lineage	SIT	SB number	Country
1H	0,96	G1	C1	2	616673757777600	Not defined	Orphan or New	New pattern	
1H	0,96	G2			616433777777600	Not defined	Orphan or New	New pattern	
4H	3,85	G3	C2	23+BCG	672773777777600	BOV_1	3529	SB0920	France
BCG-1B-16H	16,35	G4			676773777777600	BOV_1	482	SB0120	Vaccine strain
1H	0,96	G5			676773577777600	BOV_1	3710	SB1595	Italy
1H	0,96	G6			676773777774600	BOV_1	Orphan or New	SB1231	Spain
1B	0,96	G7			676673777777400	Not defined	Orphan or New	New pattern	
1H	0,96	G8	C3	27	676473777777600	BOV_1	688	SB0129	United Kingdom
12B-8H	19,23	G9			676673757777600	BOV_1	1118	SB0989	Germany
1B-1H	1,92	G10			676673777777600	BOV_1	1185	SB0897	France
1H	0,96	G11			676673757377600	Not defined	Orphan or New	New pattern	
1H	0,96	G12			676673757767600	Not defined	Orphan or New	New pattern	
1H	0,96	G13			676663757777600	Not defined	Orphan or New	New pattern	
1B	0,96	G14			670073777775600	Not defined	Orphan or New	New pattern	
1B	0,96	G15			674073777775600	Not defined	Orphan or New	New pattern	
1H	0,96	G16			674073777774600	Not defined	Orphan or New	New pattern	
4H	3,85	G17			674073777777200	Not defined	Orphan or New	SB2466	Türkiye
6B-7H	12,5	G18	C4	32	674073777777600	BOV	685	SB0288	United Kingdom
8B	7,69	G19			674073777377600	Not defined	Orphan or New	New pattern	
1B	0,96	G20			274073777777600	Not defined	Orphan or New	SB2510	Türkiye
1H	0,96	G21			674073777767600	Not defined	Orphan or New	New pattern	
1H	0,96	G22			634073777777600	Not defined	Orphan or New	New pattern	
1H	0,96	G23			674073777477600	Not defined	Orphan or New	New pattern	
2B	1,92	G24	C5	2	676773747764600	Not defined	Orphan or New	New pattern	
1H	0,96	G25	C6	1	674070177777600	Not defined	Orphan or New	New pattern	
13B	12,5	G26	C7	14	676773777017600	Not defined	Orphan or New	SB1593	Italy
1B	0,96	G27			676773747017600	Not defined	Orphan or New	New pattern	
1B-1H	1,92	G28	C8	3	200023777377600	BOV_4-CAPRAE	Orphan or New	SB0419	Sweden
1H	0,96	G29			OOO73777777600	BOV	3687	SB1625	Spain

H: Human, B: Bovis, SIT: Spoligotype International Type, C: Cluster, G: Genotype

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Table 3. Genotypes detected by 24 loci MIRU-VNTR.

Number of strains	Genotype	Cluster	Number	% rate
6B	G1			
1B	G2			
1B	G3			
3H	G4	C1	15	15.79
1H	G5			
1H	G6			
1H	G7			
1B	G8			
1H	G9			
1H	G10			
1H	G11			
1B	G12			
1H	G13			
1H	G14			
1H	G15			
2H	G16	C2	19	20
1B	G17			
2H	G18			
1H	G19			
1H	G20			
1H	G21			
1B	G22			
1H	G23			
1H	G24			
1B	G25			
2H	G26			
1H	G27			
BCG-5H	G28	C3	10	10.53
1H	G29			
1H	G30			
3H	G31			
1H	G32			
1H	G33			
1B	G34			
1B	G35			
10B	G36			
1B	G37			
4H	G38			
1H	G39	C4	39	41.05
1H	G40			
2H	G41			
1H	G42			
4B	G43			
1B	G44			
4B	G45			
1B	G46			
1B	G47			
1B	G48			
3B	G49			
1B	G50	C5	4	4.21
1H	G51	C6	1	1.05
1B	G52	C7	1	1.05
1B	G53	C8	1	1.05
4H	G54			
1H	G55	C9	5	5.27

H: Human, B: Bovis, G: Genotype, C: Cluster.

When the binary codes obtained by spoligotyping were analyzed by dendrogram, it was observed that 104 isolates were placed into 8 clusters and 29 different genotypes (Figure 1 and Table 2). The genotypes determined according to the spoligotyping results and their host and country origin are shown in Table 2.

***MIRU-VNTR typing*:** Of the 108 isolates focused on in this study, VNTR profiles could only be generated for 95 *M. bovis* isolates. Since amplicon could not be obtained in PCR studies in 7 human and 6 animal isolates, it could not be genotyped with MIRU-VNTR. A panel of 24 loci was chosen to conduct the MIRU-VNTR (Table 1).

When the obtained MIRU-VNTR data of the isolates were examined in the SITVIT2 database, it was seen that all were defined as new isolates. When the MIRU-VNTR findings of the 95 bovine and human-originated isolates were examined together with a dendrogram, they consisted of 9 clusters and 55 different genotypes. It was observed that 92 isolates (96%) had formed 6 clusters, and the remaining 3 isolates formed 3 different clusters. The most common genotype in this study was the 24 loci MIRU-VNTR code '232325253223251043123343 3', seen in 10 animal-originated isolates. The 55 different genotypes observed with 24 loci MIRU-VNTR indicate that MIRU-VNTR has a higher discriminatory power than spoligotyping (Figure 2 and Table 3). There was no human and animal-originated interspecies genotype with the same MIRU-VNTR codes. The genotypes identified according to the MIRU-VNTR results and their clusters and host origin are shown in Table 3.

ETR-C, QUB2163b, and QUB26 were the loci with the most allelic diversity in the human isolates. ETR-A, ETR-B, ETR-D (=MIRU04), ETR-E(=MIRU31), Mtub04, Mtub21, Mtub29, Mtub30, Mtub39, MIRU26, MIRU40, and QUB4156 showed less allelic diversity, and these loci had a discriminatory power (0.25≤h) (MIRU-VNTRPlus, www.miru-vntrplus.org). MIRU02, MIRU10, MIRU16, MIRU20, MIRU23, MIRU24, MIRU27, MIRU39, and Mtub34, did not show any allelic diversity. In the animal isolates, ETR-C, QUB2163b, and QUB26 were the loci with the most allelic diversity. ETR-A, ETR-B, ETR-E(=MIRU31), Mtub04, Mtub39, MIRU23, MIRU26, and MIRU40 showed less allelic diversity (0.25≤h) (MIRU-VNTRPlus, www.miru-vntrplus.org). MIRU02, MIRU10, MIRU16, MIRU20, MIRU24, MIRU27, MIRU39 and ETR-D (=MIRU04), QUB4156, Mtub21, Mtub29, Mtub30, and Mtub34 did not show any (Table 4).

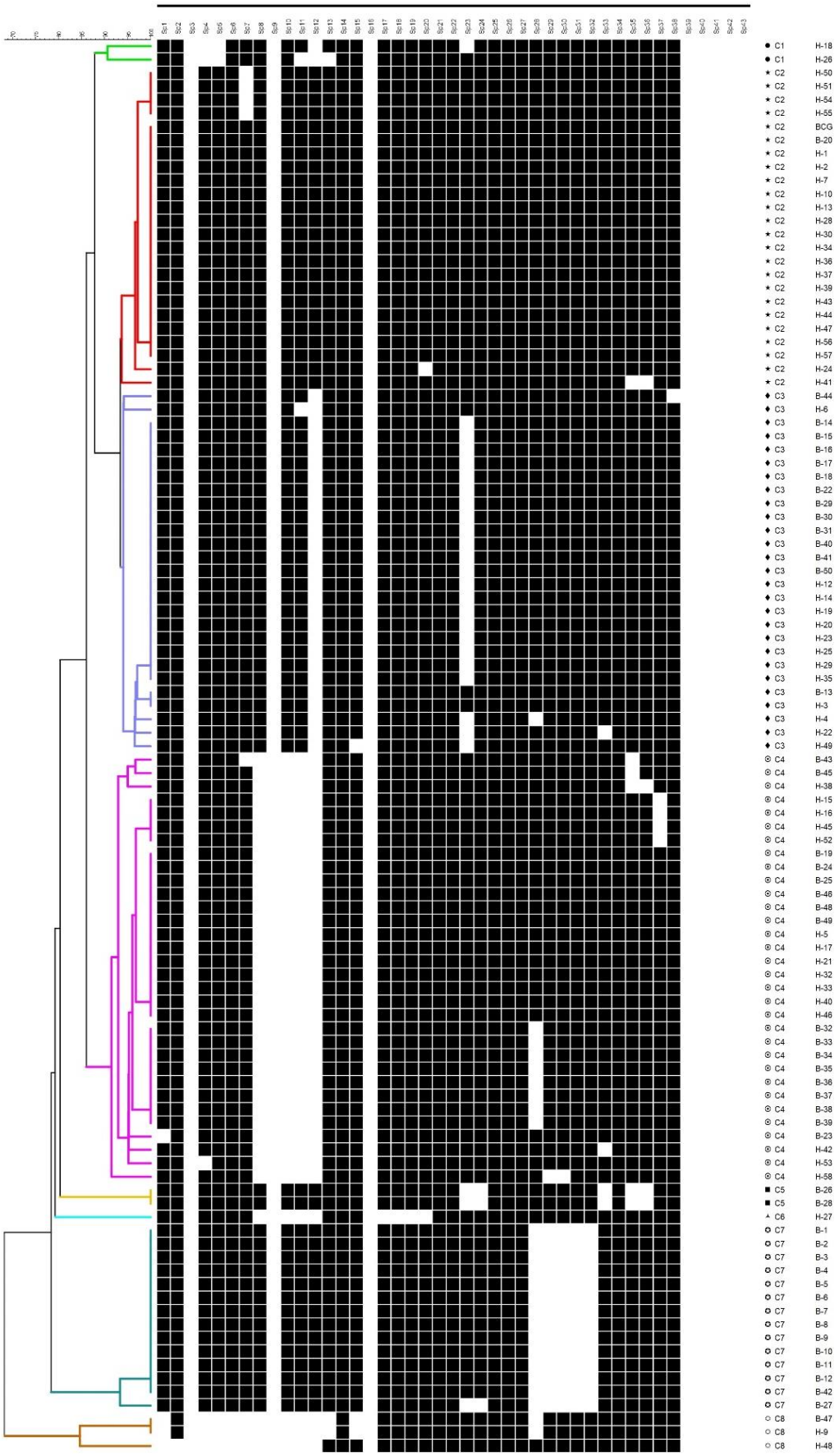

Figure 1. Dendogram of human and animal isolates by spoligotyping.

H: Human, B: Bovis, C: Cluster.

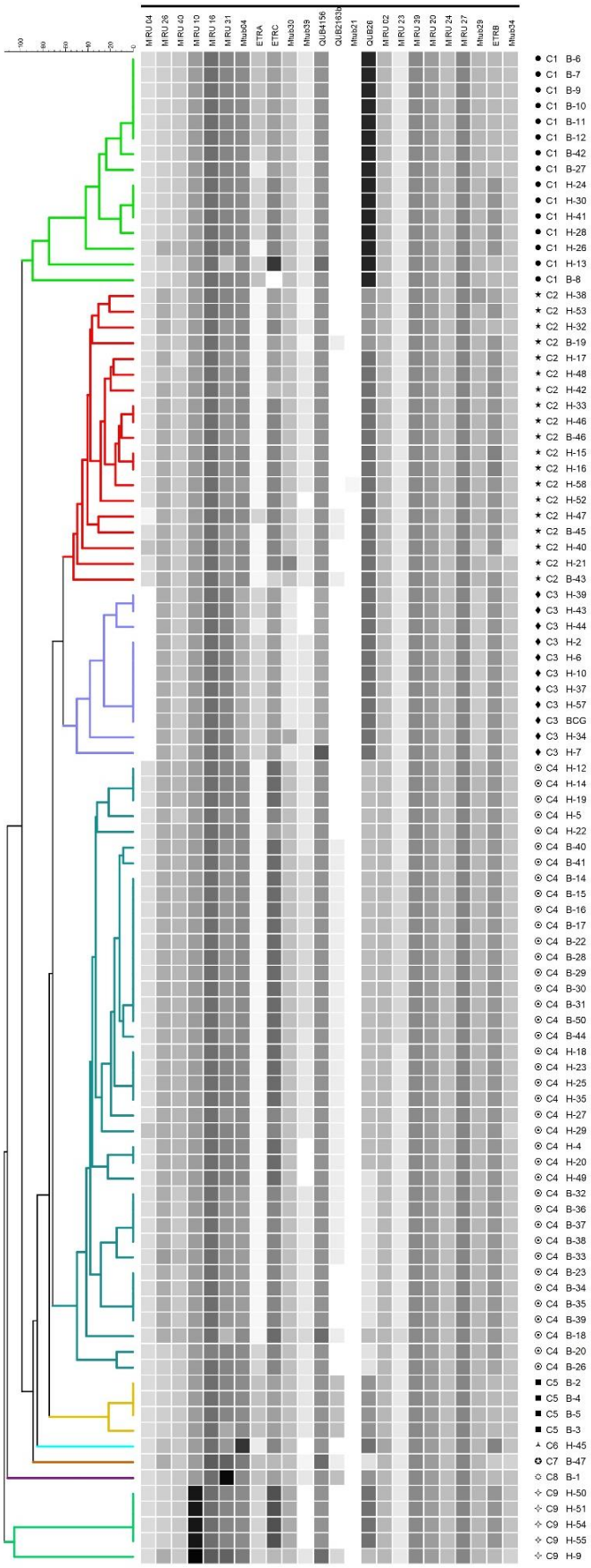

Figure 2. Dendogram of human and animal isolates by 24 loci MIRU-VNTR.

H: Human, B: Bovis, C: Cluster.

Table 4. Copy numbers and discriminatory power determined using 24 loci MIRU-VNTR.

MIRU-VNTR lokusları	human-originated isolates Allel Copy Numbers**														Discriminatory power (h*)	animal-originated isolates Allel Copy Numbers**														Discriminatory power (h*)
	0	1	2	3	4	5	6	7	8	9	10	11	12	13		0	1	2	3	4	5	6	7	8	9	10	11	12	13	
MIRU 02			51												0.000			44													0.000
MIRU 04 (ETR-D)		10	1	38	2										0.393				44												0.000
MIRU 10			46							4	1				0.164			44													0.000
MIRU 16				51											0.000			1	42	1											0.067
MIRU 20			51												0.000			44													0.000
MIRU 23					51										0.000					32	12										0.383
MIRU 24			51												0.000			44													0.000
MIRU 26				9	2	40									0.339				16	1	26	1									0.506
MIRU 27				51											0.000				44												0.000
MIRU 31 (ETR-E)	1		15	34		1									0.458	1	1	26	14		1					1					0.537
MIRU 39			51												0.000			44													0.000
MIRU 40		1	36	14											0.415			29	15												0.437
ETRA			31	20											0.466			28	3	13											0.492
ETRB				4	1	43	3								0.265			1	14	1	28										0.481
ETRC							2	15		19	10	4		1	**0.723**		1				1	1	14		13	14					**0.702**
QUB2163b		1	27	12	10	1									**0.618**			16		23		5									**0.572**
QUB26			1	14	3	27		6							**0.619**			12	14	6	3		9								**0.754**
QUB4156	9	39		2	1										0.370		42		2												0.066
Mtub04	10	2	36	2				1							0.449		11	31	2												0.426
Mtub21		11	1	38	1										0.385			1	43												0.022
Mtub29			11	39	1										0.356				44												0.000
Mtub30			9		36	5	1								0.450					44											0.000
Mtub34		1	2	48											0.095			44													0.000
Mtub39	12	2	37												0.405		2	30	12												0.446

*h=1-Σ xi2 [n/(n-1) **xi: repeats of the allele locus

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Table 5. Compatibility of molecular typing results in isolates.

Number of strains	GenotypeMTBC	Spoligotyping	MIRU-VNTR	Conformity	Number
1 H	M.bovis BCG	M.bovis BCG	BOVİS	GenotypeMTBC and Spoligotyping compatible	10
3 H	M.bovis BCG	M.bovis BCG	Failed		
1 H	BOVİS	BOVİS	Failed		
5 B	BOVİS	BOVİS	Failed		
3 H	BOVİS	M.bovis BCG	BOVİS	GenotypeMTBC and MIRU-VNTR compatible	5
1 B	BOVİS	M.bovis BCG	BOVİS		
1 H	M.bovis BCG	BOVİS	M.bovis BCG		
3 H	M.bovis BCG	Failed	Failed	Failed	4
1 B	BOVİS	Failed	Failed		
1 H	CAPRAE	CAPRAE	CAPRAE	Compatible in three tests	89
1 B	CAPRAE	CAPRAE	CAPRAE		
9 H	M.bovis BCG	M.bovis BCG	M.bovis BCG		
36 H	BOVİS	BOVİS	BOVİS		
42 B	BOVİS	BOVİS	BOVİS		
				Total	108

H: Human
B: Bovis
BOVİS: M. bovis subsp. bovis
CAPRAE: M. bovis subsp. caprae

When the molecular typing results of the 95 isolates, tested with all three methods, were evaluated, 93.7% (89/95) agreement was observed between methods. There was 95.2% (99/104) agreement for the results of 104 isolates in which the Genotype MTBC and spoligotyping tests were studied. Exactly 98.9% (94/95) agreement was found for the results of the 95 isolates in which Genotype MTBC and MIRU-VNTR tests were used. When the compatibility of the spoligotyping and MIRU-VNTR tests was examined, it was seen that genotyping results were compatible in 89 (93.7%) of 95 isolates. As a result of the study, 4 isolates could not be genotyped with spoligotyping, and 13 isolates could not be genotyped with MIRU-VNTR (Table 5).

Discussion and Conclusion

In this study, the most common genotypes from isolates of human and animal-originated were SIT1118/SB0989 (19.23%), followed by SIT482/SB0120 (16.35%), SIT685/SB0288 (12.5%), SB1593 (12.5%), SIT3529/SB0920 (3.85%), SB2466 (3.85%), SB0419 (1.92%), and SIT1185/SB0897 (1.92%).

Çavuşoğlu and Yılmaz (11) evaluated 13 *M. bovis* isolates identified with spoligotyping from MBTCs produced from clinical, human-originated samples in the Aegean region in 2017, consisting of 9 (63.6%) SIT685/SB0288, one (7.7%) SIT1118/SB0989, and one (7.7%) SIT820/SB0856. Tuzcu and Köksal (26), in their 2020 study in which 50 samples of human and cattle isolates were spoligotyped in the Çukurova region, reported the most common genotypes as SIT482/SB0120 (42.9%), SIT683/SB0140 (26.19%), SIT647/SB0418 (16.66%), and SIT685/SB0288 (9.52%). Avsever et al. (4) performed genotyping of 6 *M. bovis* isolates obtained from 4 cattle and 2 goats in the Aegean region in 2017 by spoligotyping, defining all isolates (100%) as SIT685/SB0288. Prodinger et al. (20) found SIT685/SB0288 to be the most dominant spoligotype in 34.9% of human *M. bovis* isolates in their study of 43 samples in 2014. Belakehal et al. (6) reported the genotypes SIT482/SB0120 (33.3%), SIT481/SB0121 (21.7%), and SIT665/SB0134 (11.7%), according to their spoligotyping results in 2022 with 60 cattle isolates. Melo et al. (16) obtained the genotypes SIT481/SB0121 (47.06%), SIT698/SB0295 (29.41%), SIT797/SB0852 (11.76%), and SIT482/SB0120 (5.88%) with 17 cattle isolates they spoligotyped. Genotype SIT1118/SB0989, detected most frequently in our study, was found at a rate of 7.7% in Çavuşoğlu and Yılmaz's study, first reported in Germany. The second most common genotype, SIT482/SB0120, originates from the spoligotypes of the BCG vaccine strain, identified as the most common genotype in many studies (6, 21, 26). Although detection of this genotype is expected in human-originated samples, it would be useful to confirm this with an additional method when detecting isolates of animal-originated samples since animals are not vaccinated with BCG. Spoligotyping shows cross-reactivity in isolates of animal

origin, indicating that *M. bovis* subsp. *bovis* strains as *M. bovis* BCG, whole genome sequencing should be performed for confirmation. The SIT482/SB0120 genotypes we found in our study were determined as 16 human-originated isolates and one animal-originated isolate with spoligotyping; five of the human isolates were determined as BCG vaccine strains with 24 loci MIRU-VNTR. In Tuzcu and Köksal's study, only two human isolates of the SIT482/SB0120 genotype were determined as BCG vaccine strains using 12 loci MIRU-VNTR. In comparison, 6 human and 12 animal-originated isolates were detected by spoligotyping. It has been reported that *M. bovis* isolates that show vaccine strain patterns with spoligotyping should be confirmed by additional genotyping methods (5). The third most common genotypes in our study were SIT685/SB0288 and SB1593. No information about SB1593 could be found in the SITVIT2 database. The SB1593 genotype was detected in approximately one quarter (13/49) of our animal-originated isolate samples, and it is thought to have an important place among the *M. bovis* genotypes in Turkey. In other studies, Avsever et al. found the genotype SIT685/SB0288 at a rate of 100%, Çavuşoğlu and Yılmaz at 63.6%, and Prodinger et al. at 34.9%, reporting it as the most common genotype (5,11,20). The SIT685/SB0288 genotype, rarely reported worldwide and first reported in the UK, was determined as the dominant type in the Aegean region in studies conducted in Turkey (11). The SB0419 genotype, which we found at a rate of 1.92% in our study, matched the *M. bovis* subsp. *caprae* genotype, which was first reported in Sweden. In 2011, Sayın and Erganiş identified 19 isolates as *M. bovis* subsp. *caprae* with the conventional PCR method in their study on 772 cattle, determining the presence of this genotype for the first time in Turkey (22). The SIT647/SB0418 genotype that Tuzcu and Köksal detected in 7 isolates (16.66%) of bovine-originated samples in their study in 2020 was a different *M. bovis* subsp. *caprae* genotype first reported in Belgium (26). Although it is known that the SB0419 genotype *M. bovis* subsp. *caprae*, which we detected in our study from 2 different isolates originating from a human and an animal, is a goat-derived and European-derived genotype, investigating its transition from humans and animals is required. Reporting on different genotypes in different countries shows the existence of genotypes belonging to each country. However, reporting similar genotypes in different countries suggests that genotypes may spread between countries and continents due to animal or human mobility.

When the binary codes obtained by spoligotyping were analyzed by dendrogram, it was observed that 104 isolates were placed into 8 clusters and 29 different genotypes. In their study from 2020, Tuzcu and Köksal reported that 40 bovine and 10 human *M. bovis* isolates were distributed in 6 different genotypes by spoligotyping and 4 were clustered (26). Sahraoui et al., in their 2009 study, 22 *M. bovis* spoligotypes, 8 were clustered and the remaining 14 were unique patterns by spoligotyping (21). In 2012, Parreiras et al. grouped 61 cattle isolates into 9 clusters and 17 different spoligotype patterns by spoligotyping (19). The number of clusters and genotypes obtained by dendrogram analysis of the spoligotyping findings in these studies varies according to the spoligotyping method, the number of isolates in the study, the origin of the isolates, and the dendrogram program.

When the MIRU-VNTR findings of the 95 isolates of bovine and human-originated samples were examined with a dendrogram, 9 clusters and 55 different genotypes were observed. Belakehal et al. obtained 32 different genotypes, 5 clusters, and one orphan pattern using 19 loci MIRU-VNTR typing in 42 isolates. They reported that 19 loci MIRU-VNTR showed higher discrimination power than spoligotyping (6). Melo et al. reported that they detected 2 clusters and 13 unique genotypes, each consisting of 2 isolates with 24 loci MIRU-VNTR (16). Parreiras et al. obtained 16 clusters of 61 isolates with 12 loci MIRU-VNTR typing (19). Gülcü and Hadimli indicated that they observed 29 clusters with a varying number of isolates as a result of genotyping 70 *M. bovis* isolates isolated from TB-suspected bovine tissues and organs with 12 loci MIRU-VNTR (14). Tuzcu and Köksal identified 10 different clusters with 12 loci MIRU-VNTR. The authors reported that MIRU-VNTR showed higher discrimination power than spoligotyping (26). Bolado-Martínez et al. found different MIRU-VNTR patterns for all isolates in their study of 7 and 24 loci MIRU-VNTR on 65 isolates obtained from BTB lesions (7). In most of the above studies, the MIRU-VNTR method was found to have higher discrimination than spoligotyping, which is consistent with our study. In Parreiras et al.'s study, while 17 different genotypes were determined using the spoligotyping method, 16 different genotypes were determined with the MIRU-VNTR method, and no increase in discrimination was observed. It is believed that this discrepancy might have resulted from the difference in MIRU-VNTR loci used in the study, the dendrogram program used, or other unknown reasons. The determination of such different clusters and genotypes in some studies may be due to the number of isolates obtained, whether they are of animal or human origin, the geographical region where the study was conducted, the genotyping method used, and the difference in dendrogram programs.

In our study, ETR-C, QUB2163b, and QUB26 were the loci with the most allelic diversity in the human isolates. ETR-A, ETR-B, ETR-D (=MIRU04), ETR-E(=MIRU31), Mtub04, Mtub21, Mtub29, Mtub30, Mtub39, MIRU26, MIRU40, and QUB4156 showed less

allelic diversity, and these loci had a discriminatory power (0.25≤h) (MIRU-VNTRPlus, www.miru-vntrplus.org). However, MIRU02, MIRU10, MIRU16, MIRU20, MIRU23, MIRU24, MIRU27, MIRU39, and Mtub34, did not show any allelic diversity. In the animal isolates, ETR-C, QUB2163b, and QUB26 were the loci with the most allelic diversity. ETR-A, ETR-B, ETR-E(=MIRU31), Mtub04, Mtub39, MIRU23, MIRU26, and MIRU40 showed less allelic diversity (0.25≤h) (MIRU-VNTRPlus, www.miru-vntrplus.org), while MIRU02, MIRU10, MIRU16, MIRU20, MIRU24, MIRU27, MIRU39 and ETR-D (=MIRU04), QUB4156, Mtub21, Mtub29, Mtub30, and Mtub34 did not show any (Table 4). Belakehal et al. (6) reported that the ETR-A, ETR-B, ETR-C, QUB11b, QUB11a, QUB3232, and MIRU27 loci had the highest allelic diversity (6). Melo et al. (16) indicated that the ETR-A locus showed the highest allelic diversity and that the ETR-B, ETR-C, MIRU16, MIRU27, and QUB26 loci showed moderate allelic diversity (16). Gülcü and Hadimli (14), in their study with 12 loci MIRU-VNTR, demonstrated that ETR-E (=MIRU31), MIRU26, and MIRU10 loci showed high allelic diversity, MIRU10, MIRU16, MIRU26, MIRU31, and MIRU40 loci showed moderate allelic diversity, and MIRU02, MIRU20, MIRU23, MIRU24, MIRU27, MIRU39, and ETR-D (=MIRU04) loci did not show allelic diversity (14). Tuzcu and Köksal (26) observed that MIRU26, ETR-E (=MIRU31), and MIRU40 loci showed high allelic diversity, ETR-D (=MIRU04) locus showed moderate allelic diversity and that MIRU02, MIRU10, MIRU16, MIRU20, MIRU23, MIRU24, MIRU27, and MIRU39 loci did not show allelic diversity (26). Bolado-Martínez et al. (7) investigated the efficiency of 7 and 24 loci MIRU-VNTR in their study and found that QUB3232, QUB11a, ETR-A, MIRU26, QUB26, MIRU16, MIRU27, MIRU39, MIRU02, ETR-E (=MIRU31), and QUB3336 had the highest allelic diversity; in addition, the authors of that study determined that QUB23, ETR-B, ETR-C, QUB11b, MIRU40, MIRU23, QUB18, MIRU10, MIRU04, MIRU24, and QUB15 loci showed moderate allelic diversity; in contrast, QUB1895 and MIRU20 loci did not show allelic diversity (7). Parreiras et al. (19) observed that only MIRU16 and MIRU26 loci showed high allelic diversity, while MIRU10, MIRU20, MIRU23, and MIRU39 loci did not (19). In previous studies, it was observed that ETR-A, ETR-B, ETR-C, ETR-D (=MIRU04), ETR-E (=MIRU31), MIRU26, and MIRU40 loci showed high allelic diversity, and these results are consistent with those in the present study (6, 7, 14, 16, 26). As a result of these studies, differences in the discriminating power of VNTR loci in different countries and regions were observed. MIRU02, MIRU16, MIRU20, MIRU23, MIRU24, MIRU27, and MIRU 39 loci did not show allelic variation in many studies, and these findings are also consistent with our results (7, 14, 19, 26). The presence of inconsistent loci in terms of high or low allelic diversity among some studies may be caused by many factors, such as the method used, the number of isolates, and variables present during the application of the test.

The present study shows that MIRU-VNTR produced more detailed results in terms of genotype determination compared to spoligotyping. Since the performances of the two methods in *M. bovis* genotyping were highly compatible, it was concluded that both tests can be safely used. The present study also demonstrated that both *M. bovis* subsp. *caprea* and *M. bovis* subsp. *bovis* should be considered in the epidemiology of the disease. When the genotypic distribution of the isolates was examined in this study, similar genotypes were observed in humans and animals, which shows how important is the zoonotic contagion of the disease.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Derya Altun designed and planned the project. Halil Pir provided bovine necropsy samples to obtain isolates of animal origin and contributed to sample preparation. Derya Altun performed the identification and genotyping tests. Hakan Yardımcı contributed to the interpretation of the results. Derya Altun led the writing of the draft. All authors provided critical feedback and assisted in analyzing the research and shaping the article.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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