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Original Article

Variable-number tandem repeat markers for *Mycobacterium intracellulare* genotyping: comparison to the 16S rRNA gene sequencing

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Abstract

Introduction: Characterizing *Mycobacterium intracellulare* responsible for nontuberculous mycobacterial (NTM) infections may aid in controlling outbreaks. This study aimed to compare 16S ribosomal ribonucleic acid (rRNA) sequencing and variable-number tandem repeat (VNTR) genotyping of *M. intracellulare* strains isolated from clinical samples, and to characterize VNTR clusters associated with NTM infections or cavity formation.

Methodology: Sputum samples were obtained from 77 HIV-negative patients with pulmonary disease between 2009 and 2013. One *M. intracellulare* strain was isolated from each patient and genotyped using 16S rRNA and eight loci VNTR sequencing.

Results: Single nucleotide polymorphism (SNP) genotyping identified seven point mutations at nucleotide positions 101, 178, 190, 252, 382, 443, and 490 in 16S rRNA, and four SNP patterns were identified: type 1 (16 strains), 2 (41 strains), 3 (11 strains), and 4 (1 strain); 5 strains had unique SNP patterns. VNTR genotyping identified VNTR12 as the most discriminating marker (allelic diversity 0.692). VNTR3 was the most homogeneous marker (allelic diversity 0.518), but each locus had high discriminating ability. The 77 strains were clustered according to the unpaired group method using arithmetic averages: cluster 1 (17 strains), 2 (43 strains), 3 (9 strains), and 4 (4 strains); 4strains had unique SNP patterns. Overall, over 90% strains were matched to similar SNP and VNTR groupings. VNTR clusters were associated with NTM infection ($p = 0.007$) and presence of a cavity ($p = 0.042$). Both methods distinguished four subtypes of *M. intracellulare*, which corresponded.

Conclusions: VNTRs may represent an effective, user-friendly, low-cost typing technique.

Key words: nontuberculous mycobacterial; *Mycobacterium intracellulare*; 16S rDNA sequencing; single-nucleotide polymorphism; variable-number tandem repeat genotype.

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Introduction

Mycobacterium intracellulare (*M. intracellulare*) is often responsible for nontuberculous mycobacterial (NTM) infection. As an environmental opportunistic pathogen, *M. intracellulare* is a ubiquitous inhabitant of soils, water, animal and plant surfaces, and air [1,2]. Humans acquire *M. intracellulare* by inhalation or ingestion, either directly from their normal habitat or indirectly via intake of contaminated water or food. Despite their omnipresence in the environmental niche, there have been no reported cases of human-to-human or animal-to-human transmission [3]. *M. intracellulare* is the most common cause of severe lung NTM infection, and can cause human pulmonary disease, wound infection, bacteremia, and other diseases [4,5]. *M. intracellulare* has been reported to be isolated in up to one-third of NTM in some settings [6-8].

The genetic diversity in *M. intracellulare* responsible for NTM is not yet well understood [9-11], and further characterizing the distribution and clinical profile of *M. intracellulare* strains may allow sources of the disease to be identified and allow for the monitoring of clinical outbreaks, disease relapses, and population dynamics [12]. Strain comparison of this species has been dependent upon pulsed-field gel electrophoresis (PFGE) [13,14], which is expensive, technically difficult, and time consuming, but its genome has been recently sequenced [15-17]. Now, sequence-based epidemiological characterization of *M. intracellulare* strains may be achieved [2]. Four genotypes of *M. intracellulare* have been distinguished by the polymerase chain reaction (PCR)-restriction enzyme pattern analysis heat-shock protein 65 (PRA-hsp65) technique [18,19].

Variable-number tandem repeat (VNTR) are mini satellite-like loci containing variable copy numbers and usually exist as flanking regions or non-coding region of different genes. VNTRs are scattered throughout the mycobacterial genome [20], and as a result, VNTR could be considered as a genotype tool in epidemiological research [21,22]. VNTR is an unambiguous, highly reproducible, and less costly genotyping method that has been widely used for the genotyping of several species of NTM, including *M. intracellulare* [23-25].

The number of repeats in VNTR loci can be changed by insertions or deletions, limiting the capacity of genotyping data to yield accurate phylogenetic results [26]. Characterization of single-nucleotide polymorphisms (SNPs) has been widely used in bacterial identification and genotyping [27-29], as well as SNP analysis [30]. As one of the most conserved genes, these mutable bases play an important role in phylogenetic analysis [31].

This study aimed to explore the capacity of 16S rRNA sequencing and eight loci VNTR genotyping of *M. intracellulare* strains isolated from clinical samples, and characterized *M. intracellulare* genotypes associated with NTM infections or cavity formation.

Methodology

Strains

A total of 77 clinical strains of *M. Intracellulare* were obtained from 77 HIV-negative patients with pulmonary disease between January 2009 and December 2013 at the Shanghai Municipal Center for Disease Control and Prevention (China). Of 77 HIV-negative patients with pulmonary disease, 54 were male and 23 were female, with a mean age of 53.1±16.4 years. NTM infection was detected in 48 (62.3%) patients, and colonization was detected in 29 (37.7%). A total of 42 (54.5%) patients showed a cavity. All strains isolated from patients' sputum were identified as *M. intracellulare* using the COBAS AMPLICOR *Mycobacterium tuberculosis* assay analyzer (Roche Diagnostic System, Basel, Switzerland). Age and gender of all patients were recorded. NTM pulmonary diseases were diagnosed based on the guidelines issued by the American Thoracic Society. Presence of a cavity was analyzed by X-ray. The study protocol was approved by the ethics committee of the Shanghai Municipal Centers for Disease Control and Prevention (CDC).

Preparation of mycobacterial DNA

As previously described [32], Ziehl-Neelsen stain-positive bacteria grown on Lowenstein-Jensen medium for 1–3 weeks were suspended in 1 mL of TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid [pH8.0]) and heat-inactivated for 10 minutes at 100°C, then at 0°C for 5 minutes. The bacterial specimen tube was centrifuged at 12,000×*g* for 3 minutes, and then the supernatant was transferred into another clean tube for further analysis. The amount and quality of DNA were assessed by Nano-200 micro-spectrophotometer (Hangzhou Allsheng Instruments Co., Ltd., Zhejiang, China) at 260 and 280 nm.

16S rDNA gene sequencing

A 514-bp fragment of 16SrDNA gene was amplified using the following primers: forward 5'-TGGAGAGTTTGATCCTGGCTCAG-3' and reverse 5'-TACCGCGGCTGCTGGCAC-3' (Sunny Biotech Co., Ltd., Shanghai, China). The reaction suspension (50 μL) included 25 μLof MATRIX (Tiangen Biotech, Co., Ltd., Beijing, China), 13 μLof double-distilled water (ddH_2O), 5 μL of each primer, and 2 μLof DNA template. The PCR amplification conditions were: pre-denaturation at 94°C for 5 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a single 5-minute elongation step at 72°C. DdH_2O served as a negative control, and *M. intracellulare* ATCC13950 served as a positive control. The DNA products were visualized using 1% agarose gel electrophoresis. All DNA products were sequenced by Sunny Biotech Co., Ltd., Shanghai, China. SNP stability and reliability of 16S rDNA gene sequencing were assessed by the isolation of11 strains from 4 patients over 1 year.

PCR amplification of VNTR

Eight pairs of primers were designed to amplify isolated *M. intracellulare*, as previously described [20,24] (Table 1). The PCR reaction comprised 1 μLof DNA template (approximately 20 ng) and 9 μLof mixture containing Taq (1×) PCR MasterMix (Tiangen Biotech, Co., Ltd., Beijing, China), 0.4 μL of each primer (Sunny Biotech Co., Ltd., Shanghai, China), 0.5 μLof dimethyl sulfoxide, and ddH_2O (to adjust the volume to 9 μL). The PCR amplification conditions were: pre-denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a single 7-minute elongation step at 72°C. *M. intracellulare* ATCC13950 served as a positive control. The amplified PCR products were analyzed using 1% agarose gel

electrophoresis to determine their size in base pairs. Repeat numbers were calculated according to each biomarker loci repeat size (Table 1).

Data analysis

16S rDNA sequences analysis was performed using MEGAversion 5.04, and eight loci VNTR dendrograms were constructed according to the unpaired group method using arithmetic averages (UPGMA). The discriminatory power of combined mycobacterial interspersed repetitive unit (MIRU)-VNTR loci was calculated using the Hunter-Gaston discriminatory index (HGDI), which expresses the probability of two strains in a given population appearing to be unrelated according to the typing method used [33]. Genetic diversity was assessed by allelic diversity (h) [34]. The relationship between genotyping of 16S rDNA sequences and VNTR clusters was estimated using Jaccard similarity coefficient [35]. Bionumerics software, version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyze the genetic relationships of independent strains through the construction of an UPGMA tree.

All statistical analyses were conducted using SPSS version 17.0 (IBM, Armonk, USA). Statistical significance was evaluated by Fisher's exact test for categorical variables. A p value < 0.05 was considered statistically significant.

Results

VNTR typing

Eight loci VNTRs were amplified efficiently from all strains: MIN2, and VNTR1, 2, 3, 7, 10, 12, and 13 (Table 2). Eleven strains were isolated from four patients over one year during NTM relapse, with the aim of establishing the stability of VNTR genotyping. Identical VNTR allele profiles were observed in these samples, indicating that VNTR typing was a reliable method and that the strains were genetically stable over the indicated time frames.

Genetic diversity was assessed by allelic diversity (h), as described by Selander *et al*. [34]. VNTR12

Table 1. Primer sequences, conservation, estimated size, and repeat size for PCR amplication of variable-number tandem repeat (VNTR) in *M. intracellulare* ATCC13950.

Loci name	Primer sequences	Estimated size (bp)	Repeat size (bp)	Conversation (%)
MIN2#	F: 5'-TCAGGAATGGGTCCGGTTC-3' R: 5'-AGCTCGTGACGACGGAAAC-3'	400	56	98
VNTR1*	F: 5'-TCGCCGAGGACTTCGTCT-3' R: 5'-GTCACCACGAGGAAGATCG-3'	273	57	100
VNTR2*	F: 5'-AGGGTGGTGAACGCGTAG-3' R: 5'-CTCTGGCAGCCCGATACC-3'	299	57	99
VNTR3*	F: 5'-AGAGGTGCTGCCGATTACAC-3' R: 5'-TCTTTGTCCGGTTCCTTTTG-3'	280	58	100
VNTR7*	F: 5'-TTTCATGGTTCGCCCTCTAC-3' R: 5'-GTTCGTCGGAGGTCATGGT-3'	274	53	99
VNTR10*	F: 5'-GGCTGGTTCTTTCTGGTGAC-3' R: 5'-CGCGTCAAGGAACGTCAT-3'	353	57	98
VNTR12*	F: 5'-AGACCAACCCAGAAAAGTGC-3' R: 5'-GTCGTGATACGCCGAATTG-3'	245	53	97
VNTR13*	F: 5'-GTTCAGCGAGCCGGTATCT-3' R: 5'-AGCTCTCGCAGCTTGGTTC-3'	292	50	97

F: forward; R: reverse; * These primers were previously described by Ichikawa *et al*. [24]; # These primers were previously described by Dauchy *et al*. [20].

Table 2. Variable-number tandem repeat (VNTR) allelic distribution and allelic diversity among 77 *M.intracellulare* isolates.

Loci name	Number of isolates with VNTR copy number							Allelic diversity (h) #
	0	1	2	3	4	5	6	
MIN2	2	10	13	37*	15			0.685
VNTR1	1	14	10*	38	14			0.673
VNTR2	9	4	6	46*	11	1		0.600
VNTR3	5	47*	25					0.518
VNTR7	13	4	38	18*	3			0.669
VNTR10	10	42*	3	1	13	7	1	0.647
VNTR12	5	22	17*	32	1			0.692
VNTR13	2	17	38	20*				0.640

* Denotes the profile of the reference strain ATCC13950; # Genetic diversity was assessed by allelic diversity (h), as described by Selander *et al*. [34]; Hunter-Gaston discrimination index = 0.996 [33].

exhibited the highest allelic diversity (HGDI = 0.692), and VNTR3 was the most homogeneous marker (HGDI = 0.518) (Table 2). By this method, a high discriminatory index was detected for VNTR3 and VNTR12. The overall HGDI of the eight loci of MIRU-VNTR method was 0.996. MIN2 also had a high discriminatory index (HGDI = 0.685).

The genetic relationships of the 77 independent strains were assessed by construction of an UPGMA tree. Four clusters and four singleton patterns were discriminated; cluster 2 (the most prevalent) contained 46 strains and ATCC13950 (data not shown); cluster 1 (the second-most prevalent) contained 14 strains; cluster 3 contained 9 strains; and cluster 4 contained 4 strains (Figure1).

16S rDNA gene sequencing

The 16S rDNA amplicon of the 77 *M. intracellulare* strains was sequenced. While the PCR product length predicted by the primers was 514 bp, only intact sequences 440 bp were achieved for each strain (rs1638040–rs1638480) and this segment had to be used for phylogenetic analysis. Within this sequence, seven loci base differences were detected, including nucleotide positions 101, 178, 190, 252, 382, 443, and 490 (16S rDNA). SNP patterns included four major pattern types and five singletons (Table 3). The most common pattern, SNP pattern type 2 (41 strains), included ATCC13590, which has been detected worldwide [36,37]. Type 3 (containing 11 strains) had high similarity with *M. chimaera* CIP 107892 [38]. In fact, only one base (position 403) differed between *M. intracellulare* and *M. chimaera* [39]. Type 1 (16 stains) had the same sequence as *M. intracellulare* 41, and included three base mutations, while type 2, first isolated in Malaysia, involved only one mutation [40]. Type 4 included four strains, and appeared to be a novel mutation pattern [41], suggesting that type 4 may exist only in east China.

Figure 1.UPGMA tree of the MIRU-VNTR types for 77 clinically independent *M. intracellulare* strains.

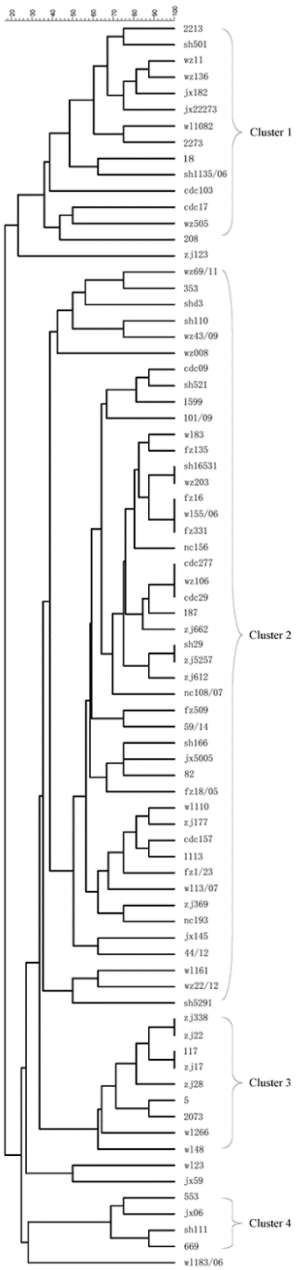

Table 3. Distribution of VNTR and SNP types in *M. intracellulare*.

VNTR cluster	SNP type					
	1	**2**	**3**	**4**	**Singletons**	**Total**
Cluster 1	13		1		3	17
Cluster 2	2	41				43
Cluster 3			9			9
Cluster 4			1	3		4
Singletons	1			1	2	4
Total	**16**	**41**	**11**	**4**	**5**	**77**

VNTR: variable-number tandem repeat; SNP: single-nucleotide polymorphism.

Comparison of VNTR and SNP typing

To assess the association between VNTR and SNP typing, Jaccard similarity coefficient was calculated [35]. As shown in Figure 2, 13 of 16 SNP Type 1 strains (81.25%) were categorized as VNTR cluster 1. All 41 SNP type 2 strains were categorized as VNTR cluster 2. Of 11 SNP type 3 strains, 9 (81.82%) were categorized as VNTR cluster 3. Three of four SNP type 4 strains (75%) were categorized as VNTR cluster 4. Overall, over 90% of strains were matched to similar SNP and VNTR groupings. The four strains with unique VNTR types also yielded unique SNP patterns (Table 3). Thus, both methods could be considered to successfully distinguish four categories of *M. intracellulare* among the 77 studied samples.

Relationship between clinical characteristics and VNTR clusters

Among the 48 patients with NTM infection, 28 (58.3%) patients had cluster 2 *M. intracellulare* strains. Cluster 1 strains were detected in 13 (27.1%) patients, and the remaining clusters or singleton strains were detected in 7 patients (Table 4). Among the 29 patients with NTM colonization, the most common cluster was cluster 2, detected in 18 (62.1%) patients, followed by cluster 3, detected in 7 (24.1%) patients; the remaining clusters or singleton strains were detected in 4 patients (Table 4). VNTR clusters were associated with NTM infection (p =0.007).

Cluster 2 *M. intracellulare* strains was observed in 21/42 (50.0%) patients with cavities. Cluster1 strains were the next-most prevalent, detected in 11 (26.2%) patients. The remaining clusters or singleton strains were detected in 10 patients (Table 4). Of the 35 patients without cavities, the most common cluster was cluster 2, detected in 25 (71.4%) patients, followed by cluster 3, detected in 4 patients (11.4%); the remaining clusters or singleton strains were detected in 6 patients (Table 4). VNTR clusters were associated with the presence of a cavity (p =0.042).

Figure 2. Genotyping of 77 clinically isolated pulmonary *M. intracellulare* strains performed using 16S rDNA sequencing and eight loci variable-number tandem repeat (VNTR) typing. b: VNTR typing method; single-nucleotide polymorphism (SNP) typing of partial 16S rRNA gene sequence. ND: not determined; NC: no cluster.

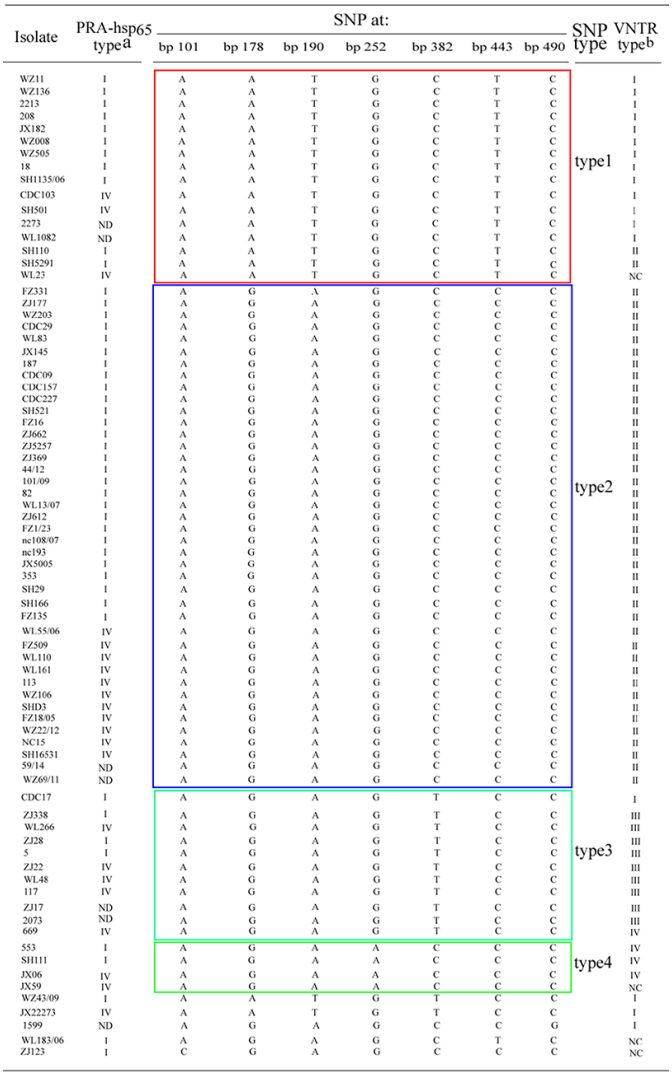

Table 3. Distribution of VNTR and SNP types in *M. intracellulare*.

VNTR cluster	SNP type					
	1	**2**	**3**	**4**	**Singletons**	**Total**
Cluster 1	13		1		3	17
Cluster 2	2	41				43
Cluster 3			9			9
Cluster 4			1	3		4
Singletons	1			1	2	4
Total	**16**	**41**	**11**	**4**	**5**	**77**

VNTR: variable-number tandem repeat; SNP: single-nucleotide polymorphism.

Table 4. Distribution of NTM infection or colonization, or presence or absence of a cavity among the different VNTR clusters.

Cluster	NTM infection n (%)	NTM colonization n (%)	P	Presence of a cavity n (%)	Absence of a cavity n (%)	P
Cluster 1	13 (27.1)	1 (3.4)		11 (26.2)	3 (8.6)	
Cluster 2	28 (58.3)	18 (62.1)		21 (50.0)	25 (71.4)	
Cluster 3	2 (4.2)	7 (24.1)	0.007	5 (11.9)	4 (11.4)	0.042
Cluster 4	3 (6.3)	1 (3.4)		4 (9.5)	0 (0.0)	
Singletons	2 (4.2)	2 (6.9)		1 (2.4)	3 (8.6)	

NTM: nontuberculous mycobacterial; VNTR: variable-number tandem repeat

Discussion

VNTR genotyping provides a rapid, effective way to estimate the presence of clonal complexes within linked strains [42]; it was recently applied to study genetic variability in clinical strains of *M. intracellulare* [20,24].

This study aimed to compare 16S rRNA sequencing and VNTR genotyping of *M. intracellulare* strains isolated from 48 patients with NTM and 29 patients without NTM. SNP genotyping identified seven point mutations in 16S rRNA, and four SNP patterns were identified (representing 97% of all tested strains). Eight loci VNTRs were amplified efficiently from all strains, MIN2, and VNTR1, 2, 3, 7, 10, 12, and 13.

The statistical power of the VNTR technique was assessed for the epidemiological characterization of *M. intracellulare*. The global discriminatory index of 0.996 confirmed the possible advantage of this technique. Interestingly, Ichikawa *et al*. [24] and Dauchy *et al*. [20] reported a Hunter-Gaston discrimination index of 0.98 in the multiple-locus VNTR analysis (MLVA) of *M. intracellulare*. In this study, 69 VNTR types were obtained from 77 *M. intracellulare* strains.

VNTR12 exhibited the highest allelic diversity, and VNTR3 was the most homogeneous marker. A higher discriminatory index was detected for VNTR3 and VNTR12 than previously reported by Ichikawa *et al*. [24]. The overall HGDI of the eight loci of the MIRU-VNTR method was 0.996. MIN2 also had a high discriminatory index, as previously described by Ichikawa *et al*. [24]. Overall, VNTR genotyping identified eight loci with high allelic diversity, and 95% were clustered into four VNTR clusters.

Identical VNTR allele profiles were observed in 11 strains from 4 patients, indicating that VNTR typing was a reliable method and that the strains were genetically stable over the indicated time frames, consistent with previous reports [20,24]. The MIN2 discrimination index was consistent with results reported by Dauchy *et al*. [20]; however, the discrimination index of VNTR3 and VNTR12 was higher, as described by Ichikawa *et al*. [24].

Linkage disequilibrium among VNTR loci has been observed in high-incidence areas of *M. tuberculosis*, suggesting that this bacterium underwent clonal evolution [43]. As clonal evolution exists in almost all bacterial strains, phylogenetic analyses based on VNTR data might not yield correct phylogenetic results [44]. High-throughput whole genome sequencing (WGS) is one of the best methods to study bacterial strain relationships [45], but WGS is expensive, labor intensive, and requires highly trained professionals. Similar to VNTR, SNP of certain genes can be used to genotype bacterial strains [27]. 16S rRNA is one of the most conserved genes, and thus is often used for bacterial identification and genotyping [46]. The mutation rate of ribosome genes is extremely low; therefore, these genes can be reliably used to represent some genotypes. Furthermore, ribosomal sequence typinghas been demonstrated to accurately summarize the relationships between bacterial genomes [47].

This study suggested that both 16S rRNA sequencing and VNTR genotyping of *M. intracellulare* can be used to distinguish four subtypes of *M. intracellulare*, which corresponded between the two methods; therefore, VNTRs may represent an effective, user-friendly, low-cost typing technique. Nevertheless, this study has some limitations. Although all the studied strains were derived from sputum, no representative animal and environmental sources were identified. The value of the VNTR selected markers may represent linkage disequilibrium and/or clonal evolution. In the future, more strains from different sources should be analyzed in order to characterize precisely the phylogeny of both environmental and pathogenic strains of *M. intracellulare*.

Conclusions

The results of SNP typing of 16S rDNA sequences and eight VNTR loci clusters were consistent with one another. VNTR clusters were related toNTM infection and the presence of a cavity. VNTR could be considered as a genotype tool in epidemiological research of *M. intracellulare*. Nevertheless, analyzing more strains

from different sources is required to confirm these results.

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Authors' contributions

KSC and YYZ contributed to conception and design; KSC, YYZ and YPP contributed to acquisition of data, or analysis and interpretation of data; KSC and YYZ were involved in drafting the manuscript or revising it critically for important intellectual content; all authors gave final approval of the version to be published.

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