Original Article

Novel Species Including *Mycobacterium fukienense sp*. Is Found from Tuberculosis Patients in Fujian Province, China, Using Phylogenetic Analysis of *Mycobacterium chelonae/abscessus* Complex*

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Abstract

Objective To identify the novel species '*Mycobacterium fukienense*' *sp. nov* of *Mycobacterium chelonae/abscessus* complex from tuberculosis patients in Fujian Province, China.

Methods Five of 27 clinical *Mycobacterium* isolates (Cls) were previously identified as *M. chelonae/abscessus* complex by sequencing the *hsp65, rpoB, 16S-23S rRNA internal transcribed spacer region (its), recA* and *sodA* house-keeping genes commonly used to describe the molecular characteristics of *Mycobacterium.* Clinical *Mycobacterium* isolates were classified according to the gene sequence using a clustering analysis program. Sequence similarity within clusters and diversity between clusters were analyzed.

Results The 5 isolates were identified with distinct sequences exhibiting 99.8% homology in the *hsp65* gene. However, a complete lack of homology was observed among the sequences of the *rpoB, 16S-23S rRNA internal transcribed spacer region (its), sodA,* and *recA* genes as compared with the *M. abscessus*. Furthermore, no match for *rpoB, sodA,* and *recA* genes was identified among the published sequences.

Conclusion The novel species, *Mycobacterium fukienense*, is identified from tuberculosis patients in Fujian Province, China, which does not belong to any existing subspecies of *M. chelonea/abscessus* complex.

Key words: Species identification; *Mycobacterium chelonae/abscessus complex*; *Mycobacterium fukienense*

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INTRODUCTION

The *M. chelonae/abscessus* complex, a rapid growing *Mycobacterium* (RGM), is an important pathogenic bacterium in the clinic when traumatic skin wounds and post-surgical soft tissue are contaminated by it[1-2]. It is also a pathogenic bacterium leading to nosocomial infections[3] and epidemics[4-5]. Recently, most studies were focused on its pathogenic features, such as the pathogen, molecular and epidemiological characteristics, and the sensitivity of the pathogenic non-tuberculosis *Mycobacterium* (NTM) to antibiotics. Subspecies including *M. immunogenum*, *M. massiliense,* and *M. bolletii* belonging to the *M. chelonae/abscessus* complex were first reported in 2001[6], 2004[7], and 2006[8], respectively. It is necessary to further identify the subspecies for policy making to guide epidemiological surveys, communicable diseases prevention and control, and rational use of antibiotics in clinical practice. Won-Jung Koh found that the sensitivity to combinated antibiotics therapy including clarithromycin is significantly higher in patients with *M. massiliense* lung disease than in those with *M. abscessus* lung disease[9], indicating that the *M. massiliense* is significantly different from the *M. abscessus*. A number of methods have been described to identify members of the *M. chelonae/abscessus* complex at the species or subspecies level, including conventional phenotypic and biochemical methods. Molecular methods such as PCR-RFLP analysis, line probe hybridization and nucleic acid sequencing are commonly used although selection of target genes is critical for accurate identification. Members of the *M. chelonae/abscessus* complex share a complete similarity in the *16s rRNA* sequence[10]. Therefore, this gene is an unsuitable target for distinguishing *Mycobacterium* species or subspecies. Previous studies considered that analysis of *hsp65*[11-13], *rpoB*[8,10-13], or *sodA*[14] genes is a suitable method for classification of *Mycobacterium* isolates at the species level, but discordant identification based on other genes is apparent[15] due to the 'complex interspecies patterns'. This phenomenon shows the importance of multiple target gene analysis meantime for accurate discrimination of species in the *M. chelonae/abscessus* complex group. Concordance among the *hsp65, rpoB,* and *sodA,* genes is presumed to be sufficient for accurate identification[15]. To date, no species has been identified with 99.8% similarity in the *hsp65* gene sequence with the *M. abscessus*. The most similar species are *M. massiliense* and *M. bolletii,* which only differ in 5 nucleotides over the entire 441 bp sequence of the *M. abscessus hsp65* gene[16]. The 5 CIs investigated in this study exhibit 99.8% homology with the *M. abscessus hsp65* gene, although a lack of homology is observed in the *rpoB, its, sodA,* and *recA* genes, indicating that the novel *M. abscessus* species exists. In this study, the biochemical characteristics of these novel isolates were analyzed and their molecular characteristics were identified with the reverse multi-line probe hybridization analysis method.

MATERIALS AND METHODS

Bacterium Strains

Of the 27 CIs, which were identified as *M. chelonae/abscessus* complex strains in a previous study, 23 isolates with identical *hsp65* PCR-RFLP patterns are correlated with the published *hsp65* PCR-RFLP pattern of *M. abscessus* ATCC19977[16], and 4 isolates share an identical pattern with the published *hsp65* PCR-RFLP pattern of the *M. abscessus* group[16] (*M. abscessus*, ATCC14472; *M. Massiliense*, CIP108297; *M. bolletii*, CIP108541). Of the 23 isolates, 5 exhibit an identical *hsp65* PCR-RFLP pattern with the *M. abscessus,* but a novel *rpoB* PCR-RFLP pattern fails to match any previously reported patterns. The *hsp65* and *rpoB* PCR-RFLP patterns of the 27 clinical isolates have been previously reported[17].

Genome DNA Extraction

Genomic DNA in 27 clinic isolates and *M. abscessus* ATCC19977 was extracted from 0.5 mL wet bacteria using a genome DNA extraction kit (TIANGEN BIOTECH, Beijing) according to the instructions of its manufacturer. The concentration of extracted nucleic acid was measured by spectrophotometry (Nanodrop Technologies, Inc, Wilmington, DE, USA).

Sequencing the Gene Fragments of hsp65, rpoB, its, sodA, and recA

Primers for amplification of the selected gene targets are listed in Table 1. The *recA* gene (430 bp) was amplified in a total reaction volume of 50 μL containing 25 μL 2×Taq PCR MasterMix (TIANGEN BIOTECH, BEIJING), 2 μL (10 μmol/μL) of each primer,

Table 1. Selected Gene Targets and Primers for Amplification

Genes	Primers	Sequence of Primers (5′→3′)	PCR Products (bp)
hsp65	Tb11	ACCAACGATGTGTGTCCAT	441
	Tb12	TTGTCGAACCGCATACCCT	
rpoB	*rpoB*F	TCAAGGAGAAGCGCTACGA	360
	*rpoB*R	ATGTTGATCAGGGTCTGC	
16s rRNA	P1	AGAGTTTGATCCTGGCTCAG	1524
	P2	AAGGAGGTGATCCAGCCGCA	
its	*ITS16s*F	ACCTCCTTTCTAAGGAGCACC	221
	*ITS23S*R	GATGCTCGCAACCACTATCCA	
sodA	*sod*F	ACATCTCGGGTCAGATCAACGAGC	464
	*sod*R	GACGTTCTTGTACTGCAGGTA	
recA	*rec*F3	GGCAARGGYTCGGTSATGC	440
	*recR*1	AGCTGGTTGATGAAGATYGC	

5 μL (30 μg/mL) DNA solution and added DD H_2O to 50 μL. The amplification reaction was performed as follows: suspention at 94 °C for 10 min, followed by 30 cycles at 94 °C for 1 min, at 60 °C for 45 s , at 72 °C for 50 s, and a final extension incubation at 72 °C for 5 min. The *hsp65* (440 bp)[18], *rpoB* (360 bp)[19], *its* (280 bp)[20-21], and *sodA* (441 bp) genes were amplified as previously described[14] and purified using PCR product purification kits (TIANGEN, China). PCR products were sequenced using BigDye Terminator cycle sequencing kits with an ABI 3 130 automatic sequencer (ABI, USA).

Phylogenetic Relationship and Gene Diversity Analysis

Gene sequences were aligned using BioEdit (BioEdit software, version 7.0.5) followed by cluster analysis using the ClustalW program. Genetic diversity was also analyzed using BioEdit by comparing the sequences of each gene target within the clinical isolates and reference strains. Phylogenic trees of each gene target were constructed using the neighbor-joining method of molecular evolutionary genetics analysis (MEGA software, version 5) with 1 000 bootstrap trails.

Phenotypic Tests

The growth rate, pigment production, growth at 42 °C, MacConkey agar-supported growth, arylsulfatase activity, nitrate reduction activity and tolerance to 5% NaCl were analyzed by phenotypic analysis as previously described[2,22].

Reverse Multi-line Probe Hybridization Analysis

A probe hybridization kit (Genotype® Mycobacterium CM, Hain Lifescience, Canada), described by Makinen[23], was used for detection and discrimination of 13 common non-tuberculosis species including *M. abscessus* and *M. chelonae* following the instructions of its manufacturer.

RESULTS

Identification of 27 Clinical Isolates

The species was identified by comparing the partial sequences of the *hsp65*, *rpoB*, *its*, *sodA, and recA* genes. Eighteen clinical isolates (CIs) were identified as *M. abscessus*, and 3 CIs (CI1, CI12, CI24) were identified as *M. massiliense*. A single CI (CI25) exhibited 100% DNA-DNA homology with the *hsp65* gene sequence of *M. massiliense*, although all the other genes analyzed exhibited 100% DNA-DNA homology with *M. abscessus*. Five CIs (CI5, CI6, CI7, CI10, CI11) exhibited 99.8% homology with the *hsp65* sequence of *M. abscessus*, while the other genes (*rpoB, sodA* and *recA*), except *its*, exhibited the homology less than 97% as compared with published data (Table 2).

Phylogenetic Relationship Analysis Based on Sequence of Each Target Gene

A phylogenetic tree of 27 CIs and type strains of selected *M. abscessus* - a relative rapidly growing *Mycobacterium* (RGM) was constructed according to the sequence of each target gene. The iIsolates were classified into group I (*n*=18), group II (*n*=4), and group III (*n*=5). According to the *hap65, rpoB, sodA,* and *recA* sequences. As shown in the phylogenetic trees, the members of each group in all the other

trees were clustered concordantly, with the exception of CI25, which was classified as *M. massiliense* based on *hsp65* but as *M. abscessus* based on *rpoB, sodA,* and *recA*. The proximity relationship of *M. abscessus* was dependent on the target gene. The phylogenetic trees based on the sequences of the *hsp65* (A), *rpoB* (B), *recA* (C), and *sodA* (D) genes are shown in Figure 1.

Genetic Diversity Analysis Based on Target Gene Sequences

The sequences of each target gene from 27 CIs and type strains of *M. abscessus* were aligned. The similarity within each cluster and the diversity between clusters were analyzed. The genetic diversity in restriction sites was analyzed for *hsp65* and *rpoB* genes. The sequence of 410 bp *hsp65* gene

Table 2. Results of BLAST Analysis Based on Sequences of 5 Target Genes from 6 Clinical Isolates with Discordant Species Identification

CIs	Original No.	*hsp65*		*rpoB*		*its*		*sodA*		*recA*	
		Spp.	Identity (%)	Spp.	Identity (%)	Spp.	Identity (%)	Spp.	Identity (%)	Spp.	Identity (%)
CI5	FJ05242	*M.ab*	*99.8*	*M.im*	96.4	*M.spp.JAN*	99.1	*M.im*	96.1	*M.ma*	91.4
CI6	FJ05517	*M.ab*	*99.8*	*M.im*	96.4	*M.spp.JAN*	98.8	*M.im*	96.1	*M.ma*	91.4
CI7	FJ05520	*M.ab*	*99.8*	*M.im*	96.4	*M.spp.JAN*	98.8	*M.im*	96.1	*M.ma*	91.4
CI10	FJ05534	*M.ab*	*99.8*	*M.im*	96.4	*M.spp.JAN*	98.4	*M.im*	96.1	*M.ma*	91.4
CI11	FJ05541	*M.ab*	*99.8*	*M.im*	96.4	*M.spp.JAN*	98.8	*M.im*	96.1	*M.ma*	91.4
CI25	FJ09083	*M.ma*	*100*	*M.ab*	100	*M.ab*	100	*M.ab*	100	*M.ab*	100

Note. *M.im:* Mycobacterium immunogenum; *M.ma:* Mycobacterium massilense; *M.spp.JAN:* Mycobacterium spp JAN; *M. ab:* Mycobacterium abscessus. CI represents the clinical isolate identification used in this study.

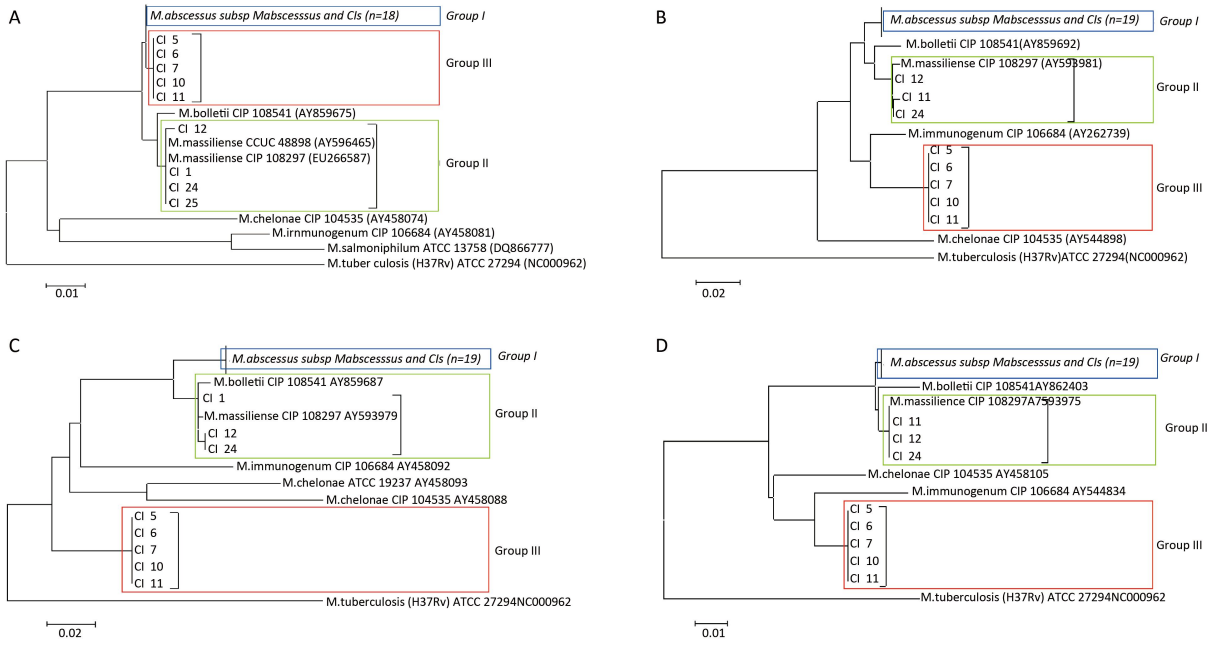

Figure 1. Construction of phylogenetic trees using the Neighbor-joining method according to the sequences of *hsp65* (A), *rpoB* (B), *recA* (C), and *sodA* (D) genes and type strains of relative RGM from selected *M. chelonae/abscessus* complex including *M. tuberculosis* H37Rv. Blue Border: Group I, *M. abscessus*; Green Border: Croup II, *M. massiliense*; Red Border: Group III, the novel species, *M. fukienense.*

revealed that 5 nucleotides (positions 277, 280, 289, 352, 502 bp defined by the published nucleotide sequence of the *M. abscessus* ATCC 19977, Genbank accession number AY489743) were distinct between *M. massiliense* and *M. abscessus* strains. The diversity at position 289 in isolates of *M. massiliense* strains destroyed one HaeIII restriction site. Seven nucleotides (positions 122, 191, 213, 272, 312, 315, 320 as defined by the published nucleotide sequence of the *M. abscessus* ATCC19977, Genbank accession number AY489743) identified as the partial *rpoB* gene (>310 bp) as shown in Figure 2 were distinct between *M. massiliense* strains and *M. abscessus* strains, and 15 nucleotides (positions 68, 77, 83, 128, 174, 176, 191, 204, 248, 250, 272, 290, 297, 306, 315) were distinct between Group III strains and *M. abscessus* strains. The diversity at positions 83, 204, and 294 of Group III destroyed two *MspI* restriction loci (marked with an ellipse in Figure 2). Two *HaeIII* restriction loci were changed at positions 68 and 297 in *rpoB* gene of the novel group strains. The sequence of its gene revealed that 3 nucleotides were distinct between *M.massiliense* strains and *M. abscessus* strains, and 10 nucleotides were distinct between Group III strains and *M. abscessus* strains. The sequence of sodA gene (>413 bp) revealed that 2 nucleotides were distinct between *M. massiliense* strains and *M. abscessus* strains, and 29 nucleotides were distinct between Group III strains and *M. abscessus* strains. The sequence of *recA* gene (>420 bp) revealed that 12 nucleotides were distinct between *M. massiliense* strains and *M. abscessus* strains, and 36 nucleotides were distinct between Group III strains and *M. abscessus* strains.

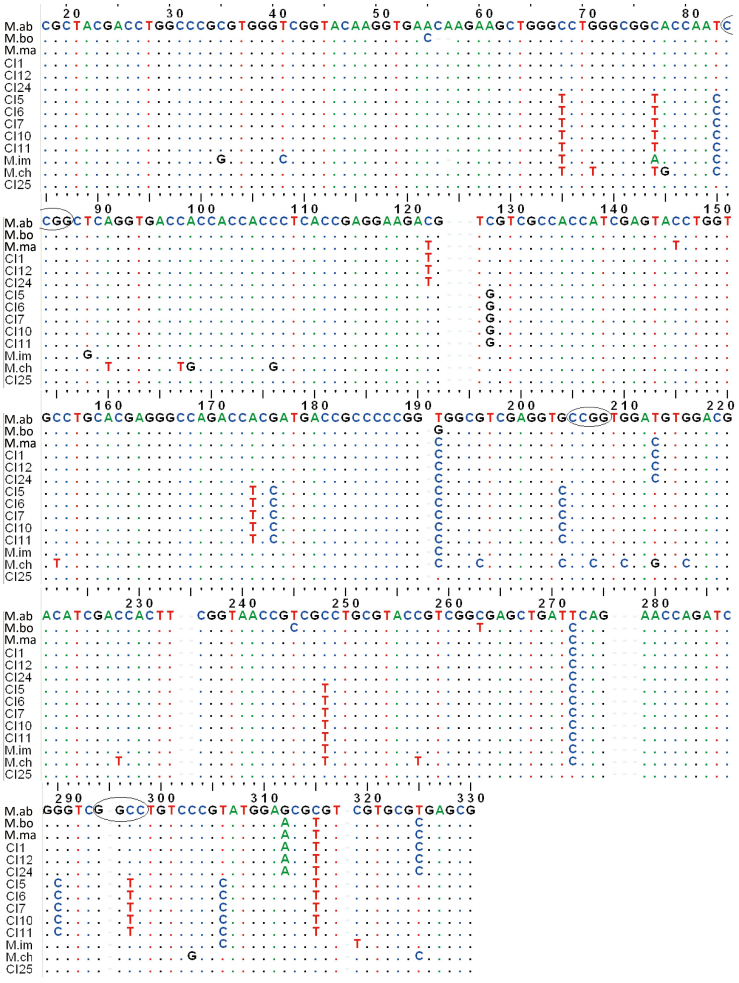

Figure 2. Alignment of partial *rpoB* gene sequences from 27 clinical isolates and type strains of selected relative RGM. Diversity at positions 83, 204, and 294 were marked with ellipse; different color represents different bases.

Phenotypic Characterization and Species Identified by Multi-line Probe Hybridization Analysis

Phenotypic characterization of the 6 clinical isolates with discordant species and type strains of the selected *M. abscessus* are shown in Table 3. All the strains were positive in Ziehl-Neelsen (Z-N) staining and could grow in Lowenstein-Jensen(L-J), L-J culture medium with p-nitrobenzoic acid (PNB) and L-J culture medium with 2-thiophenecarboxylic acid hydrazide (TCH) solid culture media, and mature colonies in less than 7 days without pigment production, and exhibited arylsulfatase activity. Five isolates, CI5, CI6, CI7, CI10, CI11 (FJ05242, FJ05517, FJ05520, FJ05534, and FJ05541) did not grow in culture medium at 42 °C and could not tolerate to 5% NaCl, thus displaying the characteristics similar to *M. chelonae* ATCC 35752. In contrast, a single isolate, CI25 (FJ09083), could grow either in culture medium at 42 °C or tolerate to 5% NaCl, thus displaying the same characteristics as the *M. abscessus* group strains *(M. abscessus* ATCC 19977 and *M. massiliense* CIP 108297). Furthermore, all the 5 isolates in this novel group were identified as *M. chelonae* using a Genotype@ Mycobacterium CM kit.

DISCUSSION

Of the 27 clinical isolates identified as *M. mechelonae/abscessus* complex strains, 23 displayed an identical *hsp65* PCR-RFLP pattern [235 bp, 210 bp (*BstII*)/145 bp, 70 bp, 60 bp, and 55 bp (*HaeIII*)] with the published *hsp65* PCR-RFLP pattern[16] of *M. abscessus* ATCC19977, and 4 showed an identical pattern [235 bp, 210 bp (*BstEII*)/200 bp, 70 bp, 60 bp and 50 bp (*HaeIII*)] with the published *hsp65* PCR-RFLP pattern of the *M. abscessus* strain group (*M. abscessus* ATCC14472, *M. Massiliense* CIP108297 and *M. bolletii* CIP108541)[16]. Of the 23 isolates, 5 exhibited an identical PCR-RFLP pattern of *hsp65* with *M. abscessus* ATCC19977. A PCR-RFLP pattern of novel *rpoB* (200 bp, 100 bp, and 80 bp), *MspI* (200 bp) and *HaeIII* (135 bp) was identified, which failed to match any reported pattern and was distinct from the other clinical *M. abscessus* strains (100 bp, 95 bp, and 80 bp), *MspI* (130 bp, 100 bp) and *HaeIII* (90 bp). In this study, the gene diversity was analyzed according to the sequences of the *hsp65*, *rpoB*, *its*, *sodA,* and *recA* genes, in which the *hsp65* gene restriction loci are located for *bstEII* and *HaeIII*, and the *rpoB* gene restriction loci are located for *MspI* and *HeaIII*. The absence of significant diversity among the 5 isolates within the *hsp65* gene restriction loci for both enzymes was demonstrated by the generation of an identical PCR-RFLP pattern of *hsp65* as compared with *M. abscessus* ATCC19977. However, the alignment of partial *rpoB* gene sequences from the 27 clinical isolates and type strains of selected relative RGM *M. abscessus* revealed the diversity at positions 68, 297, and 83, 204, 290 of Group III, resulting in the destruction of 2 *HaeIII* restriction loci and 3 *MspI* restriction loci, respectively, indicating that the 5 isolates investigated in this study are significantly different from the previously identified strains.

Table 3. Phenotypic Characterization by Multi-line Probe Hybridization Analysis

Strains	Z-N Stain	Growth at			GR	Growth at 42 °C	Result of				Probe Test
		L-J	PNB	TCH			Pigment	Ary (3d)	NO_3^-	5% NaCl L-J	
M. abscessus ATCC19977	+	+	+	+	4	-	-	+	-	+	*M. abscessus*
M. massiliense CIP108297	+	+	+	+	4	-	-	+	-	+	*M. chelonae*
M. chelonae ATCC35752	+	+	+	+	5	-	-	+	-	-	*M.abscessus*
CI5 (FJ05242)	+	+	+	+	7	-	-	+	-	-	*M. chelonae*
CI6 (FJ05517)	+	+	+	+	5	-	-	+	-	-	*M. chelonae*
CI7 (FJ05520)	+	+	+	+	5	-	-	+	-	-	*M. chelonae*
CI10 (FJ05534)	+	+	+	+	5	-	-	+	+(weak)	-	*M. chelonae*
CI11 (FJ05541)	+	+	+	+	5	-	-	+	-	-	*M. chelonae*
CI25 (FJ09083)	+	+	+	+	4	+	-	+	+(weak)	+	*M. abscessus*

Note. Z-N stain: Ziehl-Neelsen stain; L-J: Lowenstein-Jensen culture medium; PNB: Lowenstein-Jensen culture medium with p-Nitrobenzoic acid; TCH: Lowenstein-Jensen culture medium with 2-thiophenecarboxylic acid hydrazide; GR: growth rate; Ary (3d): arylsulfatase activity in the third day; NO_3^- : nitrate reduction activity; 5% NaCl L-J: tolerance to 5% NaCl in L-J culture medium.

Of the 27 clinical isolates previously identified as *M. chelonae/abscessus* complex, 21 (18 *M. abscessus* and 3 *M. massiliense*) were identified according to the sequence analysis of multiple genotypic targets which are consistent with the *hsp65* gene and partially consistent with the *rpoB*, *its*, *sodA,* and *recA* genes, and 6 resulted in different results. *M. abscessus* was the main pathogenic bacterium within the *M. abscessus* group isolated from patients in China, while the 3 *M. massiliense* isolates identified in this study are relatively new members within the group, which was first reported in Switzerland as the cause of chronic lung disease[7] and opportunistic infections after surgical transplantation[4]. However, a recent study in Taiwan concluded that *M. abscessus* complex isolates, particularly *M. massiliense,* are potential pathogens causing CNS infections[24]. The *M. massliense* detected in this study contributes to the understanding of epidemic and pathogenic characteristics of this strain in China. Genetic diversity analysis revealed that nucleotides were different at 5 positions in the *hsp65* gene sequence as compared with *M. Abscessus*, which is consistent with the variation in the *hsp65* sequence of *M. abscessus* type II reported by Konig et al.[13]. Another member of the *M. abscessus* group, *M. bolletii*[16], was not detected in this study. Five clinical isolates similar to *M. abscessus* were identified as a novel species, *Mycobacterium fukienense*. These isolates exhibiting unique genetic and phenotypic characteristics were associated with respiratory tract infections diagnosed as tuberculosis in Fuzhou Pulmonary Hospital, Fujian province, China.

A single strain (CI25) yielded a complicated pattern identified by multiple target genes analysis. Although the *hsp65* sequence was 100% identical to that of *M. massiliense*, diversity was identified at 5 positions as compared with that of *M. abscessus,* but the integrity of *BstEII* restriction loci was retained. Diversity was also identified at position 289, with destruction of a single *HaeIII* restriction locus and an identical PCR-RFLP pattern with emerging *M. abscessus*. In contrast, the *rpoB, its, recA,* and *sodA* sequences were 100% identical to type III strains. Paradoxically, these matches are described as 'interspecific composite patterns'[15], and the presumption was finally confirmed according to the analysis of two genes among the *hsp65, rpoB,* and *sodA* sequences in this study. Viana-Niero et al.[4] reported that 2 *M. bolletii* isolates are identified as *M. massiliense*, and Kim et al.[25] reported that 2 isolates belonging to type III strains based on the *hap65* sequences are identified as *M. massiliense* according to the *rpoB* and *sodA* sequences. However, it is impossible to identify another 5 isolates[15]. The *hsp65* gene exhibited 99.8% homology with *M. abscessus*, with a single nucleotide difference (G→A) at position 250 (based on the sequence of type III strains ATCC 19977, GenBank accession number AY498743). This diversity was not shown within the *bstEII* and *HaeIII* restriction loci and therefore no difference was observed in the PCR-RFLP patterns between these strains and *M. abscessus* strains. Further molecular investigation for partial *rpoB* (>310 bp), *recA* (>420 bp), and *sodA* (>410 bp) gene sequences revealed that diversity existed within 14 bp, 36 bp, and 29 bp as compared with *M. abscessus*. BLAST analysis showed the highest homology (96.4%, 91.4%, and 96.1% respectively) between *M. immunogenum* and *M. massiliense*. Similarly, the *its* sequences (>275 bp) among these isolates (*M.* abscessus, *M. chelonae,* and *M. immunogenum*) were different. Multiple target gene sequencing and cluster analysis demonstrated that the genetic and phylogenetic features of the isolates were different from those of any species that is closely related to *M. abscessus* or *M. abscessus*. Phenotypic characterization and reverse multi-line probe hybridization analysis displayed that the phenotypic characteristics and hybridization band pattern of the 5 isolates were identical with those of *M. chelonea*, supporting the conclusion that a novel species exists in tuberculosis patients in Fujian province, China.

It was reported that a single gene sequence is used as the main technique in analysis of molecular characteristics and identification of *Mycobacterium* at the species level, and the *rpoB* gene has been widely studied with its usefulness in species identification discovered as compared to *16s rRNA*[8], which has been used as a reference method, even though the DNA-DNA hybridization (DDH)[26] is the 'gold standard' for bacterial species identification. These studies concluded that *rpoB* is a powerful target gene for identification of *Mycobacterium* at the species level and is superior to the DDH[8]. It has been shown that the isolates belong to the same species if the diversity is lower than 2% between partial *rpoB* gene sequence and a confirmed species, and it is a novel species if the diversity is higher than 3%[26]. The *sodA* gene is a more powerful target gene than the *rpoB* gene for identification of *Mycobacterium* species.

In this study, the diversity in sequences of the 5

isolates gene exceeded 3%, further proving the existence of a novel species in tuboculosis patients in Fujian province, China.

In summary, analysis of multiple target genes can be used in identification of *M. mechelonae /abscessus* complex isolates and *Mycobacterium fukienense* is identifiied as a novel species.

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