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ORIGINAL ARTICLE

Identification of *Mycobacterium abscessus* species and subspecies using the Cas12a/sgRNA-based nucleic acid detection platform

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Abstract

The rapidly growing mycobacterium *Mycobacterium abscessus* is a clinically important organism causing pulmonary and skin diseases. The *M. abscessus* complex is comprised of three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii*. Here, we aimed to develop a Cas12a/sgRNA-based nucleic acid detection platform to identify *M. abscessus* species and subspecies. By designing specific sgRNA probes targeting *rpoB* and *erm*(41), we demonstrated that *M. abscessus* could be differentiated from other major mycobacterial species and identified at the subspecies level. Using this platform, a total of 38 clinical *M. abscessus* isolates were identified, 18 as *M. abscessus* subsp. *abscessus* and 20 as *M. abscessus* subsp. *massiliense*. We concluded that the Cas12a/sgRNA-based nucleic acid detection platform provides an easy-to-use, quick, and cost-effective approach for identification of *M. abscessus* species and subspecies.

Keywords *Mycobacterium abscessus* subsp. *abscessus* · *Mycobacterium abscessus* subsp. *massiliense* · *Mycobacterium abscessus* subsp. *bolletii* · FnCas12a · sgRNA probe · Identification

Introduction

Non-tuberculous *Mycobacteria* (NTM) have been recognized as important human pathogens that cause concerns for human health [1–3]. Among the NTM species, the *Mycobacterium abscessus* complex belonging to the subgroup of rapidly growing *Mycobacteria* (RGM) are important human pathogens, accounting for the majority of NTM lung infections among cystic fibrosis patients [4]. Pulmonary infections caused by the *Mycobacterium. abscessus* complex often last for many years and they tend to become refractory diseases due to their resistance to most antibiotics used in the clinic [5, 6]. The *M. abscessus* complex can be divided into three subspecies, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense* [7, 8]. It has been reported that the three *M. abscessus* subspecies have different antibiotic resistance profiles and may cause different clinical symptoms [5, 9, 10]. Therefore, rapid and accurate identification of *M. abscessus* and the precise subspecies is critical to the treatment and control of *M. abscessus* infection.

Accurate identification of *M. abscessus* at the subspecies level remains complicated. Multilocus sequence typing (MLST) is an ubiquitous method to define isolates by

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using the sequences of several housekeeping genes [11]. However, it is tedious and expensive. Some studies have reported that improved matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) could be employed to rapidly and cost-effectively identify both the *M. abscessus* complex and its subspecies in clinical laboratories [12–16]. This method is cheap, rapid, and powerful, but requires sophisticated machinery. Recently, the commercially available kit GenoType NTM-DR (Hain Lifescience, Nehren, Germany) has been frequently used not only for subspecies differentiation of *M. abscessus* but also for determination of molecular resistance [17, 18]. However, this method also needs expensive equipment. Actually, whole-genome sequencing (WGS) is the most accurate and reliable approach for the identification of bacterial species, but it is expensive and time-consuming. With the development of next generation sequencing (NGS) technology, it may be practical to employ whole-genome sequencing for identification of bacterial species in clinical diagnosis in the future. However, currently there is an urgent need to develop a rapid and accurate method to identify both the *M. abscessus* complex and its subspecies.

Here, we aimed to develop an easy-to-use, fast, and cost-effective method to identify *M. abscessus* species and subspecies. Recently, the advanced CRISPR/Cas12a-based nucleic acid detection platform has shown dramatically high sensitivity in nucleic acid detection with single base resolution [19–21]. We therefore reasoned it can be developed to identify the *M. abscessus* complex and subspecies. In the present study, by designing specific sgRNA probes targeting *rpoB* or *erm*(41), the Cas12a/sgRNA-based nucleic acid detection platform could distinguish the *M. abscessus* complex from other major mycobacterial species and identify the subspecies of *M. abscessus*.

Methods and materials

Bacteria collection and DNA extraction

In the present study, clinical mycobacterial isolates were obtained from the Shenzhen Third People's Hospital affiliated with the Southern University of Science and Technology. These isolates were previously identified using gene chip (YanengBIO, Shenzhen, China) and further confirmed by sequencing the *rpoB* gene using previously published primers [22]. The reference mycobacterial strains were obtained from the China Center for Disease Control and Prevention. All strains were cultured in Middlebrook 7H9 liquid medium. DNA was extracted using a simple boiling method [23].

PCR and sequencing of *rpoB* and *hsp65* genes

The *rpoB* and *hsp65* genes were amplified from clinical isolates DNA using previously published primers [11]. PCR was performed using Ex-Taq DNA Polymerase (TaKaRa, Dalian, China) in a 50 μL reaction volume as follows: one cycle at 94 °C for 3 min, then 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by one cycle at 72 °C for 5 min, cooling at 4 °C. PCR products were examined using 1% agarose gel electrophoresis and then directly sequenced. DNA sequences from the type strains of *M. abscessus* subsp. *abscessus* (CIP 104536^T), *M. abscessus* subsp. *bolletii* (CIP 108541^T), and *M. abscessus* subsp. *massiliense* (CIP 108297^T) were retrieved from the GenBank database.

Phylogenetic analyses

The sequences of the *rpoB* and *hsp65* genes were aligned with the ClustalW algorithm using MEGA 6.0 software. Phylogenetic trees based on the sequences of the *rpoB* and *hsp65* genes were constructed by the neighbor-joining method with 1000 bootstrap replications.

Design and preparation of sgRNA probes targeting the *rpoB* gene

The *rpoB* and *erm*(41) genes were used as the targets to identify the *M. abscessus* complex and its subspecies in this study. *rpoB* sequences of *M. tuberculosis* H37Rv and the most common NTM species (*M. abscessus* subsp. *abscessus* ATCC 19977, *M. abscessus* subsp. *bolletii* CIP 108541^T, *M. abscessus* subsp. *massiliense* CIP 108297^T, *M. intracellulare* ATCC 13950, *M. avium* ATCC 25291, *M. kansasii* ATCC 12478, *M. gordonae* ATCC 14470, and *M. fortuitum* ATCC 6841) were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). Multiple alignments of the sequences were performed using DNAMAN 8 software. We firstly designed *M. abscessus*-specific sgRNA1 probes based on the divergence of the *rpoB* gene to distinguish the *M. abscessus* complex from other mycobacterial species. Subsequently, two sgRNA probes (sgRNA2 and sgRNA3) were designed based on the diversity sequence of *rpoB* within the *M. abscessus* subspecies. Then a sgRNA4 probe targeting the C-terminal deletion of *erm*(41) was designed to distinguish *M. abscessus* subsp. *massiliense* from *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*. Primer pairs for amplification of the targets were designed and are listed in supplementary Table S1. The DNA templates for the sgRNA transcription were synthesized by Songon Biotech (Shanghai,

China). All sgRNA probes were prepared by in vitro transcription using a T7 High Yield RNA Transcription Kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. The transcribed RNA was purified using VAHTS RNA Clean Beads (Vazyme, Nanjing, China) and quantified with NanoDrop2000. All of the oligonucleotides are listed in Table S1.

PCR and Cas12a trans-cleavage assay

Target genes of *rpoB* and *erm*(41) were amplified from the strains by PCR with the primers listed in supplementary Table S1. The PCR procedures were performed using the following steps, one cycle at 94 °C for 3 min, then 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by one cycle at 72 °C for 5 min, cooling at 4 °C. The PCR products were examined using 1% agarose gel electrophoresis. The concentrations of the PCR products were measured through a Qubit dsDNA HS Assay Kit (ThermoFisher Scientific, Massachusetts, USA).

Recombinant *Francisella novicida* Cas12a (FnCas12a) protein purchased from Tolo Biotech (Shanghai, China) was used for the Cas12a trans-cleavage assay. Cas12a trans-cleavage assays were performed according to Li's previously published description [19]. In brief, an FnCas12a trans-cleavage assay was conducted in a reaction buffer consisting of 0.5 pmol FnCas12a, 100 nM purified sgRNA probes, target DNA (1 μl unpurified PCR products), 500 nM collateral ssDNA (quenched fluorescent DNA reporter), and 10 U RNase inhibitor (TaKaRa, Dalian, China) in a 20 μl volume at 37 °C for 1 h. The reaction was stopped by adding 2 μl of 0.25 M EDTA. Fluorescence emission was excited at 535 nm and detected at 560 nm using a Varioskan Flash (ThermoFisher Scientific, Massachusetts, USA), and reactions with no target DNA were taken as the background.

Results

Schematic overview of the Cas12a/sgRNA-based nucleic acid detection platform

The principle of the development of the FnCas12a/sgRNA-based nucleic acid detection platform for identification of bacteria is illustrated in Fig. 1. To improve the sensitivity of this platform, PCR or other nucleic acid amplification methods are employed to enrich target DNA. Specific sgRNA probes direct Cas12a to recognize target DNA that is complementary to the sgRNA, which thereby triggers Cas12a to trans-cleave the reporter ssDNA, allowing it to fluoresce.

Design of the sgRNA probes for identification of the *M. abscessus* complex and subspecies

In order to identify the *M. abscessus* complex, a *M. abscessus*-specific sgRNA1 probe was designed based on the divergence of the *rpoB* genes among the major *Mycobacterium* species (Fig. 2a). Alignment of the *rpoB* sequences from type strains of *M. abscessus* ATCC 19977, *M. abscessus* subsp. *bolletii* CIP 108541^{T,} and *M. abscessus* subsp. *massiliense* CIP 108297^{T} revealed different sequences that could be potential loci to distinguish *M. abscessus* subsp. *abscessus* from *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* (Fig. 2b). To determine whether these different regions were robust among *M. abscessus* subspecies, we downloaded a number of *rpoB* sequences from strains of *M. abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* from NCBI GenBank. Multiple alignment of these downloaded sequences confirmed different regions among the three *M. abscessus* subspecies (Fig. 2c). Subsequently, two specific sgRNAs (sgRNA2 and sgRNA3) were designed to target the different regions to differentiate *M. abscessus* subsp.

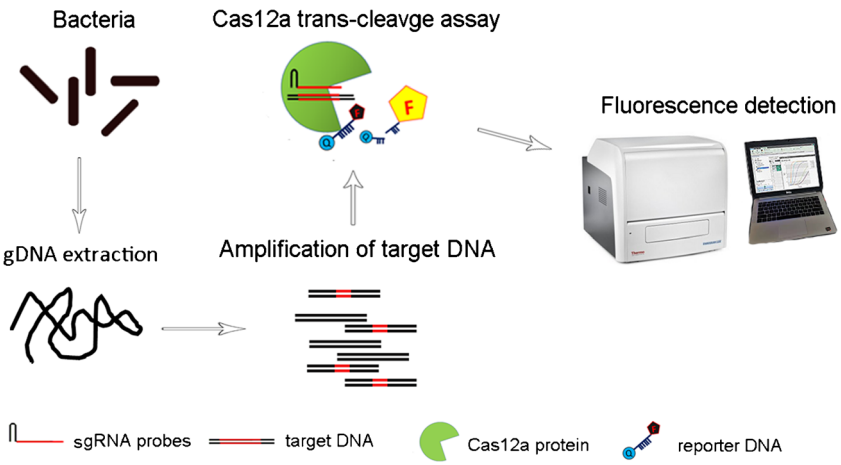

Fig. 1 Illustration of the Cas12a/sgRNA-based nucleic acid detection platform. The target DNA is specifically amplified from the extracted DNA of the bacteria by PCR or other methods. Specific sgRNA probes are designed to match the target DNA. The PCR product is mixed with the FnCas12a/sgRNA probe/ssDNA-FQ (quenched fluorescent single strand DNA reporter). Once the sgRNA probe matches the target, FnCas12a will trans-cleave the quenched fluorescent ssDNA reporter, leading to fluorescence

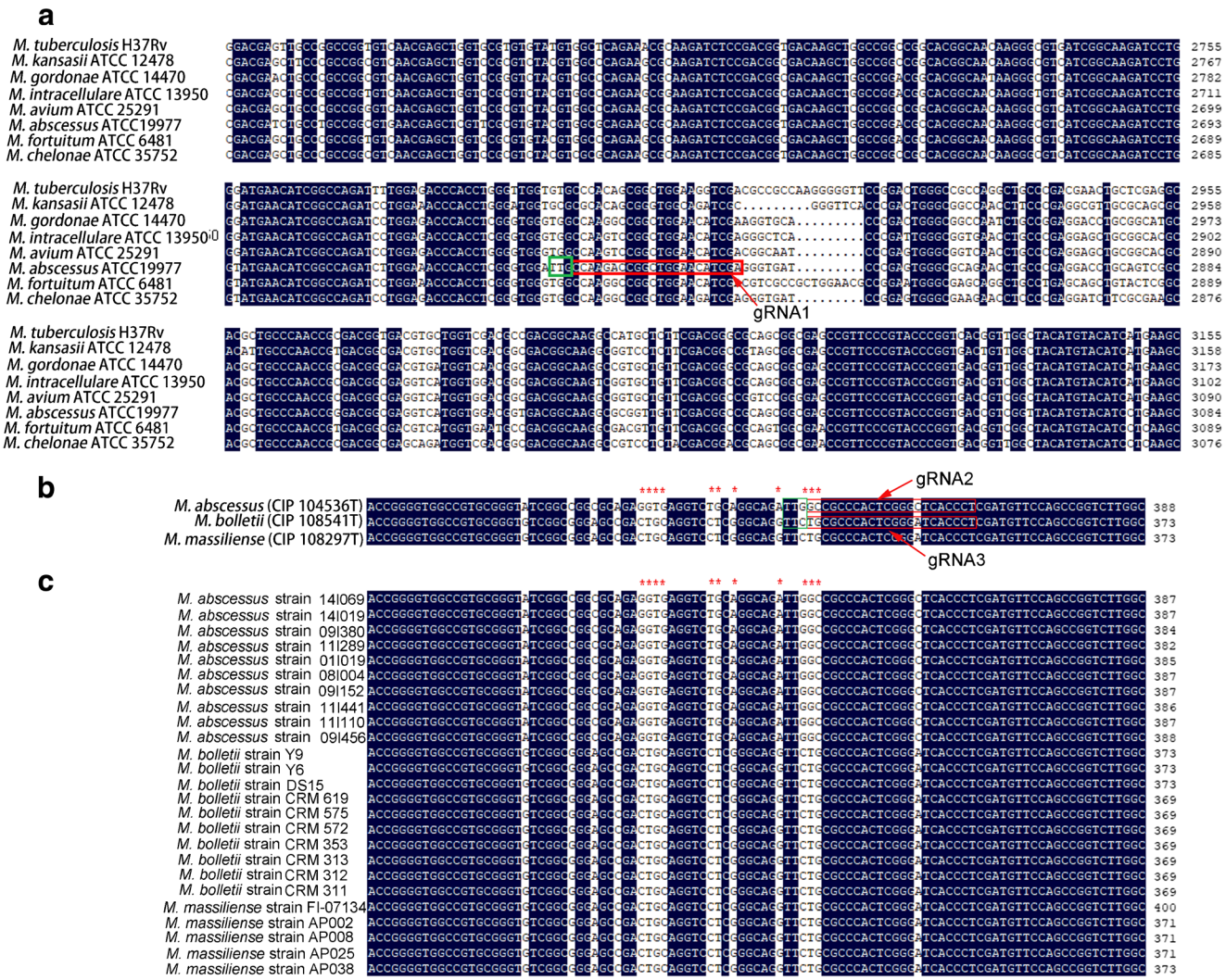

Fig. 2 Design of the sgRNA probes for identification of *M. abscessus* (**a**) and subspecies (**b**), (**c**) based on *rpoB* sequences. **a** To design a specific sgRNA probe for identification of the *M. abscessus* complex, *rpoB* sequences from the type strains of major mycobacterial species were downloaded. **b** and **c** To find the potential difference of regions for identification of *M. abscessus* subspecies, *rpoB* sequences were downloaded from many strains of *M. abscessus,* including type strains of *M. abscessus subsp. abscessus* (CIP 104536^T), *M. abscessus subsp. bolletii* (CIP 108541^T), and *M. abscessus subsp. massiliense* (CIP 108297^T). Multiple sequence alignment was performed using DNAMAN 8 software. Red boxes represent sgRNA probe targeted regions. Green boxes represent the FnCas12a recognition "TTN" motif. Red stars represent the differences among subspecies of *M. abscessus*

abscessus from *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* (Fig. 2b).

However, these probes were not reliable for differentiating *M. abscessus* subsp. *massiliense* from *M. abscessus* subsp. *bolletii* due to the extremely low *rpoB* divergence between these two subspecies. The *M. abscessus* subsp. *massiliense erm*(41) gene has been reported to contain a large C-terminal deletion [9, 24]. Therefore, a specific sgRNA4 probe targeting the C-terminal deletion region was designed to distinguish *M. abscessus* subsp. *massiliense* from *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*.

Determination of the feasibility of the Cas12a/sgRNA-based nucleic acid detection platform for identification of *M. abscessus* and *M. abscessus* subspecies

Firstly, we determined whether the designed sgRNA1 probe is able to discriminate the *M. abscessus* complex from other mycobacterial species by using reference mycobacterial strains. PCR was employed to amplify the target *rpoB* gene. As shown in Fig. 3a, according to the results of the fluorescence intensity, *M. abscessus* strains showed strong fluorescence intensity compared with control, while other reference

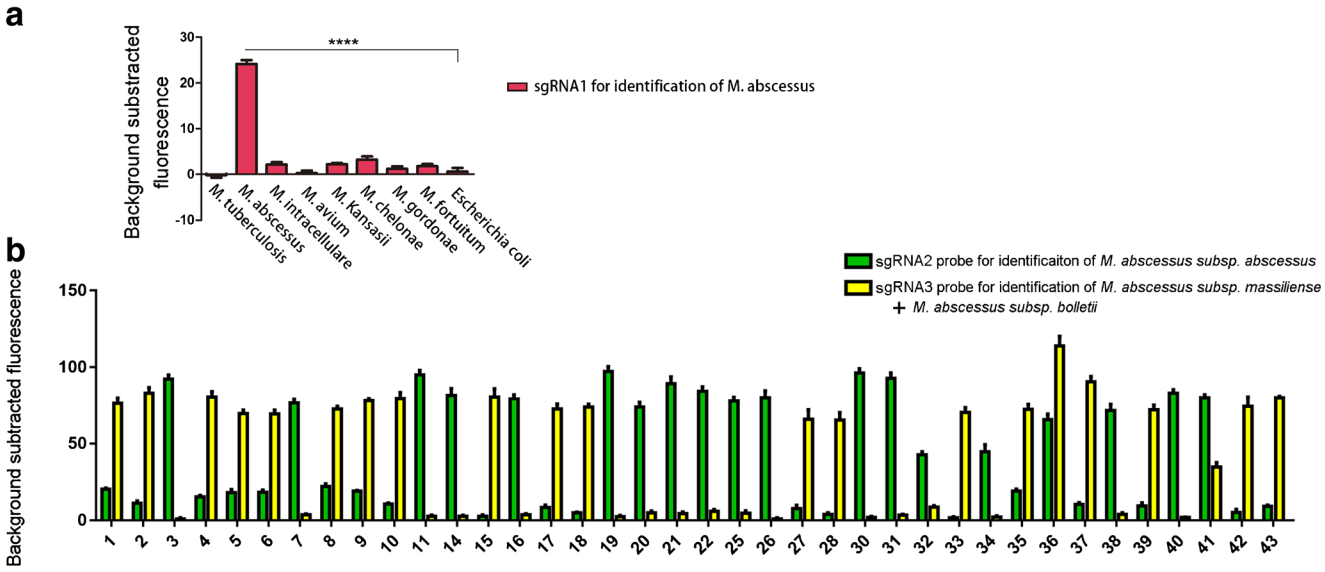

Fig. 3 Application of the FnCas12a/sgRNA-based nucleic acid detection platform for identification of *M. abscessus* species and subspecies. **a** Determination of the specificity of the designed sgRNA1 probes for identification of *M. abscessus* using the major mycobacterial reference strains and an *E. coli* negative control. **b** Identification of subspecies of *M. abscessus* among the 38 clinical *M. abscessus* isolates using sgRNA2 and sgRNA3 probes. Arabic numerals represent clinical *M. abscessus* isolates (two-tailed Student's *t* test; ****$p < 0.0001$; bars represent the mean ± SEM; n = three technical replicates)

mycobacterium strains showed no significant increase in fluorescence relative to the control. Subsequently, a number of identified clinical isolates were used to further test the specificity of the sgRNA1 probe in a blinded manner. All of the clinical *M. abscessus* strains were correctly identified without any cross-reaction, indicating the high specificity of the sgRNA1 probe (Table 1).

To determine the feasibility of sgRNA2 and sgRNA3 in discrimination of *M. abscessus* subsp. *abscessus* from *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii*, 38 confirmed clinical *M. abscessus* strains were subjected to this assay. As shown in Fig. 3b, 18 were identified as *M. abscessus* subsp. *abscessus* and 20 strains were identified as *M. abscessus* subsp *bolletii* or *M. abscessus* subsp. *massiliense* based on the fluorescence intensity, which is consistent with the results of phylogenetic trees based on the sequences of *rpoB* or *hsp65* (Fig. 4).

Table 1 Determination of the specificity of the sgRNA1 probe using clinical isolates

Organism name	Number of strains	Positive identification *n* (%)
M. tuberculosis	5	0
M. abscessus	5	5
M. intracellulare	5	0
M. avium	5	0
M. kansasii	5	0
M. chelonae	5	0
M. gordonae	5	0
M. fortuitum	5	0

Since the sgRNA2 and sgRNA3 probes were both not able to discriminate *M. abscessus* subsp. *massiliense* from *M. abscessus* subsp. *bolletii*, *erm*(41) PCR was applied to distinguish these two closely related subspecies. Both *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* subspecies are expected to produce 700 bp PCR products while the *M. abscessus* subsp. *massiliense* subspecies produces an approximately 350 bp PCR product. The agarose gel results showed that 18 strains displayed a full-size product (approximately 700 bp) while 20 strains showed a truncated product (approximately 350 bp) (Fig. 5), indicating 18 strains belong to *M. abscessus* subsp. *abscessus* or *M. abscessus* subsp. *bolletii* subspecies and 20 strains belong to *M. abscessus* subsp. *massiliense*. Meanwhile, we obtained the same results as the *erm*(41) PCR using the Cas12a-based detection platform with the sgRNA4 probe (data not shown). Taken together with the results of the Cas12/sgRNA cleavage assay, 18 and 20 strains, respectively, were identified as *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* among these 38 clinical *M. abscessus* isolates.

Discussion

The *M. abscessus* complex is one of the most important groups of rapidly growing mycobacteria that cause pulmonary infections. Although the classification of its subspecies remains controversial, the *M. abscessus* complex is currently divided into three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp.

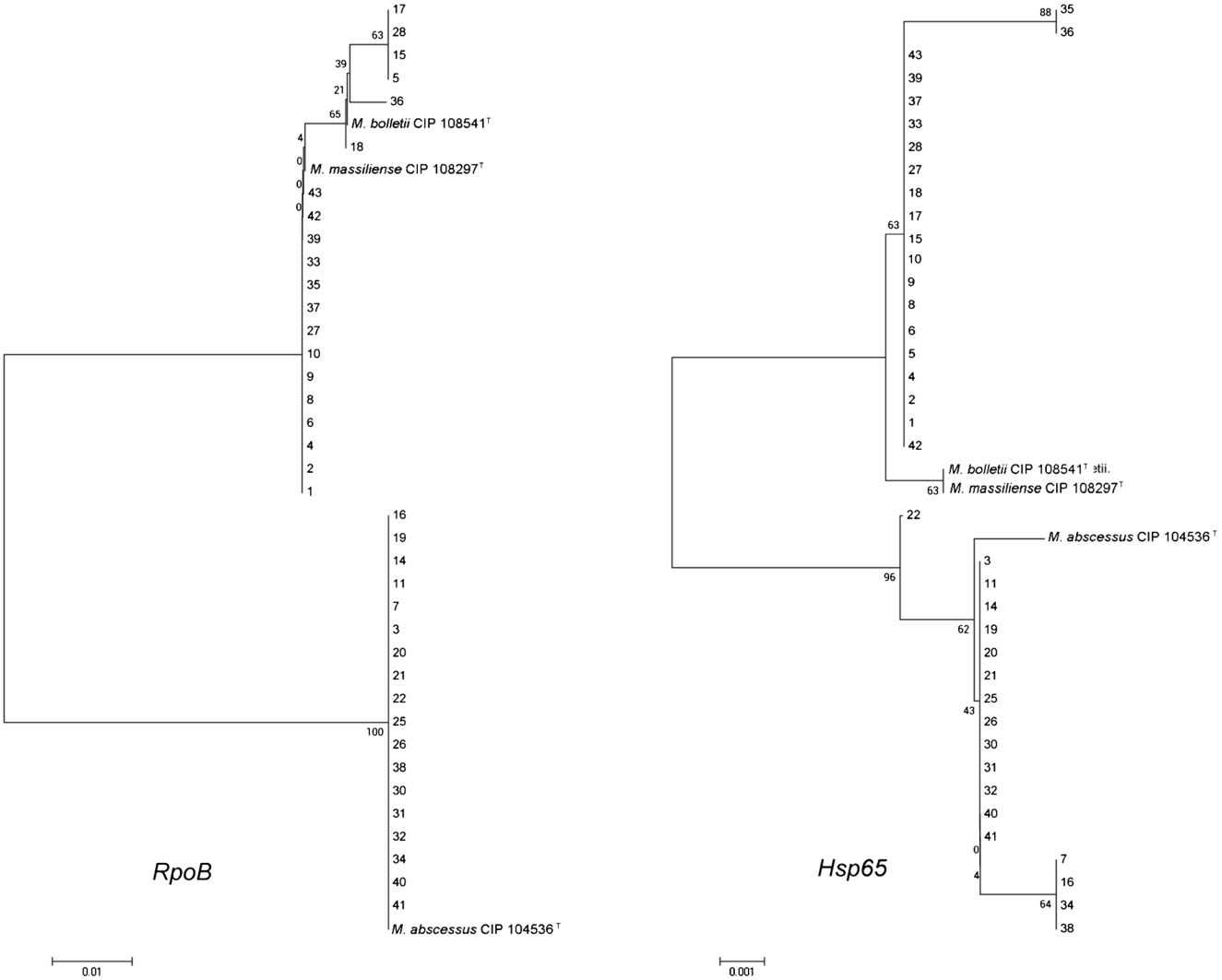

Fig. 4 Construction of phylogenetic trees based on the partial *rpoB* and *hsp65* genes of 38 clinical *M. abscessus* isolates. Both trees were constructed using the neighbor-joining method in the MEGA 6.0 program. The bootstrap values were calculated from 1000 replications. Arabic numerals represent clinical *M. abscessus* isolates

massiliense. However, accurately and quickly identifying the *M. abscessus* complex and its subspecies remains a real challenge [25].

In the present study, we described the application of the Cas12a/sgRNA-based nucleic acid detection platform for differentiation of *M. abscessus* strains at the subspecies level. Here, the *rpoB* gene was selected as the candidate gene due to its higher discriminatory power in the identification of mycobacterial species [26]. By designing a *M. abscessus* species-specific sgRNA1 probe, the *M. abscessus* complex was easily and quickly differentiated from other major mycobacterial species. A total of 38 clinical isolates were correctly identified as *M. abscessus* at the species level using the Cas12a/sgRNA-based nucleic acid detection platform, indicating the high

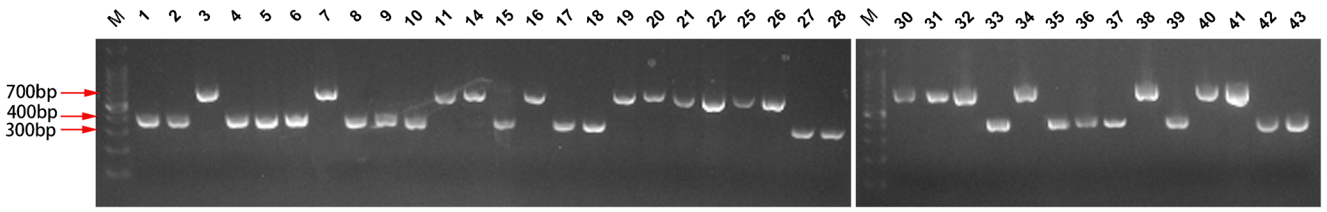

Fig. 5 The PCR results of *erm*(41) genes from the 38 clinical *M. abscessus* isolates. Arabic numerals represent clinical *M. abscessus* isolates. The *M. abscessus* subsp. *abscessus* or *M. abscessus* subsp. *bolletii erm*(41) PCR products are fully amplified to 700 bp in size. The *M. abscessus* subsp. *massiliense* amplicons are amplified to 350 bp in size. Among the 38 clinical *M. abscessus* strains, 18 produced 700 bp PCR products and 20 strains produced 350 bp PCR products

accuracy of this platform in the detection of *M. abscessus* species. Several studies have also reported the use of the *rpoB* gene to identify *M. abscessus* at the subspecies level, although intergroup lateral transfers of *rpoB* exist among the *M. abscessus* subspecies [7, 27, 28]. Interestingly, multiple sequence alignment revealed distinct regions that could be exploited to distinguish *M. abscessus* subsp. *abscessus* from *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*. By designing subspecies-specific sgRNA probes (sgRNA2 and sgRNA3) targeting the different regions, we identified 18 and 20 strains as *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* or *M. abscessus* subsp. *massiliense*, respectively. These results are consistent with the phylogenetic trees based on *rpoB* and *hsp65* gene sequences, suggesting the feasibility of identification of subspecies of *M. abscessus* merely by relying on specific detection of these distinct regions. Alternatively, we can simply design two TaqMan probes based on the distinct regions to differentiate *M. abscessus* subsp. *abscessus* from *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*.

Since the divergence of the *rpoB* sequence between the type strains of *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* is below 3%, it is not reliable for distinguishing *M. abscessus* subsp. *massiliense* from *M. abscessus* subsp. *bolletii*. Subsequently, *erm*(41) PCR as a second step was performed to distinguish *M. abscessus* subsp. *massiliense* from *M. abscessus* subsp. *bolletii*, because *M. abscessus* subsp. *massiliense* suffers from a large C-terminal deletion [9, 24]. Combining all of these results, we finally accurately identified 18 *M. abscessus* subsp. *abscessus* and 20 *M. abscessus* subsp. *massiliense* specimens among the 38 clinical isolates, and no *M. abscessus* subsp. *bolletii* strains were identified. These results suggest that *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *abscessus* are the two primary *M. abscessus* subspecies in the south of China.

There are also several limitations to this study. For example, the clinical isolates are so limited that we didn't identify any cases of *M. abscessus* subsp. *bolletii*. This assay also requires two separate PCR steps, one for amplification of *rpoB* and the other for amplification of *erm*(41). In the future, we aim to develop a Multiplex-PCR to amplify both targets in one PCR reaction, which will reduce the number of experimental steps and simplify the assay.

In conclusion, we developed an assay that could easily and quickly identify *M. abscessus* species. The assay consists of two stepwise detections. First, the *M. abscessus* complex is discriminated from other mycobacterial species using the *M. abscessus*-specific sgRNA1 probe and the Cas12a/sgRNA-based nucleic acid detection platform. Subsequently, subspecies of *M. abscessus* are identified using the subspecies sgRNA probes (sgRNA2 and sgRNA3) and *erm*(41) PCR. Since the Cas12a/sgRNA-based nucleic acid detection platform doesn't require any expensive instruments, it will be popular in clinical microbiology laboratories and can be used to accelerate early diagnosis and treatment of mycobacterial infections.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This study was approved by the Institutional Review Board of the Shenzhen Third People's Hospital.

Informed consent Informed consent was obtained from all individual participants included in the study.

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