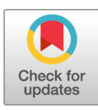

Cas12a/Guide RNA-Based Platform for Rapid and Accurate Identification of Major *Mycobacterium* Species

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ABSTRACT *Mycobacterium tuberculosis* infection and nontuberculous mycobacteria (NTM) infections exhibit similar clinical symptoms; however, the therapies for these two types of infections are different. Therefore, the rapid and accurate identification of *M. tuberculosis* and NTM species is very important for the control of tuberculosis and NTM infections. In the present study, a Cas12a/guide RNA (gRNA)-based platform was developed to identify *M. tuberculosis* and most NTM species. By designing species-specific gRNA probes targeting the *rpoB* sequence, a Cas12a/gRNA-based platform successfully identified *M. tuberculosis* and six major NTM species (*Mycobacterium abscessus*, *Mycobacterium intracellulare*, *Mycobacterium avium*, *Mycobacterium kansasii*, *Mycobacterium gordonae*, and *Mycobacterium fortuitum*) without cross-reactivity. In a blind assessment, a total of 72 out of 73 clinical *Mycobacterium* isolates were correctly identified, which is consistent with previous *rpoB* sequencing results. These results suggest that the Cas12a/gRNA-based platform is a promising tool for the rapid, accurate, and cost-effective identification of both *M. tuberculosis* and NTM species.

KEYWORDS *Mycobacterium tuberculosis*, nontuberculous mycobacteria, CRISPR/Cas12a, gRNA probe, fluorescence, identification, biosensing

Tuberculosis is a serious infectious disease that endangers human health and is mainly caused by *Mycobacterium tuberculosis* infection. In recent years, some investigations have shown an increased incidence of nontuberculosis *Mycobacterium* infections. NTMs have become recognized as important human pathogens causing public health problems (1–3). NTM infections are insensitive to most antituberculosis drugs, and the treatment regimen for patients infected with *M. tuberculosis* versus an NTM is completely different (4). However, it is difficult to distinguish *M. tuberculosis* infection from NTM infections, since they exhibit similar clinical symptoms. Therefore, the rapid and accurate identification of *M. tuberculosis* and NTM is of great significance for the early diagnosis, treatment, and control of tuberculosis and NTM infections.

The identification of *M. tuberculosis* and NTM species mainly depends on laboratory diagnosis. Conventional etiological methods used in microbiology laboratories are complex, time-consuming and labor-intensive, and have poor specificity and sensitivity, which is not conducive to the timely guidance of clinical treatment (5). In recent decades, the introduction of molecular diagnostic methods has overcome some of these disadvantages, resulting in a remarkable improvement in the direct detection of mycobacteria. A variety of molecular techniques have been developed for the identification of *M. tuberculosis* and NTM from clinical specimens and culture. These mainly include multiplex PCR (6–8), real-time PCR (9–11), DNA probe assays (12–15), PCR

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restriction fragment length polymorphism (RFLP) analysis (16–19), target gene sequencing (20–24), and microarray technology (25–27). Among them, microarray technology is currently popular because it can analyze thousands of genes at the same time and in a short time, which is very helpful for phylogenetic analysis and species identification. However, most of these techniques are not cost-effective, and some of them require expensive equipment. Moreover, most of these assays have been used for the detection or quantification of a certain *Mycobacterium* species only, such as *M. tuberculosis*, or the detection of drug resistance.

Cas12a (Cpf1) was first characterized by the Zhang Feng group as an RNA-guided endonuclease that can directly bind and cut target DNA (28). Recently, two independent groups found Cas12a possessed collateral DNA cleavage activity (29, 30). This process was immediately developed into two Cas12a-based nucleic acid detection methods called HOLMES (one-hour low-cost multipurpose highly efficient system) and DETECTR (DNA endonuclease-targeted CRISPR trans reporter) (30, 31). Both platforms could achieve attomolar sensitivity for detecting DNA targets with single-base resolution. Here, by designing a variety of species-specific gRNA probes, we attempted to rapidly and accurately identify *M. tuberculosis* and the most common NTM species (*M. abscessus*, *M. intracellulare*, *M. avium*, *M. kansasii*, *M. gordonae* and *M. fortuitum*) using a Cas12a/gRNA-based platform.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Clinical isolates, including 10 *M. tuberculosis*, 15 *M. abscessus*, 15 *M. intracellulare*, 10 *M. avium*, 10 *M. fortuitum*, 7 *M. kansasii* and 6 *M. gordonae* were obtained from Shenzhen Third People's Hospital, Shenzhen, China, and were confirmed by *rpoB* sequencing. Seven reference strains, including *M. tuberculosis* H37Rv, *M. abscessus* (ATCC 19977), *M. intracellulare* (ATCC 13950), *M. avium* (ATCC 25291), *M. kansasii* (ATCC 12478), *M. gordonae* (ATCC 14470), and *M. fortuitum* (ATCC 6841) were a kind gift from the Chinese Center for Disease Control and Prevention. Strains were cultured in Middlebrook 7H9 liquid medium and incubated at 37°C. *Escherichia coli*, *Pseudomonas aeruginosa*, *Actinobacter baumannii*, *Salmonella enteritidis* and *Klebsiella pneumoniae* were obtained from Shenzhen Third People's Hospital, Shenzhen, China, and cultured on blood agar plates.

Design and preparation of gRNA probes. Since the *rpoB* gene has been reported to be a suitable locus for the identification of *Mycobacterium* species (24, 32–34), we chose it as the target sequence for the design of gRNA probes. The *rpoB* gene sequences of *M. tuberculosis* H37Rv and the most common NTM (*M. abscessus* ATCC 19977, *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 *rpoB* gene sequences were performed using DNAMAN 8 software (Fig. 1). Species-specific gRNA probes were designed based on the divergence of *rpoB* genes (Fig. 1). We designed two specific gRNA probes for each *Mycobacterium* species except *M. fortuitum* (Fig. 1). A universal primer pair (*RpoB*-F and *RpoB*-R) for amplification of target sequences was designed based on the conserved sequences flanking the gRNA probe targeting regions (Fig. 1). All gRNA 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 DNA templates for gRNA transcription were synthesized by Sangon Biotech (Shanghai, China). The transcribed RNA was purified using VAHTS RNA Clean Beads (Vazyme, Nanjing, China) and quantified with NanoDrop 2000. All the oligonucleotides are listed in Table S1 in the supplemental material.

DNA preparation and PCR amplification. DNA was extracted from cultured strains using a TIANamp Bacteria DNA kit (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol or a simple boiling method (35). DNA concentration was determined using a Qubit dsDNA HS assay kit (Thermo Fisher Scientific, MA, USA). Target sequences were amplified by PCR using the universal primer pairs described above. PCR was performed 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, and the reactions were cooled at 4°C. PCR products were examined by 1% agarose gel electrophoresis. The PCR product concentration was determined using a Qubit dsDNA HS assay kit (Thermo Fisher Scientific, MA, USA).

Cas12a/gRNA transcleavage assay. Recombinant *Francisella novicida* Cas12a (FnCas12a) protein purchased from Tolo Biotech (Shanghai, China) was used for the Cas12a transcleavage assay. The recognition site of FnCas12a is "TTN," not "TTTN" (29), which provided more candidate loci for design of gRNA probes. Cas12a transcleavage assays were performed mainly according to Li's description (31). In brief, the FnCas12a transcleavage assay was performed in reaction buffer consisting of 0.5 pmol FnCas12a, 100 nM purified gRNA, target DNA (1 μl unpurified PCR products), 500 nM collateral single-stranded DNA (ssDNA) (quenched fluorescent single-strand 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 (Thermo Fisher Scientific, MA, USA), and reactions with no target DNA were used as the background.

Evaluation of the specificity of gRNA probes and determination of the limit of detection with reference strains. The seven mycobacterial reference strains and five other bacterial species, including

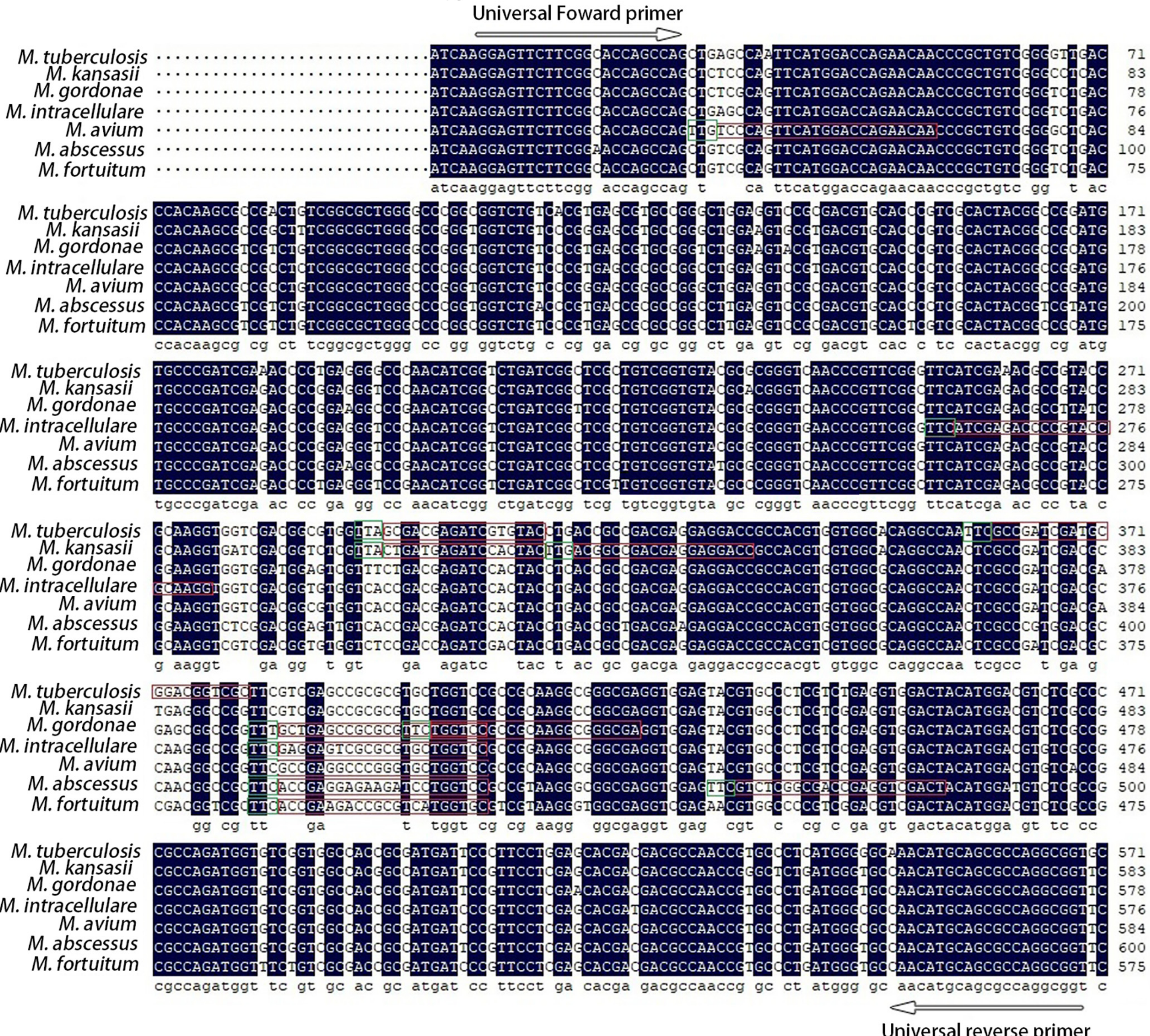

FIG 1 Design of species-specific gRNA probes based on *rpoB* sequences. *rpoB* sequences were downloaded from *M. tuberculosis* H37Rv, *M. abscessus* (ATCC 19977), *M. intracellulare* (ATCC 13950), *M. avium* (ATCC 25291), *M. kansasii* (ATCC 12478), *M. gordonae* (ATCC 14470), and *M. fortuitum* (ATCC 6841). Multiple sequence alignment was performed using DNAMAN 8 software. Conserved sequences are blank. Red boxes represent gRNA probe targeted regions. Green boxes represent the PAM motif. Universal primers used for amplification of *rpoB* fragments are indicated by arrows.

E. coli, *P. aeruginosa*, *A. baumannii*, *S. enteritidis*, and *K. pneumoniae*, were used to evaluate the specificity of gRNA probes.

The limit of detection (LOD) was determined in both DNA and quantified CFU. DNA was extracted from the reference strains using a TIANamp Bacteria DNA kit (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol and then serially diluted ranging from 10 ng to 0.01 pg. Serial dilutions with the indicated mycobacterial numbers were spiked into the artificial sputum samples. Sputum samples were treated with a 3% NaOH solution and then neutralized with a phosphate buffer followed by centrifugation. DNA was extracted from the pellets by a rapid boiling method (35). After PCR amplification, 1 μl unpurified PCR products were used for DNA detection via an FnCas12a/gRNA-based platform.

RESULTS

Schematic overview of the FnCas12a/gRNA-based platform. Recently, two revolutionary DNA detection methods named DNA DETECTR and HOLMES, respectively, were developed by two independent groups based on the Cas12a transcleavage

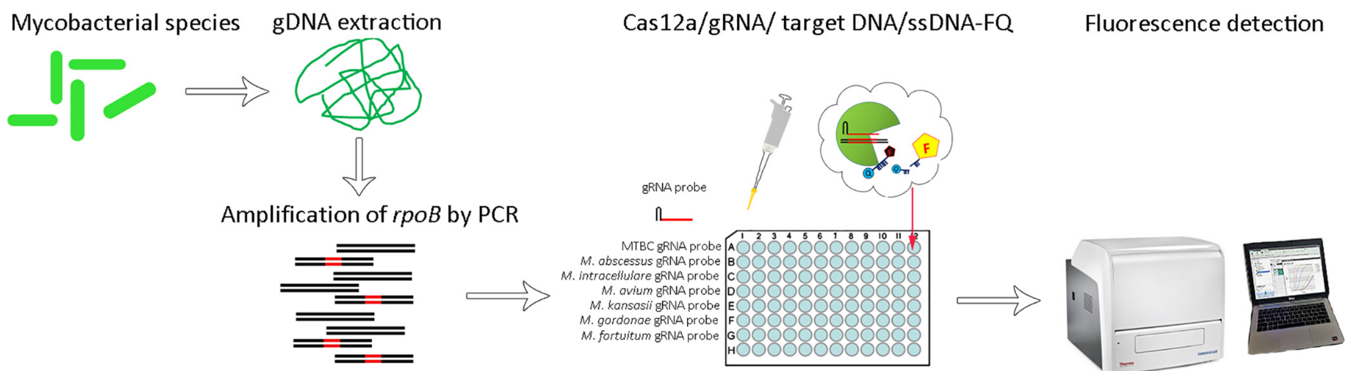

FIG 2 Illustration of FnCas12a/gRNA-based platform for identification of *Mycobacterium* species. *rpoB* fragments were specifically amplified from extracted DNA of bacteria by PCR. Species-specific gRNA probes were designed to target *rpoB* fragments. Unpurified PCR product was directly mixed with FnCas12a/gRNA/ssDNA-FQ (quenched fluorescent single-strand DNA reporter). Once gRNA probes match the target DNA and form a complex, FnCas12a will transcleave the quenched fluorescent ssDNA reporter, illuminating the fluorescence.

activity (30, 31). Both platforms could specifically and accurately detect target DNA by designing a specific gRNA, thereby achieving attomolar sensitivity for DNA detection with single-base resolution. Thus, we reasoned that a Cas12a-based nucleic acid detection assay could be developed to identify *Mycobacterium* species. Fig. 2 shows a schematic overview of the FnCas12a/gRNA-based platform for the identification of *Mycobacterium* species. Using this platform, target DNA was amplified by PCR and then incubated with FnCas12a, a gRNA probe, and quenched fluorescence reporter ssDNA. If target DNA is detected by the species-specific gRNA probe in the reaction mixture, the formation of the FnCas12a/gRNA/target DNA ternary complex transcleaves the reporter ssDNA, resulting in fluorescence.

Evaluation of the FnCas12a/gRNA-based platform with *Mycobacterium* reference strains. We selected two gRNA probes for each mycobacterial species to be analyzed, except *M. fortuitum*. The specificity of each gRNA probe was evaluated preliminarily using reference strains. From the fluorescence detection results shown in Fig. 3, each designed gRNA probe was able to identify the specific mycobacterial species without cross-reaction. In addition, we observed that fluorescence intensities varied with gRNA probe sequences (Fig. 3).

To determine the sensitivity of the FnCas12a/gRNA-based platform, DNA extracted from reference strains was serially diluted ranging from 10 ng to 10 fg. Each gRNA probe showed a similar LOD, ranging from 1 pg to 100 fg (Fig. 4A). The ratios of fluorescence intensity from LODs to the negative controls were set as the cutoffs used for subsequent clinical strain detection. To further evaluate the LOD in CFU, serial dilutions with the indicated mycobacterial numbers were spiked into the artificial sputum samples. We found the FnCas12a/gRNA-based platform had an LOD of 500 to 10,000 CFU/ml (Fig. 4B).

Application of FnCas12a/gRNA-based platform in clinical *Mycobacterium* isolates. A total of 73 clinical isolates, including 10 *M. tuberculosis*, 15 *M. abscessus*, 15 *M. intracellulare*, 10 *M. avium*, 7 *M. kansasii*, 10 *M. gordonae*, and 6 *M. fortuitum*, were examined in a blind assessment to determine the feasibility of the FnCas12a/gRNA-based platform in the identification of the major *Mycobacterium* species. For each test, *E. coli* was used as a negative control (NC). Fluorescence signals from samples were normalized against the negative controls. The fold changes in samples above or below the cutoff in FnCas12a/gRNA-based assays were considered positive or negative, respectively. Using these screening criteria, a total of 72 out of 73 clinical strains were correctly identified, showing almost 100% agreement with previous *rpoB* sequencing results (Table 1). Only one strain thought to be *M. intracellulare* was misidentified by the *M. intracellulare* gRNA probe2 (MIN gRNA probe2). We further investigated the sequence of this misidentified strain and found that it has two base mutations in the *M. intracellulare* gRNA probe2 targeting region (Fig. 5), which resulted in false negatives.

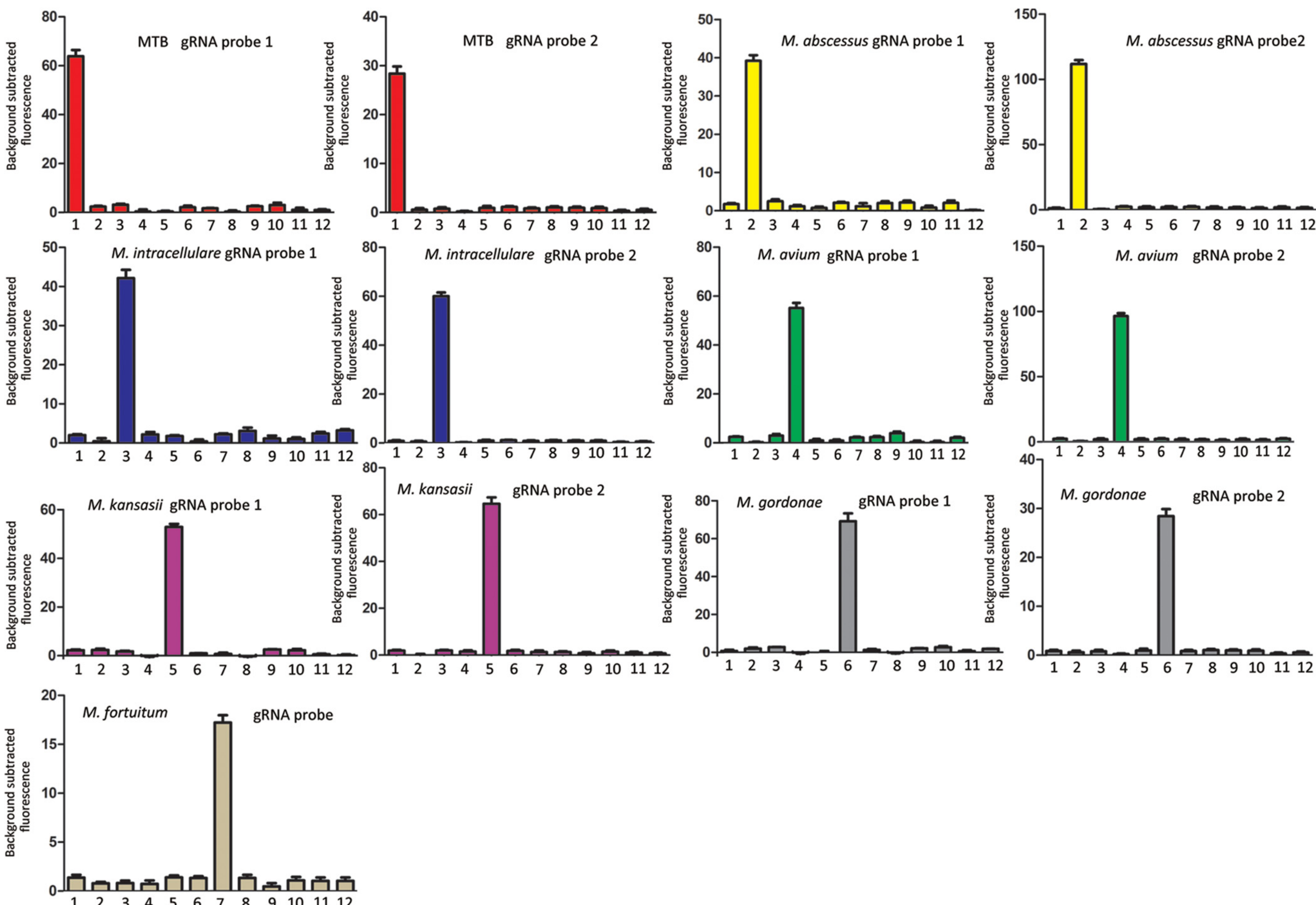

FIG 3 Determination of the specificity of the designed gRNA probes using reference *Mycobacterium* species. Bars 1 to 7 represent *M. tuberculosis* H37Rv, *M. abscessus* (ATCC 19977), *M. intracellulare* (ATCC 13950), *M. avium* (ATCC 25291), *M. kansasii* (ATCC 12478), *M. gordonae* (ATCC 14470), and *M. fortuitum* (ATCC 6841), respectively. Bars 8 to 12 represent *E. coli*, *P. aeruginosa*, *A. baumannii*, *S. enteritidis*, and *K. pneumoniae*, respectively. Error bars represent mean ± standard error of the mean (SEM), with $n = 3$ technical replicates.

DISCUSSION

Tuberculosis (TB) is one of the top 10 causes of death and the leading cause by a single infectious agent (above HIV/AIDS). Millions of people continue to fall sick with TB each year.

A report from the World Health Organization (WHO) indicates that TB caused an estimated 1.3 million deaths, and 10.0 million people developed TB disease in 2017. Nontuberculous mycobacteria (NTM) species are ubiquitous organisms distributed in ambient environments which opportunistically cause human diseases (36). In recent years, the incidence and prevalence of NTM diseases have continued to increase worldwide (37). Although the causative agents of NTM diseases vary with geographical location, the most common pulmonary NTM pathogens are *M. abscessus*, *M. intracellulare*, *M. avium*, *M. fortuitum*, *M. kansasii*, and *M. gordonae*. The clinical symptoms of NTM diseases and *M. tuberculosis* infection are often very similar. However, the mycobacterial species differ dramatically from one another in terms of treatment outcomes and antibiotic susceptibility (37–39), which seriously hinders the diagnosis and treatment of *M. tuberculosis*- and NTM-caused diseases. The most effective strategy to control mycobacterial infection is early diagnosis and treatment of the disease. Therefore, it is important to develop methods to rapidly and accurately detect and identify the infecting mycobacterial species for specific chemotherapy and better patient management.

Traditional detection methods based on growth characteristics, colony morphology,

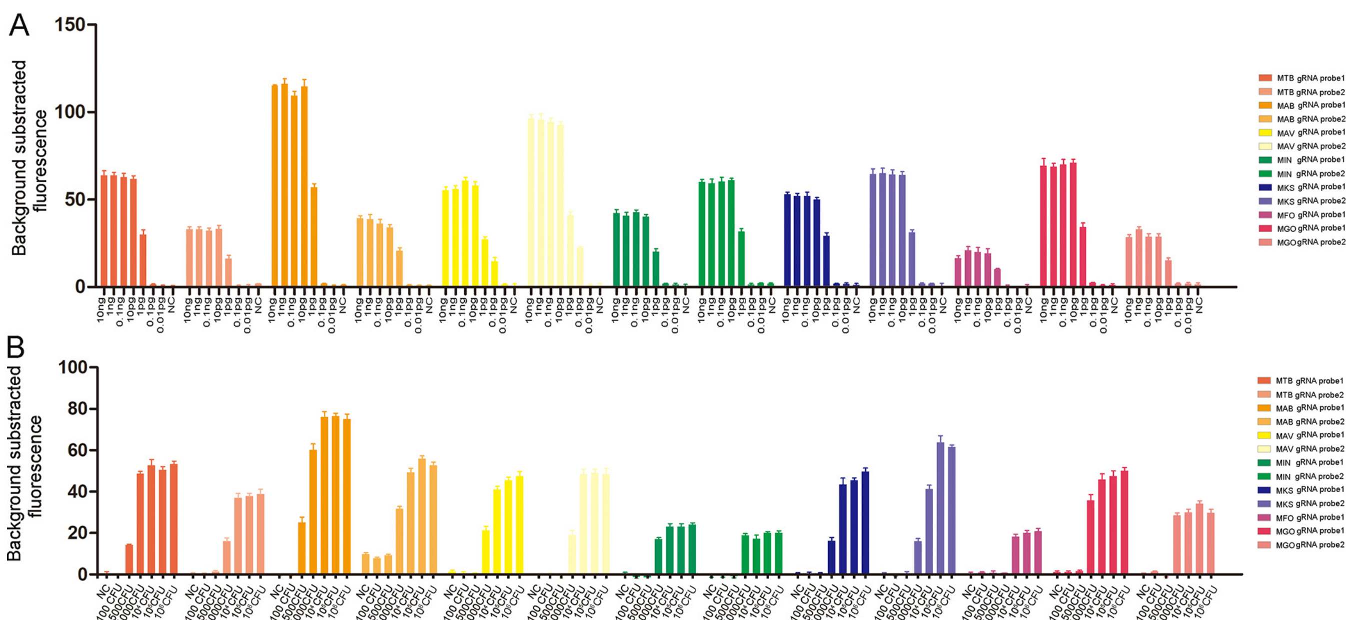

FIG 4 Evaluation of LOD with purified DNA and quantified CFU. (A) DNA extracted from pure- cultured reference strains were serially diluted in the range of 10 ng to 10 fg. (B) Serial quantified bacilli were spiked into artificial sputum. *E. coli* was used as negative control (NC). Error bars represent mean ± SEM, with $n = 3$ technical replicates.

pigment production, and biochemical reactions are laborious, tedious, and unable to categorize NTM species. Nucleic acid amplification-based methods have made great improvements in the rapid and accurate diagnosis of mycobacterial infections. Several commercially available assays based on molecular detection, such as GeneXpert *M. tuberculosis* /RIF Ultra test, Roche *M. tuberculosis* -RIF/INH test, and IS6110 PCR, are widely used for the direct detection of *M. tuberculosis* in clinical samples. Although these methods are rapid, highly sensitive, and specific, they exclusively focus on the identification of *M. tuberculosis*. The development of multiplex PCR and multiplex real-time PCR assays, which are able to detect a number of organisms in a single reaction, has dramatically accelerated the detection of mycobacterial species (8, 9, 11). However, it is difficult to identify closely related species, and the interpretations of the results are complicated. To date, Sanger sequencing is still regarded as the gold standard for identifying *M. tuberculosis* and NTM infections, but it is not cost-effective. Recently, the development of Cas13- and Cas12a-based CRISPR/Cas biosensing systems has enabled sensitive, robust, and low-cost tools for detecting nucleic acids, displaying great potential for the diagnosis of pathogens (29–31, 40). Since Cas13- and Cas12a-

TABLE 1 Identification of clinical isolates using FnCas12a/gRNA-based platform

Clinical organism	No. of strains	No. correctly identified (%)	No. misidentified	gRNA probe name
M. tuberculosis	10	10 (100)	0	MTB gRNA probe1
M. tuberculosis	10	10 (100)	0	MTB gRNA probe2
M. abscessus	15	15 (100)	0	MAB gRNA probe1
M. abscessus	15	15 (100)	0	MAB gRNA probe2
M. intracellulare	15	15 (100)	0	MIN gRNA probe1
M. intracellulare	15	14 (93.33)	1	MIN gRNA probe2
M. avium	10	10 (100)	0	MAV gRNA probe1
M. avium	10	10 (100)	0	MAV gRNA probe2
M. gordonae	10	10 (100)	0	MGO gRNA probe1
M. gordonae	10	10 (100)	0	MGO gRNA probe2
M. kansasii	7	7 (100)	0	MKA gRNA probe1
M. kansasii	7	7 (100)	0	MKA gRNA probe2
M. fortuitum	6	6 (100)	0	MFO gRNA probe

FIG 5 Partial sequences of *rpoB* gene from the two misidentified strains. Strain 25 was previously identified as *M. intracellulare* by *rpoB* sequencing. Red nucleotides indicate the *M. intracellulare* gRNA probe 2 targeting region. Black nucleotides within the red region indicate the mutated bases.

based CRISPR/Cas biosensing systems can discriminate single-base differences, we reasoned that these methods could be promising tools to identify *Mycobacterium* species.

Here, we attempted to identify *M. tuberculosis* and six major NTM species using an FnCas12a/gRNA-based platform. Through designing species-specific gRNA probes targeting the *rpoB* gene, 72 of the 73 clinical isolates were correctly identified, and there were no false-positive results, indicating the accuracy of this platform. However, there was a strain that was misidentified by the *M. intracellulare* gRNA probe 2 due to two base mutations in the targeting sequence, indicating that two base mismatches could lead to inactivation of the FnCas12a/gRNA complex. This result suggested that variation in the target sequences potentially caused detection failure or misidentification. In addition, we observed that different gRNA probes caused different fluorescence intensities. However, we did not find any nucleotide preferences for highly active gRNAs through analysis of gRNA probe sequences. Moreover, the *Mycobacterium* complex could also be identified with a package of gRNA probes (not shown). In addition, the FnCas12a-based platform exhibited high sensitivity in the detection of *Mycobacterium* species, ranging from 500 to 10,000 CFU/ml, which is comparable to real-time PCR. This method is easy to operate without any expensive equipment, which contributes to its application in poor countries and regions. The entire process of this method could be finished within 3 h, and the experimental results can be easily and intuitively read out according to the fluorescence intensity.

This study also has several limitations. For example, the clinical isolates of some NTM species may not be sufficient to test the effectiveness of gRNA probes due to the limited incidences of NTM species infection. Another limitation of this study is that we did not use direct clinical specimens to evaluate the FnCas12a/gRNA-based platform. Further study is needed on clinical samples, such as sputum, blood, tissues, and bronchoalveolar lavage fluid. In conclusion, this method allowed for the rapid, highly sensitive, and specific identification of *M. tuberculosis* and most NTM species.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.08 MB.

ACKNOWLEDGMENTS

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