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MYCOBACTERIOLOGY AND AEROBIC ACTINOMYCETES

Rapid Identification of Clinically Relevant *Mycobacterium* Species by Multicolor Melting Curve Analysis

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ABSTRACT The sustained increase in the incidence of nontuberculous mycobacterial (NTM) infection and the difficulty in distinguishing these infections from tuberculosis constitute an urgent need for NTM species-level identification. The MeltPro Myco assay is the first diagnostic system that identifies 19 clinically relevant mycobacteria in a single reaction based on multicolor melting curve analysis run on a real-time PCR platform. The assay was comprehensively evaluated regarding its analytical and clinical performances. The MeltPro Myco assay accurately identified 51 reference mycobacterial strains to the species/genus level and showed no cross-reactivity with 16 nonmycobacterial strains. The limit of detection was 300 bacilli/ml, and 1% of the minor species was detected in the case of mixed infections. Clinical studies using 1,163 isolates collected from five geographically distinct health care units showed that the MeltPro Myco assay correctly identified 1,159 (99.7%) samples. Further testing with 94 smear-positive sputum samples showed that all samples were correctly identified. Additionally, the entire assay can be performed within 3 h. The results of this study confirmed the efficacy of this assay in the reliable identification of mycobacteria, suggesting that it might potentially be used as a screening tool in regions endemic for tuberculosis.

KEYWORDS melting curve analysis, nontuberculous mycobacteria, real-time PCR, species identification

Nontuberculous mycobacteria (NTM) are ubiquitous organisms frequently isolated from environmental sources (1). Some NTM species can cause severe diseases in individuals, especially those with reduced or compromised immune function (2–5). The most common clinical manifestation of NTM infections is lung disease that resembles tuberculosis (TB) (6); however, it cannot be treated as TB because NTM harbor natural resistance mechanisms against antituberculosis drugs. In addition, NTM species differ remarkably among each other regarding pathogenicity and drug susceptibility profiles (6–8). In recent years, the mortality caused by NTM infections has increased significantly due to misdiagnosis and inappropriate therapy (9). Identification of NTM to the species and subspecies levels has thus become essential for facilitating early and precise treatment of NTM diseases.

Traditionally, mycobacteria are identified by phenotypic methods based on cell

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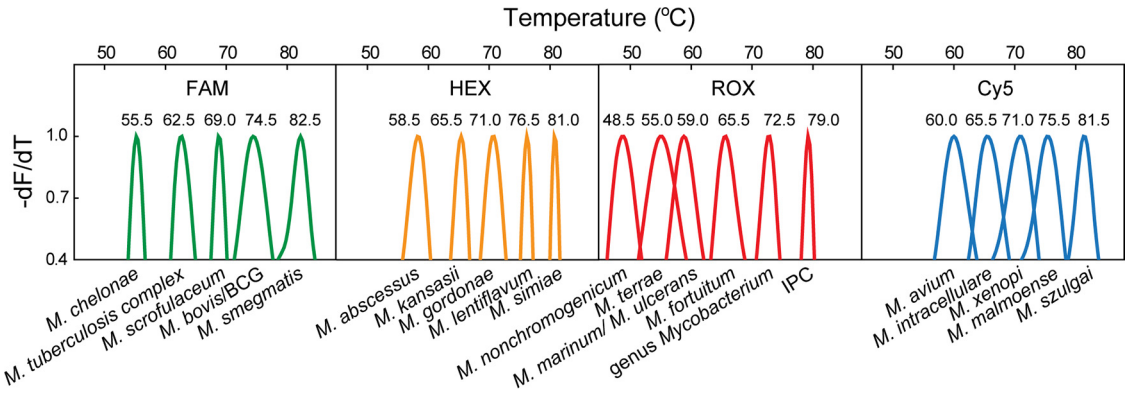

FIG 1 Melting peaks for each species in the MeltPro Myco assay in line with the 2D label strategy. To visually compare the melting temperature values of different *Mycobacterium* species, the melting curves showing the negative derivative of fluorescence intensity with respect to temperature were first normalized between 0 and 1, and then the data between 0.4 and 1 were plotted.

culturing techniques. Testing is laborious, difficult, and time-consuming. In the last decade, advances in molecular methods have enabled rapid identification of many mycobacterial species. Several molecular systems are already commercially available; for example, the line probe assay (LPA) represented by GenoType *Mycobacterium* CM/AS (Hain Lifescience GmbH, Nehren, Germany) (10) and INNO-LiPA Mycobacteria (Innogenetics, Ghent, Belgium) (11) assays can detect mycobacteria up to 30 and 17 species/complex, respectively. These assays, however, require various post-PCR manipulations and are prone to amplicon contamination. The real-time PCR-based Real Myco-ID assay (Optipharm, Osong, South Korea) is run in a closed-tube format and can thus avoid the above-mentioned problems; nevertheless, multiple PCRs are required for identification of the 17 different species (12). So far, Sanger sequencing is still regarded as the gold standard for NTM identification, yet it is often restricted to research use and may encounter difficulties with mixed-infection samples, which are not uncommon in mycobacterial disease.

The MeltPro Myco assay (Zeesan Biotech, Xiamen, China) is a newly released qualitative diagnostic assay that can identify 19 clinically relevant *Mycobacterium* species. This assay is based on a unique multicolor melting curve analysis (MMCA) (13) that can detect dozens of targets in a single reaction using a real-time PCR machine. This is achieved by using a two-dimensional (2D) labeling strategy combining fluorescence color and melting temperature (T_m) for target identification (14). One distinct feature of the MeltPro Myco assay is its ability to identify the 19 *Mycobacterium* species in a single reaction. The closed-tube detection format avoids post-PCR manipulation, making it easy to use, rapid, and less prone to carryover contamination. To assess its suitability for clinical use, we evaluated its analytical performance regarding species identification accuracy, limit of detection (LOD), and ability to detect mixed infections. We further investigated its clinical performance by testing 1,163 clinical mycobacterial isolates and 94 smear-positive sputum specimens.

MATERIALS AND METHODS

MeltPro Myco assay. The MeltPro Myco assay targets the intergenic transcribed spacer (ITS) region between the 16S rRNA and 23S rRNA genes of mycobacteria using a panmycobacterial primer set. Eighteen species-specific probes were designed to identify 17 NTM species (*Mycobacterium ulcerans* and *Mycobacterium marinum* complex were not differentiated) and the *M. tuberculosis* complex (MTBC). One additional genus-specific probe was used to identify the *Mycobacterium* genus. To differentiate *Mycobacterium bovis* and the bacillus Calmette-Guérin (BCG) vaccine from *M. tuberculosis*, a set of primers and a probe were designed to target an uninterrupted 229-bp sequence in the *M. bovis* genome, which is otherwise interrupted by a unique 12.7-kb fragment in *M. tuberculosis* (15). Moreover, a fragment of the *Arabidopsis thaliana* sucrose-proton symporter 2 (SUC2) gene was included as an internal positive control (IPC) for an indication of PCR inhibition. In total, 21 labels were assigned to 19 mycobacterial species, the genus *Mycobacterium*, and IPC (Fig. 1). The result interpretation was as follows: a positive IPC signal indicates successful amplification with no or negligible inhibition, the appearance of a genus *Mycobacterium* melting peak indicates the existence of mycobacteria, and the presence of any 19 species-specific

positive signals indicates the existence of the corresponding species; on the other hand, the absence of all species-specific melting peaks indicates the existence of mycobacterial species beyond the 19 species in the assay and the readout is *Mycobacterium* species. Finally, absence of the genus *Mycobacterium* melting peak indicates that no mycobacteria are detected.

The MeltPro Myco assay was run in a Slan-96S real-time PCR system (Zeesan Biotech). For sample detection, 5 μl of extracted DNA was added to reaction tubes prefilled with a 20-μl PCR mixture. The running program included decontamination at 50°C for 2 min, denaturation at 95°C for 10 min, 55 cycles of 95°C for 15 s, 57°C for 20 s, and 78°C for 20 s, followed by denaturation at 95°C for 2 min, hybridization at 45°C for 2 min, and temperature increase from 45°C to 90°C at a ramp rate of 0.04°C/s. When completed, results regarding species were automatically provided by a dedicated software (MeltPro Manager version 1.0; Zeesan Biotech) according to the result interpretation guidelines.

Analytical evaluation. For evaluating analytical specificity, we analyzed the most common microflora found in sputum consisting of 51 mycobacterial species and 16 nonmycobacterial species. The 51 mycobacterial strains included 22 NTM species or subspecies that could be identified by the MeltPro Myco assay and 29 other NTM species (Table 1). They were obtained from the National Center for Medical Culture Collections (CMCC) affiliated with the National Institutes for Food and Drug Control (NIFDC, China). All strains were stored in culture medium at approximately 10^6 bacilli/ml. Genomic DNA was obtained by heating lysis. Briefly, 0.5 ml of *Mycobacterium* culture in liquid medium was centrifuged at 13,000 $\times$ *g* for 10 min, and then the precipitate was suspended in 100 μl DNA extracting solution (10 mM Tris-HCl [pH 8.5], 1 mM EDTA, and 1% Triton X-100). The suspension was heated at 99°C for 20 min, followed by centrifugation at 13,000 $\times$ *g* for 10 min. The supernatant was kept at −20°C before use.

The 16 nonmycobacterial strains were obtained from our laboratory (see Table S1 in the supplemental material). These strains were provided in the form of genomic DNA of approximately 10^3 copies/μl.

For evaluating analytical sensitivity, four NTM species, i.e., *M. tuberculosis*, *M. kansasii*, *M. fortuitum*, and *M. avium*, were chosen as representatives of the four fluorescence channels; their exact concentrations were determined by direct microscopic counting using a hemocytometer. Bacterial suspensions were prepared in a serial dilution of 3×10^5, 3×10^4, 3×10^3, 3×10^2, and 3×10^1 bacilli/ml in 0.9% NaCl. Each bacterial suspension was subjected to DNA extraction using the Lab-Aid 824 MTB DNA extraction kit (Zeesan Biotech). The LOD was defined as the lowest concentration that gives no more than one negative result in 20 replicates (i.e., positive rate, $\geq$95%).

To determine the efficiency of MeltPro Myco assay in detecting mixed infections, we tested two pathogens that occur in the same fluorescence channel. *M. chelonae* and *M. tuberculosis* were chosen, as the two occur in the 6-carboxyfluorescein (FAM) channel as neighboring species. Plasmid DNA (10^3 copies/μl) containing the ITS region of *M. chelonae* was mixed with plasmid DNA (10^3 copies/μl) containing the ITS region of *M. tuberculosis* in different percentages to generate DNA templates containing 0%, 1%, 5%, 25%, 50%, 75%, 95%, 99%, and 100% *M. chelonae*. They were subjected to the MeltPro Myco assay and tested in triplicate. We also tested two pathogens occurring in different fluorescence channels. To this end, *Mycobacterium intracellulare* in the Cy5 channel was paired with *M. tuberculosis* in the FAM channel. Plasmid DNA templates containing different percentages of *M. intracellulare* were subjected to the MeltPro Myco assay, as mentioned above.

Clinical evaluation. Two batches of samples were used for clinical evaluation of the MeltPro Myco assay consisting of 1,163 clinical isolates and 94 smear-positive sputum samples.

The clinical isolates used met the following eligibility criteria, were MGIT 960 culture positive, and were consecutively collected. They were collected from five health care units. Among them, 378 isolates were from southern China (Guangzhou Chest Hospital, Guangzhou, Guangdong), 189 isolates were from southeastern China (Xiamen Center for Disease Control and Prevention, Xiamen, Fujian), 217 isolates were from central China (Henan Provincial Center for Disease Control and Prevention, Zhengzhou, Henan), 297 isolates were from northeastern China (Infectious Disease Hospital of Heilongjiang Province, Harbin, Heilongjiang), and 82 isolates were from northwestern China (Chest Hospital of Xinjiang Uyghur Autonomous Region, Wulumuqi, Xinjiang Uyghur Autonomous Region). DNA extraction from clinical isolates was carried out by heating lysis, as described previously.

Patient samples consisted of 94 smear-positive sputum samples that were consecutively collected from Guangzhou Chest Hospital. Each sample was decontaminated by using the *N*-acetyl-L-cysteine (NALC)-NaOH method, followed by neutralization with sterile phosphate-buffered saline (PBS; pH 6.8). After centrifugation at 3,000 $\times$ *g* for 15 min, the pellet was resuspended in 2 ml PBS buffer. A 1.5-ml sample of the decontaminated specimens was subjected to DNA extraction using the Lab-Aid 824 MTB DNA extraction kit.

To confirm the detection results of the MeltPro Myco assay, a variety of comparison methods were used. First, an IS*6110*-based MTBC test kit (Zeesan Biotech) was used as a screening tool to confirm MTBC-containing samples. Second, a spoligotyping assay was used to differentiate between *M. bovis* and *M. tuberculosis* (16). Third, Sanger sequencing was used to confirm all NTM species by sequencing the 5′ region of the 16S rRNA gene (~500 bp) and the ITS region between the 16S rRNA and 23S rRNA genes, as previously described (17). The sequences obtained from the two regions were subjected to BLAST analysis against the NCBI GenBank database.

To validate the mixed infections identified by the MeltPro Myco assay, PCR products from the ITS region were separated by agarose gel electrophoresis (AGE) or polyacrylamide gel electrophoresis (PAGE). DNA recovered from the bands was subjected to Sanger sequencing. For amplicons that cannot be separated by AGE or PAGE, PCR detection of species-specific genes was used to confirm the coexisting strains; *M. tuberculosis* was confirmed by the MTBC test kit that targets IS*6110*, *M. avium* was confirmed

TABLE 1 Results of strains tested with the MeltPro Myco assay

Strain	Bacterium	Results obtained by the MeltPro Myco assay				
		FAM (°C)[a]	HEX (°C)[a]	ROX (°C)[a]	Cy5 (°C)[a]	Identification[b]
95001	*M. avium*	-	-	72.3, 79.2	59.6	*M. avium*
95002	*M. intracellulare*	-	-	72.5, 79.0	65.0	*M. intracellulare*
95003	*M. xenopi*	-	-	72.5, 79.0	71.0	*M. xenopi*
95004	*M. ulcerans*	-	-	58.5, 72.4, 78.9	-	*M. marinum/M. ulcerans*
95005	*M. terrae*	-	-	55.0, 72.5, 79.0	-	*M. terrae*
95007	*M. nonchromogenicum*	-	-	48.6, 72.5, 79.0	-	*M. nonchromogenicum*
95010	*M. malmoense*	-	-	72.5, 79.0	75.8	*M. malmoense*
95013	*M. kansasii*	-	65.6	72.4, 79.0	-	*M. kansasii*
95014	*M. marinum*	-	-	58.6, 72.5, 79.0	-	*M. marinum/M. ulcerans*
95015	*M. simiae*	-	80.7	72.5, 79.1	-	*M. simiae*
95017	*M. scrofulaceum*	68.7	-	72.4, 79.0	-	*M. scrofulaceum*
95018	*M. gordonae*	-	70.8	72.5, 79.0	-	*M. gordonae*
95019	*M. szulgai*	-	-	72.5, 79.0	81.2	*M. szulgai*
95020	*M. chelonae*	55.0	-	72.4, 78.8	-	*M. chelonae*
95021	*M. abscessus*	-	58.5	72.5, 79.0	-	*M. abscessus*
95022	*M. fortuitum*	-	-	65.0, 72.5, 79.0	-	*M. fortuitum*
95023	*M. smegmatis*	82.4	-	72.5, 79.0	-	*M. smegmatis*
93009	*M. tuberculosis*	62.3	-	72.5, 79.0	-	MTBC
95048	BCG	62.3, 74.6	-	72.5, 79.0	-	*M. bovis*/BCG
95049	*M. bovis*	62.3, 74.6	-	72.4, 79.0	-	*M. bovis*/BCG
95054	*M. africanum*	62.3	-	72.5, 79.0	-	MTBC
D2PB302	*M. microti*	62.3	-	72.5, 79.0	-	MTBC
95006	*M. gastri*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95008	*M. shimoidei*	-	-	72.3, 78.8	-	*Mycobacterium* spp.
95009	*M. triviale*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95012	*M. farcinogenes*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95016	*M. asiaticum*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95024	*M. phlei*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95025	*M. thermoresistibile*	-	-	72.5, 79.1	-	*Mycobacterium* spp.
95026	*M. aichiense*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95027	*M. aurum*	-	-	72.4, 79.0	-	*Mycobacterium* spp.
95028	*M. chubuense*	-	-	72.4, 79.0	-	*Mycobacterium* spp.
95029	*M. duvalii*	-	-	72.6, 79.0	-	*Mycobacterium* spp.
95030	*M. flavescens*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95031	*M. gadium*	-	-	72.5, 79.1	-	*Mycobacterium* spp.
95032	*M. gilvum*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95033	*M. komossense*	-	-	72.4, 79.0	-	*Mycobacterium* spp.
95034	*M. neoaurum*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95035	*M. obuense*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95036	*M. parafortuitum*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95037	*M. rhodesiae*	-	-	72.5, 79.2	-	*Mycobacterium* spp.
95038	*M. tokaiense*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95039	*M. porcinum*	-	-	72.4, 79.0	-	*Mycobacterium* spp.
95040	*M. pulveris*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95041	*M. senegalense*	-	-	72.3, 79.0	-	*Mycobacterium* spp.
95042	*M. fallax*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95043	*M. agri*	-	-	72.5, 79.1	-	*Mycobacterium* spp.
95044	*M. austroafricanum*	-	-	72.4, 79.0	-	*Mycobacterium* spp.
95045	*M. diernhoferi*	-	-	72.5, 79.0	-	*Mycobacterium* spp.
95046	*M. chitae*	-	-	72.5, 79.2	-	*Mycobacterium* spp.
93467	*M. vaccae*	-	-	72.4, 79.0	-	*Mycobacterium* spp.

[a]Melting temperature of melting peak. "-" means no melting peak was detected.
[b]The identification "*Mycobacterium* spp." represents bacteria that could only be detected at the genus level by the MeltPro Myco assay.

by the detection of IS*901*, and *M. intracellulare* was confirmed by the detection of DT1 (an insertion element harboring transposase gene), as previously described (18, 19).

RESULTS

Analytical evaluation. Evaluation of analytical specificity showed that the MeltPro Myco assay could correctly detect all 51 mycobacterial strains to the species/genus level and exhibited no cross-reactivity with the 16 nonmycobacterial strains (Tables 1 and S1).

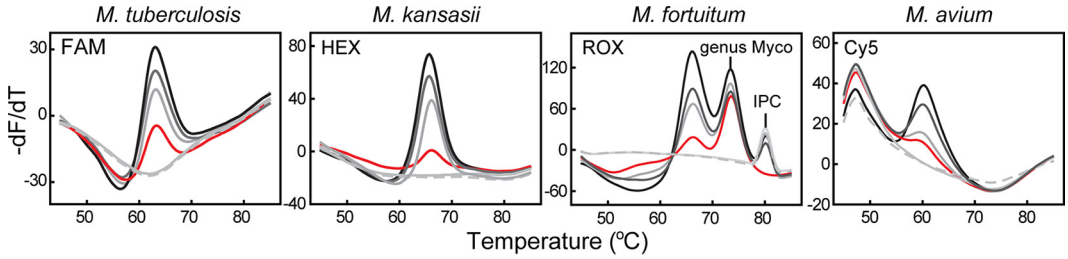

FIG 2 Analytical sensitivity of the MeltPro Myco assay. Melting curves of the four representative species, i.e., *M. tuberculosis*, *M. kansasii*, *M. fortuitum*, and *M. avium*, ranged from 3×10^5 to 3×10^1 bacilli/ml and are shown (gray lines) together with the melting curves representing the LOD (red lines) and no-template control (NTC) (dashed lines). Myco, *Mycobacterium*.

The four studied strains, *viz*. *M. tuberculosis*, *M. kansasii*, *M. fortuitum*, and *M. avium*, gave reproducible readouts in 20 replicates when the concentration was equal to or higher than 300 bacilli/ml; therefore, the MeltPro Myco assay LOD was determined to be 300 bacilli/ml (Fig. 2).

We then evaluated the ability of the MeltPro Myco assay to detect mixed infections by using two-species mixed samples as models. Our results showed that minor species could be detected at as low as 1% of the abundant species, regardless of whether the two species were in identical or different fluorescence channels (Fig. 3).

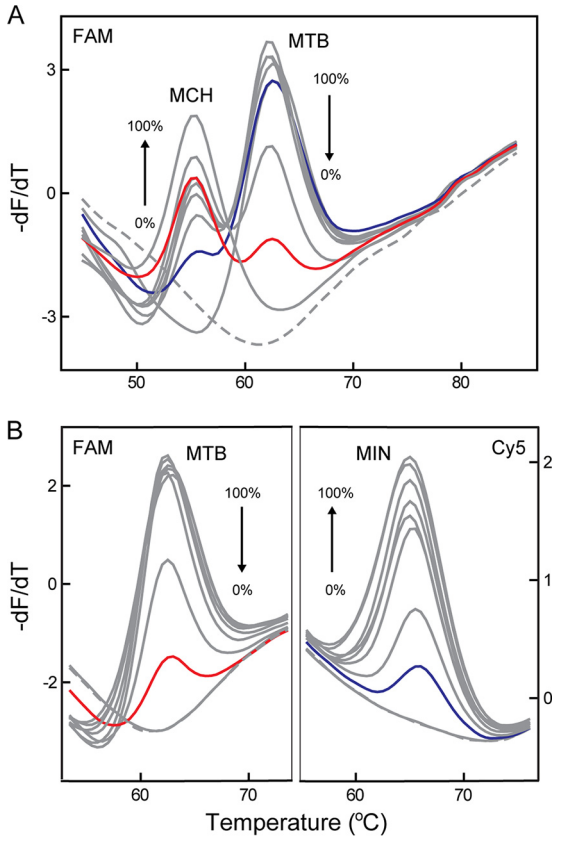

FIG 3 MeltPro Myco assay for mixed-infection detection. (A) Two coexisting species, *M. tuberculosis* (MTB) and *M. chelonae* (MCH), were detected in one channel (FAM). (B) Two coexisting species, MTB and *M. intracellulare* (MIN), were detected in two channels (FAM and Cy5). The overall template concentration was 5×10^3 copies per reaction. Melting curves of artificial plasmid templates containing two species with various ratios (0:100, 1:99, 5:95, 25:75, 50:50, 75:25, 95:5, 99:1 to 100:0) are shown (gray lines) together with melting curves of the mixed template containing 1% MTB (red line), 1% of MCH (A), or 1% of MIN (B) (blue line), and NTC (dashed line).

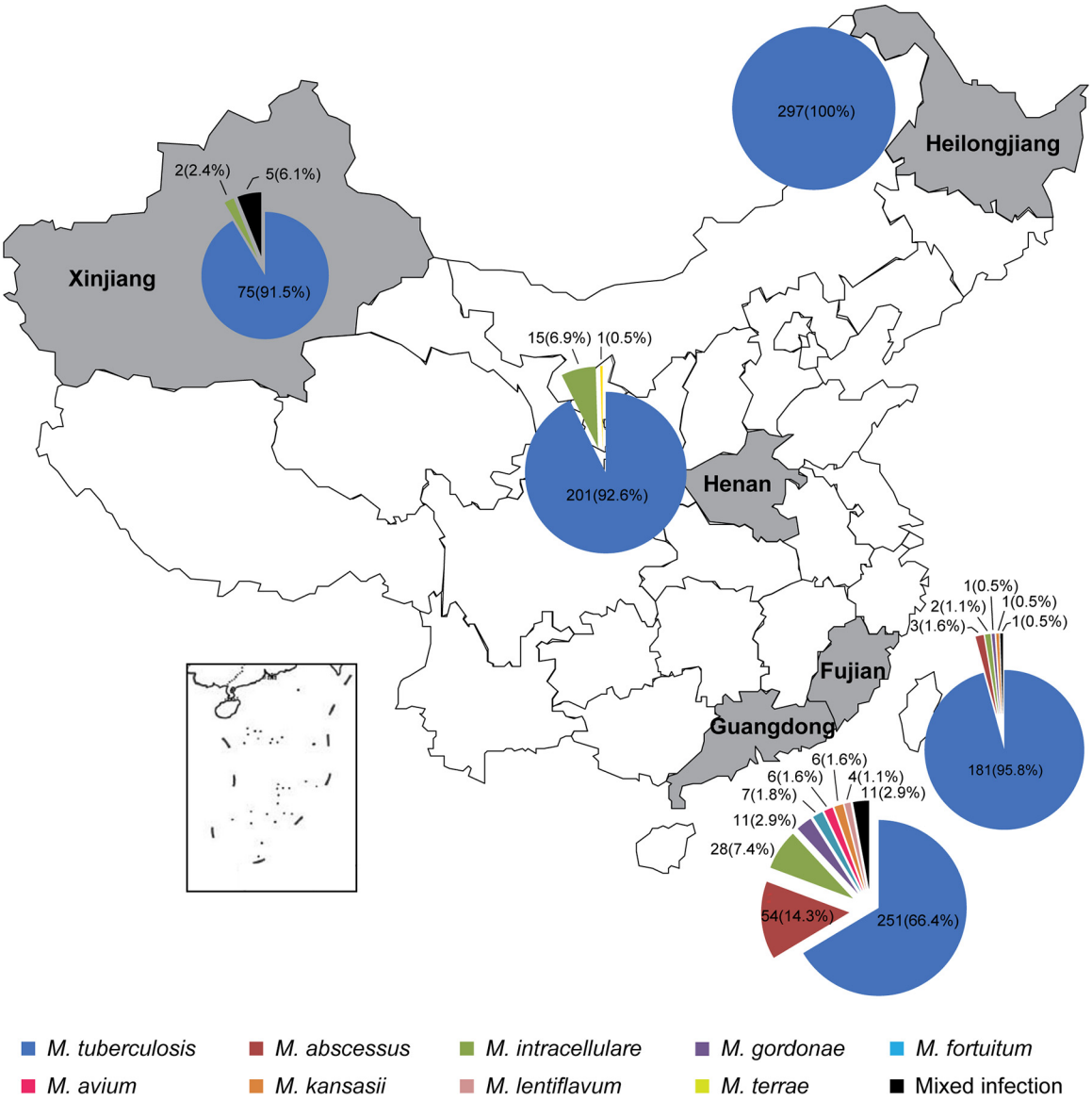

FIG 4 *Mycobacterium* species distribution over five regions in China.

Clinical evaluation. We first assessed the MeltPro Myco assay using 1,163 clinical isolates collected from five geographically distinct health care units in China. Of the 378 isolates from Guangzhou, 251 *M. tuberculosis*, 116 NTM, and 11 mixed infections were identified. Of the 189 isolates from Xiamen, 181 *M. tuberculosis*, 7 NTM, and one mixed infection were detected. From Henan, 201 *M. tuberculosis* and 16 NTM were detected in 217 samples. From the Heilongjiang samples, all 297 isolates were identified as *M. tuberculosis*. Of the 82 isolates from Xinjiang, 75 *M. tuberculosis*, 2 NTM, and 5 mixed infections were identified. In total, 1,146 samples of single infections (1,005 samples of *M. tuberculosis* and 141 samples of NTM) and 17 samples of mixed infections were identified by the MeltPro Myco assay. The species frequencies are shown with their location of origin in Fig. 4.

To verify the above-mentioned results, we first screened all samples using the MTBC test kit. The results showed that all 1,005 samples identified as *M. tuberculosis* gave positive results, whereas none of the 141 NTM and 14 of 17 mixed infections were positive.

To confirm the 141 NTM single-infection results, Sanger sequencing was used to analyze the 5′ region of the 16S rRNA gene and the ITS region between the 16S rRNA

TABLE 2 Validation of 18 mixed infections found in 1,163 clinical isolates

Sample	Mixed infection bacteria	Discrepant bp[a]	Validation assay[b]
GZ k1624623	*M. avium-M. abscessus*	38 (AGE)	ITS
GZ k1624078	*M. intracellulare-M. abscessus*	38 (AGE)	ITS
GZ k1624914	*M. tuberculosis-M. abscessus*	36 (AGE)	ITS
GZ k1621849	*M. tuberculosis-M. abscessus*	36 (AGE)	ITS
GZ k1623927	*M. tuberculosis-M. abscessus*	36 (AGE)	ITS
GZ k1620589	*M. tuberculosis-M. abscessus*	36 (AGE)	ITS
GZ k1621157	*M. tuberculosis-M. abscessus*	36 (AGE)	ITS
GZ k1617424	*M. xenopi-M. lentiflavum*	25 (PAGE)	ITS
GZ k1623332	*M. fortuitum-M. senegalense*	14 (PAGE)	ITS
GZ k1618570	*M. tuberculosis-M. gordonae*	10 (PAGE)	ITS
GZ k1621958	*M. tuberculosis-M. avium*	2	IS*6110*, IS*901*
GZ k1616819	*M. tuberculosis-M. intracellulare*	2	IS*6110*, DT1
XM TA15018	*M. tuberculosis-M. intracellulare*	2	IS*6110*, DT1
XJ 5504	*M. tuberculosis-M. intracellulare*	2	IS*6110*, DT1
XJ 5541	*M. tuberculosis-M. intracellulare*	2	IS*6110*, DT1
XJ 5551	*M. tuberculosis-M. intracellulare*	2	IS*6110*, DT1
XJ 5614	*M. tuberculosis-M. intracellulare*	2	IS*6110*, DT1
XJ 5563	*M. tuberculosis-M. intracellulare*	2	IS*6110*, DT1

[a]Discrepant base pairs between two amplicon fragments amplified from mixed strains using panmycobacterial ITS primers, as previously described (17). Agarose gel electrophoresis (AGE) or polyacrylamide gel electrophoresis (PAGE) was used to separate two different amplicons based on corresponding discrepant base pairs.

[b]For mixed infections with two different amplicons that can be separated by AGE or PAGE, both PCR products were subjected to ITS sequencing. For M. tuberculosis-M. avium and M. tuberculosis-M. intracellulare, M. tuberculosis, M. avium, and M. intracellulare were confirmed by using their respective specific genes, i.e., IS*6110*, IS*901*, and DT1.

and 23S rRNA genes. Concordant results were found for 137 of the 141 NTM samples. Of the 4 discordant samples from Guangzhou Chest Hospital, 3 samples identified as *M. intracellulare* by the MeltPro Myco assay were determined to be *Mycobacterium marseillense*, *Mycobacterium chimera*, and *M. avium* complex through sequencing. Notably, these three species belong to the *M. avium* complex, which also contains *M. intracellulare*, indicating a close relationship between them. The other sample identified as *M. fortuitum* alone by the MeltPro Myco assay displayed overlapping peaks in the sequencing chromatogram, indicating mixed species. The PCR products were then subjected to PAGE for separation of possible mixed amplicons, and two bands were recovered. Sequencing analysis revealed that they were amplicons of *M. fortuitum* and *Mycobacterium senegalense*. As *M. senegalense* was outside the species identification groups in the MeltPro Myco assay, sequencing analysis confirmed the assay results.

To confirm the 17 mixed infection results, we first used AGE or PAGE to separate and recover their ITS amplicons by taking advantage of the length differences ($>$10 bp). Otherwise, we used PCR detection of species-specific target genes. Through these approaches, all 17 samples containing mixed species detected by MeltPro Myco assay were confirmed to be correct (Table 2).

Taken together, clinical evaluation of the 1,163 clinical isolates showed that the MeltPro Myco assay correctly identified 1,159 (99.7%) samples. Three samples identified as *M. intracellulare* proved to be species that were closely related and within the *M. avium* complex, while a mixed-infection sample missed identifying one species, as it is outside the assay coverage.

To explore the suitability of the MeltPro Myco assay in detecting clinical samples, we tested 94 smear-positive sputum samples that were consecutively collected. The MeltPro Myco assay identified 92 species-resolvable samples, 1 *Mycobacterium* sp. sample, and 1 negative sample (Fig. 5). Of the 92 samples identifiable to the species level, 69 samples contained single MTBC members composed of 68 *M. tuberculosis* (73.1%) and 1 *M. bovis*, 21 samples contained single NTM species, and 2 samples contained two mixed species. The MTBC test kit gave concordant results for all *M. tuberculosis*-containing samples, while *M. bovis* identification was confirmed by spoligotyping. Sequencing analysis revealed that the *Mycobacterium* spp. proved to be *M. europaeum*,

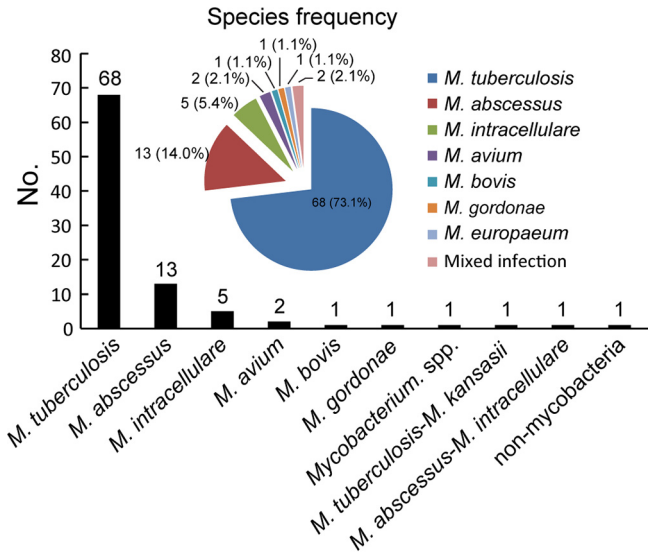

FIG 5 Distribution of the mycobacterial species detected from the 94 smear-positive sputum specimens.

an NTM species outside the coverage of the MeltPro Myco assay. The negative sample contained *Corynebacterium* species, a nonmycobacterial organism that is also beyond the coverage of the MeltPro Myco assay. Consequently, the results of the MeltPro Myco assay for the 94 smear-positive sputum samples were all confirmed to be correct.

DISCUSSION

Compared to traditional phenotypic methods, molecular identification of mycobacterial species has advantages in rapidness, accuracy, reproducibility, and especially the ability to identify mycobacteria directly from clinical raw sample. Rapid identification of mycobacteria would not only help the treatment of NTM diseases but also benefit TB control. For example, patients infected with NTM can be removed from isolation immediately without any subsequent TB contact investigation after a rapid identification.

In this study, we evaluated the analytical and clinical performances of the MeltPro Myco assay. The MeltPro Myco assay accurately identified 51 reference mycobacterial strains to the species/genus level and showed no cross-reactivity with 16 nonmycobacterial strains. Using representative NTM species of the four fluorescence channels, the MeltPro Myco assay LOD was found to be 300 bacilli/ml. In the case of mixed infections, 1% of the minor species could be detected regardless of whether the two species were in identical or different fluorescence channels. Clinical evaluation using 1,163 isolates collected from five geographically distinct health care units showed that the MeltPro Myco assay could correctly identify *M. tuberculosis* and NTM species in 1,159 samples. Further testing with direct clinical samples showed that all 94 smear-positive sputum samples were correctly identified.

The MeltPro Myco assay represents the first melting curve analysis-based system designed for the identification of NTM. The unique feature of this assay is its ability to identify 17 clinically important NTM to the species level, as well as to detect the genus *Mycobacterium*, MTBC, and further *M. bovis* BCG, all in a single and closed-tube reaction. The MeltPro Myco assay also distinguishes itself from existing systems, such as membrane hybridization assays that require various post-PCR manipulations or real-time PCR assays that need multiple reactions. The turnaround time is within 3 h, and the hands-on time is less than 20 min, as only a single template addition step is required. The assay can detect up to 94 samples with 1 positive- and 1 negative-control sample in a single run on a 96-well real-time PCR machine, facilitating its potential use as a screening tool in clinical settings.

The MeltPro Myco assay utilizes the ITS region as the target sequence in identifying 17 NTM species and detecting the genus *Mycobacterium*. The ITS region proved to be more specific than other target regions that are currently used in NTM identification. This specificity is reflected in the reduced cross-reactivity among NTM species. Of the 17 NTM resolvable species, only *M. marinum* and *M. ulcerans* could not be differentiated. The MeltPro Myco assay correctly identified all 51 common mycobacterial strains to the species/genus level and showed no cross-reactivity with 16 nonmycobacterial species. In contrast, existing systems exhibit extensive cross-reactivity among the target NTM species; for example, a sequencing-based system targeting a hypervariable segment of the 16S rRNA failed to differentiate NTM pairs, such as *M. chelonae* and *M. abscessus*, *M. kansasii* and *M. gastri*, and *M. marinum* and *M. ulcerans*, among others (20). Likewise, a DNA chip assay based on 16S rRNA listed 3 of the 17 NTM species as being cross-reactive with other species (21). A widely used membrane hybridization assay, the GenoType CMdirect version 1.0 (or GenoType *Mycobacterium* CM version 2.0), utilizes the 23S rRNA target region and had 7 of the 14 species showing cross-reactivity with other target NTM members (10). Even more cross-reactions were observed with the real-time PCR-based system Real Myco-ID, where 6 of 10 NTM species were cross-reactive among the target species (12).

The inclusion of *M. bovis* BCG in the MeltPro Myco assay could add clinical value, as *M. bovis* harbors natural resistance to pyrazinamide, a first-line drug used to treat *M. tuberculosis*. Distinguishing *M. bovis* from MTBC would prevent the unnecessary usage of pyrazinamide. Moreover, disseminated BCG infections mostly occur in patients with immune deficiencies after vaccination; thus, rapid diagnosis of BCG infections is critical due to its extremely high mortality rate (22).

The low LOD of the MeltPro Myco assay, i.e., 300 bacilli/ml, forms the basis for its application beyond cultured samples to potential smear-positive specimens that contain 5,000 to ~10,000 bacilli/ml (23). Moreover, its capability in detecting 1% of minor species supports its applicability to mixed-infection samples, which are difficult, if not impossible, for Sanger sequencing.

Clinical evaluation further supported the accuracy of the MeltPro Myco assay in mycobacterial identification. Of all 1,163 isolates, the MeltPro Myco assay correctly identified 1,159 samples when Sanger sequencing was regarded as the gold standard. Three discordant samples detected as *M. intracellulare* proved to be *M. marseillense*, *M. chimera*, and *M. avium* complex, all of which are rare NTM species that belong to the *M. avium* complex (MAC) and were not included as identifiable species in the MeltPro Myco assay. These results indicate the cross-reactivity of *M. intracellulare*-specific probes with its MAC members. According to the American Thoracic Society (ATS) recommendation (6), the differentiation between MAC members is not yet clinically significant. There is no prognostic or treatment advantage for distinguishing MAC isolates into specific species. Thus, these three MAC species could be listed as *M. intracellulare* equivalents in the MeltPro Myco assay to some extent. However, currently reported multiple outbreaks of *M. chimera* infections associated with heater cooler units following cardiac surgery (24, 25) indicated that differentiating subspecies within the MAC would be important in the epidemiological survey. The last discordant sample detected as *M. fortuitum* was confirmed to be a mixed infection of *M. fortuitum* and *M. senegalense*; however, *M. senegalense* is beyond the identifiable species of the MeltPro Myco assay. Thus, the assay detection results were correct according to the interpretation guidelines.

The large number of samples employed for evaluating clinical specificity also provided information on the incidence rate and species distribution of NTM in China. As shown in Fig. 3, South China (Guangzhou and Xiamen) showed a higher incidence rate, with more NTM species (135/567 [23.8%]) than in north-central China (Henan, Heilongjiang, and Xinjiang; 23/596 [3.9%]). These preliminary data support the hypothesis that high humidity and temperature lead to an increased prevalence of NTM (26–28). The 1,163 isolates gave an overall incidence rate of 13.6% for NTM (including mixed infections), which was somewhat lower than that reported in the fifth national

tuberculosis epidemiological survey conducted in 2010, where the incidence rate of NTM was 22.9% (29). This discrepancy could probably be attributed to different locations and sample sizes. It is worth noting that *M. abscessus*, a species of multidrug-resistant NTM, was the most common organism (14.3%) identified in southern China. Previously, *M. abscessus* was thought to be independently acquired by susceptible individuals from the environment. However, recent studies have shown that the majority of *M. abscessus* infections were acquired through transmission, potentially via vomit and aerosols (30). The high incidence rate of *M. abscessus* presents a challenge for prevention and control and highlights the importance of its identification. The MeltPro Myco assay could correctly identify all the *M. abscessus* samples. However, similar to current commercial assays, it could not differentiate the subspecies of *M. abscessus*, which are important in the development of treatment regimens (31).

Most existing molecular assays for mycobacterial identification restrict their use to clinical isolates rather than clinical samples (10–12). However, an additional and long culturing step is required to obtain these isolates. Unfortunately, there are no universal culture conditions for all NTM species. For example, NTM species, such as *M. chelonae*, *M. marinum*, and *M. ulcerans*, require low culture temperature (25 to 33°C), while *M. ulcerans* needs egg yolk for growth and also needs lengthy incubation, which will differentiate it from *M. marinum*. This drawback can be even more complex in cases where a sample consists of both fast- and slow-growing mycobacteria, as its composition can change along with the culture. These culture-derived problems also impair the performance of matrix-assisted laser desorption ionization–time of fight mass spectrometry (MALDI-TOF MS) systems developed for the identification of mycobacteria. In fact, the accuracy of MALDI-TOF MS systems varied with medium types, incubation periods, sample preparation, and repeat testing (32–35). More seriously, MALDI-TOF MS was unable to identify *M. tuberculosis* in the presence of polymicrobial culture (36). Apparently, all the above-mentioned problems could be overcome when clinical samples are simply detected without a culture step.

Taking this into account, the success of the MeltPro Myco assay in detecting 94 sputum samples has important implications. Despite the limited number of samples, the clinical samples gave richer information regarding NTM incidence in the real world than in cultured samples. Of the 94 smear-positive sputum specimens consecutively collected in Guangzhou Chest Hospital, 68 *M. tuberculosis*, 1 *M. bovis*, 21 NTM, and 2 mixed-infection samples, as well as 1 mycobacterial sample and 1 nonmycobacterial sample, were identified. In comparison, of the 378 clinical isolates consecutively collected in the same hospital, 251 *M. tuberculosis*, 116 NTM, and 11 mixed-infection samples were identified. The high incidence of NTM infections in this hospital, including mixed infections, detected in sputum (25.5%) was close to the results obtained from clinical isolates (33.6%). Similar incidences of mixed-infection detection were also found between sputum and clinical isolates (2.1% versus 2.9%). As in the clinical samples, *M. abscessus* was again the predominant NTM species, followed by *M. intracellulare* in the sputum samples. Despite these similarities, the diverse nature of the sputum samples could be seen with the detection of 1 *M. bovis* sample as well as 1 *M. europaeum* and 1 corynebacterium that were additionally detected in such a small sample size.

One limitation of this study was the lack of smear-negative specimens for evaluation. Smear microscopy is a routine and low-cost screening tool in a TB laboratory. A positive specimen can contain 5,000 to ~10,000 bacilli/ml, which is well above the LOD (300 bacilli/ml) of the MeltPro Myco assay. Therefore, it is promising for the MeltPro assay to identify mycobacteria in the smear-negative specimens. Further studies are needed regarding the outcome and cost-effectiveness for the implementation of this assay into clinical practice.

Another limitation of this study is that some species covered by the MeltPro Myco assay were not detected among the clinical samples. This is mainly due to the limited sample types, which were restricted to respiratory samples. Further study is needed on those nonrespiratory sample types, such as tissues or pus, so that a comprehensive coverage of NTM species might be achieved.

The sustained increase in NTM infection incidence in recent years and the difficulty in distinguishing NTM infection from tuberculosis constitute an urgent need to identify NTM to the species level. In this regard, the MeltPro Myco assay is appropriate due to its rapidness, ease of use, and robustness. As the MeltPro Myco assay can also directly detect smear-positive specimens, it can even be implemented as a screening tool in regions endemic for tuberculosis.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01096-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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We declare no conflicts of interest.

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