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Development and validation of real-time polymerase chain reaction and next-generation sequencing assays for liquid culture–based identification of *Mycobacterium tuberculosis* and nontuberculous mycobacteria

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Abstract

Objectives: Acid-fast bacilli (AFB) culture remains the gold standard for laboratory diagnosis of mycobacterial infections because of its high sensitivity and specificity. However, the lengthy time to cultivation, particularly among slow-growing mycobacteria, including *Mycobacterium tuberculosis* and *Mycobacterium avium/Mycobacterium intracellulare*, can result in more than a 6-week delay in clinical diagnosis and administration of optimal therapy. Although liquid AFB culture paired with molecular identification probes dramatically decreased time to cultivation and reporting, these probes were recently discontinued, with no suitable alternative for liquid culture–based identification of cultivated mycobacteria. We aimed to develop and validate a molecular approach for identifying clinical AFB isolates directly from liquid culture.

Methods: We developed and validated the tandem use of quantitative real-time polymerase chain reaction (qPCR) and next-generation sequencing (NGS) to identify clinical AFB isolates directly from liquid culture.

Results: Both laboratory-developed tests demonstrated performance characteristics of >95% sensitivity and specificity. Importantly, integration of molecular testing into the liquid-based AFB workup streamlined the workflow and reduced the time to reporting of mycobacterial identification to ≤10 days.

Conclusions: The tandem use of qPCR and NGS provides an effective alternative for the identification of mycobacteria from liquid culture following discontinuation of conventional molecular probes. These findings demonstrate the value of effective diagnostic design and test development in optimizing the delivery of accurate and timely results for infectious disease diagnosis.

KEY POINTS

- Quantitative real-time polymerase chain reaction (qPCR) and next-generation sequencing (NGS) can reliably identify clinical mycobacterial isolates directly from liquid acid-fast bacilli (AFB) cultures, with high sensitivity and specificity.
- This tandem molecular approach provides a practical alternative following the discontinuation of traditional molecular identification probes.
- Integration of qPCR and NGS into liquid AFB culture workflows can reduce time to mycobacterial identification to ≤10 days, supporting earlier diagnosis and administration of targeted therapy.

Key words

mycobacterium; molecular diagnostics; species identification; real-time PCR; next-generation sequencing; liquid culture medium; TB; MAC; NTM

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INTRODUCTION

Acid-fast bacilli (AFB)—namely, members of the Mycobacteriaceae family—are a group of bacteria that share a common in vitro characteristic (acid-fastness), yet vary widely in clinical significance and presentation.[1] In particular, the *Mycobacterium tuberculosis* (Mtb) complex is a group of the most important pathogens worldwide with respect to mortality and public health concern.[2,3] Globally, the rate of incident cases of tuberculosis (TB) has continuously risen, especially since the start of the COVID-19 pandemic, from 10.1 million in 2020 to 10.8 million in 2023.[3] While the mortality of untreated TB can be reduced from nearly 50% to 15% with the current World Health Organization–recommended regimen, the associated morbidity remains significant.[3] Notably, among the clinically isolated Mtb complex, an estimated 12.4% may be the highly related but typically zoonotic *Mycobacterium bovis* species that is intrinsically resistant to the first-line drug pyrazinamide, which may further confound TB management.[4,5]

Among nontuberculous mycobacteria (NTM), *Mycobacterium avium/Mycobacterium intracellulare* (often coupled together as MAI) and the *Mycobacterium abscessus* complex are among the most common causes of mycobacterial lung disease, demonstrating similar health burdens to TB.[6] In a 12-year retrospective study of over 1600 patients with NTM lung disease, NTM infections were significantly correlated with all-cause mortality, noncancer mortality, and mortality due to chronic airway diseases.[6]

Global efforts to curtail the public health impact of AFB infections are, in part, rooted in early diagnosis, including universal drug susceptibility testing for the Mtb complex.[3] The laboratory diagnosis of mycobacterial infections, however, is challenged by the characteristically slow time to cultivation despite the high sensitivity and specificity of conventional culture-based methods.[2] Correspondingly, advancements in AFB laboratory testing have largely focused on substantially reducing the turnaround time (TAT) to actionable results. Notably, the Cepheid Xpert MTB-RIF assay is one of the earliest US Food and Drug Administration (FDA)–cleared sample-to-answer solutions for the simultaneous detection of the Mtb complex along with *rpoB*-mediated rifampin resistance in sputum specimens of patients under investigation for TB. Early evaluations of the quantitative real-time polymerase chain reaction (qPCR)–based assay demonstrated 98% agreement with AFB culture of smear-positive specimens, with lower agreement with smear-negative samples.[7] However, while the Xpert MTB-RIF assay was able to offer a direct-from-sample testing platform for AFB, it remains limited in scope, only targeting Mtb species.

Toward more comprehensive laboratory solutions for the diagnosis of AFB infections, the development of liquid AFB culture has significantly reduced the average time to cultivation, particularly of slow-growing mycobacteria, to 12 to 16 days compared with 6 to 8 weeks in solid media.[2] Moreover, the automation of liquid cultures, such as with the BACTEC MGIT Automated Mycobacterial Detection System (Becton, Dickinson and Company [BD]) using mycobacterial growth indicator tubes (MGITs), has allowed for continuous monitoring through the use of fluorescence to detect growth in broth culture. Previously, the use of the MGIT system was often coupled with a commercially available fluorescent probe–based test for the subsequent identification of select *Mycobacterium* species or complex directly from a liquid growth–derived bacterial pellet. Unfortunately, the probes were discontinued, and no FDA-cleared alternative for direct-from-liquid growth methods for bacterial identification has been made available. While studies have reported the moderately effective off-label utility of matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) directly from liquid culture, its capacity to accurately identify mycobacterial species, including Mtb, particularly among polymicrobial growth, remains an important limitation.[8,9] Accordingly, subculture onto solid media to obtain isolated colonies becomes necessary, further delaying microbial identification and susceptibility testing.

Moreover, there have been limited methods available to differentiate *M bovis* from human Mtb isolates, which has historically been identified through its phenotypic properties, including growth pattern, niacin accumulation, and nitrate reduction. More recently, several molecular methods have been developed, including spoligotyping and single-nucleotide polymorphism analysis of either the *oxyR* or *gyrB* gene; however, commercially available solutions remain absent in the United States.[10]

To address this diagnostic gap, we concurrently validated 2 laboratory-developed tests (LDTs) using contemporary molecular techniques to be used in tandem following microbial growth in liquid culture. We aimed to capitalize on the already ubiquitous clinical applications of qPCR and the growing accessibility of next-generation sequencing (NGS) to develop tests that can seamlessly integrate into the conventional AFB culture workflow to offer solutions for the more rapid identification of cultivated mycobacterial pathogens.

METHODS

Validation samples and specimens

Primary clinical samples, including sputum and bronchoalveolar lavage, and isolates previously cultivated from primary clinical samples or ATCC type strains were used for assay development and validation. Mycobacteria isolated from clinical specimens were identified using a combination of VITEK MS MALDI-ToF (bioMérieux) and the series of Gen-probe *Mycobacterium* species direct detection probes (Hologic). Primary clinical samples and cultivated isolates were handled in a BSL-2 laboratory using BSL-3 practices. The ATCC quality control (QC) strains were grown on selective 7H11 monoplates and then seeded into the MGIT tubes.

Mtb/MAI qPCR from MGIT culture

DNA extraction and qPCR

Quantitative real-time PCR of MGIT-derived bacterial growth was performed on the BD Max System using the *Mycobacterium tuberculosis* complex, *Mycobacterium avium* complex, and *Mycobacterium* species Open System PCR Reagents (BioGX OSR) available from BioGX. The MGIT-derived bacterial pellet (0.5 mL MGIT fluid with sediment) was heat-inactivated (100 °C for 30 minutes) and homogenized using 0.5-mm glass beads and a Precellys Homogenizer (Bertin Technologies)

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with the following setting: 6000 rpm for 60 seconds × 3 cycles with a 5-second interval. After bead beating, the sample tube was centrifuged at 5000 *g* for 1 minute, and 200 µL supernatant was loaded into a BD Max Sample Buffer Tube and placed on the BD Max instrument with the appropriate cartridge. The PCR cycling conditions include an initial denaturation cycle of 99 °C for 300 seconds, followed by 40 cycles of 99 °C for 10 seconds, 60 °C for 30 seconds, and 70 °C for 16.6 seconds. For the channel settings, the gain was set at 80, and the threshold was set at 200 for all 3 targets, including MTB, MAI, and internal control. Data analysis was performed by the BD Max software, and the amplification plots were reviewed for validity prior to release of results.

BioGX OSR

The BioGX OSR is formulated as a lyophilized product for the multiplex real-time PCR-based detection of the Mtb *IS6110* gene, the MAI ribosomal RNA (rRNA) ITS region gene, and *Mycobacterium* species 16S rRNA (pan-mycobacterial) gene as an internal control. The BioGX OSR additionally includes *Drosophila* DNA that may be used as an amplification control but is not analyzed in this assay. The Mtb/MAI (MbID)–qPCR was specifically intended for use following primary microbial cultivation in MGITs and confirmation of acid-fastness by Ziehl-Neelsen (ZN) staining. The ZN-positive samples with failed amplification of the pan-mycobacterial target were repeated or further investigated.

Method validation

Assessment of performance characteristics (accuracy, precision, and analytical sensitivity) was performed using a combination of previously characterized primary clinical samples (n = 18), pure cultures of clinical isolates (n = 65), and ATCC isolates (n = 12) seeded into MGIT tubes and cultivated using the BD Bactec MGIT 960 mycobacterial detection system until flagged positive for detectable growth. All samples were tested within 3 days of positivity.

Mycobacterial identification by NGS from MGIT culture

Sample processing

A 1-mL sample from the bottom of a MGIT tube was transferred to a 2-mL screw-cap tube followed by heat inactivation at 100 °C for 30 minutes. The tube was spun at 10000 rpm for 2 minutes, and the supernatant was discarded without disturbing the pellet, which was then resuspended using 500 µL Lysis Buffer G2 (Qiagen) and transferred into another 2-mL screw-cap sample tube prefilled with 0.5-mm glass beads. The sample was incubated at 35 to 37 °C with agitation for 30 minutes, followed by bead beating using a Precellys Homogenizer (Bertin Technologies) with the following setting: 6000 rpm for 60 seconds × 3 cycles with a 5-second interval. After bead beating, the sample tube was centrifuged at 5000 g for 1 minute, and 200 µL supernatant was transferred to a clean sample tube for DNA extraction.

DNA extraction and sequencing

Genomic DNA was extracted using the EZ Tissue kit (Qiagen) according to the manufacturer's protocols. Sequencing libraries were prepared using the MagicPrep NGS System (Tecan Genomics) as previously described.[11,12] Library quality was assessed using the High Sensitivity DNA Analysis kit (Agilent), and quantification was determined using the Qubit dsDNA High Sensitivity Assay kit (ThermoFisher). Paired-end sequencing (250 bp × 2) was performed on the Illumina MiSeq platform.

Bioinformatic analysis

KmerFinder (https://cge.food.dtu.dk/services/KmerFinder/) from the Center for Genomic Epidemiology was used to identify the candidate species/subspecies and the most closely related mycobacterial reference genome. The downstream bioinformatic analyses were performed using CLC Genomics Workbench, V22 (Qiagen). Raw reads were trimmed and mapped to the reference genome from the top KmerFinder hit. The consensus sequences of *rpoB*, *groEL1* and *groEL2*, *rrs* (16S rRNA), and *gyrB* genes were generated, and matched species/subspecies were determined based on the top hits on BLAST (NCBI). The *rpoB* gene was used as the primary marker, with *groEL1*, groEL2, and *rrs* (16S rRNA) as secondary markers, following a set of defined algorithms to determine identification at the genus or species level (Figure 1 and Table 1). To identify *M bovis*, the consensus sequence of the *gyrB* gene was used as the primary marker. The species-level identification of the *M abscessus* complex and MAI requires additional analyses previously described by the laboratory.[13,14]

Method validation

A combination of positive MGIT samples from previously characterized primary clinical samples (n = 28) or seeded with ATCC isolates (n = 8) was used for the validation of MbID-NGS. Identification of *M bovis* was corroborated through the characteristic mono-resistance to pyrazinamide mediated by a specific mutation (His57Asp) in the *pncA* gene, followed by BLASTing the *gyrB* gene for confirmation.[10] In addition, a pleural fluid positive for *M bovis* and an expectorated sputum positive for *M intracellulare* subspecies *chimaera* were sequenced in triplicate for 3 separate runs to validate the precision/reproducibility.

Qualitative and statistical analyses

Qualitative concordance for the accuracy and precision studies was inclusive of absolute concordance (organism identification to the same taxonomic level) and relative concordance (organism identification to a different taxonomic level but belonging to the same/related taxonomic group). Mean, standard deviation, and percent coefficient of variation (%CV) were calculated using Microsoft Excel. Figure S1 was created using the ggplot2 package[15] with R version 4.4.2 (2024-10-31) run on RStudio 2024.12.1.

RESULTS

Liquid-based culture to expedite the cultivation of particularly slow-growing mycobacteria is limited by the few options available for subsequent identification of the cultivated microorganisms. Thus, we first developed a mycobacterial identification qPCR test (MbID-qPCR) for use with MGIT-grown bacterial suspension to identify the most common mycobacterial pathogens with high clinical consequence: Mtb and MAI. The MbID-qPCR assay uses commercially prepared

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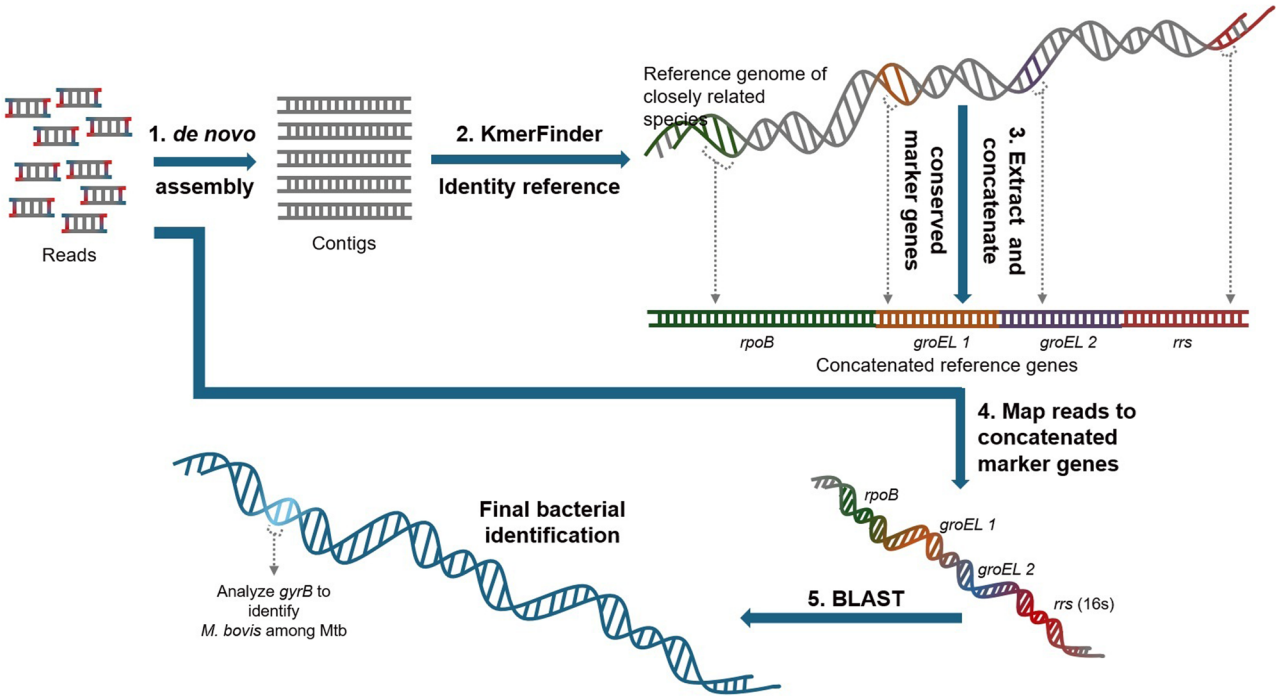


Figure 1 Analytical pipeline for identification of *Mycobacterium* species using next-generation sequencing. Reads are assembled de novo, and the contigs are used to identify a preliminary reference genome. Marker genes (mycobacterial *rpoB*, *groEL1*, *groEL2*, and *rrs* [16S ribosomal RNA]) are extracted from the preliminary reference and concatenated. Raw reads are then assembled against the concatenated mycobacterial marker genes to identify the most closely related organism. Further analysis using *gyrB* is performed on isolates identified as *Mycobacterium tuberculosis* to obtain species-level identification, primarily *Mycobacterium bovis*.

Table 1 Interpretive Guidelines for Mtb/MAI-NGS[a]

If	And	And	Then	If MTBc	Then
rpoB	***groEL1/groEL2***	***rrs* (16S rRNA)**	**Report**	***gyrB***	**Report**
≥99.0% query coverage AND ≥99.0% identity AND Single top hit	One or more top hits concordant with species-level identification using *rpoB*	One or more top hits agree with species-level identification using *rpoB*	Species-level identification (except MTBc)	≥99.0% query coverage AND ≥99.0% identity AND Single top hit	Species-level identification
≥99.0% query coverage AND ≥99.0% identity AND Multiple top hits	Single top hit AND concordant with species-level identification using *rpoB*	One or more top hits agree with species-level identification using *groEL1/groEL2*	Species-level identification (except MTBc)		Species-level identification
≥99.0% query coverage AND ≥94.0% identity AND Single top hit	One or more top hits concordant with species-level identification using *rpoB*	One or more top hits concordant with species-level identification using *rpoB*	Genus-level identification with *most closely related* species		
≥99.0% query coverage AND ≥94.0% identity AND Multiple top hits	Single top hit AND concordant with species-level identification using *rpoB*	One or more top hits agree with species-level identification using *groEL1/groEL2*	Genus-level identification with *most closely related* species		
≥99.0% query coverage AND ≥94.0% identity AND Multiple top hits	One or more top hits concordant with species-level identification using *rpoB*	One or more top hits concordant with species-level identification using *rpoB*	Genus-level identification		

Abbreviations: MAI, *Mycobacterium avium/Mycobacterium intracellulare*; Mtb, *Mycobacterium* tuberculosis; MTBc, Mycobacterium tuberculosis complex; NGS, next-generation sequencing; rRNA, ribosomal RNA.
[a]Four gene markers are used for the species-level identification of Mtb and nontuberculous mycobacteria. The *gyrB* gene is further analyzed on Mtb isolates for identification of closely related *Mycobacterium bovis*.

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open-system reagents (BioGX OSR) on the BD Max open system for rapid and automated identification of Mtb and/or MAI, along with a pan-mycobacterial target. The MbID-qPCR is specifically intended for use following primary microbial cultivation in MGITs and confirmation of acid-fastness by ZN staining. Accordingly, the pan-mycobacterial target is used as both amplification and sample QC in lieu of the manufacturer-provided *Drosophila* DNA internal control. Although rare and typically due to pipetting errors, ZN-positive samples with failed amplification of the pan-mycobacterial target are repeated or further investigated.

To validate the accuracy of the MbID-qPCR assay, MGITs were seeded with previously characterized (culture-confirmed) primary clinical samples (n = 18) or inoculated with pure isolates of Mtb and/or *M avium* or *M intracellulare* (n = 65). The MbID-qPCR assay successfully detected and identified 100% of the expected organism(s) cultivated in the MGIT (Table S1). The mean Ct values for *IS6110* (Mtb), rRNA ITS (MAI), and mycobacterial 16S rRNA (QC) were 10.0 (Ct range, 7.4-17.1; SD, 2.4), 14.3 (Ct range, 11.4-18.5; SD, 1.9), and 14.3 (Ct range, 11.1-24.6; SD, 2.56), respectively. For dual-positive samples seeded at equal concentrations, the mean difference between the Mtb and MAI Ct was minimal at 2.2 cycles with no indication of competitive inhibition.

The detection of Mtb and/or MAI at consistently low Ct values indicates that positively detected growth in MGIT is sufficient for the MbID-qPCR assay to reliably identify the target organisms. To assess the potential impact of the primary sample bacterial burden on the performance of the MbID-qPCR, we seeded MGITs with pure cultures of Mtb or MAI at inoculum concentrations ranging from 1 to 10000 colony-forming units/mL and incubated them until sufficient growth was obtained to trigger the growth indicator of the instrument. In all positively detected growth in MGIT, the MbID-qPCR assay accurately detected and identified Mtb and MAI, with all Ct values significantly below 25 cycles and no apparent dilution effect between the various inoculum concentrations (Table S2). This indicates that the sensitivity of the MbID-qPCR is independent of primary bacterial burden, and its performance is sufficient in MGIT-positive cultures.

In contrast to concerns for assay sensitivity, the high bacterial burden associated with positive MGIT tubes (as demonstrated by low Ct values above) raises concerns about potential false positivity due to carryover. A MGIT tube flagged positive by the instrument reportedly contains between 10^5 and 10^6 colony-forming units/mL.[16] Correspondingly, as a precaution to minimize the potential for specimen cross-contamination, we sought to establish an upper limit Ct for reportable identification. To determine an appropriate Ct cutoff for reporting organism identification, all Ct values of the positive samples used thus far for validation studies (accuracy and analytical sensitivity) were compiled, and the mean and standard deviation were calculated. The combined maximum Mtb/MAI Ct value was 23.0, with a mean (SD) internal control (*Mycobacterium* spp 16 S rRNA) Ct of 17.9 (5.63) (Figure S1). The calculated (combined) mean Ct + 2SD and Ct + 3SD were 24.78 cycles and 29.40 cycles, respectively. Based on the rounded-up Ct values, we established the upper Ct limit for reporting an identification result without requiring further confirmation to be 25 cycles for both Mtb and MAI; samples with Ct values >25 and ≤30 require repeat testing for confirmation, and samples with Ct values >30 for either target are considered negative for the corresponding target, except in the setting of dual positivity for both Mtb and MAI. In such circumstances, repeat testing with any detectable result (any Ct value) is considered true positive.

Next, to assess the precision of the assay, we tested MGITs that are either dual-positive or dual-negative controls in replicates. Triplicate testing in the same run demonstrated an intra-assay precision of 100%, with %CV <5% for all targets (Table S3). Using the same control samples, the assay also demonstrated an interassay precision of 100% among 12 independent runs, with similarly <5% CV. Additionally, using contrived samples seeded at the lowest detectable concentration of the MGIT system for Mtb or MAI as determined above (Table S2B), the assay also performed with 100% precision for each target, with %CV <10%.

Lastly for the MbID-qPCR assay, we sought to evaluate the analytical specificity by seeding MGITs with ATCC isolates of commonly encountered non-MAI NTM (n = 12). Among the rapid- and slow-growing mycobacterial species tested, the assay was able to accurately detect the pan-mycobacterial 16S rRNA with a mean Ct of 16.4 (Ct range, 13.1-22.6), with nondetectable Mtb *IS6110* or MAI rRNA ITS genes (Table S4). Collectively, these data demonstrate the acceptable performance of our MbID-qPCR as a rapid molecular identification tool for Mtb and/or MAI using MGIT-derived bacterial growth with 100% analytical sensitivity and specificity (Table 2).

To augment the dual-identification capacity of the MbID-qPCR, we also developed a whole-genome sequencing–based NGS assay using existing clinically validated wet-laboratory protocols for bacterial NGS—namely, automated genomic extraction and library preparation.[11,13,17,18] In contrast to our previously published analytical pipeline for 16S-based bacterial identification,[17] the new bioinformatic protocol for mycobacterial identification by NGS (MbID-NGS) uses a 4-marker approach with highly conserved mycobacterial genes: *rpoB*, *rrs* (16S rRNA), and *groEL*, which is commonly present in 2 distinct copies (*groEL*1 and *groEL*2) in genomes of *Mycobacterium* species (Table 1 and

Table 2 Performance of Mtb/MAI Multiplex MbID-qPCR[a]

Performance specifications		True, No.		False, No.		Sensitivity, %	Specificity, %
		Positive	Negative	Positive	Negative		
MbID-qPCR	Mtb	37	58	0	0	100	100
	MAI	49	46	0	0	100	100

Abbreviations: MAI, *Mycobacterium avium/Mycobacterium intracellulare*; MbID, *Mycobacterium tuberculosis* and *Mycobacterium avium/Mycobacterium intracellulare*; Mtb, *Mycobacterium tuberculosis*; qPCR, quantitative polymerase chain reaction.
[a]Analytical sensitivity and specificity were calculated using data from the accuracy and specificity studies.

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Table 3 Accuracy of MbID-NGS: MGITs Inoculated With Clinical Specimens (n = 28) or Pure Culture ATCC Isolates (n = 8) and Incubated Until Positive Detection by the BD BACTEC MGIT System[a]

Sample No.	Sample source	Culture result	Result by MbID-NGS	Concordant?
1	BAL	*M tuberculosis*	*M tuberculosis*	Yes
2	Expectorated sputum	*M avium* complex	*M avium* subsp *hominissuis*	Yes
3	Expectorated sputum	*M avium* complex	*M avium*	Yes
4	Expectorated sputum	*M avium* complex	*M intracellulare*	Yes
5	Expectorated sputum	*M gordonae*	*M gordonae*	Yes
6	Induced sputum	*M avium*	*M avium* subsp *hominissuis*	Yes
7	Stool	*M avium* complex	*M avium* subsp *hominissuis*	Yes
8	Expectorated sputum	*M avium* complex	*M avium* subsp *hominissuis*	Yes
9	Expectorated sputum	*M avium* complex	*M avium* subsp *hominissuis*	Yes
10	Expectorated sputum	*M avium* complex	*M avium* subsp *hominissuis*	Yes
11	Expectorated sputum	Probable *M avium* complex	*M intracellulare* subsp *chimaera*	Yes
12	ATCC 927	*M marinum*	*M marinum*	Yes
13	ATCC 12478	*M kansasii*	*M kansasii*	Yes
14	ATCC 19970	*M xenopi*	*M xenopi*	Yes
15	BAL	*M gordonae*	*M paragordonae*	No
16	ATCC 35799	*M szulgai*	*M szulgai*	Yes
17	Surgical swab	*M abscessus* subsp *abscessus*	*M abscessus* subsp *abscessus*	Yes
18	Expectorated sputum	*M abscessus* subsp *massiliense*	*M abscessus* subsp *massiliense*	Yes
19	Expectorated sputum	*M abscessus* subsp *abscessus*	*M abscessus* subsp *abscessus*	Yes
20	ATCC 29548	*M haemophilum*	*M haemophilum*	Yes
21	ATCC 19981	*M scrofulaceum*	*M scrofulaceum*	Yes
22	Induced sputum	*M tuberculosis* complex	*M tuberculosis*	Yes
23	ATCC 14470	*M gordonae*	*M gordonae*	Yes
24	ATCC 25177	*M tuberculosis*	*M tuberculosis*	Yes
25	Induced sputum	*M tuberculosis* complex	*M tuberculosis*	Yes
26	Pleural fluid	*M tuberculosis* complex	*M bovis*	Yes
27	Expectorated sputum	*M avium* complex	*M intracellulare* subsp *chimaera*	Yes
28	Surgical swab, hip, right	*M tuberculosis* complex	*M bovis*	Yes
29	Induced sputum	*M tuberculosis* complex	*M bovis*	Yes
30	Induced sputum	*M tuberculosis* complex	*M bovis*	Yes
31	Induced sputum	*M tuberculosis* complex	*M bovis*	Yes
32	Urine, clean catch	*M tuberculosis* complex	*M bovis*	Yes
33	BAL	*M tuberculosis* complex	*M bovis*	Yes
34	Surgical swab, aorta	*M tuberculosis* complex	*M bovis*	Yes
35	Urine, catheter	*M tuberculosis* complex	*M bovis*	Yes
36	Pleural fluid	*M tuberculosis* complex	*M bovis*	Yes

Abbreviations: BAL, bronchoalveolar lavage; *M*, *Mycobacterium*; MbID, *Mycobacterium tuberculosis* and *Mycobacterium avium/Mycobacterium intracellulare*; NGS, next-generation sequencing.
[a]Qualitative concordance for the accuracy and precision studies is inclusive of absolute concordance (organism identification to the same taxonomic level) and relative concordance (organism identification to a different taxonomic level but belonging to the same/related taxonomic group).

Figure 1). Additionally, the *gyrB* gene of isolates preliminarily identified as Mtb are further analyzed to specifically identify *M bovis* among related species.

Compared with the microbial identification obtained using conventional methods (eg, previously available molecular probes and/or MALDI-ToF from solid culture), MbID-NGS produced 97% (35/36) concordance (Table 3). In some cases, particularly among MAI, MbID-NGS was able to provide higher-resolution identification to the species or subspecies level than previously available methodologies (Table 3). In 1 instance (sample 15), MbID-NGS identified *Mycobacterium paragordonae*, while the reference method (bioMérieux VITEK MS MALDI-ToF) previously identified the highly related but distinct *Mycobacterium gordonae* species.

For isolates preliminarily identified as Mtb, the *gyrB* gene was further analyzed to distinguish from intrinsically pyrazinamide-resistant *M bovis* isolates. Identification of *M bovis* with 100% coverage and identity to a reference was corroborated by the presence of a pyrazinamide resistance-associated single-nucleotide polymorphism (pncA|p.His57Asp) in 100% of the *M bovis* isolates, which was absent in 100% of the *M tuberculosis* isolates (Table 4, Table S5).

As anticipated for culture-based sequencing, the total read count was consistently sufficient to meet the assay requirement of 0.5 million reads per sample for bioinformatic analyses, with a significantly higher mean total read count of 3988 076 reads (range, 0.91-9.24 million; SD, 3.54 million) to provide sufficient reference gene coverage (Table S5). This demonstrates that, similar to the performance of the MbID-qPCR above, positively detected growth in MGITs produces consistently sufficient bacterial load for mycobacterial identification by MbID-NGS.

Lastly, we sought to validate the precision of MbID-NGS using seeded MGITs positive for *M bovis* and *M intracellulare* subspecies *chimaera*. Consistent with the performance previously established by our laboratory for bacterial identification by NGS,[17] the intra- and interassay precision of MbID-NGS both produced 100% concordance among replicates in the same run and among different runs (Table 5). Altogether, the performance

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evaluation of the MbID-NGS produced results that were acceptable for clinical use, with >95% accuracy and precision.

Ultimately, the primary impetus behind the development of the molecular AFB assays was to provide a liquid culture–based solution for the identification of mycobacterial isolates that offer more rapid and clinically actionable results. To estimate the potential impact of implementing these laboratory-developed tests in a comprehensive molecular workflow, we compared its anticipated TAT against the standard of care using current laboratory scheduling and workflow (Figure 2). The clinical laboratory is a 5-day/week single-shift laboratory, in which positive cultures are batch-processed daily, with all subsequent positivity (later in the day or during weekend) being delayed until the following processing day. All observed growths (from solid and liquid broth cultures) are confirmed for acid-fastness using ZN staining before proceeding with identification testing. Prior to the molecular AFB assays, the clinical laboratory used MALDI-ToF for mycobacterial identification (MbID). The use of MALDI-ToF for MbID relies on sufficient growth of isolated colonies in solid culture media, which often requires serial subculture and typically results in an average of 5 to 14+ additional days from initial cultivation in MGIT broth. In contrast, direct-from-MGIT MbID-qPCR is performed on the same day it is confirmed as AFB (by ZN staining), which allows for the identification of Mtb and/or MAI with same-day reportable results. Consequently, for all other mycobacterial species, time-to-reportable identification can vary between 6 and 10 days depending on when MGIT is flagged positive relative to scheduled NGS processing (Thursday-Monday).

Altogether, with an average 12 to 16 days for mycobacterial growth in MGIT,[2] the molecular AFB workflow can produce actionable results from primary clinical samples in as little as 2 to 3 weeks, particularly for patients infected with Mtb and/or MAI, while similar results by conventional workflow may take as long as 5 weeks.

Table 4 Accuracy of MbID-NGS: Identification of *M bovis* Corroborated Through the Characteristic Mono-Resistance to Pyrazinamide Mediated by Single-Nucleotide Polymorphisms

Sample No.	Variant chromosome position, mutation	Position coverage, X	Variant frequency, %	Resistance
26	2289073, pncA_p. His57Asp	70	100.0	Pyrazinamide
28	2289073, pncA_p. His57Asp	129	100.0	Pyrazinamide
29	2289073, pncA_p. His57Asp	105	100.0	Pyrazinamide
30	2289073, pncA_p. His57Asp	70	100.0	Pyrazinamide
31	2289073, pncA_p. His57Asp	104	100.0	Pyrazinamide
32	2289073, pncA_p. His57Asp	107	100.0	Pyrazinamide
33	2289073, pncA_p. His57Asp	70	100.0	Pyrazinamide
34	2289073, pncA_p. His57Asp	59	98.3	Pyrazinamide
35	2289073, pncA_p. His57Asp	257	99.6	Pyrazinamide
36	2289073, pncA_p. His57Asp	70	100.0	Pyrazinamide

Abbreviations: MbID, *Mycobacterium tuberculosis* and *Mycobacterium avium/Mycobacterium intracellulare*.

DISCUSSION

Recent technological advancements in the clinical microbiology laboratory have revolutionized laboratory workup to significantly improve the diagnosis of infectious diseases. Here, we capitalized upon the growing applications of molecular technologies to address a long-standing limitation of AFB laboratory workup: the extended TAT of solid culture–dependent workflow. The combination of qPCR with NGS using precedent cultivation in liquid medium allows for expedited TAT with superior performance.

Quantitative PCR has been the mainstay of molecular infectious disease diagnostics for more than a decade. Because of the limited FDA-cleared options, significant efforts have been invested in laboratory-developed PCR-based assays for rapid identification of mycobacteria, both from primary samples and cultivated isolates, with

Table 5 Precision of MbID-NGS[a]

Precision		*M bovis*				*M intracellulare* subspecies *chimaera*				Acceptable?
		Total reads, No.	N50, No.	Contig count, No.	MbID-NGS	Total reads, No.	N50, No.	Contig count, No.	MbID-NGS	
Intra-assay	Replicate 1	1 365 834	89 654	877	*M bovis*	2 956 604	95 756	2594	*M intracellulare* ssp *chimaera*	Yes
	Replicate 2	3 675 490	134 725	460	*M bovis*	10 800 798	157 327	7293	*M intracellulare* ssp *chimaera*	Yes
	Replicate 3	2 825 140	103 706	611	*M bovis*	11 423 644	89 471	7379	*M intracellulare* ssp *chimaera*	Yes
Interassay	Run 1	2 825 140	103 706	611	*M bovis*	2 956 604	95 756	2594	*M intracellulare* ssp *chimaera*	Yes
	Run 2	2 236 946	80 712	517	*M bovis*	21 287 070	116 907	10 849	*M intracellulare* ssp *chimaera*	Yes
	Run 3	2 240 912	92 707	512	*M bovis*	2 985 564	167 795	2943	*M intracellulare* ssp *chimaera*	Yes

Abbreviations: *M*, *Mycobacterium*; MbID, *Mycobacterium tuberculosis* and *Mycobacterium avium/Mycobacterium intracellulare*; NGS, next-generation sequencing.
[a]*M bovis* and *M intracellulare* were sequenced in triplicate in the same run and in 3 separate runs to determine assay precision.

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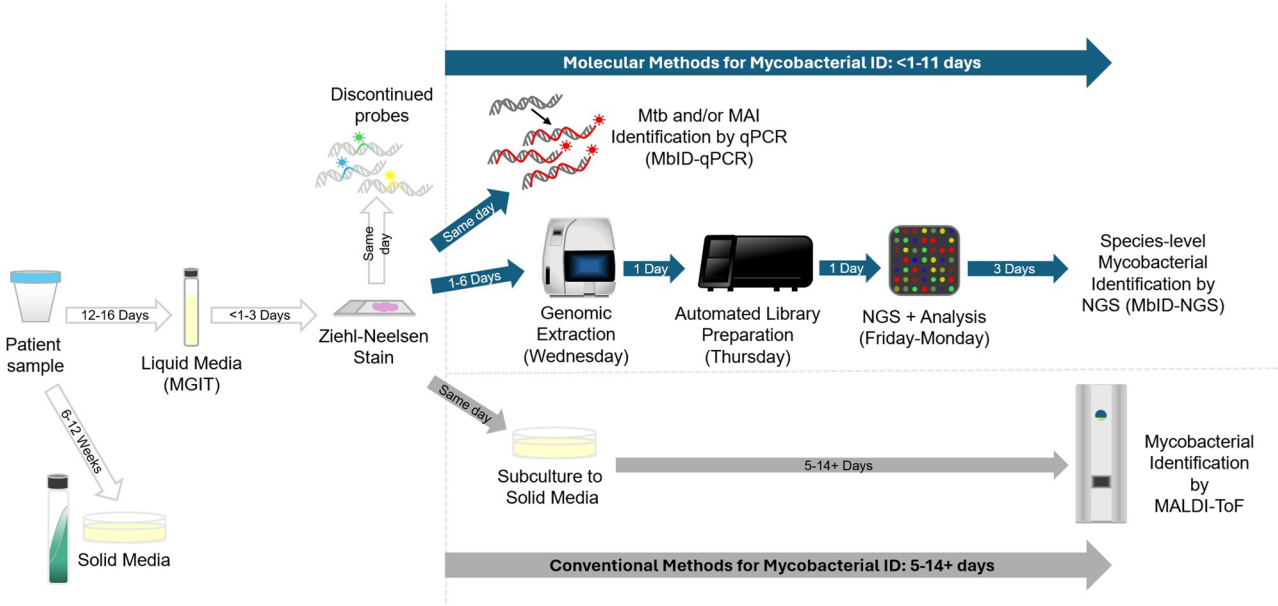


Figure 2 Integrated molecular acid-fast bacilli (AFB) identification workflow. Following confirmation of AFB using Ziehl-Neelsen (ZN) stain, liquid culture was previously tested using the discontinued fluorescent probes. Currently, liquid culture is tested by *Mycobacterium tuberculosis* and *Mycobacterium avium/Mycobacterium intracellulare* (MbID) quantitative polymerase chain reaction (qPCR) using BioGX OSR for the identification of *Mycobacterium tuberculosis* (Mtb) and/or *Mycobacterium avium/Mycobacterium intracellulare* (MAI). MbID–next-generation sequencing (NGS) is subsequently performed for non-Mtb/MAI mycobacterial species-level identification. Identification using molecular methods has an estimated turnaround time of <1 to 10 days (blue arrows) following ZN staining, while conventional methods (gray arrows) may take as long as 5 to 14+ days.

notable successes.[19,20] More recently, the capacity to develop assays for individual laboratory needs has been greatly augmented by the availability of open-system platforms that allow for customizable assay design while still maintaining the original ease of use. Indeed, the use of commercially prepared OSR in our laboratory-developed MbID-qPCR was able to produce optimal analytical performance using a sample-to-answer platform. Importantly, the use of the qPCR methodology addresses the limitations in analytical specificity previously noted by others when using alternative methodologies for direct-from-MGIT testing—namely, the use of MALDI-ToF on mixed-culture bacterial pellets.[9]

Although our MbID-qPCR assay demonstrated excellent performance characteristics with 100% sensitivity and specificity in our validation studies, a performance caveat should be noted about the use of a highly sensitive methodology on a sample source with high template burden: the risk for false positivity due to cross-contamination. While we address this risk by implementing an upper threshold for positive reporting, the risk remains and should be continuously monitored. Indeed, in our assessment of cycle threshold distribution (Figure S1), there is an apparent bimodal distribution of Ct values in positive samples during our validation, which we speculate is related to the time the MGIT was flagged positive on the instrument and the time it was subsequently tested. During validation, retrieval of a certain number of flagged positive MGITs was delayed for several days due to various reasons. In our data, this appears to yield a 5- to 10-cycle reduction in Mtb signal, correlating to a substantial increase in target template. While this was not explicitly investigated during our assay development, the effect of delayed processing should be monitored, particularly when delayed over the weekend or holiday, due to the risk of cross-contamination. Of note, a retrospective analysis of >1100 results since the implementation of MbID-qPCR showed no evidence of cross-contamination while maintaining 100% detection of Mtb and/or MAI from positive MGIT culture (ie, no compromise in assay sensitivity).

To complement the limited identification targets of qPCR, many have developed NGS pipelines to provide a larger database of identifiable mycobacterial species than even the more common alternative, MALDI-ToF, along with a higher resolution of identification.[21-23] Indeed, our MbID-NGS was able to identify 1 instance of *M paragordonae* that was previously identified by MALDI-ToF as *M gordonae*. *M paragordonae* is a recently characterized emerging pathogen that shares phenotypic properties with *M gordonae* as a slow-growing scotochromogenic *Mycobacterium*[24,25] but is not included in the current list of FDA 510(k) cleared organisms identifiable by the VITEK MS System. In the same token, however, it is worth noting that the validation of the robustness of our MbID-NGS test in its capacity to identify mycobacterial isolates was limited to the species available in the laboratory for testing. Thus, the performance of the assay in identifying less frequently isolated mycobacterial species should be continuously assessed upon clinical implementation.

The most comparable test to our MbID-NGS is the recently developed MGIT sequencing by Fukushima et al[23] using culture-positive MGIT broths sequenced on the MinION long-read next-generation sequencer. In a prospective study of 138 patients, MGIT sequencing performed with 99.1% accuracy to the species level and 84.5% to the subspecies level. In comparison, our retrospective validation of the

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MbID-NGS using the MiSeq platform demonstrated 100% accuracy and was able to identify, in particular, members of the MAI and *M abscessus* complex to the species and subspecies levels, potentially owing to the greater depth of coverage and higher sequencing accuracy that can be achieved using short-read sequencing as well as customized algorithms specifically designed and validated for each complex. Moreover, our MbID-NGS includes supplemental analysis to identify clinically significant *M bovis* strains among Mtb.

The greatest advantage of these LDTs, however, is achieved when fully integrated into an effective workflow, particularly along with our previously developed and validated assays. The clinical laboratory has a large menu of molecular LDTs that have been developed in response to arising needs, including microbial identification of atypical microorganisms and whole-genome sequencing for antimicrobial resistance prediction. While their individual utilities have been largely designed to augment existing laboratory workflows, the discontinuation of a standard-of-care test (Gen-Probe) for mycobacterial identification from liquid culture necessitated a reconsideration of the complete laboratory workup for cultivated AFB isolates. In response, the clinical laboratory sought to implement an integrated molecular AFB workflow using discreetly sufficient yet technically compatible assays that collectively produce superior performance.

There are several limitations in this study. In the MbID-qPCR assay, other species within the MAI complex, including *Mycobacterium arosiense*, *Mycobacterium colombiense*, *Mycobacterium marseillense*, *Mycobacterium timonense*, *Mycobacterium vulneris*, and *Mycobacterium yongonense*, were not included in the validation because of their rarity. Therefore, the test performance on these species is unknown. In the MbID-NGS test, we did not validate samples with mixed species. In such cases, additional metagenomics software, such as Sylph (https://github.com/bluenote-1577/sylph), is required. Importantly, even if a rapid identification of the mycobacteria is acquired with these molecular methods, it is still necessary to culture positive MGIT broths to obtain isolates for drug susceptibility testing and possibly to sort out mixed species.

The MbID-qPCR and MbID-NGS as a direct replacement to the discontinued probe-based mycobacterial ID test were developed using existing testing platforms in the laboratory. In particular, MbID-NGS utilizes previously published laboratory tools for the small-scale performance of NGS,[11] which allows it to be combined with other compatible assays (eg, NGS for bacterial and fungal identification, mycobacterial resistance prediction, *Aspergillus fumigatus* resistance prediction, and/or cytomegalovirus resistance prediction) to maximize sequencing efficiency and minimize laboratory costs.[12-14,17,18] More importantly, the 2 LDTs were able to close the utility gap of our previously published NGS-based assay for drug resistance prediction for Mtb and *M abscessus*.[13,18] The design of the new molecular AFB workflow seamlessly merges mycobacterial identification with drug resistance prediction for select species to produce rapidly actionable clinical results.

It remains notable, however, that despite the growing applications of laboratory-developed molecular assays for addressing specific laboratory needs, accessibility to the required expertise and the high costs associated with LDTs still pose significant hurdles to their broad implementation. In particular, the initial barrier for the adoption of NGS within a laboratory may be cost-prohibitive, and achieving cost-efficiency often requires sample batching of multiple compatible assays. The integrated molecular AFB workflow proposed here may be used as a model for the utility of various LDTs to achieve not only superior analytical performance but also an effective workflow that most efficiently capitalizes on existing technologies.

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Author contributions

J.R.C. and S.Y. performed the data analysis and wrote the manuscript, with input from all authors. J.R.C., S.K., B.v.B., and S.Y. designed the comprehensive workflow; S.K. led the validation of the MbID-qPCR, and B.v.B. led the validation of the MbID-NGS with support from UCLA MMPG Lab. O.G. and S.Y. supervised the development and validation of the assays.

Supplementary material

Supplementary material is available at *American Journal of Clinical Pathology* online.

Conflicts of interest

None declared.

Funding

Not applicable.

Data availability

The bacterial genomic sequences are available upon request.

Ethics statement

This study has been reviewed and approved by the UCLA Office of the Human Research Protection Program and received an institutional review board exemption.

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