Description, validation, and review of a decade of experience with a laboratory-developed PCR test for detection of *Mycobacterium tuberculosis* complex in pulmonary and extrapolumary specimens

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ABSTRACT

Rapid detection of *Mycobacterium tuberculosis* complex directly from clinical specimens is critical for patient care. Mycobacterial culture requires days to weeks for results and therefore many laboratories employ rapid molecular methods for the diagnosis of tuberculosis. There are two FDA-cleared molecular assays for the detection of *M. tuberculosis* complex in the United States and both are cleared for testing of respiratory specimens only. The detection of *M. tuberculosis* complex in extrapolumary specimens is often done using laboratory-developed PCR methods. In this work, the verification and subsequent validation of test performance over a decade is detailed for a laboratory-developed PCR assay (MTBRP) that detects *M. tuberculosis* complex from respiratory and non-respiratory specimens. The assay also provides information about potential isoniazid resistance. The performance of the MTBRP PCR assay was compared to the Cepheid Xpert MTB/RIF assay in acid-fast smear positive and smear negative specimens and mycobacterial culture for acid-fast smear positive specimens. The MTBRP assay demonstrated 99% correlation with the Xpert MTB/RIF assay using 499 respiratory specimens. The performance of the MTBRP PCR assay compared to mycobacterial culture for 867 AFB smear positive respiratory and non-respiratory specimens demonstrated a sensitivity of 100% and a specificity of 99.1%. This work provides longitudinal evidence using real-world clinical laboratory conditions and specimens to demonstrate that laboratory-developed PCR assays such as the MTBRP can provide a rapid and sensitive method for detection of pulmonary and extra-pulmonary tuberculosis from a wide-variety of smear positive species sources.

1. Introduction

The direct detection of *Mycobacterium tuberculosis* complex (Mtbc) from clinical specimens is critical for patient care and public health efforts. In 2009, a Laboratory Developed Test (LDT) designated the *Mycobacterium tuberculosis* complex rapid PCR (MTBRP) test was verified and implemented at the Mayo Clinic. The MTBRP uses real-time PCR for the direct detection of Mtbc from a variety of specimen types including respiratory and non-respiratory specimens. The inclusion of non-respiratory specimen types was intended to assist with the direct detection of extrapolumary tuberculosis. The purpose of this report is to describe the MTBRP PCR assay and to validate its performance over a decade of use. MTBRP assay results were compared with mycobacterial culture in MGIT broth plus Middlebrook agar and the FDA-cleared Cepheid GeneXpert® MTB/RIF PCR assay [1]. The GeneXpert® MTB/RIF assay is a cartridge-based, closed extraction and PCR test that has excellent sensitivity and specificity for the detection of Mtbc. The turnaround time from sample to answer is approximately 2 h and the test is simple for clinical laboratories to perform. A newer version, the GeneXpert MTB/RIF Ultra assay, has improved sensitivity over the original assay but it is not yet cleared by the FDA and it is still unavailable for use in U.S. clinical diagnostic laboratories. A limitation of the GeneXpert® MTB/RIF assay is that it is cleared for use only with sputum or concentrated sputum sediment. Other specimen sources such as...
bronchial washings and CSF can be verified “off-label” but this can be a challenge for many laboratories [2–4].

While the MTBRP was developed by a single reference laboratory, the information is potentially of wider interest because the primers and probes are commercially available which allows other labs to adopt and implement the assay if desired. This report also demonstrates the value of an LDT for testing of non-respiratory specimen types for which FDA-cleared assays may not be available. Finally, as the diagnostic laboratory community faces the possibility of expanded regulatory oversight of laboratory developed tests in the future, this report may also provide one approach to validation of an LDT assay for clinical use.

2. Materials and methods

2.1. Isolates and specimens

Type strains of Mycobacterium species were obtained from the Deutsche Sammlung von Mikroorganismen (DSMZ; Braunschweig, Germany) and the American Type Culture Collection (ATCC; Manassas, VA). Clinical specimens were collected from patients with suspected tuberculosis and excess specimen was utilized for verification of the MTBRP assay. Specimens included both respiratory sources (sputum, induced sputum, bronchial washings, bronchoalveolar (BAL) fluid, pleural fluid, tracheal secretions) and non-respiratory sources (sterile body fluids such as pleural fluid, peritoneal fluid, CSF, fresh and formalin-fixed, paraffin-embedded tissue (FFPE), urine, stool). An Institutional Review Board of the Mayo Clinic approved the use of all specimens included in this study.

2.2. Specimen processing, AFB staining, and mycobacterial culture

Specimens from non-sterile sites (respiratory specimens, stool) were digested and decontaminated using standard N-acetyl-cysteine (NALC) with 2 % NaOH treatment [5]. Specimens from sterile sources were inoculated to culture medium directly without pre-processing. All specimens were inoculated into BACTEC™ MGIT™ (Becton Dickinson, Franklin Lakes, NJ) liquid medium or VersaTREK liquid medium (ThermoFisher, Waltham, MA) with incubation for 42 days and onto Middlebrook 7H11/S7H11 solid medium biplates which were incubated at 35°C and 1400 rpm for 15 min. The liquefied specimens were inoculated to culture medium directly without pre-processing. All specimens were liquefied prior to extraction by adding 500 µL of 0.1 mm silica glass beads and 2.4-mm zirconia beads on a Disruptor Genie (Biospec Products, Bartlesville, OK) or 500 µL partial 16S rRNA gene sequencing as previously described [6].

2.3. Specimen preparation and extraction for the MTBRP PCR test

M. tuberculosis complex culture isolates were inactivated and lysed by placing a 1 µL inoculating loop-full of an isolated colony from a culture plate into a 2.0 mL tube containing 50 µL of 0.1 mm silica glass beads and 100 µL of 2.4 mm Zirconia beads (Biospec Products, Bartlesville, OK) and 500 µL of double sterilized water. The tubes were placed on a 95–100 °C heat block for 5 min and subsequently lysed on a Disruptor Genie (Scientific Industries, Bohemia, NY) for 2 min. Tubes were then centrifuged briefly at 1,000 × g to 5,000 × g to pellet the sample.

Respiratory specimens, sterile body fluids and bone marrow specimens were liquefied prior to extraction by adding 500 µL of specimen to 100 µL Proteinase K (Roche Applied Sciences, Indianapolis, IN) followed by incubation and mixing using a thermomixer (Eppendorf, Hauppauge, NY) at 55 °C and 1400 rpm for 15 min. The liquefied specimens were then heated at 95–100 °C and mechanically lysed using a mixture of 0.1-mm silica glass beads and 2.4-mm zirconia beads on a Disruptor Genie (Biospec Products, Bartlesville, OK) for 2 min.

Fresh tissue was processed by placing a small piece of tissue (approximately 0.5 cm²) into a 2.0-mL tube containing 0.1-mm silica glass beads and 2.4-mm zirconia beads, 450 µL of 1X Tris-EDTA buffer (Sigma Aldrich, St. Louis, MO) and 100 µL of Proteinase K. The samples were vortexed briefly and then placed on a thermomixer for at least 4 h at 55 °C with a mixing speed of 500 rpm. The samples were then heated at 95–100 °C for 5 min and further lysed on a Disruptor Genie for 2 min.

FFPE tissue sections were processed by placing one 50 µm section (e.g., from large tissue sections) or two 50 µm sections (e.g., from punch biopsies) into a 2.0 mL tube and processed as previously described [7]. Briefly, the sections were placed into a 1.5 mL tube containing 500 µL xylene (Sigma Aldrich) and incubated for 5 min at room temperature. The tubes were then centrifuged for 30 s at 20,800 × g and the xylene was removed with a fine-tip disposable pipette. A second incubation in 500 µL xylene at room temperature, followed by centrifugation and removal of xylene was performed. Subsequently, 500 µL of 95 % ethanol was added to the tubes, the samples were gently vortexed, and then incubated for 5 min at room temperature. The samples were then centrifuged for 3 min at 20,800 × g and the alcohol was removed using a fine-tip disposable pipette. Following removal of the alcohol, 400 µL of 1X Tris-EDTA, 100 µL Proteinase K and 50 µL 10 % SDS were added to the tube. The samples were then vortexed and placed on a Thermomixer (Eppendorf) overnight at 55 °C with a mixing speed of 500 rpm. The following day, 200 µL of the digested specimen was extracted on a MagNApure extraction platform (Roche) with a final elution volume of 100 µL.

Formed stool was processed by dipping a sterile, cotton-tipped swab into different locations of the stool specimen and then swirling the swab into a 2.0 mL bead tube containing 1 mL double sterilized water, 0.1 mm silica glass beads and 2.4 mm Zirconia beads (Biospec Products). Samples were placed on a 95–100 °C heat block for 5 min and lysed on the Disruptor Genie for 2 min.

Urine and CSF specimens were processed by adding 200 µL to a 2.0 mL tube and subsequently heating at 95–100 °C for 5 min. Following the processing above, 200 µL of the processed specimen was extracted on the MagNA Pure LC instrument (Roche Applied Sciences, Indianapolis, IN) using the LC Total Nucleic Acid Isolation kit (Roche).

2.4. MTBRP PCR assay

The target for the MTBRP assay is katG, the catalase-peroxidase gene of Mtb. A 203-bp portion of katG is amplified and detected using sequence-specific primers and fluorescence resonance energy transfer (FRET) probes. The MTBRP assay detects the members of the Mtb complex. The assay also provides information about potential isoniazid (INH) resistance mediated by the katG(S315T) mutation which is reported to be responsible for ~ 60–70 % of INH-resistant cases of Mtb [8]. The katG (S315T) mutation causes a mismatch under the FRET probes which results in a shift of the melting curve peak, when present (Fig. 1).

The MTBRP PCR is performed on the LightCycler 2.0 instrument (Roche Applied Sciences, Indianapolis, IN). Primers and probes were synthesized by TIB MOLBIOL (Table 1, available from TIB as Primer-Set [535], Adelphia, NJ). The PCR assay was performed using the LC FastStart DNA Master hybridization probe kit (Roche Applied Sciences, Indianapolis, IN), with each reaction containing 3 mM MgCl₂, 1X Roche LC FastStart mix, 0.5 µM of each primer, 0.2 µM of the fluorescein labeled probe and 0.4 µM of the red 640 labeled probe. The total volume per reaction was 20 µL (15 µl master mix plus 5 µL extracted nucleic acid). PCR amplification with real-time detection was performed using the following cycling parameters: 1 template denaturing cycle at 95 °C for 10 min, followed by 45 amplification cycles at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s. Following amplification, melting curve analysis was performed by measuring the fluorescence signal during the following cycling parameters: 95 °C for 0 s, 59 °C for 20 s, 45 °C for 20 s with a 0.2 °C/s transition, and 85 °C for 0 s with a 0.2 °C/s transition.
Table 1

| Name | Sequence 5’ to 3’ |
|------|------------------|
| TBC1 (forward) primer | 5’-GGG TCA CAC TTT CGT TAA |
| TBC2 (reverse) primer | 5’-GGA TCT CGA GGA AAT GTG TGG |
| TBC3 probe | 5’-AAG CCG TAA GGA CGG CAT C-FL |
| TBC4 probe | 5’-RED640-CCA GCG GCA TCG AGG T-PH |
| Lambda Internal Control | 5’-ATG CCA CGT AAG CGA AAA-FL |
| Lambda R primer | 5’-GCA TAA ACG AAG CAG TCG AGT |
| Lambda FL (probe 1) | 5’-GGT GCC GTC CAC TTC CGG AAT AAC-FL |
| Lambda LC (probe 2) | 5’-LC 670-GGG ATA TTT TTG ATG TGA CGG AAG CG-PH |

FL = fluorescein dye. RED640 = RED640 fluorescent dye. LC 670 = RED670 fluorescent dye. PH = phosphate.

The negative control consists of E. coli ATCC #25911 spiked into 50 % S.T.A.R. buffer (Roche Diagnostics catalog #03335208001). S.T.A.R. buffer is a general use buffer for stabilization, transport, and recovery of nucleic acids in stool specimens and is compatible with extraction on the MagNA Pure LC instrument. A negative control is included with this assay through both the MagNA Pure nucleic acid extraction and LightCycler PCR amplification processes.

A plasmid clone of the M. tuberculosis complex target sequence (katG gene) is used as a positive control and can be purchased from TIB MOLBIOL (#30-8412-01). The plasmid clone is maintained at 100 targets/µl in 1X Tris-EDTA buffer and is included with this assay through both the MagNA Pure nucleic acid extraction and LightCycler PCR amplification processes.

A plasmid clone of the M. tuberculosis complex target sequence with resistance to INH designated the RTB control (TIB MOLBIOL #30-8412-02), is also used as a positive control to confirm that a melting temperature shift will be detected due to mutation in the katG(S315T) loci. This isoniazid-resistance control (RTB) is maintained at 100 targets/µl in 50 % S.T.A.R. buffer and is included with each batch of samples in the LightCycler PCR amplification process.

An internal control (lambda bacteriophage DNA) is included in assays of FFPE to assess for inhibition in that specimen matrix. A retrospective analysis of 386,706 specimens from the Mayo Clinic Microbiology Laboratories showed that inhibition rates were <1.0 % for specimen matrices other than formalin-fixed, paraffin embedded (FFPE) tissue sections [9].

The PCR results are qualitative and a specimen is interpreted as positive for the presence of Mtb DNA when the Tm of the specimen is 64 ± 2.5 °C and the fluorescent cut-off value is >0.020 units (Fig. 1). A specimen is interpreted as positive for the presence of Mtb DNA with probable INH resistance when the Tm of the specimen is 58 ± 2.5 °C and the fluorescent cut-off value is >0.020 units. A specimen is interpreted as negative when no melting peak is seen or when a Tm fluorescent cut-off value of <0.020 is detected for a melt peak.

2.5. Analytical sensitivity (Limit of Detection)

The MTBRP analytical sensitivity was established using serial dilutions of the wild-type M. tuberculosis positive control plasmid in 1X Tris/EDTA buffer. 5 µl of each dilution ranging from 10^6 to 10^8 targets/µl were tested 5 times each and the analytical sensitivity was defined as the lowest concentration to be detected 5 out of 5 times. The analytical sensitivity in specimen matrix was confirmed by spiking 30 analyte-negative specimens (e.g., respiratory specimens) with genomic Mtb DNA at a level 1 log above the LoD as established by the TE buffer experiments followed by extraction and PCR. The specimen matrices tested were respiratory specimens (e.g., sputum, tracheal secretions, bronchoalveolar lavage (BAL) fluid), CSF, fresh tissue (e.g., organ tissue, respiratory), FFPE tissue, sterile body fluids (e.g. pleural, peritoneal), stool, and urine.

2.6. Inclusivity

The Mycobacterium tuberculosis complex species were tested to demonstrate that the MTBRP assay can detect the species within the Mtb that are generally considered to cause disease in humans (Table 2). The isolates were tested at a concentration of 10^3-10^6 CFU/mL following the extraction and PCR protocols described previously.

2.7. Analytical specificity

The analytical specificity of the assay was determined in silico by performing a BLAST search of the primer and probe sequences on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). In addition, an extensive panel of genomic DNA from potentially cross-reacting organisms, including bacteria, non-tuberculous mycobacteria, and viruses were tested to verify the specificity of the assay (Supplemental Table S1). Amplification and sequencing of the 500 bp 16S rRNA target for bacteria was utilized to confirm the presence of amplifiable nucleic acid in the specificity panel. When available, specific viral PCR assays were used to demonstrate the integrity of viral nucleic acid [10-12].

Table 2

| Analytical Reactivity (Inclusivity) Panel. |
|------------------------------------------|
| M. tuberculosis complex Isolates | Species | Source |
|------------------------------------------|---------|--------|
| M. tuberculosis | ATCC 35837 |
| M. tuberculosis | ATCC 25618 |
| M. tuberculosis | ATCC 25177 |
| M. tuberculosis | ATCC 22794 |
| M. tuberculosis | ATCC 35825 |
| M. tuberculosis | ATCC 35828 |
| M. tuberculosis | ATCC 35822 |
| M. bovis | ATCC 19210 |
| M. bovis BCG | ATCC 35737 |
| M. africanum | ATCC 25420 |
| M. microti | ATCC 19422 |

ATCC = American Type Culture Collection.
2.8. Precision studies

Intra-day assay precision of the MTBRP assay was evaluated using 3 clinical specimens (2 respiratory and 1 tissue) previously determined to be positive for wild-type M. tuberculosis and 2 clinical (respiratory) specimens positive for INH-resistant M. tuberculosis. Each specimen was tested in triplicate in the same LightCycler run. A positive and a negative control were included through extraction and PCR steps. The crossing point (Cp) and melting temperature (Tm) of each positive sample were determined and used to calculate the mean, standard deviation and % CV for each sample.

The inter-day assay precision was determined using a positive wild-type M. tuberculosis positive plasmid control extracted on 2 MagNA Pure instruments and tested using 2 LightCycler instruments operated by 7 technologists over 20 days. The inter-day assay precision of the INH-resistant M. tuberculosis peak at a Tm = 58.0 ± 2.5 °C was also determined. Plasmid controls were used to test inter-day precision because clinical specimen volumes were insufficient to permit testing over 20 days. The crossing point and melting temperature of each positive control was determined and used to calculate the mean, standard deviation and % CV for each sample.

2.9. MTBRP PCR identification of M. tuberculosis complex from culture isolates

Twenty-six Mtb culture isolates and 92 nontuberculous mycobacterial (NTM) culture isolates were used to evaluate the ability of the MTBRP PCR assay to correctly differentiate Mtb from NTM and to evaluate the detection of INH resistance mediated by the katG(S315T) mutation. The isolates were cultured from clinical specimens using standard techniques and were identified using the nucleic acid hybridization probe specific for Mtb (Hologic AccuProbe). NTM isolates were identified using either a hybridization probe specific for M. avium complex or M. gordonae, or sequencing of a 500 bp region of the 16S rDNA gene as previously described [6]. Following identification, phenotypic broth susceptibility testing of Mtb isolates was performed using the VersaTREK® platform (Thermo Fisher, Waltham, MA).

2.10. Comparison of the MTBRP PCR assay with mycobacterial culture

The performance of the MTBRP assay was established by reviewing data from clinical specimens submitted from patients whom physicians considered tuberculosis in their differential diagnosis. In order to determine the sensitivity and specificity of the MTBRP test versus mycobacterial culture data was collected for all 867 AFB smear positive specimens from a total of 10,671 clinical specimens where the MTBRP PCR assay, standard mycobacterial culture and acid-fast smear were ordered on the same specimen from November 2011 through January 2020.

2.11. Comparison of the MTBRP PCR assay with the Cepheid GeneXpert MTB/RIF assay

The correlation of the MTBRP assay with the Cepheid GeneXpert MTB/RIF assay was established using 499 respiratory specimens (acid-fast smear positive and smear negative) submitted consecutively to the laboratory over a 1 year period with sufficient volume to permit performance of both tests. The respiratory specimens were comprised of 348 sputum samples, 101 BAL samples, 32 bronchial washings, and 18 tracheal secretions. The specimens were split and processed in parallel for the two molecular assays. The Xpert MTB/RIF assay was performed according to the manufacturer’s package insert with 0.5 mL of NALC/2% NaOH decontaminated respiratory specimen processed by mixing with 1.5 mL of sample reagent, incubating for 15 min and then adding the mixture to the Xpert 1.0 cartridge (Cepheid). The MTB/RIF cartridge was inserted into the GeneXpert XVI instrument, and measurement and analysis were conducted and reported by the GeneXpert Dx software (version 4.4a).

2.12. Statistics

Categorical agreement between methods was determined using Cohen’s kappa (κ) coefficient [13].

3. Results

Analytical sensitivity. The limit of detection for the assay using the positive control plasmid spiked into Tris buffer was established at 10 copies of katG target/µL. The limit of detection was also found to be 10 copies of katG target/µL when spiked into a variety of specimen matrices (CSF, BAL fluid, sputum, induced sputum, sterile body fluids, urine, and fresh lung tissue). The recovery was ≥90 % for all specimen matrices spiked at 10 copies/µL with the exception of FFPE tissue where the recovery was lower at 63 % (Supplemental Table S2).

Inclusivity. A panel consisting of eleven isolates comprising five species within the Mtb complex was tested and all were positive by the MTBRP assay (Table 2). Several of the less common species within the complex, M. canetti, M. caprae, and M. pinnipedii, were not tested by the MTBRP assay because they were not able to be obtained. These three species are predicted to be detected by the MTBRP assay on the basis of in silico analysis of the target sequence.

Analytical Specificity. An in silico BLAST search of the primer, probe, and target sequences for the Mtb katG gene did not yield any highly similar sequences except for members of the Mtb, as predicted. In addition, a nucleic acid specificity panel consisting of genomic DNA from 94 NTMs, 66 other bacteria and 16 virus strains were tested in vitro using the MTBRP PCR assay and no cross-reaction was detected (Supplemental Table S1).

3.1. Precision

Intra-day assay precision was determined by testing 5 specimens in triplicate on the same day. Three specimens (2 respiratory and 1 lung tissue) contained the wild-type katG target and two specimens (2 respiratory) had the katG(S315T) target mutation that confers INH resistance. Crossing point cycles and melting curve temperatures are provided in Supplemental Table S3. The coefficient of variation for all specimens across 3 replicates was < 1.2 % for both the crossing points and melting temperatures.

Inter-day assay precision was determined by testing the positive control plasmid extracted on two MagNA Pure instruments and tested using 2 LightCycler instruments over 20 days. The testing was performed by 7 different clinical laboratory technologists. Crossing point cycles and melting curve temperatures are provided on Supplemental Table S4. The coefficient of variation for both the wild-type and INH-resistant positive control plasmids across the 20 days was <1.0 %.

3.2. MTBRP assay performance using culture isolates

As expected, 26/26 (100 %) of the Mtb culture isolates were positive by the MTBRP assay and 92/92 (100 %) of NTM isolates were negative by the PCR assay (Supplemental Table S5).

3.3. Comparison of MTBRP assay with the Cepheid GeneXpert MTB/RIF assay

A total of 499 respiratory specimens were tested in parallel using the MTBRP assay and the Cepheid GeneXpert MTB/RIF Assay (Table 3). Of those, 27 specimens (5 %) were positive for M. tuberculosis complex by both assays and 468 specimens (94 %) were negative by both methods. There were 2 GeneXpert MTB/RIF-positive, MTB-RPF-negative specimens and 2 GeneXpert MTB/RIF-negative, MTB-RPF-positive specimens. The
correlation of the MTBRP assay with the Cepheid GeneXpert MTB/RIF Assay was 99.2% (kappa coefficient = 0.93, positive % agreement = 93.1%, negative predictive agreement = 99.6%).

3.4. MTBRP PCR performance from clinical specimens compared with mycobacterial culture

For 867 acid-fast smear positive specimens, the PCR assay had a sensitivity of 100% and a specificity of 99.1% compared with mycobacterial culture (Table 4 and Supplemental Table S6).

3.5. Evaluation of the MTBRP assay melting curve temperature for prediction of INH resistance

The melting curve temperature of the MTBRP assay was analyzed for 204 Mycobacterium tuberculosis complex isolates where phenotypic broth critical concentration susceptibility testing was also performed in order to determine if the melting curve Tm predicted phenotypic resistance to INH (Table 5). 178 isolates produced a melt peak at 64.5 ± 2.5 °C with the MTBRP assay. Of these 178 isolates, 159 were susceptible to INH at 0.1 µg/ml, 10 were susceptible to a higher concentration of INH (0.4 µg/ml) but resistant at the lower 0.1 µg/ml concentration and 9 were resistant to INH at both 0.1 µg/ml and 0.4 µg/ml. Twenty-six isolates produced a melting peak at 58 ± 2.5 °C and 100% of these isolates were resistant to INH at both low (0.1 µg/ml) and high (0.4 µg/ml) concentrations. Sequencing of all 26 isolates with a melting peak at 58 ± 2.5 °C indicated that the katG (S315T) variant was present. Sanger sequencing of the 19 isolates that displayed phenotypic isoniazid resistance at either 0.1 µg/ml or 0.4 µg/ml but which had a melt peak at a Tm = 64.5 ± 2.5 °C indicated that the wild-type katG (S315I) codon was present suggesting that isoniazid resistance in these isolates was mediated by a target other than katG(S315T).

4. Discussion

The use of LDT PCR assays for the detection of Mtbc has been reported previously by numerous groups but, to our knowledge, none have been followed over a long period of time in order to collect actual performance data in a clinical diagnostic laboratory [14–26]. Some of the early LDTs were introduced as an alternative to the labor intensive and open PCR tests. Since that time, the Cepheid Xpert MTB/RIF PCR assay has been FDA-cleared for use in the U.S. and it offers the advantage of being simple to perform and is a closed system that reduces the risk of false positive results due to contamination events. However, the Xpert MTB/RIF assay is limited in the specimen types that can be tested with the FDA-cleared specimen types being sputum and concentrated sputum sediment. Some laboratories have verified the Xpert MTB/RIF assay for off-label use of selected specimen types such as CSF and BAL fluid but its use on other specimen types has been limited. The MTBRP LDT PCR assay was developed and verified prior to FDA-clearance of the Xpert MTB/RIF assay in the U.S. It can be used to test respiratory specimens as well as non-respiratory specimens for cases of suspected extrapulmonary tuberculosis. The MTBRP PCR assay utilizes a real-time PCR system which is closed after nucleic acid extraction so the risk of false positives results due to cross-contamination or human error is reduced compared to conventional PCR systems.

Characterization of the MTBRP PCR assay performance included determination of the analytical sensitivity and specificity of the assay versus standard mycobacterial culture and the correlation of the MTBRP assay with the FDA-cleared Xpert MTB/RIF assay. The primers, probe, and controls can be purchased from a commercial entity which enables other laboratories to use the assay if desired following their own verification of performance.

The analytical sensitivity of the MTBRP assay was established at 10 katG targets/µl in Tris buffer and sensitivity in a variety of specimen matrices including BAL fluid, CSF, lung tissue, sputum, sterile body fluids, and urine ranged from 90 to 100%. As expected, the sensitivity was lower in FFPE tissue blocks (63%) so a positive MTBRP result would indicate the presence of Mtb nucleic acid but a negative PCR result from FFPE tissue blocks would not rule out the possibility of tuberculosis. The analytical specificity of the MTBRP assay is also excellent with no false-positive results from NTMs or any other microorganisms tested.

Intra-day and inter-day precision tests indicated that the MTBRP assay produces highly reproducible cycle crossing points (Cp) and melting curve temperatures (Tm) across multiple runs per day, across multiple days and multiple instruments and when performed by multiple operators. The % coefficient of variation across all precision tests was ≤1.15%.

Using an in silico analysis, all members of the M. tuberculosis complex would be predicted to be detected by the MTBRP assay. Confirmation of this was accomplished through the Inclusivity Panel which demonstrated detection of 7 strains of M. tuberculosis and 1 strain each of M. bovis, M. bovis BCG, M. africanum and M. microti. While in clinical use, the MTBRP assay also detected a strain of M. canetti in a specimen from a patient from Djibouti, Africa. The identity of the M. canetti strain was determined using a real-time PCR assay that can distinguish between the various members of the Mtbc [27] and by whole genome sequencing of the isolate after growth in culture.

The correlation of the MTBRP assay with the Cepheid GeneXpert MTB/RIF assay for AFB smear positive specimens was high at 99.2% (n = 499 specimens).

Results from the MTBRP PCR assay performed directly from clinical specimens were compared with results from standard mycobacterial culture over approximately a decade. The results in Supplemental Table S6 indicate that the MTBRP has excellent sensitivity (100%) and specificity (99.1%) across specimen types compared with culture for acid-fast smear positive specimens.

The MTBRP assay can be used to provide limited information about INH resistance through examination of the melting curve peak temperature. A melting curve peak at 58.0 ± 2.5 °C, correlated with phenotypic
resistance to INH at both low (0.1μg/ml) and high (0.4μg/ml) concentrations determined on the VersaTREK platform. The interpretation of the melting peak at 64.5 ± 2.5 °C produced mixed results with most isolates (n = 159) found to be susceptible to INH at both low and high concentrations, but a small subset of isolates (n = 10) were resistant at 0.1 μg/ml and susceptible at 0.4 μg/ml and another subset (n = 9) were resistant at both concentrations. Therefore the presence of a peak at 58.0 ± 2.5 °C strongly correlated with INH resistance mediated by the well-known katG(S315T) mutation. The result is reported as “probable INH resistance” when this peak is present. Conversely, when the 64.5 ± 2.5 °C peak is present, no statement can be made about INH susceptibility or resistance. Phenotypic resistance to INH is known to be mediated through a number of genes (katG, inhA, ahpC, and fabG1) so phenotypic resistance in isolates that have a melting peak at 64.5 ± 2.5 °C is presumably mediated through one of the genes other than katG, the target for the MTBRP assay.

In summary, the performance of the laboratory-developed MTBRP PCR assay strongly correlates with results obtained using the FDA-cleared Cepheid Xpert MTB/RIF molecular assay in respiratory specimens. The MTBRP PCR assay also has high sensitivity and specificity compared with mycobacterial culture in acid-fast smear positive specimens from both respiratory and non-respiratory specimens allowing for the detection of both pulmonary and extrapulmonary tuberculosis from a variety of specimen sources. An advantage of the MTBRP PCR assay over the FDA-cleared assay is the ability to test a wide variety of non-respiratory specimen types. The MTBRP assay is also useful in instances where mycobacterial cultures are not performed or when only formalin-fixed, paraffin-embedded tissue blocks are available from pathology. Finally, the MTBRP assay is able to detect the various members of the M. tuberculosis complex (i.e., M. bovis and M. bovis BCG) but it doesn’t differentiate the various species within the complex. The MTBRP assay does have limitations and its performance in AFB smear-negative specimens has not been fully characterized so it should always be performed in conjunction with standard mycobacterial culture. A negative result in a smear-negative or FFPE specimen should not be used as the sole basis to rule out Mtb. Future studies will examine the performance of the assay for smear-negative specimens. The MTBRP assay produces results much faster than mycobacterial culture with PCR results available within less than 24 hrs after submission of the specimen to the laboratory for most specimen types compared with days to weeks for mycobacterial culture results. The MTBRP assay is also quite specific for M. tuberculosis complex, so a positive result provides the clinician with rapid results to assist with patient care decisions. The presence of a melting peak at 58.0 ± 2.5 °C provides an early indication of potential INH resistance. The verification and performance evaluation performed for the MTBRP assay provides a potential approach for clinical laboratories to develop and verify LDT PCR assays should enhanced regulatory review of LDT assays be required in the future.

5. Consent

The work described in this manuscript was approved by an Institutional Review Board of the Mayo Clinic.

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CRediT authorship contribution statement

Seanne P. Buckwalter: Conceptualization, Methodology, Validation, Writing – original draft. Brian J. Connelly: Validation, Writing – review & editing. Laura K. Louison: Validation, Writing – review & editing. Jolene M. Kolesch: Investigation, Writing – review & editing. Senait A. Herring: Investigation, Writing – review & editing. Ethan D. Woodliff: Data curation, Writing – review & editing. Catherine M. Bolster LaSalle: Resources, Writing – review & editing. Thomas E. Grys: Resources, Writing – review & editing. Sharon M. Deml: Investigation, Writing – review & editing. Sherri L. Wohlfel: Supervision, Writing – review & editing. Lory K. Steinmetz: Supervision, Writing – review & editing. Nancy L. Wengenack: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nancy L. Wengenack reports a relationship with Mayo Clinic Department of Laboratory Medicine and Pathology that includes: employment. Author serves on the Advisory Board of J Clin Tuberc Other Mycobact Dis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at [https://doi.org/10.1016/j.jctube.2022.100340](https://doi.org/10.1016/j.jctube.2022.100340).

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