Validation and Implementation of Real-Time PCR for Detection of MTBC/MAC DNA by: Examining Challenges and Impacts to Virginia's Public Health Laboratory and Department of Health

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SUMMARY

In 2021, the Division of Consolidated Laboratory Services (DCLS) Tuberculosis (TB) Laboratory validated a rapid, cost effective laboratory-developed test (LDT) for the detection of *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium avium* complex (MAC) DNA by real-time PCR for direct sputum specimens and culture isolates using the ABI 7500 Fast Dx instrument platform. A comprehensive study to meet clinical laboratory validation requirements for a LDT was performed on a total of 227 samples, including 120 direct respiratory specimens and 107 culture isolates grown on a variety of media including LJ, MGIT, and 7H10. DCLS evaluated performance characteristics of the real-time PCR assay including accuracy, precision, sensitivity, limit of detection and competition.

This multiplexed, real-time PCR assay provides a cost effective replacement for GenProbe Accuprobe and provides an efficient, batched alternative for nucleic acid amplification testing (NAAT) on direct clinical sputa compared to the Xpert MTB/RIF assay. Previously, the Cepheid Xpert MTB/RIF was the only MTBC NAAT performed by DCLS which was restricted for use on first-time fluorochrome smear positive patients, not on anti-tuberculosis therapy. In 2021, this previous testing algorithm resulted in a 17.5% reporting benchmark for the detection of tuberculosis cases using NAAT within 2 days of specimen receipt. Since real-time PCR implementation, all applicable sputa are tested within 2 days of receipt for MTBC and MAC regardless of fluorochrome smear result, allowing DCLS to enhance its NAAT reporting benchmark to further align with the Health People 2030 goals. To further assist clinicians in public health decisions, sputa with a MTBC DNA detected PCR result are reflexed to Xpert testing for detection of rifampin resistance. All clinical specimens proceed to culture regardless of NAAT results.

During validation testing, the optimization of extraction and PCR controls proved to be challenging and resulted in the laboratory purchasing commercial controls for the assay. This modification was successful and provided improved control stability. Additionally, validation data indicated testing one replicate was sufficient to achieve reliable results.

BACKGROUND

DCLS provides the majority of mycobacterial testing support for the Virginia Department of Health's (VDH) Tuberculosis (TB) Control Program and serves as the tuberculosis reference library for the Commonwealth of Virginia. In 2019, DCLS processed 3,507 clinical specimens for mycobacterial culture (417 MTBC; 148 MAC) and received 299 reference isolates (161 MTBC; 66 MAC). Per the TB laboratory's workflow, all acid fast bacilli (AFB) positive cultures were tested utilizing the GenProbe Accuprobe method for MTBC and MAC. Replacement of GenProbe Accuprobe with the New York State Department of Health, Wadsworth Center's MTBC/MAC real-time PCR assay is cost effective and provides rapid results for Virginia's patients. With a new testing algorithm and real-time PCR it is expected that DCLS' reporting benchmark will improve from 17.5% to at least 77%. The total cost per specimen for real-time PCR in a batched workflow of 15 specimens is $12.04, compared to $34.84 for GenProbe Accuprobe.

METHODS

DCLS utilized the New York State Department of Health, Wadsworth Center's Detection of MTBC and MAC DNA by real-time PCR SOP (version 6) as a reference method. The TB laboratory froze and stored direct specimens for validation testing for approximately 5 months. The validation was performed on 120 direct respiratory specimens and 107 isolates grown on MGIT, LJ and 7H10. Some modifications to the reference method were required for successful validation at DCLS and are summarized below.

Bicoid Inhibition Control Modification

Wadsworth Center originally developed and purified the Bicoid inhibition control plasmid utilizing the PerfectPrep Plasmid Mini Kit, which was no longer available at the time of DCLS' validation. The TB Laboratory instead utilized the Qiagen HiSpeed Plasmid Midi Kit to purify Bicoid which led to a lower plasmid concentration. Due to a lower yield, the TB laboratory used a higher concentration of Bicoid during validation, and continuously monitors cycle threshold (Ct) values to determine if adjustments are required.

Positive Extraction Control Modification

Wadsworth Center utilizes MTBC ATCC 25177 for their positive extraction (POS EXT); however, occasionally during validation, DCLS had failures in the POS EXT. DCLS utilized MTBC ATCC 27294 during validation and implementation. After further guidance from Wadsworth Center, the control preparation was further adjusted to 200µL aliquots placed in 1.5mL Sarstedt O-ring tubes with 3, 3-mm glass beads, and heat inactivated at 80°C for 1 hour after confirmation of the appropriate Ct value (~30). Previously POS EXT was heat inactivated, aliquoted in 200µL and then placed in 1.5mL Sarstedt O-ring tubes with 3, 3-mm glass beads.

Positive PCR Control Modification

Wadsworth Center utilizes an in-house strain of MTBC that is known to contain 1 copy of IS6110 that is mixed at a 1:1 ratio with MAC DNA. Due to issues with stability and repeat TbextRd-9 detection failures during validation testing, DCLS purchased ZeptoMetrix controls (*Mycobacterium avium* Serotype 2 and *Mycobacterium tuberculosis* H37Rv-9, titered BSL-3) which are mixed at a 1:1 ratio (Ct value = ~30 for each strain). Previously, DCLS made 5µL aliquots of the positive PCR control, but altered aliquots to be 200µL to further aide in control stability.

RESULTS

Accuracy

The real-time PCR assay successfully detected MTBC in 45/55 direct sputa with previous MTBC positive cultures, for an accuracy of 81.8% when compared to culture (refer to Table 1). The assay detected 100% of fluorochrome smear positive specimens and 44% of smear negative specimens for MTBC. There were 10 direct specimens with PCR results that did not correlate to the final culture result. Two were MTBC inconclusive by PCR (culture results: 1 nontuberculous mycobacteria (NTM), 1 no AFB) and 7 were MTBC detected by PCR (culture results: 4 MAC, 3 NTM); however, all 9 patients had a prior history of MTBC positive cultures and likely received anti-mycobacterial therapy. One specimen was MTBC detected by PCR but there was no prior history of MTBC for this patient.

The real-time PCR assay had a lower accuracy rate of 19.2% (5/26) for MAC when compared with culture (refer to Table 2). The assay detected 42% of smear positive specimens and 0% of smear negative specimens for MAC. Of the MAC smear positive specimens that were negative by PCR, 7/12 were also culture positive for MTBC, which could account for the fluorochrome smear positivity.

The real-time PCR assay accuracy for isolates was 100% for both MTBC and MAC across all media types tested (refer to Table 3).

Table 1: MTBC Accuracy for Direct Specimens

Culture Results	Number Tested	+ MTBC PCR Result	% Accuracy
MTBC Final	55	45	81.81
Fluorochrome Positive	37	37	100
Fluorochrome Negative	18	8	44.44
+MTBC PCR Result (%)	**-MTBC PCR Result (%)**	**Inconclusive (%)**	**Inhibited (%)**
45 (81.81)	6 (10.91)	2 (3.64)	2 (3.64)

Table 2: MAC Accuracy for Direct Specimens

Culture Results	Number Tested	+ MAC PCR Result	% Accuracy
MAC Final	26	5	19.23
Fluorochrome Positive	12	5	41.67
Fluorochrome Negative	14	0	0
+MAC PCR Result (%)	**-MAC PCR Result (%)**	**Inconclusive (%)**	**Inhibited (%)**
5 (19.23)	10(65.38)	0 (0)	4 (15.38)

Table 3: Accuracy for Isolates

Media Type	Culture Result	N	PCR Result	N	Accuracy	Number Tested
MGIT	MTBC	32	MTBC	32	100%	63
	MAC	18	MAC	18		
	NTM	13	Negative	13		
LJ	MTBC	15	MTBC	15	100%	43
	MAC	9	MAC	9		
	NTM	19	Negative	19		
7H10	MTBC	15	MTBC	15	100%	44
	MAC	10	MAC	10		
	NTM	19	Negative	19		

Competition

Titer competition studies were completed in sputum at high and low concentrations of each organism and tested against each other (MTBC high/low: 10^{-2} / 10^{-6}; MAC high/low: 10^{-2} / 10^{-5}). Low concentrations were selected to correlate to the limit of detection (LOD) for each organism. The assay successfully detected MTBC at the LOD if a high concentration of MAC was present, but did not detect MAC at the LOD if a high concentration of MTBC was present.

Additionally, competition studies were completed with positive MGIT broths (refer to Table 4). MGITs were inoculated with each organism (MTBC, MAC, and a NTM), incubated until MGIT positive, and tested against each other at a 1:1 and 10:1 ratio of each organism. The assay successfully detected the applicable target for the MGIT competition studies.

Table 4: Positive MGIT Competition Study

	1:1 Ratio			10:1 Ratio					
Sample	MTBC:MAC	MTBC:NTM	MAC:NTM	MAC:MTBC	MAC:NTM	MTBC:MAC	MTBC:NTM	NTM:MAC	NTM:MTBC
PCR Result	MTBC+ MAC+	MTBC+	MAC+	MTBC+ MAC+	MAC+	MTBC+ MAC+	MTBC+	MAC+	MTBC+

Method Comparison

Real-time PCR was equivalent for the detection of MTBC to the Xpert MTB-RIF assay and equivalent for the identification of MTBC and MAC to the GenProbe Accuprobe assay. GenProbe Accuprobe data only shown (refer to Table 5).

Table 5: GenProbe Accuprobe Method Comparison

MTBC	PCR MTBC+	PCR MTBC-	MAC	PCR MAC +	PCR MAC -
Accuprobe MTBC +	18	0	**Accuprobe MAC +**	**12**	**0**
Accuprobe MTBC -	0	10	**Accuprobe MAC -**	**0**	**10**

Precision

All sample types (sputa, MGIT, LJ, 7H10) were tested in duplicate over five days by two different analysts across five ABI 7500 Fast Dx Instruments. 100% precision was achieved for all samples on all five ABI instruments. Data not shown.

Limit of Detection

Limit of Detection (LOD) was performed with ZeptoMetrix controls that were serially diluted in water (no matrix) and also seeded into negative sputum (refer to Table 6). Samples were run in duplicate and LOD was determined by establishing the last dilution that the applicable target was detected in matrix and no matrix.

Table 6: LOD for MTBC and MAC

	Sample (titer CFU/mL)	PCR Target	LOD (CFU/mL)
No matrix	*M. avium* (6.39x10^8)	MAC	6.39x10^3
	M. tuberculosis (4.22x10^7)	MTBC	4.22x10^1
Sputum	*M. avium* (6.39x10^8)	MAC	6.39x10^3
	M. tuberculosis (4.22x10^7)	MTBC	4.22

Implementation and Laboratory Workflow Updates

The laboratory used a phased approach for implementation of the real-time PCR assay, starting with isolate testing in January 2022 to replace GeneProbe Accuprobe. Direct sputa testing was implemented in April 2022 after outreach and education was provided to local health departments. Additionally, implementation of testing in two phases allowed the laboratory to consistently and effectively train scientists on molecular testing and address issues as they arose.

Result referral periods were established for real-time PCR testing based on reference standards and to ensure efficient testing practices:

Direct Sputa: Patient with previous positive MTBC result (NAAT and/or culture) within the past 12 months

Isolates: MTBC or MAC isolate within 1 month since date of collection from previous isolate

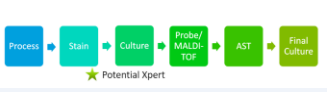

Figure 1: TB Laboratory workflow prior to real-time PCR implementation. NAAT was only performed on first time smear positive specimens that met testing requirements for Xpert.

Figure 2: DCLS daily real-time PCR workflow. Specimens and isolates are heat-inactivated and then stored at -20°C and tested the next business day.

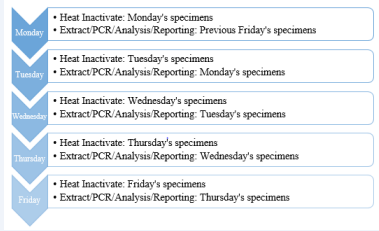

Figure 3: TB Laboratory workflow after real-time PCR implementation. MGIT tube represents workflow after a specimen has a positive AFB culture.

CONCLUSIONS

When validating a LDT NAAT, optimization of the method and associated controls is a key step to ensure successful evaluation of the assay. Utilizing commercial control material ensured stability in QC results and allowed validation testing to proceed smoothly. Implementation of the MTBC/MAC DNA by real-time PCR assay ultimately benefits public health intervention efforts and provides a rapid, cost-effective method for patient diagnosis.

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