DEVELOPMENT AND VALIDATION OF A SUITE OF MULTIPLEXED PCR ASSAYS FOR THE DETECTION AND DIFFERENTIATION OF NON-TUBERCULOUS MYCOBACTERIA

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INTRODUCTION

Current Nontuberculous mycobacteria (NTM) diagnostics rely on clinical presentation and a combination of testing modalities including radiology and culture. NTM cultures often have extended turnaround times due to the slow growth rates of these organisms. Additionally, discriminating between members of the *Mycobacterium abscessus* complex (MAbC) is important due to increasing drug resistance. Therapy to resolve NTM infections typically involves a long treatment course. PCR-based NTM diagnostics can provide a means for clinicians to attain a diagnosis and start the most appropriate therapy sooner than relying on traditional culture methods.

The primary objective of this study was to validate the ability of several in-house designed qPCR assays to detect nucleic acid originating from clinically significant members of the *Mycobacterium* genus.

Here we describe the development, validation and performance characteristics of a multiplexed, quantitative qPCR assay for MAbC, as well as two multiplexed, qualitative qPCR assays targeting members of the *Mycobacterium avium* complex (MAC) and members of the *Mycobacterium* genus (broad-range *Mycobacterium* or BR-Myco).

MATERIALS AND METHODS

Multiple qPCR primers and probes were designed to target conserved genes for organisms belonging to the MAbC and MAC. MAbC target assays were designed to specifically detect and quantify the three subspecies associated with this taxonomic group. MAC target assays were designed to qualitatively detect select organisms belonging to this complex. BR-Myco oligos targeted the *Mycobacterium* internal transcribed spacer (ITS) sequence and were optimized to detect clinically significant members of the genus outside of the MAbC and MAC. The most promising candidate assays were then optimized and multiplexed within each complex and further characterized with linearized plasmid containing the various target sequences.

The assays demonstrating the best preliminary efficiency, precision, and sensitivity were then validated in accordance with guidelines recommended by the New York State Department of Health, College of American Pathologists (CAP), and Clinical and Laboratory Standards Institute (CLSI) to establish the analytical specificity, linearity and dynamic range, analytical sensitivity (limit of detection and lower limit of quantification), intra- and inter-assay precision (reproducibility), and analytical accuracy of the test method[1-7].

BAL and sputum samples were pre-processed by bead-beating using the MagMAX™ CORE Mechanical Lysis Module. DNA was then extracted from samples using the MagMAX™ DNA Multi-Sample Ultra 2.0 Kit and KingFisher™ Flex system (Thermo Fisher). Amplification and detection were performed using TaqMan™ Fast Advanced Master Mix (Thermo Fisher) and the Applied Biosystems™ 7500 Fast instrument. Quantification was performed using linearized plasmid standards containing assay target sequences and results were evaluated in copies/mL for the quantitative MAbC assay and C_Ts for the qualitative MAC and BR-Myco assays.

References:

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RESULTS

Figure 1. Full-Process Linearity and dynamic range

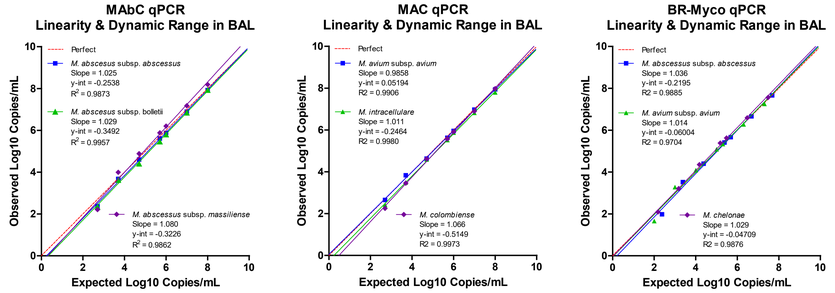

Table 1. MAbC Analytical accuracy data

	MAbC qPCR Assay			
Sample ID	Mabs-abs[1]	Mabs-boll[1]	Mabs-mass[1]	Negatives[2]
M. abscessus subsp.				
abscessus (n=48)	48	0	0	121
bolletii (n=48)	0	48	0	121
massiliense (n=48)	0	0	48	121
Unspiked Samples (n=50)	0	0	0	50

[1]Mabs-abs = *M. abscessus* subsp. *abscessus*, Mabs-boll = *M. abscessus* subsp. *bolletii*, and Mabs-mass = *M. abscessus* subsp. *massiliense*
[2]Negative samples are defined as samples spiked with non-MAbC NTM species

Table 2. BR-Myco Analytical accuracy data

	BR-Myco qPCR Assay	
Sample ID	Positives	Negatives
M. abscessus subsp.		
abscessus (n=48)	48	0
bolletii (n=48)	48	0
massiliense (n=48)	48	0
M. avium subsp.		
avium (n=27)	27	0
paratuberculosis (n=1)	1	0
hominissuis (n=1)	1	0
sylvaticum (n=1)	1	0
M. intracellulare (n=27)	27	0
subsp. *chimaera* (n=2)	2	0
M. colombiense (n=27)	27	0
M. chelonae (n=27)	27	0
M. fortuitum (n=1)	1	0
M. gordonae (n=1)	1	0
M. kansasii (n=1)	1	0
M. malmoense (n=1)	1	0
M. scrofulaceum (n=1)	1	0
M. simiae (n=1)	1	0
M. terrae (n=1)	1	0
M. xenopi (n=1)	1	0
Unspiked Samples (n=50)	0	50

Table 3. MAC Analytical accuracy data

	MAC qPCR Assay	
Sample ID	Positives	Negatives[1]
M. avium subsp.		
avium (n=27)	27	179
paratuberculosis (n=1)	1	
hominissuis (n=1)	1	
sylvaticum (n=1)	1	
M. intracellulare (n=27)	27	179
subsp. *chimaera* (n=2)	2	
M. colombiense (n=27)	27	179
Unspiked Samples (n=50)	0	50

[1]Negative samples are defined as samples spiked with non-MAC NTM species

- **Figure 1.** Linear regression of full-process sample preparations in BAL matrix resulted in the individual assay target regression metrics described when calculated in $\log_{10}$ copies/mL.
- **Table 1.** Analytical accuracy was demonstrated for MAbC qPCR by challenging the assay with a total of 144 BAL samples spiked with one of the three subspecies belonging to the MAb complex (*abscessus, bolletii,* or *massiliense*) as well as 121 BAL samples spiked with non-MAbC mycobacteria and 50 unspiked BAL samples. All samples were correctly identified.
- **Table 2.** Analytical accuracy was demonstrated for MAC qPCR by challenging the assay with a total of 86 BAL samples spiked with one of seven species / sub-species belonging to the MAC as well as 179 BAL samples spiked with non-MAC mycobacteria and 50 unspiked BAL samples. All samples were correctly identified.
- **Table 3.** Analytical accuracy was demonstrated for the BR-Myco qPCR by challenging the assay with a total of 265 BAL samples spiked with various NTM species (144 MAbC NTMs, 86 MAC NTMs, 27 *M. chelonae*, and 8 different non-MAbC / non-MAC *Mycobacterium* species) as well as 50 unspiked BAL samples. All samples were correctly identified.

RESULTS

Table 4. MAbC qPCR LoD and LLoQ[1] for *M. abscessus* subsp. *abscessus*

Probit LoD Prediction:			161 copies/mL		LLoQ:	750 copies/mL	
Expected copies/mL	*Observed copies/mL*	*Expected $\log_{10}$ copies/mL*	*Observed $\log_{10}$ copies/mL*	*Standard deviation*	*Bias*	*% Detection*	*Total analytical error*
750	776	2.88	2.82	0.25	0.06	100%	0.6
150	150	2.18	1.98	0.46	0.19	100%	1.1
100	74	2.00	1.69	0.42	0.31	95%	1.2
50	105	1.70	1.75	0.51	0.05	50%	1.1
5	33	0.70	1.51	0.00	0.82	10%	0.8

[1]LLoQ – Lower Limit of Quantification

- The MAbC assay limit of detection (LoD_{95}) for *M. abscessus* subsp. *abscessus* predicted by probit regression was 161 copies/mL (95% confidence interval of 101 to 350 copies/mL) in BAL.

Table 5. MAC qPCR LoD Confirmation

Organism	*Observed copies/mL*	*Expected $\log_{10}$ copies/mL*	*Observed $\log_{10}$ copies/mL*	*Standard deviation*	*% Detection*
M. avium subsp. *avium*	122	2.10	1.96	0.36	100%
M. intracellulare	167	2.40	2.17	0.24	100%
M. colombiense	351	2.40	2.51	0.19	100%

- The MAC assay limit of detection (LoD) was assessed by a range-finding experiment followed by confirmation of detection (≥95%) at discrete concentrations for each target in 20 spiked BAL replicates.

Table 6. MAbC Intra- and inter-assay precision

High Samples					*Medium Samples*					*Low Samples*				
Data set		*Mabs-abs[1] copies/mL*	*Mabs-boll[1] copies/mL*	*Mabs-mass[1] copies/mL*	*Data set*		*Mabs-abs copies/mL*	*Mabs-boll copies/mL*	*Mabs-mass copies/mL*	*Data set*		*Mabs-abs copies/mL*	*Mabs-boll copies/mL*	*Mabs-mass copies/mL*
Intra-assay day 1	**Mean**	412,030	287,185	211,318	Intra-assay day 1	**Mean**	41,024	28,958	17,570	Intra-assay day 1	**Mean**	4,898	3,603	1,985
	SD	52,846	38,793	11,688		**SD**	3,301	7,110	856		**SD**	1,081	623	816
	%CV	12.83%	13.51%	5.53%		**%CV**	8.05%	24.55%	4.87%		**%CV**	22.07%	17.29%	41.12%
Intra-assay day 2	**Mean**	441,219	346,506	224,495	Intra-assay day 2	**Mean**	53,109	28,718	22,830	Intra-assay day 2	**Mean**	5,869	3,004	1,617
	SD	50,830	53,966	22,645		**SD**	6,751	5,309	2,410		**SD**	1,639	1,067	549
	%CV	11.52%	15.57%	10.09%		**%CV**	12.71%	18.49%	10.55%		**%CV**	27.92%	35.51%	33.93%
Intra-assay day 3	**Mean**	394,092	234,908	199,418	Intra-assay day 3	**Mean**	41,666	24,019	17,931	Intra-assay day 3	**Mean**	4,707	1,660	1,663
	SD	51,358	44,316	16,756		**SD**	2,031	4,925	3,030		**SD**	696	174	808
	%CV	13.03%	18.87%	8.40%		**%CV**	4.88%	20.50%	16.90%		**%CV**	14.78%	10.51%	48.62%
Intra-assay day 4	**Mean**	430,111	275,463	206,516	Intra-assay day 4	**Mean**	46,973	27,580	21,302	Intra-assay day 4	**Mean**	5,528	2,417	1,277
	SD	49,109	13,802	29,099		**SD**	2,709	5,095	4,589		**SD**	971	865	512
	%CV	11.42%	5.01%	14.09%		**%CV**	5.77%	18.47%	21.82%		**%CV**	17.57%	35.77%	40.09%
INTER-ASSAY	**Mean**	419,363	286,016	210,437	INTER-ASSAY	**Mean**	45,693	27,319	19,841	INTER-ASSAY	**Mean**	5,251	2,609	1,636
	SD	49,275	54,924	21,089		**SD**	6,231	5,473	3,530		**SD**	1,136	995	666
	%CV	11.75%	19.20%	10.02%		**%CV**	13.64%	20.03%	17.79%		**%CV**	21.64%	38.13%	40.73%

[1]Mabs-abs = *M. abscessus* subsp. *abscessus*, Mabs-boll = *M. abscessus* subsp. *bolletii*, and Mabs-mass = *M. abscessus* subsp. *massiliense*

- MAbC assay precision observed for full-process samples are shown with intra-assay copies/mL %CVs results ranging from 4.88 to 48.62% across all concentrations tested and inter-assay %CVs ranging from 10.02% to 40.73% observed across all concentrations tested. MAC assay intra- assay precision (data not shown) for C_T %CVs ranged from 0.16%-4.26% across all concentrations tested and inter-assay precision %CVs ranged from 0.29%-2.97%. BR-Myco C_T %CVs intra- assay precision (data not shown) for C_T %CVs ranged from 0.14%-3.74% across all concentrations tested and inter-assay precision %CVs ranged from 0.65%-2.85%.

CONCLUSIONS

Traditional mycobacterial culture methods can have extended turn-around times and poor sensitivity relative to molecular diagnostics. The Viracor in-house designed and validated PCR-based NTM diagnostic suite described here allows clinicians to attain a prospective diagnosis and start an appropriate therapy as soon as possible. While culture methods still have a place within the mycobacterial diagnostic space (differentiation / identification and resistance / susceptibility profiles), the assays described here can effectively reduce the time-to-therapy and potentially improve health outcomes for patients diagnosed with pulmonary NTM.