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Culture-Independent Detection of Nontuberculous Mycobacteria in Clinical Respiratory Samples

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Culture-based detection of nontuberculous *Mycobacteria* (NTM) in respiratory samples is time consuming and can be subject to overgrowth by nonmycobacterial bacteria. We describe a single-reaction TaqMan quantitative PCR assay for the direct detection of NTM species in clinical samples that is specific, sensitive, and robust.

While rates of infection caused by members of the *Mycobacterium tuberculosis* complex continue to fall in developed countries (1, 2), disease caused by nontuberculous mycobacteria (NTM) is an area of growing concern (3–6). Pulmonary infection represents more than 90% of NTM cases (7) and has been described in a range of clinical contexts (8–11). Appropriate management of suspected pulmonary NTM infection requires the timely detection and identification of the etiological agent. The current "gold standard" for detection of NTM in respiratory samples relies on protracted *in vitro* culture, potentially delaying targeted therapy. It also requires samples to undergo decontamination prior to culture to lower levels of commensal microbiota (12) and is associated with variable sensitivity (13). The ability to perform a rapid quantitative screen for the presence of any NTM species would provide an important early indication of mycobacterial involvement and would be informative in cases where samples are culture negative, despite clinical or radiological signs.

To prevent false-positive results arising from the detection of closely related species (14, 15), existing molecular assays target narrow phylogenetic groups or specific pathogens (16–21), require prior mycobacterial isolation by culture (22–24), or are unable to provide accurate species-level NTM identification (25). We describe a TaqMan quantitative PCR (qPCR) assay, based on the single-copy *hsp65* gene, for the direct detection of NTM species in respiratory clinical samples.

The assay design was based on the full-length *hsp65* gene sequences that are available for 116 of the 174 currently described NTM species, including all 56 NTM species reported in respiratory disease (see Fig. S1 in the supplemental material). The PCR primers (forward, HSP171 [5′-CGCCAAGGAGATCGAGCTGG-3′], and reverse, HSP563 [5′-GGACAAGGTCGGCAACGAGGG-3′]) generate a 348-bp *hsp65* amplicon and are used in conjunction with a TaqMan probe (5′-FAM-AGAAGGCCGTCGAGAAG GTCA-BHQ-3′ [FAM, 6-carboxyfluorescein; BHQ, black hole quencher]) at an annealing temperature of 60°C (Fig. 1). A detailed description of assay development and methods is provided in the supplemental material.

In silico analysis indicated complete homology to the targeted *hsp65* gene region for 77 *Mycobacterium* species. Fourteen species had ≤2 nucleotide mismatches within the primer binding region, with a corresponding reduction in annealing temperature of up to 4.5°C. However, in all such cases, the corresponding primer binding region showed 100% sequence homology (see Tables S1 and S2 in the supplemental material). Twenty-one mycobacterial species (including *M. tuberculosis* and *Mycobacterium leprae*) and 40 assessed nonmycobacterial species had >3 nucleotide mismatches to the primer sequences, requiring an annealing temperature of <55.5°C (see Fig. S2 and Table S3 in the supplemental material).

The assay's performance was assessed using DNA extracts from 15 NTM strains and negative controls that included closely related nonmycobacterial species, common respiratory pathogens, nine *M. tuberculosis* strains, *Mycobacterium bovis*, *Escherichia coli*, and human DNA (Table 1). The assay's sensitivity was assessed using a dilution series of purified *Mycobacterium abscessus* DNA (selected based both on its clinical importance and its position within NTM phylogeny). The correlation between template concentration and cycle threshold (C_T) values was linear between 3.34×10^3 and 2.65×10^8 CFU/ml equivalents (slope, −3.31; $R^2 = 0.99$), with a reaction efficiency of 100%. Analysis using *Mycobacterium intracellulare* DNA, a species with a single-base primer mismatch, resulted in a linear range of 6.26×10^3 to 4.39×10^8 CFU/ml equivalents (slope, −3.403; $R^2 = 0.99$), with a reaction efficiency of 97%.

The potential for carryover of clinical sample components to influence assay performance was assessed in three ways. First, the amplification efficiency and dynamic range of *M. abscessus* DNA were determined following the addition of DNA extracts from

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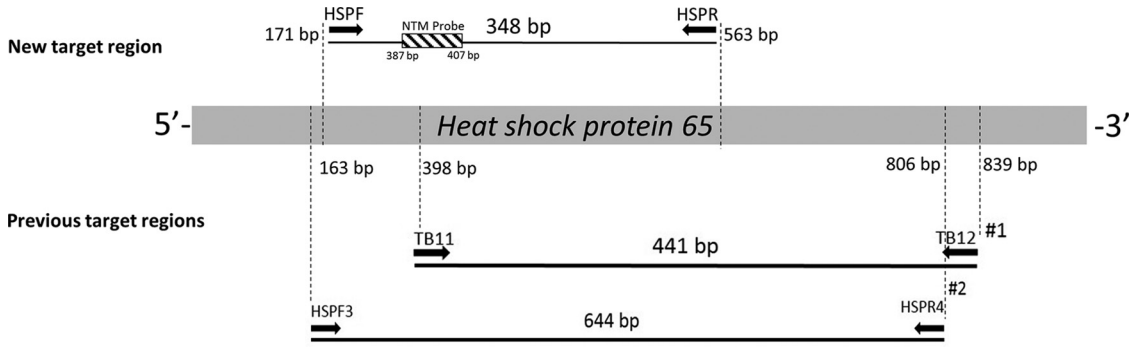

FIG 1 Primer and probe target sites. Primer binding sites for previously described NTM detection assay primers are also shown, as follows: #1, Telenti et al. (30); #2, Kim et al. (31).

culture- and qPCR-negative bronchoalveolar lavage (BAL) and sputum samples. Second, the assay's performance was assessed following the addition of purified human DNA at a concentration that substantially exceeded the levels in respiratory clinical samples. Third, the impact of the addition of horse blood prior to DNA extraction on *M. abscessus* DNA amplification efficiency was assessed, using a dilution series starting at 50% (vol/vol). In each case, no significant change in assay performance was observed

TABLE 1 Amplification data for reference and control strains

Species (strain)	Source[c]	C_T value	CFU/ml equivalent
Mycobacterial species[a]			
M. abscessus	ATCC 19977	16.9	2.65×10^8
M. avium	Clinical strain	20.1	7.56×10^7
M. chelonae	ATCC 35752	22.6	1.26×10^7
M. flavescens	Collection strain	28.3	2.42×10^5
M. fortuitum	ATCC 9820	18.6	2.08×10^8
M. goodii	Clinical strain	21.3	3.21×10^7
M. gordonae	Clinical strain	23	9.93×10^6
M. interjectum	Clinical strain	21.2	3.34×10^7
M. intracellulare	Clinical strain	23	9.99×10^6
M. kansasii	Clinical strain	24.9	2.68×10^6
M. lentiflavum	Clinical strain	21	3.81×10^7
M. marinum	Collection strain	23.3	7.72×10^6
M. simiae	Clinical strain	19	1.62×10^8
M. smegmatis	Clinical strain	20.8	4.39×10^7
M. bovis (BCG)	Collection strain	ND[d]	ND
M. tuberculosis (H37Rv)	Clinical strain	ND	ND
M. tuberculosis (Uganda 1)	Clinical strain	ND	ND
M. tuberculosis (Orygis)	Clinical strain	ND	ND
M. tuberculosis (MDR)	Clinical strain	ND	ND
M. tuberculosis (LAM/Uganda 1)	Clinical strain	ND	ND
M. tuberculosis (EAI)	Clinical strain	ND	ND
M. tuberculosis (EAI 1)	Clinical strain	ND	ND
M. tuberculosis (BJ Delhi CAS)	Clinical strain	ND	ND
M. tuberculosis (BJ Delhi CAS 1)	Clinical strain	ND	ND
Nonmycobacterial species[b]			
Rhodococcus equi	Collection strain	ND	ND
Nocardia farcinica	Collection strain	ND	ND
Corynebacterium glucuronolyticum	Collection strain	ND	ND
Staphylococcus aureus	Clinical strain	ND	ND
Pseudomonas aeruginosa	Clinical strain	ND	ND
Haemophilus influenzae	Clinical strain	ND	ND
Escherichia coli	Clinical strain	ND	ND
Streptococcus pneumoniae	Clinical strain	ND	ND
Human	Placental DNA	ND	ND

[a] South Australian pathology collection.
[b] Flinders Medical Centre pathology laboratory.
[c] ATCC, American Type Culture Collection.
[d] ND, not detected.

TABLE 2 NTM-positive respiratory samples as determined by qPCR, and corresponding diagnostic culture results

Sample type	C_T value	CFU/ml equivalent	Identification obtained with:	
			qPCR/ sequencing	Diagnostic microbiology
BAL fluid	30.5	1.36×10^5	*M. avium*	*M. avium*
BAL fluid	30.9	1.02×10^5	*M. avium*	*M. avium*
BAL fluid	32.1	4.58×10^4	*M. intracellulare*	*M. intracellulare*
BAL fluid	35.4	1.13×10^4	*M. avium*	*M. avium*
BAL fluid	34	8.70×10^4	*M. avium*	—[a]
BAL fluid	32.7	3.02×10^4	*M. avium*	*M. avium*
Sputum	31.1	3.22×10^4	*M. massiliense*	*M. massiliense*
Sputum	31.7	2.08×10^4	*M. flavescens*	—[a,b]
Sputum	31.5	2.44×10^4	*M. abscessus*	*M. abscessus*
Sputum	33.4	6.43×10^3	*M. avium*	—[a]
Sputum	35.5	1.49×10^3	*M. avium*	*M. avium*

[a] —, not detected.
[b] Sample was recorded as "insufficient for adequate assessment" for standard diagnostic analysis.

(Mann Whitney test, $P > 0.3$) (see Fig. S3 to S5 in the supplemental material).

Assay validation was performed using 42 respiratory samples from patients suspected of respiratory NTM infection, including 30 BAL samples and 12 sputum samples (of which 8 were NTM positive according to standard diagnostic testing; see Table S4 in the supplemental material). Positive results from NTM culture were confirmed by qPCR, and species identity was confirmed by DNA sequencing. However, in three cases, samples were NTM culture negative but qPCR positive. *Mycobacterium avium* was detected in the BAL sample at a concentration of 8.7×10^4 CFU/ml equivalents, while *Mycobacterium flavescens* and *M. avium* were detected in the two sputum samples at 2.1×10^4 and 6.4×10^3 CFU/ml equivalents, respectively (Table 2; see also Table S5).

Negative culture results in patients with suspected NTM infection are not uncommon, with NTM isolated from subsequent samples in some instances (26). A number of factors could contribute to discrepancies between culture-dependent and molecular analysis. For example, culture overgrowth by nonmycobacterial species can substantially reduce NTM detection, while sample decontamination techniques used to prevent this can reduce mycobacterial viability (12). In addition, NTM recovery can be reduced in patients receiving commonly used antibiotics, such as macrolides and quinolones (12).

In the case of the culture-negative, qPCR-positive BAL sample, high levels of *Haemophilus influenzae* growth were reported. While this species is fastidious, the finding suggests the potential presence of other nonmycobacterial species that might have contributed to the failure to isolate NTM through bacterial overgrowth. In the case of the culture-negative sputum sample in which *M. flavescens* was detected by PCR, *M. abscessus* had been isolated from this patient on a previous occasion (although definitive typing had not been performed), and the patient had received apparently successful eradication therapy. While the basis for discordance remains unclear, it is important to highlight that, as with all PCR-based assays, a positive result does not rely on the presence of viable bacterial cells (27, 28). DNA in nonviable bacteria or present in the extracellular environment (as might occur following successful antibiotic therapy) can also act as a PCR template (29), a factor that must be taken into account when interpreting disparities between culture and PCR-based results. In the case of the patient in whose sample *M. avium* was detected by qPCR alone, the corresponding sample was recorded as being "insufficient for adequate assessment" by culture (an outcome that is sometimes interpreted wrongly at the clinical level as a culture-negative result). However, sputum samples collected both prior to and following this sample were also found to be culture negative, suggesting a genuine discrepancy between culture and qPCR results.

Our study was unable to assess the assay's ability to detect a number of rare or recently described NTM species for which high-quality DNA sequence data are not yet available. It was further limited by a requirement for DNA sequencing to identify the source of positive PCR results, a technology that is not available in all laboratories. However, the single-reaction qPCR assay described offers substantial advantages over other molecular assays in terms of time and cost. Importantly, the assay does not amplify DNA from *M. tuberculosis*, a range of closely related nonmycobacterial species, or common airway bacteria and is unaffected by the presence of high concentrations of human DNA or blood derivatives. Our assay provides a specific, sensitive, and robust means to rapidly screen respiratory clinical samples for the presence of NTM and represents an important adjunct to conventional diagnostic approaches.

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