Materials and Methods

PCR primer and probe design

TaqMan quantitative (q) PCR assay design was based on sequence data held in GenBank nucleotide database (1). High quality, full-length *hsp65* gene sequences were available for 116 of the 174 currently described NTM species. These included all 79 species that have been reported in human infections, of which 56 have been reported in the context of respiratory disease. Sequences were aligned using CLC Sequence Viewer version 7.6.1 (CLC Bio, Aarhus, Denmark) and candidate primer and probe combination designed using Primer3Plus web interface version 2.4.0 (2).

Primer/probe design was performed initially against *M. abscessus* (NCBI Reference Sequence: NZ_CP009616.1). Based on sequence conservation across NTM species (as indicated by the sequence alignment, Figure S1) and sequence divergence in non-NTM species (Figure S2), a forward primer was designed to bind the 147-249 base region, and the reverse primer to bind the 510-580 base region of the *M. abscessus hsp65* gene.

Parameters for primer design were a length of 15-25 bases, a melting temperature of between 55-65°C, and a GC content from 35-70% (3). Candidate primers were excluded if their annealing temperature differed by >3°C, or if there were more than 3 G or C residues at the 3' end (3). Primers with complete homology to the *M. abscessus* reference sequence were retained for further assessment. Parameters for probe design were a length of 15-25 bases, a melting temperature of between 66-70°C, and a GC content from 35-70%. Candidate probes were excluded if they had runs of four consecutive G residues or 6 consecutive A residues, two or more CC dinucleotides in the middle of the probe, or a G in residue in the second position on the 5' end. Both primer and probe sequences were assessed for dimer and hairpin formation using IDT OligoAnalyzer 3.1 (http://sg.idtdna.com/calc/analyzer).

25 Candidate primer/probe combinations were assessed *in silico* for their homology to the aligned
26 NTM *hsp65* sequences, as well as to 40 other non-NTM control species representing closely
27 related taxa (*Corynebacterium glucuronolyticum*, *Nocardia farcinica* and *Rhodococcus equi*),
28 common respiratory pathogens (*Pseudomonas aeruginosa*, *Staphylococcus aureus*,
29 *Streptococcus pyogenes*, *Tsukamurella tyrosinosolvens*, *Acinetobacter baumanii*,
30 *Haemophilus influenzae*, *Bordetella bronchiseptica*) and *Mycobacterium tuberculosis* and
31 *Mycobacterium leprae*. In each case, oligonucleotide annealing temperatures were determined
32 using IDT Biophysics web interface version 1.02 (http://biophysics.idtdna.com/). Homology
33 between primers and probe and human sequences were assessed using Basic Local Alignment
34 Search Tool (BLAST) v.2.3.0 against the NCBI Human genomic plus transcript (Human G+T)
35 database.
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37 **NTM and non-NTM control strains**
38 DNA extracts from 15 NTM isolates (3 reference strains and 12 clinical isolates) (Table 1)
39 were used for assay assessment. In addition, closely related non-mycobacterial species (*R. equi*,
40 *N. farcinica*, *C. glucuronolyticum*), common respiratory pathogens (*S. aureus, P. aeruginosa,*
41 *H. influenzae, S. pneumoniae*), nine *M. tuberculosis* strains, *M. bovis* (BCG), *Escherichia coli*,
42 and human placental DNA (Bioline, NSW, Australia) were used as controls (Table 1).
43
44 **Clinical samples**
45 Forty-two isolates were obtained from patients suspected of having respiratory NTM
46 infections, comprising 30 bronchoalveolar lavage (BAL) samples and 12 sputum samples
47 (Table 2) with full ethical approval. In each case, sample portions were analysed by diagnostic
48 microbiology, in accordance with standard diagnostic practice (4). In addition, a sample aliquot
49 was frozen prior to DNA extraction and qPCR analysis.

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51 **DNA extraction**

52 DNA extraction from bacterial isolates and clinical samples was performed using GenElute
53 Bacterial Genomic DNA Kit (Sigma-Aldrich, Castle Hill, Australia). To maximise
54 mycobacterial DNA yield, bead-beating was performed in a FastPrep-24 instrument (MP
55 Biomedicals, USA) at 6.5 m/s for 45 seconds using 1x chrome bead (1mm diameter) (Diantree
56 Scientific, St Helens, Australia) and 200 µg silica:zirconium bead (0.1 mm diameter) (Diantree
57 Scientific). Bead-beating was followed by a 1 hour enzymatic lysis step using Gram-positive
58 lysis solution (Sigma-Aldrich) to which lysozyme (final concentration 50mg/mL) (Sigma-
59 Aldrich) and lysostaphin (final concentration 10mg/mL) (Sigma-Aldrich) were added. Samples
60 were mixed by vortexing before incubation at 37ºC for 1 hour. Other DNA extraction
61 procedures were as in accordance with GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich)
62 manufacturer's instructions. DNA was eluted into 100µL of DNA-free water and
63 concentrations were measured using a Qubit assay (Thermo Fisher Scientific, Scoresby,
64 Australia) according to manufacturer's instructions.

65

66 **PCR amplification conditions**

67 The TaqMan probe (Sigma-Aldrich) was 5'-labelled with FAM and 3'-labelled with Black Hole
68 Quencher. PCR reactions were performed using KAPA Probe Fast qPCR Master Mix (2x)
69 ROX Low (GeneWorks, Thebarton, Australia) in 25 µl reaction volumes. Reactions comprised
70 approximately 10 ng of DNA, each primer at a final concentration of 250 nM and probe at a
71 final concentration of 300 nM. Reactions were performed using a QuantStudio 6 RT-PCR
72 system (Thermo Fisher Scientific, Scoresby, Australia), with cycle conditions of 3 min at 95°C,
73 followed by 45 cycles of 60 s at 95°C and 45 s at 60°C.

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75 **Assessment of assay performance**

76 The sensitivity of the *hsp65* TaqMan assay was assessed using serial dilutions of *M. abscessus*
77 DNA (3.3-3.3x10^{-6} ng/µL, 10-fold increments). The efficiency of the qPCR was assessed based
78 on the coefficient of determination (R^2) of template concentration plotted against cycle
79 threshold (C_T) value, amplification efficiency, E ($E=10^{-1/slope}$), and consistency across replicate
80 reactions. C_T values were converted to DNA template concentration by comparison with a *M.*
81 *abscessus*-derived standard curve, generated concurrently. DNA concentration was then
82 converted to colony forming unit per mL (CFU/mL) equivalents based on the genome mass of
83 *M. abscessus* genome (ATCC 19977) and a single gene copy per cell. This analysis was
84 performed in triplicate.

85 The inhibitory effect of purified human DNA was assessed by adding 40 ng/µL (final
86 concentration) to the dilution series of *M. abscessus* isolate DNA. A similar procedure was
87 carried out using DNA extracted from horse blood (Thermo Fisher Scientific, Scoresby,
88 Australia) to assess blood inhibitors on the assay performance in detecting NTM. A fixed
89 concentration of *M. abscessus* DNA of 10 ng/µL (final concentration) to a dilution series of
90 horse blood, starting at 50% v/v. Assay inhibition by carryover of components from clinical
91 samples was assessed by comparison of signal obtained from 10-fold dilution series of *M.*
92 *abscessus* DNA (3.3 to 3.3x10^{-6} ng/µL) spiked with culture-negative BAL sample DNA, to a
93 final concentration of 32 ng/µL. The same procedure was carried out using DNA extracted
94 from a culture- and qPCR-negative sputum sample DNA.

95 Agreement between NTM species detection by qPCR and standard diagnostic culture was
96 assessed based on their application to a clinical sample collection. Amplicon length was
97 confirmed by gel electrophoresis on a 1% (w/v) agarose gel (Bio-Rad, Hercules, California,
98 USA). Electrophoretic bands were visualised using EZ-Vision Three DNA Dye (AMRESCO,
99 Solon, USA) in a GeneGenius Bio Imaging System (Syngene, Frederick, USA) using

100 GeneSnap software (Syngene, version 6.04), with length determined relative to HyperLadder
101 100bp Plus (Bioline). Amplicon specificity was confirmed for all positive reactions by Sanger
102 sequencing (Genomics and Molecular Pathology, SA Pathology, Bedford Park, South
103 Australia, Australia). Nucleotide sequences were assessed relative to the NTM alignment using
104 CLC Sequence Viewer version 7.6.1. All statistical analyses were performed using GraphPad
105 Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA.
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107 **References**

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