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Novel Multiplex Real-Time PCR Diagnostic Assay for Identification and Differentiation of *Mycobacterium tuberculosis*, *Mycobacterium canettii*, and *Mycobacterium tuberculosis* Complex Strains▽†

Kate Reddington,[1,3]‡ Justin O'Grady,[1,3]‡§ Siobhan Dorai-Raj,[1,3] Majella Maher,[3] Dick van Soolingen,[2] and Thomas Barry[1,3]*

Microbiology, School of Natural Sciences, National University of Ireland, Galway, Ireland[1]; National Tuberculosis Reference Laboratory, National Institute for Public Health and the Environment, Bilthoven, Netherlands[2]; and Molecular Diagnostics Research Group, NCBES, National University of Ireland, Galway, Ireland[3]

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Tuberculosis (TB) in humans is caused by members of the *Mycobacterium tuberculosis* complex (MTC). Rapid detection of the MTC is necessary for the timely initiation of antibiotic treatment, while differentiation between members of the complex may be important to guide the appropriate antibiotic treatment and provide epidemiological information. In this study, a multiplex real-time PCR diagnostics assay using novel molecular targets was designed to identify the MTC while simultaneously differentiating between *M. tuberculosis* and *M. canettii*. The *lepA* gene was targeted for the detection of members of the MTC, the *wbbl1* gene was used for the differentiation of *M. tuberculosis* and *M. canettii* from the remainder of the complex, and a unique region of the *M. canettii* genome, a possible novel region of difference (RD), was targeted for the specific identification of *M. canettii*. The multiplex real-time PCR assay was tested using 125 bacterial strains (64 MTC isolates, 44 nontuberculosis mycobacteria [NTM], and 17 other bacteria). The assay was determined to be 100% specific for the mycobacteria tested. Limits of detection of 2.2, 2.17, and 0.73 cell equivalents were determined for *M. tuberculosis*/*M. canettii*, the MTC, and *M. canettii*, respectively, using probit regression analysis. Further validation of this diagnostics assay, using clinical samples, should demonstrate its potential for the rapid, accurate, and sensitive diagnosis of TB caused by *M. tuberculosis*, *M. canettii*, and the other members of the MTC.

Tuberculosis (TB) is the leading cause of death worldwide from an infectious agent (13), with the WHO estimating that one-third of the global population is infected with *Mycobacterium tuberculosis*. In a global report from the WHO (2009), it was estimated that there were 9.27 million cases of TB in 2007, with 2 million associated deaths (41a). TB in humans is caused by members of the *Mycobacterium tuberculosis* complex (MTC). The eight closely related species of the MTC have a wide range of natural hosts, including human hosts (*M. tuberculosis*, *M. africanum*, *M. canettii*), bovine hosts (*M. bovis*), caprine hosts (*M. caprae*), rodent hosts (*M. microti*), and pinniped hosts (*M. pinnipedii*), along with the attenuated *M. bovis* strain BCG (Bacillus Calmette-Guérin), the commonly used vaccine strain. While there are a number of natural hosts, each species of the MTC has been implicated in human infection (6, 20).

Traditionally, diagnosis of TB relies on smear microscopy and culture techniques in combination with a battery of biochemical tests which are time-consuming and labor-intensive and which often yield unreliable results (18). Nucleic acid diagnostic (NAD) techniques, in particular, real-time PCR, offer rapid, reliable, and highly sensitive alternative tools for the detection of many infectious agents (25, 42). Advances in real-time PCR, such as the availability of multiple fluorophores, along with the development of nonfluorescent quenchers, have facilitated multiplexing, allowing the simultaneous detection and differentiation of multiple targets, along with internal controls, in one reaction (3).

While significant advances have been made in the diagnosis of TB using NAD techniques (19), the differentiation of members of the MTC to the species level is not routinely performed. Commercially available real-time PCR kits for the diagnosis of TB generally identify the MTC but not individual species. The high degree of nucleotide sequence homology between members of the complex makes species differentiation challenging (31). Comparative genomics revealed that the *M. tuberculosis* and *M. bovis* genomes are 99.95% similar at the nucleotide level (14), with whole-genome DNA microarrays identifying 16 regions of difference (RD1 to RD16) (4). These RDs represent regions of the genome which are deleted in *M. bovis* BCG but which are present in *M. tuberculosis* and have been used for the differentiation of members of the MTC. One RD commonly targeted for the specific detection of *M. tuberculosis* is RD9 (31); however, this RD is also present in *M. canettii* (7). There is currently no real-time PCR test which can

* Corresponding author. Mailing address: Microbiology, School of Natural Sciences, National University of Ireland, Galway, University Road, Co. Galway, Ireland. Phone: 35391493189. Fax: 35391494598. E-mail: thomas.barry@nuigalway.ie.

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‡ Both authors contributed equally to this work.

§ Present address: Department of Infection, Windeyer Institute of Medical Sciences, University College London, London W1T 4JF, United Kingdom.

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TABLE 1. Oligonucleotide primers and probes used in this study

Name	Function	Sequence (5′→3′)
MTC_IAC Fw	Forward sequencing primer, forward MTC and internal control real-time PCR assay primer	AGACCGTGCGGATCTTG
MTC_IAC Rv	Reverse sequencing primer, reverse MTC and internal control real-time PCR assay primer	CATGGAGATCACCCGTGA
MTC probe	MTC probe	HEX-ACGGATTGGTCACCCGGATT-BHQ1
IAC probe	Internal control probe	Cy5-ACGACCTTCTCGGAACCGT-BHQ2
*wbbl1*_Fw	Forward sequencing primer, forward real-time PCR assay primer	TACCAGCTTCAGTTTCCGT
*wbbl1*_Rv	Reverse sequencing primer, reverse real-time PCR assay primer	GCACCTATATCTTCTTAGCCG
wbbl1 probe	*wbbl1* probe	FAM-ATGGTGCGCAGTTCACTGC-BHQ1
M. canettii sp Fw	Forward *M. canettii*-specific primer	ATGTGGTTTCAGTACGACTTC
M. canettii sp Rv	Reverse *M. canettii*-specific primer	GATGGCAGTGTCTTATCCAA
M. canettii sp probe	*M. canettii*-specific probe	ROX-TGAGAGGTGTTGGCACGCAA-BHQ2
M. canettii seq 1.a	Forward sequencing primer 1	TGTCGGCGGCCACGT
M. canettii seq 1.b	Reverse sequencing primer 1	GAAGTCCAGCATCTTGGCGTT
M. canettii seq 2.a	Forward sequencing primer 2	TGTCGGCGGCCACGT
M. canettii seq 2.b	Reverse sequencing primer 2	ATCGTGCAGTGCGGCCA
M. canettii seq 3.a	Forward sequencing primer 3	GCAGCATTGTGGTTGACCGA
M. canettii seq 3.b	Reverse sequencing primer 3	TCCCAGCGTTGCGCCTT
M. canettii seq 4.a	Forward sequencing primer 4	TGATGCGGCTGCTCAAGC
M. canettii seq 4.b	Reverse sequencing primer 4	TGTCAAGGGACATGGGGAACT

diagnose TB while differentiating between *M. tuberculosis* and *M. canettii* as the causative agent of infection.

M. tuberculosis is the most important human pathogen in the MTC and is thought to be responsible for 95% of human cases of TB, yet it rarely causes disease in other mammals (1, 7, 9). While drug-resistant strains of *M. tuberculosis* are emerging, it is considered sensitive to antituberculosis drugs such as pyrazinamide (PZA), a first-line antibiotic that reduces patient treatment time from 9 months to 6 months (27, 35). However, *M. canettii*, which has been reported to cause TB in humans, is intrinsically resistant to PZA; therefore, the ability to differentiate it from *M. tuberculosis* is important for indicating the therapeutic regimen necessary for patient treatment (34).

M. canettii is considered to be the most phenotypically distinct member of the MTC and is considered the species from which other members of the complex may have evolved (6). *M. canettii* is phenotypically characterized by its smooth glossy white colonies; however, a small number of these colonies have been shown to revert to rough colony variants when individual colonies are replated (38). Smooth colonies are uncharacteristic of the MTC and are due to the presence of large amounts of lipooligosaccharides in the *M. canettii* cell wall (30). Like *M. tuberculosis*, *M. canettii* contains all the RDs with the exception of RD12 *M. canettii* ($RD12^{can}$), which has been targeted for the specific detection of *M. canettii* in a complex conventional PCR methodology (18).

While infection with *M. canettii* is thought to be rare and confined to eastern African countries, there is a lack of rapid diagnostic tests available to differentiate between it and *M. tuberculosis*. Cases of human TB caused by *M. canettii* have now been reported in Europe and America (12). In addition, recent reports have suggested that the number of true cases of TB caused by *M. canettii* may in fact be underrepresented (15, 34). Therefore, an ability to differentiate *M. tuberculosis* and *M. canettii* not only is important from a patient treatment perspective but also will provide important epidemiological information for clinicians.

We report on the design, development, and testing of a multiplex real-time PCR assay using novel nucleic acid diagnostics targets to detect the MTC while simultaneously detecting and differentiating between *M. tuberculosis* and *M. canettii* in one reaction.

MATERIALS AND METHODS

Diagnostics target identification. The diagnostics target genes used in this study were identified using a number of approaches. In order to identify a target for collective detection of the MTC, a number of housekeeping genes which are highly conserved throughout the *Mycobacterium* genus were evaluated. To identify novel targets for the detection of *M. tuberculosis*, approximately 3,000 genes were evaluated on the basis of regions deleted in other members of the MTC but present in *M. tuberculosis* or, alternatively, present in other members of the MTC but deleted in *M. tuberculosis*. These potential target regions were identified using the Mycobacterial Genome Divergence Database (MGDD; http://mirna.jnu.ac.in/mgdd/), which allowed identification of insertions, deletions, and single nucleotide polymorphisms between *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG. Potential target regions were also identified using the web-based version of the Artemis comparison tool, WebACT (http://www.webact.org/WebACT/home). Nucleotide sequence information was retrieved from the *M. africanum* and *M. microti* genomes (currently being sequenced by the Welcome Trust Sanger Institute) to determine, *in silico*, if the candidate diagnostics target nucleotide sequences for *M. tuberculosis* identification were specific. For the remaining members of the complex, namely, *M. canettii*, *M. caprae*, and *M. pinnipedii*, for which no nucleotide sequence information was available at the time of this study, the specificities of potential targets for these species were determined empirically and further validated through sequencing.

As the diagnostics target used in this study for the specific detection of *M. tuberculosis* was experimentally determined to also detect *M. canettii*, a novel diagnostics target specific for *M. canettii* was also required. When nucleotide sequence information for *M. canettii* became publicly available on the Sanger website, a number of large regions of nucleotide sequence were evaluated for putative species-specific nucleotide sequence diagnostics motifs using the BLAST program.

For each putative diagnostics target identified, alignments were carried out using the ClustalW multiple-sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw2/index.html), from which real-time PCR primers and probes were designed (Table 1).

Bacterial strains, culture media, and growth conditions. Sixty-four MTC isolates (26 *M. tuberculosis*, 11 *M. bovis*, 7 *M. bovis* BCG, 5 *M. canettii*, 2 *M. caprae*, 5 *M. africanum*, 5 *M. microti*, and 3 *M. pinnipedii* isolates), 44 nontuberculosis mycobacteria (NTM), and 17 other bacterial species were used in this study (see Tables S1 and S2 in the supplemental material). Of the 64 MTC isolates, 36 strains, previously characterized by a variety of methods as described in the

literature (12, 22, 23, 26, 30, 37–39), were provided by Dick van Soolingen (RIVM, Bilthoven, Netherlands). All other MTC strains, provided by Mario Vaneechoutte (University of Ghent, Ghent, Belgium), were clinical isolates collected over a 10-year period from reference laboratories in Belgium and the Netherlands. These isolates were characterized on the basis of custom techniques available at the time. Twenty-eight NTM were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [DSMZ]) and grown on Middlebrook agar/broth at either 30°C or 37°C. Fast-growing mycobacteria were cultured for 3 to 6 days, and slow-growing mycobacteria were incubated for 6 weeks or until sufficient growth was visible. All media were purchased from BD Biosciences (Oxford, United Kingdom). For the 16 remaining NTM, in addition to 4 of the 5 *Nocardia* strains used in this study, DNA was supplied by Mario Vaneechoutte and had been characterized using techniques previously described (10, 36, 41).

DNA isolation and quantification. Genomic DNA from NTM and 2 *M. bovis* BCG cultures was isolated from 1 ml of culture (Middlebrook 7H9 broth; Becton Dickinson), using a modified procedure combining mechanical lysis (IDI lysis kit; GeneOhm, Quebec City, Quebec, Canada) and purification using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Briefly, 1 ml of culture was centrifuged in a benchtop centrifuge (microcentrifuge 5415; Eppendorf) at 13,000 rpm for 3 min. The supernatant was discarded and the pellet was resuspended in 250 μl GeneOhm sample buffer. The suspension was transferred to a GeneOhm lysis tube and bead beaten (Mini-Bead-Beater-16; Stratech, United Kingdom) for 3 min. After bead beating, 200 μl was transferred to a sterile microcentrifuge tube and steps 3 to 8 of the procedure for purification of total DNA from animal tissue in the Qiagen DNeasy blood and tissue kit were followed, according to the manufacturer's instructions. The total genomic DNA samples provided by RIVM were extracted using methods previously described (40), while genomic DNA from Mario Vaneechoutte was extracted as described in a study by De Baere et al. (10). For all other bacterial species tested, DNA was provided from stocks held within the Microbiology laboratory of the National University of Ireland, Galway. DNA concentrations for all NTM and members of the MTC used in this study were determined using a PicoGreen double-stranded DNA quantitation kit (Molecular Probes, Eugene, OR) and a TBS-380 minifluorometer (Invitrogen Corporation, CA). All DNA samples were stored at −20°C before use.

Conventional and real-time PCR primers and hydrolysis probe design. Oligonucleotide primers and hydrolysis probes were designed in accordance with general recommendations and guidelines (11, 33), following the alignments of each of the nucleic acid diagnostics target genes identified in this study. All primers and probes (Table 1) used in this study were supplied by MWG-Biotech AG (Essenberg, Germany). The primers used in this study were designed to have a melting temperature (T_m) of 58 to 61°C, with all probes designed to have a T_m of 4 to 7°C higher. Hydrolysis probes were designed to be specific for each target following published design guidelines (11). These parameters were adhered to during the design of monoplex assays so that the assays could be easily multiplexed after specificity and sensitivity testing was complete.

For the MTC and internal amplification control (IAC) assay, PCR primers MTC_IAC Fw and MTC_IAC Rv (Table 1) were designed to amplify a 155-bp fragment of the *lepA* gene for all members of the MTC and *M. smegmatis*. The MTC_IAC Fw primer was located at positions 618 to 634 bp and the MTC_IAC Rv primer was located at positions 754 to 772 bp of the *M. tuberculosis* H37Rv *lepA* gene. For the *M. tuberculosis* and *M. canettii* assay, primers *wbbl1*_Fw and *wbbl1*_Rv were designed to amplify a 146-bp fragment of the *wbbl1* gene. The *wbbl1*_Fw primer was located at positions 15 to 34 bp and the *wbbl1*_Rv primer was located at positions 141 to 159 bp of the *M. tuberculosis* H37Rv *wbbl1* gene. Finally, the *M. canettii*-specific assay was designed to amplify a 128-bp fragment of a 2,869-bp region of the genome identified in this study to be specific to *M. canettii* (an *M. canettii*-specific assay could potentially be designed with sequence from anywhere in this region). This 2,869-bp region of the genome has been mapped to the *M. tuberculosis* H37Rv genome and is inserted between Rv0150c (hypothetical protein) and Rv0151c (gene name, *PE1*; a proline-glutamate [PE] family protein) at position 177,445 bp on the *M. tuberculosis* H37Rv genome.

All real-time PCR assays were initially tested in a monoplex format, evaluating their specificity and sensitivity, using probes labeled with 6-carboxyfluorescein (FAM) and black hole quencher 1 (BHQ1). After the monoplex real-time PCR assays were optimized, three of the four assay probes were labeled with different fluorescent dyes to allow multiplex real-time PCR. The MTC probe was labeled with 4,4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein (HEX) and BHQ1, the *M. canettii*-specific probe with carboxy-X-rhodamine (ROX) and BHQ2, and the IAC probe with Cy5 and BHQ2.

Conventional PCR. Conventional PCR was performed using the sequencing primers outlined in Table 1 on an iCycler iQ thermal cycler (Bio-Rad Laboratories Inc., CA). All reactions were carried out in a final volume of 50 μl containing 5 μl 10× buffer (15 mM $MgCl_2$), forward and reverse primers (final concentrations, 0.2 μM), 2 μl *Taq* DNA polymerase (1 U/μl; Roche Diagnostics, Basel, Switzerland), 1 μl deoxynucleoside triphosphate mix (10 mM deoxynucleoside triphosphate set; Roche Diagnostics), 2 μl of template DNA, and 38 μl nuclease-free water (Applied Biosystems/Ambion, TX). The cycling parameters consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C (1 min), amplification at 55°C (1 min), and extension at 72°C (1 min) and by a final elongation at 72°C for 10 min.

Sequencing. Nucleotide sequence data for real-time PCR assay design were generated in this study or were obtained from either the National Center for Biotechnology Information (NCBI) or the Sanger website (where partial nucleotide sequences for *M. canettii*, *M. africanum*, and *M. microti* were available). The primers used for the real-time PCR assays were also used in conventional PCRs to generate nucleotide sequence information for each of the assays developed. In addition, sequencing primers were designed to span the full 2,869-bp *M. canettii*-specific nucleotide sequence identified, to evaluate if this region is conserved in all *M. canettii* strains tested in this study, and to identify potential diagnostics target nucleotide sequences for probe design.

PCR products were generated as described above, followed by purification using a High Pure PCR product purification kit (Roche Diagnostics). The purified PCR products were sequenced externally (Sequiserve, Vaterstetten, Germany).

Development of IAC for real-time PCR. An IAC targeting the *M. smegmatis lepA* gene was developed for the multiplex real-time PCR. Both the IAC and MTC targets were amplified using the same primer set; however, the IAC probe, targeting *M. smegmatis*, was labeled with Cy5, while the MTC probe was labeled with HEX (Table 1). Titrations of MTC and IAC DNA were performed to determine the optimum concentration of IAC target per reaction mixture such that it was always detected without impacting detection of the primary MTC target (17). An IAC concentration of 500 genome equivalents per reaction mixture allowed the positive detection of the IAC at low concentrations or in the absence of primary target.

The *lepA* gene was chosen as the target for the IAC because sufficient sequence heterogeneity exists between the *M. smegmatis* and MTC *lepA* gene sequences for the design of independent, specific probes. There was also enough homology in the sequence flanking these probe regions to design one set of primers to amplify both MTC and IAC targets. This resulted in three rather than four primer pairs in the multiplex PCR, reducing assay complexity.

Real-time PCR. Monoplex real-time PCR was performed on a LightCycler (version 2.0) instrument (Roche Diagnostics) using a LightCycler TaqMan master kit (Roche Diagnostics). A final volume of 20 μl was used in each reaction mixture, containing 5× master mix, forward and reverse primers (final concentrations, 0.5 mM), FAM-labeled probe (final concentration, 0.2 mM), and template DNA (2 μl), and the volume was adjusted to 20 μl with the addition of nuclease-free distilled H_2O (dH_2O). The cycling parameters consisted of incubation for 10 min at 95°C to activate enzymes and denature DNA, followed by 50 cycles of 95°C for 10 s and 60°C for 30 s, followed by a cooling step at 40°C for 10 s. The temperature transition rate for all cycling steps was 20°C/s.

Multiplex real-time PCRs were carried out on the LightCycler 480 instrument using a LightCycler 480 probes master kit (Roche Diagnostics). A final volume of 40 μl was used for each multiplex reaction mixture. The optimized master mix contained 2× LightCycler 480 probes master (6.4 mM $MgCl_2$), forward and reverse primers (final concentrations, 0.5 μM), FAM-labeled probe (final concentration, 0.4 μM), HEX-, ROX-, and Cy5-labeled probes (final concentrations, 0.2 μM), and template DNA (MTC, 2 μl; IAC, 2 μl; NTM, 10 μl), and the volume was adjusted to 40 μl with the addition of nuclease-free dH_2O. The internal control DNA mixture was diluted to contain 500 genome equivalents per 2 μl, and the NTM DNA consisted of ~10^4 genome equivalents per 10 μl.

The cycling parameters used were the same as those used on the LightCycler 2.0 instrument. The temperature transition rate, referred to as the ramp rate on the LightCycler 480 instrument, was 4.4°C/s while heating and 2.2°C/s while cooling. Prior to experimental analysis on the LightCycler 480 instrument, a color compensation file was generated using the technical note outlined in the Advanced Software Functionalities of the operator manual (2).

Nucleotide sequence accession numbers. Partial *wbbl1* gene nucleotide sequences generated in this study for the five *M. canettii* strains (see Table S1 in the supplemental material) were deposited in GenBank with the following accession numbers: HQ625205 through HQ625209. Nucleotide sequences generated in this study for the proposed novel $RD^{canettii1}$ were also deposited in GenBank with the following accession numbers HQ625200 through HQ625204.

RESULTS

Diagnostics targets identification. The diagnostics nucleotide sequence target identified in this study for detection of the MTC in combination with detection of the IAC was *lepA* (Rv2404c). LepA is an elongation factor required for accurate and efficient protein synthesis capable of inducing backtranslocation of mistranslocated tRNAs. The *lepA* gene is present in all bacteria sequenced to date and codes for one of the most conserved proteins in bacteria (55 to 68% amino acid sequence similarity between bacteria), with a homologue (Guf1) being found in higher organisms (32).

For the specific detection of *M. tuberculosis* and *M. canettii*, a *wbbl1* gene nucleotide sequence target (Rv3265c) was identified. The *wbbl1* gene encodes rhamnosyl transferase, which inserts rhamnose into the cell wall and which is thought to be essential for mycobacterial viability (24). Nucleotide sequence analysis of members of the MTC revealed a 12-bp region of the *wbbl1* gene which is present only in *M. tuberculosis* and *M. canettii* and which has been deleted in all other members of the MTC.

The *M. canettii*-specific diagnostics nucleotide sequence target identified in this study is a region of the genome that appears to be deleted in all other members of the MTC. This 2,869-bp region was discovered while $RD12^{can}$ was mapped to the unfinished genome sequence of *M. canettii* available on the Sanger website. This region appears to be a novel RD specific for *M. canettii*. To date, the *M. canettii* genome is not annotated; therefore, the function of the gene(s) in this region is unknown. The *M. canettii* diagnostics target region identified was BLAST analyzed and revealed similarity to a putative ATP binding protein gene in *Nocardioides* species (query coverage, 52%; maximum indent, 73%). This region was sequenced using the primers listed in Table 1 for the *M. canettii* strains used in this study. Sequence analysis revealed 100% similarity between the five strains and the sequence available on the Sanger website.

Assay design and development. While the guidelines for primer and probe design were adhered to as closely as possible, the high GC content (60 to 65%) of the *Mycobacterium* species did have an impact on assay design. The *wbbl1*-specific probe was based on a region that was present in *M. tuberculosis* and *M. canettii* and deleted in other members of the MTC and that is very G/C rich, making probe design difficult. This probe was labeled with FAM, and double the standard probe concentration (0.4 μM/reaction mixture) was used to improve the endpoint fluorescence, sensitivity, and robustness of the assay.

Internal amplification control. In order for a result to be considered valid using the multiplex real-time PCR assay developed in this study, a positive signal must be obtained in at least one of the four detection channels on the LightCycler 480 instrument. If none of the assay targets or the IAC is detected, the result is considered invalid and the assay must be repeated (17, 28). In this study, *M. smegmatis* DNA is spiked into the PCR master mix to act as an internal control target. Equally, *M. smegmatis* cells could also be used as a process control, when they are spiked into patient samples before total genomic DNA extraction. This could then act as a control for both DNA extraction and PCR efficiency when patient samples are tested.

Specificity of diagnostic assays. The specificity of each real-time PCR assay was confirmed in both monoplex and multiplex formats using the specificity panel listed in Tables S1 and S2 in the supplemental material. The *wbbl1* assay was specific for the detection of the 26 *M. tuberculosis* and the 5 *M. canettii* strains. Figure 1A shows the detection of six *M. tuberculosis* strains (circles) and five *M. canettii* strains (triangles). The remaining members of the MTC, the NTM, and other bacteria were not detected. All 64 MTC strains were detected in the MTC assay (Fig. 1B), and 44 NTM and 17 other bacteria were not detected. The *M. canettii* assay was specific for the five *M. canettii* isolates (Fig. 1C) and did not cross-react with the specificity panel. The specificity of the IAC assay was tested using the MTC panel and was specific for *M. smegmatis* DNA. As the MTC and IAC assays are competitive, the IAC is detected only at low concentrations or in the absence of primary target. In Fig. 1B, the *M. africanum* 3 sample tested in the assay is present at a lower concentration than the other members of the MTC; hence, a stronger amplification signal is observed in the Cy5 channel (IAC) with this sample (Fig. 1D, *M. africanum* 3 represented by diamonds). A simple interpretation of the results that can be obtained from the multiplex real-time PCR developed is outlined in Table 2.

Sensitivity of the assays. The limit of detection (LOD) of each assay was evaluated in a monoplex real-time PCR format. Genomic DNA was quantified and serial dilutions with from 200,000 to 2 genome equivalents were prepared, based on the genome size of *M. canettii* (4,525,000 bp), which equates to approximately 4.9 fg DNA per cell. *M. canettii* was chosen for sensitivity testing of the multiplex assay, as it is detected in each of the primary target assays (the *M. tuberculosis*/*M. canettii*-, MTC-, and *M. canettii*-specific assays).

In the monoplex format, the dilution series was run in duplicate and a sensitivity of 2 to 20 *M. canettii* genome equivalents was determined for each assay. In the multiplex format, the lower limit of detection was established using probit regression analysis. In this analysis, 12 replicates of each of 20, 15, 12, 10, 7.5, 4, 2, and 0.2 genome equivalents were evaluated. LODs of 2.17, 2.20, and 0.73 genome equivalents for the *M. canettii*/*M. tuberculosis*-, MTC-, and *M. canettii*-specific assays, respectively, were determined with 95% probability. The IAC, at a concentration of 500 genome equivalents per reaction mixture, was detected in all samples tested.

DISCUSSION

In 1998, the Stop TB initiative was established with the ultimate goal of obtaining a world free of TB. Within this initiative, a core group, the New Diagnostics Working Group (NDWG), was founded in an effort to establish new diagnostics for TB. This working group, along with a number of collaborators, such as the Foundation for Innovative New Diagnostics (FIND), have launched a new web resource, Evidence-Based Tuberculosis Diagnosis, which highlights the importance of new diagnostics for the rapid and cost-effective detection of TB (29).

At present, literature describing TB NAD assays, in addition to commercially available TB NAD kits (5, 8, 16), is limited, and the scope and capacity of these assays to differentiate the MTC to the species level are also limited. For example, the

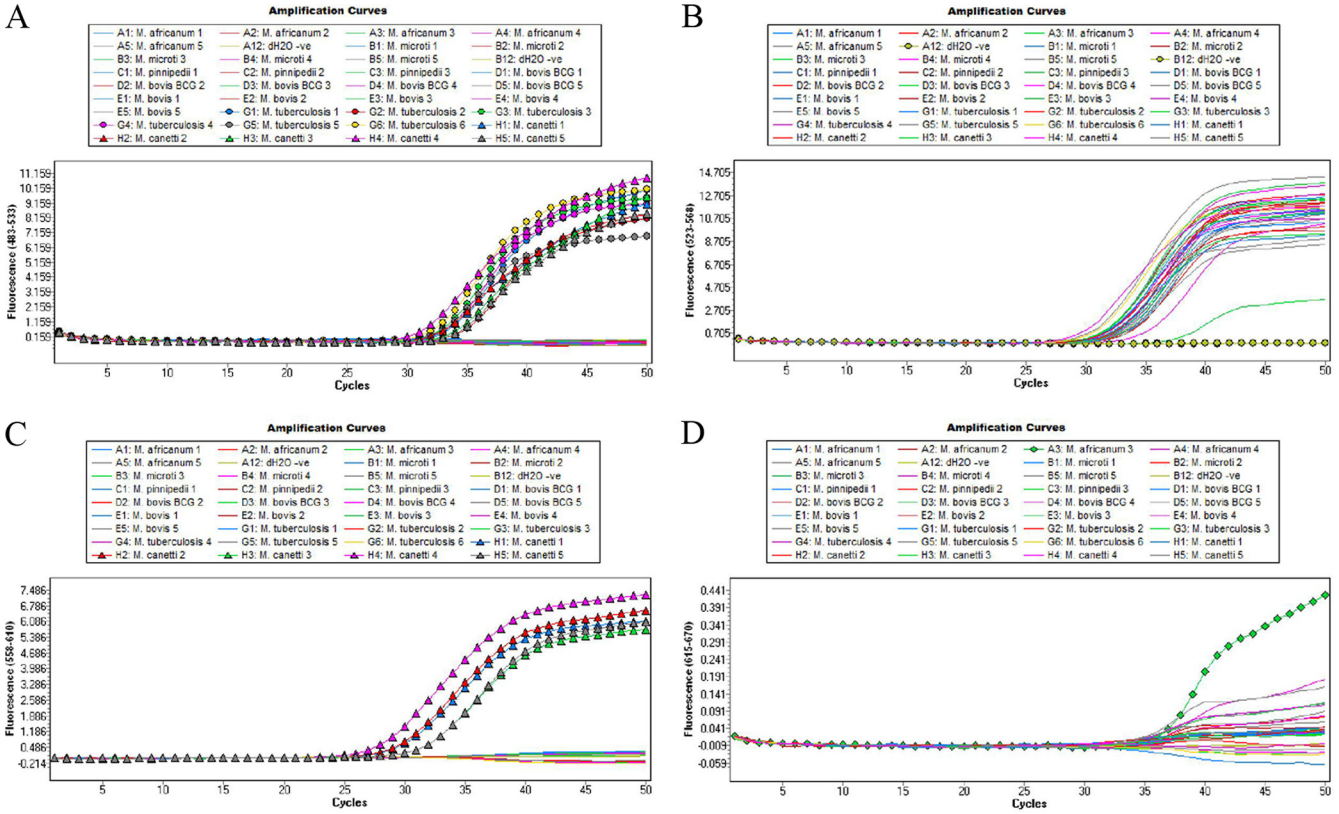

FIG. 1. (A) Real-time amplification curves for *M. tuberculosis* (circles) and *M. canettii* (triangles) using *wbbl1* gene in FAM channel (483 to 533 nm); (B) amplification curves for all MTC using *lepA* gene in HEX channel (523 to 568 nm), with the nontemplate control, highlighted with circles with lines; (C) amplification curves for *M. canettii* specific assay in ROX channel (558 to 610 nm), with the five *M. canettii* strains depicted with triangles; (D) amplification curves for IAC in Cy5 channel (615 to 670 nm), with *M. africanum* 3 highlighted with diamonds through the amplification curve. The reduction of the IAC signal is due to competition from high concentrations of primary target, resulting in competition between the MTC and IAC assays.

GenoType MTBC kit (Hain Lifescience GmbH, Nehren, Germany), which claims to differentiate the members of the MTC, does not in fact differentiate between *M. tuberculosis* and *M. canettii* or between *M. africanum* and *M. pinnipedii* (21). Recently, a study by Pinsky and Banaei (31), which describes a real-time PCR for differentiation of the MTC, fails to differentiate *M. tuberculosis* and *M. canettii* and is also unable to differentiate between *M. africanum*, *M. microti*, *M. pinnipedii*, and *M. caprae*. A capability to identify the specific MTC species causing infection is important for determining the appropriate therapeutic regimen for the patient (34).

TABLE 2. Result interpretation table

Diagnostics result profile				Result
FAM (*wbbl1* assay)	HEX (MTC assay)	ROX (*M. canettii* RD assay)	Cy5 (IAC[a])	
+	+	+	+/−	*M. canettii* present
+	+	−	+/−	*M. tuberculosis* present
−	+	−	+/−	MTC (other than *M. canettii or M. tuberculosis*) present
−	−	−	+	Not a member of MTC
−	−	−	−	Result invalid and test must be repeated

[a] The IAC is detected only in the presence of low concentrations of primary target or in the absence of primary target.

The multiplex real-time PCR assay described in this study uses novel nucleic acid diagnostics targets for the identification of the MTC, *M. tuberculosis*, and *M. canettii*. The first novel target identified was a *lepA* gene nucleotide sequence. This target was used to detect the MTC and to develop the IAC for the real-time PCR diagnostics assay. The second novel molecular target identified and evaluated in this study was a *wbbl1* gene nucleotide sequence target which enables the simultaneous detection of *M. tuberculosis* and *M. canettii* and which is a target with the same diagnostics potential as the RD9 region widely used for *M. tuberculosis* identification. The third novel diagnostics target nucleotide sequence identified allowed the differentiation of *M. canettii* from *M. tuberculosis*. As this nucleotide sequence is present in the five *M. canettii* strains used in this study and is 100% homologous, we propose this to be a novel RD ($RD^{canettii1}$).

An *M. canettii* RD which represents a region of the genome flanking RD12 which is deleted in *M. canettii* but present in *M. tuberculosis* has previously been described by Huard et al. (18). The study uses conventional PCR for differentiation of the MTC, including *M. tuberculosis* and *M. canettii*, on the basis of

PCR product size. The method requires time-consuming multiple reactions and produces results that require detailed interpretation. In the current study, we have identified a putative new RD which is present in *M. canettii* but deleted in *M. tuberculosis* and all other members of the MTC. As this region is present only in *M. canettii*, the interpretation of results is less complex.

The multiplex real-time PCR developed in this study is the first hydrolysis probe-based diagnostic tool capable of rapid detection of the MTC, combined with the detection and differentiation of *M. tuberculosis* and *M. canettii* using novel targets, to be described. This rapid, specific, and sensitive multiplex real-time PCR assay produces a diagnostic result in less than 1 h after DNA extraction. These novel genetic markers will be further optimized and validated against a more extensive range of clinical isolates, in addition to clinical samples, in the future.

While the current study has focused on identifying novel nucleotide sequence diagnostics targets and the development of a multiplex real-time PCR for differentiation of *M. tuberculosis* and *M. canettii*, work has begun in the Microbiology laboratory of the National University of Ireland, Galway, on developing a series of nucleic acid-based diagnostics assays with the ability to differentiate all species of the MTC. For example, there is a requirement to differentiate infection caused by both *M. bovis* and *M. bovis* BCG from the remainder of the members of the MTC, as they, like *M. canettii*, are intrinsically resistant to PZA (35).

Ultimately, there will be a requirement to transfer this set of assays to a platform with a capability of detecting and differentiating the MTC in a single-test diagnostics format. For example, the design of a microarray utilizing these novel MTC diagnostics targets, in combination with diagnostics targets for the determination of MTC drug resistance, could be significantly advantageous. This rapid diagnostics approach would provide the clinician with important information about the optimal therapeutic regimen required for an infected patient while at the same time provide the clinical laboratory with unambiguous MTC epidemiological data.

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