Tuberculosis (TB) in humans is caused by members of the *Mycobacterium tuberculosis* complex (MTC). The accurate identification of the MTC member causing human infection is important because the treatment of TB caused by some MTC members requires an alteration of the standard drug regimen, it can inform whether transmission is human to human or zoonotic, and it enables accurate epidemiology studies that help improve TB control. In this study, an internally controlled two-stage multiplex real-time PCR-based method, SeekTB, was developed for the accurate identification of all members of the MTC. The method was tested against a panel of well-characterized bacterial strains (*n* = 180) and determined to be 100% specific for members of the MTC. Additionally, 125 Mycobacteria Growth Indicator Tube (MGIT)-positive cultures were blindly tested by using SeekTB, and the results were compared to those of the GenoType MTBC and TBc ID tests. The SeekTB and GenoType MTBC results were 100% concordant, identifying 84 of these isolates as *M. tuberculosis* isolates and 41 as non-MTC isolates. Nine discordant results between the molecular methods and the TBc ID culture confirmation test were observed; however, nucleotide sequencing confirmed the results obtained with GenoType MTBC and SeekTB.

SeekTB is the first-described internally controlled multiplex real-time PCR diagnostic method for the accurate identification of all eight members of the MTC. This method, designed for use on cultured patient samples, is specific, sensitive, and rapid, with a turnaround time to results of approximately 1.5 to 3.5 h, depending on which, if any, member of the MTC is present.

Management decisions. This highlights the need for a rapid diagnostic assay that is capable of differentiating the members of the MTC while simultaneously providing information relating to contact and source tracing.

Currently, there is only one commercially available diagnostic assay for the differentiation of the MTC, namely, the Genotype MTBC kit (Hain Lifesciences GmbH, Nehren, Germany). While useful for identifying some members of the MTC, this kit is limited by its inability to accurately identify *M. tuberculosis*, *M. canetti*, *M. africanum*, and *M. pinnipedi* (16). There have also been a number of molecular-based assays for MTC differentiation described in the literature (9, 11, 13, 20, 21). These methods are also limited by an inability to differentiate all members of the MTC, and most of these methods require postamplification processing, which increases method complexity, analysis time, and potential contamination.

Previously, we have reported the design and development of two 4-plex real-time PCR diagnostic assays for the accurate iden-
ification of the MTC, one for the identification of M. tuberculosis and M. canettii and one for the identification of M. bovis, M. bovis BCG, and M. caprae (22, 23). In this study, additional diagnostic targets were incorporated into these 4-plex assays, resulting in two 5-plex real-time PCR diagnostic assay. By incorporating these targets, we have now devised a diagnostic method and an algorithm which allow for the accurate identification of the MTC and the differentiation of all 8 members, including the capability to differentiate between M. africanum West African 1 and M. africanum West African 2. This two-stage method, SeekTB, can be performed in approximately 3.5 h post-DNA extraction.

MATERIALS AND METHODS

Ethics statement. This study was approved by the research ethics review committee of the University of Zambia School of Medicine, Ridgeway Campus, Lusaka, Zambia. All study participants gave written informed consent in accordance with internationally recognized clinical trial standards.

Bacterial isolates used in this study. The panel of MTC isolates, nontuberculosis mycobacteria (NTM), and other bacterial species used in this study (see Tables S1 and S2 in the supplemental material) was the same panel as that described in a previous study (23), with the addition of 47 MTC isolates provided by the National Reference Center for Mycobacteria, Borstel, Germany (characterized as described previously by Alix-Begue et al. [2]). All genomic DNA samples used in this study were isolated, quantified, and stored as described previously (22).

PCR primers and hydrolysis probe design. The novel diagnostic targets used in this study were identified by using in silico comparative genomic approaches previously described (22, 23). All oligonucleotides used in this study were designed in accordance with previously reported guidelines (10, 24). All primers and probes (see Table S3 in the supplemental material) were designed with similar properties to facilitate multiplexing (23).

Conventional PCR and sequencing. Conventional PCR amplification of the RD713 region (2,798-bp M. africanum West African 1) and RD701 (340-bp M. africanum West African 2) was performed according to methods described previously by Huard et al. (14). Genomic DNA was also amplified for sequencing to confirm the identity of some clinical isolates. The sequencing primers (see Table S3 in the supplemental material) were used in conventional PCRs as previously described (22, 23). PCR products were purified and sequenced according to methods described previously by Reddington et al. (23).

Real-time PCR. Multiplex real-time PCRs were performed with a LightCycler 480 instrument using the LightCycler 480 Probes Master kit (Roche Diagnostics). The optimized PCR mix for multiplex 1 contained 2 × LightCycler 480 Probes Master mix (6.4 mM MgCl2); forward and reverse primers (0.5 µM final concentration); Cyan 500-, carboxy-X-rhodamine (ROX)-, and Cy5-labeled probes (0.2 µM final concentration) in addition to 4,4,7,2,4,5,7-hexachloro-6-carboxyfluorescein (HEX)-labeled (0.1 µM final concentration) and 6-carboxyfluorescein (FAM)-labeled (0.4 µM final concentration) probes; dimethyl sulfoxide (4%; Sigma-Aldrich, MO); and template DNA (MTC, 2 µl; internal amplification control [IAC], 2 µl; NTM, 10 µl), adjusted to a final volume of 40 µl with the addition of nuclease-free distilled water (dH2O). The M. smegmatis internal control DNA was diluted to contain 100 genome equivalents per 2 µl, and NTM DNA was diluted to contain ~104 genome equivalents per 10 µl. For multiplex 2, the optimized PCR mix was the same, with the exception of the FAM-labeled probe, where a 0.2 µM final concentration was sufficient. PCR cycling was performed as previously described (22). To avoid fluorescence leaking from channel to channel, a color compensation file was generated (3), and the noise band was set to 1.2 fluorescence units (multiplex 1) and 1.3 fluorescence units (multiplex 2) in the Cyan 500, FAM, HEX, and ROX channels.

Evaluation of SeekTB performance on clinical cultures. Genomic DNA from 125 Mycobacteria Growth Indicator Tube (MGIT)-positive culture samples isolated at the University Teaching Hospital, Lusaka, Zambia, were tested blindly. Positive MGIT cultures were identified as MTC or non-MTC by using the Tbc ID (BD Diagnostics) test, according to the manufacturer’s instructions, before DNA extraction. Isolated DNA samples were also tested by using the GenoType MTBC kit (Hain Life-sciences) according to the manufacturer’s instructions.

RESULTS

Diagnostics targets. During this study, it was determined that the wbbL1 assay, outlined previously for the specific detection of M. tuberculosis-M. canettii isolates (22), also detected M. africanum West African 1. Therefore, an additional assay for the specific detection of M. africanum West African 1 was necessary. Region of difference (RD) 713 was previously identified as specific to M. africanum West African 1, this region is 2,798 bp (14, 27). An assay targeting a 138-bp region of RD713 (primers RD_713Fw and RD_713Rv and probe RD713 [see Table S3 in the supplemental material]) was designed for the specific detection of M. africanum West African 1 and was incorporated into the first multiplex assay. The MTC-specific probe was also redesigned to improve specificity (see Table S3 in the supplemental material).

The second multiplex real-time PCR diagnostic assay was modified from a previously described multiplex assay (23), by incorporating an assay for the specific detection of M. africanum West African 2. PCR primers RD701_FWD and RD_701 and probe RD701 were designed to amplify an 81-bp region of RD 701, an RD specific to M. africanum West African 2 (7, 19, 27) (see Table S3 in the supplemental material). In members of the MTC with intact RD 701, this region is 2,885 bp, whereas in M. africanum West African 2, this region is 340 bp (27).

The incorporation of an additional diagnostic assay to each of the above-described multiplex assays resulted in two 5-plex assays capable of differentiating all 8 members of the MTC and the 2 M. africanum clades (see Fig. S1 and S2 in the supplemental material).

Specificities of the diagnostic assays. The specificities of all monoplex assays were evaluated against a panel of 180 MTC, NTM, and other bacteria (see Tables S1 and S2 in the supplemental material). Subsequently, the specificities of the assays were re-evaluated in a multiplex format. All assays were 100% specific for the target organisms in both monoplex and multiplex formats.

Using multiplex 1, the RD 713 assay was specific for the 5 M. africanum West African 1 isolates tested. The wbbL1 assay was specific for the 60 M. tuberculosis, 8 M. canetti, and 5 M. africanum West African 1 isolates tested. The MTC assay was specific for the 119 MTC isolates tested. The RDcanettii assay was specific for the detection of M. canettii isolates (22), also detected all M. bovis strains tested but detected all M. bovis and M. caprae strains tested, allowing for the specific identification of M. bovis BCG. The IAC assay was 100% specific.

A typical representation of the amplification curves generated
in each of the analysis channels in the two multiplex assays is shown in Fig. S1 and S2 in the supplemental material.

**Sensitivities of the assays.** The lower limit of detection (LOD) of the assays was determined by using probit regression analysis (22, 23). LODs of 9.04, 5.88, 0.4, and 5.08 genome equivalents for the MTC-specific (lepA), *M. tuberculosis-M. canetti-M. africanum* West African 1-specific (*wbbl1*), *M. canetti*-specific (RDcanettii1), and *M. africanum* West African 1-specific (RD713) assays, respectively, were determined.

For multiplex 2, LODs of 5.66, 6.05, 98.28, and 24.9 genome equivalents for the *M. bovis-M. bovis* BCG-*M. caprae* (*lpqT*), *M. bovis-M. caprae* (RD1), *M. caprae* (lepA), and *M. africanum* West African 2 (RD701) assays, respectively, were determined. In both multiplex assays, the IAC at a concentration of 100 cell equivalents was detected in all samples tested.

**Interpretation of results.** The interpretation of the multiplex assay results was performed as described in Table 1. When isolates are tested, multiplex 1 should always be performed first. If the sample is positive for a member of the MTC but *M. tuberculosis*, *M. canetti*, or *M. africanum* West African 1 is not identified, multiplex 2 should subsequently be performed. Multiplex 2 directly identifies *M. bovis, M. bovis* BCG, *M. caprae*, and *M. africanum* West African 2. To identify the remaining members of the MTC, namely, *M. microti* and *M. pinnipedii*, the combined results from both multiplex assays must be taken into account. Assay results can be easily interpreted according to Table 1.

### Table 1: Result scenario for each member of the MTC

<table>
<thead>
<tr>
<th>Multiplex assay</th>
<th>Result with analysis channel and target</th>
<th>Result interpretation</th>
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<tbody>
<tr>
<td>1</td>
<td><strong>Cyan 500</strong></td>
<td><strong>FAM</strong></td>
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<td>RD 713</td>
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<td>2</td>
<td><em>M. caprae</em> lepA</td>
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<sup>a</sup>+, positive signal observed; –, positive signal not observed.

<sup>b</sup>The interpretations of the results of the multiplex 2 assay take into account the multiplex 1 results.

### Discussion

The accurate differentiation of members of the MTC is necessary to (i) perform epidemiological studies, (ii) monitor whether TB transmission is human to human or zoonotic (9, 21), and (iii) administer appropriate anti-TB treatment, due to some members of the complex displaying natural resistance to PZA (1, 26).

The method described in this study, SeekTB, is composed of two 5-plex real-time PCR assays which can be performed sequentially. The first multiplex diagnostic assay has the capability of detecting the presence of the MTC isolate while simultaneously differentiating *M. tuberculosis, M. canetti*, and *M. africanum* West African 1, taking approximately 1.5 h to complete after DNA extraction. For the majority of TB cases, only the first multiplex assay would have to be performed, as *M. tuberculosis* is thought to be the causative agent of approximately 95% of human infections (6). The second multiplex diagnostic assay would be performed if a member of the MTC was detected in the sample but *M. tuberculosis, M. canetti*, or *M. africanum* West African 1 was not identified. The second multiplex assay enables the specific identification of the remaining members of the complex, namely, *M. bovis, M. bovis* BCG, *M. caprae, M. africanum* West African 2, *M. microti*, and *M. pinnipedii*. The sequential performance of both tests takes approximately 3.5 h after DNA extraction.

The specificity of the diagnostic targets used in this study was initially tested by using a panel of well-defined MTC isolates. These isolates were previously characterized by using methods such as spoligotyping, mycobacterial interspersed repetitive-units–variable-number tandem-repeat (MIRU-VNTR) analysis, IS6110-based typing methods, RD analysis, and biochemical testing in addition to morphological examination. The developed method demonstrated 100% agreement with the previously used methods, correctly identifying all members of the MTC and demonstrating the robustness of the rapid tests developed.
A further evaluation of the SeekTB method was performed by blindly testing a panel of uncharacterized positive MGIT cultures and comparing the results with those generated by using the GenoType MTBC test. The results of both molecular methods demonstrated 100% agreement; however, some discordance was observed when the results of these molecular methods were compared to culture results. The concordant molecular method results were indicative of true results in this study, as confirmed by nucleotide sequencing.

While the characterization of the MGIT-positive culture samples resulted in the identification of M. tuberculosis only, it should be noted that this is a relatively small number of isolates from one urban geographical region. MGIT-positive culture sample microbiobanks with a high likelihood of containing a variety of members of the MTC are required to further validate SeekTB (and the novel diagnostic targets used). The additional testing of a larger panel of MGIT-positive culture samples from this region and other regions worldwide will be performed in future.

When comparing the two molecular methods tested, the SeekTB method offers a number of advantages over the GenoType line probe assay: the developed method is less laborious, requiring less hands-on time; there is no need for the postamplification handling of samples, which significantly reduces the likelihood of introducing contamination; there is an enhanced sensitivity with the real-time-PCR-based SeekTB method; and SeekTB has the ability to accurately identify M. tuberculosis, M. canetti, M. pinnipedii, and both clades of M. africanum in addition to the members of the MTC identifiable by using the GenoType MTBC test.

In conclusion, the diagnostic method developed in this study, SeekTB, is the first-described internally controlled, multiplex-PCR-based method capable of rapidly and accurately identifying all members of the MTC. This method has been validated on a large panel of well-characterized MTC isolates and has been successfully used for the culture confirmation of positive African MGIT cultures. Ultimately, SeekTB needs to be further tested in the field, both on positive culture samples and directly on clinical samples, such as sputum, to demonstrate its potential and robustness for the specific identification and differentiation of isolates of the MTC.

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