Direct Comparison of the GenoType MTBC and Genomic Deletion Assays in Terms of Ability To Distinguish between Members of the Mycobacterium tuberculosis Complex in Clinical Isolates and in Clinical Specimens

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The automated GenoType MTBC assay was evaluated for the ability to detect and identify members of the Mycobacterium tuberculosis complex. In addition to 35 reference strains and 157 clinical isolates, performance of this assay was tested directly on 79 smear-positive clinical specimens. The assay proved as accurate as the reference deletion analysis for all 192 isolates and detected and identified M. tuberculosis complex members in 93.2% of the specimens containing the M. tuberculosis complex.

The Mycobacterium tuberculosis complex is composed of the closely related organisms M. tuberculosis, M. africanum, M. bovis, and M. bovis BCG and four other, rarely seen members, M. caprae, M. microti, M. canettii, and M. pinnipedii (3, 5). Rapid and reliable identification of the M. tuberculosis complex is critical in guiding public health and primary care decisions. This is because each organism exhibits a different epidemiology, host spectrum, geographic range, primary care decisions. This is because each organism exhibits a different epidemiology, host spectrum, geographic range, and drug susceptibility (14). Recently, comparative genomics of the M. tuberculosis complex identified several regions (regions of difference; RD), ranging in size from 2 to 12.7 kb, that were present in M. tuberculosis H37Rv and absent in other members of the M. tuberculosis complex. These results suggested that deletion of genomic regions has been important in generating genetic diversity within the complex (3, 14). Based on these findings, our laboratory developed a rapid PCR-based genomic deletion analysis, using six RDs that are either common to various members of the M. tuberculosis complex or specific to each one. This approach was based on an initial screening for RD1, RD9, and RD10, with a reflex test for RD3, RD5, and RD11 (14). To further improve and simplify this method, we later replaced the reflex panel with assays targeting RD4 and RD12; this modified assay was validated with 1,685 M. tuberculosis complex clinical isolates (15). More recently, a multiplex PCR-based, solid-phase reverse hybridization GenoType MTBC assay (Hain Lifescience GmbH, Nehren, Germany) was developed, based on the detection of single nucleotide polymorphisms of the gyrB gene and on the presence or absence of RD1 (17, 18). This assay provides a rapid and accurate method to identify the presence of various members of the M. tuberculosis complex (8, 13, 17, 18) when it is used with growth-positive cultures. The first goal of the present study was to evaluate the automated version of the GenoType MTBC assay on a set of well-characterized reference strains and a large number of clinically significant isolates, using PCR-based deletion analysis as a reference method. The second goal of the study was to determine the sensitivity and accuracy of the GenoType MTBC assay when it was used directly on smear-positive clinical specimens.

A total of 35 phenotypically and genetically well-characterized (10, 14, 15, 21) reference mycobacterial strains and 157 clinical M. tuberculosis complex isolates, which were obtained from samples submitted to the Wadsworth Center, were used in the study (Table 1). In addition, 79 acid-fast bacillus smear-positive specimens were analyzed. These included 59 clinical specimens that had been received for routine mycobacterial testing between January 2002 and June 2007 and that had been shown to be M. tuberculosis complex positive (Wadsworth Center) and 20 clinical specimens that had been shown to be M. tuberculosis complex negative (Florida Bureau of Laboratories) by Amplified MTD (Mycobacterium tuberculosis) direct tests (Gen-Probe Incorporated, San Diego, CA). Only one specimen per patient was used in the present analysis. The specimens were initially digested and decontaminated by a modified Petroff's NaOH method (Wadsworth Center) and by a NALC-NaOH method (Florida Bureau of Laboratories) (10, 21). Smears were prepared with 0.1 ml of the concentrated sediment, using the Ziehl-Neelsen acid-fast staining method (10). The grade of smear positivity was available retrospectively for 55 specimens, as follows: numerous, 16 specimens (29.1%); moderate, 11 specimens (20.0%); few, 18 specimens (32.7%); and rare, 10 specimens (18.2%). After inoculation for growth detection (BACTEC 12B/MGIT 960 and Lowenstein-Jensen Gruft), the leftover sediment was stored at −80°C. Growth detection for mycobacteria and identification of members of the M. tuberculosis complex by PCR-based deletion analysis of acid-fast growth-positive cultures were performed as described below.

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TABLE 1. *M. tuberculosis* complex and NTM reference strains and clinical *M. tuberculosis* complex isolates examined in this study

<table>
<thead>
<tr>
<th>Strain or isolate</th>
<th>M. tuberculosis complex reference strains (n = 25)</th>
<th>M. africanum ATCC 35711</th>
<th>M. bovis ATCC 35721</th>
<th>M. bovis ATCC 35724</th>
<th>M. bovis ATCC 35725</th>
<th>M. bovis ATCC 35726</th>
<th>M. bovis ATCC 35729</th>
<th>M. bovis ATCC 35730</th>
<th>M. bovis ATCC 19210</th>
<th>M. bovis BCG ATCC 35731</th>
<th>M. bovis BCG ATCC 35732</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. caprae IS 230177</td>
<td>M. bovis BCG ATCC 35733</td>
<td>M. caprae IS 230177</td>
<td>M. caprae IS 230177</td>
<td>M. caprae IS 230177</td>
<td>M. caprae IS 230177</td>
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<td></td>
<td>NTM reference strains (n = 10)</td>
<td>M. intracellulare ATCC 13950</td>
<td>M. kansasii ATCC 12478</td>
<td>M. gordoniae ATCC 14470</td>
<td>M. fortuitum ATCC 6841</td>
<td>M. ulcerans ATCC 19423</td>
<td>M. phlei ATCC 11758</td>
<td>M. flavesens ATCC 14474</td>
<td>M. gastri ATCC 15754</td>
<td>M. celatum ATCC 51131</td>
<td>M. heidelbergense ATCC 51253</td>
</tr>
</tbody>
</table>

Clinical *M. tuberculosis* complex isolates
Total (n = 157 [100%])
*M. tuberculosis* (n = 38 [24.2%])
*M. africanum* (n = 47 [29.9%])
*M. bovis* (n = 37 [23.6%])
*M. bovis* BCG (n = 34 [21.7%])
*M. caprae* (n = 1 [0.6%])

For clinical specimens, the GenoType MTBC assay was carried out as described below, with the following modifications (1). One milliliter of decontaminated and concentrated specimen was centrifuged at 10,000 × g for 15 min, the supernatant was discarded, and the pellet was resuspended in 300 μl of sterile distilled water. The specimen was then heat killed at 95°C for 20 min in a heat block. This was followed by a 15-min sonication step in a Transsonic 460/H (Elma, Germany) sonicator. After sonication, 5 μl of the supernatant was used immediately for amplification, while the rest was stored at −20°C.

The growth medium was then incubated at 37°C and held for 6 to 8 weeks before the sample was reported as negative. When they were positive, the strains were identified as members of the *M. tuberculosis* complex by means of a PCR-based deletion analysis; non-*M. tuberculosis* complex mycobacteria were identified using PCR restriction analysis (23). Prior to amplification by PCR, 200-μl aliquots of reference strain cultures and growth-positive liquid cultures of clinical specimens were incubated at 80°C for 1 h to heat kill mycobacterial cells. Genomes of the isolates were then analyzed by PCR for the presence or absence of five regions (RD1, RD4, RD9, RD10, and RD12) as described earlier (14, 15). Three primers were included in each assay: two primers were specific for the sequences that flanked the region, and the third was specific for an internal sequence close to one of the flanking primers. The sizes of the PCR products were used to determine the presence or absence of each region. The primer sequences and PCR product sizes are listed in Table 2. Control molecular tests were performed with the *M. tuberculosis* H37Rv ATCC 2794 strain and with a master mix with distilled water used as a negative control.

For reference and clinical isolates, the GenoType MTBC assay was carried out according to the manufacturer’s instructions, with 5 μl of heat-killed bacterial cells used in the amplification mixture (17, 18). Each biotin-labeled PCR product was denatured and hybridized to a strip with 13 specific oligonucleotide probes, using a heat-controlled washing and shaking automaton (GT-Blot 48; Hain Lifescience GmbH, Nehren, Germany). The specificity and targeted genes (in parentheses) of the probes were as follows: 1, conjugate (hybridization) control; 2, *Mycobacterium* genus-specific amplification control (23S rRNA); 3, *M. tuberculosis* complex-specific probe for identification control (23S rRNA); 4 to 12, discriminative for *M. tuberculosis* complex species ( gyrB), and 13, *M. bovis* BCG-specific probe (RD1). Six different patterns could be obtained.
TABLE 3. Results of GenoType MTBC assay used directly on 59 smear-positive and M. tuberculosis complex-positive clinical specimens

<table>
<thead>
<tr>
<th>PCR-based deletion analysis identification for growth-positive cultures (reference method) (n)</th>
<th>No. (%) of conclusive GenoType MTBC assay results with smear-positive and M. tuberculosis complex-positive clinical specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis (48)</td>
<td>44* (91.6)</td>
</tr>
<tr>
<td>M. africanum (6)</td>
<td>6 (100)</td>
</tr>
<tr>
<td>M. bovis (4)</td>
<td>4 (100)</td>
</tr>
<tr>
<td>M. bovis BCG (1)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Total (59)</td>
<td>55 (93.2)</td>
</tr>
</tbody>
</table>

* Amplification was unsuccessful for three genes (23S rRNA, RD1, and gvrB) in four specimens with the GenoType MTBC assay; therefore, a conclusive result could not be obtained.

(M. tuberculosis or M. canettii, M. africanum, M. bovis, M. bovis BCG, M. caprae, and M. microti). An evaluation sheet showing the positions of the lines and the interpretation table provided with the kit were used to evaluate the GenoType MTBC results as described elsewhere (17, 18).

In the first phase of the study, the sensitivity and specificity of the GenoType MTBC assay were evaluated with a set of reference ATCC strains and other previously well-characterized strains of the M. tuberculosis complex (n = 25) and non-tuberculosis mycobacteria (NTM) (n = 10) (Table 1). With the exception of M. canettii, which was identified as M. tuberculosis, the GenoType MTBC test correctly identified all members of the M. tuberculosis complex. The specificity of the assay was 100%, since hybridization was detected only with the mycobacterium-specific probe when the NTM were tested.

In addition, a large number (n = 157) of clinical M. tuberculosis complex isolates (Table 1) were analyzed by the GenoType MTBC test; these had been identified by PCR-based deletion analysis (10, 14, 15, 21). Of these isolates, 38 (24.2%) were identified as M. tuberculosis, 47 (29.9%) were identified as M. africanum, 37 (23.6%) were identified as M. bovis, 34 (21.7%) were identified as M. bovis BCG, and 1 (0.6%) was identified as M. caprae. These results were in full agreement with the reference deletion analysis. In addition, both the GenoType MTBC test and the deletion analysis were able to readily identify M. tuberculosis and M. africanum in two mixed cultures that also contained M. avium.

Growth detection and PCR-based deletion analysis identified 48 M. tuberculosis, 6 M. africanum, 4 M. bovis, and 1 M. bovis BCG strain in the set of 59 smear- and MTD-positive specimens. The GenoType MTBC test correctly detected M. tuberculosis complex with the M. tuberculosis complex-specific capture probe in 58 (98.3%) of these 59 specimens. The GenoType MTBC test correctly identified the presence of M. tuberculosis in 44 (91.6%) of the 48 specimens containing M. tuberculosis and in all other specimens (Table 3). For four M. tuberculosis specimens (6.7%), amplification was unsuccessful for all three target regions (23S rRNA, RD1, and gvrB), presumably due to the presence of PCR inhibitors. We did not see any correlation with the grade of smear positivity in these four specimens.

Growth detection and PCR restriction analysis identified 14 M. avium complex, 4 M. abscessus, and 2 M. kansasii strains in the set of 20 smear-positive and MTD-negative specimens. The GenoType MTBC test was correctly positive only for the conjugate (hybridization) control and the Mycobacterium genus-specific amplification control (23S rRNA) with these specimens.

Previously, our laboratory developed a PCR-based genomic deletion analysis that facilitated the rapid, reliable, and routine identification of members of the M. tuberculosis complex (14).

In the present study, the GenoType MTBC assay exhibited a sensitivity and specificity equal to those of our deletion analysis for all reference and clinical M. tuberculosis complex isolates, with the exception of M. canettii (Table 1). Thus, M. canettii cannot be differentiated from M. tuberculosis by the GenoType MTBC assay, but this test can differentiate M. canettii from other members of the complex (14). Alternatively, M. canettii can be identified rapidly by deletion analysis or by a recently identified M. canettii-specific silent mutation of the pncA gene (20). Both the GenoType MTBC test and our deletion analysis are simple to perform and can be incorporated easily into the routine clinical workflow. This is in contrast to other molecular methods (e.g., spoligotyping or DNA sequencing), which are not within the scope of many laboratories’ capabilities and are less accurate in identifying members of the M. tuberculosis complex (7).

In addition, the GenoType MTBC assay provided a reliable direct detection method for identification of members of the M. tuberculosis complex for 93.2% of the smear-positive and M. tuberculosis complex-positive specimens (Table 3), with an excellent specificity, since none of the 20 smear-positive and MTD-negative specimens containing NTM gave a false-positive result for the M. tuberculosis complex or any members of the complex with the assay. The ability to differentiate accurately among the members of the M. tuberculosis complex has important public health and primary care ramifications. First, it allows health care professionals to better determine the level and target (humans or animals) of contact tracing and to exclude pyrazinamide from the treatment regimen in cases of M. bovis infection given this organism’s natural resistance to pyrazinamide. Second, it can help physicians to identify severe side effects of M. bovis BCG treatment in patients with bladder cancer and in other immunocompromised patients (9, 19, 26). Third, it can help analysts to intercept the transmission link more rapidly in situations of outbreak due to M. bovis transmission between animal products and humans (4) or to reduce transmission of the disease between animals, especially if they belong to endangered species (2, 11). Lastly, it can help epidemiologists to accumulate more information on the epidemiology and clinical significance of various members of the complex in geographic areas where unusual members of the complex are more common than is currently recognized (6, 12, 16).

Importantly, the turnaround time to detect and identify members of the M. tuberculosis complex with the GenoType MTBC assay can be well in line with the recommended 2 to 3 weeks by the CDC, especially when the test is used directly on clinical specimens (22, 24). The direct application of the assay to clinical specimens may also fulfill the Healthy People 2010 initiative, which recommends that the average time to confirm and report 75% of tuberculosis cases be within 48 h (25). A major advantage of the GenoType MTBC assay over deletion analysis is automation of the detection phase of the assay,
which enables the parallel hybridization of 48 samples and makes this test highly suitable for laboratories managing large specimen loads.

In conclusion, the GenoType MTBC assay, though not yet U.S. FDA approved, is an automated, easy-to-use, and reliable method that can be used either in combination with broth-based culture systems or directly on smear-positive clinical specimens for the routine identification of members of the *M. tuberculosis* complex in any mycobacteriology laboratory.

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REFERENCES