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GenoType Mycobacterium Assay for Identification of Mycobacterial Species Isolated from Human Clinical Samples by Using Liquid Medium

P. Ruiz, J. Gutierrez, F. J. Zerolo, and M. Casal*

University Hospital "Reina Sofía," Department of Microbiology, Faculty of Medicine, Mycobacteria Reference Center, 14004 Cordoba, Spain

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The GenoType Mycobacterium assay was used to identify 98 mycobacteria isolates by using liquid cultures from positive BACTEC, MGIT, and ESP bottles. This system identifies 16 mycobacteria. There was complete agreement between the GenoType results and the laboratory identifications for *Mycobacterium tuberculosis* complex and other *Mycobacterium* spp. GenoType also identified mixed mycobacterial infections.

While *Mycobacterium tuberculosis* complex strains are still responsible for the majority of mycobacterium infections worldwide, opportunistic infections caused by mycobacteria other than *M. tuberculosis* have increased, mainly as a consequence of several factors such as the AIDS epidemic (2). The use of culture in liquid media in clinical mycobacteriology laboratories improves the ability to detect growth of *Mycobacterium* spp. (11). Identification of a mycobacterium growing on solid medium can be done by biochemical methods, such as thin-layer chromatography (5), gas-liquid chromatography (16), high-performance liquid chromatography (HPLC), DNA probes, or direct sequencing (3, 13, 15). Reverse hybridization assays can be performed directly from liquid culture for rapid identification of *M. tuberculosis* complex (9). Unfortunately, these tests only detect a limited number of mycobacteria species of clinical significance other than *M. tuberculosis*.

The GenoType assay (Hain Diagnostika, Nehren, Germany) allows for the identification of 16 different mycobacterial species that are most frequently isolated in the clinical laboratory. Isolation is commonly done by PCR amplification of the 16S-23S ribosomal DNA spacer region followed by hybridization of the biotinylated amplified DNA products with 16 specific oligonucleotide probes. The specific probes are immobilized as parallel lines on a membrane strip (1). The goal of this study was to evaluate the GenoType assay for the identification and differentiation of specific mycobacteria species directly from positive liquid cultures, an approach not previously published for this assay.

The GenoType assay was evaluated for specificity and speed of the procedure in a routine clinical laboratory. Ninety-eight clinical specimens from different patients were submitted for culture, decontaminated with an equal volume of *N*-acetyl cysteine–4% NaOH, and concentrated by centrifugation. The sediment was used to inoculate Middlebrook 7H11 agar, BACTEC 12B broth, MGIT broth, and ESPII Myco broth. The cultures were incubated at 37°C for 6 weeks according to the manufacturer's instructions. When a BACTEC culture had a growth index (GI) of ≈100, it was visibly positive by Ziehl-Neelsen stain. In MGIT the microscopy was positive from 100 growth units (GU), and in ESPII the microscopy was positive the same day as the culture was identified as positive by the ESP instrument. In those positive cultures with values lower than the ones mentioned, results with the stain were negative and the absence of contamination was confirmed.

Positive cultures were identified by DNA probes (Accuprobe; Gen-Probe, Inc.), conventional biochemical tests performed according to standard protocols, and HPLC (6, 10, 12). For identification with the GenoType assay, we used all positive cultures from Middlebrook 7H11 agar, MGIT positives bottles with ≥135 GU, BACTEC positive bottles with a GI of ≥30, and ESPII positive bottles on the same day that they were detected as positive.

The initial procedure for solid medium was to dilute a small amount of colonies with 1.0 ml of TE buffer (10 nM Tris-HCl [pH 8.0], 1 nm EDTA). For liquid media, 1 to 2 ml was centrifuged at 3,000 × *g* for 5 min, the supernatant was discarded, and the pellet was diluted with 1.0 ml of TE buffer. All test samples were boiled at 95°C for 15 min in order to extract DNA. Amplification was performed as follows: 5 μl of extracted DNA was added to 45.5 μl of reagent mix, which consisted of 35 μl of primer-nucleotide mix, 5 μl of amplification buffer, 5 μl of $MgCl_2$, and 0.5 μl of *Taq* polymerase. The amplification mix was placed into a Perkin Elmer 9600 thermal cycler and run for 1 cycle at 95°C for 15 min, 10 cycles at 95°C for 30 s and 60°C for 2 min, 20 cycles at 55°C for 3 s and 72°C for 3 s, and 1 cycle at 72°C for 8 min. The detection of the amplified products was performed on agarose gel.

For the hybridization step, a 50°C shaking water bath and a tray for strips were required. A volume of 20 μl of the amplified product was mixed carefully with 20 μl of denaturation solution and incubated for 5 min at room temperature. One milliliter of the hybridization buffer was added to the strips with the immobilized probes and incubated for 30 min in a 50°C shaking water bath, followed by washing twice in 1.0 ml with stringent wash solution for 1 min. This step was followed by incubation in 1.0 ml of the stringent solution for 15 min in the 50°C shaking water bath and by washing twice using Rinse

* Corresponding author. Mailing address: University Hospital "Reina Sofia," Department of Microbiology, Mycobacteria Reference Center, Cordoba University, 14004 Cordoba, Spain. Phone: 34-957-218284. Fax: 34-957-218229. E-mail: mi1carom@uco.es.

TABLE 1. Summary of mycobacterial species identification by all methods tested

Organism	No. of isolates	No. of isolates positive by:			
		GenoType	Probe	Biochemical testing	HPLC
M. tuberculosis complex (MTB)	47	47	47		
M. avium	8	8	8		
M. intracellulare	2	2	2		
M. kansasii	2	2	2		
M. gordonae	8	8	8		
M. fortuitum	4	4		4	4
M. chelonae	6	6		6	6
M. peregrinum	6	6		6	6
M. marinum	4	4		4	4
M. xenopi	2	2		2	2
M. interjectum	2			2	2
M. diernohferi	2			2	2
M. gadium	1			1	1
MTB + *M. fortuitum*	2	2		2	2
MTB + *M. avium*	1	1		1	1
MTB + *M. kansasii*	1	1		1	1

I solution (GenoType; Hain Diagnostika). One milliliter of diluted conjugate (1:100 in conjugate buffer) was added and incubated for 30 min on a shaking platform at room temperature. The conjugate was removed and the strips were washed three times with 1.0 ml of Rinse II solution. It was then aspirated and 1.0 ml of substrate was added. The sample was then incubated for 10 to 15 min in the dark without shaking. The color development was stopped by rinsing the strips twice with distilled water. The strips were transferred to a paper towel for drying. Once the strips were completely dry, the results were interpreted using the reading card provided by the manufacturer (Hain Diagnostika). Positive bands corresponded to different *Mycobacterium* species. In order to identify mixed infections, mycobacteria cultured on Middlebrook 7H11 were incubated at 28, 37, and 40°C for 60 days. Positive cultures were assessed microscopically. When more than one mycobacterial colony was present, each type was characterized using biochemical and genetic procedures or liquid chromatography (HPLC) (8).

All positive cultures were tested in the GenoType assay from both solid medium and all of the liquid media.

The GenoType assay identified 93 mycobacteria isolates at species level and 5 at genus level. There was agreement between the GenoType assay results and the laboratory identification tests for the following isolates: 47 *M. tuberculosis* complex, 8 *M. avium*, 2 *M. intracellulare*, 2 *M. kansasii*, 8 *M. gordonae*, 4 *M. fortuitum*, 6 *M. chelonae*, 6 *M. peregrinum*, 4 *M. marinum*, and 2 *M. xenopi* (Table 1). In two clinical samples, bands were present for both *M. tuberculosis* and *M. fortuitum*. Results from the 7H11 agar plates confirmed the presence of two mycobacteria strains. The same results were observed in one case each of *M. tuberculosis* with *M. avium* and *M. tuberculosis* with *M. kansasii*.

There were 2 isolates of *M. interjectum*, 2 *M. diernohferi* isolates, and 1 *M. gadium* isolate that were identified to only the genus level. The GenoType assay does not include species-specific probes for these organisms.

Of the 98 total samples, smears were positive in 45 and negative in 53 samples. All samples were identified by the GenoType assay and cultured on Middlebrook agar. The BACTEC GI for *M. tuberculosis* was ≤100 for 16 of 47 strains; for 11 strains, the GI was between 100 and 500; for 7 strains, the GI was between 500 and 900; and for 13 strains, the GI was 999. For other mycobacteria the results were as follows: for eight isolates of *M. avium* the growth index was ≤184; for two isolates of *M. intracellulare*, the GIs were 230 and 250; for two isolates of *M. kansasii*, the GIs were 214 and 58; for seven of eight isolates of *M. gordonae*, the GI was 999, and for one of these eight isolates the GI was 630; for four isolates of *M. fortuitum*, the GI was less than 100; for *M. chelonae* and *M. peregrinum*, the GI was less than 215. For *M. marinum*, *M. xenopi*, and the other coexistent strains, the GI was 999. Overall for the BACTEC 460 system, 25 isolates were identified with a GI of less than 100, 20 isolates had a GI between 100 and 500, and 40 isolates had a GI of greater than 500. Using the MGIT system, of the 47 positive isolates identified as *M. tuberculosis*, 18 isolates had a GU of ≤1,000, 14 isolates had a GU of 1,000 to 2,000, 10 isolates had a GU of 2,000 to 4,000, and 5 isolates had a GU of ≥2,000. For the other mycobacterial isolates, 22 had a GU of ≤1,000, 3 had a GU between 1,001 and 2,000, 12 isolates had a GU of 2,001 to 4,000, and 12 isolates had a GU of >4,000. On the ESPII system, 12 *M. tuberculosis* isolates were identified between 5 and 7 days, 16 were identified between 7 and 10 days, and 19 were identified between 10 and 15 days. For the other mycobacterial isolates, the identification required the following time: 5 to 7 days for 23 isolates, 8 to 10 days for 6 isolates, and 11 to 16 days for 17 isolates.

The GenoType assay was laborious to perform, but the interpretation of the results was clear and easy. DNA probe tests are more rapid than the GenoType assay and require only the extraction and hybridization step with little manipulation. The GenoType assay requires extraction, amplification, and hybridization, which require more labor. The main advantage of the GenoType assay over GeneProbe DNA probes and LiPA assays is that the GenoType assay can identify a wider range of species in a single assay, which eliminates the need to perform different tests for each species or the wait for growth on solid medium to guide the identification algorithm. DNA probe tests do not identify mixed infections containing different *Mycobacteria* strains in a single analysis, which can give false results if other assays are not performed in conjunction with the probe tests. The GenoType assay identifies coinfections by different mycobacteria, which can result in greater clinical relevance. (4, 7, 14, 17).

According to kit costs, the price for detection by the GenoType assay is about 95 to 100 Euros. The price is similar to GenProbe's test, which contains only four identification probes, compared to the GenoType assay which can identify 16 mycobacteria strains and coinfections by different mycobacterial species. However, one single test from Gen Probe is less expensive (23 Euros). A disadvantage of the restriction fragment length polymorphism method is that it requires more technical expertise in order to obtain a correct interpretation of results than the GenoType assay. A disadvantage of HPLC is that a larger quantity of culture is required in order to perform the identification.

In our laboratory, 48% of mycobacterial isolates were *M. tuberculosis* complex as identified by DNA probes. Additionally, 8.1% of the isolates were identified as *M. avium*, 2.0% as *M. intracellulare*, 2.0% as *M. kansasii*, and 8.1% as *M. gordonae* using DNA probes. The GenoType assay was able to identify these isolates directly from the positive BACTEC 12B, MGIT, and ESPII broths with a single assay. The assay could also identify *M. fortuitum*, *M. chelonae*, *M. peregrinum*, *M. marinum*, and *M. xenopi*, which comprised 4.08, 6.1, 6.1, 4.08, and 2.04% of the isolates, respectively. Overall, the GenoType assay correctly identified 94.9% of all isolates in our laboratory to the species level. The remaining 3.1% of isolates are miscellaneous organisms such as *M. interjectum*, *M. diernohferi*, and *M. gadium*, for which the GenoType assay does not have a species-specific probe. With these results, we validated the system for species identification from cultured material in the BACTEC system with a GI of ≥30, in MGIT with a GU of ≥135, and in ESPII after 5 days of incubation. The data include samples that were acid-fast bacillus smear negative.

Prior to this study, the GenoType assay had been tested only on ATCC strains grown on solid media. In this study we have validated the performance of the assay directly from positive BACTEC, MGIT, and ESPII liquid media for the following isolates: *M. avium, M. celatum, M. chelonae, M. fortuitum* 1*, M. fortuitum* 2*, M. gordonae, M. intracellulare, M. kansasii, M. malmoense, M. peregrinum, M. phlei, M. scrofulaceum, M. tuberculosis* complex*, M. avium* complex*, M. marinum*, and *M. xenopi.*

In conclusion, we found the GenoType assay to be an easy-to-interpret and rapid test to perform in the clinical setting. The advantages of the test are that (i) it provides identification of a large variety of mycobacterial species in a single test and (ii) it can identify multiple mycobacterial species within the same sample.

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