JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2004, p. 4303–4306 Vol. 42, No. 9
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.9.4303–4306.2004

Usefulness of the GenoType MTBC Assay for Differentiating Species of the *Mycobacterium tuberculosis* Complex in Cultures Obtained from Clinical Specimens

Elvira Richter,[1]* Michael Weizenegger,[2] Anne-Marie Fahr,[2] and Sabine Rüsch-Gerdes[1]

Forschungszentrum Borstel, National Reference Center for Mycobacteria, Borstel,[1] and Department of Microbiology, Laboratory Group, Heidelberg,[2] Germany

Received 22 January 2004/Returned for modification 27 February 2004/Accepted 13 May 2004

A novel DNA strip assay, GenoType MTBC, was evaluated for differentiation of *Mycobacterium tuberculosis* complex species from 77 positive liquid cultures in clinical practice. Species identification (*M. tuberculosis* [71 strains], *Mycobacterium bovis* subsp. *bovis* [5 strains], and *Mycobacterium africanum* subtype I [1 strain]) results were identical to conventional results. The sensitivity was slightly higher for this test than for the AccuProbe assay.

The *Mycobacterium tuberculosis* complex is composed of the closely related species *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and "*M. canetti*." *M. bovis* comprises *M. bovis* subsp. *bovis*, *M. bovis* subsp. *caprae*, and the *M. bovis*-derived BCG vaccine strain; *M. africanum* includes two subtypes, I and II; *M. microti* is the vole strain; and "*M. canetti*" may be merely a subspecies of *M. tuberculosis* (17, 23, 24, 25). Although the species are closely related genetically, they differ in host and geographic range, certain phenotypes, and pathogenicity. *M. tuberculosis* is the most significant pathogen for humans in Europe and America, whereas *M. africanum* is widely distributed among African patients (4, 15). Both subspecies of *M. bovis* are reported to infect humans, yet they have a broad host range, including wildlife and domestic livestock like bovines and goats (12, 26). Of particular interest is the intrinsic resistance of *M. bovis* subsp. *bovis* against pyrazinamide, one of the first-line antituberculous drugs (11, 17). The vaccine strain *M. bovis* BCG is more frequently used for bladder cancer immunotherapy and can be detected in human urine specimens from bladder cancer patients (2, 6). Thus, for those specimens, the isolation of *M. tuberculosis* complex is not necessarily an indication for antituberculous treatment. Therefore, rapid differentiation to the species and subspecies levels should be obtained not only for epidemiological purposes but also for adequate treatment of each patient.

So far, rapid identification of the *M. tuberculosis* complex has been achieved by using AccuProbe gene probes (Gen-Probe, San Diego, Calif.), which target the 16S rRNA. However, species identification still relies on the determination of a series of phenotypic and growth characteristics and on biochemical tests, which all require sufficient growth, are time-consuming, and may not be performed in every laboratory (5, 25). Recently, the GenoType MTBC (Hain Lifescience GmbH, Nehren, Germany)—a new commercially available DNA strip assay for the rapid identification of the members of the *M. tuberculosis* complex—was evaluated by use of a well-characterized collection of *M. tuberculosis* complex isolates and was proven to be useful for species differentiation (20). Moreover, due to the inclusion of an *M. tuberculosis* complex-specific oligonucleotide, this test may replace the AccuProbe tests of cultures from clinical specimens as a method for identification of the *M. tuberculosis* complex, giving the additional information provided for the species involved.

The aim of this work was to evaluate the performance of the GenoType MTBC with positive liquid cultures from clinical specimens received for mycobacterial culture in comparison to species identification by classical methods. Furthermore, the GenoType MTBC results were compared to results obtained by AccuProbe tests.

Specimens. Two German microbiology laboratories were involved in this study: (i) the National Reference Center for Mycobacteria, Borstel, Germany, and (ii) the Department of Microbiology of the Laboratory Group, Heidelberg, Germany. All samples used in this study originated from human specimens and a few veterinary specimens sent to the laboratories for routine mycobacterial culture. Specimens (pulmonary and extrapulmonary) were processed according to national and international guidelines (5, 10) by using an *N*-acetyl-L-cysteine-NaOH decontamination procedure, inoculated into BACTEC MGIT 960 tubes (Becton Dickinson and Co., Cockeysville, Md.) and onto solid slant media (Löwenstein-Jensen and Stonebrink), and incubated at 37°C for up to 6 and 8 weeks, respectively.

BACTEC MGIT 960 tubes that had been reported to be positive by the MGIT instrument were checked for the presence of mycobacteria or contamination by (i) performing an acid-fast smear and (ii) subcultivation on Trypticase soy agar with 5% sheep blood. Specimens were chosen for the test if the acid-fast smear was positive or if the acid-fast smear was negative and contamination by other microorganisms could be excluded (no growth on Trypticase soy agar within 24 h or no other microorganisms visible in the smear). In total, 77 specimens, derived from 71 tuberculosis patients and two veterinary samples (cows), were included in this study. No more than two specimens from one patient were used. Only specimens growing tuberculosis bacteria were included in the study.

* Corresponding author. Mailing address: Forschungszentrum Borstel, National Reference Center for Mycobacteria, Parkallee 18, 23845 Borstel, Germany. Phone: (49) 4537-188760. Fax: (49) 4537-188311. E-mail: erichter@fz-borstel.de.

TABLE 1. Results of GenoType MTBC, AccuProbe assay, and conventional methods used for identification of strains

GenoType MTBC result	*n*	AccuProbe assay results	Conventional identification results[a]
M. tuberculosis	71	69 positive, 2 negative	Eugonic growth, positive for niacin accumulation and for nitrate reductase, growth in the presence of TCH
M. bovis subsp. *bovis*	5	5 positive	Dysgonic growth, negative for niacin accumulation and for nitrate reductase, no growth in the presence of TCH, resistant to pyrazinamide, microaerophilic growth on semisolid Lebek agar, no change in color of bromcresol purple agar, typical pattern obtained by spoligotyping
M. africanum subtype I	1	1 positive	Dysgonic growth; negative for niacin accumulation, positive for nitrate reductase, no growth in the presence of TCH, susceptible to pyrazinamide, typical pattern obtained by spoligotyping, typical restriction pattern of the *gyrB* gene

[a] Typical eugonic growth characteristics of *M. tuberculosis* includes a rough and raised morphology. Typical dysgonic growth characteristics of *M. bovis* includes a smooth morphology.

Sample preparation. For the MGIT samples included in the study, AccuProbe assays (Gen-Probe) for *M. tuberculosis* complex were performed according to the manufacturer's instructions. In parallel, aliquots of identical liquid media were prepared for the GenoType MTBC test. For this, 1 ml was centrifuged (10,000 × *g*, 15 min, room temperature), the supernatant was discarded, and the pellet was suspended in 300 to 500 μl of distilled water. After 15 min of boiling and 15 min of sonication, the samples were used immediately for amplification or stored at −20°C.

GenoType MTBC assay. The GenoType MTBC assay is based on an *M. tuberculosis* complex-specific 23S ribosomal DNA fragment, *gyrB* DNA sequence polymorphisms, and the RD1 deletion of *M. bovis* BCG (9, 16, 22). Specific oligonucleotides targeting these sequences are immobilized on membrane strips. Amplicons derived from a multiplex PCR hybridize to these probes. The assay was performed according to the manufacturer's instructions and as described previously (20). Amplification was done with 35 μl of primer nucleotide mix, amplification buffer containing 2.5 mM $MgCl_2$ (both provided with the kit), 1.25 U of HotStarTaq polymerase (QIAGEN, Hilden, Germany) (not provided with the kit), and 5 μl of DNA in a final volume of 50 μl with the following amplification protocol: denaturation at 95°C for 15 min; 10 cycles of denaturation at 95°C for 30 s and elongation at 58°C for 120 s; an additional 20 cycles of denaturation at 95°C for 25 s, annealing at 53°C for 40 s, and elongation at 70°C for 40 s; and a final extension at 70°C for 8 min. Hybridization and detection were carried out in a Profiblot automated washing and shaking device (Tecan, Männedorf, Switzerland). Ten microliters of the amplification products was mixed with 20 μl of denaturing reagent (provided with the kit) for 5 min in separate troughs of a plastic well. After the addition of 1 ml of prewarmed hybridization buffer, the membrane strips were added to every trough. Hybridization took place at 49°C for 30 min, followed by two washing steps. For colorimetric detection of hybridized amplicons, streptavidin-conjugated alkaline phosphatase and the appropriate substrate were added. After the final washing step, the strips were air dried and fixed on a data sheet. Species were identified according to the interpretation table provided (see Table 1).

Strain differentiation. Every strain included in this study was differentiated by classical biochemical and, in part, additional molecular methods. Biochemical analyses of every strain included determination of colony morphology, nitrate reduction on modified Dubos broth, niacin accumulation test (isoniazid test strips; Difco Laboratories, Detroit, Mich.), and growth in the presence of thiophene-2-carboxylic acid hydrazide (TCH; 2 μg/ml) (5, 10). Strains without clearcut biochemical characteristics of *M. tuberculosis* (i.e., positive for niacin accumulation and nitrate reduction and growth in the presence of TCH) were additionally analyzed either by further phenotypic or biochemical tests, such as those for determining susceptibility to pyrazinamide, growth characteristics on semisolid Lebek agar, and induction of change in the color of bromcresol purple agar, which were performed as described previously (5, 14), or by molecular techniques, such as spoligotyping (8) or analysis of the *gyrB* single-base-pair mutations (16). Final differentiation of each strain was based on the combined test results according to German guidelines (5) and as described by Niemann et al. (15)

In this study, we evaluated the usefulness of the new commercially available GenoType MTBC test for identification of, and for species differentiation within, the *M. tuberculosis* complex as applied to routine mycobacterial cultures. The use of two solid cultures and one liquid culture is the "gold standard" for mycobacterial diagnostics. Liquid cultures usually yield positive results faster than solid cultures do (7, 19). Due to this, we used exclusively liquid media (BACTEC MGIT 960) for evaluation of the test. Furthermore, these cultures were derived from routine patient samples and were not inoculated with reference stock strains.

In routine work, AccuProbe assays for *M. tuberculosis* complex are performed with liquid media if the smear confirms the growth of acid-fast bacilli or if no acid-fast bacilli can be detected but contamination by other microorganisms can be excluded. Therefore, we compared the usefulness of the GenoType MTBC to that of the AccuProbe assays for positive specimens from routine culture samples by using the same liquid medium.

Seventy-seven liquid samples growing mycobacteria of the *M. tuberculosis* complex were included in this study. Both the GenoType MTBC and the AccuProbe assays for *M. tuberculosis* complex were performed with the same liquid culture. With the GenoType MTBC assay, detection of *M. tuberculosis* complex and species identification could be obtained for all 77 specimens investigated. Thus, the GenoType MTBC assay, like the AccuProbe assays, can be used for culture confirmation of tuberculosis. In contrast, two specimens were negative for *M. tuberculosis* complex by the AccuProbe assay, although the

smears were positive for acid-fast bacilli (Table 1). AccuProbe assays are faster and easier to perform than the GenoType MTBC assays are, since they can be performed within approximately 2 h and do not require an amplification procedure. In contrast, the great advantage of the GenoType MTBC is the rapid additional information provided for the species involved.

With regard to species identification, all GenoType MTBC assays gave unequivocal results and none of the strips showed patterns others than those predicted by the interpretation table added to the test. Of the 77 specimens investigated, 71 showed the pattern of *M. tuberculosis*, 5 showed that of *M. bovis* subsp. *bovis* (two specimens from cows and three human specimens; the latter were isolated from sputa [$n = 2$] and from a swab [$n = 1$]), and one showed the pattern of *M. africanum* subtype I (sputum). The identification results for all specimens were in complete accordance with the results obtained by conventional identification (Table 1). Species identification could now be achieved with liquid cultures without the necessity of subcultures on solid media and thus could be obtained at least 3 weeks earlier than the results of the biochemical tests. In particular, the identifications of the *M. bovis* and the *M. africanum* strains were markedly accelerated, as these species grow rather slowly on Löwenstein-Jensen medium, and since classical identification of these species relies mainly on negative test results (niacin accumulation and nitrate reduction), well-grown cultures are necessary for reliable results.

Classical susceptibility testing is performed with solid media (Löwenstein-Jensen medium in Germany) and requires 3 to 4 weeks of growth. Because of the threat of rising numbers of resistant *M. tuberculosis* strains, more efforts have been undertaken to accelerate susceptibility testing by using liquid media, which enables susceptibility results to be obtained within 7 to 10 days after growth of mycobacteria from the clinical specimen (1, 3, 18, 21). However, classical differentiation by biochemical techniques delays final results, including the species identification, for up to several weeks. Thus, for laboratories that perform susceptibility testing with liquid media, the introduction of a molecular test for identification of species of *M. tuberculosis* complex in liquid cultures can markedly shorten the overall time required for obtaining results.

Within the scope of this study, we did not analyze the specificity of this test by using cultures growing nontuberculous mycobacteria. The specificity of the *M. tuberculosis* complex-specific 23S rDNA fragment of this test is well known from the performance of the GenoType Mycobacteria (Hain Lifescience) test, which is based on the same fragment (13).

In conclusion, the GenoType MTBC assay was found to enable a very rapid identification of *M. tuberculosis* complex species and species differentiation within the complex in clinical practice. It can be performed efficiently with liquid medium in order to speed up mycobacterial diagnostics. It fits easily into the work flow of a routine laboratory and can be conducted in laboratories that do not carry out sophisticated biochemical tests for differentiation.

We thank B. Schlüter and I. Radzio, Borstel, Germany, and G. Glöckner and P. Johanson, Heidelberg, Germany, for excellent technical assistance, and Hain Lifescience GmbH for providing reagents for this study.

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