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Routine Identification of *Mycobacterium tuberculosis* Complex Isolates by Automated Hybridization

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Methodologies for biochemical identification of mycobacteria isolated from clinical samples are still cumbersome, taking skilled technicians 3 to 6 weeks. We describe here a 2-h identification system for mycobacterial isolates belonging to the *Mycobacterium tuberculosis* complex using a DNA probe. After 30 min of hands-off sample preparation, the 1.5-h hybridization test is totally automated in the newly developed VIDAS system (bioMérieux, Marcyl'Etoile, France), which performs solid-phase specific hybridization of 16S rRNA at 37°C. The strain collection of actinomycetes tested was composed of 662 isolates from 27 species: 461 members of the *M. tuberculosis* complex (443 *M. tuberculosis*, 10 *M. bovis*, and 8 *M. bovis* BCG isolates) and 201 isolates of other species, including 55 *M. avium-intracellulare* isolates). They were identified by traditional methods: growth rate, colonial morphology, pigmentation, and biochemical profiles. The automated probe assay displayed an excellent correlation with the reference results. The four members of the *Nocardia* and *Rhodococcus* genera tested did not cross-hybridize. This flexible random-access and automated technology was shown to suit the routine context of the laboratory by rapidly delivering the results.

Infections caused by *Mycobacterium tuberculosis* are found worldwide (5). This species, along with the *M. avium-M. intracellulare* complex, is associated with AIDS-infected patients, and such infections are on the increase (13). Consequently, the need for more rapid methods of identification of these slowly growing bacteria has emerged. To date, classical identification requires three steps: isolation of the strain by culture of the sample (which can take several weeks), biochemical identification, and, normally, susceptibility testing. These latter two steps are equally long and cumbersome.

New techniques based on the characterization of genetic elements rather than biochemical properties have been developed, leading to a substantial reduction in identification time. Such techniques are based on the detection of a specific part of the mycobacterial genome, either on isolated organisms, with DNA probes (7), or directly on the clinical sample via enzymatic amplification, i.e., PCR (10), of a genomic target and generally coupled to probe confirmation (4).

Although amplification shows great potential, direct probing of the suspect culture is more reliable at present (3, 11). Recently, commercially available nonradioactive probes have been proposed for the identification of mycobacterial species (7), but these systems are not automated and are more adapted to batch testing than to single tests.

In order to meet the laboratory requirements for automation and single testing, we present an automated instrument for the identification of culture isolates belonging to the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, also abbreviated tuberculosis [TB] complex). The bioMérieux VIDAS system, initially designed for sandwich immunoassays, in this case performed DNA hybridizations. The routine use of this system has been evaluated in the mycobacterial laboratory of the Pasteur Institute of Lyon, France, with clinical cultures and reference strains.

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MATERIALS AND METHODS

Bacterial strains. The series tested came from the Institut Pasteur de Lyon culture collections. It included clinical isolates collected in France and in the United States of America as well as reference strains (Table 1). There were 461 members of the TB complex (443 *M. tuberculosis*, 10 *M. bovis*, and 8 *M. bovis* BCG isolates) and 201 isolates of other species, including 55 *M. avium-intracellulare* isolates and 4 members of the *Nocardia* and *Rhodococcus* genera. About 4% of the members of the TB complex were *M. bovis* or *M. bovis* BCG. The eight BCG strains represent strains isolated from infants after they presented a postvaccination reaction. The clinical isolates phenotypically characterized as TB strains constituted a chronological series, whereas tested mycobacteria from other species represented a random selection of the isolates received during the same period. In this laboratory, the occurrence of clinical samples positive for mycobacteria is 3%.

Phenotypic identification of the strains. All clinical isolates were grown on Lowenstein-Jensen or Coletsos agar and examined for growth rate, gross and microscopic colony morphology, and pigmentation. They were also submitted in parallel to the TB DNA probe test. If this test was positive, only the niacin and nitrate reduction tests (tube tests) were performed (11). In other cases, isolates were subjected to the catalase (drop method), arylsulfatase, pyrazinamidase, urease, and lipase (Tween 80 hydrolysis) tests (11) and also to additional tests for certain isolates.

Genotypic identification of the strains. (i) Nucleic acids extraction. Total nucleic acids were released from culture material by a 30-min hands-off sample preparation which included sonication and chemical steps. Briefly, one or two freshly grown colonies of bacteria (3- to 5-mm diameter, i.e., 10^8 bacteria) were scraped on the end of a spatula and resuspended in a 1.5-ml Eppendorf tube in 250 μl of sterile

TABLE 1. Bacterial strains tested with the *M. tuberculosis* complex DNA probe

Sp.	No. of isolates	Origin[a]
Mycobacterium tuberculosis complex	461	
Mycobacterium bovis	10	Including ATCC 19210
Mycobacterium bovis BCG	8	
Mycobacterium tuberculosis	443	
Other mycobacterial spp.	201	
Mycobacterium asiaticum	1	ATCC 25276
Mycobacterium avium-intracellulare	55	
Mycobacterium chelonae	12	
Mycobacterium chelonae subsp. *abscessus*	4	
Mycobacterium flavescens	5	
Mycobacterium fortuitum	11	
Mycobacterium fortuitum subsp. *fortuitum*	2	
Mycobacterium fortuitum subsp. *peregrinum*	5	
Mycobacterium gastri	7	
Mycobacterium gordonae	22	
Mycobacterium kansasii	16	
Mycobacterium marinum	3	
Mycobacterium nonchromogenicum	1	
Mycobacterium phlei	5	
Mycobacterium scrofulaceum	2	
Mycobacterium simiae	1	
Mycobacterium smegmatis	6	
Mycobacterium terrae	5	
Mycobacterium triviale	3	
Mycobacterium vaccae	2	
Mycobacterium xenopi	28	
Atypical *Mycobacterium* sp.	1	
Other spp.		
Nocardia asteroides	1	ATCC 3308
Rhodococcus aichiensis	2	
Rhodococcus sputi	1	

[a] All strains are of clinical origin unless otherwise stated. ATCC, American Type Culture Collection.

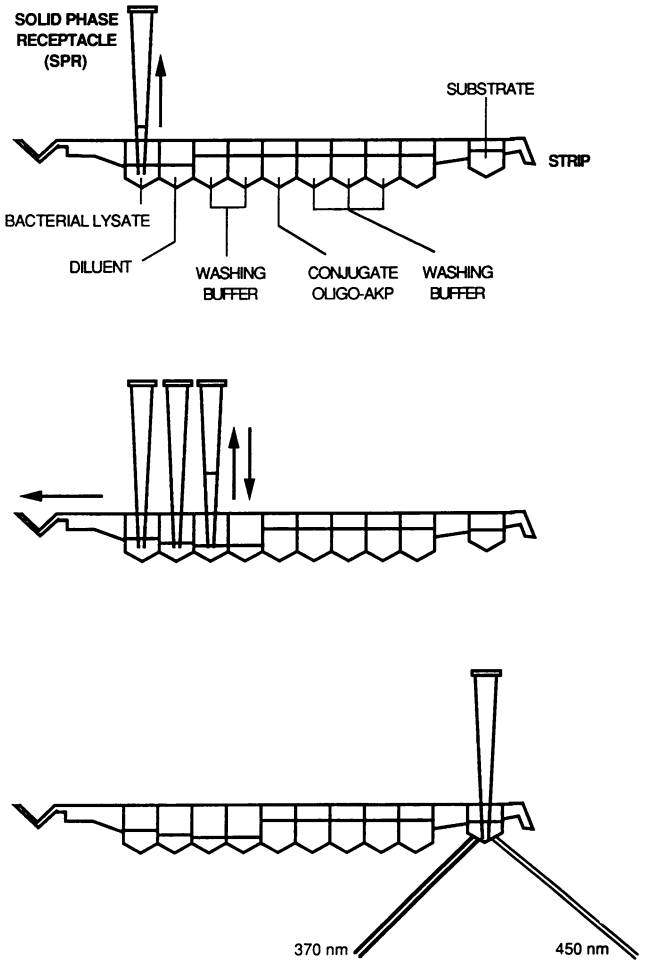

FIG. 1. Representation of the VIDAS disposable strip and SPR vessels used for nonradioactive DNA hybridization. Vertical arrows, solution pipetting; horizontal arrow, linear movement of the strip in relation to the different pipetting actions performed by the fixed SPR; nanometer-numbered lines, exciting (370-nm) and emitted (450-nm) light rays falling on the optically clear cuvette for fluorescence reading; Oligo-AKP, oligonucleotide-alkaline phosphatase conjugate.

water. A total of 50 μl of this suspension was then added to a tube containing 50 μl of lysing solution (proprietary information). The tube was put into a bath sonicator (Bioblock N 88 169-85 W-; Bioblock, Illkirch, France) for 15 min at 60°C and then incubated for 15 min at 37°C.

(ii) Automated hybridization. At the end of the sample preparation, the total volume of lysate (100 μl) was placed in the 10-well polypropylene strip of the VIDAS instrument (bioMérieux). This strip had already been filled with all the necessary reagents for hybridization in separate wells: sample diluent, labelled probe, washing buffer, and substrate. The loaded strip was placed into the tracks of the VIDAS, and all subsequent steps of the nonradioactive hybridization protocol were performed automatically for 1.5 h at 37°C. The principle of this machine is shown in Fig. 1 and was derived from immunological techniques for which VIDAS was initially commercially developed. Two DNA oligonucleotides specific for the *M. tuberculosis* complex were used in a sandwich hybridization format (8) as indicated in Fig. 2. One oligonucleotide was applied as a coating inside the solid surface of the pipette tip, the solid phase receptacle (SPR), and acted as a capture probe. A second oligonucleotide, covalently linked to alkaline phosphatase, was used as a detection probe (6). This probe is similar to that described by Böddinghaus et al. (2) and targeted towards 16S rRNA.

(iii) Automated detection. Upon completion of the assay, the alkaline phosphatase substrate, 4-methylumbelliferyl phosphate, is pumped from the final well, which is an optically clear cuvette. The enzyme catalyzes its conversion into a fluorescent product, 4-methylumbelliferone. The intensity of fluorescence of the cuvette content is measured by the VIDAS optical scanner before and after contact with the SPR. Results are subsequently analyzed automatically and expressed in relative fluorescence units (RFU) by the computer interface. Test values are generated for each sample by subtraction of the blank value (substrate cuvette reading) from the sample fluorescence. Test values are compared with a cutoff value to yield an interpretation, which is then printed by the instrument. Test values of <1,500 RFU were considered negative results. Tests values greater than or equal to 1,500 RFU were considered positive results and thus provided evidence for *M. tuberculosis* identification. A maximum of 30 tests could be processed by the VIDAS system in a single run in 2 h. In each set of

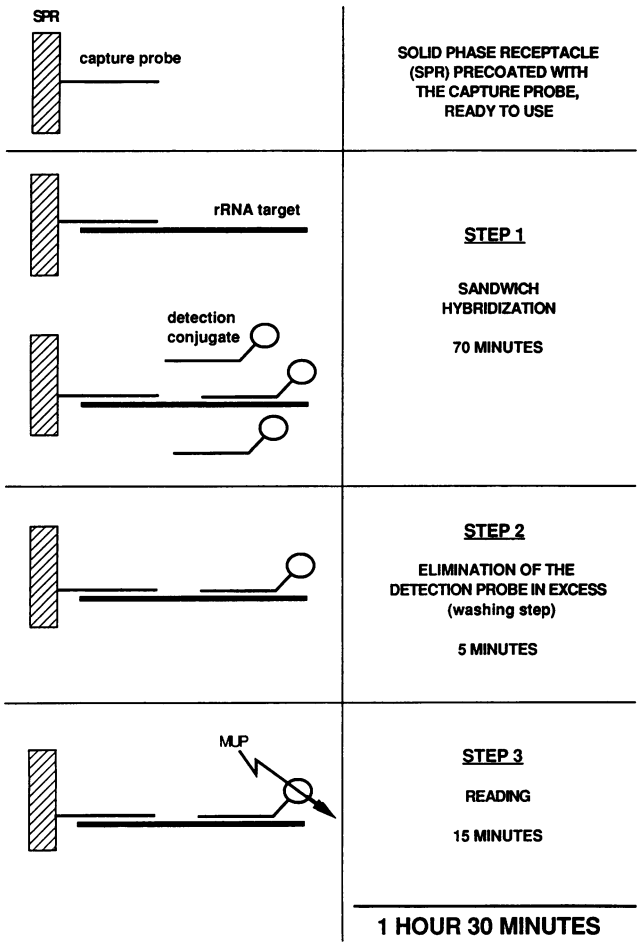

FIG. 2. Representation of the VIDAS steps for sandwich hybridization. Hatched rectangles, section of the solid surface of the pipette tip (SPR); narrow lines, oligonucleotide or oligonucleotide-alkaline phosphatase conjugate; oval, alkaline phosphatase; thick lines, rRNA target; broken arrow, representation of the alkaline phosphatase-mediated fluorescence detection; MUP, 4-methylumbelliferyl phosphate fluorescent substrate.

experiments, the same TB and non-TB isolates were used as controls.

RESULTS AND DISCUSSION

Specificity and sensitivity of the DNA probe test. The specificity of the test was demonstrated on 662 isolates from 27 species. The DNA probe reacted specifically with nucleic acids of the 461 TB complex isolates tested. Among the TB strains were five multidrug-resistant TB strains and five atypical nitrate-negative strains, demonstrating the presence of identical target nucleotide sequences in these strains.

Among the 201 non-TB strains, 199 did not respond to the test, whereas 2 strains, the reference *M. asiaticum* strain and the atypical *Mycobacterium* strain, gave false-positive signals. The positive value generated with *M. asiaticum* was 4,272 RFU. Repeated experiments (12 times) with this strain gave an average signal of 756 RFU with a range of 19 to 1,254, indicating that the initial data were probably an erratic result. If a 1,500-RFU cutoff was established, this isolate remained negative. The atypical strain also showed a discrepant positive value of 2,825 RFU. Repeated experiments (four times) gave similar results. Initially, this strain was found to be niacin and nitrate negative. Further biochemical testing indicated this strain to be a member of the *M. terrae* complex. We therefore directly sequenced on an automated sequencer a 300-bp PCR-amplified portion of the genes coding for rRNA including the polymorphic species-specific locus (data to be published elsewhere). Analysis of this fragment indicated complete identity with the *M. tuberculosis* sequence, confirming the DNA probe result (results not shown). This strain was indicated to be a member of the TB complex although it has been subjected to more complete investigation.

Taken together, test values for real-positive results (including the atypical strain) gave on average 8,506 RFU with a standard deviation of 2,892 (the range was 1,745 to 12,128), whereas the average value of the non-TB strains (excluding the erratic *M. asiaticum* value) was 250 RFU with a standard deviation of 150 (the range was 2 to 772). Considering that the identification of the atypical strain was phenotypically negative and genotypically positive and that the *M. asiaticum* isolate was negative for both tests, the VIDAS test demonstrated 100% sensitivity, 99.5% specificity, a positive predictive value of 99.7%, and a negative predictive value of 100%.

Hybridization features. The VIDAS instrument has been already adapted to the detection of nucleic acid targets obtained in vitro by PCR (1). Here we present the adaptation for the detection of natural 16S rRNA targets extracted from clinical strains of bacteria. Differences lie in the longer length of the natural target (16S is approximately 1,400 to 1,600 nucleotides in bacteria), its single-strandedness, and the presence of numerous secondary structures (9). Solid-phase hybridization of this target retains all its specificity although it is performed at a low temperature (37°C). Specificity results obtained with the *M. tuberculosis* complex probe are consistent with those obtained by Böddinghaus et al. (2) since the probe used is located in the same unique variable locus whose nucleotide sequence is specific for each species of mycobacteria (positions 161 to 215 according to the IUB numbering of *Escherichia coli*). Furthermore, we demonstrated the ability of our system to discriminate point mutations of DNA-RNA hybrids at 37°C, since rRNA of the *M. marinum* isolate did not hybridize with the probe, whereas the sequence for this species differs from that of the *M. tuberculosis* complex by only two mutations. The target sequence of *M. asiaticum* presents one single C-A mismatch with the probe (12). Given the setting of the 1,500-RFU cutoff, the mismatch was discriminated.

The use of the VIDAS system was evaluated in a routine context. It was found to be user friendly and allowed a random-access testing strategy, which is more adapted to the mycobacterial laboratory context than to batch testing. The resulting flexibility should lead to cost savings. From a practical point of view, it is important to note that rRNA targets did not leak between the tracks of the automate since results obtained for negative samples repeatedly inserted between positive samples remained negative (results not shown). Thus, extracted nucleic acids are not aerosolized at a detectable level by the instrument. The advantage of using DNA probes for identification of mycobacterial cultures instead of classical biochemical tests has been stressed by others (2, 3, 7). In addition to a substantial saving in time (from 3 to 6 weeks down to 2 h) and earlier consideration of the therapeutic choice, it results in safer procedures for the laboratory workers themselves: there is less handling of numerous tubes containing pathogenic material or highly toxic compounds, such as cyanogen bromide in the niacin test. Also, this approach should allow a better-quality result for the identification of

mycobacterial species for which the negative value of some biochemical tests has a discriminative value. Inherent difficulties in the interpretation of these tests will disappear.

The VIDAS system is a highly automated, user-friendly instrument with a minimum of manual input. Its DNA probe adaptation for *M. tuberculosis* complex isolates is highly specific and was shown to be more rapid and safer to use than conventional biochemical identification in a routine setting. This system has the potential to be developed for the identification of other mycobacterial species, in particular the *M. avium-intracellulare* complex and to be used on liquid culture enrichment.

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