JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2001, p. 1079–1084 Vol. 39, No. 3
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.3.1079–1084.2001
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Performance Assessment of New Multiplex Probe Assay for Identification of Mycobacteria

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Received 25 September 2000/Returned for modification 14 November 2000/Accepted 7 December 2000

A new DNA probe assay (INNO LiPA Mycobacteria; Innogenetics, Ghent, Belgium) for the simultaneous identification, by means of reverse hybridization and line-probe technology, of *Mycobacterium tuberculosis* complex, *Mycobacterium kansasii*, *Mycobacterium xenopi*, *Mycobacterium gordonae*, the species of the *Mycobacterium avium* complex (MAC), *Mycobacterium scrofulaceum*, and *Mycobacterium chelonae* was evaluated on a panel of 238 strains including, besides representatives of all the taxa identifiable by the system, a number of other mycobacteria, some of which are known to be problematic with the only other commercial DNA probe system (AccuProbe; Gen-Probe, San Diego, Calif.), and two nocardiae. The new kit, which includes a control probe reacting with the whole genus *Mycobacterium*, correctly identified 99.6% of the strains tested; the one discrepancy, which remained unresolved, concerned an isolate identified as MAC intermediate by INNO LiPA Mycobacteria and as *Mycobacterium intracellulare* by AccuProbe. In five cases, because of an imperfect checking of hybridization temperature, a very slight, nonspecific, line was visible which was no longer evident when the test was repeated. Two strains whose DNA failed amplification at the first attempt were regularly identified when the test was repeated. Interestingly, the novel kit dodged all the pitfalls presented by the strains giving anomalous reactions with AccuProbe. A unique feature of INNO LiPA Mycobacteria is its ability to recognize different subgroups within the species *M. kansasii* and *M. chelonae*, while the declared overlapping reactivity of probe 4 with some *M. kansasii* and *Mycobacterium gastri* organisms and of probe 9 with MAC, *Mycobacterium haemophilum*, and *Mycobacterium malmoense*, may furnish a useful aid for their identification. The turnaround time of the method is approximately 6 h, including a preliminary PCR amplification.

Interest in mycobacteria, both *Mycobacterium tuberculosis* and mycobacteria other than tuberculosis (MOTT), is rising in industrialized and in developing countries. Their rapid and accurate identification is necessary, both to decide whether measures are needed to prevent their diffusion and to make therapeutic choices. The identification of MOTT appeared, up to a few years ago, easily feasible by resorting to a limited panel of biochemical and cultural tests (14). This firm belief was supported by a poor knowledge of mycobacterial taxonomy that recognized only about 30 mycobacterial species, the majority of which often contaminate biological specimens but occasionally may also behave as significant pathogens. The growing weight of genetics in taxonomic studies has undermined that conviction and has resulted in a rapid increase in the number of recognized mycobacterial species, which is now approaching 100 (a list of bacterial names with their standing in nomenclature may be accessed at http://www.bacterio.cict.fr). In the presence of such a large number of species, the chance of having taxa with overlapping phenotypic patterns increases, and the phenotypic identification is also made more problematic by the individual variability of single organisms.

The quest for stable features on which to base classifications and identifications has led to the development of techniques for analyzing lipids present in the mycobacterial cell wall (4) or genomic regions with taxon-specific nucleotide sequences (9). From the latter approach sprang the DNA probe technology, and its commercial exploitation (AccuProbe; Gen-Probe, San Diego, Calif.) represents one of the most important acquisitions of modern mycobacteriology (6), which greatly improves the performance of many laboratories worldwide. The major drawback of AccuProbe, however, is that it detects only the mycobacteria most frequently isolated in the United States. This may represent a problem in countries, mainly in Europe, where other species not covered by AccuProbe are frequently isolated (7, 12).

Recently a new DNA probe kit (INNO LiPA Mycobacteria; Innogenetics, Ghent, Belgium) (LiPA) designed for the simultaneous identification of up to 17 different taxa, which uses reverse hybridization on 14 probes immobilized as parallel lines on membrane strips, has been made commercially available. This study aimed to assess the performance of this novel kit and tested it on a large panel of strains representing all the taxa that the LiPA kit, it is claimed, can identify, as well as

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TABLE 1. Mycobacteria tested and corresponding hybridization results

Mycobacterium	Correct identifications		Overlapping hybridizations	Misidentifications
	At species level	At genus level		
M. tuberculosis complex	15			
M. tuberculosis	12			
M. bovis	6			
M. bovis BCG	1			
M. africanum[a]	1			
M. kansasii[a]	5			
M. kansasii reacting with both the previous and the present AccuProbe	4			
M. kansasii reacting with the present AccuProbe but not the previous one	4			
M. kansasii not reacting with either the previous or the present AccuProbe[b]	2			
M. gastri[a]			2	
M. xenopi	21			
M. gordonae	8			
M. gordonae genotype 1	1			
M. gordonae genotype 2	1			
M. gordonae genotype 3	2			
M. gordonae presenting HPLC[c] profile i	4			
M. gordonae presenting HPLC profile ii	4			
M. avium	9			
M. intracellulare	3			1
MAI-X (intermediate)	10			
M. malmoense			6	
M. haemophilum[a]			1	
M. paratuberculosis[a]	1			
M. scrofulaceum[a]	25			
M. chelonae	27			
M. asiaticum		1		
M. bohemicum		2		
M. botniense[a]		1		
M. branderi[a]		1		
M. celatum type 1[a]		4		
M. celatum type 2[a]		2		
M. celatum type 3[a]		1		
M. conspicuum[a]		3		
M. flavescens		2		
M. fortuitum[a]		13		
M. genavense		1		
M. interjectum		1		
M. lentiflavum		3		
M. marinum		7		
"*M. palustre*"		2		
M. phlei		1		
M. shimoidei		1		
M. simiae		5		
M. szulgai		1		
M. terrae		7		
M. thermoresistibile		1		
M. triviale		1		
M. tusciae		1		

[a] Includes the reference strain.
[b] Kindly provided by Robert Ferguson (Albuquerque, N.M.).
[c] HPLC, high-performance liquid chromatography.

several mycobacteria known to give equivocal results with AccuProbe.

MATERIALS AND METHODS

The LiPA assay is based on the principle of reverse hybridization. Biotinylated DNA material, obtained by means of a PCR amplification of the 16S-23S ribosomal RNA spacer region, is hybridized with 14 specific oligonucleotide probes immobilized as parallel lines on membrane strips. The addition of streptavidin labeled with alkaline phosphatase and of a chromogenic substrate results in a purple-brown precipitate on hybridized lines. The kit includes all necessary reagents except the thermostable DNA polymerase (*Taq* polymerase; Stratagene Europe, Amsterdam, The Netherlands), Tris EDTA (TE) buffer, agarose, and ethidium bromide (Sigma, Milan, Italy).

The study was carried out in seven Italian laboratories skilled in the management of mycobacteria and experienced in molecular biology techniques. The strains were retrieved from the laboratory collections, subcultured on solid or in liquid media, and incubated at 37°C. Solid media included Lowenstein-Jensen or Middlebrook 7H11 agar (Becton Dickinson, Sparks, Md.). Liquid media included Bactec 12B (Becton Dickinson) or the BBL MGIT (Becton Dickinson). In addition to freshly subcultured strains, Lowenstein-Jensen cultures kept at 2 to 8°C for various times (sometimes for years) were also tested. Strains of *Mycobacterium haemophilum* and *Mycobacterium genavense* were grown on Middlebrook 7H11 enriched with bovine hemoglobin (4 μl/ml) (Becton Dickinson) or mycobactin J (0.5 μg/ml) (Rhône-Merieux, Lyon, France), respectively. Independent isolates (Table 1) were selected from species whose taxonomic status was unquestionable, having been identified by means of AccuProbe (plus the niacin and nitrate tests to differentiate members of the *M. tuberculosis*

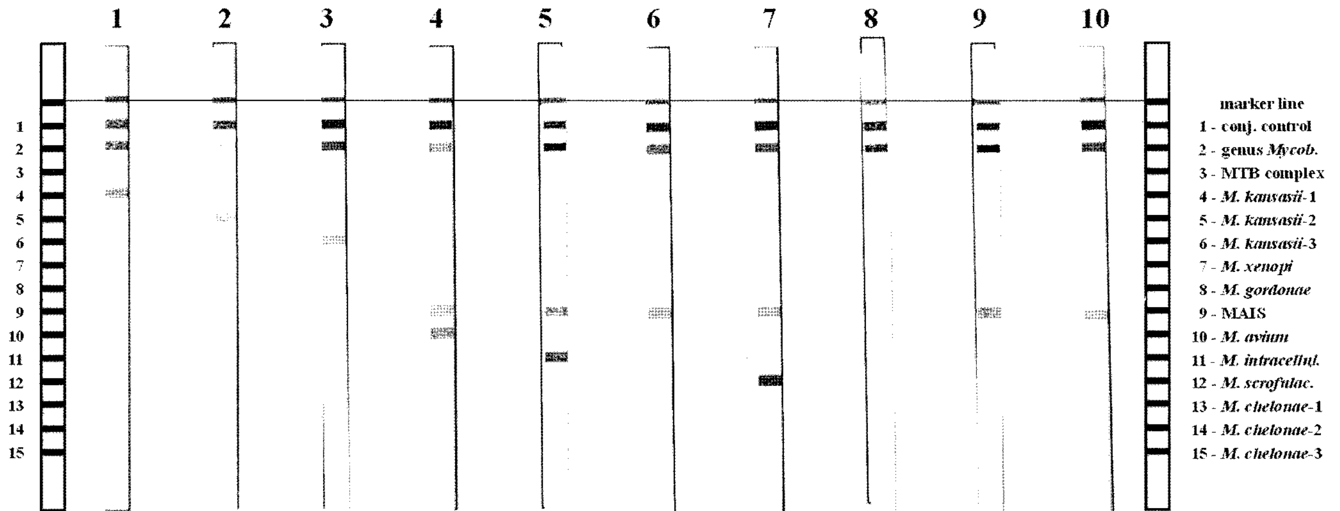

FIG. 1. Hybridization patterns of several mycobacteria. Strips: 1, *M. kansasii* group i; 2, *M. kansasii* group ii; 3, *M. kansasii* group iii, iv, or v; 4, *M. avium*; 5, *M. intracellulare*; 6, MAI-X; 7, *M. scrofulaceum*; 8, *M. celatum*; 9, *M. malmoense*; 10, *M. haemophilum*.

complex [10]), by high-performance liquid chromatography of cell wall mycolic acids (15), or by 16S rDNA gene sequencing (9); in many cases they had been identified by more than one such technique. The identifications resulting from conventional cultural and biochemical tests alone, available for a number of our strains, were not considered sufficient, and therefore the corresponding mycobacteria were not included in the present study. Several mycobacterial reference strains and two *Nocardia* strains were included as well. Of the 238 mycobacterial strains tested, 61 were outside the detection range of LiPA and 177 were inside that range, as stated by the manufacturer.

From solid media, a small amount of several colonies were resuspended in TE buffer (10 mM Tris HCl, 1 mM EDTA [pH 8]), boiled for 10 min, and centrifuged (5 min at 13,000 rpm). Subsequently 2 μl of supernatant was amplified (see below). From liquid media, 2 μl of broth culture was centrifuged (15 min at 13,000 rpm), and the pellet was resuspended in TE buffer and heat inactivated at 95°C for 30 min, followed by further centrifugation (10 s at 13,000 rpm), freezing for 30 min at −20°C, vortexing, and final centrifugation as above. Ten microliters of supernatant was used for PCR amplification. The amplification was carried out using a Perkin-Elmer 4800 thermal cycler (Boston, Mass.) in 0.2-ml tubes in the presence of *Taq* polymerase, deoxyribonucleoside 5′ triphosphates, and biotinylated primers complementary to sequences flanking the 16S-23S ribosomal RNA spacer region. An initial step of 1 min at 95°C was followed by 40 cycles of 30 s at 95°C, 30 s at 62°C, and 30 s at 72°C. The success of the amplification step was checked by gel electrophoresis, loading 10 μl of amplified product onto a 2% agarose gel, staining with ethidium bromide, and noting the presence of a single band 400 to 500 bp long.

Ten microliters of the amplification product was placed in a disposable trough (provided in the kit) along with 10 μl of denaturation solution, mixed by pipetting, and left for 5 min at room temperature. Following the addition of 2 ml of the hybridization solution, prewarmed to at least 37°C but not exceeding 62°C, and mixing by gentle shaking, a typing strip was submerged in the solution and the trough was placed into an exactly 62°C covered, shaking water bath (approximately 80 rpm, i.e., the highest amplitude of shaking suitable to exclude the possibility of any spilling in the troughs) for 30 min. The strip was then washed twice with 2 ml of stringent wash solution followed by a further incubation at 62°C for 10 min. The color development of the strip was carried out at room temperature on a shaking platform following two washes with 2 ml of rinse solution, the addition of 2 ml of conjugate (streptavidin labeled with alkaline phosphatase), and incubation at room temperature for 30 min. After incubation, the strip was washed twice with 2 ml of rinse solution and once with 2 ml of substrate buffer and was incubated for 30 min while shaking. In the final step, the strip was washed twice with 2 ml of distilled water and dried on absorbent paper.

Any hybridization was revealed by the appearance of purple-brown bands on the membrane-based strip. The identity of the reacting probes was indicated, with the help of the template provided, by the position of the colored bands (Fig. 1). On the strip are present 14 probes with different specificities (Fig. 1), as well as line 1, at the top, used as a control for the addition of conjugate and substrate solution. The staining of the genus-specific probe (line 2) serves as a positive control, confirming that the appropriate amount of amplified mycobacterial DNA was added for hybridization. One strip not tested with any strain, run in parallel with the others, was used both as a negative control (the genus *Mycobacterium* probe must remain uncolored) and to verify the hybridization temperature: one (line 9) of the expected bands (lines 1, 5, and 9) is missing when the temperature is too high (64 to 66°C), while an extra band (line 13) is present when the temperature is too low (58 to 60°C).

The probes available on the strip are summarized in Table 2. Probe 2 is genus *Mycobacterium* specific, two others (6 and 9) react with several species, and still others split *Mycobacterium kansasii* and *Mycobacterium chelonae* into subgroups. Six species are directly identified by the presence of a unique hybridization line (in addition to the genus-specific one), while a further five species are identified by the combination of the reactions of two probes, as shown in the decision scheme (Fig. 2), plus by a minimum of information about the phenotypic features of the strain. The species within the *M. tuberculosis* complex are not differentiated.

RESULTS

The DNA of both isolates of *Nocardia* was amplified, as revealed by the band visualized in agarose gel, but it did not

TABLE 2. Structure and specificity of the line-probe strip

Line probe	Declared specificity[a]
1	None (conjugate control)
2	All *Mycobacterium* species
3	*M. tuberculosis, M. bovis, M. microti, M. africanum*
4	*M. kansasii* group i
5	*M. kansasii* group ii
6	*M. kansasii* groups iii, iv, and v; *M. gastri*
7	*M. xenopi*
8	*M. gordonae*
9	*M. avium, M. intracellulare, M. scrofulaceum*, MAI-X, *M. malmoense, M. haemophilum*
10	*M. avium, M. paratuberculosis, M. silvaticum*
11	*M. intracellulare*
12	*M. scrofulaceum*
13	*M. chelonae* groups i, ii, iii, and iv
14	*M. chelonae* group iii
15	*M. chelonae* group i

[a] *M., Mycobacterium*.

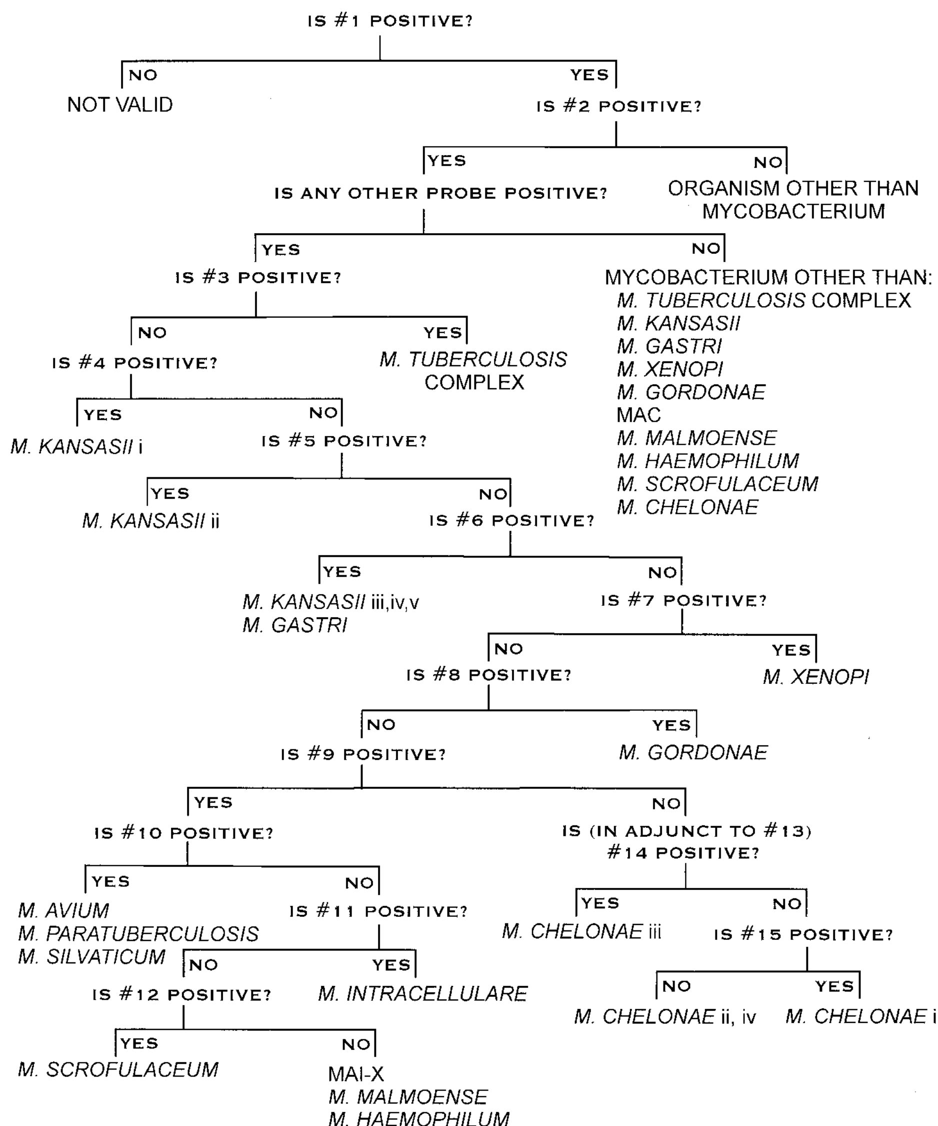

FIG. 2. Flow chart decision scheme for the interpretation of INNO LiPA Mycobacteria results.

hybridize with any of the line probes. Two strains processed within the same session, not presenting any amplification product at the gel-agarose check, succeeded in subsequent attempts and could be correctly identified. All the 238 mycobacterial strains reacted, as expected, with line probe 2, specific for the genus. In five cases (occurring in two different laboratories and in distinct batches) in which the hybridization control strip revealed, by the presence of a slight line 13, a discrepancy of hybridization temperature, a faint extra line, however easily distinguished from the clear specific ones, was present. In these cases the test was repeated and only specific lines were obtained. As little as 2°C below the requested 62°C temperature may be responsible for the appearance of slight spurious bands.

Of the 177 strains belonging to taxa in the detection range of LiPA, including several tested as old cultures, all but one were correctly identified by their reactions with the kit's probes. A single strain with slightly discrepant results had been assigned by AccuProbe to the species *Mycobacterium intracellulare*; with LiPA it did react with probe 9 covering the whole *Mycobacterium avium* complex (MAC), but not with probe line 11, the one specific for *M. intracellulare*. Among our *M. kansasii* strains, nine reacted with probe 4, four with probe 5, and two with probe 6. Of the *M. chelonae* strains, 10 hybridized with line 13, 9 with lines 13 and 15, and 8 with lines 13 and 14, thus allowing their assignment to subgroups; these subgroups, however, could not be verified since the subgroups of our strains had not been established. The declared overlapping reactions of probe line 6, with *Mycobacterium gastri*, and of line 9, with *M. haemophilum* and *Mycobacterium malmoense*, were confirmed and were at times characterized by weak lines.

DISCUSSION

AccuProbe has been in the last decade the tool preferred by a variety of laboratories to provide rapid and correct identifi-

cation of the most frequently isolated mycobacteria; it has become the de facto, provisional "gold standard" for commercial systems that identify mycobacteria by means of DNA probes. Now LiPA offers a competitive genomic assay. As expected the two assays, based on distinct designs, offer different performances. Unlike the AccuProbe, the identification of specific mycobacterium strains with the LiPA is simultaneous and does not require the preventive choice of the probe according to the colonial morphology; nor does it require new tests whenever a negative result is obtained. Eight taxa are directly detectable, including all five identifiable by the AccuProbe. Among the species not covered by the AccuProbe system but included in LiPA strips, at least two are rather common, *M. chelonae* and *Mycobacterium xenopi*, the latter being the most frequently isolated MOTT in many countries of Europe (12).

Two species are split in genotypes, *M. kansasii* and *M. chelonae*. The presence of five groups within the presently accepted species *M. kansasii* is well documented in literature (2, 13), and differences in the respective clinical significance are also reported. A first indication of the genetic heterogeneity within the species *M. kansasii* emerged several years ago when the AccuProbe that was commercially available at that time was found to fail hybridization with a number of strains displaying the phenotypic features of this species (17); a reformulated version of the AccuProbe *M. kansasii* apparently overcame the problem (18) but further, albeit rare, variants were found that still failed hybridization. Several such variants of *M. kansasii* were present in our test panel (Table 1); interestingly, the ones known to hybridize with both formulations of the AccuProbe *M. kansasii* were positive with line 4 of the LiPA; the ones known to hybridize with the reformulated AccuProbe only were positive with line 5, while the only two strains not reacting at all with any AccuProbe hybridized with line 6 of the LiPA, which includes groups iii, iv, and v. In contrast with *M. kansasii*, nothing is known of the clinical importance of the splitting of *M. chelonae* into four genotypes, which, however, indeed reflects a genetic heterogeneity.

Minor differences between AccuProbe and LiPA were observed for the strains different from *M. avium* and *M. intracellulare* but belonging to the MAC. In addition to the so-called MAI-X group (19) (otherwise known as "intermediate"), a novel mycobacterium ("*Mycobacterium palustre*") genotypically and phenotypically unrelated to MAC (16), with whose species nova description one of us is at present involved, hybridizes with the AccuProbe *M. avium* complex but not with the probes specific for *M. avium* and *M. intracellulare*. Such cross-reaction was not present with LiPA, where only the MAI-X group hybridizes with probe 9, specific for the *M. avium* complex, and not with the ones for *M. avium* or *M. intracellulare*.

The discrepant identification of a strain belonging to the MAI-X group by LiPA, and to *M. intracellulare* by AccuProbe, remains unresolved; no basis is available to make a decision, since high-performance liquid chromatography and conventional tests are unable to discriminate among such organisms. Probably only the sequencing of hypervariable, species-specific genomic regions has the potential to solve the dilemma. It is, in any case, an issue of limited value, since species differentiation within the MAC is clinically unimportant and is relevant only from the epidemiological point of view.

The hybridization of the ninth probe with a number of species may at times be confusing. It appears that the majority of doubts can be eliminated by the simultaneous positive result with line 10, 11, or 12 (Fig. 2). When such a result is absent, however, distinguishing between the MAI-X group and *M. malmoense* requires different approaches, such as mycolic acid investigations or biochemical tests; *M. haemophilum*, on the contrary, can be easily distinguished by its metabolic requirements. A correct species identification was obtained with all known genotypic (8) and phenotypic (5) variants of *Mycobacterium gordonae* that we selected to check the sensitivity of the *M. gordonae*-specific probe, and this performance was achieved with both LiPA and AccuProbe. Our four strains of *Mycobacterium celatum* type 1 did not cross-react with the probe for the *M. tuberculosis* complex, as they used to do with AccuProbe (3) before the procedure change introduced by the producer (elongation of the selection step to 10 min).

The different genetic targets of LiPA and AccuProbe explain well the alternative behavior of the two systems. The 16S-23S rRNA spacer is more polymorphic (1) than the 16S rRNA region. In terms of workload, the LiPA procedure (requiring about 6 h), is certainly more complex than the AccuProbe technique (which can be completed within about 90 min); with the latter, however, in cases of negative results, the additional time required for repeating the test with a new probe must be considered. Furthermore, the amplification step frees LiPA from the need of substantial biomass, which, on the contrary, limits the sensibility of AccuProbe.

Only one evaluation of LiPA has been reported so far (11). The previous study was carried out with strains randomly isolated in a routine laboratory, limiting the survey to the most common species. Even with this limitation, the two evaluations agree about the excellent performance of the system. We confirmed the genus specificity of LiPA, which did not react with the genus *Nocardia* (the genus most closely related to *Mycobacterium*); the species and intraspecies specificity was excellent (99.6%), considering the unresolved discrepancy with AccuProbe; also, whenever the five strains presenting (at the first testing) aberrant extra bands were included, a value close to 98% was obtained anyway.

The sensitivity of LiPA for strains in its identification range has been absolute; as this range is wider than that of AccuProbe, we infer that the diagnostic yield would also be superior, with the extent depending on the relative prevalence of *M. xenopi*, *M. malmoense*, *M. haemophilum*, *Mycobacterium scrofulaceum* and *M. chelonae*, which are not covered by AccuProbe. A positive asset of LiPA is that it is based on the very stable DNA, which makes it effective for the identification of old and nonviable cultures; in this respect AccuProbe, which targets the unstable rRNA, can be applied only to actively growing organisms. A minor limitation of the technique is represented by the requirement for stringent control of hybridization temperatures, failing which spurious bands may develop.

Our large panel of mycobacteria covered all the species in the declared identification range of the new kit, including several variants of the same species, and many others; such a selection aimed to check all forseeable limits of the probes in the new kit, and the test results were in substantial agreement with the ones declared by the manufacturer. LiPA is therefore a very specific and sensitive assay that allows, within a few

hours, the precise identification of the large majority of mycobacteria isolated in the laboratories. In our opinion, however, because of its complex procedure, the assay can be usefully implemented only in major mycobacteriology laboratories already skilled in molecular biology techniques.

ACKNOWLEDGMENTS

We thank Innogenetics Italia (Pomezia, Italy) for providing reagents and technical support, Pasquale Urbano (University of Florence, Florence, Italy) for considerable advice, and Maree Williams for formal correction of the manuscript.

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