JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2001, p. 4477–4482 Vol. 39, No. 12
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.12.4477–4482.2001
Copyright © 2001, American Society for Microbiology. All Rights Reserved.

Rapid Identification of Mycobacteria to the Species Level Using INNO-LiPA Mycobacteria, a Reverse Hybridization Assay

P. N. SUFFYS,[1]* A. DA SILVA ROCHA,[1] M. DE OLIVEIRA,[1] C. E. DIAS CAMPOS,[2], A. M. WERNECK BARRETO,[2] F. PORTAELS,[3] L. RIGOUTS,[3] G. WOUTERS,[4] G. JANNES,[4] G. VAN REYBROECK,[4] W. MIJS,[4] AND B. VANDERBORGHT[4,5]

Biochemistry and Molecular Biology Department, Oswaldo Cruz Institute, Oswaldo Cruz Foundation,[1] Reference Center Professor Hélio Fraga,[2] and Laboratory of Molecular Biology, HUCFF, Federal University of Rio de Janeiro,[5] Rio de Janeiro, Brazil, and Institute of Tropical Medicine, Antwerp,[3] and Innogenetics, Zwijnaarde,[4] Belgium

Received 25 May 2001/Returned for modification 16 July 2001/Accepted 13 September 2001

INNO-LiPA Mycobacteria (LiPA; Innogenetics, Zwijnaarde, Belgium) is a kit for the simultaneous detection and identification of *Mycobacterium* species in culture and identifies the *Mycobacterium tuberculosis* complex, the *M. avium* complex (MAC), and the following *Mycobacterium* species: *M. kansasii*, *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. gordonae*, *M. xenopi*, and the *M. chelonae-M. abscessus* complex. The assay, which targets the 16S-23S rRNA spacer region, was evaluated on 157 mycobacterial strains that had been identified by conventional techniques and PCR-restriction enzyme analysis of the *hsp65* gene (PRA). Forty-seven reference strains consisting of 37 different species and 110 human clinical isolates were submitted to the test, and all were hybridized with the *Mycobacterium* genus probe (MYC) on the LiPA strip (100% sensitivity). Ninety-four isolates hybridized to their corresponding species- or complex-specific probes; only one isolate phenotypically identified as *M. gordonae* did not react with its specific probe (99.4% accuracy). Thirty-seven MAC strains were phenotypically identified to the complex level and to the species level by LiPA as *M. avium* ($n = 18$) or *M. intracellulare* ($n = 7$) or as belonging to the *M. avium-M. intracellulare-M. scrofulaceum* complex ($n = 12$). Of the last 12 strains, 10 had *M. avium* PRA patterns and 2 had *M. intracellulare* PRA patterns. Three isolates that had been identified as a single species by conventional identification were proven to be mixed cultures by the LiPA assay. The whole procedure can be performed in 1 working day, starting with the supernatant of a small amount of bacterial mass that had been treated by freezing and then boiling.

To date, more than 70 mycobacterial species have been identified, and occasionally, isolates with unknown characteristics, mostly from immunodeficient patients, are being described. Differentiation of pathogenic and nonpathogenic mycobacteria other than *Mycobacterium tuberculosis* (MOTT) from members of the *M. tuberculosis* complex (MTC) is needed for patient management, considering that many MOTT are resistant to the antibiotics used for treatment of tuberculosis (27).

Identification of mycobacterial isolates to the species level is performed by analysis of phenotypic and biochemical characteristics of the organisms after culture in solid media, which is a time-consuming process, or by high-pressure liquid chromatography analysis, which requires expensive equipment. Development of molecular tests has speeded up diagnosis, but most methods suffer from specific drawbacks. The AccuProbe system (Gen-Probe) differentiates only the MTC, *M. avium*, *M. intracellulare*, *M. gordonae*, and *M. kansasii*. In-house PCR-based identification systems have been developed but either identify a limited number of mycobacterial species or are difficult to use on a routine basis (4, 11). Restriction enzyme analysis of PCR products of specific genes is still widely used for identification of mycobacteria to the species level (7, 19, 23) and on clinical isolates (22, 26). Sequencing of conserved genes is sensitive and accurate but still expensive and technically demanding (9, 18, 21, 28), although commercially available sequencing systems might simplify the procedure (16). Finally, recently developed high-density DNA probe systems need specialized equipment (8).

Recently, INNO-LiPA Mycobacteria (LiPA), a commercially available assay targeting the 16S-23S rRNA spacer region, was developed for the detection of *Mycobacterium* spp. and identification of members of the MTC, *M. kansasii*, *M. xenopi*, *M. gordonae*, *M. avium*, *M. intracellulare*, *M. scrofulaceum*, and *M. chelonae*. The assay has been evaluated with BACTEC 12B bottles, and LiPA results have been compared with results obtained by using DNA probes, conventional biochemical tests, and PCR-restriction fragment length polymorphism (RFLP) analysis; the assay demonstrated clear-cut results and was rapid and easy to perform (14). All samples tested were, however, local clinical isolates, and the specificity of the test was not evaluated on a large number of different species. Furthermore, genetic variability has been observed in mycobacterial isolates with distinct geographic origins (13, 17). We therefore evaluated the assay with reference strains and clinical isolates from different regions of Brazil belonging to 42 different species.

MATERIALS AND METHODS

Culture of mycobacterial strains and DNA extraction. Forty-seven mycobacterial reference strains belonging to 37 species were used in this study and are listed in Table 1. Clinical mycobacterial isolates ($n = 110$, see Table 2) were obtained as Lowenstein-Jensen cultures mainly from the National Reference Center Professor Hélio Fraga (Rio de Janeiro, Brazil) and from Laboratório

* Corresponding author. Mailing address: Laboratory of Molecular and Diagnosis of Infectious Diseases, DBBM, IOC, Fiocruz, Av. Brasil 4365, Manguinhos 21045-900, Rio de Janeiro, Brazil. Phone: 55-21-5984289. Fax: 55-21-2709997. E-mail: psuffys@ioc.fiocruz.br.

TABLE 1. LiPA patterns and LiPA interpretations obtained with 47 reference isolates of 37 different mycobacterial species

Mycobacterium species	Strain(s)	*n*	LiPA probe(s) for which strain was positive	LiPA interpretation
M. africanum	MB3[a]	1	MYC, MTB	MTC
M. agri	ATCC 27406	1	MYC	*Mycobacterium* sp.
M. aichiense	ATCC 27280	1	MYC	*Mycobacterium* sp.
M. asiaticum	ATCC 25276, Z26114[b]	2	MYC	*Mycobacterium* sp.
M. aurum	ATCC 23366	2	MYC	*Mycobacterium* sp.
M. avium	ATCC 25291	1	MYC, MAIS, MAV	*M. avium*
M. bovis	ATCC 19210	1	MYC, MTB	MTC
M. bovis bacillus Calmette-Guérin	ATCC 35736	1	MYC, MTB	MTC
M. chelonae	NCTC 946, MB41[a]	2	MYC, MCH-1	*M. chelonae* II, IV
M. chitae	ATCC 19627	2	MYC	*Mycobacterium* sp.
M. chubuense	ATCC 27278	1	MYC	*Mycobacterium* sp.
M. diernhoferi	ATCC 19340	2	MYC	*Mycobacterium* sp.
M. flavescens	ATCC 14474	1	MYC	*Mycobacterium* sp.
M. fortuitum	ATCC 6841	1	MYC	*Mycobacterium* sp.
M. gilvum	S132[b]	1	MYC	*Mycobacterium* sp.
M. gordonae	ATCC 14470	1	MYC, MGO	*M. gordonae*
M. gordonae subsp. *ureolyticum*	MIS 222[b]	1	MYC, MGO	*M. gordonae*
M. intracellulare	ATCC 13950	1	MYC, MAIS, MIN	*M. intracellulare*
M. kansasii	ATCC 12478	1	MYC, MKA-1	*M. kansasii* I
M. komossense	ATCC 33013	1	MYC	*Mycobacterium* sp.
M. mageritense	ATCC 700351[c]	1	MYC	*Mycobacterium* sp.
M. malmoense	Z10792[b]	1	MYC	*Mycobacterium* sp.
M. marinum	ATCC 927, MIS14[b]	2	MYC	*Mycobacterium* sp.
M. neoaurum	ATCC 25790	1	MYC	*Mycobacterium* sp.
M. nonchromogenicum	ATCC 19530[a]	2	MYC	*Mycobacterium* sp.
M. parafortuitum	ATCC 19686	1	MYC	*Mycobacterium* sp.
M. peregrinum	ATCC 14467	1	MYC	*Mycobacterium* sp.
M. phlei	ATCC 11758	1	MYC	*Mycobacterium* sp.
M. porcinum	ATCC 33776	1	MYC	*Mycobacterium* sp.
M. rhodesiae	ATCC 27024	1	MYC	*Mycobacterium* sp.
M. smegmatis	ATCC 19420	1	MYC	*Mycobacterium* sp.
M. szulgai	NCTC 10831, SCS74/13[b]	2	MYC	*Mycobacterium* sp.
M. terrae	ATCC 15755	1	MYC	*Mycobacterium* sp.
M. thermoresistibile	ATCC 19527	1	MYC	*Mycobacterium* sp.
M. tokaiense	T47502[b]	1	MYC	*Mycobacterium* sp.
M. triviale	ATCC 23292	1	MYC	*Mycobacterium* sp.
M. tuberculosis H37Rv	ATCC 27294	1	MYC, MTB	MTC
M. vaccae	ATCC 15483	1	MYC	*Mycobacterium* sp.
M. xenopi	MYC527[b]	1	MYC, MXE	*M. xenopi*

[a] University of Ghent, Ghent, Belgium.
[b] National Institute of Public Health and the Environment, Bilthoven, The Netherlands.
[c] University of Madrid, Madrid, Spain.

Richet (Rio de Janeiro, Brazil). Conventional identification had been performed according to standard procedures (10), and high-quality DNAs from mycobacterial reference cultures were prepared as described earlier (20). For clinical isolates, the DNA extraction procedure was simplified by suspending a loop of mycobacterial mass taken from a culture in Lowenstein-Jensen medium in 0.5 ml of 10 mM Tris-HCl–1 mM EDTA–1% Triton X-100 and submitting it to three cycles of freezing and boiling (5 min, −70°C; 10 min, 100°C). Nucleic acids and processed cultures were stored at −20°C until further use.

PCR-RFLP analysis of *hsp65*. PCR-restriction enzyme analysis of a 441-bp amplified fragment of the *hsp65* gene (PRA) was performed as modified by Telenti et al. (23). Briefly, 10 ng of purified mycobacterial DNA or that amount present in 2 μl of the supernatant of a frozen and boiled bacterial mass was amplified using 1.25 U of *Taq* polymerase by submitting the sample to 45 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C, followed by a final extension step at 72°C for 7 min. When an amplicon was present, 20 μl was digested with 10 U of either *Bst*EII or *Hae*III and the restriction pattern was analyzed by electrophoresis in a mixture of 4% agarose (Sigma) and 1% NuSieve agarose (FMC BioProducts, Rockland, Maine) and by ethidium bromide staining. Each gel had a 50- or 100-bp-ladder molecular weight marker (Gibco BRL) in at least two lanes. Patterns were compared with those published in the literature (3, 6, 22, 23) or analyzed against a pattern database constructed with GelCompar (3).

LiPA. The same amount of the DNA sample that was submitted to PCR-RFLP was added to a 40-μl PCR mixture containing deoxynucleoside triphosphates (supplied in the kit as amplification buffer), biotinylated primers and $MgCl_2$ (supplied as primer solution), and 1.25 U of *Taq* polymerase (Amersham Pharmacia, Little Chalfont, Buckinghamshire, United Kingdom). After 1 min at 95°C, amplification was performed by submitting mixtures to 30 s at 95°C, 30 s at 62°C, and 30 s at 72°C for 40 cycles. Amplification was verified in 2% agarose and by staining with ethidium bromide, and generally, a band with a size ranging from 250 to 500 bp was obtained. A 10-μl aliquot of the amplified product was mixed with 10 μl of denaturing solution and incubated for 5 min at room temperature in a hybridization trough in a 12-trough tray (both supplied with the kit). Two milliliters of hybridization solution (at 62°C) and a membrane strip were added to each sample, and the tray was then placed in a shaking water bath (Gemini twin; Robbins Scientific, Sunnyvale, Calif.) at 62°C and incubated for 30 min. This hybridization step was followed by two 1-min incubations with 2 ml of wash solution at room temperature and one 10-min incubation at 62°C. All further incubations were performed at room temperature in an orbital shaker. The strips were rinsed with 2 ml of rinse solution, followed by incubation with alkaline phosphatase-streptavidin conjugate solution for 30 min and washing with 2 ml of rinse solution and 2 ml of substrate buffer. Finally, 2 ml of substrate solution was added and the strips were incubated for 30 min; the color reaction was stopped by washing the strips twice with 2 ml of deionized water, and identification of the sample was performed by comparing the strip against an interpretation chart supplied with the kit. In each test run, one LiPA control sample was hybridized against a strip as a control for the hybridization conditions. The LiPA test was repeated when hybridization signals suggested the presence of two mycobacterial species in a sample or when discrepant results were obtained by the different

TABLE 2. Conventional identification, PRA and LiPA patterns, and interpretations obtained with 110 clinical human isolates of mycobacteria

Conventional identification	PRA identification	No. of isolates	LiPA probe(s) for which strain was positive	LiPA interpretation
MTC	MTC	1	MYC, MTB	MTC
M. kansasii	*M. kansasii* I	14	MYC, MKA-1[a]	*M. kansasii* I
	M. kansasii III	1	MYC, MKA-1	*M. kansasii* I
M. gordonae	*M. gordonae* I	3	MYC, MGO	*M. gordonae*
	M. gordonae II	1	MYC, MGO	*M. gordonae*
	M. gordonae III	6	MYC, MGO	*M. gordonae*
	M. gordonae VII	1	MYC, MGO	*M. gordonae*
	Unpublished	5	MYC, MGO	*M. gordonae*
	Unpublished	1	Ambiguous	Ambiguous
MAC	*M. avium* I	14	MYC, MAIS, MAV	*M. avium*
	M. avium I	6	MYC, MAIS	*M. avium-M. intracellulare-M. scrofulaceum* or *M. malmoense*
	M. avium II	3	MYC, MAIS, MAV	*M. avium*
	M. avium III	1	MYC, MAIS, MAV	*M. avium*
	M. avium III	4	MYC, MAIS	*M. avium-M. intracellulare-M. scrofulaceum* or *M. malmoense*
	M. intracellulare I	5	MYC, MAIS, MIN	*M. intracellulare*
	M. intracellulare I	2	MYC, MAIS	*M. avium-M. intracellulare-M. scrofulaceum* or *M. malmoense*
	Unpublished	2	MYC, MAIS, MIN	*M. intracellulare*
M. scrofulaceum	Unpublished	2	MYC, MAIS, MSC	*M. scrofulaceum*
M. abscessus-M. chelonae	*M. abscessus* I	3	MYC, MCH-1, MCH-2	*M. chelonae* III
	M. abscessus II	8	MYC, MCH-1, MCH-2	*M. chelonae* III
M. fortuitum complex	*M. fortuitum* I	2	MYC	*Mycobacterium* sp.
	M. fortuitum III	2	MYC	*Mycobacterium* sp.
	M. peregrinum I	3	MYC	*Mycobacterium* sp.
	M. peregrinum II	1	MYC	*Mycobacterium* sp.
	Unpublished	2	MYC	*Mycobacterium* sp.
M. flavescens	*M. flavescens* I	1	MYC	*Mycobacterium* sp.
	Unpublished pattern	1	MYC	*Mycobacterium* sp.
M. szulgai	*M. szulgai* I	3	MYC	*Mycobacterium* sp.
	Unpublished	2	MYC	*Mycobacterium* sp.
M. duvali	Unpublished	1	MYC	*Mycobacterium* sp.
M. lentiflavum	*M. lentiflavum* I	2	MYC	*Mycobacterium* sp.
M. simiae	*M. simiae* I	4	MYC	*Mycobacterium* sp.
M. triviale	*M. triviale* I	1	MYC	*Mycobacterium* sp.
M. terrae	Unpublished	1	MYC	*Mycobacterium* sp.
Unidentified *Mycobacterium* sp.	Unpublished	1	MYC	*Mycobacterium* sp.

[a] The MAV probe was weakly visible for one isolate.

identification procedures. Also, 3 of the 12 samples that hybridized with the genus and *M. avium-M. intracellulare-M. scrofulaceum* probes only were resubmitted to the test and had their hybridization patterns confirmed.

Sequencing and analysis. For some isolates, the amplified 16S-23S spacer region generated as described above was purified using a QIAquick PCR purification kit (Qiagen, Chatsworth, Calif.) and sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 377 sequencer (Applied Biosystems, Foster City, Calif.). Analysis of sequences was performed by comparison against sequences in GenBank using Seqed and Fasta of the Wisconsin Package (version 9.1; Genetics Computer Group, Madison, Wis).

RESULTS

No difference in signal intensity or background was observed when the LiPA assay was performed on purified DNA or on the supernatant of a heat shock-treated bacterial mass (data not shown). For all isolates analyzed by hybridization, a clear amplicon of the expected size was visible on agarose gel.

Initially, we tested 49 reference strains, including the type strains of *M. ulcerans* (ATCC 19423) and *M. simiae* (ATCC 25275), and all hybridized to the *Mycobacterium* genus probe (MYC). The *M. ulcerans* and *M. simiae* strains, however, gave an *M. intracellulare* pattern on LiPA assay, and PRA analysis demonstrated that they had been contaminated with *M. intracellulare*, so they were not included in the study. Twelve strains also hybridized to their corresponding complex- or species-specific probes (Table 1): reference strains of *M. tuberculosis*, *M. bovis*, and *M. africanum* strains hybridized correctly with the probe for the MTC, while *M. gordonae*, *M. avium*, *M. intracellulare*, and *M. xenopi* reacted with their specific probes. For *M. kansasii* and *M. chelonae*, species for which more than one probe was present on the strip, hybridization occurred with MKA-1 and MCH-1, respectively. All other reference strains, including *M. fortuitum*, reacted with the MYC probe only.

All human clinical isolates reacted with the genus-specific *Mycobacterium* probe (MYC) (Table 2). For species or species groups that have specific probes in the LiPA assay, concordant results were found: isolates of MTC ($n = 1$), *M. kansasii* ($n = 15$), *M. gordonae* ($n = 16$), *M. scrofulaceum* ($n = 2$), *M. abscessus* ($n = 11$), and the *M. avium complex* (MAC) ($n = 37$) all hybridized with the appropriate probes on LiPA assay. The *M. kansasii* isolates were positive with the MKA-1 probe, while the *M. abscessus* isolates reacted with the MCH-1 and MCH-2 probes. For one *M. kansasii* isolate, a weak cross-reaction was

visible with the MAV probe in the absence of the *M. avium-M. intracellulare-M. scrofulaceum* signal. The LiPA test could not be repeated on this isolate, and we consider this result a hybridization artifact that was probably due to a small variation in hybridization temperature, which is critical during the assay, or due to the fact that the strip was not completely submerged into the assay buffers. Clinical isolates of *M. fortuitum* ($n = 10$), *M. flavescens* ($n = 2$), *M. terrae* ($n = 1$), *M. lentiflavum* ($n = 2$), *M. duvali* ($n = 1$), *M. szulgai* ($n = 5$), *M. triviale* ($n = 1$), *M. simiae* ($n = 4$), and one unidentified *Mycobacterium* species were identified to the genus level only. One isolate identified as *M. gordonae* by conventional methods gave different results during different test runs: in two tests, only the MYC probe was positive, and in one other run, an *M. avium* isolate was found on LiPA assay.

All isolates were also characterized by PRA, as summarized in Table 2. All clinical *M. kansasii* isolates had the *M. kansasii* I-type PRA pattern, except for one isolate that had the *M. kansasii* III pattern. Of the 16 *M. gordonae* isolates, 3 had the *M. gordonae* I PRA pattern, 1 had the *M. gordonae* II pattern, 6 had the *M. gordonae* III pattern, 1 had the *M. gordonae* VII pattern, and 5 were characterized by three new PRA patterns for this species (data not shown). At the time of the study, no conventional identification procedures were performed to differentiate between isolates of *M. chelonae* and *M. abscessus*, but among the strains identified as the *M. chelonae-M. abscessus* complex, three had the PRA pattern of *M. abscessus* I and eight had the pattern of *M. abscessus* II. The two strains that reacted with the *M. scrofulaceum* probe on LiPA assay had a so-far-undescribed PRA pattern (data not shown), and their identities were confirmed twice by conventional testing. Among the isolates of the *M. fortuitum-M. peregrinum* complex, two had the *M. fortuitum* I PRA pattern, two had the *M. fortuitum* III pattern, three had the *M. peregrinum* I pattern, one had the *M. peregrinum* II pattern, and two had unpublished PRA patterns (data not shown). One strain had the *M. duvali* pattern, one had the *M. terrae* pattern, one had the *M. flavescens* pattern, and two *M. szulgai* strains had a so-far-undescribed PRA pattern (data not shown).

Thirty-seven clinical isolates that had been identified phenotypically as MAC were identified by the LiPA assay as *M. avium* ($n = 18$), *M. intracellulare* ($n = 7$), or *M. avium-M. intracellulare-M. serofulaceum* (probes MYC and MAIS positive; $n = 12$). From the 18 isolates hybridizing with the specific *M. avium* probe on LiPA assay, 14 were *M. avium* I, 3 were *M. avium* II, and 1 was *M. avium* III on PRA. Among the seven isolates reacting with the specific *M. intracellulare* probe on LiPA assay, five were identified as *M. intracellulare* I on PRA and two had this pattern but without the *Hae*III 60-bp band (data not shown). From the 12 strains that reacted with the MAIS probe only, 6 were *M. avium* I, 4 were *M. avium* III, and 2 were *M. intracellulare* I.

Initially, for seven isolates from patients, discrepant results obtained by different identification procedures were caused by mixed cultures: in two cultures, the presence of at least two different species was detected by genetic analysis, while in five others, the presence of different species in a single culture was suggested upon repetition of conventional identification. Among the cultures that had been characterized as a single species by conventional procedures but had mixed signals by the LiPA assay, one isolate was an *M. gordonae* strain that hybridized two times with the MGO, MAIS, and MAV probes on LiPA assay and had a combination of *M. gordonae* III and *M. avium* III patterns on PRA. Another isolate, phenotypically identified as *M. fortuitum*, was initially positive on two LiPA assays for the MYC, MAIS, MAV, and MIN probes but had a PRA pattern suggestive of *M. fortuitum* I with background; upon subcloning, the isolate was reidentified as *M. fortuitum*, had a clear *M. fortuitum* I PRA pattern, and was MYC probe positive only on LiPA assay. For five clinical isolates, discrepant results were obtained when conventional identification results were compared to the results obtained by PRA and the LiPA assay. One isolate that had been identified as *M. scrofulaceum* by a conventional method was identified as *M. gordonae* III and was positive for the MGO probe by the LiPA assay, an isolate identified as *M. gordonae* was identified as *M. kansasii* I and was positive for the MKA-1 probe by the LiPA assay, and three isolates conventionally identified as MAC gave genetic patterns for another species. One of the isolates of the last group had patterns for MTC by both genetic assays, one was *M. peregrinum* I and *Mycobacterium* species by PRA and the LiPA assay, respectively, and the other was *M. szulgai* and *Mycobacterium* species by PRA and the LiPA assay, respectively. For these isolates, the biochemical identification was repeated on subculture, confirming the results obtained by PRA and the LiPA assay.

DISCUSSION

In this study, we evaluated the LiPA line probe assay on both clinical isolates and reference strains. Recently, Miller et al. (14) examined the performance of the LiPA test, demonstrating correct identifications for 59 of 60 *Mycobacterium* isolates, but that study concentrated on cultures received in a single lab in the United States, half of which were *M. tuberculosis*. Genetic variability of mycobacteria has been associated with differences in geographic origin (5, 13), and although MOTT in Brazil are mostly MAC, *M. kansasii*, and *M. fortuitum* (1), all assays required for conventional identification to the species level are not routinely performed, so the number of infective or colonizing *Mycobacterium* species in the country is underestimated (2, 3). Also, an earlier study using PRA on a relatively small number of clinical isolates suggests the existence of particular Brazilian allelic types of mycobacteria (2). The LiPA assay in this study was performed on clinical isolates obtained from a laboratory that receives clinical specimens from different regions of the country.

All mycobacterial samples reacted positively with the *Mycobacterium* genus probe on LiPA assay (100% sensitivity), so, in contrast to what occurs in acid-fast staining, a single technology determines both the genus and clinically important species. All isolates for which species- or complex-specific probes are present on the strip were correctly identified by a positive reaction of the specific probes, except for one *M. gordonae* isolate. The LiPA assay for this isolate was performed three times, once on a DNA sample that yielded an unpublished PRA pattern that was observed in two other isolates that were positive for the MGO probe on LiPA assay. We have no simple explanation for this result, but PCR contamination or DNA degradation could be involved. The total accuracy for identi-

fication is therefore 99.4% (156 of 157 samples correctly identified).

The LiPA assay was able to further identify to the species level 25 out of 37 strains that had been conventionally characterized as MAC; these isolates were identified by the LiPA assay as *M. avium* and *M. intracellulare* and had PRA patterns corresponding to these species (Table 2). Twelve strains, besides being positive for the MYC probe, were positive for the MAIS probe only, and although four of these had the *M. avium* III PRA pattern that has been detected so far only in strains from Brazil (12), six had *M. avium* I and two had *M. intracellulare* I PRA patterns. Three out of the 53 isolates that could not be correctly identified by Miller et al. (14) were *M. intracellulare* strains (from a total of 15 MAC strains) that were identified by the LiPA assay as *M. avium-M. intracellulare-M. scrofulaceum*. The observation that one out of three MAC strains in our study could be identified to the species level by PRA but to the *M. avium-M. intracellulare-M. scrofulaceum* complex level only by the LiPA assay could be explained by the different genetic targets of both assays. Some strains may be particular to Brazil since certain PRA genotypes of MAC isolates have so far been reported only in this country (12, 15). We suspect these strains to be *M. avium-M. intracellulare* intermediates, based on the large number of MAC strains that have been sequenced for development of the species-specific probes (data not shown). Nonetheless, the fact that these variants have been positive for the MAIS probe demonstrates that the assay is sufficiently sensitive to pick up genetic variants within MAC.

Essentially as reported by Miller et al. (14) but in contrast to a recent study performed in Italy (24), all isolates that hybridized with the MCH-1 and MCH-2 probes were *M. abscessus* and all *M. kansasii* strains (except one) that were positive for the MKA-1 probe on LiPA assay were determined to be *M. kansasii* I on PRA. We tested only a single clinical isolate of *M. tuberculosis*, but all 26 strains tested by Miller et al. (14) hybridized with the correct probes.

The fact that *M. fortuitum* cannot be differentiated from other species for which no specific probes are present on the strip may be a limitation of the assay because specific therapeutic schemes for this organism are available (27). In Brazil, 11% of MOTT of pulmonary origin isolated are *M. fortuitum* strains (1), and about 50% of the clinical isolates that reacted only with the species-specific probe in this study were *M. fortuitum* strains. This species, however, is genetically heterogeneous (W. Mijs, L. Rigouts, F. Portaels, and R. Rossau, Abstr. 21st Annu. Congr. Eur. Soc. Mycobacteriol., 2000), so inclusion of species-specific probes in the LiPA assay may be technically complicated.

Although 18 out of the 110 clinical isolates had so-far-unpublished PRA patterns, we feel confident about the identities of 109 samples because conventional identification procedures were performed in a reference center whose staff has considerable experience in the matter. Our results also demonstrate that discordance between the results of conventional and genetic identification procedures was mostly due to the presence of two different mycobacterial species in a culture. In the present study, no single colonies were picked for resubmitting to the different identification procedures but colonies with different morphologies were sometimes observed during conventional identification (data not shown). The LiPA assay was able to detect mixtures when mycobacterial species for which specific probes are present on the strip were involved, as demonstrated by the mixed signals described in Results; the assay did not, however, allow simultaneous identification of *M. fortuitum* and another species because no species-specific probe was present for the former species. We imagine that the result obtained by different identification procedures on mixed cultures will be influenced by the relative quantity of each species present in the clinical sample, by the growth rate of the organisms, and by the fraction of the culture that is being submitted to subculturing or DNA extraction. At least for species that have a specific probe, the LiPA assay seems to be more sensitive than PRA for detecting mixed cultures. This conclusion is in agreement with the reported higher sensitivity of the line probe assay than those of sequencing and phenotypic assays for detecting mixed populations of human immunodeficiency virus (25). In any case, we suggest that preferentially clonal organisms should be used for comparison of identification procedures.

When this paper was in preparation, Tortoli et al. (24) reported an assessment of the performance of the LiPA line probe assay on 238 strains isolated in seven Italian laboratories. The kit correctly identified 99.6% of the strains tested, and the discrepant result was for an unresolved MAC strain identified by the LiPA assay as a MAC intermediate.

Conclusion. Evaluation of the LiPA assay was performed on a large number of clinical isolates and on reference isolates from different *Mycobacterium* species, which were characterized by phenotypic methods and PRA. Discordance between the results of different identification procedures was due to the presence of two different organisms in a culture. The LiPA assay, which targets the 16S-23S rRNA spacer region, was able to correctly identify 156 out of 157 isolates or strains, resulting in an accuracy of 99.4%. One *M. gordonae* isolate showed ambiguous results. A correlation was found between identifications obtained by the LiPA assay, which targets the 16S-23S rRNA spacer region, and PRA, which targets the *hsp65* gene, although allelic differences were observed. In general, the LiPA assay is a reliable genetic test for the identification and differentiation of *Mycobacterium* species.

ACKNOWLEDGMENTS

We thank Mario Vaneechoutte, Maria Jesus Garcia, Dick van Soolingen, and Richet Laboratory for donation of some of the isolates.

This study was supported by the CNPq, Faperj, and PRONEX.

REFERENCES

1. **Barreto, A. M. W., and C. E. D. Campos.** 2000. Micobactérias não tuberculosas no Brasil. Bol. Pneumol. Sanit. **8:**23–32.
2. **Barreto, A. M. W., and F. M. Martins.** 1999. Laboratório de tuberculose do CRPHF: quinze anos de atividades. Bol. Pneumol. Sanit. **7:**41–51.
3. **da Silva Rocha, A., C. da Costa Leite, H. M. Torres, A. B. de Miranda, M. Q. Pires Lopes, W. M. Degrave, and P. N. Suffys,** 1999. Use of PCR-restriction fragment polymorphism analysis of the *hsp65* gene for rapid identification of mycobacteria in Brazil. J. Microbiol. Methods **37:**223–229.
4. **De Beenhouwer, H., Z. Liang, P. de Rijk, C. van Eekeren, and F. Portaels.** 1995. Detection and identification of mycobacteria by DNA amplification and oligonucleotide-specific capture plate hybridization. J. Clin. Microbiol. **33:**2994–2998.
5. **Devallois, A., M. Picardeau, C. N. Paramasivan, V. Vincent, and N. Rastogi.** 1997. Molecular characterization of *Mycobacterium avium* complex isolates giving discordant results in AccuProbe tests by PCR-restriction enzyme analysis, 16S rRNA gene sequencing, and DT1-DT6 PCR. J. Clin. Microbiol. **35:**2767–2772.

6. **Devallois, A., K. S. Goh, and N. Rastogi.** 1997. Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene and proposition of an algorithm to differentiate 34 mycobacterial species. J. Clin. Microbiol. **35:**2969–2973.
7. **Dobner, P., K. Feldmann, M. Rifai, T. Löscher, and H. Rinder.** 1996. Rapid identification of mycobacterial species by PCR amplification of hypervariable 16S rRNA gene promoter region. J. Clin. Microbiol. **34:**866–869.
8. **Gingeras, T. R., G. Ghandour, E. Wang, A. Berno, P. M. Small, F. Drobniewski, D. Alland, E. Desmond, M. Holodniy, and J. Drenkow.** 1998. Simultaneous genotyping and species identification using hybridization pattern recognition analysis of generic Mycobacterium DNA arrays. Genome Res. **8:**435–448.
9. **Kapur, V., L.-L. Li, M. R. Hamrick, B. B. Plikaytis, T. M. Shinnick, A. Telenti, W. R. Jacobs, Jr., A. Banerjee, S. Cole, K. Y. Yuen, J. E. Clarridge, B. N. Kreiswirth, and J. M. Musser.** 1995. Rapid *Mycobacterium* species assignment and unambiguous identification of mutations associated with antimicrobial resistance in *Mycobacterium tuberculosis* by automated DNA sequencing. Arch. Pathol. Lab. Med. **119:**131–138.
10. **Kent, P. T., and G. P. Kubica.** 1985. Public health mycobacteriology: a guide for the level II laboratory. Centers for Disease Control, Atlanta, Ga.
11. **Kox, L. F. F., H. M. Jansen, S. Kuijper, and A. H. J. Kolk.** 1997. Multiplex PCR assay for immediate identification of the infecting species in patients with mycobacterial disease. J. Clin. Microbiol. **35:**1492–1498.
12. **Leão, S. C., M. R. S. Briones, M. P. Sircili, S. C. Balian, N. Mores, and J. S. Ferreira-Neto.** 1999. Identification of two novel *Mycobacterium avium* allelic variants in pig and human isolates from Brazil by PCR-restriction enzyme analysis. J. Clin. Microbiol. **37:**2592–2597.
13. **Legrand, E., K. S. Goh, C. Sola, and N. Rastogi.** 2000. Description of a novel *Mycobacterium simiae* allelic variant isolated from Caribbean AIDS patients by PCR-restriction enzyme analysis and sequencing of hsp65 gene. Mol. Cell. Probes **14:**355–363.
14. **Miller, N., S. Infante, and T. Cleary.** 2000. Evaluation of the LiPA MYCOBACTERIA assay for identification of mycobacterial species from BACTEC 12B bottles. J. Clin. Microbiol. **38:**1915–1919.
15. **Oliveira, R. S., M. P. Sircili, S. Y. M. Ueki, M. A. S. Telles, B. Schnabel, M. R. S. Briones, and S. C. Leão.** 2000. PCR-restriction enzyme analysis of a bone marrow isolate from a human immunodeficiency virus-positive patient discloses polyclonal infection with two *Mycobacterium avium* strains. J. Clin. Microbiol. **38:**4643–4645.
16. **Patel, J. B., D. G. B. Leonard, X. Pan, J. M. Musser, R. E. Berman, and I. Nachamkin.** 2000. Sequence-based identification of *Mycobacterium* species using the MicroSeq 500 16S rDNA bacterial identification system. J. Clin. Microbiol. **38:**246–251.
17. **Picardeau, M., G. Prod'hom, L. Raskine, M. P. LePennec, and V. Vincent.** 1997. Genotypic characterization of five subspecies of *Mycobacterium kansasii*. J. Clin. Microbiol. **35:**25–32.
18. **Rogall, T., T. Flohr, and E. C. Böttger.** 1990. Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA. J. Gen. Microbiol. **136:**1915–1920.
19. **Roth, A., U. Reischl, A. Streubel, L. Naumann, R. M. Kroppenstedt, M. Habicht, M. Fischer, and H. Mauch.** 2000. Novel diagnostic algorithm for identification of Mycobacteria using genus-specific amplification of the 16S–23S rRNA gene spacer and restriction endonucleases. J. Clin. Microbiol. **38:**1094–1104.
20. **Santos, A. R., A. B. de Miranda, L. M. Lima, P. N. Suffys, and W. M. Degrave,** 1992. Method for high yield preparation in large and small scale of nucleic acids from mycobacteria. J. Microbiol. Methods **15:**83–94.
21. **Soini, H., E. C. Böttger, and M. K. Viljanen.** 1994. Identification of mycobacteria by PCR-based sequence determination of the 32-kilodalton protein gene. J. Clin. Microbiol. **32:**2944–2947.
22. **Taylor, T. B., C. Patterson, Y. Hale, and W. W. Safranek.** 1997. Routine use of PCR-restriction fragment length polymorphism analysis for identification of mycobacteria growing in liquid media. J. Clin. Microbiol. **35:**79–85.
23. **Telenti, A., F. Marchesi, M. Balz, F. Bally, E. C. Böttger, and T. Bodmer.** 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol. **31:**175–178.
24. **Tortoli, E., A. Nanetti, C. Piersimoni, P. Cichero, C. Farina, G. Mucignat, C. Scarparo, L. Bartolini, R. Valentini, D. Nista, G. Gesu, C. P. Tosi, M. Crovatto, and G. Brusarosco.** 2001. Performance assessment of new multiplex probe assay for identification of mycobacteria. J. Clin. Microbiol. **39:**1079–1084.
25. **Van Laethem, K., K. Van Vaerenbergh. J.-C. Schmit, S. Sprecher, P. Hermans, V. De Vroey, R. Schuurman, T. Harrer, M. Witvrouw, E. Van Wijngaerden, L. Stuyver, M. Van Ranst, J. Desmyter, E. De Clercq, and A.-M. Vandamme.** 1999. Phenotypic assays and sequencing are less sensitive than point mutation assays for detection of resistance in mixed HIV-1 genotypic populations. J. Acquir. Immune Defic. Syndr. **22:**107–118.
26. **Wilson, R. W., V. A. Steingrube, B. A. Brown, and R. J. Wallace, Jr.** 1998. Clinical application of PCR-restriction enzyme pattern analysis for rapid identification of aerobic actinomycete isolates. J. Clin. Microbiol. **36:**148–152.
27. **Wright, P. W., and R. J. Wallace, Jr.** 1994. Nontuberculous mycobacteria with and without HIV infection, p. 183–206. *In* L. N. Friedman (ed.), Tuberculosis: current concepts and treatment. CRC Press, Inc., Boca Raton, Fla.
28. **Zolg, J. W., and S. Philippi-Schulz.** 1994. The superoxide dismutase gene, a target for detection and identification of mycobacteria by PCR. J. Clin. Microbiol. **32:**2801–2812.