Evaluation of the New GenoType Mycobacterium Assay for Identification of Mycobacterial Species

Cristina Russo,[1] Enrico Tortoli,[2]* and Donato Menichella[1]

*Microbiology Laboratory, Bambino Gesù Hospital, Rome,[1] and Regional Reference
Center for Mycobacteria, Microbiology and Virology Laboratory,
Careggi Hospital, Florence,[2] Italy*

The new commercial assay GenoType Mycobacterium, intended for the identification of mycobacteria, was evaluated with a panel of 197 strains belonging to 86 different taxa. The system, which relies on solid-phase reverse hybridization, is composed of two different kits: GenoType CM for the identification of more frequently detected mycobacterial species and GenoType AS for less common species. The sensitivity and specificity were 97.9% and 92.4% for the CM kit and 99.3% and 99.4% for the AS kit. Our results show that the system may represent a useful tool to substantially enlarge the number of mycobacteria that can be reliably identified in clinical laboratories.

Procedures for the identification of mycobacteria have greatly changed in the last years, and conventional biochemical and cultural methods are nowadays used only by a few laboratories worldwide. Even the methods based on analysis of cell wall lipids, which have played a primary role in the last 20 years of the 20th century (2, 8, 11), now seem to be in trouble because of the steady increase in the number of species within the genus *Mycobacterium* (15).

As expected, the enormous progress in genetic knowledge has brought molecular methods into the limelight. Commercial DNA probes, first marketed by Gen-Probe (San Diego, Calif.), immediately turned out to be a handy and reliable tool, suitable for accurate identification of the most common mycobacterial species in many laboratories. The sensitivity and specificity of such DNA probes are very high (6), and the only real limit of the system is the low number of species it is able to identify.

Several years ago, an important innovation was represented by the development of the reverse hybridization line probe assay. Such a system relies on paper strips on which multiple probes, each characterized by a different specificity, are immobilized. The amplified target sequence of the organism to be identified binds to the specific probe, and hybridization is revealed by the development of a colored line, while its position within the strip identifies the species.

The number of line probes in such commercial strips has risen over the last few years, and very recently a system using two different strips has been commercialized in Europe (not submitted to the U.S. FDA so far). Such a DNA probe system (GenoType Mycobacterium; Hain, Nehren, Germany) is available in two different kits, named CM (for "common mycobacteria"), which includes the most frequently encountered mycobacteria, and AS (for "additional species"), which includes mycobacteria rarely isolated from clinical samples (Fig. 1).

The aim of this study was to investigate the specificity and sensitivity of this method using several reference strains and several clinical isolates.

MATERIALS AND METHODS

The 197 strains used to test the DNA probe system included both clinical isolates from our laboratory collections and reference strains from various international collections and covered a very wide spectrum of mycobacterial species. For several taxa, intraspecific variants were also included. This was the case for *Mycobacterium gordonae*, for which, in addition to a number of typical strains, other strains were investigated, such as: isolates lacking pigmentation, isolates characterized by the uncommon high-performance liquid chromatography profile ii (3), isolates belonging to each of the three genotypes defined by microheterogeneity within the 16S rRNA gene (7), and isolates for which, in our hands, hybridization had failed with the commercial line probe assay INNO LiPA Mycobacteria (LiPA) (Innogenetics, Ghent, Belgium). Even in the case of *Mycobacterium kansasii*, of which five genetic variants have been reported (1, 12), strains belonging to the three most frequent genotypes (i, ii, and iii) were included in the study. Furthermore, two strains of *Mycobacterium fortuitum* not hybridizing with any of the LiPA probes (E. Tortoli, unpublished data) were investigated. There were 151 clinical mycobacterial isolates, 37 reference strains, and 9 strains belonging to genera other than *Mycobacterium* used in this study (Table 1).

In two-thirds of the cases, the identification of non-reference strains had been previously performed using biochemical and cultural tests (18) in combination with high-performance liquid chromatography (2), and, in the remaining cases, with LiPA (16). Moreover, on the occasion of the present study, all non-reference strains were further checked by genetic sequencing of the first 500 bp of the 16S rRNA gene (4). For 11 of these strains, genetic sequencing revealed that the first identification was not correct mainly because of the inadequacy of phenotypic tests to discriminate between species presenting similar features.

All of the isolates were processed after subcultivation on Middlebrook 7H11 agar. Only two strains of *Mycobacterium genavense* were processed directly from liquid culture.

One hundred ninety-seven strains were assayed with GenoType Mycobacterium CM (CM), whereas only 167 of them were tested with GenoType Mycobacterium AS (AS). In fact, several isolates belonging to species represented by a large number of strains were left out from the assay, as their testing with AS had only the aim of checking possible cross-reactivity.

16S rRNA gene sequencing. Amplification and sequencing were carried out with MicroSeq 500 16S rRNA gene bacterial identification kit (Applied Biosystems, Foster City, Calif.) on the DNA extracted by using PrepMan Ultra (Applied Biosystems). The identification of the sequences was obtained by comparison with the GenBank database.

GenoType. The GenoType assay was performed according to the manufacturer's instructions: DNA extraction by the ultrasonic method was followed by PCR

* Corresponding author. Mailing address: Centro Regionale di Riferimento per la Diagnostica dei Micobatteri, Piastra dei Servizi, Ospedale di Careggi, viale Morgagni 85, 50134 Firenze, Italy. Phone: 39-055-7949199. Fax: 39-055-7949010. E-mail: e.tortoli@libero.it.
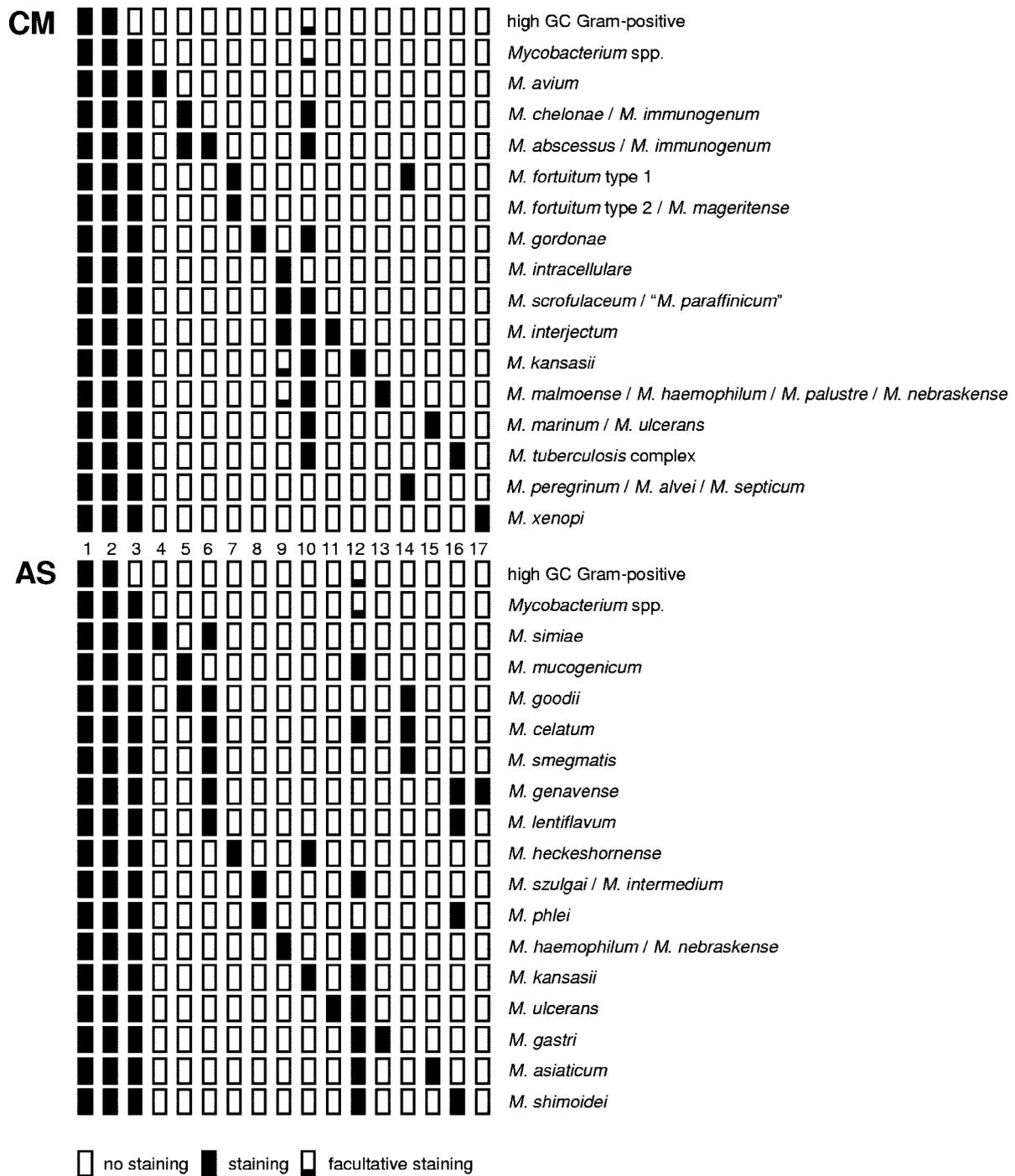
FIG. 1. Hybridization patterns produced in GenoType CM and AS with different mycobacterial species. 1, conjugate control; 2, universal probe; 3, genus *Mycobacterium* probe; 4 to 17, "species"-specific probes.

amplification of a trait of the 23S rRNA gene, as recommended. Reverse hybridization and detection were carried out on a shaking water bath (TwinCubator; Hain). The final identification was obtained by comparison of line probe patterns with the provided evaluation sheet (Fig. 1).

For calculation of sensitivity and specificity at the species level, the identifications of all strains belonging to species compatible with the ones proposed by the system were considered as correct both when these identifications were unequivocal and when they were not. For the species not included in the strips, the identification was considered as correct when only the genus-specific probe was positive.

RESULTS

The genus *Mycobacterium*-specific probe in the CM strip correctly identified 186 out of 188 mycobacteria (sensitivity, 98.9%), and that in the AS strip identified 157 out of 158 mycobacteria (sensitivity, 99.4%). Specificity was 100% for the AS strip and 88.9% for the CM strip, since one out of nine nonmycobacterial strains (*Tsukamurella* spp.) was weakly positive with the *Mycobacterium* genus probe (Table 2).

TABLE 1. Results of strains tested with GenoType CM and AS[a]

Species	Identification by (no. of strains):	
	GenoType CM	GenoType AS
M. abscessus	M. abscessus/immunogenum (5)	Mycobacterium spp. (3)
M. africanum	M. tuberculosis complex (1)	Mycobacterium spp. (1)
M. alvei*	M. peregrinum/septicum/alvei (1)	Mycobacterium spp. (1)
M. asiaticum*	Mycobacterium spp. (1)	M. asiaticum (1)
M. austroafricanum*	**None** (1)	Mycobacterium spp. (1)
M. avium	M. avium (15)	Mycobacterium spp. (8)
M. avium silvaticum*	M. avium (M. genus negative) (1)	Mycobacterium spp. (1)
MAC	**M. intracellulare** (4)	Mycobacterium spp. (4)
M. botniense	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. bovis	M. tuberculosis complex (1)	Mycobacterium spp. (1)
M. bovis BCG	M. tuberculosis complex (1)	Mycobacterium spp. (1)
M. branderi*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. brumae*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
"M. canettii"	M. tuberculosis complex (1)	Mycobacterium spp. (1)
M. caprae*	M. tuberculosis complex (1)	Mycobacterium spp. (1)
M. celatum type 1	Mycobacterium spp. (4)	M. celatum (4)
M. celatum type 3*	Mycobacterium spp. (1)	M. celatum (1)
M. chelonae	M. chelonae/immunogenum (5)	Mycobacterium spp. (3)
M. chimaera*	**M. intracellulare** (3)	Mycobacterium spp. (3)
M. chitae*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. chlorophenolicum*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. chubuense*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. confluentis*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. cookii*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. conspicuum	Mycobacterium spp. (1)	M. conspicuum (1)
M. diernhoferi*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. duvalii*	**None** (1)	None (1)
M. elephantis	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. fallax	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. farcinogenes	**M. fortuitum** type 1 (1)	Mycobacterium spp. (1)
M. flavescens*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. florentinum*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. fortuitum	M. fortuitum type 1 (9)	Mycobacterium spp. (5)
	M. fortuitum type 2/mageritense (1)	Mycobacterium spp. (1)
M. gadium*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. genavense	Mycobacterium spp. (2)	M. genavense (2)
M. gilvum*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. gordonae	M. gordonae (9)	Mycobacterium spp. (5)
	Mycobacterium spp. (1)	Mycobacterium spp. (1)
	None (1)	Mycobacterium spp. (1)
M. haemophilum*	M. malmoense/haemophilum/palustre/nebraskense (2)	M. haemophilum/nebraskense (2)
M. heckeshornense*	Mycobacterium spp. (1)	M. heckeshornense (1)
M. hiberniae*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. holsaticum*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. immunogenum	M. abscessus/immunogenum (1)	Mycobacterium spp. (1)
M. interjectum	M. interjectum (2)	Mycobacterium spp. (2)
M. intermedium	Mycobacterium spp. (1)	M. szulgai/intermedium (1)
M. intracellulare	M. intracellulare (7)	Mycobacterium spp. (5)
M. kansasii	M. kansasii (8)	M. kansasii (7)
		None (1)
M. kubicae*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. lentiflavum	Mycobacterium spp. (4)	M. lentiflavum (4)
M. mageritense	M. fortuitum/mageritense (1)	Mycobacterium spp. (1)
M. malmoense*	M. malmoense/haemophilum/palustre/nebraskense (2)	Mycobacterium spp. (2)
M. marinum	M. marinum/ulcerans (7)	Mycobacterium spp. (7)
M. mucogenicum	Mycobacterium spp. (3)	M. mucogenicum (3)
M. murale*	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. neoaurum	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. nonchromogenicum	Mycobacterium spp. (2)	Mycobacterium spp. (2)
M. novocastrense	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. obuense	Mycobacterium spp. (1)	Mycobacterium spp. (1)
M. palustre	M. malmoense/haemophilum/palustre/nebraskense (1)	Mycobacterium spp. (1)
"M. paraffinicum"	M. scrofulaceum/"paraffinicum" (1)	Mycobacterium spp. (1)
M. parafortuitum	**None** (1)	Mycobacterium spp. (1)
M. parascrofulaceum	**M. scrofulaceum/"paraffinicum"** (3)	Mycobacterium spp. (3)
M. peregrinum	M. peregrinum/alvei/septicum (6)	Mycobacterium spp. (5)

TABLE 1—*Continued*

| Species | Identification by (no. of strains): | |
	GenoType CM	GenoType AS
*M. phlei**	*Mycobacterium* spp. (1)	*M. phlei* (1)
*M. porcinum**	**M. fortuitum** type 1 (1)	*Mycobacterium* spp. (1)
*M. poriferae**	*Mycobacterium* spp. (1)	*Mycobacterium* spp. (1)
*M. rhodesiae**	*Mycobacterium* spp. (2)	*Mycobacterium* spp. (2)
M. scrofulaceum	*M. scrofulaceum*/"*paraffinicum*" (2)	*Mycobacterium* spp. (2)
M. senegalense	**M. fortuitum** type 2/*mageritense* (1)	*Mycobacterium* spp. (1)
M. shimoidei	*Mycobacterium* spp. (1)	*M. shimoidei* (1)
M. simiae	*Mycobacterium* spp. (5)	*M. simiae* (5)
M. smegmatis	*Mycobacterium* spp. (2)	*M. smegmatis* (2)
M. szulgai	*Mycobacterium* spp. (3)	*M. szulgai*/*intermedium* (3)
M. terrae	*Mycobacterium* spp. (2)	*Mycobacterium* spp. (2)
M. thermoresistibile	*Mycobacterium* spp. (1)	*Mycobacterium* spp. (1)
M. tokaiense	*Mycobacterium* spp. (1)	*Mycobacterium* spp. (1)
M. triplex	*Mycobacterium* spp. (1)	*Mycobacterium* spp. (1)
M. triviale	*Mycobacterium* spp. (1)	*Mycobacterium* spp. (1)
M. tuberculosis	*M. tuberculosis* complex (5)	*Mycobacterium* spp. (5)
M. tusciae	*Mycobacterium* spp. (1)	*Mycobacterium* spp. (1)
M. xenopi	*M. xenopi* (10)	*Mycobacterium* spp. (6)
	None (1)	
Tsukamurella spp.	**M. peregrinum**/**septicum**/**alvei** (1)	Organism with high guanidine-cytosine content (1)
Legionella pneumophila	None (1)	None (1)
Rhodococcus equi	Organism with high guanidine-cytosine content (2)	Organism with high guanidine-cytosine content (2)
Gordonia spp.	Organism with high guanidine-cytosine content (2)	Organism with high guanidine-cytosine content (2)
Nocardia asteroides	Organism with high guanidine-cytosine content (3)	Organism with high guanidine-cytosine content (3)

[a] Discrepant results are in boldface; asterisked species include one reference strain.

One hundred thirty-eight strains belonging to 33 of the 37 taxa covered by the GenoType were correctly identified. For 97 of them, the identification was unambiguous, while for 41 it fell within a group including two or more alternatives. All but one of the nonmycobacterial strains were nonreactive with the genus- and species-specific probe. The strain of *Tsukamurella* positive by the genus-specific probe also presented the pattern of *Mycobacterium peregrinum* in the CM strip. The nonmycobacterial strains, belonging to species more closely related to the *Mycobacterium* genus, hybridized as expected with the universal probe present in both strips.

With the CM strip, misidentification occurred in 12 cases (four strains belonging to the *Mycobacterium avium* complex [MAC], three *Mycobacterium chimaera* strains, three *Mycobacterium parascrofulaceum* strains, and one strain each of *Mycobacterium farcinogenes*, *Mycobacterium porcinum*, and *Mycobacterium senegalense*), while identification was missed in four cases, in which the hybridization pattern did not match any of the expected ones. The sensitivity and specificity were 97.9% and 92.4%, respectively (Table 2).

With the AS strip, there was one case of a non-sense pattern

for one strain of *M. kansasii* (out of eight) and one case of missed identification at the genus level (*Mycobacterium duvalii*). Sensitivity was 99.3%, and specificity was 99.4% (Table 2).

DISCUSSION

The GenoType Mycobacterium assay is able to identify up to 37 *Mycobacterium* species, using two strips. The technology is based on reverse-phase hybridization aimed at revealing an amplification product of a proprietary fragment of approximately 230 bp within the 23S rRNA gene.

Hybridization patterns may be characterized either by hybridization of a single probe or by hybridization of multiple probes simultaneously (Fig. 1), thus suggesting the presence of more polymorphic traits scattered within the target.

The system represents the evolution of a previous kit, based on a single strip, which practically overlaps the present CM. The most evident improvement is represented by the introduction of the AS strip, but another important aspect of the new system is the introduction of the genus *Mycobacterium*-specific probe, which has been added to the already present universal probe. The combined results of such probes allow us to distinguish, among the strains not recognized at the species level, the ones belonging to the genus *Mycobacterium* and those belonging to genetically related genera (*Nocardia*, *Gordonia*, etc.) from the other species.

Assayed with a large number of species, the system proved very reliable. The values of sensitivity and specificity, which turned out to be good with CM (98 and 92%), were excellent with AS (99% for both).

A limit of the system is represented, however, by patterns shared by more than one species (up to four; Fig. 1). In addi-

TABLE 2. Sensitivity and specificity of GenoType assay

Assay	% Sensitivity	% Specificity
Genus-specific probe		
CM strip	98.9	88.9
AS strip	99.4	100
Species-specific probes		
CM strip	97.9	92.4
AS strip	99.3	99.4

TABLE 3. Distinctive features suitable to differentiate slowly growing species which share an identical GenoType pattern

Species	Characteristic(s)
M. szulgai	No growth at 41°C
M. intermedium	Growth at 41°C
M. haemophilum	No growth on LJ[a]
M. nebraskense	Growth on LJ
M. scrofulaceum	Tween 80 negative
"M. paraffinicum"	Tween 80 positive
M. malmoense	Growth on LJ, nonchromogenic, no growth at 41°C
M. haemophilum	No growth on LJ, nonchromogenic, no growth at 41°C
M. palustre	Growth on LJ, scotochromogenic, growth at 41°C
M. nebraskense	Growth on LJ, scotochromogenic, no growth at 41°C
M. ulcerans	Identified by AS
M. marinum	Not identified by AS

[a] LJ, Lowenstein-Jensen medium.

tion to the ones declared by the manufacturer (seven on the CM strip and two on the AS strip), several ambiguous patterns, almost all concerning the CM strip, emerged from our investigation. In particular, the following species turned out not to be distinguishable: *M. parascrofulaceum* from *M. scrofulaceum*; *M. chimaera* and several intermediate MAC spp. from *Mycobacterium intracellulare*; and *M. farcinogenes*, *M. porcinum*, and *M. senegalense* from *M. fortuitum*. The differentiation of slowly growing species, hybridizing with the same probe, may be easily achieved by investigating few simple phenotypic features (Table 3), whereas it seems to be more problematic for rapid growers, which may need definitive speciation by genetic sequencing.

Published evaluations of GenoType are available only for the previous version, overlapping the present CM assay. The relevant specificity and sensitivity results are substantially in agreement with our results (9, 13, 14). The use of a larger panel, however, allowed us to add further information, such as the misidentification of species not tested before (*M. farcinogenes*, *M. chimaera*, *M. porcinum*, and *M. senegalense*). Differently from others, we did not detect any strain that could not be amplified, while, in our hands, a few strains failed to hybridize with the genus-specific probe or produced a pattern not overlapping any of those provided by the system. We are, however, surprised by an aspect which had never been pointed out before (5, 10, 17): the identification, either as *M. avium* or as *M. intracellulare*, of all the strains belonging to the MAC, including the ones genetically distinct from both *M. avium* and *M. intracellulare*.

The only other commercial method with characteristics similar to GenoType is LiPA (16). In addition to the 18 taxa identified by both systems, the combination of CM and AS is suitable for identifying 15 further species. On the other hand, with LiPA, different from GenoType, all but one the hybridization patterns were unique to single species. GenoType showed a better sensitivity in identifying the species *M. fortuitum*: all 10 strains tested were in fact correctly identified, while two of them had been missed by LiPA (E. Tortoli, unpublished data). Some limits of specificity of the *M. fortuitum* probe (cross-reactivity with *M. senegalense* and *M. mageritense*), reported for LiPA (16), were also present in GenoType.

One of the unique features of GenoType is the differentiation of two types within the species *M. fortuitum*. No clinical difference related to such variants is known so far, and the distinction appears at present to have only epidemiological value.

We would propose some suggestions in order to improve the GenoType system. The inclusion in the CM strip of a new line probe, combining all the specificities present in AS, would allow us to target the use of the AS strip only for the strains it is able to identify. Moreover, the development of new, more specific, probes for *M. avium* and *M. intracellulare* and the combination in a new line probe (targeting the whole MAC) of the present *M. avium* and *M. intracellulare* probes would substantially increase the reliability of identification of the strains included in this complex.

In conclusion, the new GenoType assay appears to be reliable and suitable to identify almost all of the most frequently isolated mycobacteria (apart from *Mycobacterium terrae* and *Mycobacterium nonchromogenicum*), thus reducing the number of strains to be submitted to reference centers. The high number of overlapping patterns in the CM strip may be a problem. It should be noted, however, that most of them concern rarely detected species.

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