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Direct Detection of *Mycobacterium tuberculosis* Complex DNA and Rifampin Resistance in Clinical Specimens from Tuberculosis Patients by Line Probe Assay▽

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The INNO-LiPA.Rif TB test (LiPA) has only been applied to a limited number of clinical specimens. To assess the utility of this test for detecting *Mycobacterium tuberculosis* complex DNA and rifampin (RMP) resistance, 420 sputum samples comprising specimens from untreated ($n = 160$) and previously treated ($n = 260$) patients from 11 countries in Asia, Africa, Europe, and Latin America were tested. DNA was extracted from sputum samples by using a modification of the Boom's method, while the *rpoB* core region was amplified by nested PCR. The results were analyzed in conjunction with those obtained by Ziehl-Neelsen (ZN) microscopy and by culture on solid media. The LiPA test was positive for *M. tuberculosis* complex DNA in 389 (92.9%) specimens, including 92.0% (286 of 311) ZN-positive and 94.5% (103 of 109) ZN-negative specimens. Of these, 30.6% were RMP resistant. In contrast, 74.3% of the specimens were positive for *M. tuberculosis* by culture, and 30.8% of them were RMP resistant. LiPA detected *M. tuberculosis* complex DNA in 92.4% (110 of 119) of the culture-positive and 100.0% (41 of 41) of the culture-negative specimens from untreated patients. There was a 99.6% concordance between the RMP resistance as determined by culture and by the LiPA test. With an optimal DNA extraction method, LiPA allows rapid detection of *M. tuberculosis* complex DNA and RMP resistance directly from sputum specimens. LiPA can still provide useful information when culture fails for various reasons. The rapid availability of this information is necessary to adjust patient treatment and avoid the risk of amplification of drug resistance.

Tuberculosis (TB) remains one of the most important diseases worldwide. In recent years, the incidence of TB has been rising, as has the prevalence of drug-resistant cases in many parts of the world (31). A high rate of drug resistance in a community would compromise the effective standardized chemotherapy and jeopardize TB control, especially in regions with high human immunodeficiency virus prevalence where the susceptibility to disease is higher.

Rifampin (RMP) is a key component for the effectiveness of the World Health Organization-recommended short-course chemotherapy. Therefore, patients in whom resistance to this drug develops have a poor prognosis, particularly when the resistance to RMP is associated with resistance to other anti-TB drugs (9). Multidrug-resistant (MDR) TB, i.e., resistance to at least RMP and isoniazid (INH), the two most potent anti-TB drugs is a problem of increasing importance in both developed and developing countries (31). Several previous studies suggested that RMP resistance could be a good indicator for MDR in some settings with high MDR prevalence (10, 28, 30, 31). Therefore, early diagnosis of the disease and rapid detection of resistance to this major anti-TB agent are essential for the optimal control of TB.

The use of molecular techniques based on PCR amplification of genes involved in resistance mechanisms, followed by the detection of key mutations associated with resistance, provides faster RMP susceptibility results than the classical methods that are based on the growth of bacilli on culture media. Unfortunately, to date, only a few molecular tests have been standardized and extensively evaluated for the rapid detection of resistance to RMP in *Mycobacterium tuberculosis* when applied to cultured isolates. One such test, the INNO-LiPA.Rif TB (Innogenetics, Belgium) is recommended for application on isolates, and its performance for this purpose has been found to be highly reliable by several studies (10, 15, 22). However, since *M. tuberculosis* DNA can be detected in clinical samples, independent studies were conducted to assess the applicability of the test to clinical specimens, which would provide even faster results. Recent reviews on the molecular detection of RMP resistance found only a few studies and small sample sizes (16, 17) for the direct detection of RMP resistance in clinical specimens. This necessitates the need for larger studies to assess the reliability of the direct application of LiPA to clinical specimens.

Here, we have analyzed an extended number of sputum samples from TB patients from diverse geographic origins to determine whether LiPA could be a reliable tool for detecting RMP resistance directly in clinical samples. This would allow its recommendation as a rapid prediction of MDR TB before culture results become available and would help limit the spread of difficult to treat bacilli in the community.

MATERIALS AND METHODS

Samples. A total of 420 sputum samples collected between 1992 and 2005 for patient care or during drug resistance surveys in 11 countries (Table 1) of Asia, Africa, Europe, and Latin America were referred to the mycobacteriology laboratory of the Institute of Tropical Medicine (ITM), Antwerp, Belgium. The

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TABLE 1. Origins and results for ZN microscopy, RMP resistance detection with the INNO-LiPA.Rif TB test, and culture on solid medium of 420 sputum specimens

Origin (*n*)[b]	No. of specimens (%)[a]						
	ZN microscopy		RMP resistance detection by LiPA			Culture on solid medium	
	Pos	Neg	RMP^s	RMP^r	Neg	Pos	Neg
Asia (285)	241	44	184	73	28	204	81
Africa (112)	60	52	72	37	3	91	21
Europe (18)	10	8	11	7	0	14	4
Latin America (5)	0	5	3	2	0	3	2
Total (420)	311 (74.0)	109 (26.0)	270 (64.3)	119 (28.3)	31 (7.4)	312 (74.3)	108 (25.7)

[a] RMP^s, rifampin sensitive; RMP^r: rifampin resistant; Neg, negative; Pos, positive.
[b] *n*, number of specimens.

majority of the specimens (76.4%) originated from Bangladesh (24, 29) and Rwanda (6). Nearly all of them were collected at registration for treatment as new or retreatment cases (relapse, default, or failure) from smear-positive patients. The Bangladesh samples from 1994 to 1995 were all from newly registered cases in the context of drug resistance survey; thereafter till 10/2000, there were only relapses, defaulters, and failures at the start of retreatment. Only one sample per patient was tested by LiPA. Overall, specimens were drawn from 160 nontreated and 260 previously treated patients.

Local specimens (from Belgium) were processed immediately upon receipt at the ITM and aliquots of decontaminated specimens were kept at −20°C. Specimens from other countries were transported to Antwerp in 1% cetylpyridinium chloride (CPC) at room temperature (26) and processed for culture upon arrival at the ITM by the Petroff method (19); aliquots were stored at −20°C until testing by LiPA. Detection of acid-fast bacilli (AFB) was done by using Ziehl-Neelsen (ZN) staining. Cultures were done on Löwenstein-Jensen and Stonebrink media, and susceptibility to RMP and INH was tested on Löwenstein-Jensen medium by the proportion method (5).

DNA release and amplification from sputum specimens. Sample preparation for PCR amplification was based on a modification of the method proposed by Boom et al. (4). Preparation of the following three reagents was a prerequisite to applying the method: (i) buffer L2 (120 g of GuSCN [Sigma, Benelux, Belgium], 100 ml of 0.1 M Tris-HCl [pH 6.4]), (ii) buffer L6 (120 g of GuSCN, 100 ml of 0.1 M Tris-HCl [pH 6.4], 22 ml of 0.2 M EDTA [pH 8.0], 2.6 g of Triton X-100), and (iii) diatom suspension (10 g of analytical-grade Celite [Janssen Chemica, Geel, Belgium], 50 ml of H_2O, 500 μl of 32% HCl).

Before testing, the specimens were inactivated by heating in a water bath at 100°C for 20 min. Then, 50 μl of the specimens was added to a 1.5-ml Eppendorf tube containing five to eight glass beads of <100 μm (Sigma) and 900 μl of buffer L6. The tube was vortexed for 5 s, and 40 μl of the diatomaceous earth (Sigma Chemical, St. Louis, MO) pretreated with 37% HCl was added. The tube was again vortexed for 5 s, gently mixed for 10 min at ambient temperature on a horizontal shaker, and then centrifuged at 12,000 × *g* for 8 min. The supernatant was then discarded, and the pellet was washed by resuspending it and spinning it twice with buffer L2, once with 70% ethanol, and once with acetone. The pellet was left to air dry for 15 min at room temperature with the tube cap loosened. The DNA was eluted by resuspending the pellet in 125 μl of TE buffer (10 μM Tris, 1 mM EDTA [pH 8.0]), followed by incubation at 56°C for 10 min and centrifugation for 2 min at 12,000 × *g*. Portions (5 μl) of the supernatant were used for PCR.

The core region of the *rpoB* gene was amplified by a nested PCR using primers OP1/OP2 (outer primers) and IP1/IP2 (biotin-labeled inner primers) as described earlier (8). Mutations associated with RMP resistance were detected by the line probe assay (LiPA). The INNO-LiPA.Rif TB kit is recommended for use only on isolates. This does not require a nested PCR since the amount of DNA is large and PCR sensitivity is not an issue. However, the amount of DNA obtainable from sputum samples is very little because of loss of material throughout the washing steps during sample decontamination and DNA extraction. This is an important limitation for samples with low bacillary content. The single PCR used in the kit is based on the primers IP1 and IP2 (8). A nested PCR was used here to avoid PCR-negative results in case of insufficient amount of DNA in the extracts as recommended (8).

RMP resistance detection by LiPA. The LiPA (INNO-LiPA.Rif TB; Innogenetics, Ghent, Belgium) is based on a reverse hybridization technique and was performed according to the manufacturer's instructions. Briefly, 10 specific oligonucleotide probes (one specific for the *M. tuberculosis* complex, five overlapping wild-type probes that cover the hypervariable core region of the *rpoB* gene, and four mutation-specific probes) are immobilized at known locations on a membrane strip and hybridized under stringent conditions with the biotin-labeled PCR product. The hybrids formed are subsequently detected by using a colorimetric reaction (8).

Statistical analysis. EpiInfo 6.04d (Centers for Disease Control and Prevention, Atlanta, GA) was used for calculation of the chi-square (χ^2) values to compare the percentages.

RESULTS

The 420 specimens were classified by microscopy examination into 311 ZN-positive and 109 ZN-negative samples. On the one hand, 92.6% (389/420) of all of the specimens were positive for *M. tuberculosis* DNA by the LiPA test (Table 1). Of these, 30.6% (119/389) were RMP resistant. On the other hand, 74.3% (312/420) of the specimens were positive by culture, 30.8% (96/312) of which were RMP resistant. LiPA detected 100 specimens missed by culture and missed 22 other specimens detected by culture. Comparable results between culture and the LiPA test were available for 256 specimens, with a 99.6% concordance between the two tests. The discrepant result constitutes one sample that was found to be susceptible by LiPA and resistant by culture. Previous studies reported up to 5% of RMP-resistant strains with no mutation in the *rpoB* core region screened by the LiPA test (1, 2, 7, 11, 28). The results for susceptibility to INH were available for 94 of the RMP-resistant specimens by the culture method, and 92.6% of them were also INH resistant.

The LiPA-positive samples comprised 92.0% of the ZN-positive and 94.5% of the ZN-negative specimens (Table 2). The LiPA test did not detect *M. tuberculosis* DNA in 7.4% of the specimens, 25 of which were ZN positive and 6 of which were ZN negative. There was no significant difference in the proportion of LiPA-positive specimens between ZN-positive and ZN-negative specimens ($\chi^2 = 0.76$; $P = 0.38$).

TABLE 2. Detection of mycobacteria by ZN microscopy and the LiPA test for 420 sputum specimens

LiPA test result	No. of specimens (%)		
	ZN$^+$	ZN$^-$	Total
LiPA$^+$	286 (92.0)	103 (94.5)	389 (92.6)
LiPA$^-$	25 (8.0)	6 (5.5)	31 (7.4)
Total	311	109	420

TABLE 3. LiPA and culture results for sputum specimens from untreated and treated patients

LiPA test result	No. of specimens (%)			
	Untreated patients (n = 160)		Treated patients (n = 260)	
	Culture$^+$	Culture$^-$	Culture$^+$	Culture$^-$
LiPA$^+$	110 (92.4)	41 (100)	179 (92.7)	59 (88.1)
LiPA$^-$	9 (7.6)	0	14 (7.3)	8 (11.9)
Total	119	41	193	67

Of the 160 specimens from untreated patients, positive culture was obtained for 74.4% (119/160) specimens, and 25.6% of the specimens remained culture negative (Table 3). LiPA detected *M. tuberculosis* DNA in 92.4% (110/119) of the culture-positive and 100.0% (41/41) of the culture-negative specimens from untreated patients (Table 3). The proportion of LiPA-positive specimens was not significantly different between culture-positive and culture-negative specimens from untreated patients ($\chi^2 = 2.02$; $P = 0.155$).

Of the 260 specimens from previously treated patients, positive culture was obtained for 74.2% of the specimens, while 25.8% of the specimens remained culture negative. LiPA detected *M. tuberculosis* DNA in 92.7% (179/193) of the culture-positive and 88.1% (59/67) of the culture-negative from previously treated patients (Table 3). The proportion of LiPA-positive specimens was not significantly different between culture-positive and culture-negative specimens from previously treated patients ($\chi^2 = 1.41$; $P = 0.235$).

DISCUSSION

The performance of LiPA for the rapid detection of resistance to RMP in TB isolates has been found to be very good in several studies. A recent systematic review of 14 studies found a sensitivity greater than 95% and a specificity of 100% for 12 of the studies (16). Importantly, the test allows the detection of RMP resistance rapidly (in 2 days) and simultaneous confirmation of the presence of *M. tuberculosis*-complex by a specific probe. The test also allows prediction of MDR in more than 95% of the cases (28). In contrast, 2 to 6 weeks are required for primary isolation of the bacilli by culture, which delays the rapidity of obtaining drug susceptibility results. Very few studies applied LiPA directly to clinical specimens (8, 10, 13, 30), and the number of specimens tested has always been small. The largest previous study included 67 specimens (8). The small sample sizes of these studies do not allow a good assessment of the degree of accuracy of the test. In the present study, 420 sputum samples from diverse geographic regions were tested, including ZN-positive and ZN-negative specimens. It is expected that the sensitivity of the test would be lower when applied directly to clinical specimens than when applied to isolates because of ZN-negative specimens tested that could give negative results. Negative results with LiPA may be due to the absence of *M. tuberculosis* complex DNA in the specimen or as a result of failure to amplify *M. tuberculosis* DNA from clinical specimens. The sensitivity of the LiPA on clinical samples depends on the sensitivity of the PCR step, while the successful PCR amplification relies not only on the optimum PCR conditions but also on the efficacy of the DNA release method. Lower PCR sensitivity may result in a failure of the DNA release method applied. The sensitivity of the various DNA release methods reported depended on the bacillary load in the specimens (12, 18, 27). The DNA release step is more likely to explain the lower sensitivity of the LiPA on clinical specimens than the PCR conditions because the PCR amplification step has been well standardized and validated (8).

It was interesting that there was no significant difference in detecting *M. tuberculosis* DNA and its resistance to RMP when LiPA was applied to ZN-positive or ZN-negative specimens. This could probably be due to a very efficient DNA release method applied (the modified Boom method) that avoids PCR inhibitors and the loss of the targeted DNA. The present study demonstrates that LiPA results are valid regardless of the ZN status of the specimen. However, the ZN status of a specimen is always considered in parallel with other examinations and the disease presentation and also with the treatment regimen administered. A ZN-positive sputum specimen indicates TB with a high bacillary load. For patients under treatment these may be dead bacilli, or for patients with treatment failure these may be resistant bacilli. ZN negativity is normally indicative of low numbers of AFB or the absence of AFB in specimens. This may be the case for non-TB patients or cured TB patients and also for TB patients treated or under treatment that still harbor resistant bacilli at numbers too low (fewer than 10,000 bacilli/ml of sputum) to be detectable by microscopy (14). A high proportion of TB patients coinfected with human immunodeficiency virus present with ZN-negative specimens (25). Moreover, transportation in CPC medium has been reported to negatively influence the ZN staining (23), even though the majority of the specimens (from Bangladesh and Rwanda) in the present study were found smear positive locally.

LiPA results were obtained within 2 days compared to a minimum of 6 weeks that would have been required for bacillus isolation by culture methods. Importantly, LiPA detected 77 specimens missed by the culture. The specificity of the LiPA has been assessed and found to be very high. Although PCR amplification can occur for other mycobacteria, the probe for *M. tuberculosis* complex included in the assay renders the test 100% specific to this complex only (8). A 100% specificity of the LiPA was reported in several studies (16). Although cross-contamination can occur, this has not been observed in either the internal or external quality control that our laboratory participates in, making this possibility an unlikely explanation for the high proportion of LiPA-positive samples. Rather, the apparent lower sensitivity of the culture may be due to the transport conditions in the CPC that could have reduced the viability of the bacilli. Further, the viability of the bacilli could also have been affected by the additional sample decontamination by the Petroff method using NaOH. Furthermore, delays in transportation and the use of suboptimal culture medium could have also affected the growth rate. In addition, another plausible cause could be the high number of patients under treatment whose specimens might have remained negative in culture because of some poorly growing resistant isolates (3). Due to these limitations, culture could not therefore be used as a suitable reference method in the present study but was only used for the purpose of obtaining isolates in order to

perform conventional drug susceptibility testing. However, as for LiPA, the sensitivity of the culture was the same regardless of the treatment status of the patients. The majority of the samples (76.4%) were from smear-positive patients from Rwanda or Bangladesh; they were taken at registration for treatment as a new or retreatment case (relapse, default, or failure). All samples were collected before the commencement of any treatment or retreatment and expected to yield positive cultures as a new case or smear-positive failure, relapse, or return after default. The majority of the retreatment cases from Bangladesh (132/185 [71.3%]) were culture positive, as would be expected. Patient's records from 19 retreatment cases from Bangladesh with negative culture and positive LiPA (RMP resistant) were available for analysis. Four of them were recorded as cured without known relapse, and the fifteen others failed or relapsed from the standard non-second-line retreatment regimen (some of whom died later, and others were treated with second-line drugs as MDR TB but not based on the LiPA result). Therefore, although LiPA could give false results in case of dead bacilli, the clinical outcome of patients with positive smears suggested that this was not the case for these 15 patients.

The high sensitivity and the rapidity of results obtained with LiPA highlight its particular usefulness in patient follow-up. However, like any other laboratory test, LiPA results must be interpreted cautiously (together with clinical assessment) by physicians. The phenomenon of transient resistance during treatment makes careful interpretation indispensable for all laboratory results, including classical drug susceptibility testing. For example, LiPA result alone is indicative but not conclusive since it does not differentiate between dead and live bacilli. Likewise, a negative culture result alone should not exclude the presence of the bacilli since culture may fail because of transport or decontamination conditions that can affect the viability of the bacilli owing to suboptimal culture conditions or in the case of resistant and/or unfit bacilli. Compared to culture methods, LiPA has an additional advantage due to its rapidity and can thus still provide useful information when culture fails for various reasons. In the case of resistance to RMP, the rapid availability of results with the LiPA is important for faster adjustment of the treatment, which would improve patient management and limit the spread of RMP-resistant bacilli. Rapid drug susceptibility results are also of particular importance in clinical trials where rapid detection of resistance to first- and second-line drugs is necessary before the inclusion of patients in any new phase III clinical trial in order to avoid the risk of the amplification of drug resistance (21).

In our study, 92% of the RMP-resistant specimens were also INH resistant (data not shown) and thus MDR, which is in agreement with previous reports suggesting RMP resistance as a good predictor for MDR in some settings (10, 28, 30), and in particular among previously treated cases, where the prevalence of MDR-TB is high and non-MDR RMP resistance is low (28, 31).

LiPA detected *M. tuberculosis* complex DNA in 100 specimens missed by culture; 21 of them were RMP resistant. Patients with these resistant specimens may not be treated accurately, and this could lead to the propagation of RMP-resistant bacilli, which could amplify the magnitude of MDR TB and compromise TB control in general. Therefore, adaptation of LiPA test so that it is applicable directly to clinical samples can be a powerful tool for the control of TB and its resistance to anti-TB drugs.

Microscopy remains the main tool for TB diagnosis in high-prevalence settings. Culture might provide a more reliable means particularly for ZN-negative patients. Unfortunately, classical culture methods are time-consuming and are not accessible to the majority of patients. In contrast to these above tests, the LiPA test simultaneously detects RMP resistance and confirms the presence of *M. tuberculosis* complex bacilli in a single test. However, its broader application is limited largely by the cost of the test and to a minor extent because the test has not yet been approved by the Food and Drug Administration for use in the United States (20).

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