JCM Accepts, published online ahead of print on 11 October 2006
J. Clin. Microbiol. doi:10.1128/JCM.01332-06

Direct Detection of *Mycobacterium tuberculosis*-complex DNA and Rifampin Resistance in Clinical Specimens from Tuberculosis Patients by the Line Probe Assay; a Large Scale Study

Hamidou Traore, Armand van Deun, Isdore Chola Shamputa, Leen Rigouts and Françoise Portaels*

Microbiology Department, Mycobacteriology Unit, Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium

*Corresponding author.

Mailing address: Mycobacteriology Unit, Department of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium.

Tel: +32-3-247 63 17, Fax: +32-3-247 63 33, E-mail: portaels@itg.be

ABSTRACT

The INNO-LiPA.Rif TB test (LiPA) has only been applied on a limited number of clinical specimens. To assess the utility of this test to detect *Mycobacterium tuberculosis*-complex DNA and rifampin (RMP) resistance, 420 sputum samples comprising specimens from non-treated (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 using a modification of the Boom's method while the *rpoB* core region was amplified by nested PCR. The results were analysed 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% (286/311) ZN-positive and 94.5% (103/109) 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% (110/119) of the culture-positive and 100.0% (41/41) of the culture-negative specimens from untreated patients. There was a 99.6% concordance between the RMP resistance 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.

INTRODUCTION

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 HIV prevalence were susceptibility to disease is higher.

Rifampin (RMP) is a key component for the effectiveness of the WHO 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 developed as well as in developing countries (31). Several previous studies suggested that RMP resistance could be a good indicator for MDR in some settings with high MDR prevalence (30) (10) (28, 31). Therefore, early diagnosis of the disease and rapid detection of resistance to this major anti-TB agent are essential for 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 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 molecular detection of RMP resistance found only 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 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 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 case (relapse, default, failure) from smear-positive patients. Bangladesh samples from 1994-95 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 start of retreatment. Only one sample per patient was tested by LiPA. Overall, specimens were drawn from 160 non-treated 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), processed for culture upon arrival at ITM by the Petroff method (19) and aliquots stored at –20°C until testing by LiPA. Detection of acid-fast bacilli (AFB) was done using Ziehl-Neelsen (ZN) staining. Cultures were done on Löwenstein-Jensen (LJ) and Stonebrink media and susceptibility to RMP and INH was performed on LJ was 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 and colleagues (4). Preparation of the following 3 reagents was a prerequisite to applying the method: **1**- *Buffer L2* (120g GuSCN [Sigma, Benelux]; 100 ml 0.1M Tris-HCl, pH 6.4), **2**- *Buffer L6*: (120g GuSCN; 100 ml 0.1M Tris-HCl, pH 6.4; 22 ml 0.2M EDTA, pH8.0; 2.6g Triton X-100), **3**- *Diatom suspension* (10g analytical grade Celite [Janssen Chemica, Geel, Belgium]; 50 ml H_2O; 500 µl 32% HCl).

Before testing, specimens were inactivated by heating in a water bath at 100°C for 20 min. Fifty microlitres of the specimens were added to a 1.5 ml Eppendorf tube containing 5 to 8 glass beads < 100µm (Sigma) and 900 µl of *buffer L6*. The tube was vortexed for 5 sec and 40 µl of the diatomaceous earth (Sigma Chemical, St. Louis, USA)

pretreated with 37% HCl was added. The tube was again vortexed for 5 sec and 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 re-suspending and spinning 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 re-suspending the pellet in 125 µl 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. Five microlitres 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 as 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 primers IP1/IP2 (8). A nested PCR was used here to avoid PCR negative results in case of insufficient amount of DNA in the extracts as per recommendation (8).

RMP resistance detection by Line Probe Assay

The Line Probe Assay (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, 5 overlapping wild-type probes that cover the hyper-variable core region of the *rpoB* gene and 4 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 using a colorimetric reaction (8).

Statistical analysis

Epi Info 6.04d (CDC, Atlanta, USA) was used for calculation of Chi square (χ^2) to compare percentages.

RESULTS

The 420 specimens were classified by microscopy examination into 311 ZN-positive and 109 ZN-negative. On the one hand, 92.6% (389/420) of all 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 of which 30.8% (96/312) 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 susceptible by LiPA and resistant by culture. Previous studies reported up to 5% of RMP

resistant strains with no mutation in the *rpo*B core region screened by the LiPA test (1, 2, 7, 11, 28). 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 of which 25 were ZN-positive and 6 were ZN-negative specimens. There was no significant difference in the proportion of LiPA-positive specimens between ZN-positive and ZN-negative specimens ($\chi^2 = 0.76$; $p = 0.38$).

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 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 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, 2-6 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 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 applying LiPA 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. This 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 high bacillary load. For patients under treatment these may be dead bacilli, or patients with treatment failure and resistant bacilli. ZN-negativity is normally indicative of low numbers of acid fast bacilli (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 (less than 10,000 bacilli/ml of sputum) to be detectable by microscopy (14). A high proportion of TB patients co-infected with HIV 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 this 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 bacilli 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 papers (16). While 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 suboptimum 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 the above limitations, culture could not therefore be the used as a suitable reference method in this study but was only used for the purpose of obtaining isolates in order to perform conventional drug susceptibility testing (DST). However, as for LiPA, the sensitivity of the culture was the same regardless 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 new or retreatment case (relapse, default, 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 15 others failed or relapsed from the standard non-second-line retreatment regimen (some of whom died later and the others were treated with second-line drugs as MDRTB 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 result obtained with LiPA highlight its particular usefulness in patients' 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 the classical DST. For example LiPA result alone is indicative but not conclusive as it does not differentiate between dead or live bacilli. Likewise a negative culture result alone should not exclude the presence of the bacilli as culture may fail because of transport or decontamination conditions that can affect the viability of the bacilli, because of suboptimum culture conditions or in case of resistant-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 case of resistance to RMP, 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 including patients in any new phase III clinical trial, to avoid the risk of amplification of drug resistance (21).

In our study, 92% of the RMP-resistant specimens were also isoniazid-resistant (data not shown), 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 those resistant specimens may not be treated accurately and 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 to be applicable directly on 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 take long and are not accessible to the majority of patients. In contrast to the 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 FDA for use in the United States (20).

ACKNOWLEDGMENTS

This work was supported by the Damien Foundation, Belgium. We are grateful to K. Fissette and P. (W.B.) de Rijk for excellent technical assistance and to J. Glynn for pertinent comments. I.C.S. acknowledges support from Ackermans & van Haaren.

Table 1: Origins and results for Ziehl-Neelsen microscopy, RMP resistance detection with INNO-LiPA.Rif TB test and culture on solid medium of 420 sputum specimens.

Origin (n)	Ziehl-Neelsen microscopy		RMP resistance detection by LiPA on specimens			Culture on solid medium	
	positive	negative	RMP^s	RMP^r	negative	positive	negative
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%)

RMP^s: rifampin sensitive

RMP^r: rifampin resistant

Table 2: Detection of mycobacteria by Ziehl-Neelsen microscopy and the LiPA test for 420 sputum specimens.

LiPA test	Ziehl-Neelsen +	Ziehl-Neelsen –	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 previously treated patients.

	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

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