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ARTICLE

Use of the INNO LiPA Rif.TB for detection of *Mycobacterium tuberculosis* DNA directly in clinical specimens and for simultaneous determination of rifampin susceptibility

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Abstract The INNO LiPA Rif.TB (Innogenetics, Ghent, Belgium) is a reverse hybridization test developed to detect genetic markers of resistance to rifampin in *Mycobacterium tuberculosis* complex. In the present study, this test was used directly on 3,763 clinical specimens by adopting a nested amplification of the target. The specificity of the system (98.4%) was optimal, but sensitivity (69.5%) was unsatisfactory. However, when use of the system was limited to smear-positive specimens, the sensitivity rose to 91.7%. As expected, the ability of the system to predict rifampin resistance was not influenced by its direct use on clinical specimens and confirmed the favorable results repeatedly reported in the literature.

Introduction

Tuberculosis continues to be a major problem in both developing and industrialized countries [1]. Culture remains the gold standard for the diagnosis of tuberculosis [2], but despite substantial improvements in recent years [3], it requires, on average, 2 weeks for positive samples to be detected. Amplification techniques may represent a useful support [4] for reaching earlier diagnoses, enabling rapid implementation of treatment and minimizing of the risk of contagion.

The INNO LiPA Rif.TB (Rif.TB) (Innogenetics, Ghent, Belgium), a reverse-phase DNA probe system that has been on the market since 1997, is suitable for detecting rifampin resistance at the genetic level. This method has been widely validated on strains grown in culture for its ability to confirm their belonging to the *Mycobacterium tuberculosis* complex (MTC) and to predict rifampin resistance [5–13]. A precise meta-analysis has been published recently [14].

If the amplification of a part of the *rpoB* gene, required by Rif.TB, is performed using a nested PCR, the method can be used directly on clinical specimens. In this case, its primary function becomes the direct detection of MTC DNA, while the concurrent information about susceptibility to rifampin is an added value. The aim of this study was to evaluate the sensitivity and specificity of Rif.TB in detecting MTC in clinical specimens and to assess the accuracy of prediction of rifampin resistance as a secondary objective. Test results were compared with those from cultures.

Materials and methods

Between February 2002 and November 2004, Rif.TB was performed, in parallel with microscopy and culture, on all clinical specimens that arrived in our laboratory with a request for nucleic acid amplification for MTC. The number of specimens, excluding those that yielded contaminated cultures (all Rif.TB negative), was 3,762 from 2,923 patients. Bronchial washings and bronchial aspirates were the most common specimens (n=1,683); the others comprised 755 sputa, 339 pleural fluid specimens, 241 CSF specimens, and 165 urine specimens, while 579 specimens came from various other body sites. Specimens were decontaminated using the standard *N*-acetyl-L-cysteine 2% NaOH procedure and seeded on both solid (Löwenstein-Jensen) and liquid (MGIT; Becton Dickinson, Sparks, MD, USA) media [2].

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Isolates were identified using commercial DNA probes (INNO LiPA Mycobacteria, Innogenetics [15]; or GenoType MTBC, Hein, Nehren, Germany [16]), and, when not identifiable by such methods, by genetic sequencing of the first 500 bp of the 16S rRNA gene [17]. Susceptibility of MTC strains was performed in liquid medium using the radiometric Bactec 460 system and instrumentation (Becton Dickinson) [18].

A preliminary amplification was carried out before performing the standard PCR required by the Rif.TB [9]. Two microliters of the decontaminated pellet, lysed by boiling and freezing and resuspended in TE buffer, was PCR processed using the Z-1044 primers provided by the manufacturer (but not included in the Rif.TB kit) that delimit a 395-bp fragment of the MTC *rpoB* gene (5′-GAGAATTCGGTCGGCGAGCTGATCC and 5′-CG AAGCTTGACCCGCGCGTACACC [13]). The amplification protocol included 1 min at 95°C, 40 cycles of 30 s at 95°C, 30 s at 62°C, and 30 s at 72°C, and, at the end, 5 min at 72°C. One microliter of the above amplification product was used as target in a second PCR, in which the two primers (5′-GGTCGGCATGTCGCGGATGG and 5′-GCACGTCGCGGACCTCCAGC [13]) included in the kit, targeting a 237-bp trait within the previously amplified region, were used. The products of nested PCR were run using 2% agarose gel electrophoresis with ethidium bromide and reported as negative whenever DNA was absent. Samples yielding DNA with an approximate length of 260-bp were tested with Rif.TB strips in the line probe assay using the automated instrumentation AutoLiPA (Innogenetics) according to manufacturer's instructions.

As recommended, a sample was considered positive for presence of MTC when the strip presented the specific hybridization band (located just below the conjugate control line at the top of the strip). The presence of other hybridization bands in any subsequent position was considered relevant only to infer information about the susceptibility to rifampin.

Statistical analysis included the determination of specificity, sensitivity, and positive and negative likelihood ratios. The likelihood ratios were preferred to positive and negative predictive values, which are strongly dependent on prevalence [19]. The significance of the difference in sensitivity between smear-positive and smear-negative samples and between respiratory and nonrespiratory samples was assessed by the chi-square test.

Results

Two hundred thirty-one specimens (6.1%) were positive by microscopy for acid-fast bacilli. Mycobacteria grew in 410 cultures (10.9%); in 342 cases, the strain belonged to the MTC (including three *Mycobacterium africanum* and three *Mycobacterium bovis* strains). The nontuberculous mycobacteria included 23 *Mycobacterium xenopi*, 18 *Mycobacterium avium*, six *Mycobacterium intracellulare*, five each of the species *Mycobacterium chelonae* and *Mycobacterium gordonae*, four strains of *Mycobacterium fortuitum*, and one strain each of the species *Mycobacterium chimaera*, *Mycobacterium fluoranthenivorans*, *Mycobacterium kansasii*, *Mycobacterium mucogenicum*, *Mycobacterium scrofulaceum*, *Mycobacterium simiae*, and *Mycobacterium terrae*.

Rif.TB was positive in 280 specimens (167 smear-positive and 113 smear-negative); of these, 237 were also positive in culture. The number of true-positive results rose from 237 to 248 when 12 Rif.TB-positive/culture-negative specimens (three smear-positive and nine smear-negative) from patients whose cultures yielded MTC in other samples were added, and when a smear-negative specimen that had grown *M. xenopi* in culture was subtracted.

A total of 172 culture-positive samples were negative in the RIF.TB; of these, however, only the 105 (15 smear-positive and 90 smear-negative) that grew MTC were considered false-negative samples. The remaining 67 samples (23 smear-positive and 45 smear-negative), which had yielded nontuberculous mycobacteria in culture, were considered true-negative samples and were added to 3,297 specimens negative by both the Rif.TB and culture. Data are presented in Tables 1 and 2.

The comparison of genotypic and phenotypic results of rifampin susceptibility testing revealed agreement in 245 cases and disagreement in three cases (one case of false susceptibility and two cases of false resistance; sensitivity 99.1%; specificity 88.9%).

All but one rifampin-resistant strain presented the same band pattern, characterized by the lack of hybridization with the wild-type probe S5 and by hybridization with the probe presenting mutation R5. In that lone strain, in contrast, although no hybridization with any mutation-bearing probe was present, hybridization with S5 was missing.

Discussion

The direct amplification of MTC from clinical specimens is a useful adjunct to microscopy and culture and is used worldwide with the goal of reaching an earlier diagnosis of active tuberculosis. Multidrug resistance (resistance to at least isoniazid and rifampin) is probably the most threatening obstacle to elimination of tuberculosis; its rapid detection is considered the most effective measure, as it allows prompt isolation of the patient, thus limiting the spread of the disease [20]. The availability of a system that combines the direct detection of MTC in clinical specimens

Table 1 Comparison of results of Rif.TB, MTC cultures, and smears

	MTC culture positive[a,b]		MTC culture negative		Sensitivity	Specificity	Positive LR	Negative LR
	Rif.TB positive	Rif.TB negative	Rif.TB positive	Rif.TB negative				
Smear-positive	170[a]	15	0	46[c]	91.89	100	infinity	0.08
Smear-negative	79[b]	90	43[d]	3,319[e]	46.74	98.72	36.55	0.54
Total	249	105	43	3,365	70.34	98.73	55.75	0.30

LR likelihood ratio
[a] Including three culture-negative samples from patients with other culture-positive specimens
[b] Including nine culture-negative samples from patients with other culture-positive specimens
[c] Including 23 samples positive in culture for nontuberculous mycobacteria
[d] Including one sample positive in culture for *M. xenopi*
[e] Including 45 samples positive in culture for nontuberculous mycobacteria

with the prediction of rifampin resistance would be therefore very useful, particularly because rifampin resistance is, in the large majority of cases, accompanied by isoniazid resistance and represents a very effective marker of multidrug resistance [21].

Although Rif.TB has been repeatedly investigated for its accuracy in predicting rifampin resistance [5–13, 22, 23], only six studies tested it directly on clinical specimens. Two papers reported a 100% yield [13, 23], while in two others [8, 24] the sensitivity was 98%. Such results are not comparable with ours, as the investigations were carried out on low numbers of selected specimens, either all positive in culture [8, 13, 23] or MTC positive in more than 60% of cases [24]. In contrast, a sensitivity clearly lower than ours was unexpectedly reported in two recent investigations, both involving exclusively MTC-positive strains [10, 25]. The discrepancy between data by Viveiros et al. [25] and that reported here is not real, as the other study aspired to evaluate the impact of Rif.TB on the management of the patients and therefore did not analyze discrepant results. On the contrary, because of our aim to assess the performance of the tests, we carefully resolved amplification results that disagreed with the culture.

From many points of view, the present study confirms the ample data available for various commercial methods for amplification of MTC [26]. In fact, once more, the sensitivity of the RIF.TB is significantly higher for smear-positive specimens than for smear-negative specimens, and for respiratory specimens than for nonrespiratory ones (Tables 1 and 2). The specificity (>98%), which overlaps that reported for other commercial amplification systems [26], is beyond expectations if we consider that in such studies, in contrast to ours, false-positive results were systematically corrected on the basis of clinical information. A higher rate of contaminations was furthermore expected because of the fully manual amplification procedure adopted by Rif.TB. In contrast, the sensitivity was satisfactory in smear-positive samples only and was clearly unacceptable in nonrespiratory (48%) and smear-negative (44%) specimens. It is noteworthy that equally disappointing data have been reported for smear-negative samples with other widely used amplification systems [27–30].

The possible presence of PCR inhibitors may explain the failure of the system to amplify a number of MTC-positive isolates, but, since Rif.TB does not include any system for monitoring inhibitors, no quantification is possible. In our

Table 2 Comparison of results of Rif.TB and culture with respect to type of specimen

Type of specimen	MTC culture positive[a,b]		MTC culture negative		Sensitivity	Specificity	Positive LR	Negative LR
	Rif.TB positive	Rif.TB negative	Rif.TB positive	Rif.TB negative				
Respiratory source	208[a]	62	27[c]	2,234[d]	77.03	98.80	64.51	0.23
Nonrespiratory source	41[b]	43	16	1,131[e]	48.81	98.60	34.99	0.52
Total	249	105	43	3,365	70.34	98.73	55.75	0.30

LR likelihood ratio
[a] Including ten culture-negative samples from patients with other culture-positive specimens
[b] Including two culture-negative samples from patients with other culture-positive specimens
[c] Including one sample positive in culture for *M. xenopi*
[d] Including 55 samples positive in culture for nontuberculous mycobacteria
[e] Including 13 samples positive in culture for nontuberculous mycobacteria

setting, the use of a different amplification system with different strains yields a prevalence of inhibitors that fluctuates around 4%. If we assume a similar percentage of the strains investigated here to be affected by inhibitors, the impact of inhibitors would only decrease the prevalence of false-negative results from 105 to 100.

The ability of Rif.TB to predict rifampin resistance was not a major goal of our study; nevertheless, sensitivity (90%) and specificity (99%) turned out to be in agreement with values reported previously, which ranged from 80 to 100% and from 92 to 100%, respectively [14]. The low prevalence of rifampin resistance in our region (4%) did not allow us to draw firm conclusions about the usefulness of this test. All but one of our cases of rifampin resistance were due to the Ser→Leu substitution at codon 531. Others have found this mutation to be by far the most frequent cause of rifampin resistance [31].

In conclusion, although the Rif.TB does not seem sufficiently sensitive to be used directly on unselected specimens, it may represent, thanks to the added value of prediction of rifampin susceptibility, a valid alternative to other amplification methods on smear-positive samples.

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