Comparison of two commercial assays for the characterization of \textit{rpoB} mutations in \textit{Mycobacterium tuberculosis} and description of new mutations conferring weak resistance to rifampicin

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Received 13 October 2008; returned 27 January 2009; revised 4 May 2009; accepted 12 May 2009

Objectives: The aim of this study was to compare the efficiency of two commercial assays, INNO-LiPA Rif.TB and MTBDR\textit{plus}, for the identification of mutations in the \textit{rpoB} hot-spot region and to assess the efficiency of these mutations in conferring resistance to rifampicin.

Methods: A collection of 126 rifampicin-resistant \textit{Mycobacterium tuberculosis} and \textit{Mycobacterium africanum} isolates and 18 rifampicin-susceptible isolates from different countries were analysed using the two hybridization assays.

Results: For 60 strains the hot-spot region of the \textit{rpoB} gene was sequenced, confirming the results of the hybridization assays and allowing the identification of new mutations. In total, 17 mutations involving 10 codons were observed, two of which are newly described (D516Y and E562G/P564L). Mutations L533P, H526L, D516Y and L511P and the double mutation E562G/P564L conferred a low level of resistance.

Conclusions: The assays INNO-LiPA Rif.TB and MTBDR\textit{plus} identified \textit{rpoB} mutations in 98.4% of cases. MTBDR\textit{plus} provided additional information due to the overlapping hybridization probes and in addition allowed the investigation of kat\textit{G} mutations for isoniazid resistance.

Keywords: antibiotics, \textit{M. tuberculosis}, hybridization, probes, rifabutin

Introduction

Rapid detection of drug resistance to both first- and second-line anti-tuberculosis drugs has become a key component of tuberculosis control programmes. Before new molecules become available for treatment of tuberculosis, rifampicin remains the most efficient drug in the modern short-course regimen due to its excellent sterilizing activity.\textsuperscript{1} Resistance to rifampicin is an indicator of possible multiresistance as nearly 90\% of rifampicin-resistant strains are also isoniazid-resistant.\textsuperscript{2}

More than 96\% of rifampicin-resistant strains show mutations in a portion of the RNA polymerase B subunit gene (\textit{rpoB}), called the hot-spot region, encompassing codons 507–533.\textsuperscript{3} There are currently two commercially available solid-phase hybridization techniques for the rapid detection of drug resistance in tuberculosis: the line probe assay (INNO-LiPA Rif.TB; Innogenetics NV, Ghent, Belgium) for the detection of rifampicin resistance; and the GenoType MTBDR\textit{plus} assay (MTBDR\textit{plus}; Hain Lifescience GmbH, Nerhen, Germany) for the simultaneous detection of resistance to isoniazid and rifampicin. Four studies that applied the line probe assay directly to clinical specimens had 100\% specificity, and the sensitivity ranged from 80\% to 100\%.\textsuperscript{4} MTBDR\textit{plus}, a development of the GenoType MTBDR assay, was released in October 2007, and a few studies have tested its performance.\textsuperscript{5–7}

We sought to test the sensitivity and specificity of the two tests in our collection of strains and to compare the results with those obtained by sequencing the \textit{rpoB} hot-spot region.

Materials and methods

Strains

One hundred and twenty-six rifampicin-resistant \textit{Mycobacterium} isolates (of which four were \textit{Mycobacterium africanum}; the remainder were \textit{Mycobacterium tuberculosis}) were recovered from 142 patients by the reference centre for mycobacteria of the Service de
Santé des Armées (Hôpital Percy, Clamart, France) in a total of 18 countries. Three reference strains were used as controls together with 18 rifampicin-susceptible isolates.

**Phenotypic measurement of antibiotic resistance**

Routinely, the susceptibility to first-line anti-tuberculosis drugs was determined using a broth-based assay (BACTEC MGIT960; Becton Dickinson) employing 1 mg/L rifampicin. Resistant strains were then further tested on Löwenstein Jensen (LJ) medium using 40 mg/L rifampicin or using rifampicin discs on LJ medium. Susceptibility to rifabutin was determined using the proportion method (1 mg/L) at the Pasteur Institute (Paris, France).

**Preparation of DNA for molecular assays**

The bacteria were recovered from an LJ stab, suspended in 1 mL of 5 mM Tris (pH 8.0), heated at 95°C for 30 min and then centrifuged at 12000 g for 5 min to remove cell debris. The supernatant was stored at 20°C. For a PCR, 2 μL was used.

**Commercial assays for the identification of rpoB mutations**

INNO-LiPA Rif.TB and MTBDRplus are based on reverse hybridization. For both assays a signal on the wild-type probe indicates the absence of a mutation in the target region (Figure 1). The existence of a frequent mutation is shown by a signal on one of the specific probes (R or Mut). The different assays were performed as recommended by the manufacturers.

**Sequencing of the rpoB hot-spot region**

To verify the accuracy of the hybridization assays, the rpoB hot-spot region was sequenced in 26 samples for which a mutation was not identified by specific probes, in 11 out of 73 samples with the S531L mutation and 23 out of 52 samples with another frequent mutation. PCR amplification was performed using primers RPOB-TR1 (TACGGTCGGCGAGCTGATCC) and RPOB3R (GTACGGCGTTTCTAGATGAACCGA). The 412 bp amplicons were purified by polyethylene glycol (PEG) precipitation and sequenced using the primer RPOB-TR1 (MWG Biotech, Heidelberg, Germany).

**Results**

One hundred and nineteen isolates were rifampicin-resistant when using the broth assay or growth on solid medium containing antibiotics. In seven additional isolates showing resistance to several other antibiotics (including isoniazid and ethambutol) low-level rifampicin resistance was only detected using rifampicin discs on LJ medium. Among the 126 isolates, 117 were multidrug-resistant (MDR; 93%) of which 4 were extensively drug-resistant (XDR; 1 from Afghanistan, 1 from Armenia and 2 from the Republic of Djibouti). Susceptibility to rifabutin was observed in nine rifampicin-resistant isolates.

To investigate the rpoB mutations, the two commercial assays were applied to all the resistant strains, giving clear results for 120 samples with the INNO-LiPA Rif.TB and MTBDRplus assays. For two strains showing weak resistance to rifampicin (MIC = 1 mg/L) a wild-type profile was observed. In four additional samples both a wild-type profile and a mutation in the hot-spot region were indicated by the hybridization data, suggesting the existence of a mixture of susceptible and resistant bacilli. To identify the mutation involved, these isolates were grown in rifampicin-containing broth and the tests were repeated. The results showed the existence of a mutation in R4b/Mut2b, R4a/Mut2a and R5/Mut3 (two samples).

For a selection of 60 samples, the rpoB hot-spot region was amplified and the product was sequenced. Table 1 shows a summary of the results obtained with the INNO-LiPA Rif.TB and MTBDRplus hybridization assays and by sequencing. Two strains showed alteration at two codons, and in five strains, all isolated in the Republic of Djibouti, a deletion covering codons 513–516 was found.

Among strains with MICs ≤1 mg/L, one had mutations outside the rpoB hot-spot region on codons 562 and 564 (E562G/P564L) and another had no mutation in the sequenced fragment of the rpoB gene, which led to a wild-type profile with the hybridization assays. The remaining weakly resistant isolates had a single mutation: L511P, D516Y, L533P (two samples) or H526L. Of the nine isolates showing susceptibility to rifabutin, five had the mutation D516V, two the mutation H526C, one the mutation D516Y and one the double mutation E562G/P564L. In total, 17 distinct mutations were observed, two of which are

![Figure 1. Localization of probes corresponding to wild-type (S, Wt) or mutated (R, Mut) sequences on the rpoB hot-spot region, identified by the probes in INNO-LiPA Rif.TB (open rectangles) or MTBDRplus (grey rectangles).](image-url)
hybridization assays showed both a wild-type and a mutant wild-type over mutant bacilli, as previously observed.8 Sequencing of the susceptible phenotype (heteroresistance). In these samples, involving the co-existence of organisms that had either the resistant or profile, whereas MLVA revealed only one genotype, demonstrated the large spectrum of the strains as revealed by multiple locus variable number of tandem repeats analysis (MLVA) genotyping (data not shown) 

Deletion 513–516 del *AA TTC ATG G* Q513K CAA→AAA Deletion 513–516 del *AA TTC ATG G* D516V GAC→GTC D516Y GAC→TAC H526N and L533P CAC→AAC and CTG→CCG H526Y CAC→TAC H526R CAC→CGC H526D CAC→GAC H526N CAC→AAC H526L CAC→CTC H526C CAC→TGC (2 nucleotides) S531W TCG→TGG S531L TCG→TTG L533P CTG→CCG E562G and P564L GAA→GGA and CCT→CTT newly described (D516Y and E562G/P564L). No mutation was found in the 18 rifampicin-susceptible strains.

In the subset of strains with clear rifampicin resistance, both INNO-LiPA Rif.TB and MTBDRplus detected a rpoB mutation in 100% of cases, and sequencing confirmed the localization of the mutation. However, if we take into account the weakly resistant samples, the overall sensitivity and specificity of these assays were both 98.4%. 

Discussion

In the present collection of strains, INNO-LiPA Rif.TB and MTBDRplus demonstrated equal sensitivity and specificity. MTBDRplus is clearly improved compared with the previous MTBDR assay and it gives a more precise localization of some rare rpoB mutations due to the existence of a larger number of probes, some of which are overlapping. In addition, whereas INNO-LiPA Rif.TB investigates only rifampicin resistance, MTBDRplus, for a slightly lower cost, also allows the detection of mutations in katG and inhA responsible for resistance to isoniazid. 

Part of our strain collection results from a collaboration with ‘Médecins Sans Frontières’, which treats patients in refugee camps in different countries. Another large source of samples is the Centre Hospitalier Bouffard in the Republic of Djibouti where patients of diverse origins are treated. The genetic diversity of the strains as revealed by multiple locus variable number of tandem repeats analysis (MLVA) genotyping (data not shown) explains the large spectrum of rpoB mutations. In four cases the hybridization assays showed both a wild-type and a mutant rpoB profile, whereas MLVA revealed only one genotype, demonstrating the co-existence of organisms that had either the resistant or the susceptible phenotype (heteroresistance). In these samples, sequencing of the rpoB hot-spot region showed an excess of wild-type over mutant bacilli, as previously observed.8

Among 129 rifampicin-resistant strains, 89.5% possess mutations in codons 531, 526 or 516, as reported in the literature, although the contribution of some specific mutations in these codons differs somewhat. We describe one isolate with mutation D516Y, which was not reported in the literature, but was detected in China (Kanglin Wan, personal communication). The deletion involving codons 513–516 (AA 514515 G) was observed in five strains from the Republic of Djibouti with very similar genotypes, and was previously described in several studies.9

Some mutations confer low-level (MIC ≤1 mg/L) rifampicin resistance. The newly observed double mutation E512G/P564L is one of these. Among the other mutations L533P is known either to have no effect or to confer weak resistance,10 whereas for other codons the resistance phenotype depends on the type of amino acid substitution (codons 511, 516 and 526). Using MTBDRplus, Hillemann et al.5 reported misidentification of a L533P mutation in a rifampicin-susceptible strain. Finally, in one strain no mutation could be found in the sequenced fragment of rpoB.

In summary we have observed in this study an excellent concordance between the rifampicin susceptibility pattern obtained with the BACTEC MGIT960 system and the two available reverse hybridization assays (INNO-LiPA Rif.TB and MTBDRplus). Seventeen different rpoB mutations affecting 10 codons could be efficiently detected, and specific probes identified the four most frequent mutations in 89.5% of samples. Therefore, sequencing can be restricted to the investigation of less frequent mutations or when the interpretation of the hybridization pattern is not straightforward.

Acknowledgements

We thank Kanglin Wan for communicating unpublished results. We are grateful to ‘Médecins Sans Frontières’ and the Centre
Hospitalier Bouffard in the Republic of Djibouti for providing samples.

**Funding**

This project was funded by the CNRS and Université Paris Sud 11, and benefited from the European Community grant FP6-012166.

**Transparency declarations**

None to declare.

**References**


