

Rifampin-Isoniazid Oligonucleotide Typing: an Alternative Format for Rapid Detection of Multidrug-Resistant *Mycobacterium tuberculosis*^{∇†}

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A reverse line blot DNA hybridization format for rapid detection of multidrug-resistant tuberculosis was developed. Simultaneous detection of rifampin and isoniazid resistance in clinical isolates of *Mycobacterium tuberculosis* was based on the same amplification/reverse hybridization principle of the widely used spoligotyping. The test involved probing nine DNA regions that are targets of common drug resistance-associated mutations in the genes *rpoB*, *katG*, and *inhA*. Addition of quaternary amine tetramethyl ammonium chloride to the hybridization buffer promoted multiple hybrid formations at a single annealing temperature irrespective of the different GC contents of probes. The assay was standardized using 20 well-documented strains from the Institute of Tropical Medicine (Belgium) and evaluated blindly in a central laboratory with 100 DNA samples that were obtained from cultured clinical isolates and shipped dried from three other countries. Compared with drug susceptibility testing, both sensitivity and specificity for rifampin resistance detection were 93.0% while for isoniazid the values were 87.7% and 97.7%, respectively. Compared with sequencing and GenoType MTBDRplus methods, sensitivity and specificity reached 96.4% and 95.5% for rifampin and 92.7% and 100% for isoniazid. Altogether, 40/45 (89%) multidrug-resistant isolates were correctly identified. Advantages of this in-house development include versatility, capacity to run up to 41 samples by triplicate in a single run, and reuse of the membrane at least 10 times. These features substantially reduce cost per reaction and make the assay an attractive tool for use in reference laboratories of countries that have a high burden of multidrug-resistant tuberculosis but that cannot afford expensive commercial tests because of limited resources.

According to the World Health Organization (WHO), about 300,000 new cases of multidrug-resistant tuberculosis (MDR-TB) occurred in 2008, representing 3% of the 9 million incident cases of TB estimated for that year (31). The spread of MDR-TB has been fostered by delays in the identification of drug-resistant strains, especially in countries with moderate or limited resources. Conventional drug susceptibility testing (DST) can be performed only once a culture is found positive and takes at least 3 to 4 weeks for definitive results. Such a delay allows transmission of resistant strains (16, 21). Therefore, the development of rapid diagnostic tests that are inexpensive and feasible for the detection of MDR-TB strains is a priority for TB control.

MDR-TB is caused by strains of *Mycobacterium tuberculosis* that are resistant to at least rifampin (RIF) and isoniazid (INH). Unlike what happens in other pathogens, where resistance is conferred mostly by mobile genetic elements, drug resistance in *M. tuberculosis* usually arises by single point mutations in antibiotic target genes (7). In particular, RIF resis-

tance is due mainly to mutations in an 81-bp hot spot region of the *rpoB* gene (27), and INH resistance is frequently associated with mutations in the *katG* and *inhA* genes (1, 12, 22, 29, 30, 32). The identification of point mutations as responsible for *M. tuberculosis* drug resistance prompted the development of hybridization-based techniques for their rapid detection. There are currently available two commercial solid-phase hybridization tests: a line probe assay (INNO-LiPA Rif TB assay; Innogenetics, Ghent, Belgium) for the detection of RIF resistance and the GenoType MTBDR assays, including the improved GenoType MTBDRplus (Hain Lifesciences, Nehren, Germany), for the simultaneous detection of INH and RIF resistance. Both assays have been evaluated in different settings, giving very good results (3, 4, 17). Moreover, the WHO has recently endorsed these assays as useful tools for rapid MDR-TB diagnosis (22).

The reverse line blot hybridization format has been widely used in the spoligotyping technique for *M. tuberculosis* lineage identification (8, 11). Similarly, the same format was developed to detect mutations that confer resistance to RIF and other antibiotics (14, 18, 19, 26). The technique is currently extensively used and is well standardized (13). Nonetheless, simultaneous hybridization of different-length probes has been shown to be difficult (23). In order to overcome this limitation we adapted a method previously applied to the simultaneous detection of point mutations using tetramethyl ammonium

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TABLE 1. Sequences of primers used in this study

Gene	Primer	Sequence ^a	PCR product length (bp)
<i>rpoB</i>	Forward	5'-CGC CGC GAT CAA GGA GT	276
	Reverse	bio-CCG TCG ACC ACC TTG CGG	
<i>katG</i>	Forward	5'-GCG GTC ACA CTT TCG GTA AG	250
	Reverse	bio-TCC TTG GCG GTG TAT TGC	
<i>p-inhA^b</i>	Forward	5'-TGA GTC ACA CCG ACA AAC	330
	Reverse	bio-GAC CCT GGT GCT CTT CTA	
<i>inhA</i>	Forward	5'-GTG GTC AGC TTC CTG GCTT	538
	Reverse	bio-AGC ATA CGA ATA CGC CGA GA	

^a Reverse primers were labeled with biotin (bio).

^b *p-inhA*, promoter region; the *p-inhA* primers were used to amplify the whole region.

chloride (TMAC), which allows probes of the same length to be hybridized at the same annealing temperature, independently of their G+C content (20). In this study we describe the design of rifampin-isoniazid oligonucleotide typing (RIOT), a reverse line blot hybridization assay for the simultaneous detection of RIF and INH resistance and a preliminary blind evaluation of this assay in clinical isolates of *M. tuberculosis*.

MATERIALS AND METHODS

Clinical isolates. Four different laboratories in Sweden, Latvia, and Argentina selected a total of 100 *M. tuberculosis* clinical isolates, of which 45 were MDR, 24 were monoresistant, and 31 were pan-susceptible (see Table S1 in the supplemental material). Twenty well-documented strains from the collection at the reference laboratory of the Institute of Tropical Medicine (ITM) in Belgium were used for standardization of the method.

DST. Drug susceptibility testing was performed by the reference standard proportion method in Löwenstein-Jensen medium according to standard procedures (5, 6) as well as with Bactec 460 (25) and Bactec MGIT 960 SIRE kits (Becton Dickinson, MD). The commercial test GenoType MTBDRplus (Hain Lifesciences, Nehren, Germany) for detection of drug resistance-conferring mutations was used according to the manufacturer's instructions.

DNA extraction. The strains were cultured on solid medium (Löwenstein-Jensen or Middlebrook agar) for 4 weeks at 37°C. One loopful of *M. tuberculosis* culture was suspended in 100 µl of distilled water and boiled for 30 min. Further, the suspension was centrifuged at 16,000 × g for 5 min, the supernatant was recovered, and 10 µl of 3 M sodium acetate, pH 4.8, and 250 µl of cold ethanol were added. After incubation at -20°C for 60 min, the sample was centrifuged, and the pellet was washed with 200 µl of 70% ethanol; the tubes were left to dry at room temperature after the supernatant was discarded. Dried DNA samples were shipped code labeled and evaluated blindly. Prior to use, pellets were resuspended in 50 µl of TE (10 mM Tris, pH 8.0, 1 mM EDTA) buffer and quantified in a Nanodrop 2000 spectrophotometer (Thermo scientific).

DNA sequencing. Rifampin-isoniazid oligonucleotide typing results were compared to those of DNA sequencing and the GenoType MTBDRplus (Hain Lifesciences, Nehren, Germany). The analysis with the GenoType MTBDRplus was carried out according to the manufacturer's instructions. Sequencing of the *rpoB* gene was performed as described previously (10), whereas *katG* sequencing was performed as follows. An approximately 700-bp fragment was amplified using 200 nM (each) *katG* primers F768 (5' CATGAACGACGTCGAAACAG) and R1458 (5' GCTACCACGGAACGACGAC). The PCR mix also contained 1.5 µM MgCl₂, 5 µl of 10× PCR buffer, 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 200 µM each deoxynucleoside triphosphate (dNTP; Applied Biosystems, United Kingdom). To each reaction mixture, 10 ng of DNA template was added, and the mixture was initially denatured at 95°C for 10 min, cycled 30 times (at 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min), and finally extended at 72°C for 7 min. Using a GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech Inc.), the PCR products were purified and subsequently used as templates for cycle sequencing. Cycle sequencing was performed with *katG* primers F768 and R1458 using a BigDye Terminator Cycle Sequencing Ready Reaction Kit and separated in an ABI 3130xl Genetic Ana-

lyzer (Applied Biosystems). Retrieved *katG* sequences were aligned to the H37Rv *katG* sequence (accession number Rv1908c) using the ClustalW algorithm.

Design of primers and probes. Primers designed for the specific amplification of the hot spot region of the *rpoB* gene and for the most prevalent *katG* and *inhA* mutations associated with INH resistance are shown in Table 1. The 5' reverse primers were labeled with biotin in order to detect the hybridization signals.

Multiplex PCR assay. The conditions used for the multiplex PCR were as follows: a 25-µl final reaction volume consisted of 66 mM Tris-HCl, 1.5 mM MgCl₂, 1.25 U of *Taq* DNA polymerase (CorpoGen, Colombia), 0.2 µM each primer, 0.3 mM each dNTP, and 20 ng of DNA. Cycling conditions began with an initial denaturation step at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s. A final extension step consisted of 10 min at 72°C. The presence of amplified DNA fragments was confirmed by gel electrophoresis using 1.5% agarose gel in TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.3).

RIOT. The oligonucleotide probes listed in Table 2, all of them 18 bases in length, were attached to a Biotin C membrane (Pall Corp.) using a previously described methodology (13, 14, 23). Three complete sets, each consisting of five RIF- and four INH-specific probes, were attached to each membrane using a miniblotted of 45 slots (Immunetics). For sample probing, 10 µl of each PCR-amplified product was diluted in 150 µl of TMAC buffer (3 M TMAC, 0.1% SDS, 1 mM EDTA, 10 mM Na₃PO₄, pH 6.8), denatured at 96°C for 5 min, and cooled on ice. The heat-denatured single-stranded PCR products were applied on the membrane mounted in the miniblotted. Of the 45 slots in the apparatus, 2 were reserved for positive (strain H37Rv pan-susceptible) and negative (water) controls, and the first and the last slots were filled with 170 µl of TMAC buffer. The remaining 41 slots were available for sample probing, and any eventually unused slot was filled with buffer. Hybridization was carried out at 52°C for 60 min. The membrane was washed twice at 52°C for 15 min in 10 ml of TMAC buffer. The chemiluminescent detection was made by autoradiography with a KPL DNA detection kit following the manufacturer's instructions (KPL, Inc.). All samples were evaluated in triplicate.

Statistical analysis. The performances of RIOT, DNA sequencing, and MTBDRplus were compared to that of conventional DST for the detection of RIF and INH resistance. Sensitivity, specificity, and accuracy were calculated. Data were analyzed using GraphPad Prism, version 5 (GraphPad Software Inc.). Cohen's kappa coefficient that allows estimation of agreement, taking into account the amount of agreement occurring at random (9, 15), and the positive and negative predictive values were used to evaluate the performance of each probe.

RESULTS

Multiplex PCR assay. In order to render the assay user friendly and to minimize hands-on time, a multiplex PCR for the simultaneous amplification of *rpoB*, *katG*, and *inhA* target genes was standardized. As shown in Fig. 1, simultaneous amplification of all gene segments was satisfactory in this multiplex assay. Although larger amplicons corresponding to the

TABLE 2. Sequences of probes used in this study

Gene	Probe name	Sequence	T_m (°C) ^a	%CG	Mutation (codon)
<i>rpoB</i>	rpoB W1	5'-CAG CTG AGC CAA TTC ATG	51.4	50	512
	rpoB W2	5'-TTC ATG GAC CAG AAC AAC	49.6	44.4	516
	rpoB W3	5'-CCG CTG TCG GGG TTG ACC	61.6	72.2	522
	rpoB W4	5'-TTG ACC CAC AAG CGC CGA	60.3	61.1	526/529
	rpoB W6	5'-CGA CTG TCG GCG CTG GGG	64.3	77.7	531
<i>katG</i>	katG S309	5'-CGG AAC CGG TAA GGA CGC	57.8	61.1	309
	katG S315	5'-GAT CAC CAG CGG CAT CGA	58.6	66.6	315
<i>inhA</i>	inhA SW15	5'-GCG AGA CGA TAG GTT GTC	52.6	55.5	-15 ^b
	inhA S94	5'-TGC ATT CGA TTG GGT TCA	52	44.4	94

^a T_m , thermal denaturation midpoint temperature.

^b Mutation in the -15 codon of the *inhA* promoter region.

inhA gene showed lower band intensity than amplification of the individual gene, the hybridization signal was not affected.

RIOT specificity, sensitivity, and accuracy. The designed platform allows the analysis of up to 41 samples in triplicate plus a positive and a negative control in a single run. Figure 2 shows a scanned image of a membrane where absence of hybridization signal indicates a mutated strain. No definite discrepancy was observed between triplicates in a single membrane, and an ambiguous hybridization signal was resolved by consensus of the other two replicates. RIOT identified 40 out of 45 (89.0%) MDR-TB isolates identified by DST. Compared with DST, the sensitivity, specificity, and accuracy rates of RIOT for RIF resistance detection were 93.0%. For INH resistance detection, the values were 87.7% sensitivity, 97.7% specificity, and 92.0% accuracy. Sequencing and MTBDRplus results showed 56 isolates with RIF resistance-associated mutation, of which RIOT detected 54. The sensitivity and speci-

ficity of our assay for RIF resistance detection compared with sequencing and MTBDRplus were 96.4% and 95.5%, respectively, with an accuracy of 96.0%. As for detection of INH-associated mutation, sensitivity and specificity were 92.7% and 100%, respectively, with an accuracy of 96.0%. Noticeably, two phenotypically RIF-resistant isolates did not have any mutation in the 81-bp hot spot region of *rpoB* as detected by DNA sequencing and RIOT. Also, one isolate susceptible to INH by DST had a mutation in codon 315 of *katG* identified by RIOT, MTBDRplus, and DNA sequencing (Table 3).

Performance of individual probes. The performance of each probe was assessed by measuring the agreement of the test in comparison with DNA sequencing by Cohen's kappa coefficient and positive and negative predictive values (PPV and NPV, respectively). Kappa values ranged from 0.92 to 1.0, indicating an overall high degree of agreement with sequencing results. In particular, probes rpoB 4W and inhA SW15 exhibiting high kappa values but low PPV should be redesigned in a future version of the assay (Table 4).

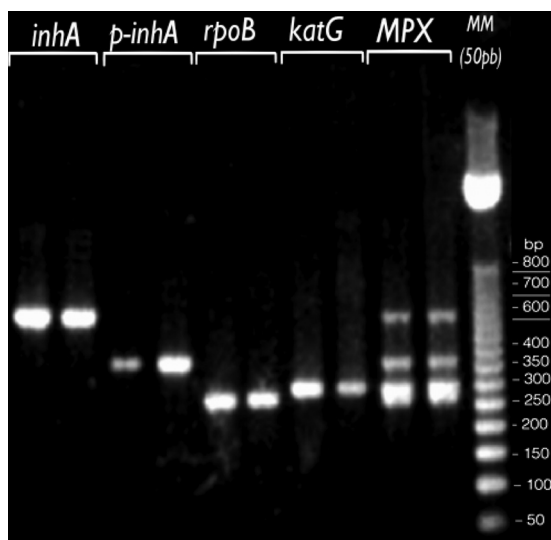


FIG. 1. Amplified PCR products of *rpoB*, *katG*, and *inhA* gene targets after electrophoresis in agarose gel. The individual amplifications of gene regions and the multiplex (MPX) assay are shown consecutively. Each pair of lanes corresponds to amplified DNA product of a clinical sample (left) and reference strain *M. tuberculosis* H37Rv (right).

DISCUSSION

We produced a tool for the rapid detection of MDR TB that is easy to perform in a laboratory with a medium level of technological capability. We named the assay RIOT, which stands for rifampin-isoniazid oligonucleotide typing because it is based on reverse line blot DNA hybridization for the simultaneous detection of mutations in genes *rpoB*, *katG*, and *inhA*. In this first version of RIOT, we used only wild-type oligonucleotide probes in order to identify any mutation in target codons. However, the platform described here is versatile enough to allow incorporation of specific probes for the most relevant mutated codons in future versions of the assay.

Designing a test aimed at detecting simultaneous point mutations in different genes is not an easy task. In our approach, we first standardized a multiplex PCR for the amplification of hot spot regions in the genes *rpoB*, *katG*, and *inhA*. Obtaining a homogeneous and robust amplification product in a multiplex PCR is critical for achieving a neat hybridization signal, and we did this by carefully adjusting DNA concentrations prior to PCR amplification. Then, we included TMAC in the hybridization buffer, which represents an innovation over a previously described rifampin oligonucleotide typing test (14)

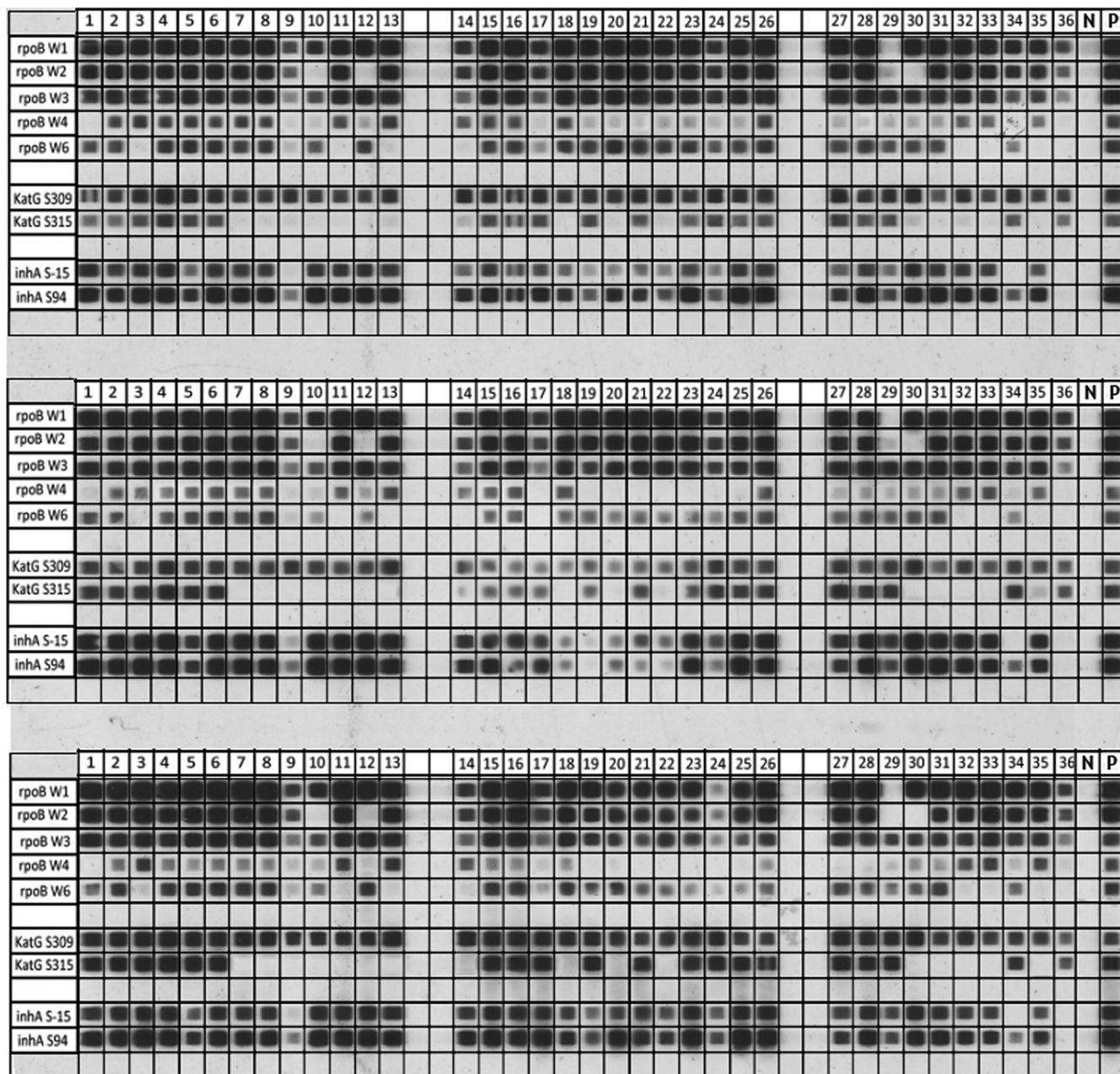


FIG. 2. Scanned image of the reverse line blot hybridization assay (RIOT). Lanes 1 to 36 correspond to clinical isolates with assorted mutations. Lane N, negative control; lane P, positive wild-type control (reference strain *M. tuberculosis* H37Rv). Blank lanes between 13 and 14 and between 26 and 27 correspond to unused slots. Three sets of nine rows each represent technical triplicates of probes for drug resistance-associated mutations. A grid designed on a transparent film is overlaid on the membrane to aid analysis of results. inhA S-15 corresponds to the probe inhA SW15.

because this compound renders the complete procedure easier to perform. To ensure that all probes are hybridized at the same temperature is an advantage for the simultaneous detection of different single-nucleotide polymorphisms. Last, we have left the platform open to further improvement as the format is versatile enough to allow inclusion of new probes to detect resistance to these and other drugs.

This molecular platform has already been applied for detecting RIF resistance (14, 18, 19, 26). However, to our knowl-

edge it has not been explored for the simultaneous detection of RIF and INH resistance. The lower sensitivity associated with INH resistance is partly due to the fact that phenotypical INH resistance is not fully covered by the mutations described to date. In an attempt to overcome this limitation, in addition to probes for *katG* codon 315 and *inhA* codon -15, we incorporated two other probes into our platform, namely, those involving codon 309 of *katG* and codon 94 of *inhA*, which were recently reported as associated with INH resistance (2, 29).

TABLE 3. Nonhybridizing probes in the RIOT assay and DST and sequencing results of 100 clinical isolates for rifampin resistance and isoniazid resistance

Type of resistance and no. of isolates	RIOT ^a (probe name)	DST result	Mutation(s) detected by sequencing/MTBDRplus (codon) ^b
RIF			
1	rpoB W1	Rif ^r	512
4	rpoB W2	Rif ^r	516
9	rpoB W4	Rif ^r	526
2	rpoB W4	Rif ^s	WT
31	rpoB W6	Rif ^r	531
2	rpoB W1/rpoB W2	Rif ^r	511/516
2	rpoB W2/rpoB W4	Rif ^r	516
2	rpoB W4	Rif ^r	526/529
1	rpoB W4	Rif ^s	526/529
1	rpoB W6	Rif ^r	533
1	rpoB W6	Rif ^r	531/536
1	None	Rif ^r	516
1	None	Rif ^r	Del 508/509
2	None	Rif ^r	No mutation
40	None	Rif ^s	No mutation
INH			
32	katG S315	Inh ^r	315
1	katG S315	Inh ^s	315
1	katG S315/S309	Inh ^r	315
1	katG S315/S309	Inh ^r	315/-15
8	katG S315/inhA SW15	Inh ^r	315/-15
1	katG S309/inhA SW15	Inh ^r	315
7	inhA SW15	Inh ^r	-15
4	None	Inh ^r	315
3	None	Inh ^r	No mutation
42	None	Inh ^s	No mutation

^a Absence of hybridization with the oligonucleotide probe.

^b WT, wild type; Del, deletion.

Although such inclusion did not add sensitivity, the performance of RIOT for INH resistance was fairly satisfactory in this preliminary evaluation.

Indeed, compared with the results of sequencing and MTBDRplus, RIOT produced reasonably accurate results for detection of INH as well as RIF resistance-associated mutations. RIOT had slightly lower sensitivity, specificity, and accuracy values than DST. It is well known that a resistance phenotype is not always associated with gene mutations (23, 29). Zhang and coworkers developed a similar multiplex probe

array including specific oligonucleotide probes aimed to detect relevant mutations in *rpoB*, *katG*, *inhA*, and *ahpC* genes. The test, which did not use TMAC in the hybridization step, showed a sensitivity and specificity of 88.5% and 100%, respectively, for RIF resistance and 86.5% and 100% for INH resistance compared with DST (24, 33). These values are in agreement with the RIOT test here described.

RIOT overall intraexperiment reproducibility was good. Any ambiguous hybridization signal could be disclosed by agreement between results of the three replicates present in each membrane. These technical triplicates proved useful to enable an accurate interpretation of the X-ray film. A fine-tuning of the design is under way, in particular, of oligonucleotide probes rpoB W4, inhA SW15, and katG S315, which is expected to avert the remaining ambiguous interpretations and restrict the number of false-positive and false-negative results, leading to a general improvement in the performance of the test. A probe specific for *M. tuberculosis* is also being included in the new design. The few results in our study that were discrepant with sequencing deserve further scrutiny.

An advantage of molecular assays like the one described here is that they can be made available in a single reference laboratory where clinical DNAs can be readily shipped without raising biosafety concerns during sample handling and transportation. In fact, the performance of our platform was evaluated blindly in Colombia with dried DNAs that had been extracted using a very simple protocol and shipped from four different countries.

Another advantage of the RIOT assay reported here is that it follows the same format of spoligotyping, a technique widely used in many reference laboratories dedicated to TB diagnosis. Additionally, it allows the simultaneous analysis of 41 DNA samples by triplicate, and the oligonucleotide attachment is easy to scale up into a standardized format. Including DNA extraction, PCR multiplex amplification and detection, its cost is approximately \$5 per sample, and the membrane can be reused at least 10 times (13), considerably reducing the cost of the assay. In this first version, RIOT was evaluated with DNA extracted from *M. tuberculosis* cultures grown from clinical samples. In the near future we will evaluate RIOT directly with smear-positive clinical samples.

All these features make RIOT an attractive tool for reference laboratories in high-MDR-TB-burden settings, particularly in countries that have only moderate or limited resources

TABLE 4. Frequency of mutations, Cohen's kappa coefficient, and PPV and NPV for RIOT assay compared to sequencing results

Probe name	No. of mutations detected		No. of false positives	No. of false negatives	Cohen's kappa coefficient	PPV	NPV
	RIOT assay	Sequencing/MTBDRplus					
rpoB W1	3	3	0	0	1.00	1.00	1.00
rpoB W2	8	9	0	1	0.98	1.00	0.99
rpoB W3	0	0	0	0	1.00	NA ^a	1.00
rpoB W4	14	12	2	0	0.96	0.86	1.00
rpoB W6	33	33	0	0	1.00	1.00	1.00
katG S309	2	0	2	0	0.93	0.00	1.00
katG S315	44	48	0	4	0.94	1.00	0.93
inhA SW15	16	16	1	1	0.96	0.94	0.99
inhA S94	0	0	0	0	1.00	NA	1.00

^a NA, not applicable.

and that do not have access to expensive commercial tests (16, 28). Further ongoing developments of the assay include the implementation of computer image analysis to reduce errors caused by subjective interpretation of the autoradiography, the introduction of mutant-specific probes, and extension of the drug resistance coverage to second-line drugs, especially those responsible for extensively drug-resistant TB (XDR-TB).

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