

## Rapid Diagnosis of Extensively Drug-Resistant Tuberculosis by Use of a Reverse Line Blot Hybridization Assay<sup>∇†</sup>

Kanchan Ajbani,<sup>1</sup> Anjali Shetty,<sup>2</sup> Ajita Mehta,<sup>2‡</sup> and Camilla Rodrigues<sup>2\*</sup>

Department of Research, P. D. Hinduja National Hospital and Medical Research Centre, Mumbai, India,<sup>1</sup> and Department of Microbiology, P. D. Hinduja National Hospital and Medical Research Centre, Mumbai, India<sup>2</sup>

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**Drug resistance in tuberculosis (TB) is a matter of grave concern for TB control programs, as there is currently no cure for some extensively drug-resistant (XDR) strains. There is concern that this resistance could transmit, stressing the need for additional control measures, rapid diagnostic methods, and newer drugs for treatment. We developed an in-house assay that can rapidly detect resistance to drugs involved in the definition of XDR-TB directly from smear-positive specimens. Two hundred fifteen phenotypically XDR-TB isolates and 50 pansusceptible isolates were analyzed using a reverse line blot hybridization (RLBH) assay. The assay was also successfully applied to 73 smear-positive clinical specimens. The RLBH assay exhibited good sensitivity for the detection of resistance to isoniazid (99%), rifampin (99%), fluoroquinolones (95.3%), and second-line aminoglycosides (94.8%). The results from application of this assay on direct smear-positive clinical specimens revealed 93% concordance with the phenotypic drug susceptibility test (DST) results for the above-mentioned drugs. The time to accurate DST results was significantly reduced from weeks to 3 days. This molecular assay is a highly accurate tool for screening for XDR-TB, which achieves a substantial reduction in diagnostic delays.**

Estimates of the incidence of *Mycobacterium tuberculosis* infections continue to increase globally, and in 1993 the WHO declared tuberculosis (TB) a global public health emergency, being the only disease so far to warrant that designation. In many countries where the incidence of TB is high, the major driving force of the epidemic is transmission. The foremost reason for this is that there are prolonged delays from the onset of the TB disease in individuals to the time of actual diagnosis. During this period, an active TB case may infect numerous other people, thereby contributing to the increase of the epidemic (20). The emergence of multidrug-resistant (MDR) tuberculosis, defined as resistance to both rifampin (RIF) and isoniazid (INH), is a compounding factor for the control of the disease. Patients harboring MDR *M. tuberculosis* strains need alternative treatment regimens involving second-line drugs that are more expensive, toxic, and less effective. Moreover, extensively drug-resistant (XDR) tuberculosis (resistance to INH, RIF, fluoroquinolones [FQs] and any 1 second-line injectable, i.e., kanamycin [KAN], amikacin [AM], or capreomycin [CM]) constitutes an emerging threat for the control of the disease over the period (4). Accurate diagnosis and initiation of early treatment are essential to reduce transmission and improve the outcome for patients with tuberculosis. Timely identification of drug resistance is especially important to avoid subjecting patients with resistance to inadequate first-line treatment for months while awaiting drug susceptibility

testing (DST) results. This exposure to inadequate therapy results in amplified drug resistance (21).

Drug resistance in *M. tuberculosis* is attributed to random mutations in the *Mycobacterium* genome. Mutations in the specific codons can therefore be exploited to rapidly detect drug resistance (8, 9, 12, 19, 22). Resistance to first-line drugs (INH, RIF) is attributed to mutations in *inhA* and *katG* for INH and *rpoB* for RIF (18, 19, 22, 23). Resistance to fluoroquinolones and second-line aminoglycosides is most frequently associated with mutations in *gyrA* and *gyrB* and in *rrs*, respectively. Studies have demonstrated that by targeting mutations in codons 90, 91, and 94 in *gyrA*, approximately 70 to 90% of all fluoroquinolone resistance can be accurately predicted (2, 5, 17). Similarly, mutations in the *rrs* gene, particularly the hot-spot region from nucleotides 1401 to 1484, can accurately predict resistance to second-line aminoglycosides (10, 16). The reverse line blot hybridization (RLBH) assay (11, 24) is a probe-based method where multiple oligonucleotide probes carrying the mutant sequence and wild-type sequence are immobilized on nitrocellulose strips and hybridized with biotin-labeled PCR products. This technique had been successfully applied for the identification of mutations related to resistance to isoniazid, rifampin, and streptomycin at our center. The assay was optimized to additionally detect mutations conferring FQ and second-line aminoglycoside (KAN, AM, CM) resistance under the same hybridization conditions, such that a single blot could be used to detect resistance to the antitubercular drugs included in the XDR-TB definition. This enabled us to reduce the time to accurate DST results.

### MATERIALS AND METHODS

**Setting.** The study was carried at the mycobacteriology laboratory at the P. D. Hinduja National Hospital (PDHNNH) and Medical Research Centre, a tertiary care center in central Mumbai, India, with a referral bias toward complicated and nonresponding cases. The study was approved by the Institutional Review Board (IRB). The laboratory receives about 10,000 samples for culture annually, with a

\* Corresponding author. Mailing address: Department of Microbiology, Research Laboratories, P. D. Hinduja National Hospital and Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400016, India. Phone: 0091 22 24447795. Fax: 099 22 24442318. E-mail: dr\_crodrigues@hindujahospital.com/.

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‡ Passed away on 6 February 2010.

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TABLE 1. Primer sequences for *inhA*, *katG*, *rpoB*, *gyrA*, *gyrB*, and *rrs*

Target region	Primer	Sequence
<i>inhA</i>	TB 92 M	5'-CCT CGC TGC CCA GAA AGG GA TCC-3'
	TB 93 M	5'-biotin-CCG GGT TTC CTC CGG T-3'
<i>katG</i>	KGF	5'-biotin-AGA GCT CGT ATG CCG GA-3'
	KGR	5'-GCG AAT GAC CTT GCG CAG ATC-3'
<i>rpoB</i>	Rpo F	5'-TCA AGG AGA AGC GCT ACG ACC TGG-3'
	Rpo R	5'-biotin-GGG TCT CGA TCG GGC ACA T-3'
<i>gyrA</i>	<i>gyrA</i> F	5'-CGA ACC GGT TGA CAT CGA GCA GGA G-3'
	<i>gyrA</i> R	5'-biotin-CAG CAT CTC CAT CGC CAA CG-3'
<i>gyrB</i>	<i>gyrB</i> F	5'-GAA GCC AAC CCC ACC GAC GCG A-3'
	<i>gyrB</i> R	5'-biotin-CTG CGC TGC CAC TTG AGT TTG-3'
<i>rrs</i>	<i>rrs</i> F	5'-CGT TCC CTT GTG GCC TGT GTG CAG-3'
	<i>rrs</i> R	5'-biotin-GTT GGG GCG TTT TCG TGG TGC-3'

culture positivity rate of 40%. Being private, tertiary laboratory susceptibility testing is done only on request, wherein of the 3,609 requests for DST from January 2008 to December 2009, 65% of isolates are MDR *M. tuberculosis*, and of these, 9.1% are XDR *M. tuberculosis*.

**Bacterial isolates.** Two hundred fifteen consecutive XDR-TB isolates tested by the mycobacterial growth indicator tube (MGIT 960) TB system were collected over a period of 24 months (January 2008 to December 2009). Fifty consecutive pansusceptible isolates tested by the MGIT 960 system were also tested using the RLBH assay. All the collected strains were stored at -70°C. DNA extraction from mycobacterial culture isolates was carried out by the cetyltrimethylammonium bromide-sodium chloride (CTAB-NaCl) method (15).

**Clinical specimens.** Seventy-three consecutive acid-fast bacillus (AFB) smear-positive clinical specimens (1+ and above) collected over a period of 5 weeks were also tested by the RLBH method. Of the 73 specimens, 31 specimens were 1+ on acid-fast bacillus smear, 15 were 2+, and 27 were 3+ on AFB smear. Smear-positive specimens were subjected to decontamination by the *N*-acetyl-L-

cysteine-sodium citrate-NaOH (6) method, and the sediment was used for DNA extraction, which was carried out using a Qiagen DNA extraction kit.

**Amplification of all targets in multiplex PCR.** Table 1 lists the PCR primer sequences, wherein the primers for *inhA*, *katG*, and *rpoB* are the same as those described previously (14). Novel primers were designed for amplifying *gyrA*, *gyrB*, and *rrs* and are also listed in Table 1. The cycling conditions for the touchdown multiplex assay for the isolates and direct clinical specimens are provided in the supplemental material.

**Designing specific sequence probes for fluoroquinolones and second-line aminoglycosides.** The initial 150 XDR-TB isolates were amplified as single targets and sequenced (1). On the basis of the mutations, specific amino acid-labeled oligonucleotide probes for fluoroquinolones and second-line aminoglycosides were designed so that they could be processed under the same hybridization and washing conditions used for the MDR blot and were covalently linked to a nylon membrane in parallel lines. Six mutant probes and 2 wild-type probes were designed for *gyrA*, and 1 wild-type and 1 mutant probe were designed for *gyrB*. For aminoglycosides, the *rrs* region was targeted with 2 wild-type probes and 3 mutant probes correlating with high-level resistance to KAN, AM, and CM (Table 2). The uniqueness and cross-reactivity of all the newly designed and modified probes were analyzed with a BLAST search (<http://www.ncbi.nlm.nih.gov>).

**Hybridization with amplified products.** Heat-denatured PCR products were applied on the membrane in the miniblotted and hybridized at 53°C for 60 min. The membrane was washed twice with gentle shaking in 250 ml 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, and 1 mM EDTA [pH 7.7])–0.5% SDS for 10 min at 60°C. The membrane was subsequently incubated at 42°C with 1:4,000-diluted streptavidin-peroxidase conjugate in 2× SSPE–0.5% SDS for 60 min, washed twice with 250 ml 2× SSPE–0.5% SDS at 42°C for 10 min, rinsed twice with 2× SSPE at room temperature (RT) for 5 min, and subjected to luminescent detection of hybrids with an enhanced chemiluminescence (ECL) detection system, followed by exposure to ECL Hyperfilm (Amersham Biosciences). The presence of a clearly visible black signal was considered a positive hybridization reaction. The results were easy to interpret (Fig. 1). For reuse, the membranes were stripped in 1% SDS solution at 80°C (twice for 40 min) and rinsed in 20 mM EDTA, pH 8.0, at RT. The membrane can be stripped and reused up to 7 times without compromising the results.

Reproducibility of the RLBH assay was validated by testing 215 clinical XDR *M. tuberculosis* isolates (of these, 150 were also sequenced and results were compared with those of sequencing [1] as well as the RLBH assay), 50 susceptible isolates, and 73 smear-positive clinical specimens (+1 and above). The results for the clinical specimens were compared with phenotypic susceptibilities determined on MGIT.

**Statistical analysis.** A 2-by-2 table was used to calculate the sensitivity and specificity of the RLBH assay in comparison with the phenotypic DST, i.e., MGIT. A *P* value (significance) of <0.05 is deemed statistically significant. To

TABLE 2. Probe sequences for fluoroquinolone and aminoglycosides

Gene and probe no.	Probe	Description	Sequence <sup>a</sup>	
<i>gyrA</i>	1	Codon 90	Wild type	5'-ACC ACC CGC ACG GCG ACG CGT C-3'
	2	Codon 90	Mutant type	5'-ACCACC CGC ACG GCG ACG <u>TGT</u> C-3'
	3	Codon 91	Mutant type	5'-ACC CGC ACG GCG ACG <u>CGC</u> C-3'
	4	Codon 94	Wild type	5'-CG ATC TAC GAC AGC CTG GTG-3'
	5	Codon 94	Mutant type I	5'-CG ATC TAC <u>GCC</u> AGC CTG GTG-3'
	6	Codon 94	Mutant type II	5'-CG ATC TAC <u>GGC</u> AGC CTG GTG-3'
	7	Codon 94	Mutant type III	5'-CGA TCT <u>ACT</u> ACA CCC TGG TG-3'
	8	Codon 94	Mutant type IV	5'-CGA TCT <u>ACA</u> ACA CCC TGG TG-3'
	9	Codon 95	Polymorphism	5'-CGA TCT ACG ACA <u>CCC</u> TGG TG-3'
<i>gyrB</i>	10	510	Wild type	5'-GTG CTA AAG AAC ACC GAA G-3'
	11	510	Mutant type	5'-CGA CCG GGT GCT AAA <u>GAG</u> CA-3'
<i>rrs</i>	12	1401	Wild type	5'-ACC GCC CGT CAC GTC ATG AA-3'
	13	1401	Mutant type	5'-ACC GCC CGT <u>CGC</u> GTC ATG AA-3'
	14	1402	Mutant type	5'-ACC GCC CGT <u>CAA</u> GTC ATG AA-3'
	15	1484	Wild type	5'-CGG CGA TTG GGA CGA AGT C-3'
	16	1484	Mutant type	5'-CGG CGA TTG GGA <u>CIA</u> AGT C-3'

<sup>a</sup> The boldface with an underscore indicates the change in the nucleotide sequence.

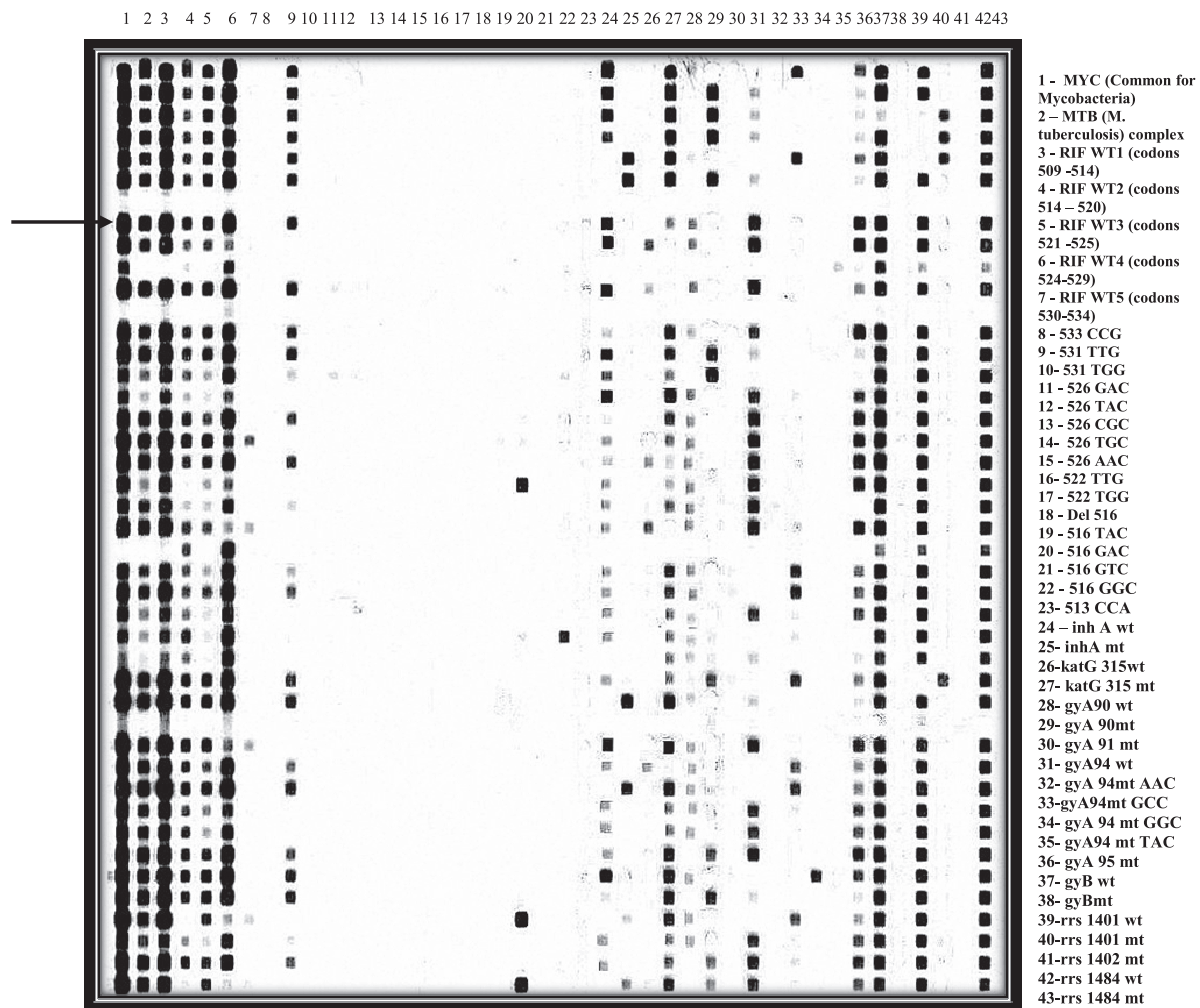


FIG. 1. RLBH performed for *rpoB*, *katG*, *inhA*, *gyrA*, *gyrB*, and *rrs*. All the different probes are blotted vertically, and patient samples (PCR products) are applied horizontally. The probe in lane 1 is a common probe for all mycobacteria, and then there is a probe for the *M. tuberculosis* complex. Then there are 5 probes for the wild-type *rpoB* gene to check for rifampin-susceptible cases and 16 mutant probes for detection of rifampin resistance. Similarly, we targeted the *inhA* wild type and mutant with a probe for the promoter region, followed by probes for the wild type and mutant at codon 315 of the *katG* gene. This is further followed by probes for the wild type and mutant for *gyrA* mutations at codons 90, 91, 94, and 95. Similarly, we also targeted the *gyrB* codon 510 region with wild-type and mutant probes. For the second-line aminoglycosides, the *rrs* region at codons 1401, 1402, and 1484 were used. To illustrate an example, the patient sample marked by the arrow has mycobacterial infection due to *M. tuberculosis* complex and shows binding with probes 3 to 6, which are for the *rpoB* wild-type codon 509 to 529. It does not show binding with probe 7 but shows binding with probe 9, indicating RIF resistance due to a position 531 TTG mutation. Further, the same sample shows binding with probe 24, which is for the *inhA* promoter wild type, and shows binding with probe 27, which is the probe for a *katG* mutation at codon 315, thereby indicating INH resistance due to a *katG* mutation. The same sample shows binding with the 28th, 31st, 36th, 37th, 39th, and 42nd probes, which correspond to the *gyrA* codon 90 wild type, *gyrA* codon 94 wild type, *gyrA* codon 95 polymorphism, *gyrB* wild type, *rrs* codon 1401 wild type, and *rrs* codon 1484 wild type, respectively, thus giving a result for that particular sample indicating resistance to INH and RIF and susceptibility to FQs and KAN, AM, and CM. WT and wt, wild type; mt, mutant.

compare two tests, e.g., RLBH and MGIT, kappa was used as a measure of agreement with the 95% confidence interval (CI).

## RESULTS

**Probe designing.** The initial 150 XDR *M. tuberculosis* isolates and 50 pansusceptible isolates were sent for sequencing to screen for mutations in *gyrA*, *gyrB*, and *rrs*, and on the basis of the mutations seen, the respective wild-type and mutant probes were designed for the fluoroquinolones and second-line aminoglycosides and used for standardizing the RLBH assay conditions.

**Evaluation of RLBH assay.** The initial 150 sequenced isolates were then used as known controls for the respective mutations. The latter 65 of the total 215 XDR *M. tuberculosis* isolates were screened only by the RLBH assay.

**Performance of the assay. (i) Rifampin resistance.** Of the total 215 XDR *M. tuberculosis* and 50 pansusceptible isolates screened by the RLBH assay, 1 isolate showed RIF resistance by MGIT (phenotypic assay) but did not show any mutation by the RLBH assay. The diagnostic sensitivity of the assay to detect RIF resistance was calculated to be 99%, with the specificity being 100%, as none of the 50 pansusceptible isolates

TABLE 3. Mutations seen in XDR *M. tuberculosis*-infected patients

Drug	Gene(s)	Mutation(s)	Amino acid change	No. of isolates
Isoniazid	<i>katG</i>	G315C	S315T	213
Isoniazid	<i>inhA</i> + <i>katG</i>	C-15T		44
Rifampin	<i>rpoB</i>	C531T	S531L	199
Rifampin	<i>rpoB</i>	A526G	H526Y	7
Rifampin	<i>rpoB</i>	G516T + A526G	D516T	2
Rifampin	<i>rpoB</i>	A516T + A526G	D516E	2
Rifampin	<i>rpoB</i>	A516T	D516E	2
Rifampin	<i>rpoB</i>	G516T	D516T	1
Fluoroquinolones				
Ofloxacin	<i>gyrA</i>	C90T + C95G	A90V	23
	<i>gyrA</i>	C90T	A90V	21
Ofloxacin and moxifloxacin	<i>gyrA</i>	T91C	S91P	2
Ofloxacin and moxifloxacin	<i>gyrA</i>	A94G + C95G	D94G	83
Ofloxacin and moxifloxacin	<i>gyrA</i>	A94G	D94G	18
Ofloxacin and moxifloxacin	<i>gyrA</i>	A94C + C95G	D94A	19
Ofloxacin and moxifloxacin	<i>gyrA</i>	A94C	D94A	12
Ofloxacin and moxifloxacin	<i>gyrA</i>	G94A + C95G	D94N	19
Ofloxacin and moxifloxacin	<i>gyrA</i>	G94A	D94N	8
Aminoglycosides				
Kanamycin and amikacin	<i>rrs</i>	A1401G		106
Kanamycin, amikacin, and capreomycin	<i>rrs</i>	A1401G		56
Kanamycin, amikacin, and capreomycin	<i>rrs</i>	G1484T		42

showed any mutation with RLBH. The kappa value was 0.99 (95% CI), indicating good agreement between RLBH and phenotypic susceptibility.

(ii) **Isoniazid resistance.** Of the total 215 XDR *M. tuberculosis* and 50 pansusceptible isolates, 1 isolate showed INH resistance by MGIT but did not bind with any mutant probes on the RLBH membrane. The diagnostic sensitivity of the assay to detect INH resistance was calculated to be 99%, with the specificity being 100%, as none of the 50 pansusceptible isolates showed any mutation with RLBH. The kappa value was 0.99 (95% CI), indicating good agreement between RLBH and phenotypic susceptibility.

(iii) **Fluoroquinolone resistance.** Of the total 215 XDR *M. tuberculosis* and 50 pansusceptible isolates, 10 isolates phenotypically resistant to fluoroquinolones did not reveal any mutation by the RLBH assay. These samples were sent for sequencing to check for the mutations in *gyrA* and *gyrB*. Sequencing did not show any mutations, indicating mutations outside the targeted region or other mechanisms of resistance, i.e., efflux mechanism. The diagnostic sensitivity of the RLBH assay to detect fluoroquinolone resistance was calculated to be 95.34%, with the specificity being 100%, as none of the 50 pansusceptible isolates showed any mutation with RLBH. The kappa value was 0.89 (95% CI), indicating good agreement between RLBH and phenotypic susceptibility.

(iv) **Second-line aminoglycosides (kanamycin and amikacin).** Of the 215 XDR-TB and 50 pansusceptible isolates, 11 isolates were phenotypically resistant to KAN and AM but did not reveal any mutation by the RLBH assay. These samples were sent for sequencing of the *rrs* region to check for the mutations. Sequencing did not show any mutations, indicating mutations outside the targeted region or other mechanisms of resistance. The diagnostic sensitivity for the detection of resistance to second-line aminoglycosides by the RLBH assay was 94.8%, with the specificity being 100%. The kappa value was

0.87 (95% CI), indicating good agreement between RLBH and phenotypic susceptibility.

The various mutations seen in the 215 XDR-TB isolates are listed in Table 3. Of all the XDR-TB isolates, 213/215 (99%) showed the *katG* S315T substitution correlating with isoniazid resistance. A total of 199/215 (92%) showed the S531L substitution in the *rpoB* gene. The mutations in *gyrA* were seen on the 90th, 91st, and 94th codons. The frequency of the D94G substitution was seen in 101/215 (46%) of XDR *M. tuberculosis* isolates. The other substitutions seen on the 94th codon were D94A and D94N, seen in 31/215 (14%) and 27/215 (12.5%) isolates, respectively. A total of 106/215 XDR *M. tuberculosis* isolates showed the A1401G mutation in the *rrs* region and were phenotypically resistant to KAN and AMK but susceptible to CM. A further 56/215 XDR-TB isolates with the same mutation showed phenotypic resistance to KAN, AMK, and CM. The G1484T mutation in *rrs* resulted in resistance to all 3 second-line injectables by the phenotypic method.

#### Applicability on direct smear-positive clinical specimens.

The results obtained on isolates were promising, but the assay was time-consuming; thus, the assay was tested on direct clinical specimens. Seventy-three specimens were tested. Of these, 8 proven by culture to be mycobacteria other than *M. tuberculosis* (MOTT) showed binding only with the mycobacterial complex probe and no binding with the *Mycobacterium tuberculosis* probe on the membrane, indicating atypical infection, which was in concordance with the results of the phenotypic MGIT method. There were 7 samples that showed inhibition with RLBH. The percent agreement for susceptibility testing between the phenotypic test and RLBH assay was calculated to be 93% for all the targeted drugs. With the application of the assay on direct clinical specimens, the turnaround time to results was reduced to 3 days (assay takes about 4 to 5 h, but times for DNA extraction and PCR amplification total the time

to results as 3 days), compared to 2 to 4 weeks in the case of the phenotypic liquid culture system.

The RLBH membrane was used 7 times without compromising the signal strength, but the strength of the signal was reduced from the 8th use, thereby causing difficulty with accurate reading of the results.

**Time to DST.** On average, the time to culture positivity for a smear 3+ positive is within 7 days (13), and thereafter, another 10 to 14 days is needed for DST results. The total time to DST for a smear 3+-positive specimen would therefore be 17 to 21 days. This is increased with smear 2+-positive specimens, wherein time to identification is 8 to 10 days, and thereafter, another 10 to 14 days is needed for DST, with the total time being 18 to 24 days. Similarly, for smear 1+-positive specimens, the time to identification is 11 days, and thereafter, 10 to 14 days is needed for DST, with the total time being 21 to 25 days. In comparison, the RLBH assay takes only 3 days from a direct smear 1+-positive specimen (including the DNA extraction, PCR amplification, and assay procedure).

## DISCUSSION

The present study is a retrospective study that shows the greater rapidity of the RLBH assay using a probe-based technique over the phenotypic methods, with sensitivities for detection of resistance to INH, RIF, FQs, and second-line aminoglycosides being 99%, 99%, 95.34%, and 94.8%, respectively. These performance characteristics are indicative of equivalence with conventional DST. In most developing countries, DST is usually performed on solid medium, such as Lowenstein-Jensen, wherein results are available after 8 to 18 weeks, during which time many patients with MDR-TB or XDR-TB may have died, transmitted their disease, or both. Conventional DST for second-line drug testing is far less standardized than DST for first-line drugs. The critical concentrations are not as clear-cut, often because the MIC values are close to the assumed critical concentrations. The RLBH assay detects the presence of *M. tuberculosis* isolates with patterns of resistance to INH, RIF, FQ, KAN, AM, and CM within a short span of 3 days directly from a smear-positive clinical specimen by detection of point mutations present in *rpoB*, *katG*, *inhA*, *rrs*, *gyrA*, and *gyrB*. This could help clinicians in initiating appropriate therapy and implement infection control measures to curb the spread of resistance. Compared with PCR-based sequencing and DNA microarray techniques, RLBH can be performed without expensive devices. Though mutations to the first-line drugs are well studied, among the Indian XDR *M. tuberculosis* isolates in a study conducted in Delhi (7), the key mutations observed were S531L in *rpoB* and S315T in *katG*, which are similar to our findings. A previous study from this center (14) done on MDR isolates revealed an array of point mutations from positions 511 to 531 in *rpoB*, which seems to have been selected out to be Ser531Leu, in the case of XDR *M. tuberculosis* isolates. Among rifampin-resistant isolates, our findings are in concordance with those of other studies (1) that are dominated by the Ser531Leu mutation among the XDR *M. tuberculosis* isolates. There are limited mutation studies for the second-line drugs. FQ resistance is correlated with mutations in the quinolone resistance-determining regions (QRDRs), predominantly in *gyrA* and in *gyrB*, and a 90% accuracy of

prediction of FQ resistance was achieved when mutations are detected at the 90th and 94th codons in this study, which are similar to the findings for Delhi (7) XDR *M. tuberculosis* isolates. Studies from outside of India also show mutations in the QRDR of *gyrA*, similar to the mutations observed among our XDR *M. tuberculosis* isolates (1). The resistance to second-line injectables can be predicted by the nucleotide changes in the 3' part of the 16S rRNA (*rrs*) gene. With this assay, 89% of the second-line aminoglycoside resistance could be detected. The key mutations were seen at A1401G and G1484T, similar to the results from characterization of XDR *M. tuberculosis* isolates from Delhi (7). This study was dominated by the A1401G and G1484T mutations in *rrs*, whereas other studies (1) have reported several other point mutations in the *rrs* gene.

Commercial assays MTBDRplus and MTBDRsl (Hain Life-sciences) (8) are strip based and use two strips per patient, which are priced at approximately \$49 (separate strips are needed for the drugs involved in the definition of MDR and FQs, plus injectables), excluding the equipment cost. In contrast, RLBH uses a single membrane to identify mutations for XDR-TB, and once it is prepared, the membrane can be stripped and reused at least 7 times without affecting the signal strength. Forty samples along with a positive control and a negative control can be tested simultaneously, and this reduces the cost of the assay, making it suitable for application in routine clinical laboratories in developing countries. Though the GeneXpert system (3), also evaluated at our center, is the new state-of-the-art technology, it can detect resistance only to rifampin, and the cost of the test at a reduced price for India is \$17, excluding the cost of the instrument, whereas our assay targets all the drugs involved in the XDR-TB definition. Thus, the present assay is an adequate tool to rapidly detect these point mutations that confer resistance to INH, RIF, FQ, KAN, AM, and CM. Besides being cost-effective, this assay enables initiation of early treatment, which could curb the spread of resistant strains, thus helping in control of tuberculosis.

The assay can detect only those mutations that are covered by the wild-type or mutant probe. The inability to detect novel mutations at locations other than the probe region or outside the amplified region would be a shortcoming, but the system is an open system, wherein newer probes can be added. Though molecular assays have a short turnaround times and are the state of the art, they cannot completely replace the conventional methods, as there are multiple genes involved in resistance and the molecular assays target only known mutations.

**Conclusion.** In this study, we have attempted to characterize the genetic mechanism of resistance in the XDR isolates, wherein we saw good concordance with the phenotypic resistance in the cases of isoniazid, rifampin, fluoroquinolones, and second-line aminoglycosides. As XDR *M. tuberculosis* is emerging as a highly threatening pathogen in a pervasive association with the AIDS epidemic, rapid tests to promptly identify resistance to first- and second-line antitubercular agents are urgently required. It has been observed that point mutations in *rpoB*, *inhA*, *katG*, *gyrA*, *gyrB*, and *rrs* can accurately predict resistance to these drugs. The RLBH assay is proposed to be an adequate tool for direct analysis of XDR *M. tuberculosis* from clinical specimens, thus fulfilling many attributes of an ideal TB diagnostic test, as proposed by the WHO for developing countries.

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There are no conflicts of interest for the study reported here.

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