INT J TUBERC LUNG DIS 7(7):652–659

Comparison of DNA sequencing, PCR-SSCP and PhaB assays with indirect sensitivity testing for detection of rifampicin resistance in *Mycobacterium tuberculosis*

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SUMMARY

SETTING: Tuberculosis Research Centre, Chennai, India.
OBJECTIVE: To rapidly identify multidrug-resistant *Mycobacterium tuberculosis* using phenotypic and genotypic methods.
DESIGN: Two genotypic assays, DNA sequencing and polymerase chain reaction single strand conformation polymorphism (PCR-SSCP), and one phenotypic assay, phage amplified biological assay (PhaB) were standardised in-house and performed on coded 101 rifampicin-resistant and 100 rifampicin-sensitive *M. tuberculosis* clinical isolates for the identification of rifampicin resistance.
RESULTS AND CONCLUSION: The results obtained using the three assays were compared with those from the conventional indirect sensitivity test. The sensitivities and specificities of DNA sequencing, PCR-SSCP and PhaB were 97% and 100%, 76% and 100%, and 97% and 84%, respectively. DNA sequencing was found to be more sensitive and specific than the other tests.
KEY WORDS: *Mycobacterium tuberculosis*; rifampicin resistance; DNA sequencing; PCR-SSCP; PhaB

ALTHOUGH it is a curable and controllable disease, tuberculosis (TB) is the only infectious disease for which the World Health Organization has declared a global emergency, in 1993.[1] Globally, it is estimated that between 2000 and 2020 nearly one billion people will be newly infected, 200 million people will get sick, and 35 million people will die from TB, if control measures are not augmented optimally.[1] The global spread of the disease is complicated by the ubiquitous appearance of drug-resistant strains, and particularly multidrug-resistant (MDR) strains defined as resistant to at least isoniazid (INH) and rifampicin (RMP).[2]

MDR-TB causes higher mortality in immunocompromised patients, who require treatment with more toxic second-line drugs and remain infectious for longer periods than those infected with drug-sensitive strains, incurring higher costs to the tuberculosis control programmes. Early detection of MDR-TB patients is therefore essential to initiate appropriate treatment. In most laboratories in developing countries, MDR-TB is confirmed by drug susceptibility testing with established bacteriological methods. However, 4 weeks are required for results to become available after obtaining the primary culture.

Various rapid phenotypic tests such as the Luciferase reporter phage assay[3] and the BACTEC MGIT 960 system,[4] and genotypic tests such as the Line probe assay[5] and real-time polymerase chain reaction (PCR),[6] have been used to detect RMP resistance of *Mycobacterium tuberculosis* by various workers.[7,8] In spite of this wide array of assays, to our knowledge no single study exists to date whereby all the assays that can be routinely performed in developing countries have been standardised in-house and performed on the same clinical *M. tuberculosis* isolates. Such a study would aid in correctly identifying the credibility of an assay when performed on the same sample under similar conditions in a routine mycobacteriology laboratory. To address this issue, the present study was undertaken, in which two rapid genotypic assays, DNA sequencing and PCR-single strand conformation polymorphism (PCR-SSCP) and one phenotypic assay, phage amplified biological assay (PhaB), were performed.

DNA sequencing is considered the gold standard among molecular techniques for the detection of mutations associated with drug resistance. Its advantages include rapidity, and precise determination of the location, type and nature of the mutations.

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Article submitted 11 November 2002. Final version accepted 17 January 2003.

Sanger's method of dideoxy sequencing uses dideoxy nucleotides to allow chain termination at each base; the sequence is defined by the length of the fragment and the base.[9] The genetic basis for RMP resistance in approximately 95% of cases is due to mutations in an 81-bp RMP resistance-determining region (RRDR) of the *rpoB* gene, corresponding to codons 507 to 533 (*Escherichia coli* numbering system).[10,11]

PCR-SSCP analysis, another molecular technique, has the advantages of apparent simplicity, visual interpretation of results, non-radioactive means of detection, rapidity and cost-effectiveness. SSCP is performed by amplifying wild type and mutant target DNA, denaturing and then electrophoresing side by side on a non-denaturing polyacrylamide gel. PCR-SSCP relies on the fact that single-stranded DNA in solution under certain conditions has a defined secondary structure. Under the appropriate conditions, the electrophoretic mobility of the single-stranded DNA is dependent not only on its length and molecular weight, but also on its overall conformation. This secondary structure is determined by the balance between destabilising thermal forces and weak local stabilising forces such as intra-strand base pairings and stackings, which are in turn determined by the primary sequence. Hence, even a single base alteration in the test sample results in a shift in migration of the test sample when compared to the wild type DNA, when run on a polyacrylamide gel.[12] PCR-SSCP can be set up easily and performed routinely.

PhaB, a phenotypic assay, is a low-cost, in-house technology that is rapid and requires no special instrumentation other than those used in a routine microbiological laboratory. In this phenotypic assay, mycobacteriophages are added to a decontaminated sputum sample/culture suspension to infect it rapidly. The sample is then treated with a virucidal solution to destroy all external phages that did not infect the host cells.[13] The phages are allowed to replicate and are then amplified by the introduction of a non-pathogenic rapid growing helper cell host (*M. smegmatis*). The progeny phage undergoing cycles of infection, replication and lysis, is seen as clear plaques in a lawn of helper cells. The number of plaques generated is directly proportional to the number of viable *M. tuberculosis* cells containing the phage. The absence of plaques in a drug-containing sample indicates that the strain is susceptible to the drug (i.e., the tubercle bacilli are no longer viable and cannot support phage replication). The presence of plaques in the drug-containing sample indicates that viable tubercle bacilli have survived (and can support phage replication), and that the strain is resistant to the antimycobacterial drug.[14,15] In this study, the above-mentioned three rapid susceptibility-testing methods were performed on selected *M. tuberculosis* clinical isolates and the results compared with those obtained with the conventional indirect sensitivity test.

MATERIALS AND METHODS

M. tuberculosis *isolates*

A total of 101 RMP-resistant (RMP^r) and 100 RMP-sensitive (RMP^s) *M. tuberculosis* clinical isolates were obtained from 201 TB patients. A laboratory reference strain of *M. tuberculosis*, $H_{37}Rv$, was used as the control. All the cultures were coded and subjected to DNA sequencing, PCR-SSCP analysis and PhaB assay to identify RMP resistance.

Indirect sensitivity test

All cultures were classified as resistant or sensitive by the conventional indirect sensitivity test using Löwenstein-Jensen (LJ) medium.[16] Briefly, the method involves preparing a 4 mg moist weight per ml culture suspension of the *M. tuberculosis* bacillary mass in a sterile bijou bottle containing 4–5 glass beads (2–3 mm). The bottle was shaken mechanically for 1 min to produce a uniform suspension. Using a 3 mm external diameter nichrome wire loop, a loopful of this suspension was inoculated onto drug-free and drug-containing LJ media (32, 64, and 128 mg of RMP per litre). All the slopes were incubated at 37°C and the minimum inhibitory concentration (MIC) was recorded at the end of 4 weeks. MIC was defined as the lowest concentration of the drug that inhibited the growth of *M. tuberculosis*. 'Growth' on an LJ slope was defined as the presence of 20 or more colonies. All cultures resistant to RMP had an MIC $\geqslant$128 mg/L, while sensitive cultures had an MIC $\leqslant$32 mg/L.

DNA sequencing

DNA extraction

One loopful of *M. tuberculosis* culture on LJ slope was inoculated into 10 ml of Middlebrook 7H9 medium (Difco, Detroit, MI) and incubated at 37°C for 4 weeks. DNA was extracted by the CTAB (hexadecyl trimethyl ammonium bromide)-NaCl method, as follows: the culture was heat inactivated at 68°C for 20 min and the cells were pelleted at 13 000 rpm for 2 min. The cells were resuspended in 500 μl of TE buffer (10 mM Tris-Hcl [pH 8.0] and 1 mM EDTA) to which 50 μl of 10 mg/ml lysozyme (Sigma, St Louis, MO) was added and incubated at 37°C for 1 h. After 1 h, 70 μl of 10% sodium dodecyl sulfate (SDS) and 6 μl of 10 mg/ml proteinase K (Sigma) were added and incubation continued for 10 min at 65°C. Following this, 100 μl of 5M NaCl was added and thoroughly mixed; 80 μl of CTAB-NaCl was added and the tubes were thoroughly mixed and incubated at 65°C for 10 min. To this mixture, 0.8 ml of chloroform: isoamyl alcohol (24:1) was added and the tubes were thoroughly mixed and spun for 5 min at 13 000 rpm. The clear aqueous phase was transferred to a fresh tube, 0.6 volumes of isopropanol was added and the tubes were left for 30 min at room temperature. The tubes were then centrifuged at 13 000 rpm

for 2 min and the supernatant was discarded. The DNA pellet was washed thoroughly in 1 ml 70% ethanol to remove traces of salts. The pellet was air-dried and dissolved in 50 μl Tris-EDTA.

PCR

PCR was performed using primers rpo3 (5′ CAGA CGTTGATCAACATCCG 3′) and rpo4 (5′ TACGG CGTTTCGATGAAC 3′) to generate a 305-bp product containing the 81-bp rifampicin resistance determining region (RRDR).[17] PCR was performed on an MJ Research Minicycler under the following conditions: denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min. The steps were repeated 30 times, followed by a final extension step at 72°C for 10 min.

Sequencing protocol

Sequencing was performed on an automated sequencer (ABI Prism model 310, Applied Biosystems, Foster City, CA) using rpo3 and rpo4 primers using the procedure described by Musser et al. with slight modification,[18] and the Bigdye terminator sequencing kit (Applied Biosystems). To 4 μl of the Terminator ready reaction mix, 1 μl of the amplified fragment (2–3 ng) and 1 μl of the primer (10 pmol/μl) were added and the volume was made up to 20 μl using deionised water. The reaction mixture was mixed well and spun briefly. The tubes were placed in a thermal cycler and heated to 95°C for 5 min. The cycle sequencing conditions were as follows: 95°C for 30 sec, 55°C for 20 sec and 60°C for 4 min. These steps were repeated for 45 cycles, followed by a rapid thermal ramp to 4°C. To this reaction mix, 2 μl of 3 M sodium acetate (pH 4.6) and 50 μl of 95% ethanol were added and mixed thoroughly. The tubes were vortexed and left at room temperature for 15 min to precipitate the extension products. The tubes were then spun for 20 min at 13 000 rpm and the supernatant was discarded. The pellet was rinsed with 250 μl of 70% ethanol and spun at 13 000 rpm for 5 min. The supernatant was again discarded. The pellet was air-dried in a vacuum centrifuge for 10 min and resuspended in 20 μl of template suppression reagent (supplied in the kit). The samples were vortexed and spun, then heated at 95°C for 2 min and immediately chilled on ice. They were vortexed and spun again and placed on ice, to load onto the ABI Prism 310 Genetic Analyzer. The data obtained were compared with the sequence obtained from the database at the Sanger Centre using the BLAST programme.[19]

PCR-SSCP

A 500 μl Middlebrook 7H9 culture suspension of *M. tuberculosis* (equivalent to 4 McFarland units) was boiled at 95°C for 20 min in a microcentrifuge tube. The tubes were then centrifuged at 13 000 rpm (Eppendorf model 5417R) for 5 min at 4°C. The supernatant was used as template DNA (around 20 ng) to perform PCR using the same conditions as described for DNA sequencing. The primers used were TR9 (5′ TCGCCGCGATCAAGGAGT 3′) and TR8 (5′ TGC ACGTCGCGGACCTCCA 3′) to obtain a 157-bp product that spans the 81-bp RRDR of the *rpoB* gene.[20] Following PCR, 3 μl of the amplified PCR fragment was denatured at 95°C for 5 min in a microcentrifuge tube in the presence of an equal amount of stop buffer (2 mM EDTA, 95% formamide and 0.05% bromophenol blue). The reaction mix was snap-cooled on ice and loaded immediately on 10% polyacrylamide gel. Electrophoresis was performed in a vertical slab gel apparatus (Bangalore Genei, Bangalore, Karnataka, India) according to the manufacturer's protocol, with 1X Tris-Borate-EDTA as the running buffer. The electrophoresis was standardised to run for 17 h at 60 V at 4°C. DNA bands in the gel were visualised by silver staining, as described by Ainsworth et al.[21] Briefly, the gel was first treated with 10% ethanol for 5 min followed by 1% nitric acid for 3 min. The gel was then stained with 0.2% silver nitrate solution containing 1 ml 10% formalin for 20 min. The gel was washed thrice with distilled water for 3 min each before exposure to the developing stain (3% sodium carbonate solution containing 0.5 ml 10% formalin). The reaction was arrested by adding 100 ml 10% glacial acetic acid. A shift in mobility of either of the DNA strands of the test sample compared to the corresponding strands from the RMP^s reference control (H_37Rv) was considered indicative of RMP resistance.

PhaB assay

Phage maintenance and propagation

Mycobacteriophage D29 was propagated and maintained in mycobacteriophage buffer.

Assay protocol

Twenty-five microliters of the culture suspension was incubated in the presence of RMP (4 mg/l) for 48 h in 1 ml Middlebrook 7H9 medium containing 5% glycerol (G-7H9). Culture suspension incubated for the same period in the absence of RMP served as control. Mycobacteriophage D29 (100 μl) was used to infect the culture suspension for 1 h at 37°C; 100 μl 10 mM ferrous ammonium sulphate (virucide) was added to kill any external phages that had not infected the tubercle bacilli. After 5 min of incubation at room temperature, 5 ml of G-7H9 medium, immediately followed by 1 ml of the helper cell suspension (*M. smegmatis* suspension equivalent to 4 McFarland units), were added to the reaction mixture, which was then poured on to a petri plate containing 3 ml of molten 1.6% agar and mixed thoroughly. After 30 h incubation at 37°C, the number of plaques generated on the plates containing the drug medium was

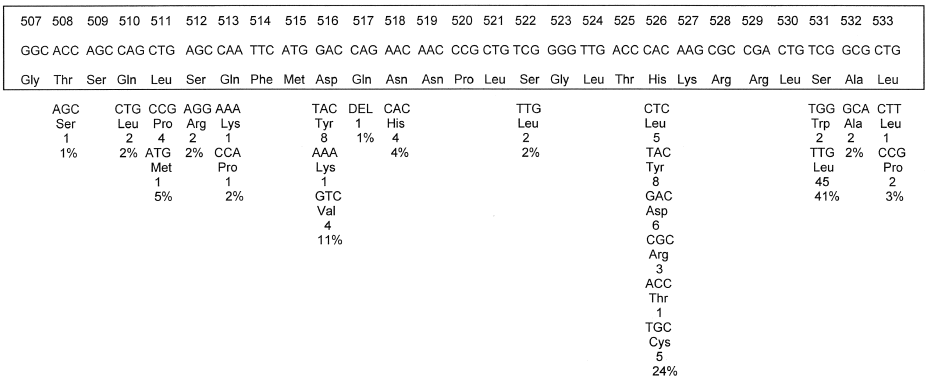

Figure 1 Mutations in the RRDR of the *rpoB* gene of *M. tuberculosis* Indian isolates. Bottom panel shows the mutated codons with corresponding amino acids. The original sequence is shown in the box. Numbers below the amino acids indicate number of isolates showing the mutation, while percentages denote the frequency of occurrence of mutations at the particular codon. RRDR = rifampicin resistance-determining region.

counted and compared with those obtained in the drug-free medium. Plaques on test plates were counted only if the control plates had a minimum of 100 plaques. Negative control with no *M. tuberculosis* cell suspension was used for every batch.

Interpretation of results

In DNA sequencing, an isolate with mutation in the RRDR was considered resistant, while an isolate with no mutation in the RRDR was considered sensitive. In PCR-SSCP, a test sample with a shift in mobility in either of the two DNA strands when compared to that of the wild type control strain $H_{37}Rv$ was considered resistant. Alternatively, if there was no difference in the migration of the strands between the test sample and the control, the isolate was considered sensitive. In PhaB, an isolate was considered resistant to RMP when the percentage of plaques in the drug-containing cultures was at least 1% of the plaques produced in the drug-free cultures, and sensitive if the percentage was less than 1%.

Statistical analysis

Epi Info version 6.0 (Centers for Disease Control and Prevention, Atlanta, GA) was used to obtain sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and kappa values. The agreement was arrived at by adding the number of true positives and true negatives and dividing them by the total number of isolates.

RESULTS

DNA sequencing

DNA sequencing was performed on a 305-bp amplified fragment of *rpoB* that spans the RRDR. Among the 101 RMP^r *M. tuberculosis* isolates, 98 showed mutations. These included 23 substitutions and one deletion (Figure 1). Two isolates had quadruple mutations, two had triple mutations, and 11 had a double mutation each, while the rest had single mutations. Two isolates had mutations just outside the RRDR, one at codon 534 (GGG to GAG) and the other at codon 535 (CCC to CAC). Three RMP^r *M. tuberculosis* isolates contained no mutation in the RRDR. No mutation was detected within or without the 81-bp RRDR of 100 RMP^s *M. tuberculosis* isolates.

The results obtained by DNA sequencing were compared with those from the conventional indirect sensitivity test (Table 1). The sensitivity of DNA sequencing was 97%. None of the sensitive isolates tested had any mutation in the RRDR thus giving a specificity of 100%. The kappa value was 0.97. DNA sequencing results were available within 24 hours after the isolation of the primary culture. The PPV and NPV of the assay were respectively 100% and 97% (Table 2).

PCR-SSCP

SSCP profiles were obtained for all 101 RMP^r and 100 RMP^s isolates. DNA strands from 77 resistant

Table 1 Concordance of DNA sequencing, PCR-SSCP and PhaB assays with results obtained by indirect sensitivity test for testing susceptibility of *M. tuberculosis* isolates to RMP

	Indirect sensitivity result		
Method and result	Susceptible	Resistant	% agreement
DNA sequencing			
Susceptible	100	3	
Resistant	0	98	99
PCR-SSCP			
Susceptible	100	24	
Resistant	0	77	88
PhaB			
Susceptible	84	3	
Resistant	16	98	91

PCR-SSCP = polymerase chain reaction single strand conformation polymorphism; RMP = rifampicin.

Table 2 Performance of DNA sequencing, PCR-SSCP and PhaB assay compared to indirect sensitivity test

Assay	Sensitivity %	Specificity %	PPV %	NPV %	Kappa	Turnaround time (hours)*
DNA sequencing	97	100	100	97	0.97	24
PCR-SSCP analysis	76	100	100	81	0.76	24
PhaB assay	97	84	86	97	0.81	78

* Time required after isolation of primary culture.
PCR-SSCP = polymerase chain reaction single strand conformation polymorphism; PPV = positive predictive value; NPV = negative predictive value.

isolates had altered mobility compared to the DNA strands from the reference strain, $H_{37}Rv$. All the 100 RMP^s isolates tested showed SSCP profiles similar to the reference strain. The results obtained by PCR-SSCP were compared with the conventional indirect sensitivity test (Table 1). The sensitivity of the assay was 76% while the specificity was 100% with a kappa value of 0.76. The PPV of the assay was 100%, and the NPV was 81%. PCR-SSCP results were available within 24 h after the isolation of the primary culture (Table 2). Figure 2 shows a representative PCR-SSCP profile.

PhaB assay

The results obtained by PhaB assay were compared with the conventional indirect sensitivity test (Table 1). The sensitivity of the assay was 97% and the specificity was 84%, with a kappa value of 0.81; the PPV was 86% and the NPV was 97% (Table 2).

DISCUSSION

The primary theme emerging from molecular genetics of *M. tuberculosis* and several other mycobacterial species is that resistance is commonly associated with simple nucleotide alterations in target chromosomal genes rather than with the acquisition of plasmids or transposons. The efficiency of the genotypic methods largely depends on the correct identification of the mutations associated with resistance. In the present study, 98 RMP^r *M. tuberculosis* isolates were identified correctly by the detection of mutations in *rpoB* gene.

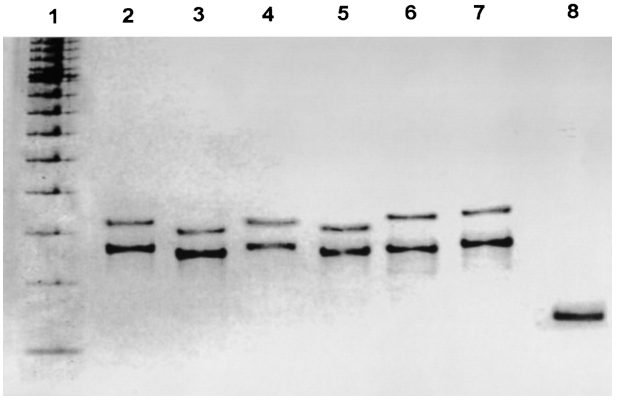

Figure 2 Gel photograph showing the SSCP pattern of *M. tuberculosis* isolates. Lane 1: 100 bp DNA ladder; Lane 2 : $H_{37}Rv$ control strain; Lane 3, 5–7: Resistant strains; Lane 4: Sensitive strain; Lane 8: Control double stranded DNA. SSCP = single strand conformation polymorphism.

Schilke et al. analysed mutations in the *rpoB* gene of *M. tuberculosis* isolates from Africa, and concluded that the pattern of mutations in them conforms to the reported pattern elsewhere for isolates from other parts of the world.[22,23] Although the mutations found in our study do conform to the pattern reported from other countries, the presence of new mutations and different relative frequencies of the occurrence of mutations indicate that the nature and frequency of these mutations vary considerably according to geographical location. This might reflect the dynamism of the organism in its ability to adapt to exposure to the drug.

In our study, three of 101 RMP^r isolates (3.03%) showed no mutation in the RRDR. Kapur et al.[10] and Hirano et al.[24] also found no mutations in the RRDR in respectively 2.5% and 5.6% of *M. tuberculosis* isolates. Resistance in these isolates could be attributed either to the presence of mutations elsewhere or to the existence of an alternative resistance mechanism to RMP in *M. tuberculosis*.

In our study, the mutations were most commonly seen in codons 531 (41%), 526 (24%), and 516 (11%). Among the different mutations seen at codon 531, the mutation of TCG (Ser) to TTG (Leu) occurred at a very high frequency (39%). Matsiota-Bernard et al.[25] and Pozzi et al.[26] also observed higher frequencies of this particular mutation of respectively 56% and 59%. Although the mutation at codon 526 (CAC to GAC) occurred at higher frequency in Italian isolates (30%)[26] and in Greek isolates (19%),[25] our study showed a low frequency (5%). However, CAC to TAC occurred at a higher frequency (7%) than CAC to GAC at codon 526. Pozzi et al.[26] compiled the results of various workers and reported that the frequency of these particular mutations ranges from 20% to 71% at codon 531 (TCG to TTG) and 0% to

30% at codon 526 (CAC to GAC). The frequencies obtained in our study fall into the above-mentioned range, conforming to the global pattern. In this study, one new mutation was observed within the RRDR, CAG (Gln) to CTG (Leu) at codon 510 (EMBL accession no. AJ457091).

Bodmer et al. found a strong correlation between the MICs of RMP and related rifamycins with certain *rpoB* variants.[27] Amino acid substitutions located at position 526 and 531 conferred high-level of resistance to RMP, rifabutin and rifapentine. No such correlation between MICs of RMP and *rpoB* mutations was seen in this study; all of our mutants, irrespective of the position of the mutation, had high MICs. Even a silent mutation at codon 532 had a high MIC (>128 mg/l). No explanation can be given for this.

PCR-SSCP was used to detect point mutations in the *rpoB* gene of *M. tuberculosis* by other workers, whereby the sensitivities and specificities ranged from 60% to 100%.[20,28] Similar results were obtained in the present study, with a sensitivity of 76% and a specificity of 100%. The PPV of the assay was 100%, indicating that any shift in migration in the test DNA can be correctly considered as resistant, but an absence of shift in migration does not necessarily mean a sensitive strain. About 50% of the resistant isolates that were misclassified as sensitive by PCR-SSCP showed a mutation at codon 531 (TCG to TTG). The rest of these isolates showed mutations at codon 526 (20%), 533 and 516 (10% each) and 522 and 513 (5% each).

Eltringham et al. performed PhaB to detect RMP resistance in *M. tuberculosis* and obtained 100% sensitivity and specificity (31/31 resistant and 46/46 sensitive).[7] Similarly, Wilson et al., using nine RMP^r (including eight MDR) and 37 RMP^s isolates, obtained 100% sensitivity and 94.6% specificity.[15] The higher specificity in his study could be attributed in part to selection of few resistant cultures. Albert et al. evaluated FASTPlaque TB-RIF™, a commercial kit, in two different laboratories using 81 RMP^r strains and 110 RMP^s strains.[29] The sensitivity, specificity, and overall accuracy for the test were 100%, 97%, and 98% at Laboratory 1, while at Laboratory 2 they were 100%, 94%, and 97%, respectively. It should be mentioned that the assay was performed using commercially available kits. Although all the above authors reported high sensitivity and specificity, the assay should be repeated in different laboratories to document the consistency of the results.

In the present study, a higher concentration of RMP, 4 μg/ml, was used in the PhaB assay because of the relatively short exposure to the drug and also to obtain an optimal break point in defining the resistance criterion. A similarly high drug concentration (5 μg/ml) was used by Albert et al. to evaluate FASTPlaqueTB-RIF™ for *M. tuberculosis*.[29] The lower specificity obtained in this study could be due to the use of media and culture conditions in which the phage was propagated. The other reason for lower specificity could be attributed to the repeated subcultures of the isolates, which might not have been an accurate representation of the actual population. To avoid false-resistant results, fresh virucide was prepared and used in the PhaB assay. Negative control was used in every batch wherein no plaque was detected. The details of the isolates that gave discrepant results are shown in Table 3.

In the present study, DNA sequencing was found to be highly sensitive and specific, and less time-consuming. No other single method holds more promise than sequencing as it has a turnaround time of just 24 hours after the isolation of primary culture.

Table 3 Details of discrepant results in PhaB assay

Specimen number	Classification by indirect sensitivity test	a Plaques Control	b Rifampicin 4 μg/ml	% of b/a	Classification by PhaB assay
48	S	94	17	18	R
49	S	296	183	62	R
54	S	175	39	22	R
57	S	187	28	15	R
58	S	245	96	39	R
60	S	164	83	50	R
62	S	132	38	29	R
63	R	127	1	0.8	S
69	S	201	200	99.5	R
81	S	98	30	30.6	R
86	S	269	17	6.3	R
94	S	273	97	35.5	R
95	S	103	32	31	R
102	S	299	234	78	R
108	S	266	204	76.6	R
139	R	297	2	0.67	S
188	S	309	278	90	R
192	R	288	0	0.35	S
195	S	105	87	83	R

S = sensitive; R = resistant.

Although PCR-SSCP also requires only 24 hours, it is less sensitive than DNA sequencing. It requires around 15% of the total cell population to be resistant to the drug for the assay to be more sensitive.[30] In addition, it may not be able to distinguish silent mutations. The limitations of DNA sequencing are that it requires technical expertise and an automated DNA sequencer; and that the efficiency of the assay depends on the frequency of accessible resistance-associated genomic mutations. Phenotypic assays have inherent advantages with an easy-to-adopt in-house technology, even in developing countries. The PhaB assay can be performed in mycobacteriology laboratories with minimum infrastructure. The sensitivity to RMP of an isolate can be obtained within 78 hours using the PhaB assay. However, the specificity of the PhaB assay was low (84%). The three methods evaluated in the present study have already been explored. However, we evaluated these assays using more isolates (101 RMP^r and 100 RMP^s) than other studies. It is possible to identify RMP resistance by DNA sequencing in clinical specimens with low numbers of organisms. The other methodology that appears promising in our study is the PhaB assay, which is relatively safe, as during the course of the assay a large percentage of bacilli are rendered non-infective through lysis by bacteriophage. It is to be noted that Wilson et al. opined that in many rapid culture techniques the numbers of infective particles increase substantially.[15]

An inherent disadvantage of all the above assays is that they are all performed using the primary isolates, which require 2–8 weeks for isolation. With the identification of almost all complements of mutations associated with resistance and a deep insight into the resistance mechanism of RMP, rapid molecular methods have great potential to be used on a routine basis for drug susceptibility testing, especially in developing countries. Newer technologies, especially those that can be performed directly on the specimens themselves, need to be explored to rapidly identify RMP resistance in *M. tuberculosis*. The findings of this study suggest that DNA sequencing is more sensitive and specific than other tests.

Acknowledgements

We are grateful to Mrs Nalini Sunder Mohan and staff, Bacteriology Department, for their excellent technical help in the laboratory, and to Dr P Venkatesan for statistical analysis.

This study was funded in part by the World Health Organization through SEARO, New Delhi, India. CM received financial support from the Council of Scientific and Industrial Research (CSIR), Government of India.

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RÉSUMÉ

CADRE : Centre de Recherche de la Tuberculose à Chennai, en Inde.
OBJECTIF : Identifier rapidement *Mycobacterium tuberculosis* multirésistant par l'utilisation de méthodes phénotypiques et génotypiques.
SCHÉMA : Deux techniques génotypiques, le séquençage de l'ADN et l'étude du polymorphisme de conformation d'une PCR à brin unique (PCR-SSCP), ainsi qu'une technique phénotypique, le test biologique d'amplification des phages (PhaB), ont été standardisées sur place et réalisées sur 101 isolats cliniques de *M. tuberculosis* classés comme résistants à la rifampicine et sur 100 isolats classés comme sensibles à la rifampicine en vue de l'identification de la résistance à la rifampicine.
RÉSULTATS ET CONCLUSION : Les résultats obtenus par les trois techniques mentionnées plus haut ont été comparés avec ceux du test indirect conventionnel de sensibilité. Les sensibilités et spécificités du séquençage de l'ADN, de la PCR-SSCP et du PhaB ont été respectivement de 97% et 100%, de 76% et 100% et de 97% et 84%. Le séquençage de l'ADN s'avère plus sensible et plus spécifique par comparaison avec les autres tests.

RESUMEN

MARCO DE REFERENCIA : Centro de investigaciones en Tuberculosis, Chennai, India.
OBJETIVO : Identificar rápidamente *Mycobacterium tuberculosis* mutirresistentes a los medicamentos utilizando métodos fenotípicos y genotípicos.
DISEÑO : Dos técnicas genotípicas, secuenciación de ADN y polymerase chain reaction single strand conformation polymorphism (PCR–SSCP) y una técnica fenotípica, test biológico de amplificación de fagos (PhaB), fueron estandarizadas en el mismo centro y aplicadas en 101 aislados clínicos de *Mycobacterium tuberculosis* codificados como resistentes a la rifampicina y en 100 otros sensibles a la rifampicina, a fin de identificar la resistencia a la rifampicina.
RESULTADOS Y CONCLUSIÓN : Los resultados obtenidos por las tres técnicas mencionadas fueron comparados con los del test convencional indirecto de sensibilidad. La sensibilidad y la especificidad fueron, respectivamente, de 97% y 100% para la secuenciación de ADN, de 76% y 100% para PCR-SSCP y de 97% y 84% para PhaB. Comparado con los otros tests, la secuenciación de ADN se mostró como el test más sensible y el más específico.