CHAPTER III

Study on drug resistance of MDR Mycobacterium tuberculosis strain by phage Technique on direct clinical samples.
INTRODUCTION

The World Health Organization (WHO) estimated that there were 9 million new cases of tuberculosis (TB) worldwide in 2014 [WHO report 2014]. The countries with the highest prevalence were India (2.6 million cases), China (1.3 million), Indonesia (680 000), Nigeria (570 000), and South Africa (380 000) [WHO report 2014]. The emergence of multidrug-resistant (MDR)-TB, and more recently, extensively drug-resistant (XDR)-TB, is a major threat to global TB control [Mattelli A et al., 2007; Migliori GB et al., 2007; Shah N et al., 2007; Zignol M et al., 2006].

MDR-TB, caused by strains resistant to at least isoniazid and rifampin, is an alarming problem for the successful management of global TB control programs, since it increases the cost of treatment and the transmission risk, and lowers the cure rate [Yew WW et al., 1995]. India is the number one country in terms of TB prevalence, and the prevalence rate of MDR-TB in the country is around 2.2% (21,000 in numbers) among new cases, 15% (43,000 in numbers) among pretreatment cases, [WHO report 2014] and an alarming 17% among patients who have undergone previous treatment and who have developed multidrug resistance. Three countries, namely China, India, and the Russian Federation were found to account for 62% of the estimated global burden of MDR-TB cases [Zignol M et al., 2006]. MDR-TB has been reported from almost all parts of the world, primarily as a consequence of poor and irregular treatment services, resulting in increased treatment costs and also an increased risk of transmission of these resistant strains of the bacilli [WHO report 2014]. Also, the cure rate for MDR-TB is lower.

The early detection of MDR-TB could help in the effective management of cases and in the overall TB control program. However, current rapid phenotypic methods for assessing drug
susceptibility of \textit{M. tuberculosis}, such as BACTEC, take 2–6 weeks after laboratory isolation of the organisms. Rapid methods based on PCR are considered an alternative approach for the detection of multidrug resistance, but presently there is confidence in the detection of rifampin resistance alone through the rpoB gene mutation, \cite{Garcia2001, Hillemann2006, Hillemann2007, Telenti1993, Watterson1998, Williams1998} variations in resistance genes in isoniazid are problematic. Further, the expensive set-up and a lack of expertise can be a hurdle for those developing countries with a high burden of TB.

Mycobacteriophages are used as diagnostic markers to improve and expedite the detection of viable mycobacteria in clinical samples, as well as for the determination of drug resistance from the primary growth. Commercial kits based on phage technology are now available for the detection of mycobacteria in clinical samples \cite{Albert2002, Alcaide2003, Mohamed2003, Muzaffar2002, Park2003, Trollip2001, Barman2007, Biswas2008, Prakash2009}. The phage-based assays depend on the ability of resistant mycobacteria to support phage replication after being exposed to drugs, while sensitive bacilli become inactivated and hence are not able to support phage replication. Extracellular phages are inactivated with a phagicidal agent, whereas intracellular phages are protected and replicate, causing their lysis and the release of a new phage progeny detected by the production of plaques on a fast-growing \textit{Mycobacterium smegmatis} (indicator strain) lawn.

There are a few reports on phage-based assays carried out on direct decontaminated sputum samples for the detection of rifampin resistance, \cite{Albert2004, Butt2004, Mogahid2014} with fewer for isoniazid resistance. The detection of multidrug resistance requires the determination of resistance to both isoniazid and rifampin. Further, phage-
based assays for streptomycin, ethambutol, and fluoroquinolones have not been reported for direct sputum samples.

The purpose of this study was to evaluate an in-house and standardized phage amplification method using direct decontaminated sputum samples for the determination of rifampin, isoniazid, ethambutol, streptomycin, and ciprofloxacin drug resistance, and to compare the results to those acquired using the gold standard proportion method.
### Literature Survey

**Major studies using Phage Assay for detection of drug resistance in* Mycobacterium tuberculosis* on clinical samples.**

<table>
<thead>
<tr>
<th>Journal</th>
<th>Year</th>
<th>Authors</th>
<th>Details about method</th>
<th>Result</th>
<th>Phage assay performed by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Int J Tuberc Lung Dis.</td>
<td>2002</td>
<td>Albert H et al.,</td>
<td>To determine the ability of FASTPlaqueTB-RIF™, a rapid bacteriophage-based test, to correctly identify rifampicin susceptibility in clinical strains of <em>Mycobacterium tuberculosis</em> after growth in the Bactec 460 semi-automated liquid culture system.</td>
<td>Rifampicin susceptibility results were available for 133 strains of <em>M. tuberculosis</em>. Using the Bactec 460 method, 42 of these strains were rifampicin-resistant and 91 strains were rifampicin-susceptible. Sensitivity, specificity and overall accuracy for the FASTPlaqueTB-RIF™ were respectively 100%, 98.8% and 99.2% for detection of rifampicin resistance; 95.3% (41/43) of the rifampicin-resistant strains were also resistant to isoniazid (multidrug-resistant).</td>
<td>FASTPlaque TB-RIF™</td>
</tr>
<tr>
<td>JPMA</td>
<td>2004</td>
<td>Butt T et al.,</td>
<td>A comparative study of 40 sputum specimens from patients of pulmonary tuberculosis, using FASTPlaqueTB-RIF™ and Bactec 460 TB system, 28 isolates were resistant to RIF and 12 isolates were susceptible.</td>
<td>Of the 40 clinical isolates of <em>Mycobacterium tuberculosis</em> tested for rifampicin (RIF) susceptibility using the Bactec 460 TB system, 28 isolates were resistant to RIF and 12 isolates were susceptible.</td>
<td>FASTPlaque TB-RIF™</td>
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</tbody>
</table>
The Bactec 460 TB system identified 24 specimens as resistant to RIF. Three specimens that revealed susceptible isolates on Bactec 460, were resistant by FASTPlaqueTB-RIF™ while four specimens which revealed resistant isolates on Bactec 460, demonstrated susceptibility to RIF by FASTPlaqueTB-RIF™. The sensitivity and specificity of FASTPlaqueTB-RIF™ were 86% and 73% respectively. The predictive values of positive and negative tests were 0.89 and 0.67 respectively. The overall accuracy of the technique was 82%. The phage assay took 48 hours to perform.

**IJMM 2002**

Krishnamurthy A et al.,

85 samples for rifampicin resistance using a novel mycobacteriophage based test (Phage assay) and radiometric BACTEC 460 TB. Of the 85 samples, 70 (82.35%) were resistant and 12 (14.10%) were sensitive by both methods. Sensitivity and specificity of 100% and 80% respectively. A good correlation was observed with conventional LJ proportion method.

**Int J Tuberc Lung Dis. 2001**

Albert H. et al.,

A comparative study of FASTPlaqueTB-RIF™ and conventional drug Rifampicin susceptibility results were available for 191 strains of *M. tuberculosis*. Eighty-one strains were found to be rifampicin resistant and
<table>
<thead>
<tr>
<th>Journal</th>
<th>Year</th>
<th>Authors</th>
<th>Study Details</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Int J Tuberc Lung Dis.</td>
<td>2004</td>
<td>Albert H et al.</td>
<td>A comparative study of the performance of the FASTPlaque (phage amplification) technology to determine RMP resistance directly from smear-positive sputum compared with isolation and the conventional indirect Middlebrook 7H11 agar proportion method.</td>
<td>The FASTPlaque direct RMP test achieved sensitivity, specificity and overall accuracy of 100% (11/11), 100% (134/134) and 100% (145/145), respectively, compared with the conventional indirect susceptibility test method (resolved data). The FASTPlaque direct RMP test reported results within 2 days from receipt of the specimen, while the conventional method took between 27 and 103 days (mean +/- SD 33.2 +/- 7.2 days).</td>
</tr>
<tr>
<td>Am Clin Lab.</td>
<td>2001</td>
<td>Trolip A et al.</td>
<td>A comparative study of the FAST PlaqueTB-MDR assay and conventional drug susceptibility method was performed.</td>
<td>Laboratory 1 sensitivity 1.00 (32/32) specificity 0.97 (60/62) overall accuracy 98% (92/94)</td>
</tr>
</tbody>
</table>

110 strains were rifampicin susceptible by conventional methods. The sensitivity, specificity and overall accuracy for the FASTPlaqueTB-RIF™ were 100%, 97% and 98% at Laboratory 1, and 100%, 94% and 97% at Laboratory 2.
Material method

Population:

Three hundred and seventy cases of acid-fast bacillus (AFB) smear-positive pulmonary TB were referred to the Microbiology Laboratory of Choithram Hospital and Research Centre (CHRC), Indore, India during the period January 2008 to August 2013. Of these cases, 62% were on anti-TB treatment and 40% had received irregular treatment for more than 2 years and showed an inadequate therapeutic response. The proportion of males was 65% and cases were in the age range of 18–65 years. The study was approved by the Research and Ethical Committee of CHRC, Indore, India. The Ethical Committee has guidelines based on the Declaration of Helsinki.

Samples:

Sputum samples were collected in wide-mouth sterile containers.

AFB smear by Auramine O fluorescent Method: AFB smear by auramine O fluorescent method was carried out on the samples (Koneman EW et al., 1997). Prior to concentration, sputum samples were checked for mycobacteria, and all 370 smear-positive samples were included in the study. The density of AFB in the smear was recorded as per WHO guidelines. Of the sputum samples included in the study, 52 were recorded as 1–3 AFB/100 high-power fields, 122 samples as 1+, 113 samples as 2+, and 83 samples as 3+.

AFB Smear: AFB smears were stained by Auramine “O” Stain and were seen under fluorescent microscope. The samples were spread evenly to cover two-third of central portion of slide & the slides were exposed to U.V light for 15-20 minutes to inactivate viable pathogens. Then the slide was heat fixed & covered with Auramine O stain for 15 minutes. The slide was than washed with
tap water & the smear was covered with decolorizing agent for 3 minutes. The slides were washed with tap water and the smears were covered with KMnO₄ for 30 seconds. The slide were washed with tap water & allowed to dry and seen under fluorescent microscope.

**Concentration Method:** The N-acetyl-L-cysteine–alkali digestion method, as described by Kubica *et al.*, [1963] was used for the concentration of sputum samples. After concentration, 5 units/ml polymyxin B (Samarth Pharmaceutical Ltd, India) and 2 mg/ml vancomycin (Sigma, USA) were added to prevent bacterial contamination.

**AFB Culture:**

The sputum samples were digested, decontaminated & concentrated by standard procedure of NaOH - NALC method [Kubica *et al.*, 1963], which is also recommended by Centers of Disease control (CDC).

The Acetylcysteine – alkali digestant was prepared by combining 50ml of 2.94% Trisodium citrate-3H2O (0.1M) with 50ml of 4% NaOH in a the sterile plastic container. To this solution 0.5g of powered N-acetyl-L-cystine (NALC) was added just before use (NaOH seves as decontaminating agent. The NALC is a mucolytic agent without bactericidal activity that liquefies mucus by splitting disulfide bonds).

1. The NaOH – NALC – Sodium citrate solution was added to specimen in a equal volume.

2. Then Homogenization of the mixture was carried out and left for 15-20 minutes (Vortex lightly or hand mix for 5-10 minutes during homogenization). Proper attention should be paid so that this digestion time does not exceed 20 minutes.
3. At the end of 15-20 minutes phosphate buffer (pH 6.8) was added up to the mark on the ring (40ml) on the centrifuge tube & then it was mixed well (Vortex lightly or hand mix).

4. Then the specimen was centrifuged at a speed of 3000 rpm for 15-20 minutes.

5. After centrifugation the tube was allow to sit for 5 min to allow aerosal to settle & then the supernatant was decanted carefully. Make sure the sediment is not lost during decanting of supernatant fluid.

6) Small quantity of phosphate buffer (pH 6.8, 1-2ml) was added and then it was vortexed for 5 minutes.

7) The resuspended pellet was used for making smear and for culture inoculation.

Identification of Mycobacteria:

The following tests were used in combination to identify *M. tuberculosis* strains and other mycobacteria (MOTT).

1) Susceptibility to p-nitrobenzoic acid (PNB): 0.5g PNB was dissolved in DMF (dimethyl formamide) and added to 1 liter LJ medium fluid during LJ preparation. The specimen was inoculated on two LJ media first one with LJ + PNB & second one with only plain LJ as control and incubated at 37°C. *M.tuberculosis* does not grow on PNB containing LJ media & other mycobacteria grow on PNB containing LJ media.

2) Niacin Test: *M.tuberculosis* accumulates largest amount of nicotinic acid and its detection is useful for definitive diagnosis of *M.tuberculosis*. One ml of autoclaved distilled water was added to culture bottle & it was left in contact for 15 minutes. Then the niacin with distilled water was poured into screw capped test tube. Then equal volume of Aniline (4%) & Cynogen bromide
(10%) was added (processed in a hood). If a Pale yellow colour develops the test is positive for *M. tuberculosis*.

**Phage Assay Protocol for Isoniazid, Rifampicin, Ethambutol, Streptomycin, and Ciprofloxacin, drug susceptibility testing directly in sputum samples from pulmonary tuberculosis cases:**

- **Stock Concentration of Drug for assay:** INH, Ethambutol, and Streptomycin (Sigma-Aldrich Chemicals GmbH, Steinheim, Germany) stock solutions (10 mg/10 ml) were made in distilled water and filtered through 0.2µm pore size membrane. Rifampicin and Ciprofloxacin stock solutions were made in dimethyl formamide (Sigma, USA). All stock solutions were aliquoted and preserved at -70°C till use.

- **Working Concentrations of Drug for assay:** Working solution was prepared in MH broth (B.D. Difco, USA).

**Table 1: The Drug concentrations used for Phage Assay**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug conc. added µg/ml</th>
<th>Final conc. in the medium µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>
- **Liquid medium for assay**: Mueller Hinton agar and broth media were used.
- **Solid Medium for assay**: Mueller Hinton agar supplemented with 0.4% glycerol and 1% glucose for plate pouring.
- **Exposure time period for mycobacteria with drug**: Decontaminated sputum specimen was exposed to Rifampicin and Streptomycin for 24 hours and other drugs for 48 hours.
- **Concentration and exposure time period of Ferrous ammonium sulphate (FAS) for killing the extracellular mycobacteriophages**: Phagicidal agent ferrous ammonium sulfate (FAS) at 30 mM (Merck) concentration was used and exposure time selected was 10 min for killing the extracellular bacteria on the basis of our earlier study [Described in chapter No 1].
- **Dilution of well content in MH Broth after FAS exposure**: The content from the tubes were transferred to 10 ml M.H. Broth to dilute out the effect of FAS and for better growth of mycobacteriophages and 1 ml was withdrawn for pour plating.
- **Pour Plate Method**: Pour plating method was used in Phage assay to get plaques on solid M.H. agar plate for counting plaques and differentiation of susceptible and resistant strain of mycobacteria. In the phage assay Ferrous ammonium sulphate exposed content in tubes were transferred to 10 ml M.H. Broth [Assay protocol: Step No.3]. After that the one ml of this broth and 1 ml of *M. smegmatis* mc² 155 cells suspension (OD: 1) were taken on Petri dishes and 7-8 ml of molted Mueller Hinton agar supplemented with 1% glucose and 0.4% glycerol were added and immediately plate was mixed clockwise and anticlockwise for 5 times to get uniform plaques. The plates were left in Laminar flow for setting for 10 minutes and then transferred to Incubator at 37°C for over night.
Mycobacteriophage D29 Preparation: Propagation of Phages was done on growth of *M. smegmatis* mc²155 over Mueller Hinton agar. Mycobacteriophage (D29) was propagated in *M. smegmatis*, as described by Sarkis & Hatfull. In brief, 100 µl of Mycobacterium *smegmatis* suspension (1.0 OD at 600 nm) was mixed with 100 µl (approximately 10⁵ pfu /ml) phage suspensions and incubated for 90 min at 37°C and then 200ul of the mixed suspension was transferred in 100 mm Petri plate. Ten ml of molten Mueller Hinton agar supplemented with 0.4% glycerol and 1% glucose and one ml *Mycobacterium smegmatis* suspension was added. The plate was mixed five times clock wise and anti clock wise and left in laminar flow for 10 min for solidification of agar and transferred to incubator at 37°C for over night. After incubation; 5 ml of Mueller Hinton broth added on the lawn of phages and kept at 37°C for over night and centrifuged at 4000 rpm for 20 min. The supernatant containing phages was passed through 0.22 µ Millipore filter membrane and stored at −20°C in deep freeze. The phage titre was determined using a standard spot test [Described in chapter No 1.].

Phage Assay Protocol:

Step 1:

The assay was carried out in 10 ml plastic screw capped tubes (Nunc, USA). Five hundred microlitre of decontaminated sputum specimen mixed with 500µl drug. Wild drug resistant strains (laboratory isolates) and *M. tuberculosis* H37Rv were included in all batches. Negative control received 500 µl decontaminated sputum specimen and 500µl Mueller Hinton broth. The tubes were incubated for 24/48 h at 37°C.
Step-2:
Two hundred microliters of mycobacteriophage D29 (10^8 pfu/ml) (mycobacteriophages suspension containing same drug concentrations) were added into the respective tubes and incubated for 90 min at 37°C.

Step-3:
The extracellular phages were inactivated with 200 µl of phagicidal agent ferrous ammonium sulfate at 30 mM (Merck, India). After that, tubes were incubated at 37°C for 10 minutes. Mixing was done by using auto pipettes.

Step-4:
All the contents of tubes were transferred to respective tubes containing 10 ml Mueller Hinton broth.

Step-5:
One ml of the tube content and 1 ml of M. smegmatis mc² 155 (OD: 1) was dispensed in sterile Petri dish (90 mm); and mixed with 10 ml of melted Mueller Hinton agar (Difco, USA) supplemented with glycerol (0.4%) and glucose (1%) The mixing done by rotating the plate clock wise and anti clock wise. The plate left for 10 min. for solidification and transferred to incubator for over night.

Step-6:
Results were analyzed by counting the plaques. In control plates the plaque count ranged from 90-150 on plate. The M. tuberculosis strains were considered resistant if reduction in plaque count was less than 80 per cent compared to control plate, and susceptible if more than 80 per cent reduction was observed.
Interpretation: The strains were considered resistant if lytic plaques were observed (< 80% reduction) on the indicator plate from those samples treated with drug; the strains were considered susceptible if more than 80% reduction in number of plaques were observed following drug treatment.

Drug susceptibility testing by proportion method:
The proportion method [Canetti G et al., 1963] was carried out using L-J medium. The recommended critical concentrations [Canetti G et al., 1963] of INH 0.2 µg/ml, rifampicin 40 µg/ml, ethambutol 2 µg/ml, streptomycin 4 µg/ml and ciprofloxacin 2 µg/ml were used. The first reading was taken after 28 days of incubation and the second on 42nd day. The percentage resistance (R) was calculated as the ratio of the number of colonies on the drug containing media to those on the control medium.

Statistical analysis: Data were analysed using Bayes-Theorem, 2×2 contingency tables to calculate sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) using StatsDirect statistical software version 2.7.2, UK. McNemar’s test was applied using Graphpad software Inc., USA (two tailed pair test) where each isolate was matched for phage assay and proportion method.
Result

Three hundred and seventy AFB-positive sputum samples were selected for the phage assay. The density of AFB in the sputum samples was 1–3 AFB/100 high-power fields in 52 samples, 1+ in 122 samples, 2+ in 113 samples, and 3+ in 83 samples.

After decontamination, the sputum samples were subjected to the phage assay and culture. The phage assay was carried out on the direct decontaminated sputum samples, whereas the proportion method was carried out on the culture isolated from the same sputum sample.

The results of the phage assay and its comparison to the proportion method are shown in Table 2.

By the proportion method on the mycobacterial isolates, mycobacterial resistance to isoniazid was found in 197 (58%), to rifampin in 143 (43%), to streptomycin in 183 (55%), to ethambutol in 106 (32%), and to ciprofloxacin in 93 (28%). The 143 mycobacterial isolates that were resistant to rifampin were also resistant to isoniazid. In addition 54 samples showed resistance to isoniazid but were sensitive to rifampin.

Concordance of the phage assay was 99.5% for isoniazid, 100% for rifampin, 99.4% for streptomycin, 99.4% for ethambutol, and 97.8% for ciprofloxacin. Discordance was seen for two, nil, one, three, and five samples in the case of isoniazid, rifampin, streptomycin, ethambutol, and ciprofloxacin, respectively.

The sensitivity of the phage assay for isoniazid was 98%, for rifampin was 100%, for streptomycin was 98%, for ethambutol was 97%, and for ciprofloxacin was 93% [Table 2]. The specificity, positive predictive value, and likelihood ratios are mentioned in Table 2.
The level of agreement between the phage assay and the proportion method was measured using Cohen's kappa index ($\kappa$) of agreement: for isoniazid this was 0.9877 (standard error (SE) 0.0546), for rifampin 1.000 (SE 0.0547), for streptomycin 0.994 (SE 0.0546), for ethambutol 0.9795 (SE 0.0545), and for ciprofloxacin 0.9636 (SE 0.0543).

Seventeen of the 370 AFB smear-positive samples did not yield growth on Lowenstein-Jensen medium and failed to give plaques in the phage assay. Thirteen of the 370 sputum samples showed bacterial/fungal contamination on the phage assay plates and did not give interpretable results. Seventeen samples had fewer than 100 plaques on the phage assay plates. Hence, the experiment was repeated using 1 ml volume of decontaminated sample, and interpretable results were obtained in 11 cases. The plaque count was persistently less than 100 on the negative control plates for six samples and hence these could not be used for comparison.

Interpretable results were obtained by the proportion method for 95.4% of the samples, while the phage assay offered results for 90.3% of the samples. However, it may be mentioned that the results were available within 48–72 h for the phage assay on the direct samples, while the proportion method took 2–6 weeks after the primary isolation.
Table 2.

Statistical Analysis of the Phage assay and its comparison with Proportion Method for the detection of Drug resistance for INH, RIF, streptomycin Ethambutol and Ciprofloxacin Direct Sputum samples

(Total Specimens: 334 Sputum AFB smear positive samples from PTB cases)

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Total Specimens</th>
<th>Susceptible by proportion method</th>
<th>Susceptible by Phage assay</th>
<th>Resistant by Phage assay</th>
<th>Resistant by proportion method but Susceptible by Phage assay</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV</th>
<th>NPV</th>
<th>LR+</th>
<th>LR-</th>
<th>Cohen's Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH</td>
<td>197 (59%)</td>
<td>137 (41%)</td>
<td>196</td>
<td>136</td>
<td>1</td>
<td>98%</td>
<td>98%</td>
<td>98%</td>
<td>98%</td>
<td>42</td>
<td>0.01</td>
<td>0.9877</td>
</tr>
<tr>
<td>RIF</td>
<td>143 (43%)</td>
<td>191 (57%)</td>
<td>143</td>
<td>191</td>
<td>0</td>
<td>100%</td>
<td>100%</td>
<td>100</td>
<td>100</td>
<td>Infinit</td>
<td>0.0</td>
<td>1.000</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>106 (32%)</td>
<td>228 (68%)</td>
<td>105</td>
<td>226</td>
<td>2</td>
<td>97%</td>
<td>97%</td>
<td>94%</td>
<td>99%</td>
<td>34</td>
<td>0.03</td>
<td>0.9795</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>93 (28%)</td>
<td>241 (72%)</td>
<td>91</td>
<td>238</td>
<td>3</td>
<td>93%</td>
<td>96%</td>
<td>90%</td>
<td>97%</td>
<td>23</td>
<td>0.06</td>
<td>0.9636</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>183 (55%)</td>
<td>151 (45%)</td>
<td>182</td>
<td>151</td>
<td>0</td>
<td>98%</td>
<td>100%</td>
<td>98%</td>
<td>infinit</td>
<td>0.01</td>
<td>0.994</td>
<td></td>
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</table>
Discussion

The conventional proportion method takes 4–6 weeks after the isolation of *M. tuberculosis*. The PCR-based method offers results within 8 h; however the set-up requires expensive equipment and skilled personnel. Attempts are now being made by international agencies to make the Gene Xpert MTB/RIF test available in developing countries [Boehme CC et al., 2010]. Further, molecular methodologies have gained credence only for the detection of rifampin resistance [Garcia L et al., 2001; Hillemann et al., 2006, 2007; Telenti A et al., 1993; Watterson SA et al., 1998; Williams DL et al., 1998]. A phage based assay was developed by Wilson et al., 1997 and was applied to rifampin and isoniazid susceptibility testing in clinical isolates of *M. tuberculosis* [Wilson SM et al., 1997]. Commercial phage-based assays are available only for the detection of resistance to rifampin [Albert H et al., 2002, 2001].

A number of research papers have documented the utility of the technique for the determination of drug resistance in mycobacterial isolates [Eltringham IJ et al., 1999a, 1999b; Gali N et al., 2003, 2006; Yzquierdo SL et al., 2006., Chauca JA et al., 2007; Traore H et al., 2007; Da Silva PA et al., 2006; McNerney et al., 2000, 2007; Butt et al., 2004]. The approach was used by Albert et al., [2004] for the detection of rifampin resistance in M. tuberculosis cells directly from sputum samples.

Today the increase in multiple drug resistance appears to be a worldwide problem, limiting the success of TB control programs, and is one of the major challenges for the directly observed therapy (DOT)-plus program. A recently updated meta-analysis describes five publications on the detection of rifampin resistance on direct decontaminated sputum samples [Minion J et al., 2010]. However, there are no published data to support the use of phage assays for the detection of isoniazid, ethambutol, streptomycin, and ciprofloxacin resistance on direct clinical samples.
Hence, the aim of the present study was to standardize an in-house phage assay to determine resistance to all of the first-line anti-TB drugs (isoniazid, rifampin, streptomycin, and ethambutol), and also to fluoroquinolones, directly on decontaminated sputum samples. Fluoroquinolones are widely used along with other anti-TB drugs in India and therefore were also included in the present study. Middlebrook 7H9 medium was replaced with Mueller–Hinton broth to make the assay economical. The cost of the commercial phage-based assay (FASTPlaque TB-RIF, Biotec Laboratories Ltd, UK) for rifampin resistance is approximately US$ 10 per test, while the cost of consumables for the in-house phage-based assay is less than US$ 1 per sample.

Seventeen of the 370 sputum samples failed to yield plaques in the phage assay and also did not show growth on Lowenstein–Jensen medium, indicating a lack of viable mycobacteria in the samples. For seven of the samples (not shown in the results), the density analysis showed only occasional AFB in the smear, and the other 10 cases were on anti-TB drugs. The other limitation for a few of the samples was the problem of contamination. Mole et al. reduced the possibility of bacterial contamination from 14.3% to 0.8% by the use of nystatin, oxacillin, and aztreonam [Mole R et al., 2007]. In the present study, a combination of polymyxin B and vancomycin was used due to the high prevalence of methicillin-resistant Staphylococcus aureus and extended-spectrum beta-lactamase producers in the local setting, but still 13 of 370 samples (3.5%) failed to give interpretable results on account of bacterial contamination.

To overcome the problem of a low plaque count in the 17 samples, the samples were reprocessed using 1 ml instead of 0.5 ml of decontaminated sputum sample, and 11 of them then gave interpretable results. However, six still yielded low plaque counts. The observation suggests phage inhibitory effects of substances present in the sputum samples, as mentioned by Albert et
The possibility of a low density of viable mycobacteria in the sputum samples also needs to be considered.

In the present study contamination was seen in 13 and indeterminate results in six, i.e., only 19/370 (5.1%) specimens. The studies by Butt et al., [2004] Albert et al., [2004] and Mole et al., [2007] reported 17–36% samples as contaminated/indeterminate by the phage assay. The improved performance in the present study appears to be due to the inclusion of vancomycin and polymyxin and the performance of the in-house assay at our end. It should be mentioned that the other studies reported only rifampin resistance and did not include other drugs (isoniazid, streptomycin, ethambutol, and ciprofloxacin).

The overall results based on sensitivity, specificity, negative and positive predictive values, likelihood ratios (LR), and agreement with the comparator based on the kappa value are good in the present study [Table 2]. The results were interpretable for 334/370 (90.3%) of the samples and were available in 48–72 h against an average period of 4–6 weeks of primary culture by the proportion method. The cost per test for isoniazid, rifampin, streptomycin, ethambutol, and ciprofloxacin sensitivity testing by the in-house phage assay was less than US$ 1. The commercially available FASTPlaque method costs approximately US$ 10 per test and has been standardized only for the detection of rifampin resistance for decontaminated sputum samples [Albert H et al., 2004]. The use of Mueller Hinton broth and agar in the present study is far more economical than the 7H11 used in the FASTPlaque assay.

The threshold for defining susceptibility was more than 80% for all the drugs in the present study. However, a more than 99% reduction in plaque count was observed for rifampin and streptomycin, and this appeared to be due to high and rapid bactericidal activity of the drugs for mycobacteria. This observation is consistent with the data from another mycobacteriophage
based assay, the luciferase-reporter phage (LRP) assay, in which a 99% reduction in the light signal was seen after an overnight incubation of susceptible isolates with streptomycin [Riksa PF et al., 1998]. These two drugs required a shorter time exposure – 24 h – compared to the other drugs, which required 48 h exposure in the present method, and the reason appears to be the rapid bactericidal activity of rifampin and streptomycin. Similar exposure times were observed in another study [Eltrigham Ij et al., 1999]. The mode of action of the drug may also be a contributory factor, e.g., activity at the transcription/translation level (streptomycin, rifampin) and at the cell wall level (e.g., isoniazid, ethambutol), as suggested by Jacobs et al., [1993].

In the case of streptomycin and rifampin, the drug concentration required for exposure in the phage assay was less than the recommended concentration for the proportion method and this also appears to be due to the rapid bactericidal activity of the drugs. The concentrations of the other drugs used in the study were higher than recommended for the proportion method, possibly because of slower drug activity. In the proportion method a lower concentration may suffice since the exposure is over a long period.

The phage assay offers a simple and rapid testing method for the detection of mycobacterial resistance using direct clinical specimens like sputum, and could be a boon for the DOT-Plus program in economically developing countries where the burden of TB is very high. The methodology does not demand expensive equipment. Our laboratory is presently working to standardize the phage assay for other anti-TB drugs and the preliminary results are very encouraging. The rapidity of the results and the cost effectiveness of the method is expected to offer great help in reducing the transmission of drug-resistant TB infections and in the effective management of MDR-TB cases.