

## Original Article

# Rapid drug susceptibility testing for *Mycobacterium tuberculosis* in Thin layer agar media

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### Abstract

There is a great need to determine the susceptibility of individual *Mycobacterium tuberculosis* strains as rapidly as possible because emergence of multidrug-resistant and extensively drug-resistant tuberculosis in developing countries. The study was conducted to evaluate the thin layer agar (TLA) media for rapid detection of resistance of *M.tuberculosis* to rifampicin (RMP) and isoniazid (INH) in clinical isolates and to determine the sensitivity and time to positivity compared to the proportion method. One hundred clinical isolates of *M.tuberculosis* were studied. For the TLA method, three compartment Petri plate containing 7H11 agar and 7H11 agar with RMP and INH. Results were compared to the proportion method for RMP and INH. The sensitivity for INH and RMP+INH was 85.7 % and 100%. The use of a TLA plate enables the rapid detection of resistance to the two prime anti-tuberculosis drugs RMP and INH in a median time of 9.60 days. TLA was a rapid method for the detection of resistance of *M.tuberculosis* in the two drugs studied. This faster method is simple to perform, providing an alternative method when more sophisticated techniques are not available in low-resource settings.

**Key words:** Rapid Drug susceptibility testing, TL7H11 media, MDR-TB, RMP

### Introduction:

Currently Tuberculosis management and control is potentially devastating threat worldwide due to emergence of MDR-TB (MDR-TB, defined as resistance to both Isoniazid [INH] and Rifampicin [RMP]) and XDR-TB (XDR-TB, defined as MDR-TB with additional resistance to any Fluoroquinolone [FQ] and to at least one of three injectable second-line anti-tuberculosis drugs used in treatment: Capreomycin [CPM], Kanamycin [KM] or Amikacin [AMK])<sup>1</sup>.

MDR-TB cases are difficult to treat and cure rates are frustratingly low. Whereas XDR-TB cases are virtually untreatable since none of the standard drugs or the reserve drugs are effective. So a new, rapid, safe and affordable diagnostic tool is needed to simplify MDR-TB and XDR-TB detection as well as drug susceptibility test (DST) for early

treatment, reducing use of inappropriate drugs and also to prevent the spread. To achieve the ambitious new goals to treat MDR-TB recommended by the World Health Organization (WHO), laboratories must develop the capacity to perform DST of first and second-line drugs to detect MDR-TB and XDR-TB using rapid methods<sup>2</sup>. Fully automated commercial systems such as the Bactec Mycobacteria Growth Indicator Tube 960 (MGIT960) have proved their reliability in the rapid detection of resistance to first and second-line drugs, with results available within on average 10 days; however, they require heavy and costly equipment that is neither universally available nor suitable for poor countries. Several new methods have been developed to reduce the diagnostic time, such as the microscopic observation drug susceptibility assay (MODS), the nitrate reductase assay (NRA) or the colorimetric redox-indicator assay<sup>3-6</sup>. One of the new low-cost methods for the diagnosis of TB is Thin layer agar, which is able to detect growth within 10 days and it does not require sophisticated equipment<sup>7</sup>.

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The present study was designed to observe the role of TLA media for detection of *MTB* and to observation of resistance to RMP and INH in *M. tuberculosis*.

#### Materials And Methods:

**Ethical aspects:** Ethical clearance was taken from Institutional review board of BSMMU and was approved on 23/4/11.

The study was carried out at the National Tuberculosis Reference Laboratory (NTRL) of National Institute of Disease of Chest and Hospital (NIDCH), Dhaka, Bangladesh, from July 2010 to June 2011. Clinically suspected pulmonary tuberculosis patients referred to the NTRL by physicians were enrolled for sputum examination. Z-N staining was done on all the sputum samples and only Z-N positive samples were processed by modified Petroff method for L-J culture and direct DST in TLA media. DST (Indirect) was done in LJ media from LJ media culture positive specimens. Direct DST was done on TLA media after observation of the bacillary load in the concentrated sputum smear made from the sediment.

#### Drug Susceptibility Test (DST)

In this study, two different methods were used to determine drug susceptibility of *M. tuberculosis* to Isoniazid (INH), Rifampicin (RMP). Para nitro-benzoic acid (PNB) was used for identification of *M. tuberculosis*. These methods were conventional DST by proportion method on L-J media and direct DST on TLA media. Reference strain of *M. tuberculosis* H37Rv was used as a susceptible control with each batch susceptibility test in conventional method. The measured amount of drugs were diluted with distilled water and sterilized by membrane filter. Required amount of stock solution of drugs were added to different media to obtain critical concentration of drugs in each medium. The critical concentrations of drugs differ in different media and the critical concentration of drugs used in this study is given in Table I <sup>8,9</sup>.

#### Direct DST method on TLA media:

AFB positive fresh sputum was used and test was done in biosafety cabinet level-II. After preparation of 1 liter media, 200 ml liquid media was taken in each of five properly labeled 250 ml sterile flask, two for control, one for PNB, one for RMP, one for INH. Then antibiotic stock solutions were added to respective flasks. While adding the drugs, flasks were shaken gently to mix the drugs properly with the media. Then the media was poured in 6 ml amounts in each pre-labeled compartment of petri plate with aseptic precaution.

#### Procedure

A smear-positive (at least 1+) sample was inoculated directly on to drug containing and drug free media. Four petri plate were used for three samples. One petri plate has three compartments. One compartment served as growth control (GC), with 1 µg/ml Rifampicin (RMP) in second compartment and 0.2 µg /ml Isoniazide (INH) in third compartment. In this way three plates were used for three samples and fourth plate was used for PNB (500µg/ml) a specific inhibitor of *M. tuberculosis* complex for these three samples. The petri plates were prepared with 6 ml of drug containing and drug free media per compartment. Before inoculations the inoculum size was adjusted on the number of AFB observed on smear microscopy of treated and concentrated sputum. Dilutions were made according to AFB load on the concentrated smear as follows: if >250 AFB/field, 10<sup>-3</sup> dilution; 25-250 AFB/field, 10<sup>-1</sup> dilution; <25 AFB/field, no dilution. Then 0.1 ml of both diluted and undiluted samples were inoculated in both drug free and drug containing media. Then incubated in 5% CO<sub>2</sub> (in closed Candle jar and water soaked tissue paper was kept in it) at 37°C for 4 weeks. Plates were read after 48 hours to check for contamination. The plates were observed twice a week to detect growth using a conventional microscope (objective 10x) for up to 4 weeks. Once the GC compartment was positive, resistance was defined as any growth appearing in the compartments with drugs as compared to the GC compartment. A susceptible strain was defined as no growth appearing in the compartments with drugs compared to the GC. No growth was observed on PNB containing compartment which indicate all the strains were *Mycobacterium tuberculosis*.

#### Conventional DST by Proportion Method on Lowenstein-Jenson Media: As per standard procedure<sup>10</sup>.

#### Results:

Out of 100 study population, 74 (74%) were new cases and 26 (26%) were re-treatment cases. All 100 clinical isolates of *M. tuberculosis* were tested for susceptibility to RMP and INH. With TLA, results were available on an average of 9.60 days of incubation compared to 54.68 days for proportion method in L-J media. The table-II shows the drug resistance pattern of isolated *Mycobacterium tuberculosis* by proportion method and direct DST method. Among the 74 new cases 4 (5.40%) were only resistant to INH and a single MDR 1 (1.35%) case was found resistant to both RMP and INH by direct DST method. Similarly by proportion method 6 (8.10%) were only INH resistant and 1(1.35%) was resistant to both RMP and INH. MDR was found only in 1 (1.35%)

case among new cases by proportion method. Among the 26 re-treatment cases 2 (7.69%) were only INH resistant and 7 (26.92%) were resistant to both RMP and INH by direct DST method. By proportion method 1 (3.84%) was only INH resistant and 6 (23.07%) were resistant to both RMP and INH. MDR were found 7 (26.92%) by direct DST method and 6 (23.07%) by proportion method respectively. Only RMP resistance was observed neither in direct DST method nor in proportion method among the study population.

The Table III shows the DST results obtained by the proportion method and TLA. Among 88 isolates of *M.tuberculosis*, 6 (6.81%) were resistant to Isoniazid by both proportion method and direct DST method. A single case (1.13 %) was found resistant only by proportion method. Agreement was 85.7% found among two methods in case of INH resistance. Hundred percent agreements were found in INH+RMP resistant cases between two methods. Mono resistance to RMP was not found by any of the method.

**Table-I:** Critical concentration of anti-TB drugs and PNB for conventional and direct DST method.

Drugs	Critical conc. for conventional DST method	Critical conc. for direct DST method
INH	0.2 µg/ml	0.2 µg/ml
RMP	40 µg/ml	1 µg/ml
PNB	500 µg/ml	500 µg/ml

**Table II:** Drug resistance pattern of isolated *Mycobacterium tuberculosis* by proportion method and direct DST method (n=99)

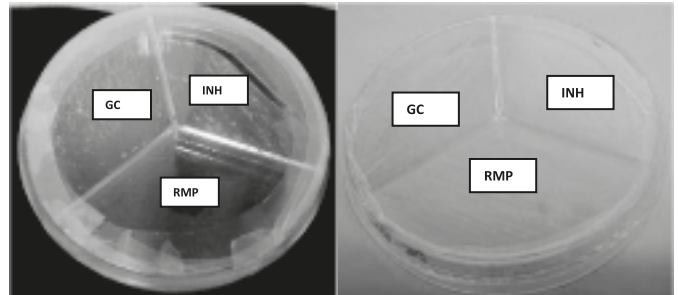
Cases	Test Method	Resistant			
		INH n (%)	RMP n (%)	INH+RMP n (%)	None n (%)
New case n=74	Direct DST method	4 (5.40)	0 (0)	1 (1.35)	63 (85.13)
	Proportion method	6 (8.10)	0 (0)	1 (1.35)	68 (91.89)
Retreatment case, n=25	Direct DST method	2 (8)	0 (0)	7 (28)	13 (52)
	Proportion method	1 (4)	0 (0)	6 (24)	15 (60)

**Table-III:** Comparison of resistance pattern of MTB to RMP and INH by direct DST and proportion method (n=88)

Anti-TB drugs	Resistant detection method					Agreement (%)
	Direct DST method, n (%)	Proportion method, n (%)	Both method n (%)	Only Proportion method, n (%)	Only direct DST method, n (%)	
INH	6 (6.81)	7 (7.95)	6 (6.81)	1 (1.13)	0 (0)	85.7 %
INH+RMP	7 (7.95)	6 (6.81)	6 (6.81)	0 (0)	1 (1.13)	100 %

Note: Proportion method in L-J media was considered as gold standard

**Drug susceptibility test by direct DST method on TLA media**



**Fig I:** Shows INH resistant strain **Fig II:** Shows both INH and RMP resistant strain

**Discussion:**

There are several methods for rapid drug susceptibility testing for mycobacteria. It is questionable whether its susceptibility testing can be rapid as well as accurate. There are several factors to be considered, like- speed in obtaining a valid result, reliability and applicability. Various studies have been carried out from the above contexts to evaluate the performance of TLA media. Drug susceptibility detection time by direct DST method in TLA media in present study was  $9.60 \pm 3.14$  days and it correlated with another study conducted by Schaberg *et al* in Germany where they showed 11 days<sup>11</sup>. On the other hand mean susceptibility detection time was  $54.68 \pm 11.33$  days in L-J media by proportion method. Thus direct DST provides good opportunity for rapid identification of MDR strain. This early detection time of DST is of great advantage to physician to choose an appropriate drug regimen and also help to prevent the spreading of MDR-TB in the community.

Susceptibility to Isoniazid and Rifampicin was higher in new cases 68 (91.89%) than re-treatment cases 15 (60%), among the 99 isolates of *MTB* in this study. Mono resistance to INH in new case was higher than re-treatment cases (8.10% versus 4%) and MDR was found in a single case (1.35%) among new cases and 6 (24%) among re-treatment cases. Similar study conducted by Van Deun *et al.* in 1999 reported that 89.04% samples were sensitive to all drugs in new cases and 66.2% in re-treatment cases. They also reported that only

INH resistance in 3.73% and MDR in 0.23% among new case and in previously treated cases 12.96% and 5.56% respectively<sup>12</sup>. INH resistance was the most common anti-tuberculosis drug resistance encountered, whether alone or in combination with other drugs<sup>1</sup>. Resistances to INH occur more frequently than for most anti-tuberculosis drugs, at a frequency of 1 in 10<sup>5-6</sup> bacilli in vitro.

Among the 99 isolates of *MTB* no mono resistant to RMP was found, RMP resistant was encountered only with combination of INH. Kamal *et al* in Bangladesh, showed similar finding<sup>13</sup>. In their study 86% RMP resistant isolates were also resistant to INH. RMP mono resistant in *MTB* is rare, except pulmonary tuberculosis in HIV patient<sup>14</sup>. RMP resistant thus generally serves as surrogate marker for dual resistant to RMP and INH<sup>15</sup>.

In present study MDR was found in a single (1.35%) case among new cases and 6 (24%) cases among re-treatment cases. Rate of MDR was higher in re-treatment cases. Inadequate drug regimen, drop out from treatment and non compliance of patient in taking drugs might be contributed to higher rate of MDR-TB in re-treatment cases. Over crowded and unhygienic living condition with the lack of infection control measures in hospital might be the other causes of higher MDR-TB in re-treatment cases.

Higher INH resistance and INH+RMP resistance detection by direct DST method in re-treatment cases were observed in this study. Very few works have been done on the direct DST method in TLA. So, the result of higher resistance by direct DST method neither could be compared nor could be explained. But detection of higher resistance pattern by direct DST method on TLA media was a qualitative test in which the observer confirmed resistance by visualizing growth under microscope; where any compartment developed microcolonies in presence of drug interpreted as resistance to that drug. Unlike proportion method, in direct DST method there were no discrete colonies to count and no calculation was required. This might be the cause of higher detection of INH resistance in TLA media in re-treatment cases. It assumes that another probable cause was dilution in direct DST method. Dilutions were made according to bacillary load in concentrated smear. But the bacillary count was subjective that's why there was chance of misdiagnose that is resistance isolate would be susceptible or susceptible would be resistance.

The proportion method was considered as gold standard. Direct DST by TLA showed 85.7% agreement with

proportion method in susceptibility testing of INH. Agreement was 100% between two methods for both RMP+INH resistant cases. Because 6 cases were detected resistant by both methods and only single case was detected resistant by only direct DST method. This single case might be either true positive or false positive. For further evaluation by MIC or genetic analysis can give the actual result.

#### Conclusion:

The TLA method showed rapid and simple detection of resistance to both RMP and INH. The method fits well into the tuberculosis diagnostic and research laboratory, since no equipment expenses are required. The rapid and reliable results make this TLA method a powerful tool for establishing appropriate treatment and reducing the impact of MDR-TB.

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