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Drug Susceptibility Testing of *M. tuberculosis* to Isoniazid and Rifampicin- A Comparison between MTT Tube Method and LJ Proportion Method

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Authors' contributions

This work was carried out in collaboration between all authors. Author RY wrote the protocol and managed the experimental process, author TW managed the literature searches, author GB designed the study, helped in the experimental process, and wrote the first draft of the manuscript, and author NB managed analyses of the study. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: To evaluate the methodology of MTT tube assay and compare it with standard proportion method for detection of drug susceptibility of *M. tuberculosis* to rifampicin (RIF) and isoniazid (INH).
Study Design: Prospective.
Place and Duration of Study: Sher-i-Kashmir Institute of Medical Sciences, Kashmir, India. One year study.
Methodology: MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay was performed on 60 clinical isolates of *M. tuberculosis*. An inoculum of 10^7CFU/ml prepared in Middlebrook 7H9 with OADC (Oleic acid, albumin, dextrose and catalase) was chosen as standard. For each drug three tubes were used, one drug containing (INH 0.2 µg/ml or RIF 1 µg/ml), second inoculum control and third blank control. The method was performed after incubating the tubes at 37°C for 4 days for RIF and 7 days for INH. Results were read visually and by spectrophotometer at 570 nm. Relative optical density units of 0.2, was taken as cutoff. Results

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of drug susceptibility were compared with those obtained by Lowenstein Jensen proportion method.
Results: For RIF, sensitivity was 88.9% and 94.4%; specificity was 100% and 97.6% for visual MTT and MTT by RODU respectively. For INH similar sensitivity of 95.1% was seen while specificity was 97.0% and 95.0% by visual MTT and MTT by RODU respectively. There was almost perfect agreement between proportion and MTT method for both drugs. Turn-around time for MTT assay was 7 days.
Conclusion: The MTT tube assay can be used for rapid drug susceptibility testing of *M. tuberculosis* to RIF and INH.

Keywords: INH; MTT; M. tuberculosis; proportion method; RIF.

1. INTRODUCTION

Tuberculosis remains one of the most important infectious diseases in the world, [1] with an estimated 2 billion people harboring the infection in latent form. According to WHO report, there were 9 million cases and 1.5 million deaths worldwide in 2013, making tuberculosis the second leading cause of death by any infectious disease after HIV [2]. A major threat to the tuberculosis control program is the emergence of multi-drug resistant (MDR) strains worldwide. Rapid diagnosis of these cases is crucial for initiating proper treatment in them.

The proportion method, described by Canetti et al. [3] is considered the gold standard drug susceptibility testing method. However; long waiting period (6-12 weeks) in obtaining results by this and other methods delays initiation of proper treatment thus facilitating transmission of drug resistant infection in the community [4]. To overcome this drawback numerous new liquid and solid medium based techniques that rapidly detect resistance have become available; no single test at present conforms to being quick, cheap and easy. Commercially available liquid culture systems like BACTEC MGIT 960, BacT/Alert 3D and molecular line probe assay have been endorsed by WHO, however due to their complexity, cost and the need for sophisticated laboratory infrastructure, utility has been limited in resource constraints settings [5, 6-11]. Simultaneous development of many non-commercial culture and DST methods like microscopic observation of drug susceptibility (MODS), thin layer agar (TLA), colorimetric redox indicator (CRI) methods, the nitrate reductase assay (NRA) and mycobacteriophage-based assays have shown promise as being rapid and inexpensive.

MTT a yellow tetrazolium salt is converted into a blue formazan by dehydrogenases of a live cell which can be read both visually and by a spectrophotometer at 570 nm [12]. Amount of formazan produced is directly proportional to the number of live cells; the reduction of the dye by bacteria when incubated in the presence of the drug indicates that the isolate is resistant to the given drug and vice versa. This principle has been utilized to assess drug susceptibility of *M. tuberculosis* as well as fungi and bacteria [13,14].

In this study colorimetric assay using MTT was compared with LJ proportion method for its ease of performance and as a rapid alternative for determining the susceptibility of *M. tuberculosis*.

2. MATERIALS AND METHODS

The study was conducted in the Department of Microbiology, Sher-i-Kashmir Institute of Medical Sciences, Kashmir, India. It was a prospective study comparing the performance of the MTT tube assay with the standard LJ proportion method for susceptibility of first line anti-TB drugs, INH and RIF.

Eighty *M. tuberculosis* strains were collected from September 2010 to March 2011. These were recovered on LJ medium after decontamination by Petroff's method, [15] from consecutive smear positive sputum of pulmonary tuberculosis cases and characterized as *M. tuberculosis* on the basis of colony morphology, growth rate, and growth on paranitrobenzoic acid and standard biochemical tests [15]. The strains were preserved in a solution of 20% glycerol at -20ºC in our repository. Of these, only 60 strains could be revived by sub-culture on LJ medium with glycerol and were included in the study. H37Rv and a known strain of *M. tuberculosis* resistant to both INH (isoniazid) and RIF (rifampicin) by the proportion method were used as controls. These strains were provided kindly by Director NJIL and OMD, Agra. INH, RIF and MTT were procured from Sigma to perform LJ Proportion and MTT tube method.

2.1 LJ Proportion Method

Drug susceptibility testing of *M. tuberculosis* isolates against RIF (40.0 µg/ml), and INH (0.2 µg/ml) was done by the standard proportion method on LJ medium [3].

2.2 MTT Assay

It was done by MTT tube method as standardized by Raut et al. [16]. Middlebrook 7H9 with 10% OADC (Oleic acid, albumin, dextrose and catalase) purchased from Hi Media and 0.01% glycerol was used for the test. Working stock solutions of RIF (20 mg/ml in DMSO) and INH (10 mg/ml in sterile distilled water) were prepared, dispensed in 0.1 ml aliquots and stored at -70°C till further use. Solutions were prepared from stock by diluting with 7H9 broth supplemented with OADC to achieve concentration of 2 µg/ml for RIF and 0.4 µg/ml for INH.

For the assay, 3-4 week old subcultures of *M. tuberculosis* on LJ medium were used as most of the cells are metabolically active at that time producing maximum amount of dehydrogenase enzyme which causes reduction of the tetrazolium dye [16,17]. An inoculum size of 10^7CFU/ml was taken as standard [16,17,18]. For RIF testing, to each drug containing tube (DCT), 0.5 ml inoculum was added to 0.5ml of RIF solution of 2 µg/ml (final concentration 1 µg/ml). For INH testing to each DCT, 0.1ml inoculum was added to 0.9 ml of INH solution of 0.4 µg/ml (final concentration 0.2 µg/ml). [16] For each isolate, drug free controls (DFC) containing 0.5 ml of standard inoculum and 0.5 ml of plain 7H9 broth were added. Blank controls (BC) were put for each batch containing only solution and media. Day 4 was taken as cutoff for RIF and day 7 for INH susceptibility testing by MTT assay [16,17]. Incubation time for INH was prolonged and the inoculums size reduced as it acts only on actively multiplying bacteria [16].

MTT in a concentration of 5 mg/ml was prepared in phosphate buffered saline at pH 7.2. Ten microlitre of this solution was added to each tube (DCT, DFC and BC on respective days of reading) and incubated at 37ºC for 4 hours. One ml of solubilizing solution containing 0.1N HCL in isopropanol was added to the tubes and contents mixed thoroughly. After half to one hour of incubation at room temperature, color change to purple in each tube was recorded visually. The strain was labeled resistant if color change was seen in both DFC and DCT, sensitive if color change was seen in DFC and no color change in DCT. Reading was also taken by spectrophotometer at 570 nm by measuring optical density (OD). The relative optical density unit (RODU) was calculated by dividing OD of DCT by OD of the DFC [16,17]. RODU of ≤0.2 was taken as sensitive and ≥0.2 resistant. H37Rv was used as sensitive strain (SS).

The two methods were compared taking proportion method as the gold standard. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using standard formulae. To measure agreement between the two methods Kappa value was used. Chi-square test was used to calculate statistical significance using SPSS-17 software.

Ethical clearance was sought from the institute's ethics committee.

3. RESULTS

Of the 60 *M. tuberculosis* isolates, 42 tested sensitive and 18 resistant to RIF by proportion method. Out of 18 resistant isolates, 16 were resistant by visual MTT and 17 by MTT by RODU whereas of 42 sensitive isolates (by proportion method), all the 42 isolates were sensitive by visual MTT but only 41 came sensitive by MTT by RODU with an overall sensitivity of 88.9% for visual MTT which was lower than MTT by RODU; 94.4%. However the difference in sensitivity was not statistically significant (*P*=0.31). Specificity was found to be 100% by visual MTT and 97.6% by MTT by RODU for RIF. The difference between the two tests was again not significant (*P*=0.50). PPV for RIF was found to be 100% for visual MTT and 94.4% for MTT by RODU, with the difference in the PPV between the two tests being statistically significant (*P*=0.03). NPV for visual MTT (95.5%) was slightly lower than that for MTT by RODU (97.6%), with the difference between the two not being statistically significant (*P*=0.44). The Kappa value of 0.918 for visual MTT and a value of 0.921 for MTT by RODU as compared to proportion method show that they are in perfect agreement Table 1.

Table 1. Concordance for RIF and INH between MTT visual method, MTT by RODU and proportion method

Rifampicin		**Proportion method (n=60)**		**Kappa-value**
		R=18	**S=42**	
Visual MTT	R	16	0	.918
	S	2	42	*P*<0.0001
MTT by RODU	R	17	1	.921
	S	1	41	*P*<0.0001
Isoniazid		**Proportion method (n=60)**		**Kappa-value**
		R=20	**S=40**	
Visual MTT	R	19	1	.925
	S	1	39	*P*<0.0001
MTT by RODU	R	19	2	.889
	S	1	38	*P*<0.0001

Value of Kappa between 0.81-1.00 implies that there is almost perfect agreement between the two methods. P –value<0.05 is statistically significant.

For INH, of the 60 isolates, 40 tested sensitive and 20 resistant by proportion method. Out of 20 resistant isolates by proportion method, 19 were resistant both by visual MTT and MTT by RODU. Thus, in case of INH-resistant isolates, results of visual MTT perfectly matched those of MTT by RODU. But for sensitive isolates, visual MTT could identify 39 out of 40 isolates and MTT by RODU could identify 38 out of 40 isolates. The overall sensitivity for visual MTT and MTT by RODU was 95.1%. Overall specificity of visual MTT was 97% and MTT by RODU was 95%; difference between the two not being statistically significant (*P*=0.72). PPV for INH for visual MTT (95%) was found to be higher than that for MTT by RODU (90.5%). However, the difference in PPV between the two tests was not found to be statistically significant (*P*=0.28). On the other hand NPV for visual MTT (97.5%) was similar to that for MTT by RODU (97.4%). The Kappa value of 0.925 for visual MTT and a value of 0.889 for MTT by RODU as compared to proportion method show that they are in almost perfect agreement Table 1.

The results obtained with MTT in our study were available on an average in 7 days (4 for RIF and 7 for INH) as against 4-6 weeks for proportion method on LJ medium. The cost of proportion method with RIF and INH was 34.24 INR per sample whereas the cost of MTT tube method with RIF and INH was 38.14 INR per sample; only marginally higher than the gold standard and well within the affordable range of most people in our country.

4. DISCUSSION

MTT is readily soluble in water or phosphate buffer and both powder and solutions are stable at 2-8ºC for extended period of time. It is less expensive than the Alamar blue reagent (resazurin salt) and results can be determined visually as well as spectrophotometrically [16]. Also visualization of color change from yellow to purple avoids any possible confusion [18].

Guidelines for detection of RIF resistance in *M. tuberculosis* using MTT dye in microplates were established in 1998 by Mshana et al. [13] However, performing the test in microtitre plates could pose a grave hazard because of generation of aerosols. In the present study, we performed the assay in tubes with screw caps as has been done by other investigators [16,17,19].

In our study, RODU values were used for interpretation of the test in addition to color change as variations between the clinical isolates to reduce MTT is due to the in vitro differences in the growth characteristics and the differences in the proportion of drug sensitive and resistant strains [17]. Although MTT by RODU was found to be more sensitive (94.4%). than visual MTT (88.9%) for RIF the difference was not significant. Although Lemus et al. [20] reported a higher sensitivity of 100% for MTT by RODU; various other studies have reported results similar to ours [17,18]. As far as specificity is concerned, it was found to be 100% by visual MTT and 97.6% by MTT by RODU; the difference again not being significant similar to what was seen by other investigators [17,18,19].

PPV was found to be 100% and 94.4% (visual MTT and MTT by RODU respectively) and NPV was 95.5% and97.6% (visual MTT and MTT by RODU respectively). The difference in the PPV between the two tests was found to be statistically significant. Thus, visual MTT alone can be used as a tool to detect RIF resistance. Although Foongladda et al. [17] reported a higher PPV of 100% for MTT by RODU, our results are comparable to those of Raut et al. [16] (97.5% for MTT by RODU). On the other hand no significant difference was seen in NPV between the two methods, similar to many other studies [16,17].

For INH, overall sensitivity for visual MTT and MTT by RODU was 95.1%; results that correlate well with those reported by many authors [16,17,18,20]. Specificity was 97% and 95% (visual MTT and MTT by RODU), the difference not being significant. Although similar specificity was reported by other researchers [18,20]; Raut et al. [16] reported a low specificity of 87.5% for both visual MTT and MTT by RODU whereas Foongladda et al. [17] reported a higher specificity of 100% for MTT by RODU. PPV for visual MTT (95%) was not found to be significantly higher than that for MTT by RODU (90.5%). Although the results of visual MTT are comparable to those of Raut et al. [16] for MTT by RODU they are lower than those of Raut et al. [16] and Foongladda et al. [17] The NPV for visual MTT and MTT by RODU was similar (97.5% and 97.4%) in our study, hence these can be safely used to rule out INH resistance. Foongladda et al. [17] reported a NPV of 96.8% for MTT by RODU, similar to ours. However, Raut et al. [16] reported a low NPV of 77.7% for both visual MTT and MTT by RODU.

It takes a minimum of 2 work days to prepare medium for proportion method as against one work day for MTT assay. The number of tubes used for testing one isolate is less (n=6) in MTT assay than in proportion method (n=16). The susceptibility results with MTT tube method are available in a week's time as compared to 4-8 weeks taken by the proportion method. The cost of MTT tube assay when compared to the gold standard LJ proportion method is only marginally higher with a difference of 4 INR.

5. CONCLUSION

MTT can be used for detection of drug resistance in resource poor settings due to its high level of agreement with gold standard, ease of performance, rapidity and low cost.

CONSENT

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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