Resazurin Tube Method: Rapid, Simple, and Inexpensive Method for Detection of Drug Resistance in the Clinical Isolates of *Mycobacterium Tuberculosis*

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ABSTRACT

Background: Tuberculosis (TB) remains a serious public health problem worldwide. The emergence of drug resistance and multidrug resistance (MDR) has become the main threat to TB treatment and control programs. Rapid detection is critical for the effective treatment of patients. In recent times, a new method using the colorimetric indicator resazurin has been proposed for drug susceptibility of *Mycobacterium tuberculosis*. **Materials and Methods:** In this study, the resazurin reduction assay was adapted to screw cap tubes. Using the Resazurin Tube Method (RTM), a total of 100 clinical isolates were tested against Rifampicin (RIF) and Isoniazide (INH). By visual reading, the *minimum inhibitory concentrations* (MICs) were obtained after eight days. The results obtained were compared with the gold standard proportion method. **Results:** Excellent results were obtained for RTM with a sensitivity of 100% for both RIF and INH, with a specificity of 98.7 and 95.3%, respectively. Kappa is the measure of agreement between the RTM and proportion method (PM) for RIF and INH, which was found to be 0.972 and 0.935 for RIF and INH, respectively. **Conclusion:** The RTM appears to be a reliable method for the rapid and simultaneous detection of MDR-TB and *drug susceptibility testing* (DST) of *M. tuberculosis*. It is simple, inexpensive, and with no biohazard risk involved.

Key words: Drug susceptibility, Mycobacterium tuberculosis, RTM

INTRODUCTION

Tuberculosis (TB) is an increasing public health problem and has an increasing incidence in developing countries.[1] with India being the country with the highest burden of TB, according to the World Health Organization (WHO) statistics, which give an estimated incidence figure of 2.3 million cases of TB in India, out of a global incidence of 9.4 million cases.[2] The rapidly increasing number of TB cases due to multidrug resistant tuberculosis (MDR-TB) strains or extensive drug resistant tuberculosis (XDR-TB) strains, has become a major threat to TB control programs.[3] Early detection of drug resistance is one of the essential steps in the management of TB. A fast detection method for the resistant strains will allow implementation of adequate treatment and contribute to controlling the dissemination of these resistant strains. Many rapid and reliable phenotypic as well as genotypic methods to detect susceptible and resistant *M. tuberculosis* strains have been introduced in the recent past.[4] However, they require expensive equipment, media or commercial products, and skilled personnel, which are not always available in most developing countries. The standard method used for drug susceptibility testing (DST) is the conventional proportion method (PM). Although this method in cost-effective, it is highly time consuming, cumbersome, and tedious to perform. Consequently diagnosis and treatment is usually carried out based on the microscopy results. As drug resistance is very common in developing countries, such a type of diagnosis may lead to faulty or improper treatment.[3] Therefore, development of alternative, inexpensive, and rapid methods are needed urgently.

In recent times, a new method, using the colorimetric indicator resazurin has been proposed for drug sensitivity

Access this article online

Quick Response Code:

Website: www.jgid.org

DOI: 10.4103/0974-777X.145239

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testing of *M. tuberculosis*.[5] Resazurin is a redox indicator dye that is reduced to resorufin by metabolically active cells. There is a direct correlation between the reduction of resazurin in the growth medium and proliferation of live organisms. The resazurin is blue in color when it is in an oxidized state and turns pink when reduced by viable cells.[6] The resazurin microtiter assay (REMA) plate method has been used successfully for determination of the minimum inhibitory concentration (MIC).[5,7,8] In all these previous studies, this assay was performed in a microtiter plate. Owing to the use of open plates, there is always a risk of spillage and generation of aerosols, which may pose a major risk to laboratory workers. To decrease these hazards and make the assay technique more appropriate for routine use in a moderately equipped laboratory, in the present study the assay has been performed in screw-capped tubes. The study aims to evaluate the resazurin reduction assay with this modification and study the feasibility of its routine use in the DST of *M. tuberculosis*.

MATERIALS AND METHODS

A total of 100 consecutive clinical isolates of *M. tuberculosis* from pulmonary TB patients from our hospital were tested by the resazurin tube method (RTM) for two frontline antituberculous drugs Rifampicin (RIF) and Isoniazide (INH). The results were compared with those obtained by the gold standard proportion method (PM).

Mycobacterial isolates

All the isolates originating from pulmonary tuberculosis patients were defined as *M. tuberculosis* according to the growth rates, pigmentation, colony characteristics, and routine biochemical tests, such as, the niacin test, nitrate reduction test, catalase tests, and so on.[9]

Antibiotics

Rifampicin and INH were obtained in powder form (Hi-Media). Stock solutions were prepared at 10 mg/ml in methanol for RIF and 1 mg/ml in sterile distilled water for INH. They were stored at −70ºC until further use.[5]

Growth indicator

The resazurin reagent was obtained as resazurin sodium salt powder (Hi-Media). The working solution was prepared at a concentration of 0.01% wt/vol in sterile distilled water. This was stored at 4ºC for up to seven days.[5]

Medium used

The resazurin tube method was performed in Middlebrook 7H9 broth containing 0.1% Casitone, 0.5% glycerol and 10% Oleic acid, Albumin, Dextrose, and Catalase (OADC) supplement.

Resazurin tube method

Drug susceptibility testing (DST) by using RTM was performed by adapting the technique proposed by Palomino *et al.,*[5] with minor modifications as follows: The assay was performed in sterile, three-inch screw-capped test tubes. Stock solutions of RIF and INH were thawed and dilutions carried out. Serial two-fold dilutions of each drug in 0.5 ml of 7H9 medium were prepared in sterile screw-capped tubes at concentrations of 2.0 to 0.06 μg/ml for RIF and 1.0 to 0.03 μg/ml for INH. The growth control tube containing 0.5 ml of inoculum in 0.5 ml of 7H9 medium without antibiotics and the sterility control tube, containing 1 ml of 7H9 medium without inoculum were included with every strain tested. Inoculum of 8 ml was prepared by suspension of colonial growth from three- to four-week-old L-J slopes in a tube containing 7H9 medium, with several glass beads. The tube was vortexed for two minutes and the sediment was allowed to form for 15 minutes. The supernatant was transferred to another sterile tube and the turbidity was adjusted to match a McFarland No.1 standard, with an approximate concentration of 10^7CFU/ml. This was taken as the standard inoculum size for the entire study.

The tubes were inoculated with 0.5 ml of standardized inoculum suspension. All the tubes were screw-capped properly and the entire rack was incubated at 37ºC under normal atmospheric conditions for seven days. After seven days of incubation, 150 μl of the resazurin indicator was added to each tube and incubated overnight. On the following day, the tubes were observed for color change. Color alteration from blue (oxidized state) to pink (reduced state) indicated bacterial growth. MIC was defined as the lowest drug concentration that inhibited bacterial growth. Breakpoint drug concentrations were defined according to Palomino *et al*.[5] For the RIF, the strain was considered resistant if MIC was ≥ 0.5 μg/ml and for INH it was ≥ 0.25 μg/ml.

Proportion method

A standard economic variant of 1% proportion method was performed according to the established procedure on

an L-J medium. The critical concentrations for RIF and INH were 40 μg/ml and 0.2 μg/ml, respectively.[10]

Statistical analysis

The performance of RTM in comparison with PM was evaluated in terms of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). The agreement between the methods was estimated by the Kappa statistic. The kappa value, a measure of test reliability, was interpreted as follows: 0.2, poor; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, good; 0.81 to 1, excellent.[11]

RESULTS

The results of the RTM were obtained after eight days of incubation. Complete color change from blue to pink was observed in all growth control tubes, whereas, color change was not observed in the sterility control tubes. The reference strain *M. tuberculosis* H37Rv, tested by RTM, showed no color change in all the drug-containing tubes, indicating no growth and confirming its sensitivity to both the drugs [Figure 1]. The MIC of RIF and INH for 100 *M. tuberculosis* clinical isolates was determined by visual reading of the assay tubes. For RIF, out of 77 susceptible isolates by PM, 76 had an MIC of ≤ 0.25 μg/ml in the RTM. One isolate showed an intermediate resistance with an MIC at 0.5 μg/ml. This was retested by both the methods and the same results were obtained. The MICs of RIF for the remaining 23 resistant isolates by PM were > 2.0 μg/ml. For INH, of the 65 isolates found to be susceptible by PM, 62 had an MIC of ≤ 0.125 μg/ml. Two isolates showed an intermediate MIC of 0.25 μg/ml in the RTM. However, one isolate, susceptible by PM, was found to be resistant by RTM giving a discordant result, with an MIC of >1.0μg/ml. These three isolates were retested by both the methods, with the same results. The MICs of INH for the remaining 35 resistant isolates by PM were >1.0 μg/ml [Table 1].

Nineteen strains resistant to both RIF and INH were categorized as MultiDrug Resistant (MDR) strains. According to RTM, the MICs for RIF and INH were ≥ 0.5 μg/ml and ≥ 0.25 μg/ml, respectively [Figure 2].

These results indicate that the sensitivity of RTM was 100% for both the drugs, whereas, the specificity was 98.7% for RIF and 95.3% for INH. Similarly, the negative predictive values (NPV) of RTM for RIF and INH were 100%, whereas, the positive predictive values (PPV) were 95.8% and 93%, respectively. Kappa was a measure of agreement between the RTM and PM for RIF and INH, and that was found to be 0.972 and 0.935 for each, respectively [Tables 2 and 3].

Table 1: MICs of RIF and INH for 100 *M. tuberculosis* isolates determined by RTM

Proportion method	No. of isolates for which MIC (μg/ml) of Rifampicin was determined by RTM						
	≤0.06	0.125	0.25	0.5	1.0	2.0	≥2.0
Resistance (n=23)	0	0	0	0	0	0	23
Susceptible (n=77)	70	3	3	1	0	0	0
	No. of isolates for which MIC (μg/ml) of INH was determined by RTM						
	≤0.03	0.06	0.125	0.25	0.5	1.0	≥1.0
Resistance (n=35)	0	0	0	0	0	0	35
Susceptible (n=65)	48	10	4	2	0	0	01

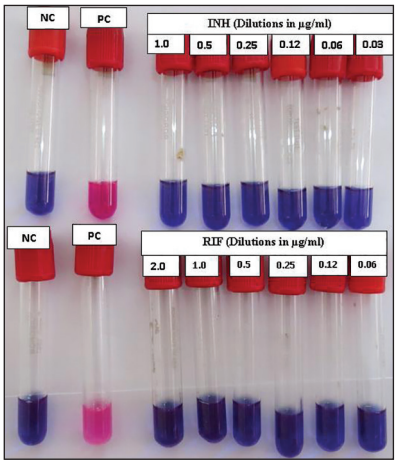

Figure 1: Drug sensitivity testing of *Mycobacterium tuberculosis* H37RV strain by RTM for INH and RIF, showing susceptibility for both. (PC: Positive control; NC: Negative control)

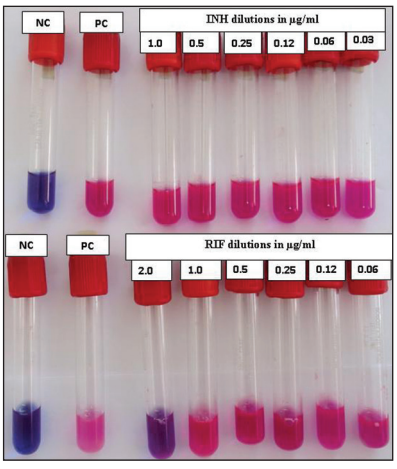

Figure 2: Drug sensitivity testing of MDR *Mycobacterium tuberculosis* strain by RTM, showing resistance to INH and RIF with MICs >1.0 μg/ml and 2.0 μg/ml, respectively. (PC: Positive control; NC: Negative control)

Table 2: Rifampicin susceptibility test results of *M. tuberculosis* strains by RTM compared to PM

		RTM		Total %
		Resistant %	Sensitive %	
Proportion method (PM) (n = 100)	Resistant	23 (100)	0 (0)	23 (100)
	Sensitive	1 (1.3)	76 (98.7)	77 (100)
Total		24 (24)	76 (76)	100 (100)

Fisher's exact test applied. $P = 0.00$; The inter-rater reliability for the raters was found to be Kappa = 0.972 ($P < .0.001$)

Table 3: INH susceptibility test results of *M. tuberculosis* strains by RTM compared to PM

		RTM		Total
		Resistant %	Sensitive %	
Proportion method (PM) (n = 100)	Resistant	35 (100)	0 (0)	35 (100)
	Sensitive	3 (4.6)	62 (95.4)	65 (100)
Total		38 (38)	62 (62)	100 (100)

Fisher's exact test applied. $P = 0.00$; The inter-rater reliability for the raters was found to be Kappa = 0.972 ($P < .0.001$)

DISCUSSION

For tuberculosis case management, it is important to have a prompt diagnosis of infection, with its drug sensitivity profile. This is also essential for the control of the spread of tuberculosis, especially drug-resistant tuberculosis in a particular area. Delay may lead to improper treatment and development of resistance to other anti-mycobacterial drugs as well.[12] Delays have proved to be fatal in HIV-TB coinfected patients also.[13] Drug sensitivity testing in new cases is important in the directly observed treatment, short-course (DOTS) strategy. The turnaround time for DST is very important for TB patients, to receive early and appropriate treatment. Therefore, an inexpensive and rapid method for detection of drug resistance is desirable for low-resource countries. Colorimetric methodologies have been suggested as an important tool for the detection of resistant strains in developing countries.[14] Methods like the Resazurin Microtiter Assay (REMA), Nitrate Reduction Test (NRT), and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, take approximately eight to ten days to obtain results.[7] This period is similar to that taken by advanced high cost methods like BACTEC and MGIT. The REMA plate method has proven to be, in recent experiences, a reliable method for the detection of DST of *M. tuberculosis*.[8,12,15] One important concern with this type of test is its biosafety, as the test is performed in open plates, with liquid media, which could generate aerosols.[7,8,12,16] It has recently been shown that this format can be adapted to screw-capped tubes, to avoid this situation. Abate *et al*., Coban *et al.,* and Raut *et al.,* had used screw-capped tubes for their colorimetric assays.[17,18,19] In the present study also, the resazurin reduction assay was performed in screw-capped tubes and excellent results were obtained. Use of more amounts of media and reagents for this method increased the cost of the test marginally, but provided more nutrients for a better growth of the organisms. According to Abate *et al*. and Raut *et al.,* color change was better appreciated in the tubes.[17,19]

The RTM is simple to perform and inexpensive, giving results after one week, with excellent concordance with PM, which takes several weeks to confirm the results. In this study RTM has also shown high levels of agreement with the conventional PM. The results have been easily determined visually by reading the change to a stable color, from blue to pink.

Cost is an important factor in DST of *M. tuberculosis*. Resazurin is cheaper than Alamar blue and MTT.[6] Use of resazurin greatly reduces the cost of this method. Therefore, it will be feasible to implement this method in laboratories with limited resources.

Martin A. used the resazurin reduction assay and demonstrated its potential for testing *M. tuberculosis* susceptibility to second-line drugs.[8] Campanerut *et al.,* used this assay efficiently in the detection of Pyrazinamide resistance.[15] Zheling *et al.,* compared five different methods for Pyrazinamide susceptibility testing and concluded that the REMA method is superior to other methods. They have also suggested its use as an alternative method to the MGIT method.[12] According to Raut *et al.,* the concordance of the colorimetric assay with PM was excellent as compared to the real-time polymerase chain reaction (RTPCR), and therefore, recommended the colorimetric assay for resource-constrained laboratories.[20] The colorimetric assay, by using MTT as the redox indicator, has been tried in the sample directly without the need for isolation of the *Mycobacteria*.[21] This kind of modification with RTM on sputum samples collected under program conditions will definitely shorten the time required for drug susceptibility reporting, by three to five weeks.

In the present study, all the isolates recorded as resistant by RTM were also resistant by PM. It shows that if a strain is read as resistant by RTM, it is most likely to be resistant, indicating the use of RTM for rapid screening of the resistant isolates.

Our results demonstrated that the RTM has an excellent test performance for RIF and INH, with good sensitivity

and specificity values when compared with PM, the gold standard. However, results with four clinical isolates tested by RTM showed discrepancies. Differences in the medium, type of media, and proportion of drug-resistant bacteria in the test population, may have contributed to these discrepancies. Out of these four strains three strains showed MIC equal to their cut-off values [Table No. 1]. Based on such types of observations, some authors have categorized such isolates as partially resistant organisms.[22,23] Such types of results could allow identification of patients due to the low level of resistant strains, making possible the use of a drug up to near the maximum level of concentration, which could reach in the human body.

Rifampicin resistance is considered to be a strong predication of MDR-TB, especially in countries where drug-resistant TB is highly prevalent.[2] In the present study, RIF susceptibility testing by RTM showed a very good agreement of RTM with PM [Table 2]. This observation strongly indicates the potential use of this simple and inexpensive assay for TB control programs in areas with high levels of TB endemicity, where the rate of MDR-TB is also on the rise.[3] Therefore, a better and faster diagnostic assay for the management of MDR-TB cases in such areas is a priority. The rapid detection of MDR cases is also of utmost priority to start prompt and appropriate treatment for the rapid cure and control of the spread of MDR-TB in the community.[3] Out of 100 isolates tested in this study, 19 strains were resistant to RIF and INH by both RTM and PM. Thus, 100% MDR-TB case detection was possible by RTM. Resazurin reduction assays have been used successfully in the recent past for the rapid detection of MDR-TB.[16] Our results obtained by RTM showed capability in the rapid detection of MDR strains and fully agreed with the results of previous studies.[5,16]

CONCLUSIONS

In conclusion a method like RTM has clear clinical advantages by the simultaneous detection of MDR-TB and susceptibility testing of the clinical isolates of *M. tuberculosis,* and is worth trying in resource-poor countries. It is easy to implement, reliable, and inexpensive. If implemented, this assay will help clinicians in the effective treatment and management of drug-resistant tuberculosis. There is no need for any special equipment or reagent. With the use of screw-capped tubes, it also eliminates the biohazard risk.

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How to cite this article: Patil SS, Mohite ST, Kulkarni SA, Udgaonkar US. Resazurin tube method: Rapid, simple, and inexpensive method for detection of drug resistance in the clinical isolates of *Mycobacterium Tuberculosis*. J Global Infect Dis 2014;6:151-6.

Source of Support: Nil. **Conflict of Interest:** None declared.