Resazurin assay for rapid drug susceptibility testing of Mycobacterium tuberculosis

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Abstract

Introduction: Tuberculosis, a disease of antiquity continues to cause high morbidity and mortality. In Sustainable Development Goals and End Tb Strategy proposed by WHO includes Drug Susceptibility Testing as major component. Conventional methods are time consuming and molecular methods are rapid but expensive and demanding. Resazurin assay is a rapid colorimetric redox indicator method of Drug Susceptibility Testing.

Materials and Methods: 67 strains of M. tuberculosis isolated from pulmonary tuberculosis patients were tested using indirect absolute concentration method by resazurin assay and compared with drug incorporated Lowenstein – Jensen media for Isoniazid and Rifamycin.

Results: There was 91.5 % and 91.7% concordance between both methods for INH and Rifamycin respectively. Resazurin assay for INH was 100% sensitive and 61.54% specific. Sensitivity and specificity for RMP was 98.04% and 62.5% respectively. The duration required for INH was 7.0 ± 2.07 and for RMP 6.58 ± 1.58 days.

Conclusions: Resazurin assay is sensitive, rapid, cheap, technically simple drug susceptibility method.

Keywords: *M. tuberculosis*, resazurin assay, drug susceptibility.

Introduction

Tuberculosis has existed for millennia and still remains a major global health problem. Even though with timely diagnosis and appropriate treatment tuberculosis can be cured, it caused 1.4 million deaths in 2015. Emergence of Multidrug, Total drug resistance among *M. tuberculosis* is amplifying the global problem. 85% of the disease burden is seen in developing countries of Asia (55%) and Africa (30%). India and China alone represent 35%. People living with HIV are also likely to develop TB up to 37% more than HIV negative individuals.[1] Resistance to anti-tuberculosis drugs has been a problem since the era of antibiotics began. The prevalence of MDR estimated is about 2-3% in new cases and up to 12-17% among retreatment cases. Early detection of drug resistant tuberculosis allows prescription of appropriate anti-tubercular regimens leading to more efficient control of the disease. The increase in the incidence of MDR tuberculosis has clearly established the need to develop reliable, rapid, cost effective drug susceptibility testing techniques.[2] In September 2015 WHO has replaced Millennium Development Goals (2000-2015) with Sustainable Development Goals (SDGs) and "End TB Strategy" for the period 2016 -2030. One of the indicator for monitoring implementation of this strategy at global and National levels is 100% coverage of Drug Susceptibility Testing [DST] for tuberculosis patients.[3] DST methods can be divided into slow vs rapid, conventional or growth based vs molecular resistance mutation detection and direct vs indirect.

Conventional Drug Susceptibility testing methods are growth based proportion method / resistance ratio / absolute concentration methods using Lowenstein – Jensen (LJ) media or agar based media, which need 3-4 weeks. They are slow, reliable, cheap and are useful in drug resistance monitoring. Automated methods or nucleic acid based techniques are rapid, expensive, need the skills, supplies, equipment and other infrastructure facilities, so difficult to implement in resource poor countries. Molecular mechanism of drug resistance are not completely understood, they may fail to detect all those resistant strains in which no associated mutations have been identified to date. They are useful for detecting MDR cases. Thus recently several rapid and inexpensive colorimetric methods like MTT, TTC, Resazurin, NRA assays have been evaluated.[4]

Resazurin, (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide) is non-proprietary product, the main component of which is Almar blue. Almar Blue itself is weakly fluorescent until it is irreversibly reduced to the pink colored and highly red fluorescent resorufin indicating cell viability. It is used as an oxidation- reduction indicator in cell viability assays for bacteria, mammalian cells and also for measuring aerobic respiration.[5] In this study we have evaluated the Resazurin assay for detection of Rifamycin (RMP) and Isoniazid (INH) resistance among the *Mycobacterium tuberculosis* strains isolated from pulmonary tuberculosis cases.

Materials and Methods

The study was cleared by Institutional Ethical committee. Sample size for the present study was calculated using the formula

$N = Z^2 (1-\alpha/2)\, p\, (1-P) / D^2$

P = With anticipated population proportion 10%.

D= Allowed error 5.%, Confidence level 95%.[6]

Accordingly 67 strains isolated from the pulmonary tuberculosis cases on Lowenstein –Jensen media and identified by colony characteristics, rate of growth and standard biochemical tests as *M. tuberculosis* were included. No history of prior anti-tubercular therapy was recorded. - All the isolates were subjected to indirect method of susceptibility testing by absolute concentration method using Lowenstein – Jensen medium and Resazurin assay using Middlebrook 7H9 broth with OADC. Standard H37Rv (ATCC 27294) was also included.

Inoculum preparation

Using nichrome loop moist growth of less than fifteen days old was swept from LJ slope and discharged into 4ml of Middlebrook 7H9 broth plus OADC supplement contained in 7ml screw capped bottles. Uniform suspension was prepared by shaking on mechanical shaker along with --- six glass beads of 3mm diameter. The turbidity adjusted to No-1 MacFarland standard. (~ 3 X 10^7*M. tuberculosis* CFU /ml). Dilute this to 1:5 in Middlebrook 7H9 broth with OADC supplement. (~6 X 10^6 CFU /ml).

Media preparation

Lowenstein –Jensen base, Middlebrook 7H broth dehydrated medium and OADC supplement were procured from Hi – Media Pvt Ltd. Final medium prepared as per the manufacturer's instructions. Pure forms of Isoniazid and Rifamycin were also procured from Hi –Media Pvt. Ltd. Stock solutions of these drugs were prepared with distilled water as solvent for INH and DMSO for RMP. Drug incorporated media were prepared in both LJ and Middlebrook 7H9 broth with OADC to achieve same final concentration.

INH - 0.1, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 µg /ml.

RMP – 2, 4, 8, 16, 32, 64, 128 µg /ml.

For determining MIC, batch wise testing with five strains in each was done. Standardized inoculum (0.1ml =~6 X 10^5 CFU) of test strains and also H37RV was inoculated on all concentrations of drug containing LJ and o one drug free LJ (growth control). Inoculated LJ slopes were incubated at 37^0C. All the slopes were examined daily for first one week to see any contamination. Preliminary readings were taken after two weeks and final readings after 4 weeks. The lowest concentration of the drug showing ≥ 99 % growth inhibition as compared to drug free medium was considered as the MIC of that drug for that strain.

Standardized inoculum (1.0ml) of each test strain and H37Rv was inoculated into five sets of 09 ml --- Middlebrook 7H9 broth with OADC containing various drug concentrations and five drug free media t to achieve ~6 X 10^5 CFU/ ml. Then all these were incubated at 37^0C.

Preparation of Resazurin dye

Resazurin dye was procured from Hi-Media Pvt. Ltd. Master solution (0.01%) was prepared in sterile distilled water. The dye solution can be stored in dark at 4°C for two weeks.

Resazurin assay performed for each strain on 2, 5, 7, 10 and 15th days. For each strain, 30µl of the resazurin dye solution is added to one set of tubes with drugs along with one drug free inoculated tube and incubated at 37^0C overnight. Change in the color from blue to pink means growth of the isolate. Color developed by test isolate is compared with color present in growth control tube. Slightest change to pink is recorded. The lowest concentration of the drug that prevents such color change is recorded as MIC of that drug for that strain. Any change in MIC after further incubation was also documented. The MIC value, which remained consistent was recorded for final analysis. Minimum duration required for final results was documented. Contamination seen in both methods was also recorded.

Randomly ten strains were numbered and given to another technician in the laboratory to test for reproducibility. Results documented were analyzed.

Results

Table 1: Showing drug resistant profile for INH and Rifamycin by both methods

	INH		RMP	
Method	**L J**	**Resazurin**	**L J**	**Resazurin**
Susceptible	59 (88.1%)	54 (80.6%)	56 (83.6%)	51 (76.1%)
Resistant	08 (11.9%)	13 (19.4%)	11(16.4%)	16 (23.9%)

For INH, $\chi^2 = 0.90$, p=0.34 Not significant

For RMP, $\chi^2 = 0.74$, p=0.38 Not significant

Table 2: Comparison of Resazurin assay for INH with conventional LJ method

L J medium	Resazurin assay		Total
	Susceptible	Resistant	
Susceptible	54 (91.5%)	05 (8.5%)	59
Resistant	00	08 (100%)	08
	54	13	67

$\chi^2 = 32.11$, p<0.0001 Highly significant

Variable	Value
Sensitivity	100
Specificity	61.54
Positive Predictive Value	91.53
Negative Predictive Value	100

Table 3: Comparison of Resazurin assay for RMP with conventional LJ method

LJ medium	Resazurin assay		Total
	Susceptible	Resistant	
Susceptible	50 (89.3%)	06 (10.7%)	56
Resistant	01(9.1%)	10(90.9%)	11
	51	16	67

$\chi^2 = 28.27$, p<0.0001 Highly significant

Variable	Value
Sensitivity	98.04
Specificity	62.50
Positive Predictive Value	89.29
Negative Predictive Value	90.91

Graph 1: Comparison of susceptibility to INH & RMP by both methods

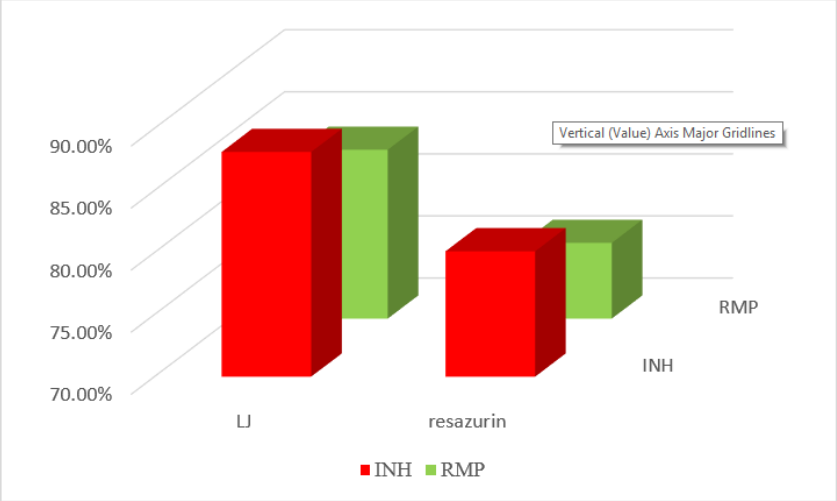


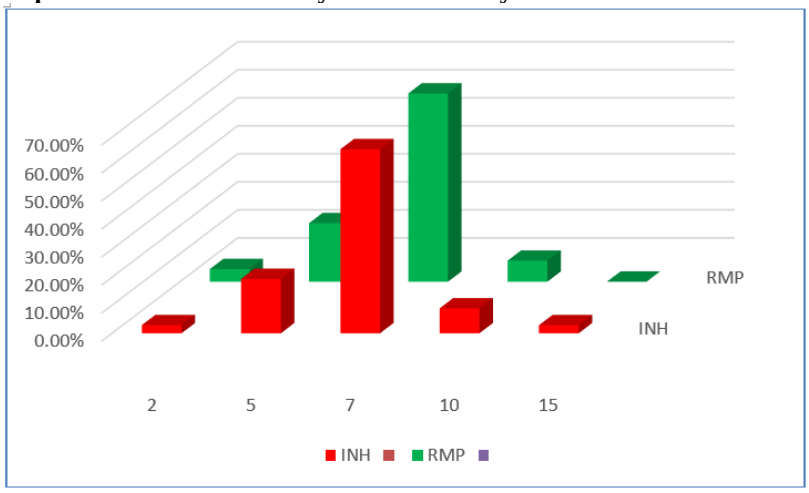
Table 4: Duration required by Resazurin assay for both drugs

Drugs	Duration in days				
	02	05	07	10	15
INH	02 (2.9%)	13(19.4%)	44 (65.7%)	06(8.9%)	02(2.9%)
RMP	03 (4.5%)	14 (20.9%)	45 (67.2%)	05 (7.5%)	00

Day INH RMP
7 59(88.1%) 62(89.9%).
10 65(97.01%) 67(100%)

Average days for INH (Mean ± SD)	7.0 ± 2.07
Average days for RMP (Mean ± SD)	6.58 ± 1.58

Graph 2: Duration required for INH & RMP by resazurin assay



Comparison of Resazurin assay for drug susceptibility testing of *M. tuberculosis* for Isoniazid and Rifampicin showed 59 (88.1%) strains as susceptible to INH by using LJ, and 54 (80.6%) by Resazurin assay. Similarly 56 (83.6%) strains showed susceptibility for RMP by using LJ and 51 (76.1%) by Resazurin assay. Thus there was 91.5% concordance between two methods for INH and 91.7% for RMP.

Statistical analysis

Resazurin assay in comparison with LJ medium for INH showed sensitivity (100%), specificity (60.54%) with PPV for resistance (91.53%) and NPV for resistance (100%). Similarly for Rifampicin Resazurin assay showed sensitivity of (98.04%), specificity (62.50%) with PPV (89.29%) and NPV (91.7%) for resistance respectively. As final results by LJ based assay were available after 28 days, consistent results by Resazurin assay for INH were available for 59 (88.1%) and 62 (89.9%) strains for RMP by 7 days. Average days required were 7 ± 2.07 days for INH and 6.58 ± 1.58 days for RMP. Contamination observed on LJ was 01 (1.49%) and 3 (4.47%) for Resazurin assay.

Discussion

Early detection of drug resistance among *M. tuberculosis* plays important role in patient treatment and prevention of spread of drug resistant strains. Various Colorimetric tests have been evaluated for detecting drug resistance as rapid and cheap methods. In this study of resazurin assay we observed resistance for INH (19.4%), RMP (23.9%) as compared to conventional method using LJ medium INH (11.9%), RMP (16.4%) respectively, but this difference was statistically insignificant (INH -χ^2 = 0.90, p=0.34, RMP - χ^2 = 0.74, p=0.38). But 100% correlation of results for INH and RMP was reported by Franzblau SG et al.[7] Similarly Palomino JC et al[8] also has reported 100% correlation between resazurin assay and LJ medium by proportion method. Yajko DM et al[9] reported 98% agreement for INH and Rifamycin between Resazurin assay and agar proportion method.

Emergence of multidrug resistance is of great concern for control of tuberculosis especially in developing countries. So drug susceptibility testing on strains isolated from new cases and also from treatment failure patients becomes important. Method adopted for susceptibility testing has to be reliable, cheap and rapid. To evaluate these features Resazurin assay was compared for INH and Rifamycin. Resazurin assay for INH susceptibility testing showed sensitivity of 100% and specificity of 61.54%, PPV (91.53%) and NPV (100%). But Ang CF et al[10] has reported Resazurin assay as 100% sensitive and 91.7% specific. Montoro et al[11] has also reported it as 100% sensitive and 96.4% specific. Farina P et al[12] has reported Resazurin assay as 95% sensitive and 93% specific. Whereas Martin A et al[13] observed higher specificity of 100%, while sensitivity was 99.1%.

Similar to our results for Rifamycin susceptibility testing by Resazurin assay, Ang CF et al[11] has reported 100% sensitivity and 91.7% specificity. But Montoro et al has reported 100% sensitivity and 98.4% specificity, whereas Palomino et al[8] has observed 100% sensitivity and specificity, which is also reported by Martin A et al.[13]

Early reports play important role in the optimization of treatment by timely recognizing the drug resistant cases. Convention agar or egg based media need 4-6 weeks for the final results. But in our study of Resazurin assay the average days required were 7 ± 2.07 days for INH and 6.58 ± 1.58 days for RMP. For INH 59 (88.1%) of results were available on 7th day and 62 (89.9%) on 10th day. Similarly 65 (97.01%) and 67(100%) of the results were available on 7th and 10th day respectively for Rifamycin. Affolabi D et al[14] observed that 100% results by 8th day. Franzblau SG et al[7] has also reported 100% results on 8th day. Kumar M et al[15] also observed 100% results on 8th day.

For reproducibility nine strains among ten, which were tested by another technician - were found to have identical results. Only one strain showed one dilution higher MIC. Thus the Resazurin assay produced reproducible results.

Thus resazurin assay shows good correlation with conventional LJ based method, it is rapid, simple to perform. It does not need any costly instruments. Since we use liquid media for its assay, it may generate aerosols, thus biosafety cabinets for processing and like we did use of screw capped bottles will minimize it. This method is evaluated by IUATLD for primary and second line anti-tubercular drugs to be used in resource poor countries.

The MICs of discordant results designated as resistant by Resazurin assay were found to be at cut off value. As Resazurin assay is colorimetric assay, slight change in the colour is more appreciated than the conventional method of growth inhibition. Similar observations were made by Franzblau et al and Herrera L et al who observed most discrepancies were seen with strains whose MICs fell at one or two dilutions higher or lower than cut off value. They also suggested to implement cut off value for three categories, i.e. susceptible, resistant and partially resistant for isolates with borderline MICs.[4] Taneja NK et al[16] has used this method for screening of antitubercular drugs against dormant as well as actively growing *M. tuberculosis* bacteria and reported that for dormant bacteria susceptibility testing can be done under hypoxia induced in-vitro. Affolabi D et al also used this assay to perform PNB susceptibility to differentiate *M. tuberculosis* and NTM.[17]

Thus Resazurin assay can be adopted as reliable, cheap, rapid, simple and reproducible method to identify and rapid drug susceptibility testing for primary and secondary drugs.

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