

Evaluation of 2,3,5-Triphenyl tetrazolium chloride (TTC) assay for drug susceptibility testing of *M. tuberculosis*

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Abstract

Background: Control of tuberculosis is hampered by emergence of drug resistance such as MDR, XDR & TDR. Treatment of this disease will be facilitated if early reliable drug susceptibility profile is available. Various commercial systems are available but in resource poor country like India cheaper colorimetric methods are evaluated. One such method is using 2,3,5-Triphenyl tetrazolium chloride (TTC), which is an oxidation–reduction dye indicating cellular viability and growth. Colorimetric methods used in liquid media are shown to give better results than using them in Lowenstein–Jensen medium (LJ).

Material and Method: A total of 82 strains isolated from patients suffering pulmonary tuberculosis and identified as *M. tuberculosis* were subjected to DST for Isoniazid and Rifampicin by Indirect drug susceptibility testing using absolute concentration method. 1% TTC was incorporated in Middlebrook 7H9 broth along with the drugs and compared with drug containing LJ.

Results: Among the 82 strains, 9 (10.9%) strains were resistant to INH by both methods. Whereas 06 (7.3%) were resistant only in TTC. (sensitivity-100%, specificity -60%, positive predictive value- 91.8% and negative predictive value of 100%, P<0.0001). Similarly 11(13.4%) strains showed resistance to RMP by both methods, 08 (9.8%) were resistant by only TTC and 01 (1.2%) only by conventional method. (Sensitivity- 98.4%, specificity- 57.9%, PPV-88.6%, NPV-91.7%, P<0.0001).

Conclusion: Results were available for 60 (73.2%) and 70 (85.4%) of strains by TTC method for INH and RMP respectively in 07 days as against 28 days in conventional method.

Keywords: *M. tuberculosis*, Susceptibility Testing, TTC Assay.

Introduction

Tuberculosis, a global health problem is an air borne infectious disease. Each year there are 10.4 million new cases of TB, and close to four million deaths. All countries are affected, but 85% of these cases occur in Africa (30%) and Asia (55%). While India and China alone represent 35%. TB is closely associated with HIV/AIDS. People living with HIV represent over 10% of annual TB cases and are 37 times more likely to develop TB than people who are HIV negative.⁽¹⁾

In 1991 a WHO resolution was set to control this disease but it was realised that specified targets could not be met by 2005, So stop TB partnership with MDG designed to achieve the targets were set for the period 2006–2015, which is now again modified with paradigm shift for 2016-2020.⁽²⁾

Resistance to anti-tuberculosis drugs has been a problem since the era of antibiotics began. In early 1990s MDR emerged. Its prevalence is estimated to be 2-3% in new cases and 12-17% among retreatment cases. India ranks second, next only to China for MDR burden.⁽²⁾ The rapid detection of antimicrobial resistance is important in the effort to control the increase in resistant *Mycobacterium tuberculosis*. Drug susceptibility testing (DST) to detect MDR plays important role in the management. Currently used methods of DST are proportion method, absolute

concentration method or resistance ratio method using Lowenstein–Jensen (LJ) or agar based media. However these methods are lengthy, time consuming requiring 3-4 weeks.⁽³⁾ Similarly automated systems like BACTEC, MGIT etc. molecular methods are also available, which are rapid, expensive so cannot be implemented in developing countries.^(4,5)

Recently several rapid and inexpensive colorimetric methods have been introduced, such as Nitrate reductase assay, Resazurin, Almar blue, 2, 3, 5-triphenyl tetrazolium chloride (TTC), 2,5- diphenyl tetrazolium bromide (MTT), 2,3, bis (2-methoxy-4-nitro -5-sulfophenyl)-2-H tetrazolium hydroxide (XTT) etc. with high sensitivity and specificity.⁽⁶⁾ So in this study we have evaluated 2,3,5-triphenyl tetrazolium chloride (TTC) assay for detection of Rifampicin (RMP) and Isoniazid (INH) resistance among *M. tuberculosis* strains isolated in our laboratory.

Material and Method

The present study was permitted by the Institutional ethical committee. Sample size for the study was calculated using the formula –

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

P - With anticipated population proportion -10%.

D - Allowed error 5% Confidence level 95%.⁽⁷⁾

Accordingly 82 strains of *M. tuberculosis*, which were isolated from pulmonary tuberculosis cases on LJ media and identified as *M. tuberculosis* using Standard biochemical tests were included. No history of anti-tubercular therapy was collected. Fresh isolates (< 15 days old) were subjected to indirect method of susceptibility testing by both methods. Standard ATCC H37Rv (27294) was also included.

Inoculum preparation: Using 22 SWG nichrome loop (wire diameter 0.7 mm), volume 2mm³ (approx. 2mg moist growth) swept from LJ slope is then discharged into 0.4ml sterile distilled water contained in 7ml screw capped bottles containing 6 glass beads of 3mm diameter. Suspension is prepared by shaking on mechanical shaker. The turbidity is adjusted to No-1MacFarland (~ 3 X10⁷ CFU /ml). Dilute this to 1:5, which is the standardized inoculum.⁽⁸⁾

Media preparation: LJ medium preparation: LJ base procured from Hi -media. Pvt. Ltd. Prepared as per the manufacturer's instructions. For TTC assay 5 sets of tubes were used containing Middlebrook 7H9 broth with OADC supplement, also procured from Hi-Media. India, Pvt. Ltd. Pure forms of drugs were also procured from Hi-Media. India, Pvt. Ltd. Stock solutions were prepared and used to achieve the final concentrations in media.

Drug incorporation: INH and RMP incorporated LJ prepared to achieve final pre-inoculation concentration of the drug and same final concentrations of drugs were achieved in Middlebrook 7H9 broth with OADC.

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 was done. 5 strains were tested in each batch. Standardised inoculum once prepared was inoculated on drug containing LJ and also drug containing Middlebrook 7H9 broth +OADC along with one drug free LJ and drug free Middlebrook 7H9 broth plus OADC.

0.5 ml of standardised inoculum of each strain was inoculated on the LJ slope with all the concentrations of INH and RMP, along with one drug free LJ slope. All concentration of drugs were also inoculated with standardised inoculum of H37Rv strain. All inoculated LJ slopes were incubated at 37°C. The inoculated slopes were examined daily for first week. Then twice a week. The lowest concentration of drug showing ≥ 99%

growth inhibition as compared to drug free medium for that strain is its MIC. Initial readings were recorded after two weeks and final readings after four weeks of incubation. Resistance defining criteria followed were as per the WHO guidelines for solid media & liquid media.

Interpretation: The strain showing MIC of ≥ 1µg/ml for INH, MIC ≥ 40µg/ml for RMP is considered as resistant.⁽⁸⁾

TTC assay - To 5ml of broth dispensed in each test tube, 0.5 ml of various concentrations of drug solutions were added to achieve the final specified concentrations. For each strain of *M. tuberculosis* five sets of these drug incorporated media were used. 0.1ml of standardised inoculum was inoculated into each of these test tubes. In each batch one drug free Middlebrook 7H9 broth + OADC was inoculated with each test strain and one set of all drug concentrations were inoculated with H37RV strain. Then they were incubated at 37 °C.

TTC solution preparation: TTC powder was procured from Hi media. Pvt. Ltd. Master solution was prepared at the concentration of 5 mg/ml by dissolving in sterile distilled water. Sterilised by filtration. Stored in refrigerator at 4°C. TTC assay performed on 2, 5, 7, 10 & 15 days.⁽⁹⁾

For TTC assay, to one set of inoculated tubes 10 µl of TTC solution was added and Incubated at 37°C for 4 hours. Colour change in each tube was recorded. Any change in colour from colourless to pink indicates the bacterial activity. Lowest concentration of drug that prevents colour change is recorded as its MIC. Always the colour in the test was compared with colour developed in drug free medium. For all the strains TTC assay was done on all five times to note any change in the results after further incubation. The MIC value, which remained consistent was recorded for final analysis. Minimum duration required for final results was recorded. Thus for INH any strain showing MIC of ≥0.1 µg/ml, for RMP ≥4µ/ml were recorded as resistant to that drug. Both methods were observed for contamination.

Then TTC method was compared with LJ method as gold standard.

Randomly we selected 15 strains of *M. tuberculosis* among the tested and MIC was recorded again using both methods by different technician for reproducibility.

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

Method	INH		RMP	
	LJ	TTC	LJ	TTC
Susceptible	73(89.02%)	67(81.7%)	70(85.4%)	63(76.8%)
Resistant	09(10.9%)	15(18.3%)	12(14.6%)	19(23.1%)

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

LJ medium	TTC method		Total
	Susceptible	Resistant	
Susceptible	67(91.8%)	06(8.2%)	73
Resistant	00	09(100%)	09
Total	67(81.7%)	15(18.3%)	82

Sensitivity = 100%

Specificity = 60%

PPV = 91.8%

NPV = 100%

P ≤ 0.0001

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

LJ method	TTC method		Total
	Susceptible	Resistant	
Susceptible	62(75.6%)	08(9.7%)	70
Resistant	01(8.3%)	11(91.7%)	12
Total	63	19	82

Sensitivity: 98.4%

Specificity: 57.9%

PPV: 88.6%

NPV: 91.7%

P ≤ 0.0001

Table 4: Duration required by TTC assay for both drugs

Drug	Duration in days				
	2	5	7	10	15
INH	04(4.9%)	08(9.8%)	57(69.5%)	12(14.6%)	01(1.2%)
RMP	3(3.7%)	13(15.9%)	54(65.9%)	10(12.2%)	02(2.4%)

Results by seven days

INH – 69 (84.1%)

RMP -70 (85.45)

Mean- 5 ± 1.98 days

Results

Comparison of TTC assay for drug susceptibility testing of *M. tuberculosis* showed 9 (10.9%) strains as resistant to INH by LJ, and 15 (18.3%) by TTC assay. Similarly 11(14.6%) strains showed resistance for RMP by LJ and 19 (23.15%) by TTC method. Thus there was 91.8% concordance between two methods for INH and 90% for RMP. Statistical analysis of TTC assay for INH showed sensitivity (100%), specificity (60%), with PPV for resistance (91.8%) and NPV for resistance (100%).

Similarly for Rifampicin TTC assay showed sensitivity (98.4%), specificity (57.9%) with PPV (88.6%) and NPV (91.7%) respectively. As final results by LJ based assay were available after 28 days, consistent results by TTC assay for INH were available for 69 (84.1%) and 70 (85.4%) strains for RMP by 7 days. Average days required were 5±1.98 days. Contamination observed on LJ was 01 (1.22%) and 3 (3.6%) for TTC assay.

Discussion

As management of tuberculosis is through chemotherapy using combination of drugs, emergence of multidrug resistant strains have challenged this strategy. Treatment of tuberculosis without the benefit of susceptibility pattern increases the risk of treatment failure and then spread of these resistant strains, and also the development of resistance to additional drugs. So there is need for simple, rapid, and affordable methods for detection of antibiotic susceptibility to define appropriate treatment regimens.

Basis of TTC assay is colourless TTC dye is reduced by dehydrogenase enzyme present in living cells to produce insoluble red TTC formazan crystals. This property is exploited as tool in cell biology for measuring the metabolic activity, drug susceptibility against, bacteria, fungi, etc.⁽¹⁰⁾

In our study we observed that TTC gives 91.8% concordant results for INH and 90% for RMP, which are consistent with Foongladda S. et al, who showed sensitivity of 92.35% for INH and 94.1% for RMP. They have shown higher specificity than ours for both

INH and RMP (99.53% and 100%). They observed PPV for resistance for INH and RMP as 97.7% and 100% respectively.⁽¹¹⁾ Pottathil S et al have shown that direct TTC assay could demonstrate sensitivity and specificity of 99.2% and 82.4% respectively with PPV and NPV of 99.2% and 88.5%.⁽¹²⁾

De Logu A et al has reported 100% correlation for all primary drugs.⁽¹³⁾ Similar observations of 100% sensitive and specific results are reported by Caviedes L et al.⁽¹⁴⁾ Abate G et al. performed colorimetric assay on direct clinical samples and found that the susceptibility results obtained with MTT concurred fully with findings obtained using the standard assay on 7H10 agar medium.⁽¹⁵⁾ Franzblau et al found 87.9% correlation between colorimetric method and BACTEC 460 system.⁽¹⁶⁾

Mohammedzah A et al showed that TTC method is 100% sensitive and 92% specific.⁽⁹⁾ We found specificity of 60% and 57.9% because 06 strains for INH and 07 strains for RMP, showing discordant results had their MIC near cut off concentrations. So in LJ method they showed MIC below cut off concentration so recorded as susceptible. where as in TTC method as TTC being colorimetric method showed change in colour so were considered as resistant. Similarly De Loqu A et al showed One hundred percent of their isolates of *M. tuberculosis* demonstrating agreement between the susceptibility and resistance to isoniazid, rifampicin, and ethambutol obtained by the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) method and standard National Committee for Clinical Laboratory Standards approved standard method (M24-T2).⁽¹⁷⁾ Similarly Ghanaat J et.al observed TTC assay for INH and RMP to be 100% sensitive and 92% specific.⁽¹⁸⁾

Availability of results at the earliest plays important role in patient management. In our study mean duration required was 5 ± 1.98 days for both drugs together. Abate G et al has also reported the duration required as 9-12 days.⁽¹⁹⁾ Whereas Caviedes L. et al reported shortest duration of 5 days.⁽¹³⁾ Other workers Mohammadzadeh A. et al, Ghanaat J. et al and Raut et al have reported 4-7 days.^(9,18,20) Martin A et al has reported the 8days.⁽²¹⁾ Coban AY et al have also observed that the results were available by 7-10 days.⁽²²⁾ Reproducibility was 100%, as all strains showed consistent results.

Thus TTC assay is a rapid, sensitive and specific method for in-vitro drug susceptibility testing for *M. tuberculosis*. The technique is economic and easy to perform. No special instruments are required. Assay is reproducible. Being liquid culture method Bio-safety cabinet of class –II A is to be used.

References

1. Global Tuberculosis Report 2016. WHO/HTML / TB /2016.13 (www.tbfacts.org).

2. Global plan to end TB 2016-2020, Stop TB partnership www.stoptb.org.
3. Mahadev B, Srikantaramu N, James P, Mathew PG, Bhagirathi R. Comparison between Rapid colorimetric Mycobacterial isolation and susceptibility testing method and conventional method using L J medium. Ind. J. Tub. 2001;129-134.
4. Gerdes R, Domehl SC, Nardi G, Gismondo MR, Welscher HM, Pfyffer GE. Multicenter evaluation of mycobacterial growth indicator tube for testing susceptibility of Mycobacterium tuberculosis to first line of drugs. J. clin. Microbiol.1999;37:45-48.
5. Roberts GD, Goodman NL, Heifets L, Larsh HW, Linder TH, McClatchy JK, McGinnis MR, Siddiqi SH, Wright P. Evaluation of BACTEC radiometric method for recovery of mycobacteria and drug susceptibility testing of Mycobacterium tuberculosis from acid fast smear positive specimens. J. clin. Microbiol.1983;18:689-696.
6. Mengatto L, Chiani Y, Imaz MS. Evaluation of rapid alternative methods for drug susceptibility testing in clinical isolates of Mycobacterium tuberculosis. Mem Inst Oswaldo Cruz Rio de Janeiro 2006;10(5):535-542.
7. Lwanga SK, Lemeshow S. Sample size determination in health studies – A practical manual WHO. Geneva. 1991:25.
8. Canetti G, Fox W, Khomenko A, Mahler HT, Menon NK, Mitchison DA. Advances in techniques of testing mycobacterial drug susceptibility test in tuberculosis control programme. Bull WHO. 1969;41:21-43.
9. Mohammadzadeh A, Farnia P, Ghazvini K, Behdani M, Tahereh R, Ghanaat J. Rapid and low cost colorimetric method using 2,3,5 – triphenyl tetrazolium chloride for detection of multidrug – resistant Mycobacterium tuberculosis. J Med Microbiol 2006;55:1657-1659.
10. Rampersad SN. Multiple applications of Almar blue as an indicator of metabolic function and cellular health in cell viability bioassays. Sensors. 2012;12:12347 -12360.
11. Foongladda S, Roengsanthia D, Arjattanakool W, Chuchottaworn C, Chairprasert, Franzblau SG. Rapid and simple MTT method for rifampicin and Isoniazid susceptibility testing of *M. tuberculosis*. Int J Tubercle Lung Dis. 2002; 6:1118-1122.
12. Pottathil S, Singh V, Nair A. Isoniazid and Rifampin drug susceptibility testing: application of 2,3,5-triphenyl tetrazolium chloride assay and microscopic observation drug susceptibility assay directly on Ziehl –Neelsen smear positive sputum specimens. Braz J infect Dis. 2016;20(1):33-40.
13. De Logu A, Borgna R, Uda P, Sanna A, Pellerano ML Saddi B, The 2,3-bis (2- methoxy -4-nitro-5 sulfophenyl) -2H- tetrazolium (XTT) assay as rapid colorimetric method for determination of antibiotic susceptibility of clinical Mycobacterium tuberculosis isolates in liquid medium. Clin Lab, 2003;49(7-8):357-365.
14. Cavides L, Delgado J, Gilman RH. Tetrazolium microplate assay as a rapid and in-expensive colorimetric method for determination antibiotic susceptibility of *M. tuberculosis*. J Clin Microbiol 2002;40:1873-1874.
15. Abate G, Aseffa A, Selassie A, Goshu S, Fekade B, Wolde Meskal D. Direct colorimetric assay for rapid detection of Rifampicin resistant Mycobacterium tuberculosis. J Clin Microbiol 2004;42:871-873.
16. Franzblau SG, Witzig RS, McLaughlin J, Torres P, Madico G, Hernandez M, Dengan T, Cook M, Quenzer VK, Ferguson RM, Gilman RH. Rapid, Low – Technology, MIC determination with clinical Mycobacterium tuberculosis isolates by using the

- microplate Almar blue assay. *J Clin Microbiol* 1998;36(2):362-366.
17. De logu A, Pellerano ML, Sanna A, Pusceddu MC, Uda P, Saddi B. Comparison of the susceptibility testing of clinical isolates of *Mycobacterium tuberculosis* by the XTT colorimetric method and the NCCLS standard method. *Int J Antimicrob Agents*. 2003;21(3):244-250.
 18. Ghanaat J, Ghazvini K, Rashed T, Farnia P, Mohammadzadeh A, Behdani M. Use of 2,3,5-triphenyl tetrazolium chloride for detection of drug resistance in *M. tuberculosis*. *ESCMID -16,2006*;1-4.
 19. Abate G, Mashana RN, Miorner H. Evaluation of a colorimetric assay based on 3-(4-5-Dimethylthiazol -2-yl)-2-5- diphenyl tetrazolium bromide (MTT) for the rapid detection of rifampicin resistance in *M. tuberculosis*. *Int J Tuber Lung Dis*, 1998;2:1011-1016.
 20. Raut U, Narang P, Menditatta DK, Narang R, Deotale V. Evaluation of rapid MTT tube method for detection of drug susceptibility of *M. tuberculosis* to Rifampicin and Isoniazid. *Ind J Med Microbiol*, 2008;26(3):222-227.
 21. Martin A, Paasch F, Docx S, Fissette K et al. Multicentre laboratory validation of the colorimetric redox indicator (CRI) for the rapid detection of extensively drug resistant (XDR) *M. tuberculosis*. *J. Antimicrob Chemother*. 2011;66:827-833.
 22. Coban AY, Akbal AU, Uzun M, Durupinar B. Evaluation of four colorimetric susceptibility tests for the rapid detection of multidrug resistant *Mycobacterium tuberculosis* isolates. *Mem Inst Oswaldo Cruz*. 2015;110(5):649-654.