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Comparison of the modified Wayne's test with the MGIT 960 for detection of Pyrazinamide resistance in Mycobacterium tuberculosis isolates.

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ABSTRACT

Pyrazinamide (PZA) has an excellent sterilizing activity against semi dormant tubercle bacilli. When used in combination with Rifampicin the treatment has been shortened from 1year to 6 months. PZA is a pro drug which requires an acidic environment for its conversion into active form of Pyrazinoic acid. The in vitro drug susceptibility for PZA is not performed routinely as the culture medium should be acidified which in turn affects the growth of Mycobacterium tuberculosis. Currently, the Mycobacterial Growth indicator tube is considered to be the Gold standard for PZA drug susceptibility testing. In the present study, the performance of the Modified Wayne's enzymatic test which detects the presence of the PZase enzyme required for the conversion of PZA into its active form was assessed by comparing it with the MGIT. 130 Mycobacterium tuberculosis culture isolates were tested by both the methods and their results were compared. The Wayne's Enzymatic assay showed an overall efficiency of 97.69%.The assay can be a good substitute for the MGIT in resource limited settings as the test is performed using reagents and chemicals readily available in a functional TB culture and Drug Susceptibility Laboratory .

Keywords: PZA, Modified Wayne's enzymatic assay, Drug Susceptibility testing, MGIT

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INTRODUCTION

Pyrazinamide (PZA) is an important anti TB drug used in the treatment of both drug sensitive and drug resistant form of Tuberculosis. The drug has an excellent sterilizing activity against semi dormant tubercle bacilli. When used in combination with Rifampicin, the treatment has been shortened from 1year to 6 months[1]. Pyrazinamide is a pro drug which requires an acidic Ph (5.5) for its conversion into the active form Pyrazinoic acid 1. Hence, many Mycobacterial laboratories do not routinely undertake Drug Susceptibility testing of Pyrazinamide as the culture medium has to be acidified which in turn affects the growth of Mycobacterium tuberculosis [2]. There are two methods widely used for PZA susceptibility testing. In the proportion method, Middlebrook 7H10 agar medium at pH 5.5 is used, with 25-50 µg/ml PZA. Colony counts, on the drug-free and drug containing medium, determine susceptibility. However, the pH of 5.5 is detrimental to Mycobacterial growth and a significant number of test results cannot be determined because of poor growth or lack of growth. The other method is the radiometric BACTEC 460TB method. Here, the BACTEC 12B medium is modified by reducing the pH to 6.0. At this pH, mycobacteria grow better than at pH 5.5. To compensate for the increase in the pH, the concentration of PZA is increased to 100 µg/ml. PZA susceptibility testing with BACTEC 460TB system has proven to be satisfactory and has been recommended by the Clinical and Laboratory Standards [3] Institute (CLSI, previously known as NCLS).The BACTEC MGIT 960 PZA test method is developed on the same principle as the BACTEC 460 method except that it is a non-radiometric method and results are automatically interpreted by the instrument. Results obtained by the MGIT method correlate well with those obtained by the BACTEC 460 method. Most laboratories have now replaced the 460TB system with the nonradiometric Bactec MGIT 960 (BT960) system (Becton Dickinson, Sparks, MD). The BACTEC MGIT 960 PZA susceptibility test is a qualitative method by which results are obtained within the 4th – 21st day after [4]. The medium used is modified 7H9 with a reduced pH of 5.9. Growth is detected by the oxygen sensor at the bottom of the tube in a manner similar to that in a regular MGIT tube. The machine detects and reports resistance to the drug based on the number of growth units in the growth control and the test medium. There are reports citing technical problems with *in vitro* testing of *M. tuberculosis* with PZA. None of the methods described give 100% agreement when compared with the 460TB reference method, and most cite problems with false resistance [5-9]. The size, quality of inoculum and the concentration of the Pyrazinamide drug used seem to be the contributing factors for the non reproducible results or false resistance results reported by the MGIT 960 as indicated in many studies.

The Pyrazinamidase (PZase) enzyme encoded by the pncA gene is responsible for converting the prodrug into its active form [10]. Mutations in the pncA gene led to complete loss or reduction in PZase enzyme activity [11]. Mycobacterium tuberculosis isolates which were found to be resistant to Pyrazinamide had reduced or no PZase activity. The PZase enzyme activity can be detected using the Wayne's enzymatic test [12] which monitors the hydrolysis of PZA to the active acid form, pyrazinoic acid (POA), through the color change of a ferrous ammonium phosphate solution added to the medium.

MATERIALS AND METHODS

Cultures:

130 cultures of Mycobacterium tuberculosis which were stored at the National Reference Laboratory at National Tuberculosis Institute, Bangalore were used to evaluate the performance of Modified Wayne's Enzymatic assay. The Mycobacterial growth Indicator tube, MGIT 960 (BT960) system (Becton Dickinson, Sparks, MD) was used as the gold standard against which the results of the enzymatic assay were compared.

Drug susceptibility testing of PZA by MGIT (960):

For the PZA drug susceptibility testing, growth from the Lowenstein Jensen medium was sub cultured into fresh MGIT tubes. Once the tubes flagged positives, they were picked up and PZA DST was set up. The media for performing the PZA drug susceptibility testing consists of the BACTEC MGIT 960 PZA medium tube (containing 7ml of Modified Middlebrook 7H9broth), ,the PZA drug kit (consisting of the lyophilized a PZA drug , 20,000µg) and growth supplement. The lyophilized drug was reconstituted with 2.5ml of sterile distilled water. A freshly positive MGIT tube was used for the test. As per the manufacturers instructions, the test was set up in a two AST Set Carrier where the Growth Control and PZA tubes were placed in sequence. The test has a 21 day protocol and the machine declares results as "Resistant" or "Susceptible".

Modified Wayne's Enzymatic Assay:

The assay was performed as described by Singh etal, 2006 [14]. Heavy growth of *Mycobacterium tuberculosis* isolates was required for performing the test.

Test Medium:

Middlebrook 7H9 broth was prepared to which 400µg of PZA drug powder and 1.5% of agar agar was added and autoclaved at 121 ^{0}C for 20 minutes. The medium was allowed to cool to 600 C after which the growth supplement was added to the medium. 4ml of the molten medium was then poured into Mc Cartney bottles and kept upright to solidify. After solidification, the entire batch of media was placed in the incubator at 37^0 C for sterility check. If the medium appeared slightly colored, contamination was suspected and was discarded.

Inoculation:

Medium was inoculated by stabbing it 3-4 times with 2-3 loopfulls of growth. The inoculated tubes were incubated at 37^0 C for 4 days. On the 4th day, the tubes were tested for the PZase enzyme. 1ml of freshly prepared 1% Ferrous ammonium sulphate was added to the medium.

Interpretation:

The tubes were observed for 20 mins. Pink to red color band in the sub surface of the medium indicates that the enzyme is active and is able hydrolyze the PZA drug into Pyrazinoic acid. The isolate was declared sensitive to PZA. In case of absence of a pink color band within 20 mins, the tubes were placed at 4^0 C and observed after 4 hrs. The result was declared to be sensitive if a pink color band appeared in the medium and resistant if there was no development of pink color.

Pink to red color band: Sensitive
Absence of color band: Resistant

RESULTS

Of the 130 isolates tested for Pyrazinamide drug susceptibility testing, 88 strains were found to be resistant and 39 were found to be sensitive to the PZA by both the methods. Three isolates showed discrepant results. The MGIT results were considered to be gold standard against which the PZase assay results was compared. To resolve the discrepancy both the tests were repeated, however, the same results were obtained and the discrepancy could not be sorted. Parameters such as Specificity, Sensitivity, Predictive value for Resistance, Predictive value for sensitive and overall efficiency of the Modified Wayne's assay was calculated. 2 false resistant and one 1 false sensitive result was observed. The specificity, sensitivity, positive & negative predictive value was more than 95%. The Wayne's enzymatic assay showed an overall efficiency of 97% proving to be reliable technique for detecting in vitro PZA resistance. The isolates were also categorized based on the states from which the samples were received and is found in Table 2.

Table No 1: Performance of PZA enzymatic assay against the MGIT960 as gold standard.

Parameter	MGIT	Modified Wayne's
Total resistance	89	90
Total sensitive	41	40
True Resistant	88	
False Resistant	2	
True Sensitive	39	
False Sensitive	1	
Specificity	95.12	
Sensitivity	98.97	
Predictive value for resistance	97.77	
Predictive value for sensitive	97.5	
Overall efficiency	97.69	

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Table No 2 : origin of isolates

Sl.No	State	Number & % of isolates received from different states
1	Maharashtra	30 (23)
2	Madhya Pradesh	6 (4.61)
3	West Bengal	8 (6.15)
4	Rajasthan	12 (9.23)
5	Jammu & Kashmir	21 (16.15)
6	Karnataka	25 (19.23)
7	Orissa	28 (21.53)
Total		130

The DST results of second line anti TB drugs for the isolates which were randomly picked for the study was available; hence their Drug resistance pattern was compared with the PZA susceptibility pattern. The second line DST pattern for the isolates which showed discrepant results in the PZase enzymatic assay was not analyzed. Mycobacterium tuberculosis isolates which were classified as MDR and showed resistance to Ofloxacin and any one of the aminoglycosides such as Amikacin, Kanamycin or Capreomycin are called Extensively Drug Resistant TB. The second line DST pattern fro 127 isolates are given in table No 3.

Table No 3: PZA susceptibility pattern among XDR's

PZA DST	2nd LINE DST RESULTS				
	K+O	A+O	C+O	K+A+O	K+A+C+O
PZA resistant (56)	15	1	0	8	32
PZA sensitive (18)	0	0	11	0	7
TOTAL:	74				

Table No 4: PZA susceptibility pattern among Non XDR's

PZA DST	2nd LINE DST RESULTS				
	K	A	C	O	K+A+C
PZA resistant (32)	3	3	0	18	8
PZA sensitive (21)	0	0	0	16	5
TOTAL:	53				

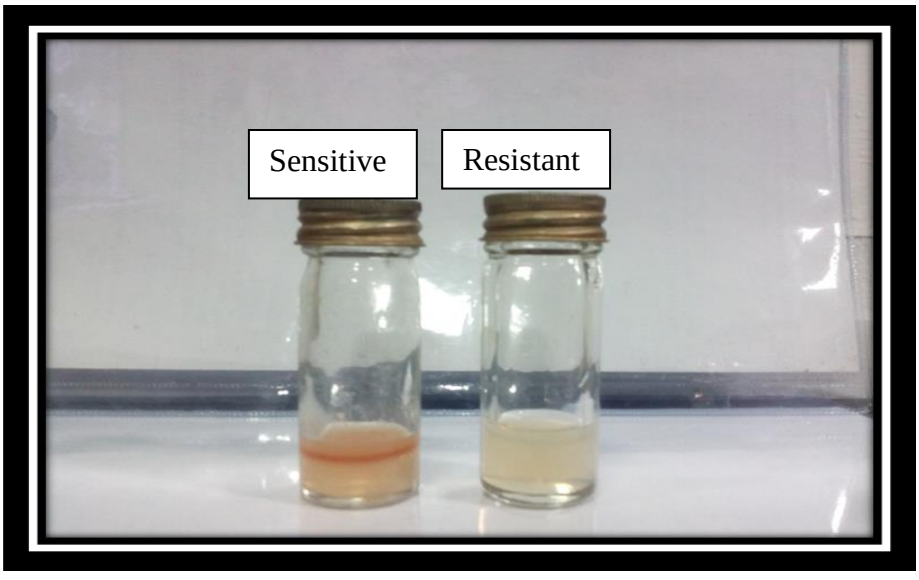

Fig 1: Modified Wayne's test: 20mins after adding 1% Ferrous ammonium sulphate

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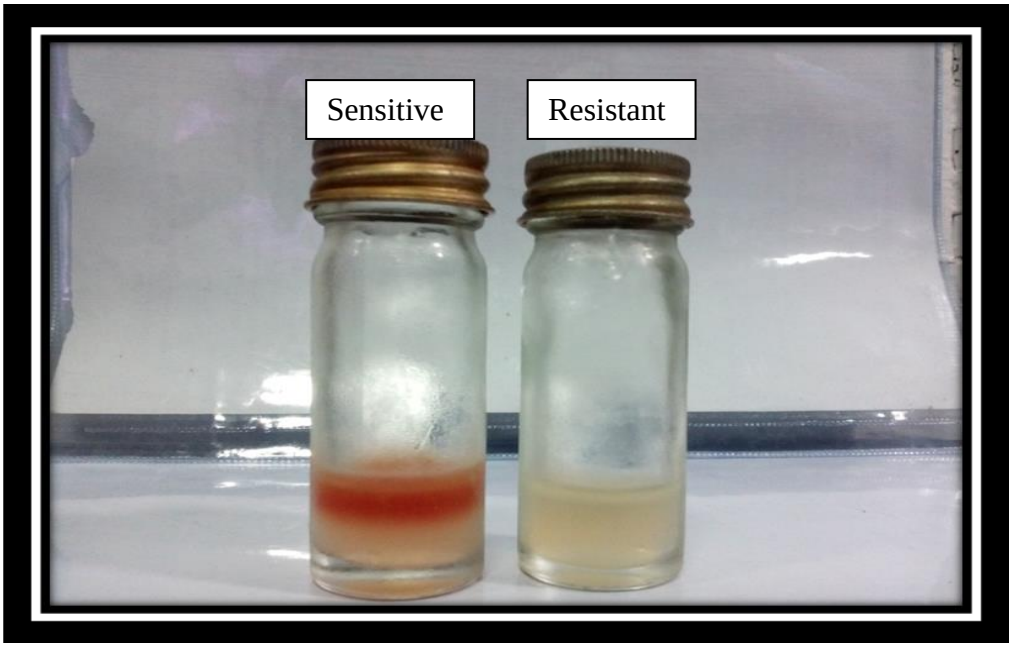

Fig 2 : Modified Wayne's test: after adding 1% Ferrous ammonium sulphate and 4hrs of refrigeration

DISCUSSION

The concordance between the MGIT 960 and PZase activity test in the current study was 97.69 per cent as indicated in Table No 1. Singh *et al* 13 reported 92.52 per cent concordance by BACTEC 460 and BacT/ALERT 3D system. However in another study on comparison between MGIT 960 and Wayne's method there was 97 per cent concordance between the two methods [11]. In our study 1 isolate was found to be resistant by MGIT 960 but was sensitive in the Wayne's enzymatic assay. In the study by Claudio Piersimoni *etal* [14], the MGIT 960 was observed to over report Pyrazinamide resistance as compared to the radiometric BACTEC 460 method. The authors claim that the large innoculum being used for the MGIT 960 was responsible for giving false resistance results. The inoculum containing large number of actively growing Mycobacterium tuberculosis cells would increase the pH of the medium thereby inactivating the action of Pyrazinamide. The authors have suggested that the inoculums size can be reduced to over come this difficulty to report correct results. Alternatively, the critical concentration of Pyrazinamide may be increased to 300µgm/ml as suggested by Heifets based on the Henderson Hasselbach equation [15-16].There have been studies which have reported that isolates which are resistant to PZA need not necessarily be negative for the PZase enzyme. The pncA gene may not show any mutation indicating an alternative mechanism of resistance, such as an elevated efflux pump mechanism [16-19]. In the current study, pncA sequencing was not performed to confirm resistance in the single isolate which showed resistance by the MGIT assay. This resistance may be due to large innoculum size or other factors contributing to increase in pH resulting in non conversion of the Pyrazinamide drug into its active form of Pyrazinoic acid.

On comparing the PZA drug susceptibility testing results with the second line Anti TB drugs DST pattern is was observed that 74 isolates (58%) were XDR and 53 isolates (41%) were non XDR's.

CONCLUSION

In conclusion, based on the results of the present study the PZase test has shown good correlation with the gold standard MGIT960 method. The PZA drug susceptibility testing by MGIT960 may not be affordable for all Mycobacterial culture and DST laboratories. In which case, the Modified Wayne's enzymatic assay can be performed. The classical Wayne's method may be difficult to interpret as very faint pink band is formed. This problem is overcome by the modified method as the medium is semitransparent and there is a very clear demarcation between positive and negative test result. The Modified Wayne's assay should be easy to implement in clinical TB laboratories, and it is performed using reagents and chemicals readily available in a functional TB culture and Drug Susceptibility Laboratory [20]. Particularly, the Modified Wayne's assay is

simple, rapid, accurate, inexpensive, and robust alternative for PZA susceptibility testing, not requiring sophisticated equipment and able to be implemented in low-resource countries.

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