Comparison of MGIT 960 & pyrazinamidase activity assay for pyrazinamide susceptibility testing of *Mycobacterium tuberculosis*

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*Background & objectives:* Pyrazinamide is an important front line antimycobacterial drug, which is also being used in the treatment of multi drug resistant tuberculosis along with second line drugs in DOTS plus programme. Conventional testing of pyrazinamide on solid medium is difficult as it is active at acidic pH. Therefore, there is a need for a rapid and simple method for susceptibility testing of pyrazinamide. This study was carried out to compare pyrazinamide susceptibility testing by MGIT 960 and two rapid pyrazinamidase activity tests.

*Methods:* Pyrazinamide susceptibility was tested in 136 clinical isolates of *Mycobacterium tuberculosis* by MGIT 960 and pyrazinamidase activity was tested by classical Wayne's method and modified PZase agar method.

*Results:* There was 88.9 per cent concordance between MGIT 960 and classical Wayne's method and 93.38 per cent with modified method for pyrazinamidase activity. Using MGIT 960 results as gold standard the sensitivity and specificity of Wayne's method was 88.15 and 90 per cent respectively and that of modified method was 89.4 and 98.3 per cent.

*Interpretation & conclusions:* Our study demonstrates that the modified pyrazinamidase activity test can be used as a screening test to detect resistance to pyrazinamide specially in resource limited settings but confirmation of susceptibility should be done by standard methods like MGIT 960.

*Key words* MGIT 960 - *Mycobacterium tuberculosis* - pyrazinamide

Pyrazinamide (PZA) is an important front line drug used in conjunction with isoniazid and rifampicin in short course chemotherapy to treat tuberculosis. It is a synthetic pyrazine analogue of nicotinamide and a prodrug, which is converted to the active form pyrazinoic acid by an enzyme pyrazinamidase (PZase). Rapid detection of drug susceptibility profile of *Mycobacterium tuberculosis* is important for patient management and in prevention of development of resistance. Susceptibility testing of PZA is technically difficult and not well standardized. Among the methods used, proportion method on solid medium (agar base or egg base) is universally accepted as gold standard. However, it has limitations as PZA has maximum *in vitro* activity at pH 5.5 at which many isolates of *M. tuberculosis* fail to grow and it takes 21 to 42 days to report the results. Moreover, the result depends on inoculum size and the most common problem is
false resistance when heavy inoculum is used which can lead to alkalization of medium\(^5\). To conquer these problems various semiautomated and automated methods have been developed which use liquid based medium at \(pH\) 5.9. These methods are BACTEC 460 TB system (Becton Dickinson Microbiology Systems, Sparks, Md.), MB/BacT system (Organon-Teknika, Durham, N.C.), ESP culture system II (AccuMed International, Westlake, Ohio), MGIT 960 (Becton Dickinson Microbiology Systems, Sparks, Md.) and BacT/ALERT 3D system (Bio-Merieux, Durham, NC.). These systems have shortened the turn around time.

Another method used for detection of PZA susceptibility is pyrazinamidase (PZase) activity test. It detects the presence of enzyme PZase required for the activation of the drug. In sensitive strains the enzyme is present and can be used as a marker of sensitivity while in resistant strains the enzyme is absent. PZase activity can be tested by Wayne’s method\(^6,7\) or thin layer chromatography method\(^8\). Wayne’s method is rapid but there is subjective error as a very faint band is formed that is difficult to interpret. A modified method by Singh \(et\ al^9\) has also been reported recently.

We undertook this study to compare MGIT 960 PZA susceptibility test with pyrazinamidase activity assays in 136 clinical isolates of \(M.\) \(tuberculosis\). Pyrazinamide susceptibility by MGIT 960 System has been shown to be as reliable as BACTEC 460 TB System\(^10\) and does not require radioactive material.

**Material & Methods**

A total of 136 isolates of \(M.\) \(tuberculosis\) were included in this study. All these isolates were obtained from clinical specimens received in the Department of Microbiology, SMS Medical College, Jaipur, for mycobacterial culture by MGIT 960 from January 2006 to February 2008. Positive MGIT tubes were inoculated on Lowenstein-Jensen (L-J) media and isolates were characterized as \(M.\) \(tuberculosis\) by standard biochemical reactions\(^2\) like Para nitro benzoic acid on LJ medium, niacin test, nitrate test, catalase test, etc. \(M.\) \(bovis\) species was excluded by susceptibility to Thiophen-2-carboxylic acid (TCH).

**PZA susceptibility testing by MGIT 960 system:**

Reagents - For PZA susceptibility the reagents were supplied by Becton Dickinson Diagnostic Instrument Systems, Sparks, USA. The culture media tube contained 7 ml of Middlebrooke 7H9 broth with \(pH\) adjusted at 5.9 and a fluorescent indicator in silicon rubber base. The drug was supplied in lyophilized form, reconstituted by adding recommended amount of sterile distilled water. The supplement was ready to use solution containing bovine serum albumin, catalase, polyoxyethylene stearate and oleic acid.

Test procedure - Two tubes were used for each isolate; one was drug free (control tube) and other containing 100 µg of PZA (test tube). To each tube 0.8 ml of supplement was added aseptically. The inoculum for sensitivity testing was prepared according to manufacturers instruction from the positive MGIT culture tube within one to five days of flagging as positive. From day one to two following positivity an undiluted inoculum was used, while from day 3 to 5 suspension was diluted 1:5 with sterile saline. The positive tubes that were more than 5 days old were subcultured in fresh MGIT culture tube. With a sterile pipette 0.5 ml of prepared inoculum was added into MGIT PZA tube and 0.5 ml of 1:10 dilution of this inoculum was used for control tube.

Interpretation - The test is based on ability of \(M.\) \(tuberculosis\) isolate to grow in drug containing tube compared to a drug free tube (growth control tube). The MGIT 960 instrument continuously monitors tubes for increased fluorescence, automatically interprets these results and reports as susceptible, resistant or invalid in 4 to 21 days.

\(M.\) \(tuberculosis\) \(H_37\)Rv strain was tested with each lot of MGIT 960 PZA medium and drug set for quality control. All positive MGIT tubes were checked for contamination by subculture on blood agar. Smear was prepared from positive tubes and stained by Ziehl-Neelson stain to confirm the presence of acid fast bacilli and Gram stain was done to rule out contamination.

**Pyrazinamidase activity test:** For PZase activity test the conventional method described by Wayne’s\(^9\) and modification described by Singh \(et\ al^9\) were used.

Wayne’s method - The medium consists of Dubos broth base, agar and sodium salt of pyruvic acid. PZA was added at a concentration of 100 µg/ml and distributed in glass tubes and sterilized by autoclaving. Test was done as per method described\(^7\).

Modified PZase agar method - The medium was composed of Middlebrooke 7H9 broth, glycerol and agarose. The PZA was added at a final concentration of 400 µg/ml. Four milliliter of this medium was distributed in glass tubes and sterilized by autoclaving. For each
isolate two tubes were used. A heavy inoculum (3 to 4 loopful of culture) of actively growing culture was inoculated into both of the agar butts. The tubes were incubated at 37°C. After 4 days of incubation 1ml of freshly prepared 1 per cent ferrous ammonium sulphate solution was added to one tube and refrigerated for 4 h. Any change of colour from pink to red was considered as positive. If the tubes were negative or doubtful the test was repeated after seven days of incubation with second tube.

Formation of a pink band in the subsurface of agar which diffuses into the medium indicates positive result. M. avium was used as positive control and M. bovis as negative control.

Resolution of discordance - Isolates with discordant results for any of three tests were retested. To resolve the discordance all the three tests were repeated once. For PZase test three tubes were inoculated and were read after 4, 7, and 10 days of incubation.

**Results**

All the resistant control strains used with each batch of test failed to produce PZase and were found to be resistant by MGIT 960 method also. The known susceptible control strains produced PZase and were susceptible by MGIT 960 method.

Of the 136 isolates, 67 were resistant and 52 were sensitive by all three methods. Discordant results were seen in 17 samples (Table). Four samples were resistant by both Wayne’s method and modified method but susceptible by MGIT 960. Another four samples were susceptible by MGIT 960 and modified method but resistant by Wayne’s method. Eight samples showed PZase activity by both methods while were resistant by MGIT 960 method, and one sample showed resistance by both MGIT 960 and modified method but was susceptible by Wayne’s method.

On repeat testing of the discordant isolates results generated by MGIT 960 system yielded the same results as those initially reported. After increasing the incubation time to 10 days two of the isolates with PZase negative in initial state turned positive by Wayne’s method and three by modified method.

The PZase activity was compared with PZA susceptibility test by MGIT 960 method. The sensitivity, specificity, and accuracy of Wayne’s method were 88.15, 90 and 88.97 per cent respectively and those for modified method were 89.4, 98.3 and 93.38 per cent. The positive predictive value was 91.78 and 98.5 per cent respectively for Wayne’s method and modified method (Table). The overall concordance was 87.5 per cent on initial testing, which increased to 93.3 per cent after repeat testing.

The average time of reporting PZA susceptibility results by MGIT 960 was 7.9 days and 82.5 per cent of samples were reported in 4 to 10 days.

**Discussion**

The need to test isolates of M. tuberculosis for PZA susceptibility has become more apparent as it is being used not only as front line drug but also in treatment of MDR-TB in DOTS plus programme along with other drugs. Use of PZA in treatment regimen has reduced the duration of therapy to 6 months. It has good sterilizing activity and kills the semi-dormant tubercle bacilli residing in acid environment. However, testing for susceptibility to PZA is a laborious procedure and required extreme specialization.

In our study 60 isolates were susceptible to PZA by MGIT 960; Wayne’s method identified 54 isolates (90%) and modified method 59 isolates (98.3%) as susceptible to PZA. Miller et al. reported 94.7 per cent and Trevedi and Desai found 94.6 per cent PZase positive strains as susceptible to PZA. Similar results were reported by Lu et al.

Seventy six isolates were reported as resistant to PZA by MGIT 960 in present study, of which 67

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<th>Modified method</th>
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**Performance parameters:**

| Sensitivity (%) | 88.15 | 89.44 |
| Specificity (%) | 90 | 98.3 |
| Accuracy (%) | 88.97 | 93.38 |
| Predictive value for resistance (%) | 91.78 | 98.5 |
| Predictive value for susceptibility (%) | 85.71 | 88.05 |
(88.15%) strains were reported resistant by Wayne’s method and 68 (89.44%) by modified method. The sensitivity reported by others ranged between 79-100 per cent.19,20,21.

The concordance between the MGIT 960 and PZase activity test in our study was 93.3 per cent while Lu et al19 reported 98 per cent concordance and Singh et al18 reported 92.52 per cent concordance by BACTEC 460 and BacT/ALERT 3D system respectively. In another study on comparison between MGIT 960 and Wayne’s method there was 97 per cent concordance between the two methods18.

In our study 8 isolates were resistant by MGIT 960 but were PZase positive. It is reported that isolates highly resistant to PZA are not always PZase negative19. This confirms the hypothesis that besides mutation in pnc gene an alternative mechanism of resistance to PZA exists, which may be related to the drug uptake20. Therefore, care should be taken in defining susceptibility solely on basis of PZase activity test.

PZase test is rapid, economical, and easy to perform in comparison to MGIT 960. The Wayne’s method is difficult to interpret as very faint pink band is formed. This problem is overcome by modified method as the medium is semitransparent and there is a very clear demarcation between positive and negative test result. There are some limitations of PZase activity test as the test can be used only for M. tuberculosis as some non-tubercular mycobacterium species are resistant to the PZA although they posses PZase21,22. Moreover, the test does not give the information about the concentration of PZA to which the culture is susceptible23. However, these tests can be recommended as a useful screening method for determining resistance to PZA9,24 particularly the modified method. MGIT 960 is a standard, rapid and reliable method but is not available in resource limited setting due to its expensive equipment and high running reagent cost.

To conclude, PZase activity by modified method is suitable for use in clinical microbiology laboratory. It is technicly easier to perform and interpretation is clear as pink band can be visualized easily. The test can be used as routine screening method for testing of resistance to PZA. Those samples in which tubes were negative on day 4, the second tube should be further incubated for 10 days before reporting. However, the PZase positive samples should be confirmed for susceptibility by MGIT 960 or other conventional methods.

References


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