Evolution of *In vitro* Drug Susceptibility Testing of Pyrazinamide

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Pyrazinamide is an important drug used in anti tubercular treatment which helps in killing old and semi dormant bacilli which are not metabolically active. Drug susceptibility tests are routinely performed before initiating multidrug drug resistant tuberculosis treatment. However, many Tuberculosis laboratories do not undertake drug susceptibility testing of Pyrazinamide as it is difficult to perform. *In vitro* drug susceptibility testing of Pyrazinamide requires acidic conditions which hinders the growth of TB bacilli. Hence, Nicotinamide a drug analog of Pyrazinamide was utilized for performing susceptibility testing. A literature review was undertaken and an inventory of in vitro tests for the drug susceptibility testing of Pyrazinamide was conducted. Media specifications and drug concentrations used for the susceptibility testing have also been described in chronological order. Solid culture method was used earlier, which gave consistent results but was time consuming. Liquid culture systems which had a shorter time of detection were also assessed and many studies were undertaken to evaluate their performance with respect to Pyrazinamide testing. The use of growth indicators and biochemical tests for Pyrazinamide testing has also been described.

**Key words:** Multi drug resistant tuberculosis, Pyrazinamide susceptibility testing, Nicotinamide, LJ Medium, MGIT 960.

Pyrazinamide (PZA) is an important first line anti Tuberculosis (TB) drug which is used along with Isoniazid, Rifampicin and Ethambutol in the DOTS as recommended by the World Health Organization (WHO) PZA is responsible for killing most of the persisting TB bacilli during the initial intensive phase of chemotherapy, allowing treatment to be shortened from 9 months to 6 months¹. It is interesting to note that PZA is the most active synthetic analog of vitamin B3 (Nicotinamide) that had inhibitory activity against *Mycobacterium tuberculosis* ². Studies of the analogs of Nicotinamide also led to the discovery of another powerful anti TB drug Isoniazid (INH) and Ethionamide².

PZA is a prodrug which enters passively into Mycobacterium tuberculosis and is converted to its active form Pyrazinoic acid (POA) by the intracellular bacterial enzyme Pyrazinamidase (PZase)². Since the efflux mechanism is down regulated in old, dormant bacilli POA begins to accumulate within the bacterial cell. POA exits from the cell by passive diffusion .When the extracellular pH is acidic a small portion of POA becomes uncharged HPOA which permeates through the membrane easily. The acid facilitated POA influx is apparently stronger than the weak POA efflux, so that there is an accumulation of POA in *Mycobacterium tuberculosis* cells. The gene responsible for the enzyme has been identified and mutations in this gene give rise to an altered enzyme which is unable to convert the drug into its active form thereby conferring resistance to the bacilli. Missense mutations causing amino acid substitutions, nucleotide insertions, deletions ,
nonsense mutations in the pncA structural gene or in the putative promoter region of pncA gene have been identified to produce alterations in the active site of the PZase enzyme. PZA was first demonstrated to have antitubercular activity in mouse by Krushner et al. in 1948 and by Malone et al. in 1952. Tarshis & Weed in 1953, Shwartz & Mayo, Stenken & Wolvisky in 1954 showed that PZA was devoid of in vitro activity against Mycobacterium tuberculosis. However, later studies by McDermott & Tompsett in 1954 revealed that PZA had in vitro activity only in acid medium of pH 5 – 5.5.

MATERIALS AND METHODS

In vitro methods used for Pyrazinamide susceptibility testing was searched using the internet. Key words such as “Pyrazinamide”, “PZA susceptibility testing” and “antitubercular drug susceptibility” testing were used for the search. Research articles which described novel methods of testing Pyrazinamide susceptibility testing were only reviewed.

In vitro susceptibility testing utilizing solid media

Based on previous studies which revealed the fact that PZA was active on Mycobacterium tuberculosis three acidic solid medium for drug susceptibility testing of PZA were conventionally being used. Steeken & Smiths acid medium (US veterin admin 1960), Lowenstein Jensen (LJ) medium acidified with citric acid and LJ medium acidified with Hydrochloric acid. All the three media had a pre inspissation Ph of 5.4-5.5. It was also found that the LJ medium acidified with Hydrochloric acid yielded the most satisfactory result.

In 1967 Stottmeir, Beam & Kubica developed a method for determining the susceptibility of tubercle bacilli to PZA in 7H10agar medium in Petri dish. The technique involved altering the contents of the buffer salts to provide a final Ph of 5.5. The plates were recommended to be read at 20 days of incubation to show the Minimal inhibitory concentration of PZA for Mycobacterium tuberculosis to be 18 – 22 micro perm.l. The study also showed that the acid environment of Ph 5.5 causes 50% inhibition in the colony count of Mycobacterium tuberculosis and a considerable reduction in colony size when compared with growth at ph 6.8.

In 1969, G.Cannetti & others described the proportion sensitivity method of drug susceptibility testing for first and second line drugs. The concentrations of the drugs and the inoculum used were described in detail. The LJ medium used for the PZA susceptibility testing was 4.9. Many strains grew well at this Ph. The inocula used for the PST was also 10 times more concentrated than the usually used for the other drugs. The results of the PZA susceptibility testing was found to be less reliable than that of the other drugs leading to misclassification of resistant strains as sensitive.

Brander in 1972, performed DST of PZA using Nicotinamide a drug analog of Pyrazinamide as suggested by Tan Thiam Hok in 1962. Nicotinamide did not require acidification of the medium for its activity and the growth of Mycobacterium tuberculosis was inhibited at 5mg/ml at neutral Ph. This was utilized to differentiate Mycobacterium tuberculosis from Non Tuberculous Mycobacteria by Kestle & Abbot and by Kubica in 1967. In Tan Thiam Hoks’s method LJ medium of pH around 7 was used and the concentration of Nicotinamide was 100 times greater than is needed for growth inhibition in the acid medium employing PZA.

Butler & Kilburn in 1982 described a method employing the commercially available 7H10 medium at Ph 5.5 with the use of ADC instead of the routinely used OADC. Oleic acid had some inhibitory effect on the growth of Mycobacterium tuberculosis, but avoiding OADC also did not always result in growth on some control plates. In 1954, McDermott and Tompsett studied the PZA activity in Tween Albumin and in Oleic acid – Albumin broth and determined the MIC macroscopically (visible bacterial growth in 2 weeks).

In vitro susceptibility testing utilizing liquid media

A rapid method to obtain growth of Mycobacterium tuberculosis is by using liquid culture. Traditionally, liquid cultures such as 7H9, Middlebrooks etc were used in the TB laboratory for preservation or for maximizing growth obtained from solid media. In recent times, many automated liquid culture systems have been developed and their performance have been evaluated against the
Table 1. Performance of automated liquid culture systems

<table>
<thead>
<tr>
<th>S. No</th>
<th>Author, publication year</th>
<th>Media used</th>
<th>Drug &amp; Concentration used</th>
<th>No. of isolates tested</th>
<th>Results/ Major findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1988, Max Salfinger &amp; Heifets</td>
<td>BACTEC vs Wayne’s test</td>
<td>PZA 100µg/ml</td>
<td>428</td>
<td>Correlation for susceptible 98.2%, for resistant 100%</td>
</tr>
<tr>
<td>2.</td>
<td>1995, Mark Miller et al 13</td>
<td>BACTEC – Two pH 5.5 &amp; 5.8</td>
<td>MIC of PZA At pH 5.5 &amp; 50 µg/ml At pH 5.8 &amp; 400µg/ml</td>
<td>36</td>
<td>90% of strains had an MIC of 50 µg/ml at pH 5.5 &amp; 5.8</td>
</tr>
<tr>
<td>3.</td>
<td>1996, R.R. Cutler et al 14</td>
<td>BACTEC vs Biphasic LJ with semi solid Krishners media &amp; Wayne’s test</td>
<td>BACTEC- Ph 5.9 &amp;100mg/l Biphasic LJ- Ph 5.2 &amp; 66mg/l</td>
<td>34</td>
<td>BACTEC- 80% *RPV % LJ– ^SPV 66% RPV 50% Wayne’s test- 75%SPV</td>
</tr>
<tr>
<td>4.</td>
<td>1999 M. Mestdagh 15</td>
<td>BACTEC vs Wayne’s test</td>
<td>PZA 100µg/ml Wayne’s test &amp; pncA mutations</td>
<td>62</td>
<td>92.8% of isolates which showed PZase negative showed resistance on BACTEC.</td>
</tr>
<tr>
<td>5.</td>
<td>1982 Butler and Kilbur 16</td>
<td>Middlebrook and Cohn 7H10 medium</td>
<td>PZA 25 µg/ml Ph5.5</td>
<td>76</td>
<td>90% of the strains grew at Ph5.5 when ADC was used to pretest the growth supportive ability.</td>
</tr>
<tr>
<td>6.</td>
<td>2004 A. Krishnamurthy et al 17</td>
<td>Wayne’s test vs BACTEC &amp; LJ proportion method</td>
<td>BACTEC - 100µg/ml LJ - 100µg/ml</td>
<td>130</td>
<td>Sensitivity- 83.33% Specificity- 100%</td>
</tr>
<tr>
<td>7.</td>
<td>2007, P. Singh et al 18</td>
<td>BACTEC vs BACTEC &amp; LJ proportion method &amp; Wayne’s test</td>
<td>BACTEC - 100µg/ml LJ - 100µg/ml</td>
<td>107</td>
<td>LJ as gold standard; Sensitivity of BACTEC &amp; Wayne’s test- 100% &amp; 82.85%, Specificity – 98.61%</td>
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<td>8.</td>
<td>2008, Shabhada et al 19</td>
<td>MGIT 960 vs Wayne’s test &amp; pncA mutations</td>
<td>PZA 100µg/ml</td>
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<td>Sequencing as gold standard BACTEC sensitivity-97%, specificity- 100%, Wayne’s test- sensitivity 92%, specificity 81%</td>
</tr>
</tbody>
</table>

*RPV – resistance predictive value; *SPV-susceptible predictive value
traditional “gold standard” for TB culture work which is LJ medium. Studies which have evaluated such automated liquid culture systems are given below in Table 1.

**Calorimetric methods using Nicotinamide to detect Pyrazinamide resistance**

During recent years many studies have evaluated the accuracy and feasibility of calorimetric methods using different growth indicators for detecting resistance in *Mycobacterium tuberculosis*. These methods appear to be faster than the conventional DST method and are less expensive than the various automated systems available\(^{20}\). Calorimetric methods are based on the color change which occurs as a result of reduction of the colored indicator added to the culture medium. Resistance is detected as change of color in the medium. Different indicators have been evaluated, such as MTT-(3- 4,5- dimethylthiazol- 2-yl), XTT (2,3-bis2-methoxy-4-nitro-5-sulfophenyl-2H-tetrazolium-5-carboxanilide), Alamar blue, Malachite green and Resazurin.\(^{20,31}\) The DST was performed in microtitre plates with turn around times between 7 and 14 days. The calorimetric assays are performed on culture isolates which means that extra time of 2- 6 weeks would be required for primary isolation.

**Use of biochemical tests**

The Nitrate reductase assay (NRA) is routinely performed as part of the biochemical identification of *M. tuberculosis* in most TB laboratories. The technique is based on the property of *M. tuberculosis* to reduce nitrate to nitrite which is revealed as a color change of the culture media using Griess method\(^{30}\). With the NRA it was possible to obtain results in 7-14 days. Martin _et al._ in 2005 have shown in a multicentric evaluation study that NRA may be utilized to conduct DST for anti TB drugs\(^{21}\). Based on the same the principle they also evaluated DST for PZA using Nicotinamide (1000, 500,250mg/L) at neutral pH\(^{22}\). The results were compared with MGIT960 which gave sensitivity and specificity of 91% and 94% respectively. Mirabal _et al._ in 2010 also reported similar results when the NRA was used for PZA testing\(^{23}\).

**Use of MODS**

The Microscopic observation drug susceptibility assay (MODS) uses liquid media and is fast to perform. The method is based on the principle that *M. tuberculosis* grows faster in liquid medium and its characteristic growth ‘cord formation’ can be observed through an inverted light microscope. Several studies have used this method to detect resistance to Isoniazid and Rifampicin. Ghiraldi _et al._ in 2011, utilized the MODS assay for PZA susceptibility testing where concentrations of 6.25 – 3200mg/ml was used\(^{24}\). The specificity and sensitivity of MODS and Broth Microdilution method was 100%.

**CONCLUSION**

There are no reliable tests available for the drug susceptibility testing of Pyrazinamide. This is because of the difficulty in developing a test with PZA concentrations equivalent to in vivo concentrations of 16-60µg/ml which are attainable in blood of tissue\(^{25}\). These low concentrations are active in vitro only at pH 5-5 – 5.6 ,and most TB bacilli are inhibited at such low Ph. Studies have shown that more than 10% of the isolates do not grow at this Ph\(^{25,35,36,37}\). In order to overcome this problem Salfinger _et al._ suggested using a high concentration of PZA, of 300 or 400µg/ml at Ph 6.0 – 6.2 in 7H12 broth\(^{22}\). They also reported that some strains even grew well at this pH than at the pH normally used for TB bacilli cultivation. Since the in vitro concentration of PZA is very high, the test results cannot be correlated with pharmacokinetic parameters. Zhang _et al._ have made theoretical assumptions based on Henderson –Hasselbach equations in regard to concentrations of PZA needed to inhibit growth of *M. tuberculosis* at different pH values. The equation predicted a Ph of 6.0 for an MIC of 200µg/ml. The paper by Zhang _et al._ alerts clinical laboratories of the possible causes of discrepancies in PZA test results when acidic pH and too low PZA concentrations was used\(^{12, 26}\). Heifets suggested based on earlier studies that at Ph 6.0-6.2 and a concentration of 300µg/ml in 7H12broth and 900-1200µg/ml in the new agar medium containing 10% foetal bovine serum provides the most reliable reports for distinguishing PZA susceptible and PZA-resistant *M. tuberculosis* isolates\(^{25}\). Tripathy and D.A. Mitchison had employed LJ medium at acidic Ph for Pyrazinamide susceptibility testing and defined the resistance based on four criteria. A minimal inhibitory concentration of 200µg/
ml or more employing an innoculum containing approximately 0.4mg (moist wt) of bacilli per ml, and a 10-colony end point, and proportions of 20% or more on 25µg/ml, or 5% or more on 50µg/ml and 1% or more on 100µg/ml. The efficiency of the four definitions were of the same order28.

In 2007, H.Zhang et al characterized Pyrazinamidase/Nicotinamidase and found that the monomeric protein had an optimal enzymatic activity at Ph 7.027. In contrast to earlier studies, they demonstrated that both the enzymes had similar hydrolytic activity for the substrates Nicotinamide & Pyrazinamide27.

To avoid the problem of acidic pH in PZA susceptibility testing, Brander in 1972 had developed a PZA susceptibility testing using high concentrations of Nicotinamide (500, 1000, 2000µg/ml) at neutral Ph in LJ medium. Although this method gave reliable results it was not widely adopted as reported by Y.Zhang & D.Mitchison in 20032. They also pointed that it would be prudent to use the Brander technique in epidemiological studies for determining the prevalence of PZA resistance.

Although the MGIT 960 has been considered to be the “gold standard” for PZA susceptibility, the study by Pamela Chedore et al points out that the system is prone to give erroneous results28. The false results are mainly due to the use of a lower drug concentration (100µg/ml) supplied by the manufacturer and variations in the size of the inoculum28, 30.

In conclusion based on the studies which were reviewed, it is felt that PZA resistance may be determined by using a combination of two in vitro tests22,31,34,40; the Wayne’s PZase enzymatic assay29 and DST using Nicotinamide at high concentrations on LJ medium. A rapid alternative for the DST would be to use any of the calorimetric tests such as MTT, Alamar blue, Resazurin, or Malachite green in liquid medium.

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