Comparative Evaluation of Löwenstein-Jensen Proportion Method, BacT/ALERT 3D System, and Enzymatic Pyrazinamidase Assay for Pyrazinamide Susceptibility Testing of *Mycobacterium tuberculosis*\(^\text{V}\)

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Pyrazinamide (PZA) is an important first-line antituberculosis drug because of its sterilizing activity against semidormant tubercle bacilli. In spite of its very high in vivo activity, its in vitro activity is not apparent unless an acidic environment is available, which makes PZA susceptibility testing difficult by conventional methods. The present study was, therefore, planned to assess the performance of the colorimetric BacT/ALERT 3D system and compare the results with those from conventional tests, i.e., the Löwenstein-Jensen (LJ) proportion method (pH 4.85) and Wayne’s pyrazinamidase (PZase) assay, using 107 clinical isolates. The concordance among all of these tests was 89.71% after the first round of testing and reached 92.52% after resolution of the discordant results by retesting. Prolonged incubation of the PZase tube for up to 10 days was found to increase the specificity of the PZase test. The concordances between LJ proportion and BacT/ALERT 3D, LJ proportion and the PZase assay, and BacT/ALERT 3D and the PZase assay were found to be 99.06%, 93.46%, and 92.52%, respectively. Using the LJ results as the gold standard, the sensitivities of BacT/ALERT 3D and the PZase assay were 100 and 82.85%, respectively, while the specificity was 98.61% for both of the tests. The differences in the sensitivities of BacT/ALERT 3D and the PZase assay were significant (P = 0.025). The mean turnaround times for the detection of resistant and susceptible results by BacT/ALERT 3D were 8.04 and 11.32 days, respectively. While the major limitations associated with the PZase assay and the LJ proportion method are lower sensitivity in previously treated patients and a longer time requirement, respectively, the BacT/ALERT 3D system was found to be rapid, highly sensitive, and specific.

Pyrazinamide (PZA) is a nicotinamide analog which is used as a frontline drug to treat tuberculosis. PZA has a special place in modern tuberculosis therapy, as it appears to kill a population of semidormant tubercle bacilli persisting in the body (22). The addition of PZA to the favored short-course regimen of isoniazid plus rifampin has facilitated shortening of the treatment duration from 9 months to 6 months. In spite of this remarkable role of PZA, it still remains a paradox because of its incompletely understood mode of action (35–39), which is also reflected in the difficult procedures for its susceptibility testing (11, 13, 40).

Multidrug-resistant strains of *Mycobacterium tuberculosis* have been emerging worldwide in both high- and low-income countries. The need for rapid methods of the diagnosis and determination of drug susceptibility is particularly important. While the procedures for susceptibility testing of the most of the first-line and second-line drugs have been well standardized in both liquid and solid media (6), the main problem with the susceptibility testing of PZA is the requirement of acidic pH for PZA activity (8, 16, 20, 26, 28).

The difficulty in obtaining PZA susceptibility results in acidic media by routinely used procedures for some *M. tuberculosis* strains makes the detection of pyrazinamidase (PZase) activity an interesting alternative to the conventional procedures (15, 19). This assay detects the presence of active PZase enzyme by the hydrolysis of PZA to pyrazinoic acid as evidenced by a color change (33). The application of various rapid phenotypic methods such as radiometric BACTEC 460 (12), fluorimetric Mycobacteria Growth Indicator Tube (MGIT) 960 (Becton Dickinson) (1, 23), ESP Culture System II (Trek Diagnostic Systems, Westlake, OH) (18), and the colorimetric BacT/ALERT 3D system (bioMérieux Inc., Durham, NC), previously designated MB/BACT (Organon Teknika, Boxtels, The Netherlands) (2, 4), has been reported to be useful for rapid and reliable susceptibility testing of *M. tuberculosis* isolates. The performances of these systems have been evaluated in comparison to one another as well as with the conventional proportion method for first-line drugs, including rifampin, isoniazid, ethambutol, and streptomycin (1–4, 12, 17, 18, 21, 23, 25, 34). A similar experience for PZA in MB/BacT is limited (2, 4, 24, 29). So far,
there has been no study comparing the performances of the Löwenstein-Jensen (LJ) proportion method, Wayne’s enzymatic PZase assay, and the BacT/ALERT 3D system for PZA susceptibility testing. The present study was, therefore, undertaken to evaluate the BacT/ALERT 3D system and compare the results with those of the Löwenstein-Jensen (LJ) proportion method and the enzymatic PZase assay for PZA susceptibility testing of M. tuberculosis.

MATERIALS AND METHODS

M. tuberculosis isolates. A total of 107 biochemically characterized clinical isolates of M. tuberculosis (32) with known LJ proportion susceptibility testing results (36 resistant and 71 susceptible to PZA) were included in this study. They were collected during the period of March 2004 to August 2005 from different parts of India and belonged to treated as well as untreated patients (78 previously treated and 29 untreated cases).

The PZase-susceptible reference strain M. tuberculosis H₃₇Rv (TMC-102) was obtained from the Mycobacterial Repository Center at the National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Agra, India. These isolates were subcultured on LJ medium.

PZA susceptibility testing by the LJ proportion method. For this method, dilutions prepared from the standard (1 mg/ml) clump-free suspension (13) were inoculated onto LJ medium as described previously (6, 30). An isolate was considered resistant if it yielded a growth equal to or more than 1% at a PZA concentration of 100 μg/ml compared to the drug-free acid control (pH 4.85) (30). For every batch of medium and every round of susceptibility testing, one susceptible and one resistant control were used to ensure reproducibility.

PZA susceptibility testing by the BacT/ALERT 3D system. In this method, the Mycobacteria Process bottles with 7H9 medium were supplemented with enrichment fluid (oleic acid, glycerol, and bovine serum albumin) and 2 ml of acidifying solution (0.067 M KH₂PO₄) was added to each bottle. PZA (Sigma) stock solution was added to the bottles so as to attain a final concentration of 100 μg/ml. Equal amounts of sterile distilled water were added to the drug-free control bottles. A 0.5-ml bacterial suspension adjusted to a 0.5 McFarland turbidity was used as an inoculum for the drug-containing bottles, and a 100% diluted suspension was inoculated in the drug-free control bottles (1% proportional control). The bottles were loaded in the instrument’s incubation module at 37°C. If the bottle containing PZA flagged positive at the same time as or before the bottle containing the drug-free proportional control, the isolate was considered resistant. The test was considered valid only if the results of the proportional growth control were available within 20 days. Reference strain M. tuberculosis H₃₇Rv was used as the susceptible control, and isolates found to be PZase negative and resistant by LJ proportion sensitivity testing were used as the resistant controls. All positive bottles were smeared and stained with the Ziehl-Neelsen method to confirm the presence of acid-fast bacilli.

PZase assay. The PZase assay was done as described by Wayne (33), with slight modifications briefly described below.

(i) Preparation of modified PZase agar. 7H9 broth base (2.35 g) (Difco) was dissolved in 450 ml water, to which 2 ml glycerol (instead of sodium pyruvate, which gives yellowish tint to the medium), PZA (Sigma) at a final concentration of 400 μg/ml (instead of 100 μg/ml as described previously [33]), and 1.5% agarose were added. Four-milliliter aliquots of this melted PZase agar were distributed in glass tubes and autoclaved. The tubes were then kept upright to allow the agarose to distribute in the glass tubes and autoclaved. The tubes were then kept upright to allow the agarose to distribute in the glass tubes. The tubes were then kept upright to form the butt. Modifications in medium formulation for the PZase assay were basically aimed at preparing the semitransparent media with higher PZA concentrations so that even a faint pink band could be detected easily against white background.

(ii) Inoculation of modified PZase agar and interpretation. A heavy loopful (8 to 10 mg) of actively growing culture was carefully inoculated on the surface of the modified PZase agar medium and incubated at 37°C for 4 days. One milliliter of ferrous ammonium sulfate (1%) was added to each tube after incubation and observed for an initial 4 h for the appearance of a pink band (positive) in the subculture agar. PZA-resistant isolates of M. tuberculosis found to be negative by the PZase test earlier were used as negative controls, and the PZA-susceptible strain M. tuberculosis H₃₇Rv was used as positive control with this modified PZase medium. The PZase assay was interpreted independently by two observers who did not know the results of PZA susceptibility testing by either the LJ proportion method or the BacT/ALERT 3D system.

TABLE 1. Comparison of PZA susceptibility testing results for LJ proportion method, BacT/ALERT 3D system, and PZase assay.

<table>
<thead>
<tr>
<th>Test</th>
<th>Result for indicated testa</th>
</tr>
</thead>
<tbody>
<tr>
<td>LJ proportion method</td>
<td>S  S  S  R  R  R</td>
</tr>
<tr>
<td>BacT/ALERT 3D system</td>
<td>S  S  R  S  R  R</td>
</tr>
<tr>
<td>PZase assay</td>
<td>S  R  S  S  R</td>
</tr>
</tbody>
</table>

a S, susceptible; R, resistant.

Resolution of discordance. Isolates with discordant result for any of the three tests were restested in order to resolve the discordance.

Analysis of data. Using LJ proportional susceptibility testing results as the gold standard (9), the performance parameters of the BacT/ALERT 3D system and the PZase assay were determined as recommended by Enarson et al. (10) and also as described by Barreto et al. (2). Fisher’s exact test (two-tailed) at a 5% level of significance was applied to determine the statistical significance of the difference between the sensitivities of both of these tests.

RESULTS

All of the controls yielded reproducible results by all three methods. The results of 107 test isolates by different methods/systems showed that 67 isolates were susceptible and 29 were resistant to PZA by all three methods whereas 11 isolates showed discordant results (Table 1). Among the isolates with discordant results, six were resistant by LJ and the BacT/ALERT 3D system but susceptible by the PZase assay. One isolate was resistant by the LJ proportion method only, whereas another was resistant by BacT/ALERT 3D only. Three isolates were resistant by the PZase assay only.

Isolates with discordant results were restested, and the results of the repeat test are shown in Table 2. Upon repeat testing, one isolate initially found to be resistant by the LJ proportion method only turned susceptible while another isolate resistant by the BacT/ALERT 3D system only was again found to be resistant at 100 μg/ml but susceptible at 300 μg/ml. Two isolates which were PZase negative in the first test became positive when incubated for 10 days in the repeat test, whereas a third isolate still remained negative. Six PZase-positive isolates with a resistant phenotype on LJ medium as well as BacT/ALERT 3D were indeed found to be the cases of a mixed population of resistant bacilli among a predominantly (45 to 80%) susceptible population.

Using repeat LJ results as the gold standard, the performance parameters of the other two systems were determined (Table 3). The sensitivity, specificity, and accuracy of BacT/ALERT 3D were 100% (35/35), 98.61% (71/72), and 99.06% (106/107), respectively, and those for the PZase assay were 82.85% (29/35), 98.61% (71/72), and 93.46% (100/107), respectively (Table 3). The difference between the sensitivities of BacT/ALERT 3D and the PZase assay was statistically significant (P = 0.025), whereas the specificities of both tests were equally high. The overall concordance among all three methods was 89.71% (96/107) after the first round of testing and increased to 92.52% (99/107) after restesting.

"S, susceptible; R, resistant."
TABLE 3. Performance parameters of BacT/ALERT 3D system and PZase assay after resolution of the discordant results

<table>
<thead>
<tr>
<th>Isolate</th>
<th>T/U</th>
<th>Result of initial test</th>
<th>Result(s) of repeat test</th>
<th>% Resistance on LJ medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LJ PM</td>
<td>PZase assay</td>
<td>BacT</td>
</tr>
<tr>
<td>7</td>
<td>T</td>
<td></td>
<td>S</td>
<td>−</td>
</tr>
<tr>
<td>21</td>
<td>U</td>
<td>S</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td>36</td>
<td>U</td>
<td>S</td>
<td>−</td>
<td>S</td>
</tr>
<tr>
<td>37</td>
<td>T</td>
<td>R</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>42</td>
<td>T</td>
<td>S</td>
<td>−</td>
<td>S</td>
</tr>
<tr>
<td>74</td>
<td>T</td>
<td>R</td>
<td>+</td>
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<td>79</td>
<td>T</td>
<td>R</td>
<td>+</td>
<td>R</td>
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<tr>
<td>92</td>
<td>T</td>
<td>R</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td>103</td>
<td>T</td>
<td>R</td>
<td>+</td>
<td>R</td>
</tr>
</tbody>
</table>

* R, resistant; S, susceptible; +, positive; −, negative.  
b T, treated case; U, untreated case.  
LJ PM, Löwenstein-Jensen proportion method.  
PZase assay, BacT, PZA susceptibility testing by the BacT/ALERT 3D system at a 100-μg/ml concentration.  
Negative at the end of 20 days also.  
ND, not done if the tube at 4 days was positive.  
Resistant to a PZA concentration of 100 μg/ml but susceptible at 300 μg/ml.

DISCUSSION

PZA is a frontline antituberculous drug with sterilizing activity against semidormant tubercle bacilli. Susceptibility testing of M. tuberculosis against PZA is difficult because the drug is active only in a relatively low-pH environment which causes nearly 50% inhibition in the colony count of M. tuberculosis and a considerable reduction in colony size compared with a neutral-pH environment (28). In several studies, a lack of PZase activity has been observed to be correlated with PZA resistance (5, 19, 21, 31). Therefore, the detection of PZase activity at neutral pH by Wayne’s PZase assay has been used for PZA susceptibility testing. However, the detection of weak PZase activity by this assay is difficult because of a very faint band in some isolates in conventional PZase agar medium, which has been overcome by modifying the assay.

The major utility of the PZase assay lies in its high specificity, as observed in our study (71/72, 98.61%) and as reported by others as well (17, 31). In our study, the sensitivity of the PZase assay was 82.85% (29/35). Various levels of sensitivity of the PZase assay in different studies have been reported. Trivedi and Desai (31), Davies et al. (7), and Krishnamurthy et al. (17) have reported 96% (170/177), 79% (15/19), and 83% (5/6) sensitivities, respectively.

PZA-resistant isolates with a positive PZase test have been reported in previous studies by several authors (5, 7, 17, 31). This is correlated with the observation that resistance to PZA has been described for M. tuberculosis isolates with normal PZase activity encoded by the wild-type pncA gene, thus constituting an inherent limitation of the test (14, 27, 38). In the present study also, 6 out of 35 PZA-resistant isolates were classified as sensitive by the PZase test. The presence of PZase activity in these PZA-resistant isolates could be due to the presence of mixed populations, as confirmed by the presence of a 45 to 80% sensitive population in these isolates observed by CFU on LJ medium. All of these patients had a history of previous antituberculous treatment. After analyzing the data of PZA resistance in treated versus untreated cases, we observed that 42% (33/78) of isolates were resistant among treated patients whereas this figure was only 6.9% (2/29) for the untreated ones.

The particular specificity of the PZase assay in both of the groups was 82% (27/27), respectively. The sensitivity of the PZase assay in isolates from treated cases was 82% (27/33). Considering the excellent specificity of the PZase assay in both of the groups but the lower sensitivity among the treated cases, we propose that the PZase assay should be considered more reliable in the untreated cases, as very few PZA-resistant isolates are expected among such patients. Further, a positive PZase assay in isolates from previously treated patients should be confirmed by the LJ proportion method or by other suitable phenotypic methods, such as BacT/ALERT 3D.

The discordance in results could be due to the different cutoff levels used, i.e., 100 μg/ml versus 300 μg/ml, or due to the smaller amount of PZase activity, as seen by positive results after longer incubation (Table 2). Such an observation for the PZase assay has also been reported by Kantor et al. (15). It would be advisable to inoculate two PZase agar tubes simultaneously. If the result in one tube remains negative at the end...
of 4 days, another tube should be held for 10 days in order to increase the specificity and predictive value for resistance. This will not, however, compromise the sensitivity of the test as the isolates resistant to PZA remain negative even after longer incubation. However, adequate data and more studies are required to make such a generalization or recommendation.

The sensitivity and the specificity of the BacT/ALERT 3D system in the present study are comparable to those in earlier reports (2, 4, 29). The BacT/ALERT 3D system was shown to be rapid as results were available after 10.92 (median, 10.32) days on average (range, 2.57 to 20.29 days). All of the isolates grew well, except one which was found to be susceptible at the end of 25 days by this system. The mean time required for generation of resistant results (8.04 days) was considerably lower than that for susceptible results (11.32) and was comparable to that reported in other studies using the MB/BacT system (4) as well as other nonradiometric systems, such as BACTEC MGIT 960 (1, 23). The delay in results for susceptible isolates in our study might be due to the employment of a 1:100-diluted inoculum for drug-free controls in accordance with the principle of the proportion method, which is also used as a criterion of resistance in LJ medium. Moreover, the time reported by the earlier studies with either BACTEC 460 or other nonradiometric systems did not include the time required for flagging the seed bottle positive before it is used as an inoculum. In our study, we prepared inocula from actively growing LJ cultures. However, as reported by Bemer et al. (4), the use of the undiluted growth control is particularly recommended in the case of PZA, as acidified medium delays the time of detection of susceptible results. In that case, an isolate is interpreted as drug resistant when a drug-containing test bottle gives a positive signal no later than 3.5 days after the undiluted control has flagged positive (4, 34).

Rapid and reliable susceptibility testing for PZA is very important. Since PZA is administered in the early intensive phase of antituberculosis treatment, any method giving rapid results of PZA susceptibility testing has its own merit. Considering the requirement of long times for the LJ proportion method as well as the inability of some isolates to grow on acidified medium (28, 31), the PZase assay, in spite of its being relatively less sensitive, appears to be a useful screening method for PZA susceptibility testing. However, considering the possibility of a mixed population of resistant and sensitive bacilli in isolates from previously treated patients, a positive result in such a case should be confirmed either by the proportion method or by any other rapid method, if available. Isolates negative at 4 days should be incubated for 10 days before it is concluded that they are negative. Further efforts may be made to convert this method into some semiquantitative technique. The BacT/ALERT 3D system, in addition to its higher sensitivity and specificity, can offer the extra advantages of rapidity and a better success rate; however, a higher running cost and expensive equipment are its limitations.

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