Evaluation of a biphasic media assay for pyrazinamide drug susceptibility testing of *Mycobacterium tuberculosis*

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**Objectives:** Pyrazinamide is a key first-line tuberculosis drug. Reliable drug susceptibility testing (DST) data are of clinical importance, but *in vitro* testing is challenging since the activity of pyrazinamide is pH sensitive. The BACTEC MGIT 960 is considered the principal reference technique, but Wayne’s test is an alternative, although it may be difficult to interpret. A further alternative is the use of a biphasic media assay (BMA). The objective of this work was to evaluate the BMA against the MGIT method and with screening of *pncA* gene mutations.

**Methods:** Twenty strains were inoculated in tubes containing 2 mL of Löwenstein–Jensen (LJ) medium and 2 mL of semi-solid Kirchner medium with a critical concentration of 66 mg/L pyrazinamide at a pH of 5.2 or 5.5, incubated for 2 weeks and visually read. The results obtained were compared with MGIT 960 and DNA sequencing.

**Results:** Results were obtained in duplicate for 19 strains. One strain failed to grow on two occasions and only one result was available. Reproducibility was 95%. Eleven of the 19 strains were susceptible to pyrazinamide, whereas 7 were resistant. One strain was susceptible initially and pyrazinamide resistant on repeat testing. At pH 5.5, two strains reported as susceptible at pH 5.2 gave resistant results.

**Conclusions:** The BMA might serve as a reliable low-cost DST alternative for pyrazinamide, particularly in laboratories using locally made solid media for DST. Its major drawback is the time to result. A reliable and affordable test method for the detection of pyrazinamide resistance is needed, especially in settings where multidrug-resistant tuberculosis is increasing. Proficiency testing should be routinely introduced wherever pyrazinamide DST is performed.

**Keywords:** TB, anti-tuberculosis drugs, *pncA*

**Introduction**

The increasing problem with multidrug-resistant (MDR; resistance to at least isoniazid and rifampicin) tuberculosis (TB) worldwide makes accurate and reproducible drug susceptibility testing (DST) more important than ever. Pyrazinamide is a key first-line drug in the treatment of TB, with a clear role in treating drug-susceptible cases as well as in MDR-TB (pyrazinamide-S-MDR-TB), due to the drug’s sterilizing activity against dormant persister bacteria; these are not killed by other anti-TB drugs. The inclusion of pyrazinamide shortened the duration of anti-TB therapy from 9–12 to 6 months and reduced the risk of relapse in drug-susceptible TB cases.\textsuperscript{1} Recent studies showed efficacy in pyrazinamide-susceptible MDR-TB patients, where pyrazinamide in combination with two or three bactericidal anti-TB agents, including newly developed anti-TB drugs such as bedaquiline, potentially shortened the duration of therapy from ~2 years to 1 year.\textsuperscript{2} To identify the most effective drug regimen for both drug-susceptible TB and MDR-TB patients, reliable pyrazinamide DST is of great importance.

DST on either solid or liquid media of the first-line anti-TB drugs rifampicin and isoniazid usually does not present major problems in the TB laboratory. *In vitro* testing of pyrazinamide is, however, more challenging since the activity of pyrazinamide correlates with the acidity of the culture medium, making the drug most active at a pH of 5.5 and almost inactive at neutral pH;\textsuperscript{3,4} at the same time the low pH is inhibitory to the *in vitro* growth of *Mycobacterium tuberculosis*. Furthermore, Zhang et al.\textsuperscript{2} showed that a large inoculum (10\textsuperscript{7–8} bacilli/mL) increased the pH from 5.5 to 7 in the culture medium, thereby inactivating the effect of pyrazinamide and producing false resistance results. In contrast to other first and second-line anti-TB drugs, DST on solid media is generally not recommended for pyrazinamide; thus, the susceptibility to pyrazinamide in clinical *M. tuberculosis* isolates is most often not tested in laboratories performing DST on solid media.
Consequently, pyrazinamide was, until 2013, not included in the WHO-coordinated quality assurance programme for proficiency testing, whose task is, apart from offering external quality assurance, to evaluate the inter-laboratory DST reproducibility of first-line and key second-line anti-TB agents. Reliable DST testing of pyrazinamide is technically difficult. The lack of data, or at least of quality-assured data, for pyrazinamide activity in clinical isolates is clearly problematic in a situation in which MDR-TB is increasing. The growing public health problems with resistant TB definitely require reliable in vitro pyrazinamide susceptibility test data, to identify an effective combination of anti-TB drugs.

The BACTEC MGIT 960 system (Becton Dickinson Biosciences, Sparks, MD, USA) is considered one of the reference techniques for rapid testing of drug susceptibility of TB to first-line anti-TB drugs. The MGIT system utilizes an acidified culture medium at pH 5.9 and modified test protocols specially adapted for pyrazinamide DST.

DNA sequencing and a recently developed line probe assay (Nipro-LIPA) may be alternatives for rapid detection of pncA mutations conferring pyrazinamide resistance, usually with a high degree of correlation to the phenotypic DST results, but have not been introduced for routine use.

The classical Wayne’s test is a rapid low-cost method that detects the presence or absence of pyrazinomidas (PZase), encoded by the pncA gene, in M. tuberculosis. PZase is required to metabolize pyrazinamide into its active form pyrazinoic acid (POA) and mutations in pncA may lead to a dysfunctional PZase that cannot convert pyrazinamide into POA. In the assay, the PZase activity in pyrazinamide-susceptible tubercle bacilli is easily observed as a pink band in the agar medium, but to give clear results the test usually demands large bacterial inoculums freshly harvested from solid media.

An additional methodology is to perform pyrazinamide DST in biphasic medium. Originally developed by Marks in the early 1960s and modified by Yates in the 1980s, it has been in use at the National Mycobacterium Reference Laboratory (NMRL) in London ever since, performing well in the Internal Quality Assessment scheme in place there. The methodology is as follows.

Tubes containing 2 mL of Löwenstein–Jensen (LJ) medium and 2 mL of semi-solid Kirchner medium with a critical concentration of 66 mg/L pyrazinamide at a pH of 5.2 are inoculated and then incubated for 2 weeks. After that period, the tubes are read visually. If there is no growth on the control tube, the DSTs were re-incubated for an additional week and read again.

The present work was a collaboration between the Public Health England National Mycobacterium Reference Laboratory in London (PHE-NMRL) and the supranational TB reference laboratory at The Public Health Agency of Sweden (SRL-PHAS). Here we report the first evaluation of the biphasic media assay (BMA) against the BACTEC MGIT 960 reference technique, by MIC determinations of 20 well-characterized clinical isolates of TB, together with the screening of pncA gene mutations using Sanger sequencing.

**Methods**

**Strains**

The NMRL in London received a total of 20 well-characterized pyrazinamide-susceptible and -resistant clinical M. tuberculosis isolates, coded 1–20, from SRL-PHAS and analysed these with their BMA standard procedures for pyrazinamide DST. All nine pyrazinamide-susceptible strains had wild-type pncA genes, whereas the 11 pyrazinamide-resistant strains had mutations in the same gene.

**Preparation of the two-phase medium, with solid and semi-solid layers**

(i) Solid phase:

(a) 600 mL of LJ medium with glycerol (LJG) was prepared and its pH lowered to 5.2 with 5 N hydrochloric acid.

(b) 9 mL of a 0.22% w/v solution pyrazinamide, sterilized by membrane filtration, was added to 300 mL of LJG medium for the control.

(c) 9 mL of sterile water was added to a further 300 mL of LJG medium for the control.

(d) 1 mL amounts of the media were placed in small screw-capped bottles and left to inspissate (85°C for 1 h) in a vertical position.

(ii) Semi-solid phase:

(a) 1 L of Kirchner medium was prepared; 3 g of sodium pyruvate was added and pH electrometrically adjusted to 5.2 with N hydrochloric acid.

(b) 1 g of pure agar was added and dissolved by steaming.

(c) Divided into two batches of 500 mL; 15 mL of the pyrazinamide solution was added to one and 15 mL of sterile water to the other.

(d) Both batches were sterilized by autoclaving and, when cool, 30 mL of Middlebrook OADC enrichment or horse serum was added to both.

(iii) Final medium:

(a) 2 mL amounts of test and control semi-solid medium were added to corresponding bottles containing the solid medium.

(b) The bottles containing the biphasic medium were stored at 4°C.

(iv) Additionally, a batch of media was prepared in the same way, but pH was adjusted to 5.5.

(v) The critical concentration of pyrazinamide is 66 mg/L.  

**Inoculation of the biphasic media**

Twenty cryovials were received from Sweden and sub-cultured in BACTEC MGIT 960 tubes. Purity plates were inoculated simultaneously and read 48 h later to rule out contamination. None of the tubes was contaminated. When positive, the pyrazinamide test was performed using these seeds.

Around 1 mL of the culture was extracted from the tube, placed in a bijou containing glass beads and vortexed for 1 min. The vortex cultures were left to settle for 20 min and then used to inoculate the DSTs.

For each sample, a purity LJ slope and a pair of tubes (one pyrazinamide-containing tube and one control) with 50 μL of the suspension were inoculated. Subsequently, a dilution of the inoculum was obtained by adding 50 μL of the suspension to a tube containing 2 mL of Middlebrook 7H9. This dilution was then used to inoculate an additional pair of tubes, one with and one without pyrazinamide. At the test then included an LJ slope, a control BMA tube, a pyrazinamide-containing tube, a tube containing 2 mL of Middlebrook 7H9, another control BMA tube and another pyrazinamide-containing tube.

The results obtained in Stockholm were unknown to the London team.

The tubes were then incubated for 2 weeks and read visually. If there was no growth on the control tube, the DSTs were re-incubated for an additional week and read again.

Once a full set was finished, the process was repeated from the start using a new batch of media.

After the second results were available, they were compared and the test was repeated a third time for those that failed to grow in one or both previous sets, were discrepant or difficult to interpret.

**Reading and reporting**

Reading was performed with the naked eye or with the assistance of a magnifying glass.
Colonies should be distributed evenly throughout the semi-solid medium in one or both of the control tubes.

Pyrazinamide-susceptible strains give growth in the drug-free control tubes only or they grow significantly better in these tubes. Resistant strains grow in all four tubes.16

Susceptibility testing and MIC determination with BACTEC MGIT

The DST inoculum was prepared from bacterial growth on LJ egg medium at 37°C, not older than 3 weeks. Briefly, two 1 μL loops of bacteria were suspended in 3 mL of PBS in a small glass tube with glass beads. The bacterial suspension was homogenized using a vortex or an ultrasonic water bath to disperse clumps. Thereafter the suspension was left to sediment for 20 min and the upper 2 mL was transferred to a new tube and left to sediment for another 15 min.

Prior to inoculation of the BACTEC MGIT pyrazinamide medium culture tubes (pH 5.9), the bacterial suspension was adjusted to a McFarland turbidity of 0.5 and diluted in PBS according to the pyrazinamide test protocol from the manufacturer. Susceptibility to pyrazinamide at the critical concentration of 100 mg/L was assessed using the Becton Dickinson pyrazinamide kit.

The MIC of the pyrazinamide-resistant isolates was determined at SRL-PHAS. Stock solutions (16.8-67.2 g/L) of pyrazinamide (Sigma-Aldrich, Germany) were made with sterile water and heated by immersing the container in hot tap water until completely dissolved. By adding 100 μL of the stock solution to the MGIT culture tube, test concentrations of 200–800 mg/L were obtained.

The MIC was determined as the lowest concentration to which the growth unit (GU) value of the pyrazinamide-containing culture tube was <100 when the 1:10 diluted drug-free control had reached GU = 400.

pncA sequencing

The 561 nt pncA gene, along with surplus regions of ~200 nt up- and downstream of the gene, was sequenced according to Jureen et al.9

Results

Results were obtained in duplicate for 19 strains. One strain failed to grow on two occasions and only one result was available. Reproducibility in the duplicate testing was 95%; 18/19 strains showed the same result in the BMA. Of these, 11 were susceptible to pyrazinamide, whereas 7 were resistant. One strain was susceptible the first time tested and resistant on repeat testing. The strain for which only one result was available was susceptible to pyrazinamide.

These results were compared with those obtained in the BACTEC MGIT tests in Stockholm. The full set of results can be found in Table 1.

Three strains showed a different result in the two locations, London reporting false susceptibility to pyrazinamide for all three at pH 5.2. The corresponding sensitivity and specificity of the BMA was 73% and 100%, respectively. The strain tests were repeated using media at pH 5.5 and the same critical concentration of pyrazinamide, to assess how pH affected the strains' behaviour with respect to the drug. Ten of these were interpreted as pyrazinamide susceptible and 10 as pyrazinamide resistant at pH 5.5 in the BMA. Two strains, previously pyrazinamide susceptible at the lower pH (5.2), were determined to be resistant. In agreement with previous reports, a subtle change in pH (from 5.5 to 5.8) increased resistance.6 Results of this experiment can be found in Table 1. Since the pH in the phagolysosome to which M. tuberculosis is exposed varies considerably from 4.5 to 6.2 and the activity of the drug decreases with minor pH increases,17 in vitro testing is difficult and really hard to correlate with what happens within the macrophage.

Discussion

Reliable information on pyrazinamide susceptibility is of great importance for guidance of therapy of patients with both drug-susceptible and pyrazinamide-susceptible MDR-TB. This is the first evaluation of a BMA for the determination of pyrazinamide susceptibility. At present, the main recommended reference technique for this purpose is the broth-based BACTEC MGIT 960, for which a commercial pyrazinamide test kit is available, but, in the global perspective, may not be an alternative to DST in many TB laboratories with limited resources. The BMA is a pyrazinamide DST developed in house that can be produced locally and might serve as a reliable low-cost alternative in those laboratories already preparing their own drug-containing media.

The major drawback of the BMA is the time to result, which is 2–3 weeks compared with ~7–10 days for the BACTEC MGIT. As a drawback, poor or no mycobacterial growth may not be detected until several weeks of incubation, which can delay DST results even further. When the pH was changed from 5.2 to 5.5, an improvement was observed in the detection of pyrazinamide resistance and the sensitivity of the BMA was increased from 73% to 91%, while the excellent 100% specificity was kept. There are not many validated phenotypic DST alternatives for the determination of pyrazinamide activity. Initially the determination of pyrazinamide susceptibility was usually tested at different pH in LJ medium acidified with hydrochloric acid,18–20 but problems with failing growth at a pH range between 5.0 and 5.5 was a limitation of the method. Improvements have been tried by the use of Middlebrook 7H10 agar and optimization of pH close to 5.7.21 Interestingly, in our study all M. tuberculosis strains grew at both pH 5.2 and 5.5 in the BMA, but the lower pH caused an increase in pyrazinamide susceptibility results, which was not observed at pH 5.5. The MGIT 960 tube assay operates at pH 5.9 so it is likely to overscore resistance if not performed carefully.

The PZase test may be a rapid and simple low-cost alternative to the pyrazinamide DST in many laboratories, but has shown varying accuracy in different studies and can theoretically cause false resistance due to inoculums that are too small, or false pyrazinamide susceptibility in heteroresistant isolates.6,12,20,22 Detection of mutations in the M. tuberculosis pncA gene as indicators of resistance has been reported in a meta-analysis to have a specificity of 93% and sensitivity of 87%.23 Molecular tests are expensive and not yet commercially available and must be evaluated carefully against a phenotypic gold standard before being brought into routine use.

A reliable and affordable test method for the detection of pyrazinamide resistance is needed, especially in settings where MDR-TB is increasing, and proficiency testing should be made available and introduced as a routine wherever pyrazinamide DST is performed.

In contrast to the BACTEC MGIT system, where problems with false pyrazinamide resistance seem to occur more frequently,24 the BMA showed lower sensitivity, probably due to the low pH, causing false susceptibility to pyrazinamide in 3 of the 20 strains in this study. As shown in Table 1, this study indicates that the
Table 1. Phenotypic and genotypic pyrazinamide susceptibility results obtained in both centres; the BMA was performed at pH 5.2 and 5.5 using a critical concentration of 66 mg/L.

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PZA, pyrazinamide; INH, isoniazid; RIF, rifampicin; S, susceptible; R, resistant; WT, wild-type; MGIT, mycobacteria growth indicator tube; B 460, BACTEC 460; NA, not applicable; frshft, frameshift; inf ins, in-frame insertion; rpts, repeats.

Three discrepant strains at pH 5.2 are highlighted in bold.
sensitivity of BMA may be increased without compromising the specificity of the test if the pH in the test medium is adjusted to 5.5.

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Transparency declarations
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