

Revisiting problems and solutions to decrease *Mycobacterium tuberculosis* pyrazinamide false resistance when using the Bactec MGIT 960 system

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Abstract

Pyrazinamide (PZA) is a first-line key drug used in combination with other agents for the treatment of tuberculosis (TB). Phenotypic and molecular assays for testing susceptibility of *Mycobacterium tuberculosis* (Mtb) to PZA have been developed, with the assay in liquid medium at acidic pH in the Bactec MGIT 960 (M960) system being routinely used in the mycobacteriology laboratories. However, false resistance to PZA by this method was reported to occur by several investigators, mostly due to high Mtb inoculum, which may impair drug activity by increasing the pH of the medium. In this study, a revision of the literature on the issue of false resistance in the M960 PZA assay was performed. In the reports examined, all improvements of the M960 test proposed to decrease false resistant results were based on the use of reduced inoculum densities of Mtb cells, to be easily translated into laboratory practice.

Key words

- tuberculosis
- *Mycobacterium tuberculosis*
- pyrazinamide
- drug susceptibility
- false resistance

PYRAZINAMIDE FOR TREATMENT OF TUBERCULOSIS

Tuberculosis (TB) is the main cause of death due to a single infectious agent worldwide, and drug-resistant forms of the disease are a major risk to global health security [1]. The current anti-TB therapy consists of a combination of the first-line drugs isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) given daily for 2 months, followed by RIF and INH administered daily for 4 months. Poor adherence to TB treatment leads to emergence of multidrug-resistant (MDR) *Mycobacterium tuberculosis* (Mtb) strains (resistant at least to INH and RIF) and extensively drug-resistant (XDR) strains (MDR strains resistant to any fluoroquinolone and to at least one injectable second-line drug, kanamycin, amikacin or capreomycin). At global level, in 2017 the World Health Organization (WHO) estimated that 558 000 people developed TB that was resistant to RIF, and that of these, 82% had MDR-TB [2].

PZA is a cornerstone anti-TB drug active in the first two months of therapy. It is likely that during the early inflammatory stages of the TB disease, PZA kills intracellular and semidormant tubercle bacilli living in the acidic environment of the phagolysosomes of activated macrophages. In the 1980s, the substitution of PZA for streptomycin (SM) in the regimen INH-RIF-EMB-SM shortened the course of TB therapy from 9-12 months to the current 6 months regimen, INH-RIF-EMB-PZA. The ability of PZA to shorten the *in vivo* therapy is of great importance. In this view, this drug is currently being considered in future anti-TB regimens in combination with new agents, including bedaquiline, delamanid, pretomanid, moxifloxacin, levofloxacin, linezolid in phase II and III trials [2].

MECHANISMS OF ACTION AND RESISTANCE OF PZA

The activity of PZA depends on the acidity of the culture medium, with the drug being active at pH lower

than 6. PZA is a prodrug which enters Mtb cells by passive diffusion. Inside the cytoplasm, the drug is converted into its active form pyrazinoic acid (POA) by the pyrazinamidase (PZase), an enzyme encoded by the *pncA* gene. Then, POA is expelled out of the cell through passive diffusion [3]. Under external acidic conditions, the POA is protonated and enters the cell by passive diffusion and a defective POA efflux mechanism. Following intracellular accumulation, POA kills Mtb by various mechanisms including disruption of membrane energy production, inhibition of trans-translation and inhibition of panthotenate and coA biosynthesis.

Resistance to PZA is mostly generated by mutations in the *pncA* gene, but mutations in *panD* and *rpsA* genes may also be involved [4]. Several studies indicated that MDR Mtb strains were more likely to harbor PZA resistance than non-MDR strains, with estimated prevalences being 16.2% for all TB cases and 60.5% for MDR cases [5]. The estimated global burden is 1.4 million PZA-resistant cases annually, including about 270 000 in MDR patients. Recently, the WHO categorized PZA in the group C agents for use in longer MDR-TB regimens, to be added to group A (levofloxacin or moxifloxacin, bedaquiline, linezolid) and B (clofazimine, cycloserine or terizidone) regimens, and when medicines from groups A and B cannot be used [6].

DRUG SUSCEPTIBILITY TESTING OF PZA

Drug susceptibility testing (DST) of PZA is challenging. Two main phenotypic assays are known, including the automated Bactec MGIT 960 system (MGIT; Becton Dickinson, Sparks, MD) performed by monitoring the growth of Mtb in liquid medium at pH 5.9 containing 100 µg/ml of PZA, and the classic and modified Wayne's methods, which assess activity of PZase on the basis of colorimetric changes at 100 µg/ml and 400 µg/ml of PZA, respectively [5]. The PZA MGIT test is the more commonly used of these two methods, but false resistance in a multicenter study was recently reported, largely due to high Mtb inoculum, which may impair PZA activity by increasing the pH of the medium [7]. For this reason, the WHO considers the DST to PZA not reliable, and considers an acceptable practice to administer the drug in a regimen even when a laboratory demonstrates resistance [8]. However, PZA is counted as an effective agent when DST results confirm susceptibility [6].

In the last years, genotypic PZA assays have also been developed, based on DNA sequencing to detect *pncA* mutations, which have been shown to correlate with PZA resistance [3, 9-10]. The sequencing of *rpsA* and *panD* genes provided only a 2% increase in sensitivity, and from 8% to 10% resistant strains without *pncA*, *rpsA* and *panD* mutations may point to alternative mechanisms of resistance [10]. Even though *pncA* sequencing is a powerful tool for molecular diagnosis of PZA resistance, a study reported that the countrywide prevalence of *pncA* mutations in Mtb isolates phenotypically resistant to PZA varied from 45 to 100% [11]. Furthermore, in a large investigation of genetic surveillance conducted on 7094 patients in highly endemic countries, the sensitivity for *pncA* sequencing compared with MGIT

testing adjusted for the results of the Wayne's test was only 54% [12]. The diversity of single nucleotide polymorphisms across the *pncA* gene complicates the development of rapid molecular diagnostics, because not all mutations confer resistance, and not all susceptible strains lack mutations [5, 7]. For these reasons, also these molecular techniques are not yet approved by WHO for PZA DST [5, 11]. In this view, the whole-genome sequence (WGS) may be useful to improve diagnosis. Indeed, the sensitivity of this technique for PZA DST was reported to be 91.3% in a recent study performed on 10 209 Mtb isolates assayed by WGS and various phenotypic DST [13]. However, this technology is not yet routinely available in most laboratories worldwide, thus in consideration of this limitation, it is still necessary to perform culture-based PZA DST [7].

PREVENTION OF FALSE PZA RESISTANCES WHEN USING THE MGIT SYSTEM

Erroneous results indicating false PZA resistances when using the MGIT system were reported by several investigators.

In 2009, Pandey *et al.* found that the MGIT test showed 54.2% false PZA resistance in comparison with *pncA* sequencing [14].

In 2010, Chedore *et al.* reported that 42% of 57 strains diagnosed as resistant by MGIT were false resistant in comparison with *pncA* sequencing, and with results obtained by the radiometric system BACTEC 460 TB (BACTEC) (Becton Dickinson), that was considered the gold standard [15]. Erroneous results by MGIT were attributed to various reasons including high concentration, volume and heterogeneity of the inoculum. Indeed, i) the concentration of inoculum was 2.5 times greater than in the BACTEC method, ii) the volume inoculated was 5 times greater than in BACTEC, iii) the bacilli were inoculated by a pipette in MGIT and by a fine-needle syringe in BACTEC, resulting in uneven distribution of bacilli due to "clumping". It was suggested to retest all PZA-resistant isolates by the BACTEC, but this system was later withdrawn from the market by the manufacturer to avoid disposal of the radioactive waste, and was replaced by the MGIT system.

In 2011, Simons *et al.* found that in 69 strains resulted to be PZA resistant by MGIT, 68.1% were false positives after a second MGIT analysis, and that *pncA* mutations could identify these false resistances correctly [16]. Also these investigators pointed out that false phenotypic PZA resistance was due to large inocula which increased pH and thereby inactivated PZase. To improve DST, they recommended repeating the MGIT test, giving attention to the inoculum size, and performing mutation analysis as an adjunct to the MGIT assay.

In 2013, the Swedish Institute for Communicable Disease Control reported that over the course of proficiency testing exercises, two out of six clinical laboratories showed pronounced problems with false resistance results, with a positive predictive value of only 63% and 45%, respectively [17].

Piersimoni *et al.*, in two papers published in 2013 [18] and 2016 [19], respectively, retested a total of 82 PZA-resistant strains by the BACTEC standard assay,

by reducing the inoculum volume of the MGIT test tubes from 0.5 ml to 0.25 ml. By this approach, false resistance decreased by 71.1% and 51.4%, respectively, in comparison with standard MGIT assay. It was suggested that PZA resistant isolates should undergo a repeat DST using the reduced inoculum of 0.25 ml. In case of resistance, *pncA* gene sequencing should be performed, otherwise, the isolate should be reported as susceptible. Some investigators also suggested to reassess the critical concentration for PZA susceptibility [20, 21]. In the Piersimoni's studies, both reduced inoculum and PZA testing at 200 µg/ml reduced false MGIT-resistances, however, only reduced inoculum allowed a clear separation between true- and false-resistant isolates.

In 2017, Morlock *et al.*, during the course of a multicenter study coordinated by the US Centers for Disease Control and Prevention, also mitigated false PZA positivities by MGIT through the use of reduced inoculum densities [7]. Twenty genetically and phenotypically characterized isolates were tested in ten independent laboratories by the standard PZA MGIT method (0.5 ml of positive MGIT tubes transferred to test tubes containing 100 µg/ml of PZA) and 1:2.5 and 1:5 dilutions of the positive tubes. By this approach, the percentages of resistant results that were false-resistant declined from 55.2% for the standard MGIT test to 28.8% and 16.0% for the 1:2.5 and 1:5 diluted inocula, respectively. These investigators reported that some strains were "always susceptible" regardless of inoculum concentration while other strains called "predominantly susceptible" showed high degree of false resistance when the standard method was used.

Finally, in 2017, Mustazzolu *et al.* tested 106 WHO strains with known *pncA* mutations by a slightly modified MGIT standard test [22]. A positive MGIT seed tube was allowed to settle for 20 to 30 min, then 1 ml was taken from the top surface, instead of lower down; of this volume, 0.5 ml was transferred to a 100 µg/ml

PZA-containing test tube, and 0.5 ml of 1:10 dilution from the positive tube was used to inoculate the growth control tube without PZA. Using this easy variation, 0.9% of strains were falsely resistant and no strains were falsely susceptible. Furthermore, when 10 of those strains were tested by 17 Italian independent laboratories (a total of 68 resistant and 102 susceptible strains), false resistance was reported to be 4.7% by the standard method and 1.2% by the modified MGIT method.

CONCLUSION

Overall, these observations provide compelling evidence that to decrease false resistant results it is necessary to reduce the inoculum in the MGIT PZA assay currently used worldwide. This purpose can be achieved by changing the method of drafting the initial inoculum, or changing PZA critical concentration. As suggested by Morlock *et al.* [7], clinical laboratories should validate their modified method prior to routine implementation. However, large controlled studies using both MGIT and molecular methods should be performed by investigators and/or by the manufacturer of the current test, to modify the MGIT package of the PZA assay.

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Author contribution statement

AM and LF conceived and wrote the study; CP, AI, FG and BC conceived the study and critically revised the manuscript.

Conflict of interest statement

None.

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