

Potential for Erroneous Results Indicating Resistance When Using the Bactec MGIT 960 System for Testing Susceptibility of *Mycobacterium tuberculosis* to Pyrazinamide[▽]

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Received 9 September 2009/Returned for modification 12 October 2009/Accepted 9 November 2009

During susceptibility testing of 743 isolates of *Mycobacterium tuberculosis* to pyrazinamide (PZA) using the Bactec 960 system, 57 (7.7%) isolates showed PZA resistance. Repeat testing of resistant isolates with the Bactec 460 reference method confirmed 33 (4.4%) of these isolates as resistant, and 24 (3.2%) were susceptible. Erroneous results for resistance with the Bactec 960 were confirmed by testing the 24 discordant isolates for pyrazinamidase and mutations in the *pncA* gene.

Pyrazinamide (PZA) is an important component of the multidrug regimen used to treat tuberculosis (TB). With increasing worldwide prevalence of drug-resistant TB, it is vital for laboratories to accurately detect resistance to first-line antimicrobials.

The CLSI-recommended method for PZA testing (4) is the Bactec 460TB radiometric system (Becton Dickinson, Sparks, MD). Most laboratories have now replaced the 460TB system with the nonradiometric Bactec MGIT 960 (BT960) system (Becton Dickinson, Sparks, MD). Both methods utilize an acidified Middlebrook broth and a critical concentration of 100 µg/ml.

PZA is a prodrug which in *Mycobacterium tuberculosis* is converted to its active form, pyrazinoic acid (POA), by the enzyme pyrazinamidase (PZase) (5, 7). The absence of a functional PZase enzyme in an *M. tuberculosis* strain therefore indicates resistance to PZA. The *pncA* gene coding for PZase in *M. tuberculosis* has been sequenced, and mutations in this gene have been shown to be responsible for resistance to PZA (5, 8, 12). Tests both for PZase activity and for the detection of mutations in *pncA* may be utilized as alternative methods for the detection of PZA resistance in *M. tuberculosis*.

All new *M. tuberculosis* isolates are tested for susceptibility to first-line drugs, including PZA, at the Public Health Laboratory, Toronto. During the report period, any isolate demonstrating PZA resistance by the BT960 was retested using the 460TB. If the 460TB PZA result was discordant, these isolates were further tested for the presence of PZase activity and mutations in the *pncA* gene, and testing in the BT960 was repeated.

PZA susceptibility testing in the BT960 system was performed according to the manufacturer's instructions (2). Briefly, isolates of *M. tuberculosis* in Mycobacteria Growth Indicator Tubes

(MGITs) were used as the test inocula. A drug-free control tube was inoculated with a 1:10 dilution of the inoculum, and the PZA test tube was inoculated with 0.5 ml of the inoculum and 0.1 ml of PZA. The tubes were monitored with the BT960 instrument until the growth control tube flagged positive. At that time, the instrument read the PZA test tube as either resistant (growth unit [GU] ≥ 100) or susceptible (GU < 100). A blood agar purity plate from the inoculum was incubated for 3 days.

PZA testing in the BT 460TB system was performed according to the manufacturer's instructions (13). Briefly, a drug-free control vial and the PZA test vial were each inoculated with 0.1 ml of inoculum from an actively growing culture. The vials were incubated and were read on the BT460 instrument daily until the growth index (GI) in the control vial was ≥ 200. At that time, the GI in the test vial was calculated as a percentage of the GI in the control. A result of ≥ 11% indicated resistance, < 9% indicated susceptibility, and from 9 to 11% was borderline. A blood agar purity plate from the inoculum was incubated for 3 days.

Isolates with results that were discordant between the two systems were tested for the presence of PZase activity and for mutations in the *pncA* gene at the National Reference Centre for Mycobacteriology, Winnipeg, Manitoba, Canada, by standard methodologies (12, 14) and with repeat testing in the BT960.

During the report period, 743 PZA susceptibility tests were performed using the BT960. Of these, 57 (7.7%) showed PZA resistance. Thirty-three of these isolates were confirmed to be PZA resistant using the Bactec 460 system and were eliminated from the discordant-data analysis. The remaining 24 PZA-resistant isolates (3.2% of the total) were susceptible to PZA with the 460TB and were considered discordant. Follow-up testing of these isolates showed the presence of PZase in all isolates, and all were negative for mutations in the *pncA* gene. Repeat PZA testing of the discordant isolates with the BT960 gave a second resistant result for 10 isolates and a susceptible result for 14 isolates, indicating lack of reproducibility. The

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[▽] Published ahead of print on 18 November 2009.

460TB result was considered the gold standard for reporting results.

There are reports citing technical problems with *in vitro* testing of *M. tuberculosis* with PZA. None of the methods described give 100% agreement when compared with the 460TB reference method, and most cite problems with false resistance (1, 10, 11, 15).

The effects of inoculum concentration, volume, and homogeneity, as well as the lack of reproducibility in BT960 PZA tests, have been cited (1, 5, 6, 9). However, we are not aware of a study where BT960-resistant strains were tested with the 460TB plus PZase and molecular testing.

There are several differences in the inocula used for testing PZA in the two systems. First, in the 460TB, the ratio of inoculum to medium is 1:42, whereas in the BT960 system, the ratio is 1:16.6. The concentration of inoculum in the test medium in the BT960 is thus more than 2.5 times greater than that used in the 460TB. The volume of inoculum used in the BT960 is 0.5 ml, versus 0.1 ml of inoculum used in the 460TB. The higher concentration and volume of inoculum used in the BT960 has a higher likelihood of containing organisms resistant to PZA, as it is estimated that in *M. tuberculosis*, between 1 in 10⁷ and 1 in 10¹⁰ cells are resistant to any drug (9).

Second, there is variability in the concentration of the inoculum used in the BT960 test according to the day of test setup. For days one and two after the culture flags positive, there is no dilution of the MGIT seed vial, but for days 3 to 5, the inoculum is diluted 1:5. This may lead to considerable variation in the amount of the organism in the inoculum and could cause the lack of reproducibility found during repeat testing.

Third, the inoculation method differs between the two systems. For the 460TB, a fine-needle tuberculin syringe is used, and for the BT960, the inoculum is dispensed with a disposable pipette tip, which may result in uneven distribution of bacilli due to "clumping."

The sensitivity of the PZase assay is reported to vary between 79 and 96% (1, 5, 7). All of the discordant isolates that were tested showed the presence of PZase. Sequencing of the *pncA* gene that encodes PZase has shown that 74% to 97% of all PZA-resistant strains of *M. tuberculosis* carry a mutation in several different regions of the gene (7, 8, 12). None of the discordant strains were found to have *pncA* gene mutations.

These results corroborate the 460TB results as PZA susceptible and the BT960 results as falsely resistant. For laboratories which perform a large number of susceptibility tests with the BT960 system, this discordance could lead to a significant number of false resistant and major error (3) results for PZA, as well as therapeutic issues in patient management.

Due to the potential for false resistant results during PZA testing with the BT960, laboratories should consider retesting all PZA-resistant isolates with the 460TB reference method before reporting results. Since PZA is considered an essential component of first-line TB therapy, it is important that laboratories find a successful algorithm to provide rapid and accurate susceptibility results for PZA.

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