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Evaluation of a Radiometric Method for Pyrazinamide Susceptibility Testing of *Mycobacterium tuberculosis*

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Pyrazinamide susceptibility testing of *Mycobacterium tuberculosis* requires an acid environment. By controlling the method of acidification and the quality and quantity of the inoculum, the test can be performed with the BACTEC radiometric system (Johnston Laboratories, Towson, Md.). We acidifed BACTEC 7H12 medium with buffered phosphoric acid and adjusted the test inoculum to 1/10 of that usually employed in BACTEC protocols; after 5 days of growth we correctly identified 36 of 36 strains susceptible to 50 μg of pyrazinamide per ml. All 18 resistant strains were classified as pyrazinamide resistant. (Susceptibility or resistance had been determined by standard plate assays.) The test was able to detect small resistant populations in artificial mixtures of 1 or 2% resistant bacteria with a susceptible strain (10 mixtures each). We tested 70 *M. tuberculosis* strains in acidified BACTEC 7H12 medium and by the plate dilution test at pH 5.5. All strains grew in the BACTEC medium, but three strains failed to grow on plates and were not tested further; the results of both methods agreed for the remaining strains.

Antimicrobial susceptibility testing of *Mycobacterium tuberculosis* with pyrazinamide (PZA) is technically difficult because PZA is active only in an acid environment (5, 7, 10). McDermott reported an eightfold decrease in the MIC of PZA for *M. tuberculosis* with a threefold increase in hydrogen ion concentration between pH 6.0 and pH 5.5 (7). The acid environment (pH 5.5) required for PZA susceptibility test media also reduces the growth rate of the bacteria, with up to 50% of strains failing to grow (10). A recent modification by Butler and Kilburn (2) of the 7H10 agar method of PZA susceptibility testing—omission of oleic acid from the medium and prescreening of albumin enrichment for growth-supporting ability—has greatly improved the assay. Yet even with this improvement 10% of strains fail to grow, and all strains show some inhibition of growth in comparison to growth at pH 6.8.

M. tuberculosis strains resistant to PZA frequently lack the ability to deaminate PZA into pyrazinoic acid, the antibacterial moiety of PZA (1, 5, 6, 12). To avoid the use of acid culture media and the resulting poor growth of *M. tuberculosis*, various susceptibility tests depending on pyrazinamidase activity have been described, including the detection of pyrazinoic acid by high-pressure liquid chromatography (9, 14). These methods fail to give information regarding the proportion of a test population that is PZA resistant, and they are also independent of the PZA concentration. Furthermore, even strains highly resistant to PZA may not produce pyrazinamidase (3).

The BACTEC radiometric method (Johnston Laboratories, Towson, Md.) of susceptibility testing of *M. tuberculosis* generally employs a 100-fold inoculum difference between growth control and drug test vials. This allows the quantitative determination of a 1% resistant population (11). We have studied the use of the BACTEC 460 radiometric system for PZA susceptibility testing of *M. tuberculosis*, but had difficulty documenting drug activity at pH 5.5 (J. J. Tarrand, A. Spicer, and D. H. M. Groschel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C226, p. 349). Heifets and Iseman recently reported a method of acidification of Middlebrook 7H12 broth that allows vigorous growth at pH 5.5 (4). This acidification technique, after further modification, greatly facilitated the development of a semiquantitative assay for PZA susceptibility of *M. tuberculosis* with the BACTEC 460 instruments.

MATERIALS AND METHODS

Seventy PZA-susceptible strains of *M. tuberculosis* were used, including patient isolates from our institution and from the mycobacterium collection of the Consolidated Laboratories of the Commonwealth of Virginia as well as the PZA-susceptible strain H37Rv (TMC no. 102; Trudeau Mycobacterial Collection, National Jewish Hospital and Research Center, Denver, Colo.). PZA susceptibility was confirmed by the plate method of Stottmeier et al. (10) as modified by Butler and Kilburn (3). The 18 resistant strains included 1 obtained from the Trudeau Mycobacterial Collection (TMC no. 311), 7 from the Mycobacteriology Division of the Centers for Disease Control, and 10 pyrazinamide-resistant strains we produced by UV irradiation and selection on PZA plates as previously described (11). All strains had comparable growth rates as determined by growth curves in BACTEC 7H12 medium.

Preparation of broth inocula and 1% resistant mixtures. Both PZA-resistant and -susceptible strains were grown for 2 to 3 weeks on Löwenstein-Jensen medium at 37°C in an 8% CO_2 atmosphere. Working in a Baker biological safety cabinet, approximately 2-mg samples of *M. tuberculosis* were transferred from Löwenstein-Jensen medium and homogenized in 2.3 ml of 0.02% Tween 80–0.2% fatty-acid free albumin (pH 6.8) with 5-mm sterile glass beads on a Vortex mixer for 1 to 2 min at the highest energy setting. The closed tubes were held firmly near the middle, and the beads were allowed to "walk up" the tube. After 10 mixing cycles the suspension showed a turbidity of approximately 1 McFarland standard. The suspension was allowed to settle for 10 min. The supernatant was diluted 1:10 in 5 ml of Middlebrook 7H9 broth, and the tube was incubated on a New Brunswick Scientific model TC1 roller (0.2 rpm) at 37°C

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for 48 h, resulting in an approximate turbidity of a McFarland standard of 0.5. During bacterial transfer at least 0.3 ml was left in the syringe to minimize the effect of clumping. These cultures were used for the susceptibility test as described below and for the preparation of 1% resistant mixtures.

M. tuberculosis H37Rv was used to prepare susceptibility test mixtures containing a known percentage of PZA-resistant organisms. For the preparation of 1% mixtures, 0.1-ml samples of Middlebrook 7H9 broth cultures of the resistant test strains and the susceptible H37Rv strain were inoculated into two separate BACTEC 7H12 medium vials (Middlebrook 7H12 medium with ^{14}C-labeled substrate) and incubated on the roller for 24 h. Both strains reached a growth index (GI) of about 300. The GI of the resistant strain was divided into 1% of the GI of the susceptible strain, and the result multiplied by two (the number of milliliters per vial). This yielded the volume in ml to be added to the susceptible strain to achieve a 1% mixture. This mixture was used immediately to perform the drug susceptibility test.

PZA susceptibility testing by the BACTEC method. The following procedure was adopted for PZA susceptibility testing. Inocula consisted of 0.1 ml per BACTEC 7H12 vial of 1:10 and 1:100 diluted *M. tuberculosis* suspensions with a turbidity equivalent to the McFarland standard of 0.5 (approximately 1×10^6 to 3×10^6 CFU/ml); the 1:10 dilution was added to a BACTEC 7H12 vial labeled "drug" and 1:100 dilution was added to a vial labeled "control." To the drug vial, 0.1 ml of acidifying solution containing 1,000 μg of PZA per ml was added, and to the control vial 0.1 ml of the acidifying solution without PZA was added. The acidifying solution (buffered phosphoric acid) was prepared by adding 1 ml of 85% phosphoric acid and 3 g of anhydrous dibasic potassium phosphate to 60 ml of magnesium-Tween albumin broth (0.4% $MgSO_4 \cdot 7H_2O$, 0.02% Tween 80, 0.6% fatty acid-free albumin). The acidifying solution was filter sterilized and stored at 4°C.

Immediately after inoculation, the headspace gas in the vials was exchanged on the BACTEC 460 instrument with air containing 10% CO_2. The vials were incubated at 35°C for 7 days without shaking, and daily GI readings were made.

Susceptibility testing by the plate method. PZA susceptibility testing was carried out with modified Middlebrook 7H10 medium as described by Stottmeier et al. (10) and modified by Butler and Kilburn (3). Middlebrook albumin-dextrose complex (ADC) enrichment was either obtained from BBL Microbiology Systems (Cockeysville, Md.) or prepared in our laboratory with fatty acid-free albumin (8). The malachite green concentration was reduced from 0.0025% to 0.0006%. Media were stored in polypropylene bags in the dark and used within 2 weeks of preparation. Plates were inoculated with 10^{-2} and 10^{-4} dilutions of the test bacteria, and drug susceptibility testing was performed as described by Vestal (13) with PZA concentrations of 50 μg/ml. Colonies were counted after 4 to 6 weeks of incubation at 35°C.

RESULTS

In early experiments with the radiometric PZA susceptibility test, we prepared inocula directly from Löwenstein-Jensen medium slants with glass beads and adjusted the suspension to a turbidity of a McFarland standard of 1. Except for the addition of a buffer (2 M monopotassium phosphate [anhydrous], 1 M glycine, 0.1 N HCl) that lowered the pH to 5.51 (standard deviation [SD], ±0.06; n = 45), we attempted to use conditions identical to those recommended for other antituberculous agents in the BACTEC system. Under these conditions PZA demonstrated little effect, but when the eight resistant strains were subcultured on day 10 into fresh BACTEC 7H12 medium at pH 6.8 they showed considerable more metabolic activity than susceptible strains. This method, however, proved to be very insensitive (13 of 18 resistant strains detected). Even an increase of PZA concentration from 50 μg/ml to 150 and 400 μg/ml did not increase the sensitivity of the assay.

Since growth of *M. tuberculosis* at pH 5.5 in BACTEC 7H12 vials generally leveled off with cumulative GIs of 3,000 (out of a possible 8,000), we thought that at this pH the normal medium components might be insufficient to support further growth. Addition to the medium of individual filter-sterilized medium components failed to increase growth; only magnesium sulfate and albumin allowed continued growth to a GI of about 4,000.

Various inoculum sizes and PZA concentrations were assayed in a checkerboard fashion (Fig. 1). PZA inhibited susceptible strains only with a low inoculum. Increasing the cell mass of *M. tuberculosis* reversed PZA inhibition of growth. To test whether the bacterial cells absorbed PZA or metabolic products interfered with PZA activity, UV-killed and washed bacteria or sterile filtrates from rapidly growing cultures were added to drug vials inoculated with 1:100 diluted inoculum. Both additives abolished PZA activity. Binding of PZA by mycobacteria could not be demonstrated spectrophotometrically. To avoid possible test interference due to inoculum quality (too many cells, dead cells, or metabolic products), in subsequent experiments the inoculum was reduced and inocula were prepared from a rapidly growing (48-h) culture of *M. tuberculosis* in Middlebrook 7H9 broth. This greatly improved the reproducibility of PZA susceptibility tests.

We turned our attention to the buffers used and studied different acidification conditions. The glycine buffer prolonged the multiplication time of the H37Rv strain from 1.02 days (SD, ±0.05; n = 12) at pH 6.8 to 1.5 days (SD, ±0.3; n = 10) at pH 5.5. After publication of the report by Heifets and Iseman (4) we performed the PZA susceptibility test with the proposed phosphoric acid buffer with a 1:10 diluted inoculum for the drug vial and a 1:100 diluted inoculum in the control vial. Now resistance was easily detected, but only with resistant strains and not with 1% resistant in susceptible bacteria mixtures. Furthermore, 33 of 100 determinations with susceptible strains showed falsely resistant results. Concerned about the possible influence of pipetting errors with the acidification solutions, we tested the effect of a 10% increase in volume (100 versus 110 μl) with a Hamilton syringe. Whereas a 10% increase in the amount of glycine buffer resulted in little change in pH, a 10% increase in the phosphoric acid buffer lowered the pH considerably from 5.59 (SD, ±0.1; n = 9) to 3.7 (SD, ±0.1; n = 3). A new buffer was formulated to reduce pH changes due to small pipetting errors and to provide magnesium and albumin, additives that had been shown earlier to enhance growth at low pH. With this buffer, a 1:10 diluted inoculum (0.1 ml) of *M. tuberculosis* (McFarland standard of 0.5) in the drug vials, and a 1:100 diluted inoculum (0.1 ml) in the control vials the PZA susceptibility test became reliable. Based on our results and using the plate assay as a baseline we interpreted resistance as a three-fold or greater difference in growth between the drug and the control vial on day 5; now 18 of 18 100% resistant and 36 of 36 100% susceptible strains were identified correctly. All 10 1% mixtures and all 10 2% mixtures of resistant and susceptible mycobacteria were

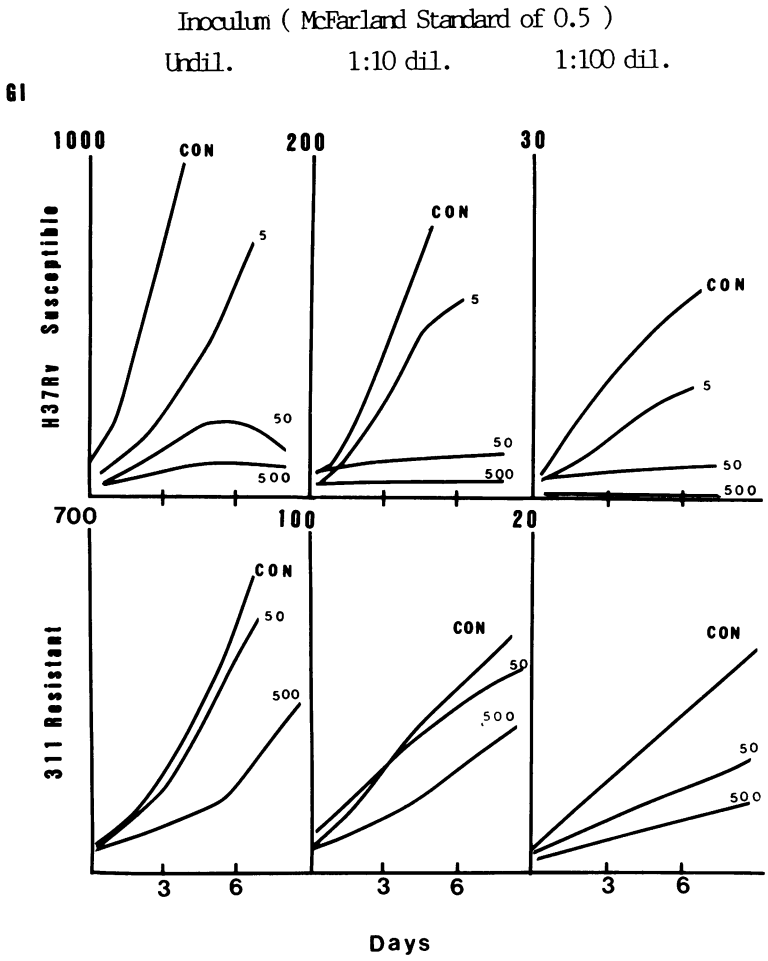

FIG. 1. Effect of inoculum size on growth of susceptible (H37Rv) and resistant (311) *M. tuberculosis* strains in the absence (CON) and presence of PZA (5, 50, 500 μg/ml). Curves represent average daily readings of two vials per group. One GI unit equals approximately 7.5 Bq of ^{14}C (as $^{14}CO_2$).

determined to be resistant. When day 5 drug vial GI readings were divided by day 5 control vial GI readings, the ratio averaged 7.0 (SD, ±1.6; n = 10) for 1% resistant mixtures and 6.2 (SD, ±1.2; n = 10) for 2% mixtures. With susceptible strains the ratio of GI of drug vials over control vials on day 5 was 1.34 (SD, ±0.66; n = 36).

The plate susceptibility assay showed all resistant strains to be 90 to 100% resistant. Susceptible strains never showed growth in the presence of PZA. Of 70 susceptible strains 3 failed to grow on the low-pH control plates and were excluded from further PZA susceptibility testing. The plate results with mixtures of resistant and susceptible *M. tuberculosis* strains were highly variable; 1% mixtures were determined to have an average resistance of 4.8% (SD, ±6.9; n = 59), and 2% mixtures averaged 8.8% (SD, ±13.0; n = 32).

DISCUSSION

In the presence of PZA, growth of mycobacteria continues to a substantial degree in 7H12 medium, even at a pH of 5.5, where drug activity would be expected. With a 100-fold inoculum difference between drug and control vials, growth in the drug vials declines before the controls grow substantially. Eventually, growth in the control vials declines as well. This cessation of growth and subsequent decline in metabolism is independent of the total GI counts and occurs well before substrate exhaustion. The addition of unlabeled palmitate extends growth for no more than 1 day. Thus, the medium fails to support abundant growth at the low pH. This occurs irrespective of the acidification buffer used and necessitates testing within 6 days of incubation. A buffered phosphoric acid acidifier shows the best growth support, but the buffered 7H12 medium is subject to a slow, constant drift of the pH to the alkaline over several days (pH 6.0 on day 5). Growth eventually declines, and the reproducibility of GI readings between drug and control vials decreases. Although magnesium sulfate and albumin improve growth, no alteration of the formulation of 7H12 broth supports growth for more than 7 days at a low pH. Poor growth is not a peculiarity of PZA-resistant *M. tuberculosis*. When tested at pH 6.8, our susceptible strains had an average doubling time of 1.02 days (SD, ±0.05; n = 12), whereas PZA-resistant strains doubled in 1.1 days (SD, ±0.2; n = 18). All 70 of our strains also grew in 7H12 medium acidified with glycine buffer to pH 5.5. In contrast, 3% of these strains failed to grow on plated media.

Inoculum size has a marked effect on growth of *M. tuberculosis* in the presence of PZA in both plate and broth media. This effect was originally observed by Stottmeier et

al. on plate media (10). A dilute inoculum of suspectible bacteria can be protected from PZA activity by the presence of many dead bacteria or spent culture medium. Similarly, a large inoculum of viable bacteria can interfere with PZA inhibition of growth. High PZA concentrations (500 to 1,000 μg/ml) or protease K treatment of culture media can overcome this inhibition. Thus, only a fresh, dilute inoculum of *M. tuberculosis* can be used for PZA susceptibility testing.

Since *M. tuberculosis*, whether PZA susceptible or resistant, will reach peak or stationary growth at pH 5.5 by day 5 or 6, it is important that test conditions allow clear differentiation between resistant and susceptible strains and 1% resistant mixtures within 5 days. This can be achieved by diluting the inoculum with a turbidity of a McFarland standard of 0.5, 1:10 for the drug vial and 1:100 for the control vial. It is important to control the pH during the susceptibility test. The acidification buffer described by Heifets and Iseman (4) promotes excellent growth at pH 5.5. However, pipetting errors with this buffer resulted in considerable pH variations and necessitated an increase in the buffering capacity.

We were able to show complete agreement between vial and plate susceptibility test results with 50 μg of PZA per ml. Due to a strong dependence on inoculum size for reliable test results, we recommend discontinuing any tests with a day 1 drug vial GI below 1 or above 100 units. With experience, this range requirement can be met easily, and test exclusion will be rare. Spectrophotometric or turbidimetric measurements for inoculum adjustments may be useful.

Although 1% resistant populations can be accurately detected, the test cannot be used to calculate the percentage of resistance in a clinical isolate. The relationship between drug vial and control vial growth is nonlinear. One percent resistant mixtures produced a drug/control GI ratio of 7.0 at day 5. Doubling the percentage of resistant organisms changed this ratio to 6.2, and 100% resistant strains had a ratio of 11.3 (SD, ±3). Drug vials containing 100% resistant strains frequently reached a GI of 999 (the limit of the BACTEC 460 scale) by day 5; these data can still be interpreted. Univariant discriminant analysis can be used to pick up an optimal discriminant between the resistant and susceptible populations. However, data obtained with our recommended system with the modified phosphoric acid buffer did not show enough overlap for this analysis. The resistance criteria adopted (growth in the drug vial at least three times that in the control vial) lie approximately 2.5 SD above the mean drug to control GI ratios found for the susceptible strains (1.34; SD, ±0.66; $n = 36$), and 2.5 SD below the combined mean of the drug to control GI ratios for the 1 and 2% resistant populations (6.6; SD, ± 1.49; $n = 20$). Thus, misclassification of susceptibility results should occur infrequently.

In our laboratory, inocula for *M. tuberculosis* susceptibility testing are frequently grown in static 7H9 broth culture held in a slanted position to increase surface area. Whether cultures prepared in this manner could remove the necessity for glass bead grinding for PZA susceptibility testing has not been determined. Supernatants from these cultures contain well-dispersed mycobacteria with little evidence of clumping and provide good inocula for susceptibility testing.

The technique described here provides accurate discrimination of 1% PZA-resistant *M. tuberculosis* from a fully susceptible population. Thus it fulfills the requirements for susceptibility testing of mycobacteria and can be performed with only minor modification of presently used methods.

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