

Radiometric Method for Pyrazinamide Susceptibility Testing of *Mycobacterium tuberculosis* in Egg-Yolk-Enriched BACTEC 12A Medium

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A radiometric method for testing the susceptibility of *Mycobacterium tuberculosis* to pyrazinamide in egg-yolk-enriched 12A medium (pH 5.5) is described. We obtained 100% agreement between the 7H10 agar method with 25 µg of pyrazinamide per ml and the modified radiometric method with a drug concentration of 50 µg/ml in tests of 90 clinical isolates.

Pyrazinamide (PZA) is one of the first-line drugs used in treating tuberculosis. The *in vitro* susceptibility of *Mycobacterium tuberculosis* to PZA is difficult to determine because the bacilli grow poorly at the acid conditions (pH 5.5) required for optimal drug activity (6). Some strains of *M. tuberculosis* will not grow at this low pH unless a heavy bacterial inoculum is used or the bacilli are first adapted for growth at low pH (5, 8). Stottmeier et al. (8) described a procedure with Middlebrook 7H10 agar at pH 5.5 to test the susceptibility of *M. tuberculosis* to PZA. Butler and Kilburn (1) improved the acidified 7H10 agar method by eliminating from the medium the oleic acid that inhibited 17% of the strains tested. In spite of this improvement, 10% of the strains failed to grow on 7H10 medium at pH 5.5, with or without oleic acid. A second approach to PZA susceptibility testing was a radiometric method that used 7H12 liquid medium with the pH reduced to 5.5 after cultures reached the exponential phase of growth (5). This method was not always successful. The investigators found that some drug-resistant strains failed to grow at the lowered pH. Tarrand et al. (10) modified the radiometric procedure further by lowering the pH of 7H12 medium to 5.5 (after exponential growth was attained) by using buffered phosphoric acid solution. They observed that this buffered acid maintained a more stable pH than did the simple acidification solution used previously.

Egg yolk or its extracts are used to support growth of *M. tuberculosis* (4, 7). When added to 7H12 (12A) medium, egg yolk neutralized the mycobacteriostatic effect of cetylpyridinium chloride used for primary isolation of *Mycobacterium* species from sputum (R. W. Smithwick, personal communication). In a preliminary study, we noted that the addition of egg yolk to BACTEC 12A medium at pH 5.5 enhanced the growth of *M. tuberculosis* strain H37Rv. This suggested that PZA susceptibility testing of tubercle bacilli with the BACTEC radiometric system (Johnston Laboratories, Towson, Md.) might be accomplished without either a special adaptation of the bacilli to low pH or an increase in inoculum size.

Spontaneous PZA-resistant mutants of *M. tuberculosis*, strains H37Rv and H37Ra, were isolated for preliminary tests and later used as controls for the evaluation tests. The 10 selected strains of H37Rv and H37Ra (Table 1), together

with 95 clinical isolates of *M. tuberculosis* (a total of 105 strains), received by the Mycobacteriology Laboratory, Centers for Disease Control, Atlanta, Ga., were used to evaluate this new method.

All strains were subcultured on Lowenstein-Jensen (L-J) medium to prepare fresh inocula for both the radiometric and the agar plate PZA susceptibility tests. Pyrazinamide (Sigma Chemical Co., St. Louis, Mo.) was added at final concentrations of 25, 50, 100, and 200 µg/ml to both 7H10 agar and 12A radiometric liquid medium. The 7H10 agar at pH 5.5 (Gibco Laboratories, Grand Island, N.Y.) was supplemented with albumin-dextrose complex enrichment (containing no oleic acid; Difco Laboratories, Detroit, Mich.) as described by Butler and Kilburn (1). Enriched, PZA-free 7H11 agar (Difco), pH 6.8, was also used to detect strains that would not grow at low pH on agar.

Before each test, liquid egg yolk was removed aseptically from alcohol-washed, fresh whole eggs, and 0.1 ml of egg yolk was added to each BACTEC vial containing 2 ml of 12A medium. After the 12A medium and yolk were carefully mixed, 0.1 ml of sterile phosphoric acid solution (approximately 2.5% in water) was added to each bottle, and the pH of several vials was checked. If the pH was not 5.5, the concentration of the acid solution was adjusted and the pH was rechecked. This working solution of phosphoric acid could be stored at 4°C and used for up to 8 weeks. Plain 12A medium without egg yolk was also adjusted to pH 5.5 by using a 1:63 dilution of working solution of phosphoric acid, described previously (5).

The radiometric drug susceptibility test used was a modification of the procedure described by Heifets and Iseman (5). A suspension of each test strain was prepared from L-J medium by adding a loopful of culture to 3 ml of diluting fluid (Johnston Laboratories) in a test tube (16 by 125 mm) containing 1.5 g of sterile glass beads (3-mm diameter) and mixing on a test tube mixer. The turbidity of this undiluted suspension was adjusted visually to approximate that of a McFarland 0.5 standard (11).

A 1:100 dilution of this inoculum was also prepared and used to inoculate pH 5.5, PZA-free control 12A medium with and without egg yolk. The undiluted suspension was inoculated to (i) all the 12A vials containing PZA, (ii) the pH 5.5 control vials without PZA, and (iii) a plain pH 6.8 vial of 12A used to determine that the strain would grow on 12A medium. The vials were tested with the BACTEC 460 for

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TABLE 1. MICs of PZA for 10 selected isolates of *M. tuberculosis* H37Rv and H37Ra that vary in PZA susceptibility^a

Strain no.	MIC ($\mu\text{g/ml}$)	
	7H10 agar	Radiometric (12A with egg yolk)
H37Ra		
1	25	50
2	25	50
3	200	200
4	200	200
H37Rv		
5	25	25
6	25	25
7	100	200
8	200	200
9	100	200
10	100	200

^a The MIC determinations in both 7H10 agar and egg yolk-supplemented 12A broth medium used the modified method of proportions of Canetti et al. (2).

detecting radiolabeled CO_2 . The vials were read daily until the pH 5.5 control (1:100 dilution of the inoculum) with egg yolk reached a growth index of 30 or greater, at which time the test was interpreted.

Each strain was tested also by the PZA procedure on 7H10 (low-pH) medium as described by Stottmeier et al. (8) and modified by Butler and Kilburn (1). Middlebrook 7H9 (Difco) broth cultures of each strain were diluted 10^{-2} and 10^{-4} , and each dilution was inoculated to 7H10 control medium (low pH) and to the same medium containing the four test concentrations of PZA. A 7H11 (pH 6.8) agar plate inoculated with each strain served as a growth control. The inoculated agar plates were incubated for 3 weeks at 37°C .

The susceptibility patterns of 10 pilot cultures, selected to include a variety of mutants both susceptible and resistant to PZA, are shown (Table 1). The preliminary determinations

TABLE 2. Comparison of the PZA susceptibility of 100 strains of *M. tuberculosis* with 7H10 agar and egg-yolk-supplemented 12A medium

Radiometric 12A (egg yolk) susceptibility test		No. of strains in:	
PZA concn ($\mu\text{g/ml}$)	Interpretation	7H10 agar susceptibility test (PZA concn, 25 $\mu\text{g/ml}$)	
		Susceptible	Resistant
25	Susceptible	59	0
	Resistant	29	12
50	Susceptible	88	0
	Resistant	0	12

of the MICs of PZA in 12A for each of the 10 strains of H37Rv and H37Ra (Table 1) correlated well with the MICs determined by the 7H10 agar method (pH 5.5). The resistant strains in this and all subsequent tests proved to be resistant to up to 200 μg of PZA per ml in the radiometric procedure. Strains 5 and 8 of H37Rv were used as susceptible and resistant controls, respectively, for all subsequent studies. An example of a strain that would not grow in 12A at pH 5.5 without egg yolk is the H37Ra PZA^r mutant (Fig. 1); this same strain grew on 7H10 only as minute colonies. Suspensions of 100 strains of *M. tuberculosis*, diluted to contain 10^4 to 10^5 bacilli, were inoculated into 12A medium (pH 5.5), with and without egg yolk. Of the 100 strains, 72 grew both with and without egg yolk, but 28 that grew in 12A with egg yolk failed to grow in 12A without egg yolk. Thus, in the absence of egg yolk, 28% of the strains could not have been tested for susceptibility to PZA. A comparison of results from 7H10 agar plate and radiometric (with egg yolk) susceptibility test procedures is shown (Table 2). Five of the original 105 strains were not used for evaluation because two were subsequently identified as non-*M. tuberculosis* (one *M. bovis*, one *M. kansasii*), one would not grow on 7H10 (low-pH) medium, one would grow on neither L-J medium

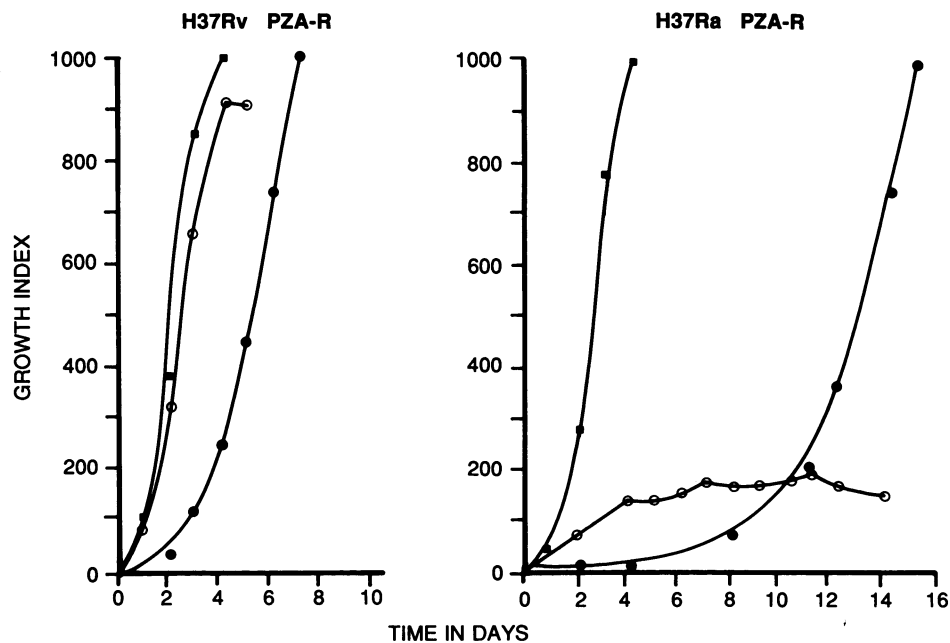


FIG. 1. Comparison of growth of two PZA-resistant (PZA-R) strains of *M. tuberculosis* on standard 12A medium, pH 6.8 (■), and on 12A medium at pH 5.5 with (●) and without (○) egg yolk supplement.

nor 12A medium with egg yolk, and one strain was contaminated. The PZA concentration of 25 µg/ml in the conventional method was compared with both 25 and 50 µg/ml in the radiometric method. The results with PZA at 50 µg/ml in egg-yolk-enriched 12A BACTEC broth agree completely with susceptibility test results obtained by the agar plate method with PZA at 25 µg/ml (Table 2). The average time for completing all radiometric tests was 10 days, compared with 21 days for the conventional method.

An acid environment is required to demonstrate *in vitro* activity of PZA. Strains susceptible to PZA contain the enzyme pyrazinamidase, which converts PZA to pyrazinoic acid. Pyrazinoic acid, the active product that kills the bacteria, also requires an acid environment for activity (5).

Crowle et al. (3) used an *in vitro* macrophage model with its natural acidic environment to demonstrate inhibition of tubercle bacilli by several antituberculosis drugs. The BACTEC radiometric method with liquid 12A medium for PZA susceptibility tests appeared promising (5). However, Tarrand et al. (10) noted that in 12A medium at pH 5.5, susceptible strains of *M. tuberculosis* first showed an increase in growth index and then declined. This decline in growth index was also seen in some of our controls (without egg yolk) inoculated with the 1:100-diluted suspension. This diluted control was critical to enable rough quantitation of the 1% resistant population needed to designate a strain of *M. tuberculosis* resistant to a test drug by the radiometric procedure (9) and to signal the end of the test, i.e., the time when the diluted control exhibits a growth index of ≥ 30 . Tarrand et al. also stated that no modification of 12A medium supported growth of *M. tuberculosis* for more than 7 days at low pH (10). In our study, the addition of egg yolk to 12A medium significantly improved the growth of tubercle bacilli at pH 5.5 so that we could use small inocula to obtain a reliable PZA susceptibility test. We recommend using three vials of 12A medium with egg yolk at pH 5.5: (i) an undiluted control (McFarland 0.5), (ii) a 1:100 dilution of the control, and (iii) a bottle containing PZA (50 µg/ml) inocu-

lated with undiluted suspension of culture. This method should allow PZA susceptibility tests to be done for virtually all strains of *M. tuberculosis*.

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