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New Agar Medium for Testing Susceptibility of *Mycobacterium tuberculosis* to Pyrazinamide

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A new agar medium to perform pyrazinamide (PZA) susceptibility testing with *Mycobacterium tuberculosis* has been developed. This medium has an acidic pH of 6.0 instead of the usual for agar media, pH 6.8, to provide optimal conditions for PZA activity, and it also differs from conventional Middlebrook 7H10/7H11 agar in that animal serum (fetal or calf bovine or fetal equine serum) is used instead of oleic acid-albumin-dextrose-catalase to support good growth of *M. tuberculosis* at the low pH of 6.0. A critical concentration of 900 or 1,200 μg of PZA/ml in this medium made it possible to differentiate between PZA-susceptible and PZA-resistant clinical isolates. This agar medium has the following advantages compared to a liquid medium: it allows determination of the actual proportion of PZA-resistant bacteria in the isolate and it is simple and inexpensive. In addition, it has the potential of being used for a direct susceptibility test with PZA, but this approach will require further confirmation. Further studies to develop critical concentrations of other drugs for this low-pH medium, as well as to investigate the possibility of cultivation in regular (non-CO_2) incubators, are in progress.

Pyrazinamide (PZA) is one of the first-line drugs in the standard treatment regimen currently used for tuberculosis patients. It is recommended in the United States (12), and it is desirable for any country with high prevalence of drug resistance (3), that a drug susceptibility test for pretreatment isolates from new tuberculosis patients be performed, at least with the first-line drugs. A variety of techniques can be used for testing antituberculosis drugs other than PZA, including the agar proportion method in its direct and indirect versions. The only PZA test approved by the Food and Drug Administration in the United States is the radiometric method using the special PZA liquid medium at pH 6.0 in the BACTEC-460 system (10). This method is quite expensive and may not be affordable for many laboratories, especially in developing countries. Unlike the test with other drugs by the agar proportion method, the test in a liquid medium does not provide any information on the actual proportion of the resistant bacteria in the patient's isolate and it cannot be used as a direct test. The previous suggestion of using the agar proportion method for a test with PZA (1, 2) did not lead to this test finding its way into the clinical laboratory practices, because of very poor growth of *Mycobacterium tuberculosis* isolates at pH 5.5 (11).

The aims of this study were (i) to develop an acidic agar medium that would satisfy the requirement for PZA inhibitory activity and provide, at the same time, good growth of *M. tuberculosis* and (ii) to evaluate this medium with PZA-susceptible and PZA-resistant *M. tuberculosis* laboratory strains and clinical isolates.

MATERIALS AND METHODS

Antimicrobial agent. PZA was purchased from Sigma Chemical Co. (St. Louis, Mo.). The necessary solutions were made in distilled water. Three solutions were made to have the final concentrations of 300, 900, and 1,200 μg/ml in the agar medium (see below).

Culture medium preparation. The commercially available Middlebrook 7H10 agar base (BBL, Becton Dickinson and Co., Cockeysville, Md.) was dissolved in deionized water at 14.4 g per 660 ml. Then 4.7 g of monopotassium phosphate (KH_2PO_4) was added to acidify the medium. In addition, 0.72 g of casein hydrolysate and 4.0 ml of glycerol were added. After being autoclaved at 121°C for 12 min, the medium was split into four sterile flasks, 160 ml each. The flasks were placed into the water bath to cool down to 54°C. After that, 20 ml of the sterile animal serum (fetal or calf bovine serum [FBS and CBS] or fetal equine serum [FES]; Sigma Chemical Co.) mixed with 20 ml of the PZA solution (or distilled water for the control) was added to a total volume of 200 ml per flask. The final concentration of the serum was 10%, and the final pH was 6.15 ± 0.1. For the purpose of growth comparison, similar media were made with oleic acid-albumin-dextrose-catalase (OADC) or albumin-dextrose-catalase (ADC) instead of the animal serum.

The media were poured into the 100- by 15-mm four-segment plastic dishes, one segment for the drug-free medium and the three remaining segments for the agar containing three PZA concentrations. After completion of the quality controls for sterility and ability to support growth, the plates were stored at 4°C, protected from light, for a period not longer than 8 weeks.

Drug susceptibility test. A culture of *M. tuberculosis*, cultivated in 7H9 broth at 37°C for a period of 4 to 7 days, was adjusted, using the same medium, to the optical density of McFarland standard no. 1. Two dilutions of this suspension, 10^{-2} and 10^{-4}, were used as an inoculum, 0.1 ml per segment, to inoculate two plates. The plates were sealed in individual polyethylene CO_2-permeable bags (XPEDX, Denver, Colo.) and incubated right side up (agar down) at 37°C in the presence of 5 to 7% CO_2 for a period of 21 days. Afterwards, the plates were removed from the incubator and placed on the bench upside down (agar up) at room temperature for at least 4 h (or overnight) to eliminate the condensate. The plates were examined without opening the polyethylene bags using a dissecting microscope. The colonies on each segment were counted, and the numbers of colonies on drug-containing segments were compared with that on the drug-free control.

Strains. Quality control (QC) strains were *M. tuberculosis* $H_{37}Rv$, susceptible to all antituberculosis drugs (ATCC 27294), and *M. tuberculosis* ATCC 35828, monoresistant to PZA. Other laboratory strains included three susceptible to all drugs, Erdman, Atencio, and 9719, as well as two PZA-resistant mutants developed by us from susceptible strains ($H_{37}Rv$ and 9719) by selection in the presence of 1,200 μg of PZA/ml on agar plates at pH 6.0. In addition to the laboratory strains, 53 clinical isolates were included in this study. Twenty-four of these strains were isolated from newly diagnosed patients and were reported by our clinical laboratory as susceptible to PZA. Our clinical laboratory has identified 29 clinical isolates as resistant to PZA based on the conventional test in the BACTEC broth at pH 6.0. All 53 isolates, along with two QC strains (susceptible $H_{37}Rv$ and PZA-resistant ATCC 35828), were retested in this study by the BACTEC radiometric method using three PZA concentrations, 100, 300, and 900 μg/ml, to determine the MIC, as described previously (4).

RESULTS

Effect of ADC, OADC, and animal sera on growth at low pH. It was previously speculated that oleic acid may inhibit bacterial growth at low pHs, and therefore it was suggested that ADC be used instead of OADC for 7H10/7H11 agar media to obviate the growth inhibition at the acidic pH of 5.5 (1, 2). We

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TABLE 1. Growth recovery of three laboratory strains on four types of agar medium

Supplement[a]	Growth recovery[b] (CFU) for indicated strain at pH:					
	6.8			6.0		
	$H_{37}Rv$	Erdman	Atencio	$H_{37}Rv$	Erdman	Atencio
OADC-1	56/32	Not done	70/29	72/71	29/13	76/77
OADC-2	50/63	77/68	55/13	Not done	Not done	Not done
ADC	45/31	73/60	82/102	5/6	3/9	39/72
FES	55/68	57/91	100/101	57/66	52/41	Not done
FBS	65/65	64/72	96/92	65/52	60/66	84/94

[a] OADC-1 was prepared in the laboratory; OADC-2 was purchased from Remel (Lenexa, Kans.).
[b] Results of two counts from two different plates for each pH and strain are shown.

have compared the growth rates of three strains ($H_{37}Rv$, Erdman, and Atencio) on pH 6.0 agar medium supplemented with 10% of OADC, ADC, FES, or FBS, all obtained from Sigma. For this purpose, we prepared two sets of agar plates (to have duplicates) containing each of the supplements, one having standard pH 6.8 and one with pH 6.0. These plates were inoculated simultaneously with 0.5 ml of the bacterial suspension adjusted to the optical density of McFarland standard no. 1 and than diluted 10^{-6} to have approximately 100 to 200 CFU per plate.

The results of experiments with three laboratory strains are shown in Table 1. These preliminary data showed no significant difference in levels of recovery of *M. tuberculosis* on the pH 6.8 agar in the presence of different supplements. At pH 6.0, the recovery of growth (number of CFU per plate) on media supplemented with FBS or FES was equal to, and sometimes even greater than, that on the standard OADC-containing medium and no less than that on the media with pH 6.8. At the same time, at pH 6.0 growth on the media supplemented with ADC was partially suppressed compared to growth on the media supplemented with either OADC or FBS. In addition, the size and appearance of colonies at the 3-week reading on the pH 6.0 medium with FBS were no different from those at pH 6.8, whereas a reduction in size on the pH 6.0 media supplemented with OADC or ADC was observed.

PZA susceptibility test results with PZA-resistant mutants. The agar proportion method should provide the opportunity for determining the actual proportion of resistant bacteria in the population. To investigate the applicability of this option to the PZA susceptibility test, we conducted experiments with artificially prepared mixtures containing various proportions of PZA-resistant bacteria with the original susceptible strains. For this purpose, we developed PZA-resistant mutants by selection from two pansusceptible strains ($H_{37}Rv$ and 9719) on agar plates containing 1,200 μg of PZA/ml. Mixtures contained 10, 25, or 50% PZA-resistant bacteria. These mixtures were tested along with the original susceptible strains and their resistant mutants. All five cultures were tested by two methods, one using BACTEC PZA broth at 100, 300, and 900 μg/ml and the other using agar plates at 300, 900, and 1,200 μg/ml.

The broth-determined MICs of PZA for two susceptible strains ($H_{37}Rv$ and 9719) were ≤100 μg/ml in the BACTEC pH 6.0 broth medium. While the growth of one of these strains (9719) was completely inhibited by all drug concentrations incorporated in the agar medium, a substantial proportion (35.9%) of another strain ($H_{37}Rv$) was not inhibited by 300 μg/ml in agar (Tables 2 and 3). Growth of both PZA-resistant mutants (bottom rows in Tables 2 and 3) was not inhibited by any of the drug concentrations used for either medium; the mutants showed full resistance to all concentrations used in the BACTEC broth (MIC > 900 μg/ml). Suspensions prepared with the intention of having 10, 25, or 50% PZA-resistant bacteria in the mixtures showed proportions of resistant bacteria grown on the agar plates approximating those in the prepared mixtures. This correlation is indicative of the ability to indicate the proportion of the PZA-resistant bacteria in a specimen, even if such proportion is as low as 10%.

PZA susceptibility test with clinical isolates. Tables 4 and 5 include data obtained with 53 clinical isolates and 2 QC strains, a total of 55 cultures tested in this series of experiments. The results of the test in agar medium supplemented with CBS were compared with those of the BACTEC method using different PZA concentrations. Tables 4 and 5 analyze the results for 900 and 1,200 μg/ml of PZA in agar medium versus 300 or 900 μg/ml in the BACTEC medium. This analysis indicated 100% agreement for 25 PZA-susceptible strains (including $H_{37}Rv$ strain) tested with PZA at either 900 or 1,200 μg/ml incorporated in the agar medium versus 300 μg/ml in the BACTEC medium (Table 4). From a total of 30 strains (29 clinical isolates and 1 QC strain [ATCC 35828]) identified as resistant to PZA at 300 μg/ml by the BACTEC method, resistance to PZA in agar medium was observed for 29 strains (including the QC strain) with a concentration of 900 μg/ml

TABLE 2. Evaluation of two PZA susceptibility testing methods using mixtures of the original susceptible strain $H_{37}Rv$ and its PZA-resistant mutant

% Resistant bacteria in mixture	% Resistant bacteria on agar plates with PZA concn (μg/ml) of:			MIC (μg/ml)	
	300	900	1,200	Agar	BACTEC
0	35.9	0.7	0	900	<100
10	42.7	23.8	18.9	>1,200	>900
25	42.6	26.6	23.9	>1,200	>900
50	57.0	78.5	43.5	>1,200	>900
100	98.6	74.1	96.3	>1,200	>900

TABLE 3. Evaluation of two PZA susceptibility testing methods using mixtures of the original susceptible strain 9719 with its PZA-resistant mutant

% Resistant bacteria in mixture	% Resistant bacteria on agar plates with PZA concn (μg/ml) of:			MIC (μg/ml)	
	300	900	1,200	Agar	BACTEC
0	0	0	0	<300	<100
10	13.8	8.6	8.6	900	>900
25	18.1	16.2	16.5	>1,200	>900
50	35.9	36.9	40.0	>1,200	>900
100	97.8	96.1	86.2	>1,200	>900

TABLE 4. Comparison of the PZA susceptibility testing of *M. tuberculosis* strains on agar plates versus in the BACTEC broth with PZA at 300 μg/ml

BACTEC result	No. of strains with indicated result on agar plates at PZA concn (μg/ml) of:				Total
	900		1,200		
	Susceptible	Resistant	Susceptible	Resistant	
Susceptible	25	0	25	0	25
Resistant	1	29	3	27	30
Total	26	29	28	27	55[a]

[a] Includes 53 clinical isolates and 2 QC strains.

(96.7%) and for 27 strains (including the QC strain) with 1,200 μg/ml (90%).

When the breakpoint of 900 μg/ml was used in the BACTEC system (Table 5), the agreement in results for susceptible strains (including $H_{37}Rv$) was 92.9% (26 of 28 strains) for PZA at 900 μg/ml in agar medium and 96.4% (27 of 28 strains) for PZA at 1,200 μg/ml in agar.

In addition, we have compared two cultivation conditions for the PZA test in the low-pH agar medium with 1,200 μg of the drug/ml: in the CO_2 incubator (in an atmosphere containing about 7% CO_2) and in a regular incubator without CO_2. No difference between these two conditions has been found, either in regard to the ability of the low-pH agar medium to support growth of the tested strains or in the results of the PZA test. These preliminary data indicated the need for further investigation of the possibility of using this low-pH agar medium for cultivation and drug susceptibility testing of *M. tuberculosis* cultures in regular incubators without CO_2.

DISCUSSION

The most reliable among all currently available methods for a test of the susceptibility of *M. tuberculosis* to PZA is the radiometric BACTEC technique (10), especially when three PZA concentrations are used to determine the MIC (4). There were reports that this method may give false-positive and false-negative results when a single concentration, 100 μg/ml, is used (4–6). Nevertheless, the reliability of this method (with 300 μg/ml) has been confirmed by detection, using DNA sequencing, of mutations specific for PZA resistance in the pyrazinamidase gene, *pncA*, in strains identified by the BACTEC technique as PZA resistant but not in susceptible strains (8, 9). Therefore, the BACTEC PZA test has been used in this study as a "gold standard" for evaluation of the PZA susceptibility test in the new agar medium. The BACTEC method, though reliable, has certain disadvantages. One of them is that it can be used only as an indirect method, which requires initial isolation of a pure culture; and it cannot be used as a direct test with acid-fast bacillus-positive specimens. The total turnaround time of this method is about 4 weeks. Among other problems associated with the BACTEC technology are high cost, the need for disposal of a substantial volume of ^{14}C-radiolabeled culture vials (which is not permitted in many countries), and the fact that most of the tuberculosis laboratories in the world do not use, and cannot afford to use, the BACTEC system. In addition, the BACTEC method does not provide information on the actual proportion of the PZA-resistant bacteria in the patient's isolate. While a number of alternative methods for susceptibility testing with other drugs are widely available, the BACTEC method is considered by many investigators as the only reliable technique for a test with PZA.

Previously, there were attempts to develop an agar-based PZA susceptibility test, and one of them involved using an ADC supplement instead of OADC in a pH 5.5 agar medium (1, 2). This method did not find its way into clinical laboratories because of insufficient growth of *M. tuberculosis* isolates on this medium.

We have detected some suppression of growth of *M. tuberculosis* on the acidic agar medium (pH 6.0) supplemented with either ADC or OADC. We found, on the other hand, that the acidic pH did not have such a negative effect when the medium was supplemented with an animal serum (FBS, CBS, or FES). Moreover, the growth of *M. tuberculosis* on the agar medium supplemented with the animal serum at pH 6.0 was even better than that on the conventional 7H11 agar, at pH 6.0 or 6.8, supplemented with OADC and especially with ADC. Previously we have shown a correlation between the pH of the medium and the PZA concentration necessary to inhibit growth of *M. tuberculosis*. For example, 50 μg/ml was required to inhibit growth at pH 5.5 in a liquid medium, but the same effect could have been achieved with 300 to 400 μg/ml at pH 6.0 (7).

In the present study we found that the growth of 25 PZA-susceptible *M. tuberculosis* strains was inhibited in a pH 6.0 agar medium (supplemented with an animal serum) with a concentration of 900 or 1,200 μg of PZA/ml but that good growth occurred in the drug-free controls. At the same time, 29 out of 30 PZA-resistant strains produced sufficient growth in the presence of PZA at 900 μg/ml in this medium. The predictive value of the agar method by comparison with the conventional BACTEC method (100 and 300 μg/ml) was 100% for susceptible strains and 96.7% for resistant strains. We have also demonstrated that a test by the proportion method using the pH 6.0 agar medium can provide clear information on the proportion of PZA-resistant bacteria in the inoculum.

Based on the results presented in this report, we are proposing a new agar medium, different from the Middlebrook 7H11 agar in pH (6.0 instead of 6.8) and in the growth supplement (animal serum instead of OADC). This medium can be used for susceptibility testing of the *M. tuberculosis* isolates with PZA by an agar proportion method. The best results were obtained when CBS (10%) and 900 μg of PZA/ml (as a critical concentration) were incorporated into this medium. Further studies with a larger number of strains are needed to make a final choice between 900 and 1,200 μg/ml of PZA as the critical concentration. One of the advantages of the agar proportion test with PZA on this medium over the conventional PZA test in the BACTEC system is that it is less expensive. Materials to prepare a biplate containing PZA and drug-free agar cost about 56 cents. The total direct cost, including labor, is about $1.00. The agar medium can be prepared in-house, while the supplies alone for the BACTEC PZA test cost about $12, according to the catalog, and have to be purchased from the manufacturer.

TABLE 5. Comparison of the PZA susceptibility testing of *M. tuberculosis* strains on agar plates versus in the BACTEC broth with PZA at 900 μg/ml

BACTEC result	No. of strains with indicated result on agar plates at PZA concn (μg/ml) of:				Total
	900		1,200		
	Susceptible	Resistant	Susceptible	Resistant	
Susceptible	26	2	27	1	28
Resistant	0	27	1	26	27
Total	26	29	28	27	55

A susceptibility test with all first-line antituberculosis drugs in the United States is now recommended for all new patients, but this requirement cannot be implemented for PZA, one of the first-line drugs, in laboratories that are not equipped with the BACTEC-460 system. Introduction of the new medium described in this report will allow performance of the PZA susceptibility test in any laboratory that is capable of performing the agar proportion susceptibility test with other drugs.

One of the important advantages of the agar proportion test with PZA is that it has a potential of being used not only as an indirect test with previously isolated cultures but also as a direct test with raw specimens. Such an approach may shorten the total turnaround time to only 3 weeks, a time that is well known from experience in applying the direct test with other drugs to acid-fast bacillus-positive sputum specimens (4). The validity of a direct PZA test by the agar proportion method requires additional studies. Whether the direct or indirect PZA susceptibility test is done by the agar proportion method, only this technology can provide information on the actual proportion of resistant bacteria in the patient's isolate. Other promising directions for studies with the low-pH (pH 6.0) agar medium, supplemented with animal sera, include the possibility of cultivation in a regular (without CO_2) incubator and evaluation of these conditions for testing the susceptibility to drugs other than PZA.

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