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Acidometric Agar Plate Method for Ampicillin Susceptibility Testing of *Haemophilus influenzae*

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The need for an accurate and rapid method of testing ampicillin susceptibility of *Haemophilus influenzae,* especially strains isolated from patients with meningitis and septicemia, is indisputable. Various methods have been employed for this purpose. Each has advantages and disadvantages. This report describes a modification of the capillary acidometric procedure in which an agar plate is substituted for a tube. All beta-lactamase results obtained by this modified technique correlated with minimal inhibitory concentrations determined in liquid media and the chromogenic cephalosporin substrate method. This modified acidometric agar procedure is a simple, inexpensive, accurate, and rapid way to determine *H. influenzae* susceptibility to ampicillin.

Since 1974, patients with meningitis due to ampicillin-resistant *Haemophilus influenzae* have been reported (2, 3, 6). Thus, ampicillin is no longer the initial drug of choice and has been replaced by chloramphenicol alone or in combination with ampicillin (recommendation of the Committee on Infectious Diseases of the American Academy of Pediatrics). Due to the toxic nature of chloramphenicol, most clinicians wish to discontinue its use as soon as possible; this makes testing for susceptibility to ampicillin an important procedure. Methods such as minimal inhibitory concentration (MIC) determination and agar diffusion are available but require a minimum of 18 h after isolation of the infecting organism. Since the resistance of *H. influenzae* to ampicillin was found to be due to the production of penicillin beta-lactamase, rapid methods for detection of this enzyme have been employed, such as iodometric (1; P. C. Fleming and B. Markowsky, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 15th, Washington, D.C., Abstr. no. 387, 1975), chromogenic cephalosporin (4), and acidometric capillary tubes (5). This report describes a modification of the capillary acidometric method in which an agar plate is substituted.

MATERIALS AND METHODS

Organisms. A total of 67 strains of *H. influenzae* were tested in this investigation. These consisted of 37 resistant (17 obtained from Children's Hospital National Medical Center) and 30 susceptible strains. The organisms were isolated from cerebrospinal fluid blood, ear, eye, nose, throat, sputum, and peritoneal cavity.

Acidometric agar plate. To 100 ml of distilled water, 1.5 g of agar and 0.2 ml of 0.5% phenol red solution were added and boiled until the agar dissolved. Using 1 N NaOH, the pH was adjusted between 8.5 and 9.0, confirming with pH paper. Then 20-ml portions were dispensed into test tubes and kept at 2 to 8°C (stable at least 6 months). When needed, a portion of medium was melted and cooled to 50 to 55°C. Penicillin G powder was then added to yield a concentration of 5,000 U/ml. The tube was mixed well, and the pH was rechecked and adjusted if necessary (pH 8.5 to 9.0). This solution was poured into a petri dish and allowed to solidify. A clump of colonies was placed in a discrete spot on the plate, as were positive and negative controls (ampicillin-resistant and -susceptible *Staphylococcus aureus* or *H. influenzae,* respectively). The plate was incubated at 35°C or at room temperature for a maximum of 1 h.

MIC determination. For measurement of MIC, Trypticase soy broth with 3% Fildes enrichment (Baltimore Biological Laboratory) was used. Two-milliliter portions of the broth were dispensed into sterile tubes, and twofold serial dilutions of ampicillin were prepared. Inoculum was harvested from overnight growth on chocolate agar that was suspended in sterile water and adjusted by comparison with half the no. 1 McFarland standard. The tubes were incubated at 35°C for 24 h after delivery of a 0.05-ml inoculum.

RESULTS

Resistant strains produced a bright-yellow zone around the colonies due to the release of penicilloic acid, whereas the color of the area around susceptible organisms remained unchanged (Fig. 1). By using chromogenic cephalosporin substrate, all 37 resistant strains were confirmed as beta-lactamase producers. The appearance of the yellow zone was slightly sooner when cultures were incubated at 35°C than at room temperature; however, none of the resistant strains took longer than 30 min to produce

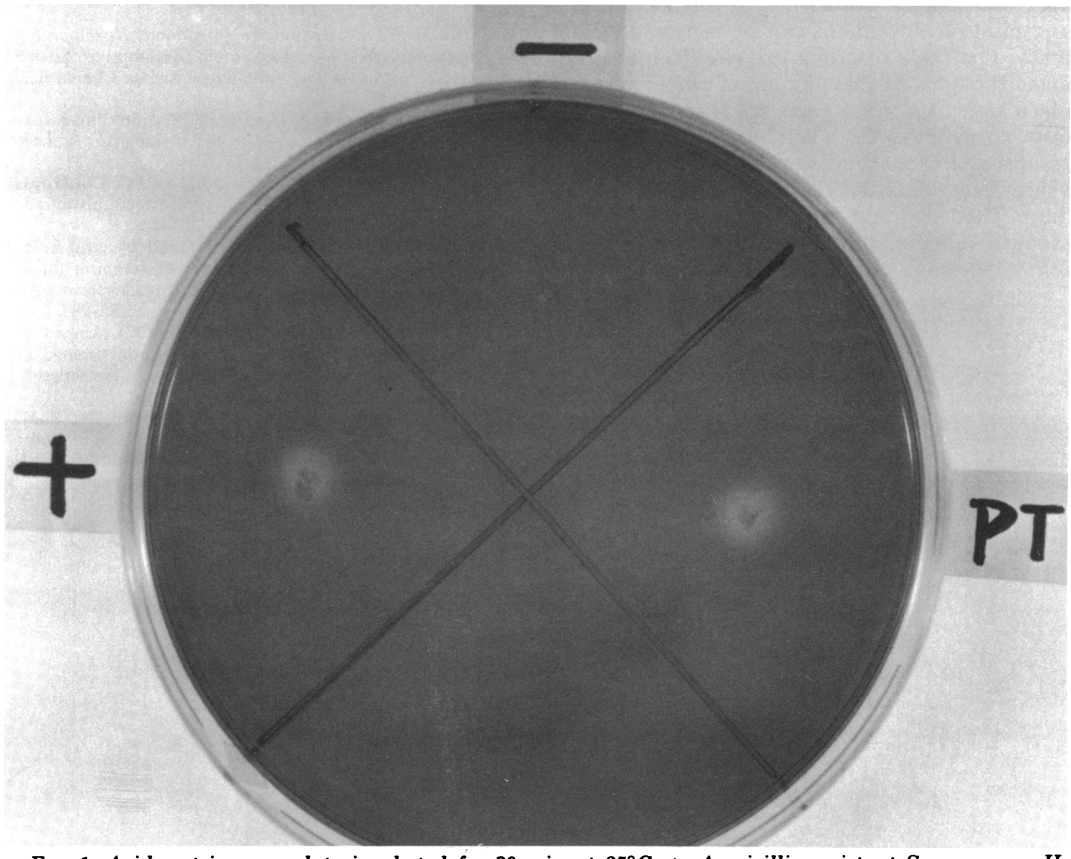

FIG. 1. *Acidometric agar plate incubated for 30 min at 35°C. +, Ampicillin-resistant S. aureus or H. influenzae; −, ampicillin-susceptible S. aureus or H. influenzae; and PT, ampicillin-resistant H. influenzae from patient.*

a yellow zone, regardless of temperature. Most produced a yellow zone within 10 min. This zone persisted over a 24-h period, becoming gradually larger but not extending over the entire surface of the plate. The optimum pH was found to be between 8.5 and 9.0. At pH values lower than 8.5, reactions may be difficult to read, and at pH values greater than 9.0, the reaction is slower. MIC determinations indicated that resistant strains were inhibited by ampicillin concentrations greater than or equal to 8.0 μg/ml, and susceptible strains showed MIC values less than 0.5 μg/ml.

DISCUSSION

Rapid methods for the detection of beta-lactamase can provide susceptibility data within 1 h after the appearance of colonies (1, 4, 5; Fleming and Markowsky, 15th ICAAC, Abstr. no. 387). Each of these methods has its advantages and disadvantages, and this report offers an alternative to them.

The iodometric method of Fleming and Markowsky (15th ICAAC, Abstr. no. 387), although quick and easy to interpret, is cumbersome to perform, and the paper used in the test varies in starch content, making some papers work better than others. The iodometric variation of Catlin (1) is less cumbersome than that of Fleming and Markowsky, but the reagents (starch and phosphate-penicillin solutions) must be prepared daily. The chromogenic cephalosporin (4) reagents are not available in the United States, thus making this a very expensive test. The capillary acidometric technique (5) is a very delicate one, and one must take special care to insure that no air bubbles are trapped between the colony and the phenol red solution. This solution containing penicillin G is stable only 7 days at −60°C, so much is wasted unless a great number of tests are run. After 24 h, nonspecific positive reactions occur with this method.

The reagents (portions of media and penicillin G powder) used in this modified acidometric

agar method are stable and readily available, and multiple specimens can be run on a single plate. The color reactions are easy to interpret, and although a certain amount of diffusion takes place after 24 h, results are still readable and no false positive results occur. All beta-lactamase results by this method correlated with the MIC values and chromogenic cephalosporin procedure. This modified acidometric method is an accurate, simple, and rapid way to measure *H. influenzae* susceptibility to ampicillin.

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