

Simple Screening Method for β -Lactamase-Positive and -Negative Ampicillin-Resistant *Haemophilus influenzae* Isolates

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A simple, inexpensive method for screening for β -lactamase-positive and β -lactamase-negative ampicillin-resistant *Haemophilus influenzae* isolates was developed. Disks containing 10 μ g of cloxacillin yielded no zone of inhibition when placed on chocolate agar plates inoculated with β -lactamase-positive (16 strains) or ampicillin-resistant (≥ 1 μ g/ml) β -lactamase-negative (10 strains) *H. influenzae*, whereas ampicillin-susceptible (≤ 0.5 μ g/ml; 36 strains) *H. influenzae* almost always (92%) showed a zone of inhibition.

Until 1973, all *Haemophilus influenzae* isolates were susceptible to the drug of choice, ampicillin. Significant β -lactamase-mediated resistance to ampicillin is now seen in up to 35% of *H. influenzae* isolates in some areas today (6, 9, 12, 13, 15). Sporadically, reports have been published of *H. influenzae* isolates which are resistant to β -lactams but which do not produce β -lactamase(s) (2, 11, 16). Some of these nonenzymatically resistant strains have been the cause of treatment failure (2, 7, 11). With the introduction and use of the newer β -lactamase-stable β -lactams, selection will likely increase the frequency of such nonenzymatic resistance to *H. influenzae*.

Clinical screening of *H. influenzae* for the presence or absence of β -lactamase is rapidly done by using a variety of β -lactamase tests. The chromogenic cephalosporin, nitrocefin, is particularly convenient (8, 10). Such testing easily distinguishes between β -lactamase-positive *H. influenzae* and those strains which do not produce β -lactam-hydrolyzing enzymes. An efficient, economical method of screening for low-level and non- β -lactamase-mediated β -lactam resistance in *H. influenzae* has not been developed. In this paper, we describe a method effective for screening for both β -lactamase-positive and β -lactamase-negative ampicillin-resistant *H. influenzae* isolates.

Sixty-one *H. influenzae* isolates were collected from a variety of clinical sources. A total of 36 isolates were susceptible to ampicillin (MIC, ≤ 0.5 μ g/ml), 15 isolates were β -lactamase positive, and 10 isolates were resistant to ampicillin by agar dilution (MIC, ≥ 1.0 μ g/ml) but did not contain β -lactamase by nitrocefin assay. All resistant and β -lactamase-positive isolates were obtained from recent clinical isolates of Foothills Hospital (Calgary, Alberta, Canada). The nonenzymatically resistant isolates came from Foothills Hospital, Edmonton General Hospital (Edmonton, Alberta), and the University of Alberta Hospital (Edmonton).

MICs were determined by using an agar dilution method (3). Colonies from overnight cultures on chocolate agar plates were picked and diluted to 10^7 CFU/ml in 0.9% NaCl. This solution was used as the inoculum for a Steers replicator (14) (Cathra Diagnostic Equipment Inc., St. Paul, Minn.). Agar dilution plates were made with Mueller-Hinton agar base supplemented with 1% hemoglobin and 1% CVA (all GIBCO Laboratories, Burlington, Ontario, Canada). The pH of the agar was adjusted to 7.2. Doubling antibiotic dilutions

were used. Plates were incubated with 5% CO₂ at 35°C for 18 to 24 h. Six-millimeter commercial disks (Oxoid Ltd., Nepean, Ontario, Canada) containing either 10 μ g of ampicillin, 5 μ g of oxacillin, or 5 μ g of cloxacillin were used for disk susceptibility testing. Cloxacillin disks with from 5 to 15 μ g of antibiotic were prepared in our laboratory. Disk susceptibility testing was done by the method of Barry and Thornsberry (1). Plates for disk diffusion testing contained chocolate agar. All strains were identified as *H. influenzae* based on satellitism and the prophylin production test (4).

The MIC of ampicillin for inhibition of *H. influenzae* versus the zone diameter of disks containing 10 μ g of cloxacillin was compared for all test strains (Fig. 1A). A zone of inhibition was noted for all but three isolates requiring for inhibition an ampicillin MIC of ≤ 0.5 μ g/ml. Those isolates requiring for inhibition an ampicillin MIC of 1.0 μ g/ml or greater had no visible zone of inhibition. All β -lactamase-positive isolates failed to show any zone of inhibition with the disks containing 10 μ g of cloxacillin.

The MIC of ampicillin versus the zone diameter of disks containing 10 μ g of ampicillin was compared for all strains tested (Fig. 1B). These results showed that a disk containing 10 μ g of ampicillin was useful for distinguishing β -lactamase-positive strains from those which do not have such an enzyme. However, there was little ability with this method to distinguish low-level (1 to 2 μ g/ml) ampicillin-resistant isolates from fully susceptible *H. influenzae* strains.

The use of disks containing 10 μ g of cloxacillin as a screening method for ampicillin resistance in *H. influenzae* was efficacious with this series of isolates. All *H. influenzae* isolates requiring for inhibition MICs of 1.0 μ g/ml or greater showed no zone of inhibition around the disks containing 10 μ g of cloxacillin. Susceptible strains of *H. influenzae* showed a zone of inhibition in 92% of cases. A disk containing 10 μ g of cloxacillin, therefore, provides important information about ampicillin susceptibility in *H. influenzae*.

The disk concentration was critical. For 20 prototype strains (including susceptible, nonenzymatically resistant, and β -lactamase-positive isolates), the cloxacillin concentration in the disks was varied from 5 to 15 μ g by 2- μ g increments. The effective concentration for the test was from 9 to 11 μ g per disk. Above and below these concentrations the test was ineffective (data not shown). Commercial disks containing 5 μ g of either oxacillin or cloxacillin appeared in some batches to provide information equivalent to that shown in Fig. 1A. However, repetition of such data was not possible with different lots of the disks. The actual

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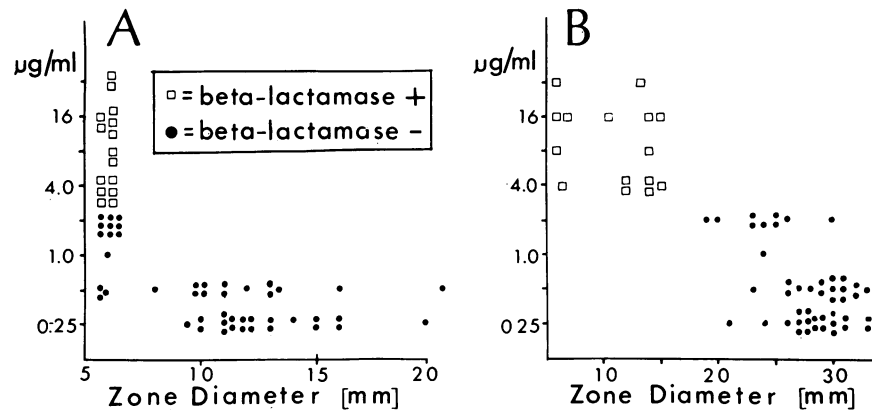


FIG. 1. MIC of ampicillin required to inhibit 61 *H. influenzae* isolates versus zone diameter of 6-mm disks containing 10 µg of cloxacillin (A) or ampicillin (B). MICs were done by agar dilution.

concentration of the drugs in some disk batches varied enough to produce differences in the results.

Although this test provides an efficient, economical method of determining ampicillin resistance in *H. influenzae*, the importance of β -lactamase resistance in the species requires that this screening test be used in conjunction with a test for β -lactamase activity.

It has been suggested previously that susceptibility testing for *H. influenzae* can be accomplished by using disks containing 10 µg of ampicillin (1). The results we have gathered show that low-level nonenzymatic ampicillin resistance (1.0 to 2.0 µg/ml) is not readily distinguished by this method. Low levels of ampicillin resistance have been shown in other species to be of clinical importance (5). Clinical treatment failures have been reported with β -lactamase-negative *H. influenzae* isolates (2, 7, 11). Systemic infection with *H. influenzae* often involves the meninges, and β -lactam penetration into the cerebrospinal fluid is generally poor. It is important, therefore, to be able to distinguish low-level resistant isolates.

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