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NOTES

Susceptibility of *Haemophilus influenzae* to Ampicillin as Determined by Use of a Modified, One-Minute Beta-Lactamase Test

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Fifty-seven cultures of *Haemophilus influenzae* were tested for production of beta-lactamase by use of a modified, 1-min test. Only the 24 cultures that were ampicillin resistant, whether type b or not, yielded positive results for the enzyme; all ampicillin-susceptible isolates were uniformly negative. The beta-lactamase procedure is simple to perform and requires only commonly available reagents.

Within the past 2 years ampicillin-resistant strains of *Haemophilus influenzae* have been isolated from critically ill patients as well as carriers (3, 7); hence, rapid and accurate susceptibility testing of this organism has become important. Unfortunately, Mueller-Hinton agar, which is employed in the "Kirby-Bauer technique," is nutritionally inadequate for growth of *Haemophilus*, and this precludes the use of the standard disk diffusion procedure for susceptibility studies of the organism. Moreover, alternatives such as testing by disk diffusion on chocolate agar or by the broth dilution minimal inhibitory concentration (MIC) technique yield unreliable (4) or highly method-dependent results (9), respectively.

Ampicillin resistance of *H. influenzae* has been reported to be mediated by production of beta-lactamase (1-3, 5, 8), and applicability of two tests for detection of this enzyme in cultures of the organism have already been reported. One test is based upon detection of a pH change that results when penicilloic acid is produced from penicillin by a cellular plug of the organisms interphased with the antibiotic in a capillary tube (8); the procedure was originally used for testing staphylococci (6). The second test for detection of beta-lactamase from *H. influenzae* uses an iodometric technique performed in small tubes or in wells of a microtiter plate (1). This report presents a modification of the capillary tube test, as performed by Thornsberry and Kirven (8), for detection of beta-lactamase from *H. influenzae*. The modified technique is simpler, more rapid, and more economical to perform than either of the other two methods cited. It differs from the capillary tube method in that the modified test entails the addition of the penicillin-phenol red test solution to saline suspensions of the test organisms in a test tube. This modification increases the surface exposure of the organisms to the antibiotic, thereby increasing the overall reaction rate of the beta-lactamase, if present. Final and definitive results are thus provided within 60 s in lieu of the 30 min recommended for the capillary tube technique. The penicillin substrate was also modified slightly in that the injectable (buffered) form of the antibiotic is used instead of pure potassium penicillin G, and the antibiotic is used at a concentration of 200,000 U/ml instead of 1,000,000 U/ml. The latter modifications are intended to provide a more readily available and more economical reagent.

Fifty-seven *H. influenzae* cultures were tested by the modified, 1-min technique. Forty-one cultures were isolated from the nasopharynx of children during prevalence studies in Maryland (7) and Virginia. The remaining 16 cultures were submitted to this laboratory from various hospitals in the eastern United States and were isolated from patients with systemic disease.

Beta-lactamase test reagent. The penicillin-phenol red test reagent for the beta-lactamase test was prepared by reconstituting a vial containing 1,000,000 U of injectable, buffered potassium penicillin G (Chas. Pfizer & Co., Inc., N.Y., N.Y., or Eli Lilly and Co., Indianapolis, Ind.) with 4.5 ml of distilled water and adding

0.5 ml of a 0.5% aqueous solution of phenol red. A drop of 1 M NaOH was then added to impart a violet color (pH 8.5) to the solution.

Inoculum for beta-lactamase test. Bacteria for the beta-lactamase test were removed with an inoculating loop from overnight cultures grown on chocolate agar (containing 3.6% GC agar base, 1% hemoglobin, and 1% IsoVitaleX, BBL, Cockeysville, Md.), and the cells of each culture were suspended in 0.5 ml of normal saline in a sterile, disposable test tube (12 by 75 mm). Enough growth was added to the saline to produce a cloudy-to-turbid appearance equivalent to that of a MacFarland no. 5 standard.

Beta-lactamase test. Three drops of freshly made beta-lactamase test reagent was added to the 0.5 ml of turbidity-adjusted saline suspension of test organisms, and the contents were immediately mixed by gently shaking the test tube. Tests in which the solution changed from violet to an intense yellow color within 1 min were interpreted as being positive for beta-lactamase. Two tubes served as controls. A negative control contained only 0.5 ml of saline plus 3 drops of the test reagent; the second or positive control contained 0.5 ml of saline and 1 drop of a 400,000-U/ml solution of penicillinase (Neutrapen, Riker Laboratories, Northridge, Calif.) plus 3 drops of the test reagent.

All positive test results were promptly apparent, most being evident immediately upon addition of the test reagent, though a few required up to 45 s for development of the full, intensely yellow color. All tests in which no color change occurred, or in which the change was only to a red or pale pink color, were interpreted as being negative for beta-lactamase. All tests negative at the end of 1 min remained negative for at least 10 h, after which some turned to light yellow, presumably due to spontaneous hydrolysis of the penicillin.

To determine the correlation between beta-lactamase production with susceptibility to ampicillin, all cultures were tested by the tube dilution MIC susceptibility technique. The inoculum for the MICs was prepared by growing six to ten colonies of the test culture for 18 to 24 h at 35 C in Trypticase soy broth (BBL) containing 5% Fildes enrichment (Difco Laboratories, Detroit, Mich.). The growth was then diluted to 10^4 colony-forming units in 10% Fildes-enriched Trypticase soy broth, and a 0.5-ml volume of the diluted culture was added to each tube of 0.5 ml of ampicillin trihydrate (Bristol Laboratories, Syracuse, N.Y.), which had been serially diluted in Trypticase soy broth. The series of inoculated ampicillin tubes was incubated at 35 C, and the lowest concentration of ampicillin that completely inhibited growth (turbidity) upon a 24-h incubation was recorded as the MIC.

Table 1 shows the correlation between results of the 1-min beta-lactamase test and the MICs of the isolates. All *H. influenzae* with an MIC of 7.8 μg/ml or greater yielded positive tests for beta-lactamase by the modified test, regardless of whether the isolates were type b or not.

To determine the minimal amount of enzyme required to produce a positive beta-lactamase test by the modified procedure within 1 min, a twofold serial dilution gradient of penicillinase (Neutrapen, Riker Laboratories) ranging from 50,000 to 1.5 U was prepared in 0.5-ml volumes of cell-free normal saline (pH 6.4), and 3 drops of the penicillin-phenol red reagent was delivered to each tube by Pasteur pipette. All tubes containing as little as 97.7 U of Neutrapen penicillinase produced a positive reaction (intense yellow color) immediately, or within 1 min. The tests were conducted at 24 C.

Current literature indicates that only a small number of ampicillin-resistant *H. influenzae* have been analyzed for production of beta-lactamase. To my knowledge, all ampicillin-resistant *H. influenzae* type b isolates tested have uniformly been found to produce the enzyme. In the present study, all *H. influenzae* isolates that were ampicillin-resistant (including five "non-b" isolates) were found to produce the enzyme. Thornsberry and Kirven (8), however, in evaluating the applicability of the capillary tube technique for detection of beta-lactamase from *H. influenzae,* found two out of five "non-b" ampicillin-resistant strains that did not produce detectable amounts of the enzyme by their technique.

If forms of resistance other than beta-lactamase production exist among *H. influenzae,*

TABLE 1. *Correlation between susceptibility of H. influenzae isolates and results of modified beta-lactamase test*

No. of strains	Type	Susceptibility		1-min beta-lactamase test
		MIC (μg of ampicillin/ml)	Interpetation	
11	b	>125	Resistant	Positive
2	b	62.5	Resistant	Positive
1	b	31.3	Resistant	Positive
2	b	15.6	Resistant	Positive
3	b	7.8	Resistant	Positive
5	b	0.5	Susceptible	Negative
10	b	<0.24	Susceptible	Negative
5	Non-b	62.5	Resistant	Positive
3	Non-b	0.5	Susceptible	Negative
15	Non-b	<0.24	Susceptible	Negative

even among "non-b" strains, such an occurrence will necessarily limit the significance of beta-lactamase tests as presumptive evidence of ampicillin resistance or susceptibility. The importance of such tests when performed on clinically significant isolates should not be minimized, however, since a positive test surely indicates that ampicillin therapy will most probably prove unrewarding.

The results of the modified beta-lactamase test demonstrate that the technique is sensitive, and that susceptibility of *H. influenzae* to ampicillin, in vitro, can be accurately predicted within 1 min.

Stability of the test solution after preparation is approximately 1 week, when refrigerated at 2 to 4 C, or until the pH of the solution demonstrates that the penicillin has been spontaneously hydrolyzed. The great simplicity, low cost, and readily available reagents required to perform this test make the technique practical, even in the smallest and most limited laboratory setting. The test can be conveniently performed from the excess cell suspensions, such as are ordinarily prepared for serotyping by agglutination technique, though care should be taken that sufficient amounts and turbidity of organisms be used. Bacterial inoculum for the test need not be fresh or actively growing cells, since consistent results were obtained when cultures up to 10 days old were retested. In this study, all bacterial inocula for the beta-lactamase test were obtained from growth on chocolate agar plates. Growth harvested from other types of media were not evaluated.

Applicability of the test procedure to detection of beta-lactamase directly from centrifuged sediment of purulent cerebral spinal fluid is an important possibility and merits investigation. If the modified test demonstrates the desired specificity and sensitivity in detecting beta-lactamase from resistant organisms in such specimens, physicians could quickly be provided with laboratory data helpful in early selection of appropriate antibiotic therapy for meningitis patients.

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