

Iodometric Detection of *Haemophilus influenzae* Beta-Lactamase: Rapid Presumptive Test for Ampicillin Resistance

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Strains of *Haemophilus influenzae* type b sporadically isolated from clinical specimens are ampicillin resistant due to production of a β -lactamase. This enzyme which inactivates ampicillin and penicillin G is not produced by ampicillin-susceptible strains. Various characteristics of β -lactamase production and ampicillin resistance of three *H. influenzae* type b isolates were investigated. A sensitive iodometric test was employed to detect β -lactamase; positive results were obtained in 5 min with 10^9 bacteria taken from cultures on a nutritionally adequate agar medium. This simple chemical test will enable the hospital laboratory to obtain presumptive evidence of ampicillin resistance on the same day that *H. influenzae* is isolated.

Ampicillin is commonly used for therapy of children with systemic disease caused by *Haemophilus influenzae*. The occasional treatment failures reported earlier were attributed to host-related conditions rather than to genetic resistance of the bacteria (1, 9, 16). However, recent reports have documented the occurrence of clinically significant ampicillin resistance (4-6, 10, 12, 19). Fifteen cases of disease caused by ampicillin-resistant *H. influenzae* were recognized between November 1973 and June 1974, and all but one of the patients showed a poor response to ampicillin therapy (6). The minimum inhibitory concentrations (MIC) of ampicillin for these bacteria ranged from 8 to 32 μ g of ampicillin per ml (5, 6).

The marked influence of the method and conditions of in vitro testing on the apparent susceptibility or resistance of *H. influenzae* to ampicillin has been clearly demonstrated (11). Moreover, it is generally recognized that diffusion tests using ampicillin disks on agar medium may indicate resistance, although the strain actually is susceptible as judged by the MIC of ampicillin in broth (9, 11, 12, 16, 18). Many hospital laboratories do not perform susceptibility tests on *H. influenzae* because of the earlier confidence that type-b isolates are invariably susceptible to ampicillin, and because of technical difficulties encountered in cultivating and testing this nutritionally demanding species (11, 18).

Ampicillin resistance of some isolates of *H. influenzae* was found to be related to the

production of β -lactamase (10). Iodometric methods have successfully detected β -lactamase production by various other bacteria (7, 8, 13-15). Therefore, a rapid iodometric test was investigated for possible use in screening *H. influenzae* isolates for presumptive ampicillin resistance.

MATERIALS AND METHODS

Haemophilus influenzae type b. Tests of ampicillin susceptibility and β -lactamase production were performed on 20 strains. Sixteen strains isolated during 1968 to 1971 were from specimens of spinal fluid (12 isolates), blood (three isolates), and vaginal secretion (one isolate) obtained from patients at the Milwaukee Children's Hospital. *H. influenzae* strain RAB was provided by John B. Robbins of the National Institutes of Health. Three ampicillin-resistant strains were made available by Clyde Thornsberry of the Center for Disease Control: strain 74-71518 was from an ear culture of a patient in Austin, Texas; strains 74-64148 and 74-90383 were cultured from spinal fluid specimens from patients in Maryland and Tallahassee, Fla., respectively.

The identity of each *H. influenzae* strain was confirmed by its requirements for both hemin (X factor) and nicotinamide adenine dinucleotide (V factor). The immunologic specificity of the type b capsules of all strains was determined by immunofluorescence or by capsular swelling (2).

Strains were preserved for study by freezing of bacterial suspensions at -60 C.

Penicillins. Sterile sodium ampicillin came from two different sources (Polycillin-N, Bristol, and Penbritin-S, Ayerst). Potassium penicillin G was a sterile product from Parke-Davis. Antibiotics were reconstituted with the recommended volumes of cold sterile

physiologic saline solution (0.15 M NaCl) and were used immediately.

Media. The modified Levinthal agar medium used for all cultures was composed of Trypticase soy broth (BBL) with 1% (wt/vol) purified agar (Difco); after sterilization at 121 C for 20 min, it was cooled to 50 C and supplemented aseptically with filter-sterilized solutions of glucose (0.1% wt/vol final concentration), 10% (vol/vol) Levinthal extract of sheep blood (to give a final concentration of 1% blood, 2), and 0.05% (vol/vol) of solution 7 (previously described, 3) to give final concentrations of 5.0 μ g of nicotinamide adenine dinucleotide per ml, 5.0 μ g of thiamine hydrochloride per ml, and 5.0 μ g of calcium pantothenate per ml.

The fluid medium was Mueller-Hinton broth (BBL) supplemented aseptically with Levinthal extract and solution 7, as above.

Susceptibility test. Freshly prepared solutions of ampicillin were pipetted into appropriate volumes of the Levinthal-supplemented broth to give concentrations ranging from 0.05 to 9.0 μ g of ampicillin per ml, and 0.5-ml volumes of each dilution were pipetted into sterile tubes. Suspensions of *H. influenzae*, taken from 18-h modified Levinthal agar cultures, were diluted to give a slight visible turbidity, which experience had shown comprised 2×10^7 to 5×10^7 bacteria per ml. Two serial 1:100 dilutions were made, and 0.5 ml of the final bacterial dilution was pipetted into each ampicillin dilution. For tests of inocula containing 10^4 and 10^5 bacteria per ml, the titration series included additional antibiotic concentrations (12, 20, 30, 40, 50, and 60 μ g of ampicillin per ml). The tubes were incubated in a 37 C water bath and were inspected for bacterial turbidity after 20, 24, and 48 h. The MIC was recorded as the lowest concentration of ampicillin which inhibited the development of visible growth for 24 h. The 20-h reading was not used because in several instances duplicate tests of resistant strains gave the same MIC after 24 h, whereas 4 h earlier one of the two showed a one-tube lower reading. One 4-mm diameter loopful of broth was sampled from each tube after the 24-h incubation and was streaked on modified Levinthal agar. The ensuing growth revealed the purity of the ampicillin broth cultures, and the minimum bactericidal concentration of ampicillin was determined on the basis of absence of growth.

β -Lactamase test. The starch indicator was a solution containing 1.0 g of soluble starch (Merck, reagent for iodometry) in 100 ml of distilled water, dissolved by heating in a boiling water bath. It was prepared fresh each day or two. Desirable properties of the starch were given by Novick (13).

The iodine reagent contained a mixture of 2.03 g of iodine and 53.2 g of potassium iodide which was dissolved in 100 ml of distilled water and stored in a brown glass bottle.

The pH 5.8 buffer was a mixture of 6.25 g of KH_2PO_4 and 0.696 g of K_2HPO_4 dissolved in 1 liter of distilled water. The pH 7.0 and 8.0 buffers were made from this 0.05 M solution by adding the necessary quantities of NaOH.

A solution containing 10,000 U of penicillin G per ml of phosphate buffer was freshly prepared and dispensed in 0.5-ml volumes in small tubes or in wells

of a microtiter plate. The test bacteria were removed with a large inoculating loop from an 18- to 24-h modified Levinthal agar culture and suspended in the penicillin solution to make a density of at least 10^9 cells. (A 10^{-2} dilution of this opaque bacterial suspension was visibly turbid.) After 1 h (or longer) at room temperature, two small drops of starch indicator were added to the suspension, followed after mixing by one small drop of iodine reagent. A blue color immediately developed due to the reaction of the iodine with the starch. The reaction mixture was further rotated for up to 1 min. Persistence of the blue color for longer than 10 min constituted a negative test and indicated that the penicillin molecules had not undergone β -lactam ring cleavage (14). Rapid decolorization occurred if the penicillin was hydrolyzed; such a positive reaction indicated β -lactamase activity, provided that no spontaneous hydrolysis of the penicillin had occurred before or during the test. Two tubes of penicillin solution without bacteria served as controls; one tube received drops of starch and iodine at the beginning of the hour, and the second control tube was held until the end of the hour when the reagents were added to the tests. Retention of the blue color by both controls for the duration of the test period testified to the absence of spontaneous penicillin hydrolysis or of possible hydrolysis by enzymes of microorganisms that might contaminate the starch or buffer solutions. A positive control for β -lactamase activity employed a penicillin-resistant *Staphylococcus aureus* clinical isolate.

RESULTS

Correlation between ampicillin resistance and β -lactamase production. Twenty strains of *H. influenzae* type b were examined for susceptibility to ampicillin using a broth dilution method (Table 1). Three strains were 10-fold more resistant than the others. The MIC values were slightly lower than those reported by some investigators (6, 11), probably because of different media and inoculum sizes. Supplementation of the Levinthal extract medium with nicotinamide adenine dinucleotide, the labile V factor, provided luxuriant growth and correspondingly effective ampicillin activity. The actual bacterial inocula for the tests of Table 1, determined by spreading 0.05-ml volumes over the surfaces of duplicate Levinthal agar plates (3), ranged from 1,000 to 3,800 colony-forming units per ml. Such an inoculum provided a stringent test of bacterial resistance to ampicillin (12). Inocula containing higher numbers of bacteria, which have been used by others (4, 19), increase the apparent resistance of β -lactamase-producing bacteria by accelerating the destruction of ampicillin in the broth dilution tests. Data given in Table 1 show that MIC values for ampicillin-resistant bacteria were artificially increased 20-fold by use of

TABLE 1. Correlation between results of tests for ampicillin susceptibility and β -lactamase activity of 20 clinical isolates of *H. influenzae* type b and 11 separate single-colony isolates of strain 74-71518

Clinical isolates (no. of strains)	74-71518 colonies (no. of isolates)	Minimal inhibitory concn of ampicillin (μ g/ml)	β -Lactamase activity
0	0	0.05	
13	5	0.1	Absent
3	1	0.2	Absent
1	0	0.4	Absent
0	0	0.8	
2 ^a	0	1.5	Present
1 ^c	1	3.0	Present
0	4	6.0	Present

^a Strain 74-64148 inoculum was 1,280 cells per ml; MIC values of 3.0 and 30.0 were found in tests using 12,800 and 128,000 cells per ml, respectively.

^b Strain 74-90383 inoculum was 1,220 cells per ml; MIC values of 3.0 and 40.0 were found in tests using 12,200 and 122,000 cells per ml, respectively.

^c Strain 74-71518 inoculum was 1,440 cells per ml from a culture of selected β -lactamase-producing colonies; MIC values of 9.0 and 60.0 were found in tests using 14,400 and 144,000 cells per ml, respectively.

about 10^5 bacteria per ml instead of 10^3 per ml. The MIC and minimum bactericidal concentration of ampicillin were the same for 12 of the susceptible strains, but for the other five the minimum bactericidal concentration was two-fold higher than the MIC.

The three ampicillin-resistant strains produced β -lactamase (Table 1), as did four strains tested in another laboratory by a different iodometric method (10). In contrast, β -lactamase activity was not exhibited by the 17 susceptible clinical isolates which I examined, or by two other susceptible *H. influenzae* strains (10). Furthermore, six substrains, independently isolated from strain 74-71518, which had spontaneously lost the ability to produce active β -lactamase (as described below) were converted to ampicillin susceptibility (Table 1). Five other substrains isolated in the same manner retained the ability to produce β -lactamase and remained ampicillin resistant.

Genetic stability of β -lactamase production. The rapid iodometric test facilitated screening of numerous subcultures to determine whether β -lactamase production was a stable property of the ampicillin-resistant bacteria. *H. influenzae* strain 74-71518 was genetically unstable compared to strains 74-64148 and 74-90383. This was determined by tests of subcultures from 10 colonies of each strain picked from cultures on Levinthal extract agar which contained various concentrations of

acridine orange and ethidium bromide. As controls, 10 colonies were picked from cultures of each strain on agar lacking the dyes. Seven of the colonial isolates, all from strain 74-71518, were β -lactamase negative. Three of these were from the control plate, suggesting that loss of the property was occurring spontaneously. Accordingly, 64 additional colonies of strain 74-71518 were tested which had not been in contact with the dyes; tests of 29 were negative.

In view of the possibility that the original 74-71518 culture was a mixture of two strains of different origin, four of the β -lactamase-positive colonies together with four negative isolates were transferred serially on agar for 12 days, each culture being initiated from a single isolated colony. Their phenotypic properties remained unchanged at the end of this process. However, these repeatedly purified cultures of β -lactamase-producing bacteria when cultivated in broth for 2 to 3 days continued to segregate β -lactamase-negative cells. Three colonies of 20 picked from a subculture on Levinthal agar had lost β -lactamase activity. This persisting instability and the relatively high frequency of loss are consistent with the possibility that the gene which mediates β -lactamase synthesis is plasmid borne in *H. influenzae* strain 74-71518, as it is in various ampicillin-resistant enteric bacteria (15).

Ampicillin-resistant laboratory mutants. Samples from the ampicillin susceptibility tests were inoculated on Levinthal extract agar for determinations of minimum bactericidal concentration. Growth occurred where the *H. influenzae* had survived during incubation for 24 h in broth containing ampicillin. A number of colonies derived from ampicillin-susceptible clinical isolates were picked and tested for susceptibility and β -lactamase. Two moderately resistant mutants were found which multiplied on Levinthal agar containing 0.5 μ g of ampicillin per ml, a concentration which inhibited the parent strains. In contrast to the ampicillin-resistant clinical isolates, these two laboratory mutants were inhibited by 1.0 μ g of ampicillin per ml and did not produce β -lactamase. A rapid loss of viability of the cultures, and the occurrence of cellular lysis in the absence of ampicillin, suggested that the resistance of these mutants might be related to altered peptidoglycan synthesis, a mechanism of resistance likely to be of little clinical significance.

Methodological considerations. A 60-min period of incubation was used for the tests of β -lactamase activity recorded in Table 1, and duplicate tests of negative strains were incu-

bated for 2 h. However, the method was slightly modified as required for examining the sensitivity of the iodometric test, the appropriate pH buffer for routine use, and the stability of the cell-associated enzyme. Very dense suspensions of 18-h cultures on Levinthal extract agar were prepared in 0.15 M NaCl solution and further dilutions were made in phosphate buffers. Equal volumes of bacteria and antibiotic (final concentration, 10,000 $\mu\text{g/ml}$) were mixed at time zero, distributed in 0.5-ml volumes into separate containers, and tested by adding the starch indicator and iodine reagent after various times of incubation at room temperature (25 C).

The results given in Table 2 were obtained with strains 74-64148 and 74-90383. Corresponding cellular suspensions of strain 74-71518 hydrolyzed penicillin slightly more rapidly. The data verify the sensitivity of the rapid iodometric test described in the Materials and Methods section.

Additional tests were performed with a suspension of strain 74-90383 which was diluted in buffers to yield a final density of about 2×10^8 bacteria per milliliter in each penicillin reaction mixture. The results (Table 3) show that the enzyme was active over the pH range examined and indicate that the pH 7.0 phosphate buffer is satisfactory for routine tests. The suspensions of bacteria which were held at 25 C for 4 h before being mixed with penicillin G retained their β -lactamase activity, indicating that the enzyme is stable for the duration of a test. Also, the enzyme is relatively heat stable. The β -lactamase associated with strain 74-90383 was partially active after exposure of bacteria at 80 C for 10 min (Table 2), but completely inactivated after 10 min at 90 C.

TABLE 2. Effect of number of bacteria on the time required for iodometric detection of penicillin G hydrolysis at pH 5.8 by the β -lactamase of *H. influenzae* strains 74-64148 and 74-90383^a

Bacteria (no./ml)	Incubation time (min)					
	2	5	10	20	40	60
10^{10} ^b	+	+	+	+	+	+
10^9	0	+	+	+	+	+
10^8	0	0	0	D	+	+
10^7 ^c	0	0	0	0	0	0

^a Test results recorded as +, extensive hydrolysis indicated by decolorization within 1 min; 0, no color change observed within 5 min; D, different results (+ or 0) with suspensions of different strains.

^b A portion of the suspension of strain 74-90383, heated for 10 min in a water bath adjusted to 80 C, was 0 after incubation for 40 min, but + after 60 min.

^c Reaction mixtures incubated for 2 and 3 h remained 0.

TABLE 3. Effect of pH and age of the bacterial suspension on the time required for iodometric detection of penicillin hydrolysis by the β -lactamase of *H. influenzae* strain 74-71518^a

pH of buffer	Time elapsed before test of suspension (h)	Penicillin substrate	Incubation time (min)			
			2	5	10	20
5.8	0	Ampicillin	0	+	++	++
	0.5	Penicillin G	0	0	0	++
	4	Penicillin G	0	0	0	++
7.0	0	Ampicillin	0	+	++	++
	0.5	Penicillin G	0	0	+	++
	4	Penicillin G	0	0	+	++
8.0	0	Ampicillin	0	0	+	++
	0.5	Penicillin G	0	0	+	++
	4	Penicillin G	0	0	+	++
		Penicillin G	0	0	+	++

^a Test results recorded as ++, extensive hydrolysis indicated by decolorization within 1 min; +, partial hydrolysis indicated by decolorization occurring between 1 and 5 min; 0, no color change observed within 5 min.

To determine whether the β -lactamase remained cell associated or diffused freely into the surrounding fluid, a dense suspension of bacteria in 0.15 M NaCl solution was held at 25 C for 50 min with occasional vigorous agitation in a Vortex mixer. The bacteria were then sedimented by centrifugation at 3 C. The clear supernatant fluid, mixed with penicillin G in pH 7.0 buffer, gave a negative iodometric test after incubation for 20 min, but was positive after 40 min (not tabulated). In contrast, the sedimented bacteria, when suspended in fresh saline to the original volume and mixed with a similar solution of penicillin G, produced extensive hydrolysis in 2 min.

The hydrolysis of ampicillin by β -lactamase was slightly more rapid than hydrolysis of penicillin G at pH 5.8 and 7.0 (Table 3). The bacteria-free controls indicated that significant spontaneous hydrolysis of ampicillin did not occur during the period of 20 min required for completion of the tests. However, ampicillin was found to be unsuitable for use as substrate for routine tests because controls revealed significant spontaneous hydrolysis after 1 h at 25 C, and solutions prepared with chilled saline and retained at 5 C for 4 to 6 h showed immediate decolorization of the blue starch-iodine mixture.

DISCUSSION

H. influenzae type b earlier provided a classic example of an ampicillin-susceptible pathogen. Recently, however, clinically significant ampicillin resistance has been recognized (4-6, 10,

12, 19). Where the resistance of these type b isolates has been examined, the mechanism has involved inactivation of ampicillin by β -lactamase. If the genetic information which specifies the synthesis of this enzyme has been acquired recently, it may have been introduced as a unit from another organism, for example, by transfer of a plasmid from a β -lactamase-producing gram-negative bacterium (15). Plasmid-borne properties tend to be unstable in some isolates owing to the spontaneous loss of the extra-chromosomal deoxyribonucleic acid fragment during bacterial multiplication. The tendency of *H. influenzae* strain 74-71518 to segregate β -lactamase-negative cells at a frequency higher than 1% offers some support for this hypothesis.

Transfer of such a plasmid into *H. influenzae* type b is probably a very rare event in nature; it may have occurred only once. The resulting ampicillin-resistant clone remained able to multiply and persist successfully in its natural habitat, the human pharynx. The subsequent transmission from one healthy human carrier to another provides for geographical dispersal of representatives of the resistant pathogen and for their occasional production of disease in susceptible children.

If β -lactamase production is responsible for the clinically significant ampicillin resistance of *H. influenzae*, as it is for various other pathogens, the chemical test for penicillin inactivation will give presumptive evidence of ampicillin resistance. Bacteria to be tested by the rapid iodometric method may be taken directly from a primary culture which has grown out on a suitable agar medium inoculated with a specimen of blood or spinal fluid.

The characteristics of β -lactamase production by *H. influenzae* are ideal for detection by this test. Like the β -lactamases of most other gram-negative bacteria (15), the enzyme is produced constitutively (10). The data of Table 2 confirm that induction is not required. Although the enzyme remains cell associated, it is present in an accessible site, thereby allowing prompt initiation of activity when a dense suspension of intact bacteria is mixed with penicillin. Other tests showed that the β -lactamase of cell suspensions is relatively stable and that rapid hydrolysis of penicillins occurs at 25 C over a broad pH range (Table 3).

This simple test will facilitate the recommended surveys of the prevalence of ampicillin-resistant *H. influenzae* (12). Where a β -lactamase-producing strain is responsible for disease of a patient being treated with ampicillin, the positive test will indicate that alternative therapy should be considered.

After this manuscript was submitted, a note by Thornsberry and Kirven (17) appeared. It described a rapid test for the β -lactamase of *H. influenzae* in which the formation of penicilloic acid is indicated by a transition of phenol red from violet (pH 8.5) to yellow. This test appears to be as useful as the iodometric test, although somewhat less sensitive because a higher concentration of penicillin G (10^6 U/ml compared to 10^4 U/ml) is used so that the formation of acid will be sufficient to change the pH indicator.

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