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NOTES

New Method for Detecting In Vitro Inactivation of Penicillins by *Haemophilus influenzae* and *Staphylococcus aureus*

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A new technique for detecting penicillinase production in *Haemophilus influenzae* and *Staphylococcus aureus* was compared with the capillary procedure for detecting beta-lactamase and the Bauer-Kirby disk susceptibility procedure. Isolates were classified similarly by all three procedures.

Ampicillin-resistant strains of *Haemophilus influenzae* have appeared with increasing frequency since reported by Thomas et al. in 1974 (13). Khan et al. (5) showed that these resistant strains produced beta-lactamase (penicillinase). Subsequently, a variety of assays for beta-lactamase have been used to detect ampicillin-resistant strains of *H. influenzae*. These assays have involved the use of an iodometric technique with a starch indicator (3, 8), chromogenic cephalosporin as the enzymatic substrate (4, 6), or other colorimetric methods (1, 11, 14).

We have developed a method to detect the ability of strains of *H. influenzae* and *Staphylococcus aureus* to neutralize penicillins. Isolates that neutralize penicillin as determined by our technique were also classified as penicillin resistant by the standard Bauer-Kirby antibiotic susceptibility procedure and had beta-lactamase detected by the capillary technique. Since our method does not require special reagents, it may be applicable in other laboratories required to test haemophilus isolates for penicillinase production.

Fifty-four isolates of *H. influenzae* from clinical specimens were studied. Additional verification of the new procedure was performed by testing 40 isolates of *S. aureus*.

Spore-seeded plates were prepared by a modification of the method of Sabath et al. (12). Five-milliliter portions of nutrient agar were distributed into screw-capped tubes. The caps were tightened and the tubes were stored in a refrigerator. To prepare the plates, stored agar was melted and cooled to about 57°C. One drop of Subtilis spores (Difco Laboratories, Detroit, Mich.) was added to the melted agar aseptically. The suspension was mixed thoroughly and poured onto the plate (100 by 15 mm).

The streak method was performed by using heavy inocula of the test organism picked from pure culture with a bacteriological loop. The streak patterns were made in two ways. (i) Unknown and non-penicillinase-producing control organisms were streaked in a cross pattern, but disconnected at the center to avoid contamination (Fig. 1A). (ii) Unknown and control organisms were streaked about 8 to 10 mm apart in a parallel line as shown in Fig. 1B. Antibiotic disks were placed as illustrated, and the plates were incubated at 37°C for 4 h or until growth was clearly visible. The inactivation of ampicillin was well demonstrated in cross-streaked patterns by inhibition zones that resembled a pair of kidney beans (Fig. 1A). The *Bacillus subtilis* grew along the streak of the resistant isolate due to the inactivation of ampicillin. On the other hand, if two isolates were streaked in parallel, the pattern illustrated in Fig. 1B appeared. The inhibition zone on the side of the resistant isolate was reduced noticeably.

The capillary technique for detecting beta-lactamase production (11, 14) and the Bauer-Kirby agar diffusion procedure (2) were used as reference methods. A slight modification of the Bauer-Kirby method was used for susceptibility tests of *H. influenzae* (15). Inocula were adjusted to contain 10^6 to 10^7 colony-forming units/ml. The medium was prepared from Mueller-Hinton agar with 5% chocolatized rabbit blood and 1% IsoVitaleX (Baltimore Biological Laboratory, Cockeysville, Md.).

The results of the classification of our isolates by the three test procedures are presented in Table 1.

All of the isolates that produced beta-lactamase as detected by the capillary tube method were demonstrated to neutralize penicillin by the use of our technique. These isolates were

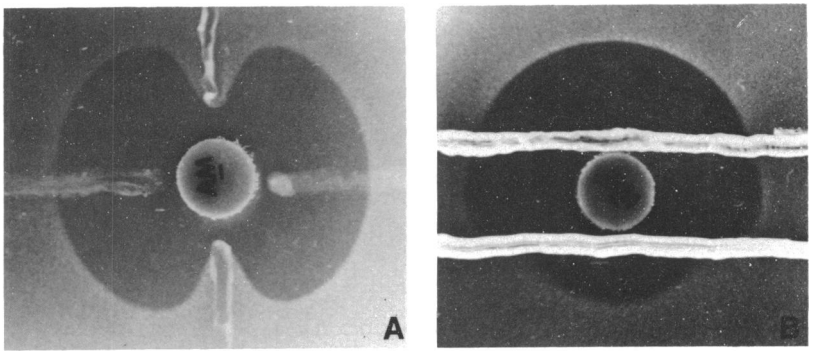

FIG. 1. *Patterns of the streak method illustrate the different effects of ampicillin-resistant and -susceptible H. influenzae isolates on inhibition zones. (A) Cross-streak pattern. The resistant isolate (upper-lower streak) caused distortion at the spherical inhibition zone, and the susceptible isolate (left-right streak) showed no effect on the zone. (B) Parallel streak pattern. The inhibition zone on the side of a resistant isolate (lower) was much smaller than the zone near the susceptible isolate (upper).*

TABLE 1. *Classification of isolates by Bauer-Kirby, capillary tube, and new streak methods*

Organism	Bauer-Kirby method	No. of isolates	Capillary tube method		Streak method	
			No. of beta-lactamase producers	No. of not beta-lactamase producers	No. of penicillinase producers	No. of not penicillinase producers
H. influenzae	Ampicillin resistant	13	13	0	13	0
(54 isolates)	Ampicillin susceptible	41	0	41	0	41
S. aureus	Penicillin resistant	21	21	0	0	0
(40 isolates)	Penicillin susceptible	19	0	19	0	19

also classified as penicillin resistant on the basis of the Bauer-Kirby technique. The classification of isolates that were not beta-lactamase producers was also consistent among the three techniques.

We believe our techniques have several useful features. The results are available more rapidly than are the Bauer-Kirby results. There is no need to purchase penicillin powder or prepare chromogenic cephalosporin powder, as is necessary for other methods (4, 14). Even when the tests are performed infrequently, preparation of spore-seeded plates from stored agar tubes can be completed quickly. Special antibiotic disks are not necessary for our technique because the method uses the same antibiotic disks as does the Bauer-Kirby procedure.

Since several antibiotic disks can be used on the same plate, it appears possible to test the inactivation of a variety of antibiotics. Previous studies have compared penicillinase production with resistance of gram-negative enteric organisms and *Bacteroides fragilis* (7, 9, 10). Preliminary tests have encouraged us to extend the use of our technique for studies of these other organisms as well.

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