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Iodometric Spot Test for Detection of Beta-Lactamase in *Haemophilus influenzae*

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A simple iodometric spot test for detecting beta-lactamase activity in *Haemophilus influenzae* 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.

The emergence of *Haemophilus influenzae* strains that produce beta-lactamase (5, 10) has stimulated many investigators to develop a variety of rapid assays for detecting this enzyme activity, e.g., an iodometric technique with a starch indicator (2, 9), chromogenic cephalosporin as the enzyme substrate (4, 6, 7), or other acidometric methods (3, 8, 11). The iodometric method is based on the fact that the intact (active) penicillin molecule does not bind iodine, whereas the beta-lactamase-inactivated product, penicilloic acid, does bind iodine. Thus, a positive reaction indicates that iodine, being bound to penicilloic acid, is unavailable for further reaction with starch, and therefore no purple color develops in testing. Since certain paper that has absorbed iodine turns purplish-brown, and since the stability of penicillin is better in dry form than in solution, we have taken advantage of these features to develop a simple iodometric spot test. Isolates of *H. influenzae* that inactivate penicillin as determined by our technique were also classified as penicillin resistant by the Bauer-Kirby antibiotic susceptibility procedure (1) and produced beta-lactamase when tested by the capillary technique (11). Since our method does not require the preparation of special reagents, it may be applicable in smaller laboratories required to test *Haemophilus* isolates for penicillinase production.

Fifty-four isolates of *H. influenzae* from clinical specimens were studied. Additional verification of the procedure was performed by testing 60 isolates of *Staphylococcus aureus*.

Reagents for this test include the following: (i) potassium penicillin G for injection, USP (buffered), 10^6 U (a small amount should be stored in dry form under desiccation); and (ii) bibulous paper, Scientific Products Div., cut to 2.5 by 2.5 cm. To perform the test, two pieces of paper were placed in a petri dish, one as a positive control and the other to test the organism. A small amount of penicillin G powder (ca. 0.02 mg) was delivered to the center of each paper by a small spatula. Two drops (ca. 0.08 ml) of distilled water was then added to each paper to dissolve the penicillin powder. The paper should be barely damp and not dripping; otherwise, the inactivated penicilloic acid would spread onto the whole paper and would not be demonstrated at the concentrated spot. A loopful of inoculum from two to three colonies on a culture plate was then smeared at the center of the paper. A 10-min enzymatic reaction initiated by the organism on penicillin at room temperature was sufficient to demonstrate the presence of penicilloic acid. Four to five drops of Gram iodine were then put onto each paper. To ensure that the iodine solution was evenly absorbed, a pair of forceps was used to hold the edge of the paper while rinsing twice. Finally, the rest of the iodine solution was drained off onto the side of the petri dish, and the papers were observed within 5 min.

A slight modification of the Bauer-Kirby susceptibility method was used for *H. influenzae* (12). Inocula were adjusted to contain 10^6 to 10^7 colony-forming units per ml. The medium was prepared from Mueller-Hinton agar with 5% chocolatized rabbit blood and 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.).

The result for the beta-lactamase producers was a white spot in the center of the paper with purplish-brown surroundings. For the nonproducers, a purplish-brown color appeared on the entire paper (Fig. 1). Occasionally, in a negative reaction, a yellowish color would show on purplish brown background due to the carrying over of the color from the original colonies; however, a positive reaction should always show a pure white color.

As shown in Table 1, all of the isolates that produced beta-lactamase as detected by the capillary tube method were demonstrated to inactivate penicillin by the use of our technique.

TABLE 1. *Classification of isolates by Bauer-Kirby, Capillary tube, and iodometric spot methods*

Organism	Classification by Bauer-Kirby method	No. of isolates	Capillary tube method		Iodometric spot method	
			No. of beta-lactamase producers	No. of beta-lactamase nonproducers	No. of beta-lactamase producers	No. of beta-lactamase nonproducers
H. influenzae	Ampicillin resistant	13	13	0	13	0
	Ampicillin susceptible	41	0	41	0	41
S. aureus	Penicillin resistant	31	31	0	31	0
	Penicillin susceptible	29	0	29	0	29

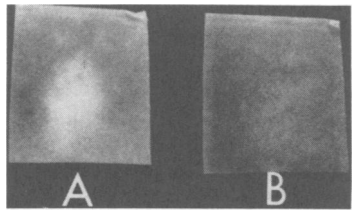

FIG. 1. *The iodometric spot test illustrates the different effects of beta-lactamase-producing and -nonproducing H. influenzae isolates on penicillin. (A) Positive reaction shows white spot on the paper that did not bind to iodine. (B) Negative reaction shows purplish brown, indicating the even binding of iodine to the paper.*

These isolates were 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 that our technique has several useful features. The reaction is completed within 10 min. There is no need to prepare reagents or to adjust pH, as the purchased potassium penicillin G is already buffered with sodium citrate, and Gram iodine is readily available in most microbiology laboratories. In addition, the penicillin powder is inexpensive and stable in dry form for at least 6 months or more. It is particularly useful when the tests are performed infrequently.

Although our data indicate that this technique could be used for detecting beta-lactamase activity of *S. aureus*, the technique needs further evaluation before being used as a routine procedure. The reason is that some strains of this species produce minute amounts of constitutive enzymes and may not yield positive test results until the enzyme is induced.

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