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Chromogenic Cephalosporin Spot Test to Detect Beta-Lactamase in Clinically Significant Bacteria

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A chromogenic cephalosporin assay for β-lactamase in which an impregnated filter paper is used is reported. Nitrocefin (Glaxo) is dissolved in buffered dimethyl sulfoxide, and 0.5 ml is used to impregnate a filter paper in a petri dish. An isolated bacterial colony is applied to the paper with a loop, and a pink reaction within 15 min indicates β-lactamase production. Results of this test on clinical isolates were correlated with standardized penicillin and ampicillin susceptibility tests. A correlation of 100% was observed with *Staphylococcus aureus* (428 resistant and 88 sensitive strains) and *Haemophilus influenzae* (161 sensitive and 15 resistant strains). Of 45 isolates of *Bacteroides fragilis*, 1 was falsely negative for β-lactamase and 1 was falsely positive; the remainder were all positive and were penicillin resistant. Of 27 strains of *Bacteroides melaninogenicus*, 14 were β-lactamase positive, and 12 of these were penicillin resistant.

Detection of bacterial resistance to penicillin or ampicillin by disk diffusion or antibiotic dilution studies generally requires overnight incubation. Since the mechanism of resistance to these antibiotics is usually the elaboration of a beta-lactamase, susceptibility can be rapidly determined by detecting the presence or absence of this enzyme in bacterial isolates. Several rapid methods for detecting β-lactamase have been developed, including the iodometric (6, 15), acidometric (3, 6), and chromogenic cephalosporin (7) procedures. In the chromogenic cephalosporin method the presence of β-lactamase causes this pale yellow compound to turn pink. The test has been reported to be useful in detecting β-lactamase in staphylococci (7, 15), *Haemophilus influenzae* (4, 13), enteric gram-negative rods (7), and *Neisseria gonorrhoeae* (9), but *Bacteroides* species have not been studied. Rosenblatt and Neumann (10) recently evaluated a rapid slide iodometric test with *Bacteroides* species and found that it detects β-lactamase produced by *Bacteroides melaninogenicus* but not that produced by *Bacteroides fragilis*. In this paper we report a simplified chromogenic cephalosporin spot test, in which filter paper impregnated with the reagent is used and which sucessfully detects the β-lactamase of *B. fragilis* and other clinically significant bacteria.

MATERIALS AND METHODS

Cultures. A total of 772 clinical isolates were tested for β-lactamase production; these included 516 *Staphylococcus aureus*, 45 *B. fragilis*, 27 *B. melaninogenicus*, 28 *H. influenzae*, and 156 *N. gonorrhoeae* strains. The isolates were tested directly from the original inoculated plates.

Method for determination of β-lactamase. The chromogenic cephalosporin assay for β-lactamase production is a modification of the method described by O'Callaghan et al. (7). A 5-mg amount of Nitrocefin (87/312) (Glaxo Research, Ltd., Greenford, Middlesex, England) is dissolved in a solution containing 0.5 ml of dimethyl sulfoxide and 9.5 ml of 0.1 M phosphate buffer, pH 7.0. This is dispensed in 0.5-ml portions and frozen at −20°C. A 0.5-ml portion of this 500-μg/ml solution is removed from the freezer, thawed, and used to impregnate a Whatman no. 1 filter paper disk (diameter, 7 cm) in a petri dish. This impregnated filter paper is generally usable for 1 day or until the paper dries out. An isolated bacterial colony from the surface of an agar plate is applied to the impregnated filter paper with a loop, and a pink reaction developing within 15 min indicates β-lactamase production.

Additional methods. Anaerobic bacteria were presumptively identified by the antibiotic disk method of Sutter and Finegold (11), and their penicillin susceptibility was determined by the broth disk method of Wilkins and Theil (14). Isolates were considered sensitive if they were inhibited by 1.2 μg of penicillin per ml. The remaining bacteria were identified by standard methods. Susceptibility of *S. aureus* to penicillin was determined by the standardized Kirby-Bauer method (1). Susceptibility of *H. influenzae* to ampicillin was determined by a modified Kirby-Bauer test as recommended by Thornsberry and Kirven (12).

RESULTS

Table 1 shows the correlation of the results of the chromogenic cephalosporin β-lactamase tests and susceptiblity to penicillin and ampicillin. There was 100% correlation with *S. aureus* (428 resistant and 88 sensitive strains). One of

TABLE 1. *Correlation of β-lactamase test with penicillin and ampicillin susceptibility for S. aureus, B. fragilis, B. melaninogenicus, and H. influenzae*

Organism	β-Lactamase test result	No. of strains			
		Penicillin resistant	Penicillin sensitive[a]	Ampicillin resistant	Ampicillin sensitive[b]
S. aureus	+	428 (7)[c]	0		
	−	0	88		
B. fragilis	+	43 (1)	1		
	−	1	0		
B. melaninogenicus	+	12	0		
	−	2	13		
H. influenzae	+			15	0
	−			0	161

[a] For *S. aureus*, Kirby-Bauer zone ≥29 mm; for *Bacteroides* spp., minimal inhibitory concentration ≤1.2 μg/ml.

[b] Kirby-Bauer zone ≥20 mm.

[c] Numbers in parentheses are the number of delayed positive strains (reaction took up to 15 min to turn positive).

43 resistant isolates of *B. fragilis* was negative for β-lactamase production, and 1 sensitive strain was positive. The remainder were all positive and were penicillin resistant. Of 27 strains of *B. melaninogenicus*, 12 were β-lactamase positive and penicillin resistant. Of the 15 β-lactamase-negative strains, 2 were resistant to penicillin. There was excellent correlation of the chromogenic cephalosporin test and ampicillin susceptibility of *H. influenzae*.

In addition, 156 isolates of *N. gonorrhoeae* were screened by this test method, and all were negative. Two known penicillin-resistant strains were obtained from the San Francisco Department of Public Health, and both were positive for β-lactamase production.

In the test described here, most of the β-lactamase-positive bacteria developed a pink color within a few seconds; however, seven isolates of *S. aureus* and one of *B. fragilis* took up to 15 min to demonstrate β-lactamase production.

DISCUSSION

Beta-lactamase production correlates with penicillin and ampicillin resistance in bacteria such as *S. aureus*, *H. influenzae*, *N. gonorrhoeae*, and *Bacteroides* species. By using the chromogenic cephalosporin method described here the susceptibility of these bacteria to these two antibiotics can be reliably reported more rapidly than by any diffusion or dilutional method. In many instances this result can be reported before identification to species is completed. This is especially pertinent for anaerobic gram-negative rods which may take several days for definite identification. Once a *B. fragilis* isolate is finally identified, it is predictable that the isolate is penicillin resistant, but it may be more useful to a physician to have the susceptibility result first and the specific identity later. Beta-lactamase production by an anaerobic gram-negative rod does not verify that the isolate is *B. fragilis* since other species of *Bacteroides*, especially *B. melaninogenicus*, may possess this enzyme. Therefore, identification to species of beta-lactamase-positive anaerobic gram-negative rods is necessary.

The basis for the chromogenic cephalosporin reaction is the development of a pink color when the β-lactam ring of the cephalosporin compound is hydrolyzed by β-lactamase to form the corresponding cephalosporanic acid (7). It has been shown previously that *B. fragilis* species hydrolyze cephalosporins much more actively than penicillins (2, 8). Thus, the test described here appears superior to methods which use penicillin as a substrate and are consequently unable to detect beta-lactamase produced by *B. fragilis* (2, 8, 10). In our studies false-positive tests were extremely rare, but O'Callaghan et al. (7) noted false-positive tests when the cephalosporin reagent was added to broth cultures that were highly alkaline (pH 10) or that contained serum, albumin, thiols, cysteine, glutathione, mercaptoethanol, or dimercaprol. Therefore, caution may be necessary when testing colonies obtained from media containing any of these substances. The modification described here (i.e., impregnating filter paper disks with reagent) is similar to the filter paper test of Kovacs for oxidase production and makes testing of many different colonies more convenient for technologists (5).

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