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Pyridinium-2-Azo-*p*-Dimethylaniline Chromophore, a New Chromogenic Cephalosporin for Rapid Beta-Lactamase Testing

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A new chromogenic cephalosporin, pyridinium-2-azo-*p*-dimethylaniline chromophore, was evaluated for use in a rapid paper strip or tube test for the detection of beta-lactamases from a variety of microorganisms. A 1-min pyridinium-2-azo-*p*-dimethylaniline chromophore paper strip test was found to be a convenient and accurate method for the detection of beta-lactamase-producing strains of *Haemophilus influenzae* and *Neisseria gonorrhoeae*, although it could not be relied upon to detect beta-lactamases produced by staphylococci, enteric organisms, or *Bacteroides fragilis*.

The advent of ampicillin resistance in *Haemophilus influenzae* type b and *Neisseria gonorrhoeae* in the 1970s (1, 2, 6) has led to widespread use of rapid beta-lactamase tests as a means of determining susceptibility or resistance to beta-lactam antibiotics (3, 4, 12). Beta-lactamase testing is also valuable for *Staphylococcus aureus* isolates whose penicillin minimal inhibitory concentration by microdilution testing is 0.06 to 0.125 U/ml. Certain staphylococcal isolates with minimal inhibitory concentrations in this range produce beta-lactamase and should be considered resistant to penicillin and ampicillin irrespective of the minimal inhibitory concentration. The phenomenon of late emergent growth of certain *Enterobacter* species due to delayed induction of beta-lactamase has been recognized during the clinical trials of certain of the rapid antibiotic susceptibility test instruments (11). Beta-lactamase testing is a potentially useful strategy for determining the resistance properties of clinical isolates of *Enterobacter* spp.

Several methods have been described for performing beta-lactamase tests. These include traditional acidometric or iodometric methods (4, 5, 9, 10) which generally use penicillin as a substrate and detect production of penicilloic acid as evidence of enzyme activity. A new approach to beta-lactamase detection has been the use of chromogenic cephalosporins, the first of which was nitrocefin, or 87/312 (7, 8), Glaxo Research, Ltd., London, England. Nitrocefin changes from a yellow to a red color when the beta-lactam ring is disrupted. More recently, another somewhat similar chromogenic cephalosporin, pyridinium-2-azo-*p*-dimethylaniline chromophore (PADAC; Calbiochem, La Jolla, Calif.) has been described (10).

PADAC has a chromophore moiety at the 3-substituted position of the cephalosporin nucleus. The parent compound has a distinct purple or violet color which changes to a bright yellow when the beta-lactam ring is opened, and the chromophore moiety is simultaneously released (10). Although hydrolysis of the beta-lactam ring of PADAC can be quantitatively determined spectrophotometrically, the visible color change of a PADAC solution seems adequate for the qualitative detection of beta-lactamase activity, as in the testing of clinical isolates.

We describe here two different methods for beta-lactamase testing with PADAC. A rapid paper strip test was devised for the screening of *H. influenzae* and *N. gonorrhoeae*. To perform this version of the PADAC test, a 250-μg/ml aqueous solution of PADAC was used to moisten a Whatman no. 2 filter paper strip approximately 1 by 5 cm in size. Colonies of bacteria were removed from a culture plate and applied to an area of the moist PADAC-saturated strip. Development of a definite yellow color on the violet-colored strip within 1 min was indicative of the presence of constitutive beta-lactamase.

A second version of the PADAC test used 200 μl of a 100-μg/ml aqueous solution of PADAC placed in a small test tube. An equal volume (200 μl) of a turbid bacterial suspension was added, and the mixture was incubated for 30 min at 37°C. A positive test was indicated by the development of a yellow color, and a negative test was evidenced by the mixture remaining purple. This version of PADAC testing was intended for those organisms having inducible beta-lacta-

TABLE 1. Results of beta-lactamase testing by PADAC and iodometric strip tests

Organism	No. positive/no. of isolates tested	
	PADAC	Iodometric
H. influenzae (25)[a], ampicillin resistant	25/25	25/25
H. influenzae (45), ampicillin susceptible	0/45	0/45
N. gonorrhoeae (15), penicillin resistant	15/15	15/15
N. gonorrhoeae (28), penicillin susceptible	0/28	0/28
B. fragilis (22), penicillin resistant	16/22	0/22
E. coli (18), ampicillin resistant	7/18	14/18
E. coli (29), ampicillin susceptible	0/29	1/29
K. pneumoniae (32), ampicillin resistant	0/32	27/32
Enterobacter spp. (30), ampicillin and cephalothin resistant	14/30	25/30
S. aureus (23), penicillin resistant	0/23	21/23
S. aureus (4), penicillin susceptible	0/4	0/4

[a] Number of isolates.

mases, which might require an incubation period for expression.

These two PADAC methods were evaluated by parallel testing with a rapid starch-iodine paper strip test (5) on a group of *H. influenzae*, *N. gonorrhoeae*, *Bacteriodes fragilis*, *Staphylococcus aureus*, and several representatives of the *Enterobacteriaceae* with previously determined susceptibilities to penicillins and cephalosporins. Table 1 summarizes the results obtained by parallel testing of all isolates. All beta-lactamase-producing *Haemophilus* and *Neisseria* isolates were easily detectable by either the PADAC strip or starch iodine test. No false-positives were seen with either method when applied to these two genera. Of 22 penicillin-resistant *B. fragilis* isolates, 16 were detected by the PADAC method, whereas none was positive by the iodometric strip test.

Members of the *Enterobacteriaceae* and *S. aureus* were examined using both versions of the PADAC test and the starch-iodine strip test. The PADAC strip and tube tests were negative with 29 ampicillin-susceptible strains of *Escherichia coli*. One apparent false-positive occurred with the iodometric paper strip test with an *E. coli* isolate. Only 7 of 18 ampicilin-resistant *E. coli* isolates yielded positive PADAC strip tests, and only 1 of 18 was positive by the tube test; whereas 14 of 18 isolates were positive by the iodometric test. The PADAC tests were not able to recognize the production of beta-lactamase by 32 strains of ampicillin-resistant *Klebsiella pneumoniae*, although 27 of 32 isolates were detected by the starch-iodine test. The cephalosporinases produced by 30 *Enterobacter* spp. isolates were not detected by the PADAC tube method, whereas 14 isolates were positive by the PADAC strip test and 25 isolates were positive by the iodometric test.

No false-positives were seen when penicillin-susceptible *S. aureus* isolates were tested by any of the methods. Of 23 penicillin-resistant *S. aureus* isolates, penicillinase production was detected in 21 by the iodometric strip test, whereas none was detected by either PADAC method.

Extension of the incubation period of the PADAC tube test in an effort to increase sensitivity for testing of organisms with inducible enzymes resulted in the development of a third color reaction, i.e., a loss of all color. This reaction was observed both with organisms which produced beta-lactamases and with those which did not. The addition of a non-beta-lactamase-producing test strain of *S. aureus* to a PADAC solution which had been reacted with purified beta-lactamase caused the yellow solution to become colorless. Conversely, the addition of purified beta-lactamase to a PADAC solution which had been rendered colorless by a penicillin-susceptible *S. aureus* isolate failed to yield a yellow color. These observations suggest a direct attack on the chromophore group by certain organisms, with or without hydrolysis of the beta-lactam ring. The addition of equimolar quantities of ascorbic acid, a potent reducing agent, also produced a colorless solution. Therefore, the most likely mechanism appears to be a reduction of the diazo bond of the chromophore, perhaps resulting in a hydrazo linkage, or complete cleavage of the bond, either of which would result in a colorless solution. Thus, if the PADAC solution is rendered colorless by a microorganism, it is not possible to determine whether beta-lactamase was present.

Our data suggest that a PADAC strip test may be useful for beta-lactamase testing of either *H. influenzae* or *N. gonorrhoeae* strains which produce abundant and freely reactive type III beta-lactamase (2). However, PADAC does not appear to be as sensitive as nitrocefin (7, 8) to the presence of naturally occurring beta-lactamases of species of *Enterobacter*, *Klebsiella*, *Bacteroides*, and *Staphylococcus* and thus cannot be considered reliable for testing these latter microorganisms.

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