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Rapid, Inexpensive Method for Specific Detection of Microbial β-Lactamases by Detection of Fluorescent End Products

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A rapid method was developed for specific detection of microbial β-lactamases which uses ampicillin and cephalexin as substrates. The end products (open β-lactam ring forms) generated after separately incubating either substrate with β-lactamase-producing organisms initially were separated from the unhydrolyzed substrates by high-voltage electrophoresis at pH 2.1. The end products of both antibiotics were highly fluorescent and could be analyzed visually and semiquantitatively under a long-wave UV lamp. Application of 5 μl of the same incubation mixture onto filter paper without subsequent electrophoretic separation also resulted in development of fluorescence after brief heating at 120°C for 5 min. This spot test differentiates penicillinase activity from cephalosporinase activity and distinguishes between β-lactamase and acylase activities, since the end products of acylase [the common side chain, D(−)-α-aminophenylacetic acid, and the intact β-lactam nuclei, 6-aminopenicillanic acid and 7-aminodeacetoxycephalosporanic acid] are not fluorescent. This method was relatively rapid, inexpensive, and more sensitive than the chromogenic cephalosporin (nitrocefin) method when 21 strains of 7 gram-positive species and 77 strains of 29 gram-negative species of bacteria were tested.

β-lactamases which hydrolyze the amide bonds of the β-lactam ring of sensitive penicillins and cephalosporins (Fig. 1 and 2) are widely distributed among microorganisms (14) and play an important role in microbial resistance to β-lactam antibiotics. Chemical methods used for detecting microbial β-lactamases include (i) the acidimetric method, which employs a pH color indicator to detect the decrease in pH resulting from the formation of a new carboxyl group; (ii) the iodometric method, which is based on decolorization of a starch-iodine complex by the end products of β-lactamase hydrolysis, which act as reducing agents to reduce iodine in the complex; and (iii) the chromogenic cephalosporin method, which is based on a color change after the hydrolysis of a chromogenic cephalosporin substrate (16). The microbiological assay methods (16) are based on the loss of antibacterial activity after the hydrolysis of the β-lactam ring. Microbial acylases which remove the acyl side chains of susceptible penicillins or cephalosporins (Fig. 1 and 2) are also produced by many microorganisms (20). The cleavage of acyl side chains from β-lactam antibiotics often results in a decrease in pH and reduction of antibiotic activity, so acidimetric and microbiological methods may not differentiate β-lactamase activity from acylase activity (18). Although microbial β-lactamases do not act exclusively on penicillins or on cephalosporins, many show a predominance of either penicillinase or cephalosporinase activity (14, 16, 17); thus, chemical or microbiological methods which utilize either a penicillin alone or a cephalosporin alone can give false-negative results for β-lactamase activity (1, 5, 6, 9, 10, 12, 15).

In this study, we found that some penicillins and cephalosporins, such as ampicillin and cephalexin, yielded fluorescent end products after hydrolysis by β-lactamase. These fluorescent end products could be detected on filter paper under a long-wave UV lamp after brief heating at 120°C for 5 min. For the detection of microbial β-lactamases, we used ampicillin to detect penicillinase activity and cephalexin to detect cephalosporinase activity. The nitrocefin test (13) is specific and is the simplest and most rapid of the available tests for β-lactamase (16). We performed our fluorescent spot test concurrently with the nitrocefin test under the same conditions. Since β-lactamase activity toward ampicillin and cephalexin generated fluorescent end products (D-phenylglycylpenicilloic acid and D-phenylglycyldeacetoxycephalosporoic acid) and acylase activity produced nonfluorescent end products [D(−)-α-aminophenylacetic acid, 6-aminopenicillanic acid, and 7-aminodeacetoxycephalosporanic acid], we could distinguish β-lactamase activity from acylase activity. In addition, by using β-lactam substrates representing both penicillin and cephalosporin antibiotics, we could determine the specificity of β-lactamases of various species of gram-positive and gram-negative bacteria.

(This work was reported in part at the 23rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Las Vegas, Nevada, October 1983 [K. C. S. Chen, J. S. Knapp, and K. K. Holmes. Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 23rd, Las Vegas, Nev., abstr. no. 86, 1983].)

MATERIALS AND METHODS

Chemicals. Compounds (acid forms) related to β-lactam antibiotics, including the acyl side chain and the β-lactam nuclei, were purchased from Sigma Chemical Co., St. Louis, Mo., and separately dissolved in 0.04 M sodium phosphate buffer, pH 7.5, to a final concentration of 0.02 M, except for amoxicillin (0.01 M; prepared in 0.02 M sodium phosphate buffer) and D(−)-α-aminophenylacetic acid (0.005 M; prepared in 0.01 M sodium phosphate buffer). Nitrocefin was a gift from Glaxo Research Ltd., Greenford, Middlesex, England, prepared and used at a concentration of 50 μg/ml as described previously (16).

Preparation of inocula for detection of β-lactamase. *Haemophilus ducreyi* and *Haemophilus influenzae* were grown

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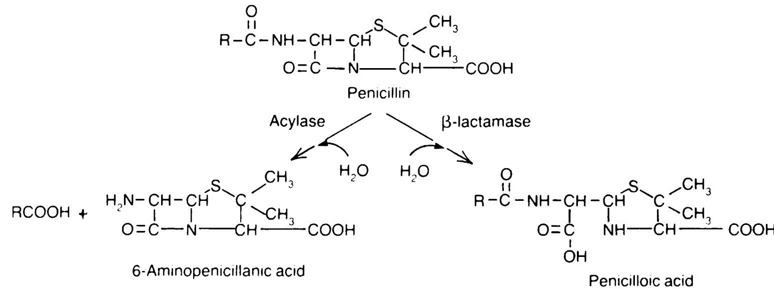

FIG. 1. Hydrolysis of penicillins by acylase and β-lactamase.

on GC agar base (BBL Microbiology Systems, Cockeysville, Md.) with supplements as described previously (19). *Bacteroides* spp. were grown anaerobically on Columbia base agar (BBL) with supplements as described previously (21). The other microorganisms described previously (2) were grown aerobically on GC medium base (Difco Laboratories, Detroit, Mich.) containing 1% defined supplement (22) at 37°C overnight, except for *Neisseria gonorrhoeae*, which was grown in a CO_2 incubator (19).

Portions (50 μl) of each β-lactam antibiotic were separately placed in a microcentrifuge tube (200 μl; Stockwell Scientific, Monterey Park, Calif.). Approximately half of a loopful (diameter, 2 mm) of growth of each strain was removed from the agar plate, dispensed in each substrate by brief agitation on a vortex apparatus, and incubated for 1 h at 37°C or for 5 and 15 min at room temperature for the rapid spot test described below. Uninoculated substrate controls were prepared in the same manner. After incubation, the tubes (except the uninoculated substrate control tubes and tubes for the rapid spot test) were centrifuged in a Microfuge (model 152; Beckman Instruments, Inc., Fullerton, Calif.) for 1 min.

Detection of open β-lactam ring end products by the spot test. After 1 h of incubation at 37°C, a 5-μl volume of supernatant fluid from each tube, including each uninoculated substrate control tube, was applied separately onto Whatman 3MM paper and heated at 120°C in an oven for 5 min. The fluorescent intensity of each test spot was then compared with its uninoculated substrate control spot under a long-wave UV lamp and classified as negative, weakly positive, or positive.

For the rapid spot test, 5 μl of uncentrifuged bacterial suspension from each inoculated tube after 5 or 15 min of incubation at room temperature and 5 μl from each uninoculated substrate control tube were applied onto the paper and heated at 120°C for 5 min. For microorganisms which showed strong autofluorescence (e.g., *Pseudomonas* spp.), we applied the tip of the Eppendorf pipettor containing 5 μl of suspension to the filter paper and allowed the fluid to be withdrawn from the tip by capillary action. This caused the bacteria to remain concentrated at the point of application, so that central bacterial autofluorescence could be differentiated from peripheral fluorescence of the end products. The fluorescent intensity of the rapid spot test was classified as described for the 1-h spot test.

Detection of open β-lactam ring end products by HVE. After 1 h of incubation, a 5-μl volume of supernatant fluid from each tube was separately applied onto Whatman 3MM paper which was subjected to high-voltage electrophoresis (HVE) at pH 2.1 and 80 V/cm for 30 min (4). The paper was dried at 90°C for 15 min and viewed under a long-wave UV lamp. The fluorescent intensity of each test was compared with that of its uninoculated substrate control and classified as negative, weakly positive, or positive. The paper was then stained with ninhydrin-cadmium acetate (7) to reveal the unhydrolyzed substrate. The color intensities of the end products were further classified as negative or as weakly, moderately, or strongly positive.

Detection of β-lactamase by the nitrocefin test. The nitrocefin test (13) was performed under the same conditions as the spot test. Approximately half of a loopful of growth of each organism was dispersed in 50 μl of nitrocefin (50 μg/ml) in a well of a microtitration plate (Linbro Division, Flow Laboratoris, Inc., Hamden, Conn.) and incubated for 1 h at 37°C or for 5 and 15 min at room temperature for the rapid test.

RESULTS

Detection of β-lactamase by identification of fluorescent end products. In initial studies, penicillins (ampicillin and amoxicillin) and cephalosporins (cephalosporin C, cephaloglycin,

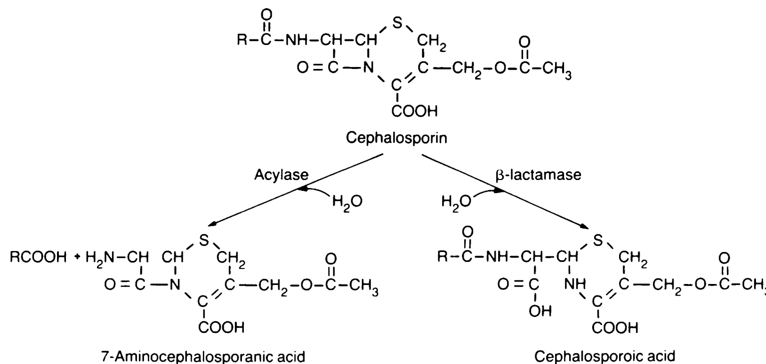

FIG. 2. Hydrolysis of cephalosporins by acylase and β-lactamase.

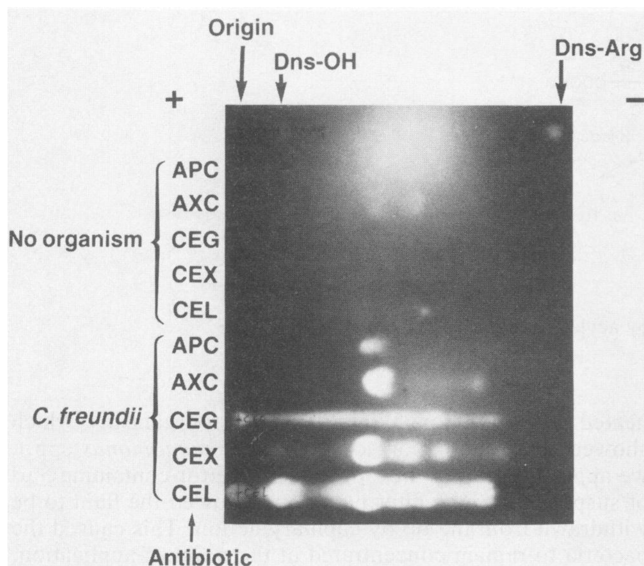

FIG. 3. HVE analyses at pH 2.1 of the end products of ampicillin (APC), amoxicillin (AXC), cephaloglycin (CEG), cephalexin (CEX), and cefadroxil (CEL) produced by *C. freundii* NRL 5329. Each uninoculated substrate control (100 nmol) and its reaction mixture after incubation with the organism for 1 h at 37°C were applied separately on a Whatman 3MM paper, subjected to HVE at pH 2.1 (4) at 80 V/cm for 30 min, and dried at 90°C for 15 min. The end products were visualized with a long-wave UV lamp. The electrophoretic mobility was toward the cathode (−). Fluorescent markers (3) were dansyl acid (Dns-OH; neutral marker at pH 2.1) and dansyl arginine (Dns-Arg). Similar patterns were obtained if each substrate (100 nmol) was incubated with 1 μg each of purified β-lactamase from *E. cloacae* or *B. cereus* for 1 h at 37°C.

cephalexin, and cefadroxil) containing a primary amino group on the acyl side chain were separately incubated with *Citrobacter freundii* for 1 h at 37°C. Supernatant fluid from each reaction mixture was separated by HVE at pH 2.1 (4). The product and the unhydrolyzed substrate were revealed by ninhydrin-cadmium acetate stain (7) after drying at 90°C for 15 min. The ninhydrin-cadmium acetate stain showed distinct spots of the end product and the unhydrolyzed substrate for all β-lactam antibiotics tested except cephaloglycin and cephalosporin C (the end products trailed toward the cathode; data not shown). Subsequent studies showed that before ninhydrin-cadmium acetate staining each major end product (as detected later by ninhydrin-cadmium stain; cephaloglycin produced no distinct major end product) and some minor end products, except from cephalosporin C, were highly fluorescent under a long-wave UV lamp, whereas the unhydrolyzed substrates were not fluorescent (Fig. 3). The fluorescent pattern produced by *C. freundii* (Fig. 3) for each substrate was found to be identical to that produced by purified β-lactamase from *Enterobacter cloacae* or *Bacillus cereus* (Sigma; 100 nmol of each substrate incubated with 1 μg of each enzyme for 1 h at 37°C). Trace amounts of the fluorescent open β-lactam-ring forms of ampicillin, amoxicillin, and cefadroxil detected in the uninoculated substrate control were attributable to spontaneous hydrolysis during incubation and contamination of the commercial sources with the open-ring forms themselves, and this background of fluorescence was easily distinguished from the amount of fluorescent end product by the microbial β-lactamases. The minor fluorescent end products of cephalexin and cefadroxil after incubation were presumably due to acid degradation of each major end product during HVE at pH 2.1, since better cooling of paper during HVE reduced their formation (results not shown). Therefore, all five β-lactam substrates which produced fluorescent end products (open β-lactam-ring forms) during incubation with known β-lactamases could be used for detection of microbial β-lactamases.

We further found that the end product of each β-lactam substrate shown in Fig. 3 could be detected on the filter paper without subsequent electrophoretic separation after brief heating at 120°C for 5 min. Thus, a simple spot test could be employed for detection of microbial β-lactamases.

Selection of β-lactam substrates for differentiation between penicillinase and cephalosporinase activities of β-lactamase by the spot test. To detect β-lactamases with a predominance of penicillinase or cephalosporinase activity and to detect weak β-lactamase producers with the spot test, two substrates which produced the least fluorescent background (due to nonenzymatic hydrolysis during incubation and the end product contaminants present in the commercial sources) were selected. Ampicillin produced less fluorescent background than amoxicillin (results not shown) and was chosen as the substrate for penicillinase, despite the fact that its open β-lactam-ring form was less fluorescent than that of amoxicillin (Fig. 3). Likewise, cephalexin was chosen as the substrate for cephalosporinase. A summary of the fluorescent spot test method is provided in Fig. 4.

Differentiation of β-lactamase and acylase activities by the spot test and HVE. To determine whether the spot test method could distinguish β-lactamase activity from acylase activity, the end products of acylase (the common side chain, D(−)-α-aminophenylacetic acid, and the intact β-lactam nuclei, 6-aminopenicillanic acid and 7-aminodeacetoxycephalosporanic acid, 100 nmol each) were separately applied onto Whatman 3MM paper. None of the end products of acylase were fluorescent either by the spot test method or the HVE method (Fig. 5A and B). However, all produced color after ninhydrin-cadmium acetate staining (Fig. 5C). Therefore, both the spot test method and the HVE method can distinguish β-lactamase activity from acylase activity.

Nonfluorescent substrate	Hydrolysis	Fluorescent product	Fluorescence development	Long-wave UV lamp
Ampicillin (50 μl, 0.02 M, pH 7.0)	1/2 loopful of bacteria ⟶ Penicillinase activity	D-Phenylglycyl-penicilloic acid	5 μl on paper ⟶ 120°C, 5 min	Fluorescence
Cephalexin (50 μl, 0.02 M, pH 7.0)	1/2 loopful of bacteria ⟶ Cephalosporinase activity	D-Phenylglycyl-deacetoxycephalosporoic acid	5 μl on paper ⟶ 120°C, 5 min	Fluorescence

FIG. 4. Summary of the fluorescent spot test for detection of microbial β-lactamases.

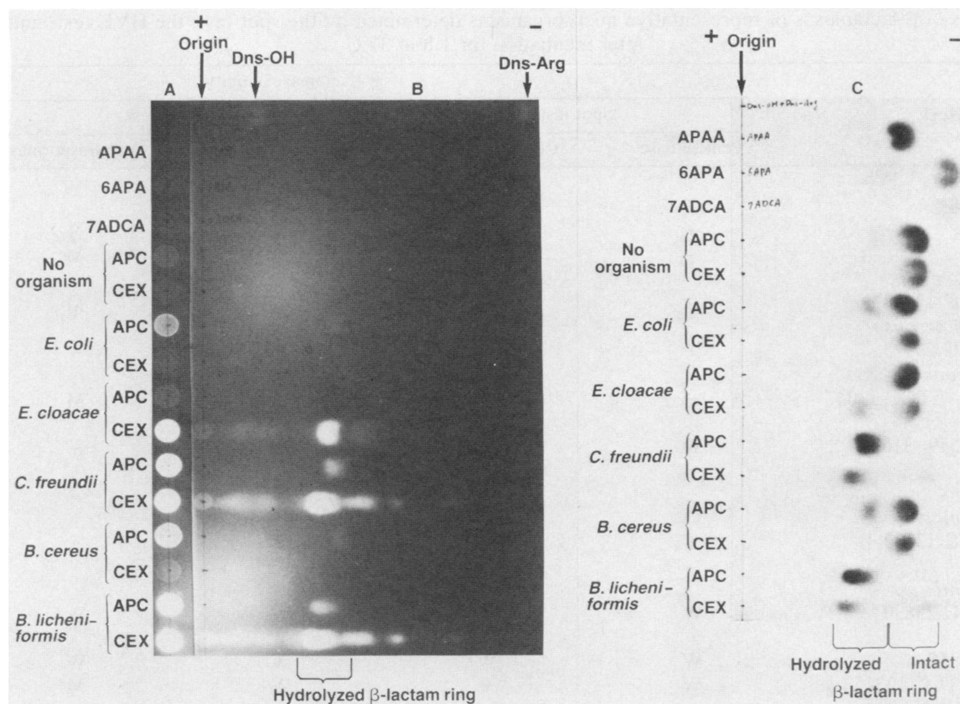

FIG. 5. (A) Activity of β-lactamase in *E. coli* ATCC 27549, *E. cloacae* ATCC 13047, *C. freundii* NRL 5329, *B. cereus* ATCC 27348, and *B. licheniformis* ATCC 25972 determined by the spot test. (B) HVE test viewed under a long-wave UV lamp. (C) HVE test after ninhydrin-cadmium acetate stain, using ampicillin (APC; for penicillinase activity) and cephalexin (CEX; for cephalosporinase activity) as substrates. Each uninoculated substrate control (100 nmol) and its reaction mixture after incubation with the organism for 1 h at 37°C were applied separately to Whatman 3 MM paper, subjected to HVE at pH 2.1 (4) at 80 V/cm for 30 min, and dried at 90°C for 15 min. The end products (100 nmol each) of the hydrolysis by acylase of ampicillin or cephalexin [D(−)-α-aminophenylacetic acid (APAA), 6-aminopenicillanic acid (6APA), and 7-aminodeacetoxycephalosporanic acid (7ADCA)] gave no fluorescence.

Semiquantitation of β-lactamase activity. The spot test result for β-lactamase activity with a given substrate was classified as weakly positive (W) when the fluorescent intensity of the spot was faint but discernibly greater than than of the uninoculated substrate control and as positive (+) when bright, blue-green fluorescence was observed.

The HVE test result for β-lactamase activity with a given substrate was classified as weakly positive (W) when faint fluorescence was observed at the position corresponding to that of the end product after HVE and the color intensity of the end product spot after ninhydrin-cadmium acetate stain was slightly but discernibly greater than that of the end product in the uninoculated substrate control (produced by spontaneous nonenzymatic hydrolysis during incubation and from end product contamination of commercial sources). The HVE test result for β-lactamase was classified as moderately positive (M) when bright, blue-green fluorescence was observed at the position corresponding to that of the end product but the color intensity of the end product after ninhydrin-cadmium acetate stain was less than that of the remaining substrate spot. The HVE result was strongly positive (S) when bright, blue-green fluorescence was observed at the position corresponding to that of the end product spot and the color intensity of the end product spot after ninhydrin-cadmium acetate stain was greater than that of the remaining substrate spot.

Distribution of β-lactamase activities among representative microorganisms. The spot test method was used for the detection of β-lactamase produced by one strain each of *Escherichia coli*, *E. cloacae*, *C. freundii*, *B. cereus*, and *B. licheniformis*, using ampicillin (for penicillinase activity) and cephalexin (for cephalosporinase activity) as substrates (Fig. 5). The results of the spot test (Fig. 5A) were confirmed by HVE visualized under UV light (Fig. 5B) and with a ninhydrin-cadmium acetate stain (Fig. 5C). Fluorescence was produced by *E. coli* during incubation with ampicillin for 1 h at 37°C (Fig. 5A), and the end product (D-phenylglycylpenicilloic acid) was detected under UV light (Fig. 5B) and further confirmed by ninhydrin-cadmium acetate stain (Fig. 5C). The penicillinase activity of this *E. coli* strain was determined to be weakly positive by both the spot test and the HVE method by the criteria described above, whereas no cephalosporinase activity was detected by either method. The fluorescent end product, D-phenylglycyldeacetoxycephalosporoic acid, was produced by *E. cloacae* during incubation with cephalexin for 1 h at 37°C (Fig. 5A). The end product was visualized under UV light after HVE (Fig. 5B) and further confirmed by ninhydrin-cadmium acetate stain (Fig. 5C). The cephalosporinase activity of *E. cloacae* was classified as positive by the spot test and as moderately positive by the HVE method, using the criteria described above; no penicillinase activity was detected by either method. By the spot test and the criteria described above, the penicillinase and cephalosporinase activities of *C. freundii* and *B. licheniformis* were both classified as positive, whereas the penicillinase activity was positive and cephalosporinase activity was negative in *B. cereus* (Fig. 5A). By the HVE method and the criteria described above, the penicillinase and cephalosporinase activities of *C. freundii* and *B. licheniformis* were both classified as strongly positive; the

TABLE 1. Activities of β-lactamases of representative microorganisms determined by the spot test, the HVE test, and the nitrocefin test after incubation for 1 h at 37°C

Microorganism[a]	β-Lactamase activity[b]				
	Spot test		HVE test		Nitrocefin test
	Penicillinase	Cephalosporinase	Penicillinase	Cephalosporinase	
Gram-negative enteric					
Citrobacter freundii					
NRL 5329	+	+	S	S	+
ATCC 10787	–	+	–	M	+
Enterobacter aerogenes					
NRL 9817, ATCC 13048	–	+	–	M	+
Enterobacter agglomerans					
NRL 9819; ATCC 29915	+	–	S	–	+
Enterobacter cloacae					
NRL 5335, 9818; ATCC 13047	–	+	–	M	+
Escherichia coli					
ATCC 21986, 27549, 31027	W	–	W	–	+
Klebsiella oxytoca					
NRL 9979	+	–	S	–	+
Klebsiella pneumoniae					
NRL 9976; ATCC 13883, 27799	+	–	S	–	+
Morganella morganii					
NRL 5334; ATCC 25830	W	W	W	W	+
Proteus mirabilis					
ATCC 14273, 29855	W	W	W	W	–
Proteus vulgaris ATCC 13315	W	+	W	M	–
Providencia rettgeri					
ATCC 9250, 31052	–	–	–	–	–
Salmonella typhimurium ATCC 13311	–	–	–	–	–
Serratia marcescens					
ATCC 8100, 17991	–	+	–	S	+
Serratia rubidaea ATCC 181	W	+	W	S	+
Shigella dysenteriae					
ATCC 13313	–	W	–	W	+
Shigella sonnei					
ATCC 11060	–	+	–	M	+
Gram-negative nonenteric					
Branhamella catarrhalis					
NRL 32674, 32681, 32763	+	+	S	S	+
NRL 30069, 30071, 32589	–	–	–	–	–
Eikenella corrodens					
ATCC 1073, 23834	–	–	–	–	–
Haemophilus ducreyi					
V-1157, 1158, 1169	+	+	S	M	+
V-1152, 1168	–	–	–	–	–
Haemophilus influenzae					
AS1115, 902	+	+	S	S	+
AS1117, Ela; ATCC 19418	–	–	–	–	–
Neisseria gonorrhoeae					
NRL 33044, 33047, 33050	+	+	S	S	+
NRL 8327, 30483, F62	–	–	–	–	–
Pseudomonas aeruginosa					
SM31302-31311	W	W	W	W	–
Pseudomonas cepaciae					
BM1, 2	+	+	S	S	+
Pseudomonas fluorescens					
BM3; ATCC 25289	–	+	–	S	+
Pseudomonas maltophilia					
BM4, 5; ATCC 13637	+	+	S	S	+
Pseudomonas putida					
BM6, 7; ATCC 25571	–	+	–	S	+
Gram-negative anaerobic					
Bacteroides bivius					
ATCC 29303	+	+	S	M	+
Bacteroides capillosus					
ATCC 29799	–	–		–	–

Continued on following page

TABLE 1—*Continued*

Microorganism[a]	β-Lactamase activity[b]				
	Spot test		HVE test		Nitrocefin test
	Penicillinase	Cephalosporinase	Penicillinase	Cephalosporinase	
Bacteroides fragilis					
ATCC 23745, 25285	W	+	W	M	+
Gram-positive					
Bacillus cereus					
ATCC 13061	+	–	S	–	+
ATCC 27348, 14579	+	–	M	–	–
Bacillus circulans ATCC 4513	+	–	S	–	–
Bacillus licheniformis					
ATCC 9789, 14409	+	W	S	W	+
ATCC 25972	+	+	S	S	+
Bacillus subtilis					
ATCC 9799, 14410, 14415	W	–	W	–	–
ATCC 14807	+	–	S	–	–
Staphylococcus aureus					
ATCC 12598, 25923	–	–	–	–	–
BM U17, Su3	+	–	S	–	+
BM Me19	+	–	S	–	–
Staphylococcus epidermidis					
ATCC 12228, 14990	+	–	S	–	+
Streptococcus faecalis					
ATCC 11420, 12984, 19433	–	–	–	–	–

[a] Strain numbers are those of the American Type Culture Collection (ATCC), the Neisseria Reference Laboratory (NRL), Stephen A. Morse (SM), Barbara H. Minshew (BM), and Arnold L. Smith (AS). Strains of *Haemophilus ducreyi* were described previously (19). The growth conditions for each microorganism are described in the text.

[b] The activities of penicillinase and cephalosporinase in each microorganism as determined by the spot test and by the HVE test were recorded as follows. The spot test result for β-lactamase activity with a given substrate was classified as weakly positive (W) when the fluorescent intensity of the spot was faint but discernibly greater than that of the uninoculated substrate control, and as positive (+) when bright, blue-green fluorescence was observed. The HVE test results for β-lactamase activity with a given substrate were classified as follows: weakly positive (W), faint fluorescence was observed at the position corresponding to that of the end product after HVE, and the color intensity of the end product spot after ninhydrin-cadmium acetate stain was slightly but discernibly greater than that of the end product in the uninoculated substrate control (produced by spontaneous nonenzymatic hydrolysis and from contamination); moderately positive (M), bright, blue-green fluorescence was observed at the position corresponding to that of the end product spot, but color intensity of the end product spot after ninhydrin-cadmium acetate stain was less than that of the remaining substrate spot; strongly positive (S), bright, blue-green fluorescence was observed at the position corresponding to that of the end product spot, and the color intensity of the end product spot after ninhydrin-cadmium acetate stain was greater than that of the remaining substrate spot.

penicillinase activity and cephalosporinase activity of *B. cereus* were classified as moderately positive and negative, respectively (Fig. 5B and C).

Activities of β-lactamases in 21 strains of 7 gram-positive species and 77 strains of 29 gram-negative species of bacteria were determined by the spot test, the HVE test, and the nitrocefin test after incubation for 1 h at 37°C (Table 1). The results of the nitrocefin test agreed well with those of the spot test (confirmed by the HVE test; Table 1). Some β-lactamases which acted predominantly against ampicillin, such as a few species of gram-positive bacteria, were not detected by the nitrocefin test (Table 1). Therefore, our fluorescent method was more sensitive than the chromogenic cephalosporin (nitrocefin) method.

Activities of β-lactamases in selected β-lactamase producers listed in Table 1 were further assessed by the rapid spot test and the nitrocefin test after incubation for 5 and 15 min at room temperature, and the results are listed in Table 2. The incubation time needed for a positive reaction for the fluorescent spot test appears to be the same as, or shorter than, that needed for the nitrocefin test (Table 2). Some clinically important microorganisms such as *N. gonorrhoeae* and *H. influenzae* produce β-lactamases which could be detected by the rapid spot test immediately after organisms were suspended in the ampicillin substrate solution, without further incubation (results not shown), just as they could be detected by the nitrocefin test under similar conditions.

DISCUSSION

This fluorescent spot test method can identify β-lactamase activity per se and is not influenced by acylase activity. For example (Table 1) two strains of *Providencia rettgeri* which produce acylase (K. C. S. Chen and K. K. Holmes, unpublished data) but not β-lactamase were nonreactive in all β-lactamase assays, including the nitrocefin test; a strain of *Pseudomonas maltophilia* (BM4) which produces both acylase and β-lactamase (K. C. S. Chen and K. K. Holmes, unpublished data) was reactive in all the tests shown in the table. The test can also detect and differentiate between penicillinase activity and cephalosporinase activity. The fluorescent spot test requires 5 min of heating at 120°C to maximize the fluorescent potential of the end products; the mechanism by which heating enhances fluorescence is not understood.

Stock solutions of the substrates (ampicillin and cephalexin, acid forms) for the spot test were easily prepared as 0.02 M solutions in 0.04 M sodium phosphate buffer, pH 7.5. These solutions both had a pH of 7.0, and the pHs of both solutions fall to 6.5 (within the optimal pH ranges of microbial β-lactamases [18]) upon complete hydrolysis by *C. freun-*

TABLE 2. Activities of β-lactamases of selected microorganisms determined by the rapid spot test and the nitrocefin test after incubation for 5 and 15 min at room temperature

Microorganism[a]	β-Lactamase activity[b]					
	Rapid spot test				Nitrocefin test	
	5 min		15 min		5 min	15 min
	Penicillinase	Cepahlosporinase	Penicillinase	Cephalosporinase		
Gram-negative enteric						
Citrobacter freundii						
NRL 5329	+	+	+	+	+	+
ATCC 10787	−	−	−	+	−	+
Enterobacter aerogenes						
NRL 9817, ATCC 13048	−	−	−	+	−	−
Enterobacter agglomerans						
NRL 9819; ATCC 29915	+	−	+	−	−	+
Enterobacter cloacae						
NRL 5335, 9818; ATCC 13047	−	−	−	+	−	−
Klebsiella oxytoca						
NRL 9979	+	−	+	−	−	−
Klebsiella pneumoniae						
NRL 9976; ATCC 13883	+	−	+	−	−	−
Serratia marcescens						
ATCC 8100, 17991	−	−	−	+	−	+
Serratia rubidaea						
ATCC 181	−	−	−	−	−	−
Shigella sonnei						
ATCC 11060	−	−	−	+	−	−
Gram-negative nonenteric						
Branhamella catarrahalis						
NRL 32674, 32681, 32763	+	−	+	−	+	+
Haemophilus ducreyi						
V-1157, 1158, 1169	+	−	+	−	+	+
Haemophilus influenzae						
AS1115, 902	+	−	+	−	+	+
Neisseria gonorrhoeae						
NRL 33044, 33047, 33050	+	−	+	−	+	+
Pseudomonas cepaciae						
BM1, 2	−	−	−	+	−	+
Pseudomonas fluorescens						
BM3; ATCC 25289	−	−	−	+	−	+
Pseudomonas maltophilia						
BM4, 5	+	+	+	+	+	+
Pseudomonas putida						
BM6, 7; ATCC 25571	−	−	−	+	−	+
Gram-positive						
Bacillus cereus						
ATCC 13061	+	−	+	−	+	+
ATCC 27348, 14579	−	−	+	−	−	−
Bacillus circulans						
ATCC 4513	−	−	W	−	−	−
Bacillus licheniformis						
ATCC 9789, 14409	−	−	+	W	−	+
ATCC 25972	+	−	+	+	+	+
Bacillus subtilis						
ATCC 14807	−	−	+	−	−	−
Staphylococcus aureus						
BM U17, Su3	−	−	+	−	−	+
BM Mel9	−	−	+	−	−	−
Staphylococcus epidermidis						
ATCC 14990	+	−	+	−	+	+
ATCC 1228	−	−	+	−	−	+

[a] Strain numbers are those of the American Type Culture Collection (ATCC), the Neisseria Reference Laboratory (NRL), Barbara H. Minshew (BM) and Arnold L. Smith (AS). Strains of *Haemophilus ducreyi* were described previously (19). The growth conditions for each microorganism are described in the text.

[b] The activities of penicillinase and cephalosporinase in each microorganism as determined by the spot test were recorded as follows. The spot test result for β-lactamase activity using a given substrate was classified as weakly positive (W) when the fluorescent intensity of the spot was faint but discernibly greater than that of the uninoculated substrate control; the result was positive when bright, blue-green fluorescence was observed.

dii. The solutions could be stored at 4°C for 5 days or at −20°C for months without detectable increases in fluorescent background as checked by the spot test. Some organisms fluoresced slightly on paper, but this fluorescence was confined to the center of the applied spot and could be easily distinguished from the fluorescence of end products which diffused radially by capillary action of the paper from the center of application. Therefore, the spot test could be performed without prior removal of the organisms by centrifugation. Unless the β-lactamase sought shows no substrate specificity, the ampicillin and cephalexin substrates should be incubated separately with the organism. When penicillin and cephalosporin substrates are mixed together, the substrate which is not hydrolyzed may act as a competitive inhibitor of the other substrate. For example, *Serratia marcescens* ATCC 8100 and 17991 exhibited only cephalosporinase activity as detected by the spot test and the HVE test when ampicillin and cephalexin were incubated separately with the organism (Table 1). However, cephalosporinase activity was not detected in either strain by the spot test or the HVE test when both strains were incubated with a mixture of equal volumes of ampicillin and cephalexin substrate solution for 1 h at 37°C (results not shown).

The spot test described in this paper, using ampicillin and cephalexin as substrates, provides a rapid, inexpensive method for specific detection of microbial β-lactamases by detecting the presence of fluorescent end products. The test may have several applications. For example, incubation with human sera can result in a color change for nitrocefin (13). Therefore, nitrocefin is not suitable for detection of β-lactamase in the presence of certain body fluids. We found that eight of eight human sera mixed with an equal volume of nitrocefin solution converted nitrocefin to a red color after 10 min of incubation at 37°C, but produced no fluorescence after incubation with an equal volume of ampicillin or cephalexin solution for 1 h at 37°C (results not shown). Thus, the methods described here have the potential for direct detection of microbial β-lactamases in clinical specimens.

The detection of fluorescent end products offers an economical alternative for clinical laboratories which test large numbers of microorganisms for β-lactamases. For example, the routine β-lactamase nitrocefin tests of staphylococcal isolates require a noninhibitory concentration of a semisynthetic penicillin as an inducer for the enzyme (8). In our fluorescent spot test method, with sufficient incubation time (e.g., ≥15 min; Table 2) ampicillin could serve not only as a substrate but also as an inducer for these gram-positive bacteria of clinical importance (11). The potential of this method for testing clinical isolates for β-lactamases which may have a predominance of penicillinase or cephalosporinase activity deserves further evaluation.

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