Enhancement of Fluorescence Development of End Products by Use of a Fluorescence Developer Solution in a Rapid and Sensitive Fluorescent Spot Test for Specific Detection of Microbial β -Lactamases

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A fluorescent spot test method for specific detection of microbial β -lactamases as previously published (K. C. S. Chen, J. S. Knapp, and K. K. Holmes, *J. Clin. Microbiol.* 19:818-825, 1984) was improved by the use of a fluorescence developer solution. The fluorescence developer solution used in this study consisted of 0.78 M sodium tartrate buffer containing 12% formaldehyde at a final pH of 4.5. An addition of 1 volume of fluorescence developer solution to 5 volumes of ampicillin or cephalaxin substrate solution incubated with β -lactamase-producing organisms, followed by heating the mixture at 45°C for 10 min resulted in enhancement of fluorescence of the end products of β -lactamase activity. This provides a more sensitive assay for microbial β -lactamases and offers the potential for direct detection of β -lactamases in clinical specimens.

Microbial β -lactamases which hydrolyze the amide bonds of the β -lactam ring of β -lactam antibiotics, rendering the antibiotics inactive, play an important role in microbial resistance to penicillins and cephalosporins.

In early studies, products of alkaline hydrolysis of ampicillin and cephalaxin were found to be highly fluorescent at pH 4.2 and 5.0, respectively, in the presence of formaldehyde after heating at 100°C for 30 min, and this was the basis of fluorimetric assays for those two β -lactam antibiotics (1, 2). In the present study, we found that when the reaction mixture of hydrolysis of ampicillin or cephalaxin by β -lactamases was heated at 45°C for 10 min with one-fifth of its volume of 0.78 M sodium tartrate buffer containing 12% formaldehyde (pH 4.5), only the end product became fluorescent, permitting development of an assay for β -lactamase which was more sensitive than the previous one (3).

This improved assay described in the present study not only retains all the features for specific detection of microbial β -lactamases, such as simultaneous detection and differentiation of penicillinase and cephalosporinase activities, and distinguishing β -lactamase activity from acylase activity in microorganisms, as previously described (3) but also provides a great potential for direct detection of β -lactamases in clinical specimens.

MATERIALS AND METHODS

Chemicals. Tris, L(+)-tartaric acid, DL-malic acid, citric acid, succinic acid, glutaric acid, and DL-lactic acid were purchased from Sigma Chemical Co., St. Louis, Mo. Formaldehyde (37% [wt/vol]) in H₂O was from Aldrich Chemical Co., Inc., Milwaukee, Wis. Cotton-tipped applicators were from Hardwood Products Co., Guilford, Maine. The other chemicals were purchased as described previously (3). Unless otherwise stated, ampicillin, amoxicillin, and 6-aminopenicillanic acid were all dissolved in 25 mM Tris-hydrochloride (pH 7.6) to a final concentration of 10 mM (pHs were all adjusted to 7.0), and cephaloglycin, cephalaxin,

cefadroxil, 7-aminocephalosporanic acid, and 7-aminodeacetoxycephalosporanic acid were all dissolved in 25 mM sodium phosphate buffer (pH 7.5) to a final concentration of 10 mM (pHs were all adjusted to 7.0). D(-)- α -Aminophenylacetic acid (5 mM) was prepared in either 12.5 mM Tris-hydrochloride buffer or 12.5 mM sodium phosphate buffer at a final pH of 7.0.

Preparation of fluorescence developer solution for open β -lactam ring end products resulting from hydrolysis of β -lactam substrates by microbial β -lactamases. Fluorescence developer solution for open β -lactam ring end products was prepared as follows: 0.78 M sodium tartrate buffer was prepared in 12% (wt/vol) formaldehyde solution, with a final pH of 4.5. Developers using other organic acids such as malic acid, citric acid, succinic acid, glutaric acid, acetic acid, and lactic acid were prepared similarly as described above.

Preparation of inocula for detection of β -lactamase. The growth conditions for each microorganism used in this study were essentially the same as previously described (3). Portions (250 μ l) of each β -lactam antibiotic were separately placed in a disposable culture tube (12 by 75 mm). A cotton-tipped applicator which contained approximately a loopful (diameter, 2 mm) of growth of each strain removed from the agar plate was mixed in each substrate tube and incubated for either 5 min at 45°C or 15 min at 37°C. Uninoculated substrate controls were prepared in the same manner.

Fluorescent spot test for detection of β -lactamase by detection of open β -lactam ring end products. After 5 min of incubation at 45°C or 15 min at 37°C, a 50- μ l volume of fluorescence developer solution was added to each tube, including each uninoculated substrate control tube; the reaction mixture was mixed briefly with the applicator and further incubated at 45°C (water bath) for 10 min. After the incubation, the cotton-tipped applicator from each tube, including each uninoculated substrate control tube, was separately touched onto Whatman 3MM filter paper to form a spot with a diameter of about 8 mm. The fluorescent intensity of each spot was then compared with its uninoculated

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ated substrate control spot under a long-wave UV lamp and classified as negative, weakly positive, or positive.

Detection of β -lactamase by the nitrocefin test. The nitrocefin test (4) was performed under the same conditions as the fluorescent spot test described above except 250 μ l of nitrocefin (50 μ g/ml) was dispensed in each tube, including each uninoculated control tube. The color (red) intensity of each test tube was compared with that of the uninoculated nitrocefin control tube after incubation and classified as negative or positive.

RESULTS

Detection of β -lactamase by fluorescent spot test with fluorescence developer. Substrate solutions of penicillins (ampicillin and amoxicillin) and cephalosporins (cephaloglycin, cephalexin, and cefadroxil) were not fluorescent under UV light. The solutions of their corresponding open β -lactam ring end products resulting from β -lactamase hydrolysis described previously (3) also were not fluorescent under UV light. After addition of fluorescence developer solution to the intact substrates and their corresponding open β -lactam ring end products, followed by heating each reaction mixture at 45°C for 10 min, only the end products became fluorescent. For example, neither substrate solutions of ampicillin and cephalexin nor their open β -lactam ring end products resulting from hydrolysis by purified β -lactamase from *Bacillus cereus* or *Enterobacter cloacae* (3) were fluorescent (Fig. 1). After addition of fluorescence developer solution and heating at 45°C for 10 min, fluorescence was developed only for the end products of ampicillin and cephalexin after β -lactamase hydrolysis.

Differentiation between β -lactamase and acylase activities and between penicillinase and cephalosporinase activities of β -lactamase by fluorescent spot test with fluorescence developer. To determine whether the fluorescent spot test with fluorescence developer could distinguish β -lactamase activity from acylase activity, solutions of the end products of acylase activity upon ampicillin, cephaloglycin, and cephalexin (the compounds which have the common side chain D(-)- α -aminophenylacetic acid and the different intact β -lactam nuclei 6-aminopenicillanic acid, 7-aminocephalosporanic acid, and 7-aminodeacetoxycephalosporanic acid, respectively) and upon amoxicillin and cefadroxil (the compounds which have the common side chain D(-)-*p*-hydroxyphenylglycine and the different intact β -lactam nuclei, 6-aminopenicillanic acid, and 7-aminodeacetoxycephalosporanic acid, respectively) were all prepared as described under Materials and Methods. These five solutions were separately incubated with fluorescence developer solution at a 5-to-1 ratio (vol/vol) and heated at 45°C for 10 min. None of the end products of acylase were fluorescent (results not shown). Therefore, when ampicillin, amoxicillin, cephaloglycin, cephalexin, or cefadroxil was used as substrate for β -lactamase, the fluorescent spot test with fluorescence developer could distinguish β -lactamase activity from acylase activity. Ampicillin was chosen to detect penicillinase activity because it produced less fluorescent background than amoxicillin, and cephalexin was chosen to detect cephalosporinase activity because it produced less fluorescent background than cephaloglycin or cefadroxil.

Improvement of fluorescence development with fluorescence developer for fluorescent spot test. As shown in Fig. 2, the fluorescent intensity of end products produced by β -lactamase with a fluorescence developer was severalfold greater than that of the fluorescent spot test without a

FIG. 1. Fluorescence development of intact ampicillin (APC) and cephalalexin (CEX) substrate solutions and their corresponding hydrolyzed end products produced by β -lactamases; results of experiments with and without the use of fluorescence developer are shown. APC solution (3 mM in 25 mM Tris hydrochloride buffer [pH 7.0]) and CEX solution (3 mM in 25 mM sodium phosphate buffer [pH 7.0]) were separately hydrolyzed by purified β -lactamases from *B. cereus* and *E. cloacae*, respectively, as previously described (3). For experiments with developer, the fluorescence developer solution was added at a 1:5 ratio (vol/vol) to the intact substrate solutions, APC and CEX, and to their corresponding hydrolyzed mixtures. Each solution was heated at 45°C for 10 min. Intact APC and CEX showed no fluorescence, and each corresponding hydrolyzed mixture showed strong fluorescence under a long-wave UV lamp. For experiments without developer, H₂O was added at a 1:5 ratio (vol/vol) to the intact substrate solutions, APC and CEX, and to their corresponding hydrolyzed mixtures, and heated under the same conditions as described above. Intact APC and CEX showed no fluorescence (results not shown), and corresponding hydrolyzed mixtures also showed no fluorescence.

fluorescence developer performed as previously described (3).

The steps followed in the fluorescent spot test for detection of microbial β -lactamase with fluorescence developer are summarized in Fig. 3.

Detection of β -lactamases among the selected microorganisms. The spot test result for β -lactamase activity with a given substrate was classified as weakly positive when the fluorescent intensity of the spot was faint but discernibly brighter than that of the uninoculated substrate control and as positive when bright, blue-green fluorescence was observed. The spot test with a fluorescence developer was used for the detection of β -lactamase produced by one strain each of *E. cloacae*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, and *Staphylococcus aureus*, with ampicillin (for penicillinase activity) and cephalexin (for cephalosporinase activity) as substrates (Fig. 4). Fluorescence was not produced by *E. cloacae* during incubation with ampicillin for 5 min at 45°C (Fig. 4A), indicating no penicillinase activity in the organism, while fluorescence was produced by the same organism during incubation with cephalexin for 5 min at 45°C

FIG. 2. Fluorescent intensity of twofold dilutions of intact ampicillin (APC) and cephalixin (CEX) substrate solutions and of their corresponding hydrolyzed end products produced by β -lactamases; results of experiments with and without the use of fluorescence developer are shown. The same substrate solutions, ampicillin and cephalixin and their hydrolyzed end product mixtures, as described for Fig. 1, were separately diluted serially in twofold dilutions with 25 mM Tris-hydrochloride buffer (pH 7.0) for ampicillin or 25 mM sodium phosphate buffer (pH 7.0) for cephalixin. Fluorescence was developed for each solution either with fluorescence developer, as described in Fig. 1, and application of the reaction mixture (5 μ l) on Whatman 3mm filter paper, or without fluorescence developer by addition of H₂O instead of fluorescence developer solution, application of the reaction mixture (5 μ l) on the filter paper, and heat treatment of the filter paper at 120°C for 5 min as previously described (3). Fluorescence was visualized with a long-wave UV lamp.

(Fig. 4A), indicating the predominance of cephalosporinase activity of the β -lactamase produced by that organism. Fluorescence was produced by *N. gonorrhoeae* and *H. influenzae* during incubation separately both with ampicillin and with cephalixin for 5 min at 45°C (Fig. 4A), indicating that the β -lactamase produced by these two strains had both penicillinase and cephalosporinase activities. Strong fluorescence was produced by *S. aureus* during incubation with ampicillin, but only weak fluorescence was produced during incubation with cephalixin for 5 min at 45°C, indicating the predominance of penicillinase activity, with weak cephalosporinase activity of the β -lactamase produced by that organism. The increase of fluorescence of end products resulting from use of fluorescence developer is also shown in Fig. 4 (compare Fig. 4A and B), and these increases became more obvious when both ampicillin and cephalixin were used at lower concentrations (3 mM) for incubation with those four strong β -lactamase producers.

Activities of β -lactamases in the selected microorganisms

(20 strains of 11 gram-negative species and 7 strains of 4 gram-positive species) were determined by both the fluorescent spot test (10 mM of both substrates were used) and the nitrocefin test after incubation for 5 min at 45°C or 15 min at 37°C (Table 1). For gram-negative bacteria, the sensitivity of the fluorescent spot test conducted at 45°C for 5 min was essentially the same as that of the same test conducted at 37°C for 15 min. For some of the gram-positive bacteria, the test was more sensitive when conducted at 37°C for 15 min (Table 1). The sensitivity of the nitrocefin test conducted at 45°C for 5 min was less than that conducted at 37°C for 15 min for certain gram-negative and gram-positive bacteria (Table 1).

Our fluorescent spot test with fluorescence developer was more sensitive than the nitrocefin method for certain gram-negative and gram-positive bacteria (Table 1). Several tests which were falsely negative (positive fluorescent spot test, negative nitrocefin test) by the nitrocefin method for gram-negative bacteria turned positive after incubation for 1 h at

FIG. 4. Activities of β -lactamases produced by *E. cloacae* ATCC 13047, *N. gonorrhoeae* NRL 33044, *H. influenzae* AS1115, and *S. aureus* BMSu3, determined by the fluorescent spot test with (A) or without (B) fluorescence developer with ampicillin (APC; for penicillinase activity) and cephalexin (CEX; for cephalosporinase activity) as substrates. For experiments with developer, the fluorescence developer solution (50 μ l) was added to each uninoculated substrate control (250 μ l of 3 mM ampicillin in 25 mM Tris hydrochloride buffer [pH 7.0] and 250 μ l of 3 mM cephalexin in 25 mM sodium phosphate buffer [pH 7.0]) and its reaction mixture after incubation with the organism for 5 min at 45°C. Each reaction mixture was heated for 10 min at 45°C and applied on Whatman 3MM filter paper with a cotton-tipped applicator. For experiments without developer, H₂O (50 μ l) was added to the substrate control (250 μ l each) and its corresponding reaction mixture after incubation with the organism. Each reaction mixture was applied on Whatman 3MM filter paper with a cotton-tipped applicator. Fluorescence was visualized with a long-wave UV lamp and heated at 120°C for 5 min, as previously described (3).

37°C, but tests which were falsely negative for gram-positive organisms remained negative even after incubation for 1 h at 37°C (e.g., *B. cereus* ATCC 14579, *Bacillus subtilis* ATCC 14807, and *S. aureus* BMMe19; results for 1-h incubation not shown).

DISCUSSION

When the alkaline degradation products of ampicillin and cephalexin were heated at 100°C for 30 min at acidic pH and in the presence of formaldehyde, a highly fluorescent yellow

FIG. 3. Summary of steps in the fluorescent spot test for detection of microbial β -lactamases, enhanced by the use of fluorescence developer solution.

TABLE 1. Activities of β -lactamases of selected microorganisms determined by the fluorescent spot test with fluorescence developer solution and the nitrocefin test after incubation for 5 min at 45°C and 15 min at 37°C

Microorganisms ^a	β -Lactamase activity by ^b :					
	Fluorescent spot test with incubation for:				Nitrocefin test with incubation for:	
	5 min at 45°C		15 min at 37°C		5 min at 45°C	15 min at 37°C
	Penicillinase	Cephalosporinase	Penicillinase	Cephalosporinase		
Gram-negative enteric bacteria						
<i>Citrobacter freundii</i>						
NRL 5329	+	+	+	+	+	+
ATCC 10787	—	+	—	+	—	+
<i>Enterobacter cloacae</i>						
NRL 5335	—	+	—	+	—	—
ATCC 13047	—	+	—	+	—	—
<i>Klebsiella oxytoca</i>						
NRL 9979	+	—	+	—	—	—
<i>Klebsiella pneumoniae</i>						
NRL 9976	+	—	+	—	—	—
ATCC 13883	+	—	+	—	—	—
<i>Serratia marcescens</i>						
ATCC 8100	—	+	—	+	+	+
ATCC 17991	—	+	—	+	+	+
<i>Shigella sonnei</i>						
ATCC 11060	—	+	—	+	—	—
Gram-negative nonenteric bacteria						
<i>Haemophilus ducreyi</i>						
V-1157	+	+	+	+	+	+
V-1158	+	+	+	+	+	+
<i>Haemophilus influenzae</i>						
AS1115	+	+	+	+	+	+
AS902	+	+	+	+	+	+
<i>Neisseria gonorrhoeae</i>						
NRL 33044	+	+	+	+	+	+
NRL 33047	+	+	+	+	+	+
<i>Pseudomonas cepaciae</i>						
BM1	W	+	W	+	—	+
BM2	W	+	W	+	—	+
<i>Pseudomonas maltophilia</i>						
BM4	+	+	+	+	+	+
BM5	+	+	+	+	+	+
Gram-positive bacteria						
<i>Bacillus cereus</i>						
ATCC 13061	+	W	+	W	+	+
ATCC 14579	+	—	+	—	—	—
<i>Bacillus licheniformis</i>						
ATCC 9789	—	—	+	W	—	+
ATCC 25972	+	+	+	+	+	+
<i>Bacillus subtilis</i>						
ATCC 14807	—	—	+	—	—	—
<i>Staphylococcus aureus</i>						
BMSu3	+	W	+	W	+	+
BMMc19	+	—	+	—	—	—

^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 *H. ducreyi* and the growth conditions for each microorganism were previously described (3).

^b The activities of penicillinase and cephalosporinase in each microorganism as determined by the fluorescent 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 brighter than that of the uninoculated substrate control; the result was positive when bright, blue-green fluorescence was observed. Both ampicillin (for penicillinase activity) and cephalixin (for cephalosporinase activity) were used at a concentration of 10 mM, while nitrocefin was used at a concentration of 50 μ g/ml as described in Materials and Methods.

product was formed (1, 2), and this provided the basis for a sensitive fluorimetric assay for the total concentration of ampicillin or cephalixin in human plasma (1, 2). However, it is impossible to adopt these procedures for detection of microbial β -lactamases, since the open β -lactam ring end product resulting from β -lactamase hydrolysis cannot be distinguished from the unhydrolyzed substrate after alkaline hydrolysis of the reaction mixture. In this present study, we

have designed a method for improving development of fluorescence for the open β -lactam ring end products of ampicillin and cephalixin after β -lactamase hydrolysis at lowered temperature (45°C) and for a shorter time (10 min). This method could detect and distinguish penicillinase and cephalosporinase activities of β -lactamases, and as with the previous method (3), acylase activity did not interfere with the assay.

Fluorescent intensity of the end product of either ampicillin or cephalixin resulting from β -lactamase hydrolysis was about the same when fluorescence developer solution was prepared either from dicarboxylic acid (with or without hydroxyl group, such as tartaric acid and malic acid or succinic acid and glutaric acid, respectively) or from hydroxytricarboxylic acid (such as citric acid) except that tartaric acid produced the least fluorescent background for both substrate controls. Fluorescence developer prepared from monocarboxylic acid (with or without hydroxyl group, such as lactic acid or acetic acid, respectively) produced less fluorescence for end products of both ampicillin and cephalixin than tartaric acid; therefore, fluorescence developer solution used in this study was prepared from tartaric acid.

Stock solutions of the substrates could be easily prepared and stored at -20°C for several months without detectable nonenzymatic hydrolysis as previously described (3). Stock solution of the fluorescence developer could also be easily prepared and stored at 37°C for several months without losing the capacity for enhancing fluorescence development. The use of a cotton-tipped applicator for transporting the bacteria from agar plate to the substrate solution and the use of the same applicator for spotting the reaction mixture onto Whatman 3MM filter paper made this procedure suitable for routine testing for β -lactamases in clinical isolates.

Since a 45°C water bath was required for the fluorescence development of open β -lactam ring end products resulting from β -lactamase hydrolysis, it is feasible to incubate the substrate with the organism at 45°C for 5 min to speed the enzymatic reaction itself. This rapid fluorescent spot test, involving incubation at 45°C for 5 min with the substrates, worked as well as incubation at 37°C for 15 min for the gram-negative bacteria tested but not for all the gram-positive bacteria, presumably because induction of β -lactamases in some organisms in the presence of β -lactam substrate required longer incubation (3).

As previously described (3), incubation of ampicillin or cephalixin with human sera, followed by addition of fluorescence developer solution and heat treatment as described in this study for detection of β -lactamase did not result in fluorescence of the substrate (results not shown), while nitrocefin changed color readily in the presence of human sera. Thus, the fluorescent spot test described in this study has greater potential than the nitrocefin test for direct detection of β -lactamases in sera and perhaps in other clinical specimens. For example, we have recently reported the identification of penicillinase-producing *N. gonorrhoeae* in male urethral exudates within 25 min by detection of

β -lactamase in Gram-stain-positive exudate with the rapid fluorescent spot test described in this study (5). Among 208 men with culture-proven gonococcal urethritis, the fluorescent spot test was positive for urethral exudates from 92 of 101 men from whom penicillinase-producing *N. gonorrhoeae* were isolated and 4 of 107 men from whom non-penicillinase-producing *N. gonorrhoeae* were isolated (5). The sensitivity of the spot test in these patients was 91%, the specificity was 96%, and the positive predictive value was 96%. The potential of direct detection of other β -lactamase-producing pathogens in clinical specimens by this method deserves further exploration.

In summary, these experiments show that the use of a fluorescence developer containing formaldehyde and tartaric acid (a dihydroxydicarboxylic acid with a pK_{a_2} of 4.23) results in a more sensitive assay for the end products of β -lactamase activity on ampicillin and cephalixin. This increased sensitivity for detection of the end products of β -lactamase activity may enhance the sensitivity of the assay for β -lactamase in infected body fluids.

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