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Simple Assay of β-Lactamase with Agar Medium Containing a Chromogenic Cephalosporin, Pyridinium-2-Azo-*p*-Dimethylaniline Chromophore (PADAC)

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A new β-lactamase assay method with agar plates containing pyridinium-2-azo-*p*-dimethylaniline chromophore (PADAC) (50 μM), a β-lactamase-labile, chromogenic cephalosporin, was examined. On the PADAC plates inoculated with β-lactamase-producing gram-negative bacteria (10^4 CFU per spot) and incubated at 37°C, a yellow zone showing hydrolysis of PADAC by β-lactamase was formed around the colony. The zone diameter increased with incubation time. Examination with *Enterobacter cloacae* GN7471 revealed that β-lactamase activity was present in the agar around the colony, decreasing exponentially with increasing distance from the colonial margin; this suggests that the PADAC hydrolysis zone is formed by an extracellular enzyme. At 18 h, significant correlations were obtained between the zone diameters of the 10 species (clinical isolates) examined and their periplasmic β-lactamase activities determined spectrophotometrically. The addition of clavulanic acid (0.5 to 10 μg/ml) inhibited zone formation on the PADAC plates inoculated with type IIIa, Va, Vb, PSE-1, and Ic β-lactamase producers. When the clinical isolates were tested on plates with clavulanic acid (2 μg/ml), inhibition was observed in 41 to 58% of the *Escherichia coli*, *Serratia marcescens*, and *Pseudomonas aeruginosa* isolates and in all isolates of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Proteus vulgaris*. Thus, the use of the inhibitor made it possible to detect penicillinase or type Ic cephalosporinase producers. These results proved that the PADAC plate might be a useful tool permitting easy, semiquantitative determination of β-lactamase activity.

The clinical use of many β-lactams is associated with the emergence of resistant strains, and their resistance, mainly caused by β-lactamase production (7), has long been an issue to resolve in the clinical field. Various methods for rapidly detecting β-lactamase have been devised. A typical detection method is the test based on a change of color following hydrolysis of a chromogenic cephalosporin in the disk or solution containing the bacterial colony (2, 4, 6, 8). However, this is a qualitative means of enzyme detection and seems to be less practical for use in the clinic than expected. The quantitative assay for β-lactamase activity requires laborious procedures, including enzyme isolation and purification (plus extracellular release of the enzyme by ultrasonic or other treatment in the case of gram-negative bacteria) and spectrophotometric determination.

We have searched for a new simple method to assay β-lactamase and found that on agar containing pyridinium-2-azo-*p*-dimethylaniline chromophore (PADAC), one of the chromogenic cephalosporins used conventionally for qualitative detection of β-lactamase (2), a yellow zone showing enzyme hydrolysis of PADAC (PADAC hydrolysis zone) is formed around the colony of β-lactamase-producing, gram-negative bacteria, giving semiquantitative results of enzyme activity. This paper describes a new method of estimating β-lactamase with PADAC agar.

MATERIALS AND METHODS

Bacterial strains. Fourteen β-lactamase-producing, gram-negative strains producing known types of enzymes (3) (used as the standard strains) were provided by the Laboratory of Drug Resistance in Bacteria, School of Medicine, Gunma University (Gunma, Japan). The other strains were all gram-negative clinical isolates collected and identified in various hospitals in Japan in 1985 and 1986. All bacterial strains were suspended in 100 mM phosphate buffer (pH 7.0) containing 25% glycerin and stored at −80°C until use.

Antibiotics. PADAC was supplied by Hoechst AG (Frankfurt, Federal Republic of Germany). Clavulanic acid, a β-lactamase inhibitor (5), was obtained from Beecham Yakuhin K.K. (Tokyo, Japan).

Preparation of PADAC-containing agar plates. PADAC powder was dissolved in dimethyl sulfoxide, and the solution was diluted with autoclaved 100 mM phosphate buffer (pH 7.0) to make a 500 μM PADAC solution (1% dimethyl sulfoxide concentration). Separately, heart infusion agar (Eiken Chemical Co., Tokyo, Japan) was prepared with 90% of the prescribed volume of distilled water, autoclaved, and maintained at about 50°C. The PADAC solution was mixed 1:9 (vol/vol) with this agar, and the mixture was stirred and immediately poured in 16-ml portions into plastic petri dishes (9 by 15 mm; Nipro Co., Osaka, Japan). The prepared agar plates (hereafter called PADAC plates) were stored below 4°C and used within 2 weeks after preparation. The PADAC plates were used after exposure to dryness at 37°C for about 30 min. For the detection of penicillinase, PADAC plates, each containing 0.5, 1, 2, 5, or 10 μg of clavulanic acid per ml, were made.

Incubation of bacteria on PADAC plates and determination of PADAC hydrolysis zone size. A fresh overnight culture of each test strain in heart infusion broth was diluted with the fresh broth, and a 5-μl inoculum (10^4 CFU per spot) of each strain was applied onto PADAC plates with an inoculum-replicating apparatus (Microplanter; Sakuma, Tokyo, Japan). After incubation at 37°C, the diameter of the PADAC hydrolysis zone formed around the grown colony was determined; the zone diameter was read by placing the incubated

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plate on a scale paper bearing concentric circles that differed by 1 mm in diameter.

When no hydrolysis zone was formed around the colony, the result was expressed as 6 mm, which is the average diameter of bacterial colonies incubated for 18 h.

β-Lactamase assay. β-Lactamases for determination of periplasmic enzyme activity were prepared as follows without using any inducer. Each β-lactamase-producing strain was precultured on heart infusion agar at 37°C, and after 18 to 24 h of incubation, the colony was scraped from the agar and suspended in 3 ml of 50 mM phosphate buffer (pH 7.0). The suspension was centrifuged at 9,000 × *g* for 15 min at 4°C, and cells were harvested, washed with 3 ml of the above-mentioned buffer, and resuspended in another 3 ml of the same buffer. The cells were then disrupted by ultrasonic treatment (40 W, 5 min, 60% duty cycles) with a sonicator (model 375; Heat Systems-Ultrasonics Inc., Farmingdale, N.Y.) in an ice bath. Cell debris was removed by centrifugation at 12,000 × *g* for 15 min at 4°C, and the supernatant obtained was used as the crude enzyme (a total protein concentration of about 2 mg/ml).

The periplasmic β-lactamase activity was determined at 35°C by spectrophotometry (double-beam UV spectrophotometer, model 200-20; Hitachi, Ltd., Tokyo, Japan). The substrate used was 50 μM PADAC dissolved in 50 mM phosphate buffer (pH 7.0). The crude enzyme (50 μl) was added to 3 ml of the substrate solution, and a decrease in A_{570} was determined (rate assay). The enzyme activity was calculated from the molar absorption coefficient for PADAC (57,000). The protein concentration was determined by the method of Waddell (9), measuring a difference between A_{215} and A_{225}. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of substrate for 1 min.

The β-lactamase activity of *Enterobacter cloacae* GN7471 in the agar medium around the colony was determined as follows. A fresh overnight culture of the strain in heart infusion broth was diluted with the same broth, and the inoculum was applied onto heart infusion agar, in the same manner as described above, and incubated at 37°C. At 5, 8, 11, and 24 h after the start of incubation, three agar cubes (3 by 3 by 2.5 mm) were cut off so that the distances from the marginal end of the colony to the centers of the cubes were 1.5, 4.5, and 7.5 mm. Each agar cube was allowed to react with 3 ml of 50 μM PADAC solution in 50 mM phosphate buffer (pH 7.0) while the cube was shaken at 35°C for 30 min, and the enzyme activity in the reaction mixture was calculated from a decrease in absorbance (endpoint assay) by the above-mentioned spectrophotometric method. Fresh heart infusion agar was used as the control.

RESULTS

Formation of PADAC hydrolysis zone and time course in zone diameter. Figure 1 shows the PADAC hydrolysis zones formed after 18 h of incubation on the PADAC plates inoculated with seven standard β-lactamase-producing strains of gram-negative bacteria.

Figure 2 presents both the time courses in sizes of the PADAC hydrolysis zones (zone diameters) on the PADAC plates inoculated with *Escherichia coli* W3630(Rms212) (producing type IIIa β-lactamase), *E. cloacae* GN7471 (type Ia), and *Citrobacter freundii* GN7391 (type Ia) and the time courses in numbers of viable cells per inoculation spot. The zone diameters of these strains showed a lag phase for the first 3 to 8 h of incubation, although such a lag phase was not seen in the growth curve of any strain. Thereafter, the zone diameter of each strain increased in proportion to the incubation time. Even after 12 h, when cell growth entered a stationary phase, the zone diameter showed a linear increase until 18 h after the start of incubation. The slopes of the curves of the three strains were almost equal to one another.

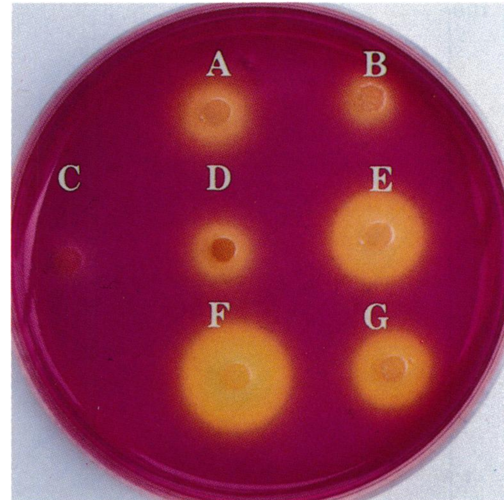

FIG. 1. Yellow zones showing hydrolysis of PADAC by β-lactamases (PADAC hydrolysis zones) formed around colonies of β-lactamase-producing strains after 18 h of incubation. (A) *Escherichia coli* W3630(Rms212) (producing β-lactamase type IIIa); (B) *Escherichia coli* W3630(Rms213) (type Va); (C) *Escherichia coli* W3630(Rte16) (type Vb); (D) *P. aeruginosa* M1(Rms139) (type PSE-1); (E) *E. cloacae* GN7471 (type Ia); (F) *S. marcescens* GN10857 (type Ia); (G) *C. freundii* GN346 (type Ia).

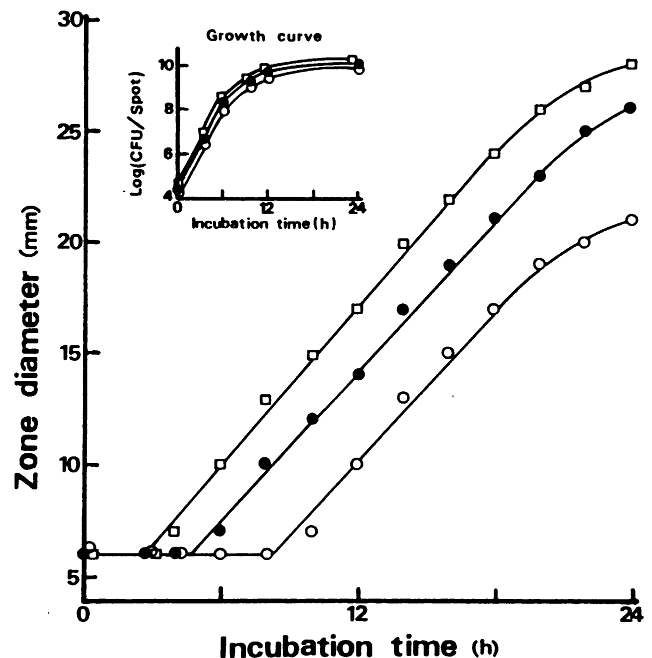

FIG. 2. Time courses in size of PADAC hydrolysis zone and viable cell count on PADAC plate inoculated with *Escherichia coli* W3630(Rms212), producing β-lactamase type IIIa (○), *E. cloacae* GN7471, producing type Ia (●), and *C. freundii* GN7391, producing type Ia (□).

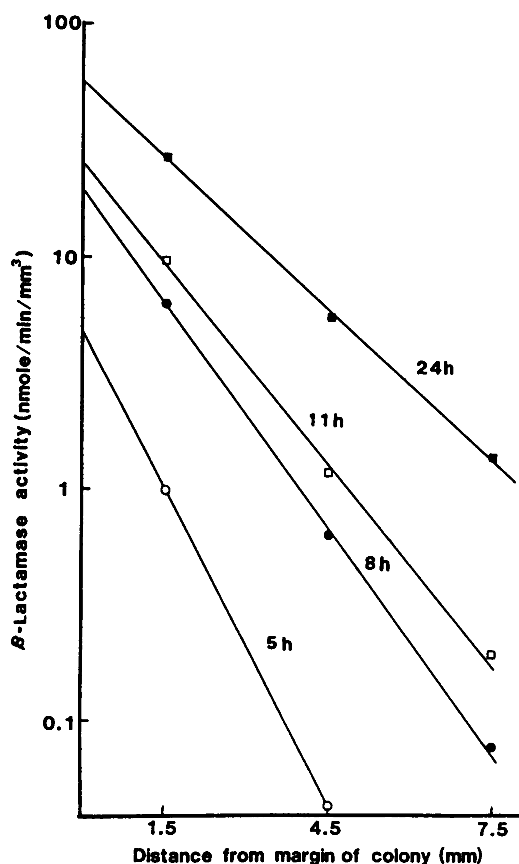

FIG. 3. Distribution of β-lactamase in agar medium (without PADAC) around the colony of *E. cloacae* GN7471 after 5 to 24 h of incubation.

As thus shown, the zone diameter changes with incubation time; therefore, for further studies, 18 h, at which time the hydrolysis zone size was large enough to be determined, was used as the incubation period.

β-Lactamase activity in agar medium around colony. The enzyme activity of *E. cloacae* GN7471 in the agar medium (without PADAC) around its colony was determined. Figure 3 shows the enzyme activity obtained as a function of the distance from the center of each agar cube to the colonial margin at various time points of incubation. The enzyme activity decreased exponentially as the distance from the colonial margin increased. Generally, the concentration of a diffusing material decreases exponentially as the distance from the center of the material increases. Therefore, this result suggested that the β-lactamase produced by the bacteria diffused according to a concentration gradient. The *y* intercepts increased with the incubation time, although the increases were not proportional to the time periods. This result suggests that the enzyme activity was increasing in the colony, the center of the cell growth.

Correlation between periplasmic β-lactamase activity and PADAC hydrolysis zone diameter. Table 1 shows the correlation between the periplasmic β-lactamase activity and the diameter of the PADAC hydrolysis zone after 18 h of incubation for 14 β-lactamase-producing, gram-negative strains whose enzyme types are known and for 10 clinically isolated, gram-negative species (13 to 37 isolates per species).

The 14 standard strains used were 4 penicillinase producers [*Escherichia coli* W3630(Rms212) (producing type IIIa), W3630(Rms213) (type Va), and W3630(Rte16) (type Vb) and *Pseudomonas aeruginosa* M1(Rms139) (PSE-1)] and 10 cephalosporinase producers (*E. cloacae* GN7471 and GN5797, *Citrobacter freundii* GN7391 and GN346, *Serratia marcescens* GN10857, *Morganella morganii* GN5407 [all type Ia], *Escherichia coli* GN5482 [type Ib], *P. aeruginosa* GN10362 [type Id], and *Proteus vulgaris* GN7919 and GN76 [both type Ic]). With these standard strains, the range of zone diameters was 6 to 24 mm, and the range of periplasmic enzyme activity was from 0.0017 to 0.597 U/mg of protein. The correlation coefficient, calculated by the least-squares method, was 0.974, and a significant, positive correlation was obtained between the logarithm of the periplasmic

TABLE 1. Correlation between diameter of PADAC hydrolysis zone and periplasmic β-lactamase activity

Organism(s)	No. of strains	Zone diam (mm)[a]	β-Lactamase activity (U/mg of protein)[b]	Result with regression equation[c]		Correlation coefficient (*r*)
				A	B	
Standard strains[d]	14	6–24	0.0017–0.597	0.324	8.23	0.974
Clinical isolates						
Escherichia coli	26	6–18	0.0005–0.316	0.270	8.16	0.810
Klebsiella pneumoniae	15	6–20	0.0010–0.337	0.363	7.98	0.947
Klebsiella oxytoca	13	6–21	0.0008–0.476	0.373	8.14	0.947
Enterobacter aerogenes	14	6–23	0.0019–0.514	0.271	7.25	0.954
Enterobacter cloacae	37	6–22	0.0004–0.850	0.384	9.21	0.974
Citrobacter freundii	18	6–22	0.0015–0.299	0.329	7.92	0.943
Serratia marcescens	27	9–21	0.0030–0.339	0.286	7.63	0.789
Morganella morganii	16	6–22	0.0008–1.430	0.449	9.71	0.974
Proteus vulgaris	16	6–20	0.0002–0.240	0.425	10.14	0.879
Pseudomonas aeruginosa	17	6–16	0.0007–0.118	0.365	9.43	0.777

[a] Diameter of PADAC hydrolysis zone, measured after 18 h of incubation.

[b] Periplasmic β-lactamase activity was determined by spectrophotometry with PADAC as the substrate.

[c] Regression equation: log Y (units per milligram of protein) = A × X (millimeters) − B, where Y is the periplasmic β-lactamase activity, A is the slope, X is the zone diameter, and B is the *y* intercept.

[d] Fourteen β-lactamase-producing strains with known enzyme types, including *Escherichia coli* W3630(Rms212) (type IIIa), W3630(Rms213) (type Va), W3630(Rte16) (type Vb), and GN5482 (type Ib), *P. aeruginosa* M1(Rms139) (type PSE-1) and GN10362 (type Id), *E. cloacae* GN7471 and GN5797 (type Ia), *C. freundii* GM7391 and GN346 (type Ia), *S. marcescens* GN10857 (type Ia), *M. morganii* GN5407 (type Ia), and *Proteus vulgaris* GN7919 and GN76 (type Ic).

TABLE 2. Inhibitory effect of clavulanic acid on PADAC hydrolysis zone

Organism	Type of enzyme[a]	Diam of PADAC hydrolysis zone (mm) with the following concn of clavulanic acid (μg/ml):					
		0	0.5	1	2	5	10
Escherichia coli W3630(Rms212)	IIIa	16	7	6	6	6	6
Escherichia coli W3630(Rms213)	Va	11	10	8	7	7	7
Escherichia coli W3630(Rte16)	Vb	8	6	6	6	6	6
Pseudomonas aeruginosa M1(Rms139)	PSE-1	17	15	15	13	12	12
Escherichia coli GN5482	Ib	17	17	17	17	17	17
Enterobacter cloacae GN7471	Ia	21	21	21	21	21	21
Citrobacter freundii GN7391	Ia	24	24	24	24	24	24
Citrobacter freundii GN346	Ia	19	19	19	19	19	20
Serratia marcescens GN10857	Ia	22	22	22	22	22	22
Proteus vulgaris GN7919	Ic	21	13	11	10	9	8

[a] According to the classification of Richmond and Sykes (7), except PSE-1 of *P. aeruginosa* M1(Rms139), which was classified by Inoue (1).

enzyme activity and the zone diameter. The correlation was not dependent on the type of enzyme produced.

With the clinical isolates, a good correlation was obtained for each species, showing correlation coefficients of 0.777 to 0.974. Among the species examined, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Klebsiella oxytoca*, *E. cloacae*, *C. freundii*, and *M. morganii* showed especially good correlations. In the data for *E. cloacae* and *Proteus vulgaris*, a fair amount of scatter was noted, especially at small zone diameters, where the β-lactamase activities varied by an order of magnitude. However, the correlation coefficients were 0.88 or more, and the enzyme activities of *E. cloacae* and *Proteus vulgaris* at a zone diameter of 6 mm were 0.0006 to 0.0012 and 0.0005 to 0.0014 U/mg of protein, respectively, with 95% confidence limits. *S. marcescens* strains (all having zone diameters greater than 6 mm) and *P. aeruginosa* included a few isolates whose enzyme activities deviated from the theoretical values calculated from the regression equation. The β-lactamase activities of the 10 species at zone diameters of 6 and 20 mm, calculated from the regression equation, were 0.0005 to 0.0036 and 0.0633 to 0.507 U/mg of protein, respectively, thus varying in ranges by nearly an order of magnitude.

Inhibition of formation of PADAC hydrolysis zone by clavulanic acid. Table 2 shows the effects of clavulanic acid (0.5 to 10 μg/ml), a penicillinase inhibitor, on the diameters of PADAC hydrolysis zones obtained with 10 standard β-lactamase-producing strains. The formation of the PADAC hydrolysis zones on the plates inoculated with type IIIa and Vb penicillinase-producing strains [*Escherichia coli* W3630(Rms212) and W3630(Rte16)] was inhibited by 0.5 μg of clavulanic acid per ml. The inhibitory effects of clavulanic acid on type Va penicillinase [*Escherichia coli* W3630 (Rms213)] were not as marked as those on the two types mentioned above. The *P. aeruginosa* strain [M1(Rms139)] producing type PSE-1 penicillinase was affected in a manner dependent on the dose of clavulanic acid. Among the six cephalosporinase-producing strains, the type Ic producer (*Proteus vulgaris* GN7919) was affected by clavulanic acid, but the other strains producing type Ia or Ib were not affected.

The effects of clavulanic acid on the formation of PADAC hydrolysis zones were also examined with clinical isolates found to produce β-lactamase (isolates which showed PADAC hydrolysis zone diameters greater than 6 mm). The dose of clavulanic acid used was 2 μg/ml, which inhibited types IIIa, Va, Vb, and Ic severely and type PSE-1 moderately. Inhibition of the formation of the PADAC hydrolysis zone by clavulanic acid was observed with 15 of the 19 *Escherichia coli* isolates, all 9 *K. pneumoniae* isolates, 7 *K. oxytoca* isolates, and 1 each of the 10 *Enterobacter aerogenes*, 11 *C. freundii*, and 7 *M. morganii* isolates; the PADAC plates of these strains showed no formation of a PADAC hydrolysis zone, thus suggesting that the enzyme activities disappeared. Of the 27 *S. marcescens* isolates, 12 isolates were affected; however, none of the 12 isolates showed complete loss of enzyme activity, and all retained zone diameters of about 9 mm. For *P. aeruginosa*, 6 of the 16 isolates showed inhibited formation of the PADAC hydrolysis zone. None of the 28 *E. cloacae* isolates were affected.

DISCUSSION

This β-lactamase assay method using PADAC-containing agar plates permitted the estimation of enzyme activity from the diameter of a PADAC hydrolysis zone formed around the colony of β-lactamase-producing bacteria on PADAC plates. As the results show, the diameters of the PADAC hydrolysis zones of all the gram-negative strains examined were significantly correlated with their periplasmic β-lactamase activities determined spectrophotometrically. The assay results by this method are less precise than those by spectrophotometric determination, but they are semiquantitative in that the enzyme activity is estimated from the zone diameter. This method is therefore considered to be superior to conventional qualitative methods to detect β-lactamase with chromogenic cephalosporins.

When the time courses of PADAC hydrolysis zone size were examined, the continued increases in zone size were noted with the three strains used, even after cell growth entered the stationary phase. PADAC has no β-lactamase inducer activity (unpublished data), and therefore, the following two explanations are proposed to account for this result: (i) diffusion of β-lactamase was slow and did not keep up with the bacterial growth rate, and (ii) cells were lysed at the stationary phase, releasing periplasmic β-lactamase into the agar. We suppose that both phenomena could have occurred.

β-Lactamase activity was noted in the agar medium (without PADAC) around the colony of a β-lactamase-producing strain (*E. cloacae* GN7471), and the pericolonial enzyme activity, determined spectrophotometrically, decreased exponentially with increasing distance from the colonial margin. In addition, when *E. cloacae* GN7471 was incubated on agar without PADAC, β-lactamase activity in the agar was detected in a fraction having a molecular weight of 20,000 or more and obtained by ultrafiltration (data not shown). These results suggest that (i) β-lactamase is released

from bacteria extracellularly and (ii) the PADAC hydrolysis zone is formed by diffusion of the extracellular enzyme into the agar. The production of extracellular β-lactamase is not considered to be due to any effect of PADAC or bacteriolysis, because the extracellular enzyme activity was noted in the agar without PADAC and during a logarithmically increasing period of bacteria. It is therefore reasonable to think that extracellular β-lactamase is produced under usual incubation conditions.

Between the diameter of the PADAC hydrolysis zone and the periplasmic β-lactamase activity, a good correlation was obtained with each of the strains used that produce known types of enzyme. Accordingly, the β-lactamase activity of a strain with an unknown enzyme activity can be estimated by using as the standard curve a regression line prepared from the data with β-lactamase-producing strains with known enzyme activities. In this case, the standard curve should be prepared for each species, because the regression line varies with the species. This variation among the species used seems to result from differences in growth rates of bacteria and in extracellular enzyme release rates. There is also some variation among strains, and it should be noted that readings from the standard curve are not always consistent with spectrophotometrically obtained values.

Control strains recommended for preparation of the standard regression line are those having wide ranges of β-lactamase activities and PADAC hydrolysis zone sizes; strains showing a greatly different growth or extracellular enzyme-releasing rate should not be included. The use of more than 10 strains is desirable. It is not necessary to identify the enzyme type of each strain, because a good correlation can be obtained even if the strains produce different types of enzymes. For determination of periplasmic enzyme activity, PADAC must be used as the substrate; otherwise, the enzyme activity does not correlate with the zone diameter.

PADAC, used as the substrate, is not a chromogenic cephalosporin selectively hydrolyzed by penicillinase or cephalosporinase. For a test strain producing both penicillinase and cephalosporinase, the PADAC hydrolysis zone is formed by reactions with both enzymes. To solve this problem, we prepared PADAC plates to which clavulanic acid, a selective penicillinase inhibitor, was added, and we observed the formation of the PADAC hydrolysis zone on plates inoculated with bacterial strains having known types of β-lactamase. The addition of clavulanic acid (2 μg/ml) to the plates inhibited type IIIa penicillinase strongly but did not inhibit the chromosomally mediated type Ia or Ib cephalosporinase.

When tested similarly with clinical isolates, inhibition of PADAC hydrolysis zone formation was noted in some of the isolates examined. Among them, the *Escherichia coli* and *S. marcescens* isolates are considered to produce penicillinase, because their PADAC hydrolysis zones were inhibited by clavulanic acid. The *S. marcescens* isolates were affected by clavulanic acid, but the degree of inhibition was not as severe as that for *Escherichia coli*. There is a possibility, therefore, that the *S. marcescens* isolates produce chromosomally mediated cephalosporinase as well as penicillinase. Many of *S. marcescens* strains thus produce multiple β-lactamases. Some types of penicillinase, including SHV-1, K1, and IIIa, are inhibited strongly by clavulanic acid, but type IIIa is the most common one seen in clinically isolated bacteria. Accordingly, the use of clavulanic acid in PADAC plates would make it possible to distinguish most penicillinase producers from strains producing only cephalosporinase (type Ia or Ib), especially the penicillinase producers that do not produce a large quantity of cephalosporinase simultaneously.

The assay with PADAC plates has several problems. (i) Its use is limited to gram-negative bacteria because of the antibacterial activity of PADAC against gram-positive bacteria. (ii) A standard regression line should be prepared for each species, which makes the assay tedious, requiring us to identify each test organism. (iii) Certain factors affect the increase of the PADAC hydrolysis zone. For example, mutants whose growth rates differ greatly from that of the parent cannot be compared by this assay, and the different extracellular β-lactamase-releasing rates also affect the correlation between the zone diameter and periplasmic enzyme activity.

In addition, although it is not a disadvantage, the incubation period for inoculated PADAC plates must be carefully set, since the PADAC hydrolysis zone size increases with time. For the present examination, 18 h, at which time the zone size showed a linear increase and became large enough to be determined, was selected as the incubation period. In the present results, the marginal end of the PADAC hydrolysis zone was not as clearly defined as that of the inhibition zone formed by an antibiotic, but this problem would be solved by using a higher concentration of PADAC (e.g., 100 μM instead of the 50 μM used in the present study). PADAC, having little antibacterial activity against gram-negative bacteria (2), has no effect on bacterial growth even at 100 μM, and the use of PADAC at this dose would give a more clearly defined hydrolysis zone, leading to more precise measurement of zone diameters.

The advantage of this assay with PADAC plates is its simplicity; that is, this assay enables us to determine the β-lactamase activities of β-lactamase-producing gram-negative bacteria semiquantitatively, simply by measuring the diameter of the PADAC hydrolysis zone formed around the colony of a test strain on the plate. It would be highly practical and useful, especially in screening large numbers of isolates and clones for production of β-lactamase. Such tests are performed, for example, by spreading mutagen-treated bacteria over the PADAC plate. There is no need to overcoat the colony with a reaction mixture or soft agar, which conventional methods require; this will make it easy to pick up the grown colony. Furthermore, the method can be used for screening β-lactamase inhibitors and for estimating their inhibitory activities. The availability of the PADAC plate would be increased by using selective β-lactamase inhibitors to classify enzyme types and by using enzyme inducers to estimate the inducibility.

We now plan to estimate the β-lactamase activities of bacteria with PADAC plates containing various concentrations of antibiotics. This would give us information about the enzyme induction in and release from bacteria during susceptibility tests.

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