Convenient Test for Screening Metallo- β -Lactamase-Producing Gram-Negative Bacteria by Using Thiol Compounds

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A simple disk diffusion test was constructed for detection of IMP-1-type metallo- β -lactamase-producing gram-negative bacteria. Two Kirby-Bauer disks containing ceftazidime (CAZ) and a filter disk containing a metallo- β -lactamase inhibitor were used in this test. Several IMP-1 inhibitors such as thiol compounds including 2-mercaptopropionic acid, heavy metal salts, and EDTA were evaluated for this test. Two CAZ disks were placed on a Mueller-Hinton agar plate on which a bacterial suspension was spread according to the method recommended by the National Committee for Clinical Laboratory Standards. The distance between the disks was kept to about 4 to 5 cm, and a filter disk containing a metallo- β -lactamase inhibitor was placed near one of the CAZ disks within a center-to-center distance of 1.0 to 2.5 cm. For IMP-1-producing strains, the growth-inhibitory zone between the two disks expanded, while no evident change in the shape of the growth-inhibitory zone was observed for CAZ-resistant strains producing serine β -lactamases such as AmpC or SHV-12. As a result, 2 to 3 μ l of undiluted 2-mercaptopropionic acid or mercaptoacetic acid able to block IMP-1 activity gave the most reproducible and clearest results, and CAZ-resistant strains producing AmpC or extended-spectrum β -lactamases were distinguishable from IMP-1 producers by this test. A similar observation was made with IMP-1-producing clinical isolates such as *Serratia marcescens*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Acinetobacter* spp., and *Alcaligenes xylosoxidans*. The specificity and sensitivity of this test were comparable to those of PCR analysis using *bla*_{IMP}-specific primers. Therefore, this convenient test would be valuable for daily use in clinical laboratories.

Carbapenem-resistant gram-negative bacterial species such as *Serratia marcescens* and *Pseudomonas aeruginosa* have emerged in Japan, and these isolates usually produce IMP-1 metallo- β -lactamase (7, 9, 13, 15, 17). The *bla*_{IMP} genes responsible for the IMP-1 production are usually mediated by integrons carried by transferable large plasmids (1). About 4.4% of *S. marcescens* strains and 1.3% of *P. aeruginosa* strains have already acquired IMP-1 productivity in Japan (manuscript in preparation), and transmissions of the *bla*_{IMP} gene cassette have been observed among various gram-negative rods (18). Since IMP-1 producers tend to demonstrate a wide range of resistance to various broad-spectrum β -lactams including the oxymino cephalosporins, cephamycins, and carbapenems, early recognition of IMP-1 producers is very important for rigorous infection control (3). The worldwide spread of this kind of organism is becoming a general concern, since several metallo- β -lactamase-producing gram-negative bacteria have recently been reported outside Japan (5, 11, 19). Indeed, PCR analyses usually give reliable and satisfactory results (18), but this method is of limited practical use for daily application in clinical laboratories because of the cost. Thus, the development of a simple and inexpensive testing method for screening of IMP-1 producers has become necessary.

MATERIALS AND METHODS

Bacterial strains. Clinically isolated ceftazidime (CAZ)-resistant (MIC, >64 μ g/ml) gram-negative bacterial strains were used in the test. Several of these

isolates were later found to carry the *bla*_{IMP} gene by PCR. A well-characterized extended-spectrum β -lactamase (ESBL) (SHV-12) producer and AmpC hyperproducers were also used as the control strains. A list of the bacterial strains tested in this study is shown in Table 1.

Evaluation of metallo- β -lactamase inhibitors. CuCl₂, FeCl₂, EDTA, and thiol compounds including mercaptoacetic acid, 2-mercaptopropionic acid, and mercaptoethanol were used and evaluated for IMP-1 inhibition, because these agents

TABLE 1. Strains used in this study

Strain	β -Lactamase produced	Source or reference
<i>Serratia marcescens</i> MKDM17	IMP-1	17
<i>Serratia marcescens</i> HKY414	AmpC (hyperproduction)	This study
<i>Klebsiella pneumoniae</i> MKD115	IMP-1	17
<i>Klebsiella pneumoniae</i> HKY402	SHV-12	This study
<i>Pseudomonas aeruginosa</i> MKAM12	IMP-1	17
<i>Pseudomonas aeruginosa</i> Pa9	AmpC (hyperproduction)	This study
<i>Pseudomonas putida</i> MSGD1	IMP-1	17
<i>Acinetobacter</i> sp.	IMP-1	This study
<i>Alcaligenes xylosoxidans</i> MNG10131	IMP-1	17
<i>Enterobacter cloacae</i>	IMP-1	This study
<i>Enterobacter aerogenes</i>	IMP-1	This study
<i>Citrobacter freundii</i>	IMP-1	This study
<i>Escherichia coli</i>	IMP-1	This study
<i>Proteus vulgaris</i>	IMP-1	This study

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FIG. 1. Inhibitory effects of 2-mercaptopropionic acid (2-MPA) on IMP-1 producers and non-IMP-1 producers. Three CAZ-resistant strains belonging to the gram-negative bacterial species *P. aeruginosa*, *S. marcescens*, and *K. pneumoniae* and producing IMP-1 metallo- β -lactamase or serine- β -lactamases (SHV-12 or AmpC) were tested. For each IMP-1 producer, a distinct growth-inhibitory zone appeared between the KB disk containing CAZ and the filter disk containing 2-MPA (left column). No change is evident around the two KB disks containing CAZ with or without 2-MPA for each serine β -lactamase producer (right column).

have been reported to block metallo- β -lactamase (2, 6, 12, 16). A colony of each bacterial strain was suspended and diluted with Mueller-Hinton (MH) broth to 10^6 CFU/ml and spread on an MH agar plate with a cotton swab according to the protocol recommended by the National Committee for Clinical Laboratory Standards (14). Two commercially supplied Kirby-Bauer (KB) disks, each containing 30 μ g of CAZ (Eiken Co. Ltd., Tokyo, Japan), were then placed on the plates. The distance between the two CAZ disks was kept at about 4 to 5 cm, and a filter disk was placed near one of the CAZ disks within a center-to-center distance of 1.0 to 2.5 cm. Two to five microliters of each inhibitor solution was added to the filter disk on the agar, and each agar plate was incubated at 37°C overnight. The concentration and amount of each inhibitor solution added to the filter disk were as follows: for CuCl_2 , 100 mM (5 μ l); for FeCl_2 , 100 mM (5 μ l); for EDTA, 100 mM (5 μ l); and for thiol compounds, an undiluted solution (2 to 3 μ l).

PCR analysis. CAZ-resistant strains used in this study were tested by PCR analysis to confirm the presence of the *bla*_{IMP} gene according to the method of Senda et al. (18) by using a new set of PCR primers (5'-ACCGCAGCAGAGTCTTTGCC-3' and 5'-ACAACCAGTTTGCCTTACC-3').

RESULTS AND DISCUSSION

Among the metallo- β -lactamase inhibitors used in this study, 2-mercaptopropionic acid gave the clearest results, be-

FIG. 2. Appearance of growth-inhibitory zone in IMP-1-producing strains by use of CAZ and 2-mercaptopropionic acid (2-MPA). Various levels of growth inhibition were observed in the IMP-1-producing gram-negative bacterial species tested. Marked growth inhibitions were observed in *Acinetobacter* sp., *Alcaligenes xylosoxidans*, *Enterobacter aerogenes*, *E. coli*, *Proteus vulgaris*, and *Pseudomonas putida*, whereas weak and ambiguous growth inhibitions were observed in *C. freundii* and *E. cloacae*.

FIG. 3. (A) Inhibitory effects of FeCl_2 on IMP-1 producers and non-IMP-1 producers. A slight expansion of growth-inhibitory zones between two disks was observed for all three IMP-1 producers (arrowheads). No change in the shape of the growth-inhibitory zone was evident for any serine- β -lactamase producer. (B) Inhibitory effects of EDTA on IMP-1 producers. Growth-inhibitory zones between two disks appeared for all three IMP-1 producers (arrowheads) when 5 μl of 500 mM EDTA solution was added to the filter.

cause this chemical agent blocked IMP-1 activity very strongly even at a low concentration (6). Mercaptoacetic acid also gave a clear result, but its inhibitory effect was slightly weaker than that of 2-mercaptopropionic acid. By using 2-mercaptopropionic acid, apparent growth-inhibitory zones were observed with all IMP-1-producing strains tested, including *S. marcescens* MKDM17, *Klebsiella pneumoniae* MKD115, and *P. aeruginosa* MKAM12, while no distinct change in the appearance of the growth-inhibitory zone was observed for CAZ-

resistant strains producing AmpC or SHV-12 (Fig. 1). Of 3,222 *S. marcescens* isolates and 2,533 *P. aeruginosa* isolates, 141 and 88 isolates carrying *bla*_{IMP}, respectively, demonstrated an expansion of the growth-inhibitory zone by 2-mercaptoacetic acid, and *Escherichia coli* strains producing Toho-1 or MEN-1 that show resistance to cefotaxime were distinguishable from IMP-1 producers by this method (data not shown). The other IMP-1 producers belonging to the gram-negative bacterial species also showed results similar to those observed with IMP-1-producing *S. marcescens* and *P. aeruginosa* strains, as shown in Fig. 2. However, relatively weak and ambiguous growth-inhibitory zones appeared for IMP-1-producing *Citrobacter freundii* and *Enterobacter cloacae*, even when two disks containing CAZ and 2-mercaptoacetic acid, respectively, were placed as close together as 1 cm (from center to center) (Fig. 2). This may be due to the hyperproduction of AmpC and/or to a change in membrane permeability in these bacteria. Further study is needed to improve the method for these strains, though IMP-1-producing strains of *C. freundii* and *E. cloacae* are still very rare.

Heavy metal salts such as CuCl_2 and FeCl_2 usually formed ring-shaped areas of precipitation around the filter disk and demonstrated their own bactericidal activity, while the growth-inhibitory zone expanded to the disk containing CAZ, as shown in Fig. 3A. The inhibitory effects of both heavy metal salts were similar, but the results were ambiguous in several strains. HgCl_2 itself has rather strong bactericidal activity and

yielded better results than CuCl_2 and FeCl_2 in the preliminary tests. However, the use of Hg^{2+} salt is not recommended from the viewpoint of human health and environmental conservation.

EDTA also created a growth-inhibitory zone between the two disks, but its appearance and reproducibility were relatively poor in several strains, even when a thick EDTA solution (500 mM) was added to the filter disk (Fig. 3B).

CAZ seemed to be the most suitable substrate for this test, because IMP-1 producers usually demonstrated high-level resistance to CAZ (MIC, $>64 \mu\text{g/ml}$) in our previous study (17, 18), and a marked inhibitory effect of thiol compounds was usually observed, as shown in Fig. 1 and 2. Indeed, any kind of broad-spectrum β -lactam disk can be used in this test, but IMP-1 producers usually demonstrate various levels of resistance to imipenem (IPM) (MIC, 4 to $>128 \mu\text{g/ml}$). However, the inhibitory effect of thiol compounds tends to be ambiguous, especially in strains that demonstrate reduced susceptibility to IPM (MIC, 4 to $8 \mu\text{g/ml}$) when the KB disk (IPM) is used (data not shown).

The emergence of gram-negative bacterial species with acquired resistance to various broad-spectrum β -lactams is becoming a worldwide clinical problem. Strains producing TEM- or SHV-derived ESBLs (4, 10) usually demonstrate high-level resistance to broad-spectrum oxyimino β -lactams such as CAZ and cefotaxime. Moreover, several *K. pneumoniae* strains that showed resistance to cephamycins as well as oxyimino cephalosporins were also found to produce AmpC-type β -lactamases such as MOX-1 (8). In Japan, furthermore, the emergence of carbapenem-resistant gram-negative bacterial strains in species such as *S. marcescens* or *P. aeruginosa* is becoming a clinical threat. Some of these isolates produce IMP-1 metallo- β -lactamase, and these strains tend to demonstrate a wide range of resistance to various broad-spectrum cephalosporins, cephamycins, and carbapenems (7, 9, 15, 17). Recently, gram-negative bacterial strains that were speculated to produce metallo- β -lactamases very similar to IMP-1 were also isolated in the United Kingdom, Italy, and Singapore (5, 11, 19). Thus, there is a need to develop a simple and specific method to distinguish IMP-1 producers from other bacteria showing a similar antibiotic resistance profile through the production of AmpC, ESBLs, or Toho-1-type β -lactamases. Indeed, PCR analysis usually gives satisfactory results in the detection of IMP-1 producers (7, 17), but it is not suitable for daily testing in clinical laboratories due to the cost. Therefore, the method described in this study is very helpful for screening IMP-1-producing strains in daily clinical laboratory testing.

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