2.2. Susceptibility testing and screening for ESBL-producing strains

Minimum inhibitory concentrations (MICs) of various β-lactam antibiotics were determined by the agar-dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines [11]. Isolates were suspected to be ESBL producers when they were not susceptible to either cefotaxime, ceftazidime or ceftriaxone (MICs of either of these antibiotics were more than 8 μg ml^{-1}). The suspected strains were stocked for further evaluation. For these strains, a double-disk test using Kirby-Bauer disks containing cefotaxime, ceftazidime, or ceftriaxone and amoxicillin/clavulanic acid was performed to confirm the production of ESBL [12].

2.3. PCR

To determine the genotype of ESBL, PCR was performed by the following method using the TEM-, SHV- and Toho-1-specific primers (Table 1). Each strain was suspended in 500 μl of distilled water at the concentration of MacFarland 0.5 and boiled at 100°C for 10 min. After centrifugation, 10 μl of the supernatant was subjected to PCR reaction as a template. PCR amplification and visualization of the amplified product were performed as described elsewhere [13]. In order to distinguish SHV-derived ESBLs from non-ESBLs such as SHV-1 and LEN-1 [14], transconjugants which had transferred resistance to ceftazidime from the original strains were subjected to PCR. For strains whose transconjugants were hard to obtain, PCR products were digested with *Nhe*I to give two fragments, 756 bp and 295 bp in size, in most SHV-derived ESBLs [15]. PCR reactions for each strain were repeated at least three times.

2.4. Nucleotide sequencing of PCR products

When a PCR reaction using TEM- or SHV-specific primers yielded products of the same apparent size as positive control, some of them were subjected to direct sequencing by the dye-terminator method by using the Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) with the ABI PRISM® 377 XL DNA Sequencing System (Applied Biosystems, Foster City, CA, USA). In addition to the primers used in the PCR reaction, we used several primers for nucleotide sequencing (Table 1). To determine the nucleotide sequence of both ends of the PCR product, we used the TA cloning kit (Invitrogen, NV, Leek, The Netherlands) according to the manufacturer's recommendation. Nucleotide sequences and predicted amino acid sequences were analyzed with FASTA at the National Institute of Genetics in Japan.

3. Results and discussion

Twenty-eight of 16 805 *E. coli* isolates and 41 of 9794 *K. pneumoniae* isolates were resistant to cefotaxime, ceftazidime or ceftriaxone (MIC ≥ 8 μg ml^{-1}). Among them, 15 *E. coli* and 34 *K. pneumoniae* were judged to be genuine ESBL producers by a double-disk test. Forty-four strains were isolated from inpatients, and five from outpatients. Most of 49 strains were recovered mainly from urine (24 strains) or sputum samples (19 strains). When we began this survey in January 1997, there were no standardized criteria for the screening of ESBLs in *K. pneumoniae* and *E. coli* as described in the latest NCCLS guidelines. It is probable that more ESBL producers would have been detected if we had used the NCCLS criteria of lower breakpoints. Relatively wide and frequent use of cephamycins and carbapenems in Japan might also contribute to the low isolation rate of ESBLs.

The genotypes of ESBLs determined by PCR were shown in Table 2. Of 15 *E. coli* ESBL producers, 12 (80%) were Toho-1-type, one was TEM-derived and two were SHV-derived. On the other hand, 12 Toho-1-type and 22 SHV-derived ESBL producers were found in *K. pneumoniae*. One *K. pneumoniae* strain R131 yielded positive PCR results with both TEM- and SHV-specific primers, and nucleotide sequence analysis revealed that

Table 1
Primers used in this study for PCR and nucleotide sequencing

PCR primers	Sequencing primers
TEM-specific	TEM
T1; 5′-CCGTGTCGCCCTTATTCC-3′	T1, T2 and T3; 5′-CAGTCACAGAAAAGCAT-3′
T2; 5′-AGGCACCTATCTCAGCGA-3′	T4; 5′-CTGGCGAACTACTTACTC-3′
	T5; 5′-TAGGCGGAGGTAGGTCAG-3′
SHV-specific	T6; 5′-TACGAAAAGACACTGACC-3′
S1;5′- ATTTGTCGCTTCTTTACTCGC-3′	
S2; 5′-TTTATGGCGTTACCTTTGACC-3′	SHV
	S1, S2, and S3; 5′-GCGGGTGGATGCCGGTG-3′
Toho-1-type	S4; 5′-CGGCGGGCTGGTTTATCG-3′
To1; 5′-ACGCTACCCCTGCTATTT-3′	S5; 5′-TAAATCACCACAATGCCC-3′
To2; 5′-CCTTTCCGCCTTCTGCTC-3′	S6; 5′-GTCGGCAAGGTGTTTTTC-3′

Table 2
Types of ESBLs detected among *E. coli* and *K. pneumoniae* clinical isolates

	Types of ESBLs		
	Toho-1-type	TEM-derived	SHV-derived
E. coli	12 (6)	1 (1)	2 (2)
K. pneumoniae	12 (6)	0	22 (5)

Number of isolates. Numbers of hospitals and clinics where ESBL producers were isolated are indicated in parentheses.

this strain produced TEM-1 and SHV-2 [16]. Notably, 13 of 22 SHV-derived ESBL producers of *K. pneumoniae* were isolated in one hospital. More than 90% of *K. pneumoniae* isolates in this hospital were ESBL producers during the survey period. A detailed epidemiologic study is underway, and judicious usage of antibiotics is recommended in this hospital.

All the TEM- or SHV-specific PCR products of *E. coli* strains HKY560, HKY600 and HKY741 were subjected to direct nucleotide sequencing. In *K. pneumoniae*, we analyzed nucleotide sequences of SHV-specific PCR products yielded from nine strains: HKY472, HKY496, HKY502, HKY514, HKY525, HKY534, HKY615, HKY730, and R131, which were representative strains of the five different hospitals where SHV-derived ESBLs were isolated. Susceptibility profiles for various β-lactam antibiotics of these strains and the type of the ESBL genes were shown in Table 3. The only TEM-specific PCR product of *E. coli* strain HKY560 was partially identical to the sequence of the gene that encoded TEM-26 reported previously [17]. The susceptibility profile of the strain HKY560 for various β-lactam antibiotics was also suggestive of a TEM-26 producer (Table 3). Surprisingly, the nucleotide sequence analysis of the SHV-specific PCR products of two *E. coli* and eight *K. pneumoniae* strains revealed that all encoded SHV-12 [18], although some had silent point mutations in the coding region. It appears that SHV-12 is the dominant SHV-derived ESBL in Japan so far. Strains HKY496, HKY502 and HKY615 showed resistance to cephamycins (Table 3). This suggested another resistance mechanism such as β-lactamase production distinct from ESBLs, or deficiency of outer membrane porin [19]. Further evaluation of these strains is planned.

Although this survey was not nationwide and might not correctly reflect the actual distribution of ESBLs in Japan, these results have some important epidemiological implications. As the previous sporadic reports indicated, Toho-1-type ESBLs were the most prevalent type in this survey. So far as we know, this is the first report in Japan to identify TEM- and SHV-derived ESBLs including TEM-26, SHV-2 and SHV-12 among *E. coli* and *K. pneumoniae*. In particular, SHV-12 was identified at a relatively high frequency. These results clearly indicate that distinct types of ESBLs, which are different in both their substrate specificity and genetic origin, co-exist in Japan. Under these circumstances, we emphasize the importance of characterizing distinct genotypes of ESBLs and to be continuously alert to the trends of ESBLs in Japan at a multi-institutional level. In addition, uniform interpretation is required in reporting ESBL-producing strains. Consequently, we have adopted the NCCLS guidelines. However, some of the Toho-1-type ESBL-producing *K. pneumoniae* strains in this survey showed MICs of ceftazidime below or equal to 1 μg ml^{-1} (data not shown). The clinical implications of ESBL-producing bacteria, including whether or not these Toho-1-type ESBL-producing *K. pneumoniae* strains are actually resistant to ceftazidime, should be cautiously evaluated in the clinical settings. We believe that, in addition to the detection of ESBLs, more information is needed, such as the specific susceptibility profile for various β-lactams and the site of infection. These factors are essential for the proper choice of antibiotics.

Table 3
MICs of representative strains for various β-lactam antibiotics

Strains	K or E[a]	Genotype of ESBL	MIC (μg ml^{-1})[b]									
			ABPC	PIPC	CER	CTX	CAZ	AZT	S/C	CMZ	LMOX	IPM
HKY560	E	TEM-26	≧64	≧64	≧64	16	≧64	≧64	2	2	≦1	≦1
HKY600	E	SHV-12	≧64	≧64	≧64	16	≧64	≧64	8	2	≦1	≦1
HKY741	E	SHV-12	≧64	≧64	≧64	32	64	≧64	4	2	≦1	≦1
HKY472	K	SHV-12	≧64	≧64	≧64	≧64	≧64	≧64	≧64	≧64	≧64	2
HKY496	K	SHV-12	≧64	≧64	≧64	32	≧64	≧64	8	≧64	8	≦1
HKY502	K	SHV-12	≧64	≧64	≧64	8	≧64	≧64	8	≧64	8	≦1
HKY615	K	SHV-12	≧64	≧64	≧64	32	≧64	≧64	8	≧64	4	≦1
HKY514	K	SHV-12	≧64	≧64	≧64	32	≧64	≧64	8	2	≦1	≦1
HKY525	K	SHV-12	≧64	≧64	64	8	≧64	≧64	32	≦1	≦1	≦1
HKY534	K	SHV-12	≧64	≧64	≧64	16	≧64	≧64	32	2	≦1	≦1
HKY730	K	SHV-12	≧64	≧64	≧64	16	≧64	≧64	8	2	≦1	≦1
R131	K	SHV-2	≧64	≧64	≧64	≧64	32	8	8	≦1	≦1	≦1

[a]K, *Klebsiella pneumoniae*: E, *Escherichia coli*.
[b]ABPC, ampicillin; PIPC, piperacillin; CER, cephaloridine; CTX, cefotaxime; CAZ, ceftazidime; AZT, aztreonam; S/C, cefoperazone/sulbactam; CMZ, cefmetazole; LMOX, moxalactam; IPM, imipenem.

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Worldwide proliferation of carbapenem-resistant gram-negative bacteria

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Worldwide proliferation of carbapenem-resistant gram-negative bacteria

Sir—Imipenem was approved for clinical use in 1987 in Japan, followed by panipenem and meropenem in 1993 and 1995, respectively. These carbapenems have been used in clinical settings as a last resort for their broad-spectrum antibacterial activity and stability against various β-lactamases produced by gram-negative bacteria. However, carbapenem-resistant *Serratia marcescens* and *Pseudomonas aeeruginosa* emerged in Japan nearly 10 years ago.[1,2] These strains produce a novel metallo-β-lactamase (IMP-1) that is mediated by transferable R-plasmids.[3] IMP-1-producing gram-negative bacteria have been proliferating in Japan and have also been identified in European countries by G Cornaglia and colleagues (March 13, p 899)[4] and in Singapore by T H Koh and colleagues (June 19, p 2162).[5]

According to our 1996–97 survey of IMP-1-producing gram-negative bacteria in Japan, 34 (1·3%) of 2533 *Ps aeruginosa* and 144 (4·4%) of 3222 *Ser marcescens* isolates produced IMP-1 through acquisition of R-plasmids that carry bla_{IMP} gene. We also identified other IMP-1-producing gram-negative bacterial species, including *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Ent cloacae*, *Proteus vulgaris*, and *Providencia rettgeri*, as well as *Ps putida*, *Ps fluorescens*, *Burkholderia cepacia*, *Alcaligenes xylosoxidans*, and *Acinetobacter* spp. Conjugational transfer of wide host-range R-plasmids bearing the bla_{IMP} gene is the mechanism of the dissemination of bla_{IMP} gene cassette onto various gram-negative bacterial species.

Gram-positive cocci such as meticillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Ent faecium*, and vancomycin-resistant *Ent faecalis* have developed multiple antibiotic resistance, and penicillin-resistant *Streptococcus pneumoniae* is also developing resistance to newly developed macrolides and fluoroquinolones. These gram-positive cocci have been disseminated worldwide, causing life-threatening infections. Fortunately, carbapenems, fluoro-quinolones, and aminoglycosides are still effective against gram-negative bacteria. However, about 20% of *Ps aeruginosa* isolates have fluoro-quinolone resistance and nearly 5% also have amikacin resistance in Japan. International travel and transport give drug-resistant bacteria a free ride. Can we expect an explosive proliferation of IMP-1-producing gram-negative bacterial species, as seen with other resistant species? The spread of carbapenem-resistant gram-negative bacteria raises the spectre of a clinical Armageddon.

To cope with bacteria that have acquired multiple antibiotic resistance, we need operational surveys and monitoring of multiple-drug-resistant gram-negative bacterial species and gram-positive cocci by WHO and national health authorities, and guidelines for prudent use of broad-spectrum antimicrobial agents in each clinical setting.

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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 μl 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 oxyimino 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 μg/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. $CuCl_2$, $FeCl_2$, 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
Serratia marcescens MKDM17	IMP-1	17
Serratia marcescens HKY414	AmpC (hyperproduction)	This study
Klebsiella pneumoniae MKD115	IMP-1	17
Klebsiella pneumoniae HKY402	SHV-12	This study
Pseudomonas aeruginosa MKAM12	IMP-1	17
Pseudomonas aeruginosa Pa9	AmpC (hyperproduction)	This study
Pseudomonas putida MSGD1	IMP-1	17
Acinetobacter sp.	IMP-1	This study
Alcaligenes xylosoxidans MNG10131	IMP-1	17
Enterobacter cloacae	IMP-1	This study
Enterobacter aerogenes	IMP-1	This study
Citrobacter freundii	IMP-1	This study
Escherichia coli	IMP-1	This study
Proteus vulgaris	IMP-1	This study

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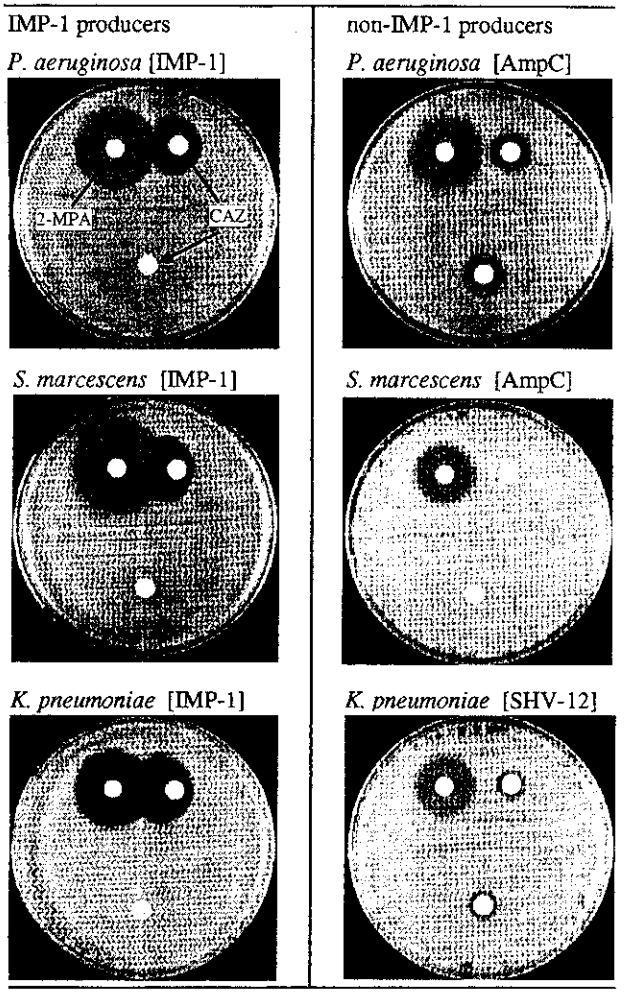


FIG. 1. Inhibitory effects of 2-mercaptopropionic acid (2-MPA) on IMP-1 producers and non-IMP-1 producers. Three CAZ-resistant strains belonging to the gram-negative bacterial species *P. aeruginosa*, *S. marcescens*, and *K. pneumoniae* and producing IMP-1 metallo-β-lactamase or serine-β-lactamases (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 μg 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 μl); for $FeCl_2$, 100 mM (5 μl); for EDTA, 100 mM (5 μl); and for thiol compounds, an undiluted solution (2 to 3 μl).

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′-ACCGCAGCAGAGT CTTTGCC-3′ and 5′-ACAACCAGTTTTGCCTTACC-3′).

RESULTS AND DISCUSSION

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

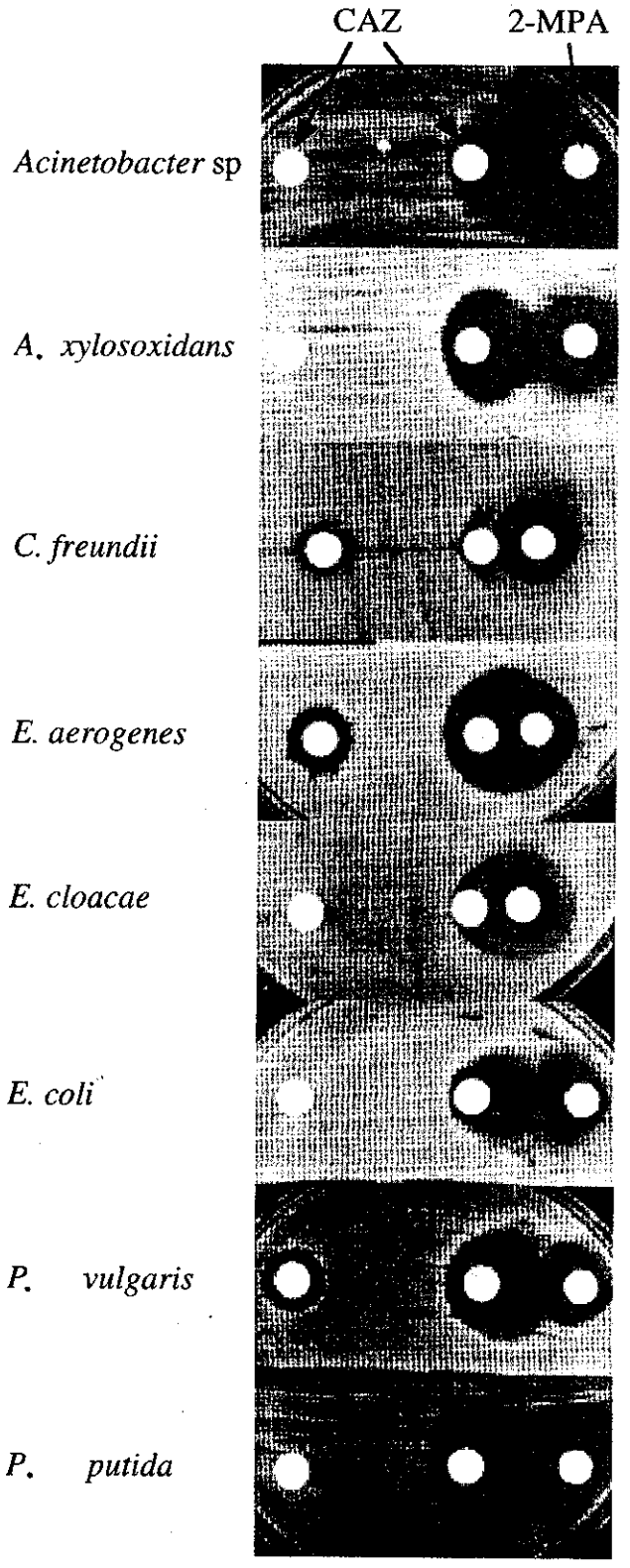
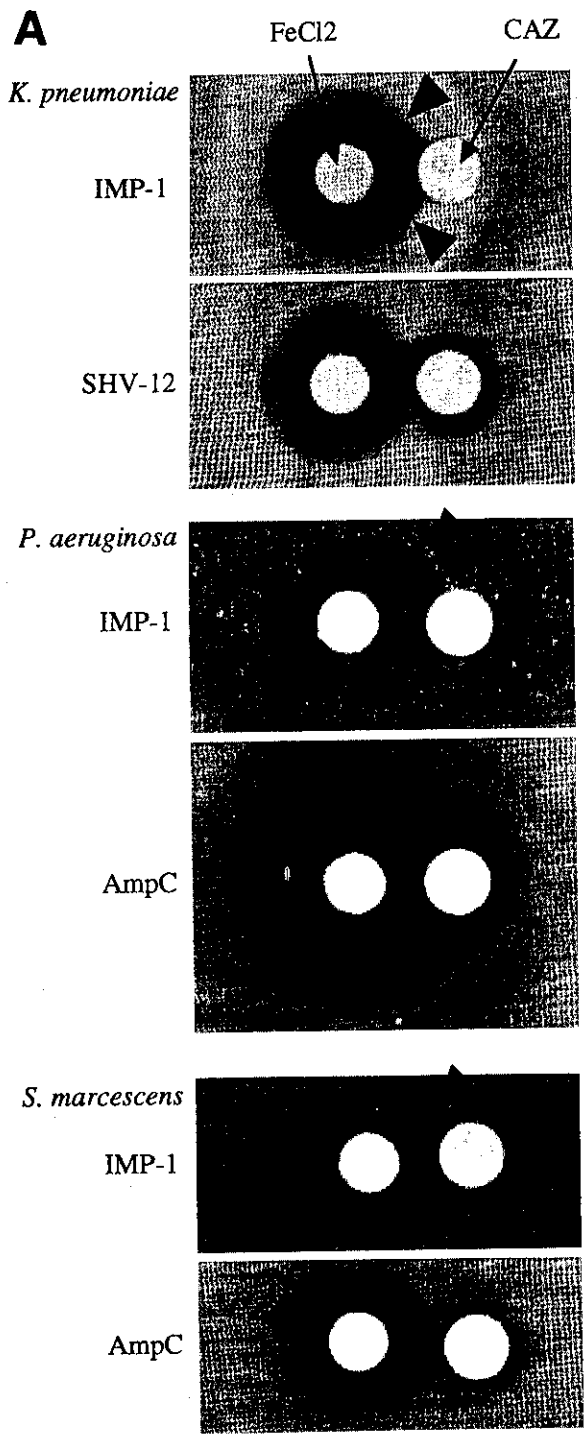


FIG. 2. Appearance of growth-inhibitory zone in IMP-1-producing strains by use of CAZ and 2-mercaptopropionic acid (2-MPA). Various levels of growth inhibition were observed in the IMP-1-producing gram-negative bacterial species tested. Marked growth inhibitions were observed in *Acinetobacter* sp., *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*.



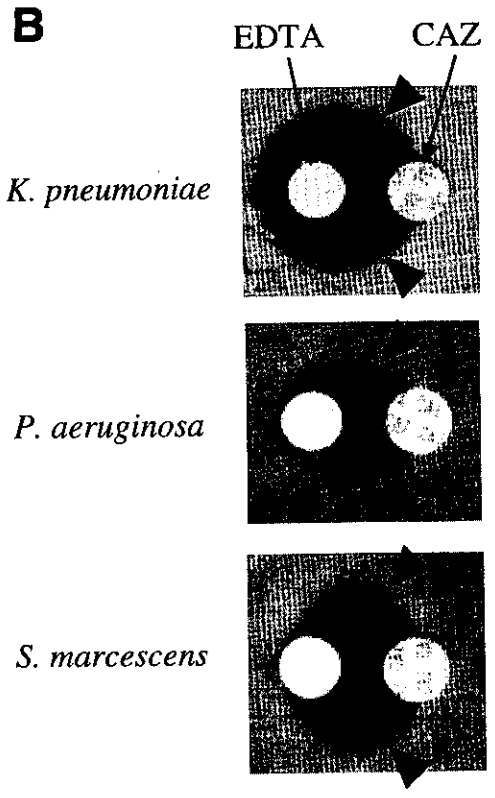


FIG. 3. (A) Inhibitory effects of $FeCl_2$ on IMP-1 producers and non–IMP-1 producers. A slight expansion of growth-inhibitory zones between two disks was observed for all three IMP-1 producers (arrowheads). No change in the shape of the growth-inhibitory zone was evident for any serine-β-lactamase producer. (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 μg/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 μg/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 μg/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.

ACKNOWLEDGMENT

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III. 遺伝子検査法の実際(耐性遺伝子の検出と型別)

4. メタロ-β-ラクタマーゼの検出法

柴田尚宏*1 荒川宜親*2

要旨

メタロ-β-ラクタマーゼは，ペニシリン，セフェム，カルバペネムやクラブラン酸，スルバクタムなどのβ-ラクタマーゼ阻害剤などβ-ラクタム環をもつほとんどすべての抗生物質を加水分解する性質をもつ強力な酵素である。わが国では，高度医療の発展に伴いメタロ-β-ラクタマーゼ産生臨床分離菌の増加が憂慮されており，その迅速検査法が求められている。従来のdisk拡散法，微量希釈法では，セファマイシン系薬に耐性を示す菌がメタロ-β-ラクタマーゼを産生する株なのか基質拡張型のAmpC型β-ラクタマーゼを産生する株なのか区別することは難しい。今回これらを区別することを中心に2-メルカプトプロピオン酸を使用したdisk拡散法とメタロ-β-ラクタマーゼ遺伝子特異的なプライマーを用いたPCR法を紹介する。

Summary

Metallo-β-lactamases have a hydrolysing activity against various β-lactams including carbapenems, and β-lactamase inhibitors such as clavulanic acid and sulbactum. Infections caused by metallo-β-lactamase producing gram-negative bacteria are becoming a serious problem in Japanese clinical settings despite the recent progress in medical technology. Current screening methods can not distinguish metallo-β-lactamase producers from those producing AmpC β-lactamase. Here we show a convenient method of detecting metallo-β-lactamase producers using a thiol compound (2-mercaptopropionic acid) as well as a PCR method with metallo-β-lactamase gene specific primers.

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Key words IMP-1型メタロ-β-ラクタマーゼ，2-メルカプトプロピオン酸，PCR法，disk拡散法，integron

はじめに

グラム陰性桿菌による感染症はAIDS患者や担癌患者，高齢者などや，最近さかんに行われつつある臓器移植などの際に障害となっている。近年とくにカルバペネム耐性菌の出現が問題となりつつある。

1991年，伝達性のカルバペネム耐性緑膿菌がWatanabeらによりはじめて報告されたが，当時とし

Gene examination methods (detection and genotyping of resistant genes) —Metallo-β-lactamase.

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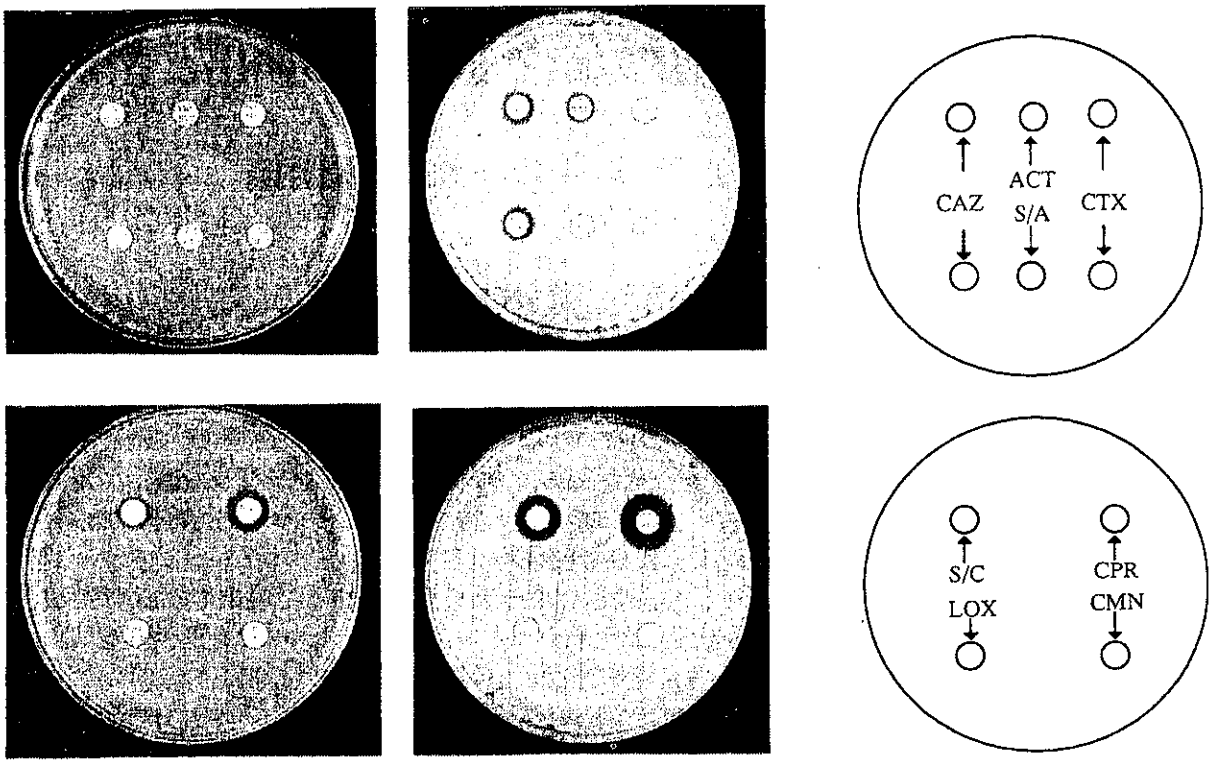


1. IMP-1 産生株(*P. aeruginosa*)　2. Amp-C 型 β-ラクタマーゼ産生株(*P. aeruginosa*)

図1　Double disk 法による 1. IMP-1 産生株と, 2. Amp-C 型 β-ラクタマーゼ産生株の表現型
この disk 法では両者は区別できない。

ては例外的な問題と考えられた[1]。

同年 Osano らにより愛知県の臨床分離 *Serratia marcescens* TN9106 株よりカルバペネムを含む広範囲に β-ラクタム剤を分解するメタロ-β-ラクタマーゼが発見され，IMP-1 と名付けられた[2]。この遺伝子は TN9106 株では染色体上にコードされていたが，Ito らの研究によって他の菌株ではプラスミド上にコードされて拡がっていることが明らかとなった[3]。さらに 1995 年に Arakawa らにより，カルバペネム耐性 *S. marcescens* AK9373 株から初めて IMP-1 型メタロ-β-ラクタマーゼ(*bla*$_{IMP}$)遺伝子を担う新しいインテグロン構造が発見され[4]，Senda らによりグラム陰性桿菌の間でそれが菌種を超えて拡散していることが明らかとなった[5]。また 1996 年 1 月から 1997 年 8 月の厚生省研究班の予備調査によると約 300 施設から臨床分離された *S. marcescens* 3,222 株中 141 株(4.4%)，*Pseudomonas aeruginosa* 2,533 株中 34 株(1.34%)で IMP-1 型メタロ-β-ラクタマーゼ遺伝子が検出された。

今後グラム陰性桿菌感染症において，IMP-1 型メタロ-β-ラクタマーゼ(*bla*$_{IMP}$)産生菌の増加が懸念され，その迅速診断法の確立が求められている。現在 PCR 法によって耐性遺伝子を検出することは可能である。しかし，PCR 法は一般病院の検査室ではコストその他の点で実施が難しい。また従来の disk 拡散法は安価で簡便ではあるが，メタロ-β-ラクタマーゼなどクラス B 群の β-ラクタマーゼと AmpC 型 β-ラクタマーゼを含むクラス C 群の β-ラクタマーゼとを鑑別することは困難な場合が多い(**図1**)。

そこで別の新しい安価で簡便な検査方法が必要となってきた。

I. メタロ-β-ラクタマーゼとは

メタロ-β-ラクタマーゼは 1966 年に *Bacillu cereus* で発見されたのが最初である[6]。また *Stenotrophomonas maltophilia* などでも報告されている[7]。現在までに約 20 種類が同定され[8]，Ambler の分子分類では，クラス B に分類，Bush らによる機能分類ではグループ 3 に分類されている[9][10]。

この酵素が他のクラスのセリン型 β-ラクタマーゼと全く異なるのは活性中心に亜鉛をもつことである。メタロ-β-ラクタマーゼはその活性中心の亜鉛

に配位結合した不安定状態の水分子が，β-ラクタム環を加水分解し開裂させる酵素で，カルバペネムを分解するほか，ほとんどすべてのペニシリン，セフェム系抗生物質を分解し，さらにスルバクタム，クラブラン酸といったβ-ラクタマーゼ阻害剤をも分解するという憂慮すべき性質を示す。

II. IMP-1型メタロ-β-ラクタマーゼ産生菌の種類と分離材料

菌種としては，腸内細菌科，ブドウ糖非発酵菌群が一般的である。

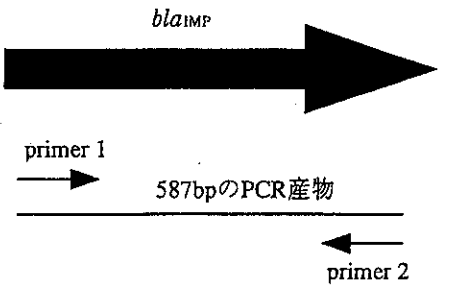
腸内細菌科では，*Serratia marcescens*，*Klebsiella pneumoniae*，*Escherichia coli*，*Providencia rettgeri* などが，ブドウ糖非発酵菌群では *Pseudomonas aeruginosa*，*Pseudomonas putida*，*Pseudomonas fluorescens*，*Alcaligenes* などがある。このうち分離頻度としては，*S. marcescens* と *P. aeruginosa* が多い。

これらグラム陰性桿菌は臨床的には，尿，呼吸器由来検体，膿・分泌液などから分離されることが多い。基礎疾患としては，悪性疾患が多く，外科手術後，抗癌剤投与，カテーテル留置の患者からしばしば分離され，日和見感染であることが多い。

III. PCR法によるメタロ-β-ラクタマーゼ産生菌の同定

IMP-1型メタロ-β-ラクタマーゼをコードする遺伝子を増幅して検出するのがPCR(polymerase chain reaction)法である。DNAの変性，アニーリング，Taqポリメラーゼ反応を繰り返すことにより，検体のDNA量が少量でも約2時間で約100万倍まで増幅が可能である。このPCR法は既に臨床の現場で広く取り入れられ，検出，培養の困難な，あるいは時間のかかる結核，非定型抗酸菌，マイコプラズマなどの診断に利用されている。

荒川らにより決定されたIMP-1型メタロ-β-ラクタマーゼをコードする遺伝子(*bla*IMP)の全塩基配列をもとにプライマーを作製した。著者らは図2のプライマーと反応条件にてPCR反応を行っている。反応条件はサーマルサイクラーの機種によって若干異なる。このPCR産物は587bpのサイズである。IMP-1型メタロ-β-ラクタマーゼ陽性菌と陰性菌の電気泳動写真を示す(図3)。ただし，メタロ-β-ラクタマーゼ産生菌がすべてPCR法で検出できるわけではなく，問題点がいくつか残っている。



primer 1	5'- ACCGCAGCAGAGTCTTTGCC -3'
primer 2	5' - ACAACCAGTTTTGCCTTACC -3'

滅菌蒸留水	30 μl
10×buffer	5 μl
dNTP	4 μl
primer 1	0.5 μl
primer 2	0.5 μl
Taq polimerase	0.25 μl
DNA template	10 μl

Total 50μl

(DNA templateの調整)
MacFarland 0.5の濃度にした菌液を100℃，10分間ボイルし，4℃，13,000rpmで5分間遠心する。
その上清を使用する。

1. denature 94℃ 2分 →
2. denature 94℃ 1分 →
   anealing 55℃ 1分 →
   extension 72℃ 1分半
   (30サイクル)
3. extension 70℃ 5分
4. 4℃

図2 IMP-1型メタロ-β-ラクタマーゼ遺伝子同定のプライマーとPCR反応条件，サーマルサイクラーの設定

第一に反応自体の問題がある。緑膿菌などの場合では原因不明の理由でPCR反応がうまくいかない例もしばしばみられる。著者らが普段行っているボイル法による粗抽出法の場合だとうまくPCR反応が進まないことがある。この場合には全DNAを定法により抽出してからPCR法を行えば成功することもあるが煩雑である。逆に非特異的PCR産物が生成し，電気泳動をした際目的のバンドが特定できないことがある。その場合には，アニーリング温度を少し上・下させてみると非特異的産物が減少する場合もある。

第二に検査にコストがかかることである。実際には1検体あたり2000円から3000円の実費がかかる。

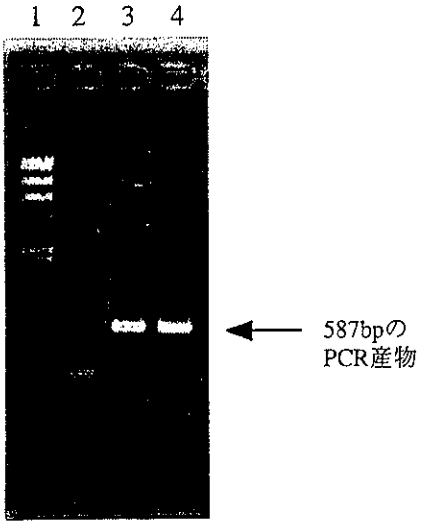


図3 PCRの結果のアガロース電気泳動写真
1. λ Hind III, **2.** negative control,
3, 4. metallo-β-lactamase 産生菌(*S. marcescens*)

一般の細菌検査室にすべてサーマルサイクラーが設置されているわけではなく，さらにある程度の熟練も要する。また同時に大量の検体を扱うのに適さない。そこでPCR法にかわる，安価で簡便な誰にでもできるスクリーニング法が必要となる。

IV. 2-メルカプトプロピオン酸を使用したDisk拡散法によるメタロ-β-ラクタマーゼ産生菌の同定

"安価に誰にでも"の答えの一つに最近われわれの開発した2-メルカプトプロピオン酸を使用したDisk拡散法がある[11]。以前よりメタロ-β-ラクタマーゼがメルカプト酢酸[12]やEDTAによって阻害されることが知られていた。今回メルカプト酢酸より2-メルカプトプロピオン酸の方がより強い阻害活性を示すことがGotoらにより発見されたため[13]，これを検出法に利用することを試みた。2-メルカプトプロピオン酸はメルカプト基-SHをもつチオール化合物の一種であり，揮発性の液体である。この検出法の概略図(図4)と2-メルカプトプロピオン酸の構造式を示す(図5)。この方法の原理は2-メルカプトプロピオン酸が培地中に拡散する時メルカプト基-SHがメタロ-β-ラクタマーゼの活性中心の亜鉛に結合し，酵素の加水分解を阻害し，その結果，菌の感受性が増し，隣接した抗生物質のdiskとの間に発育阻止帯が形成される。具体的にはNCCLSの方法に従い，菌液をMüllar-Hinton培地に塗り，次に図のご

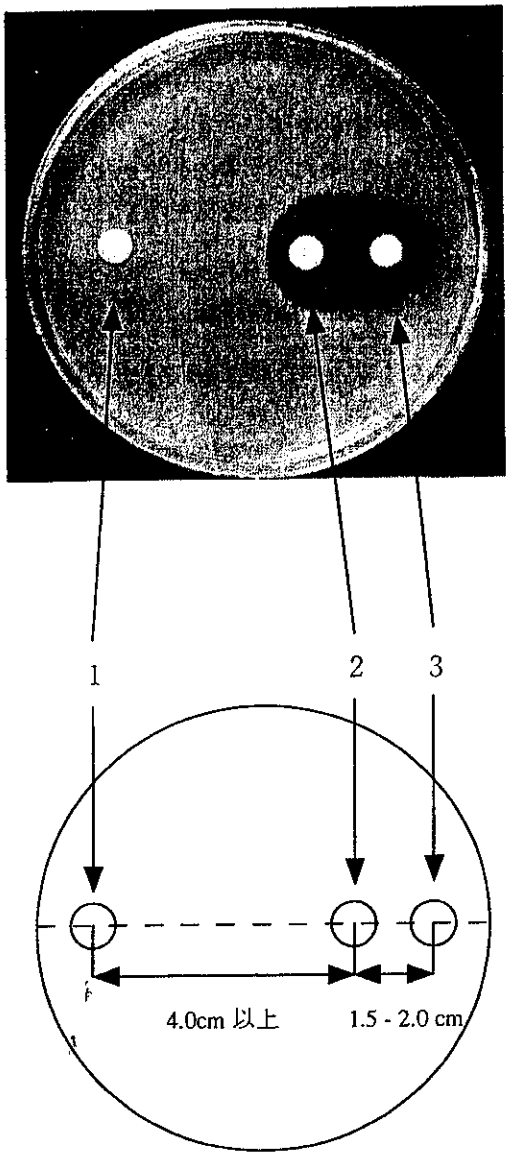


図4 Disk法の模式図
小円はdiskを表す。1，2はCAZのdisk，3はメルカプトプロピオン酸を添加したdisk。それぞれ図のようにMüllar-Hintonの平板培地に置く。

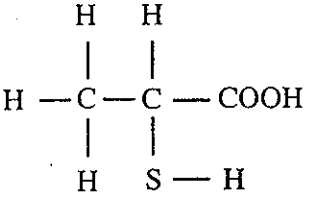
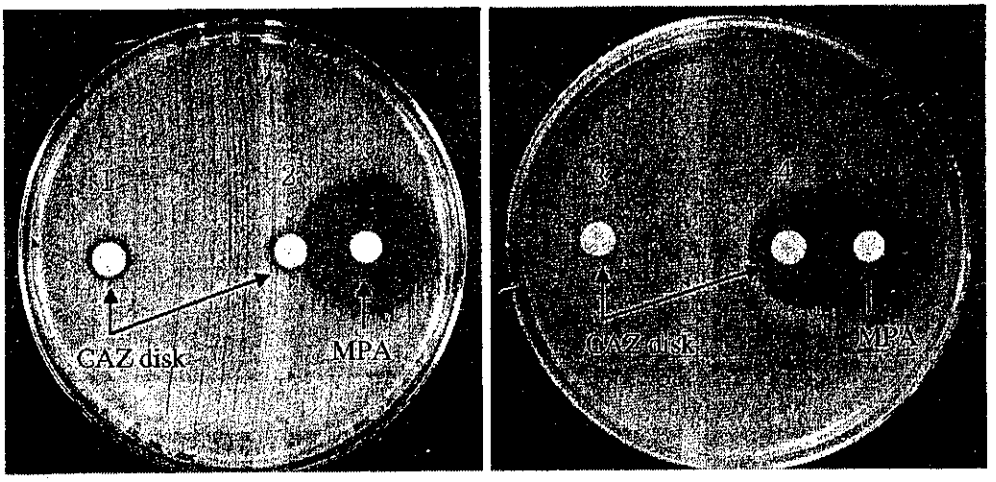


図5 2-メルカプトプロピオン酸の化学構造式
(2-mercaptopropionic acid)



A. メタロ-β-ラクタマーゼ非産生菌 (*Ps. aeruginosa*)　　B. メタロ-β-ラクタマーゼ産生菌 (*E. coli*)

図6　2-メルカプトプロピオン酸(MPA)を用いたdisk法によるメタロ-β-ラクタマーゼ非産生菌(左)と産生菌(右)のdisk拡散法による検出

A. メタロ-β-ラクタマーゼ非産生菌では，1と2の抗生物質のdiskの周囲の形状に変化が認められない。

B. メタロ-β-ラクタマーゼ産生菌では，阻害剤の影響により3の単独のdisk周囲の形状と比較し，4のdiskの周囲の形状のごとく大きさも形も変化する。

とく間隔をおいてCAZのdiskと空のdisk(または濾紙片)を置く。2-メルカプトプロピオン酸は揮発性の液体なので，培地上に置いた空のdiskにマイクロピペットを用いて2～3μl添加し，37℃に一夜静置する。メタロ-β-ラクタマーゼ非産生菌では2つのCAZのdiskの周囲の発育阻止帯の形状は同じであるが，メタロ-β-ラクタマーゼ産生菌では，阻害剤のdiskの近傍のCAZのdiskの周囲の発育阻止帯の形状と単独に置いたCAZのdiskの周囲の形状が異なるのが観察される(図6)。この方法は現在までのところほとんどのIMP-1型メタロ-β-ラクタマーゼ産生菌で有用であり(図7)，IMP-1型以外のメタロ-β-ラクタマーゼでも応用可能である。

ただし，拡張型AmpC型β-ラクタマーゼを同時に産生している株では，CAZ diskよりIPM diskの方がはっきりとした結果が得られる場合も多い。問題点としては，2-メルカプトプロピオン酸は揮発性であるために，毎回検査の時に添加しなければならないことが挙げられる。

以上クラスB，Cβ-ラクタマーゼ産生株の鑑別方法を述べた。クラスA，B，C β-ラクタマーゼ産生株の鑑別の流れを図8に示す。

V. 最近の国内と海外におけるメタロ-β-ラクタマーゼ産生菌の状況

国内で発見されたIMP-1型メタロ-β-ラクタマーゼ遺伝子(*bla*$_{IMP}$)は多くの場合伝達性の薬剤耐性プラスミド(R-plasmid)に存在しており，さらに数種類の遺伝子カセットが連なったインテグロン構造上に位置していることが明らかとなっている[4]。インテグロンとはインテグラーゼにより部位特異的に耐性遺伝子などの遺伝子カセットを集積する能力を持つ構造である(図9)。

このインテグロンに担われたIMP-1型メタロ-β-ラクタマーゼ遺伝子(*bla*$_{IMP}$)カセットはさらに染色体上とプラスミド上とを移動可能である。このIMP-1型メタロ-β-ラクタマーゼは最近までは日本でしか確認されておらず，Hirakataらは病院内でのIMP-1の存在を調査し長崎大学病院では経時的増加はみられなかったと報告している[14]。しかし，最近各地の医療施設から散発的に分離されており，また，近年諸外国でもカルバペネム耐性グラム陰性桿菌の報告がされはじめている。英国では，Woodfordらがメタロ-β-ラクタマーゼ産生緑膿菌を報告しており[15]，イタリアでもCornagliaらにより報告がある[16]。Kohらはシンガポールにおける*K. pneumoniae*で

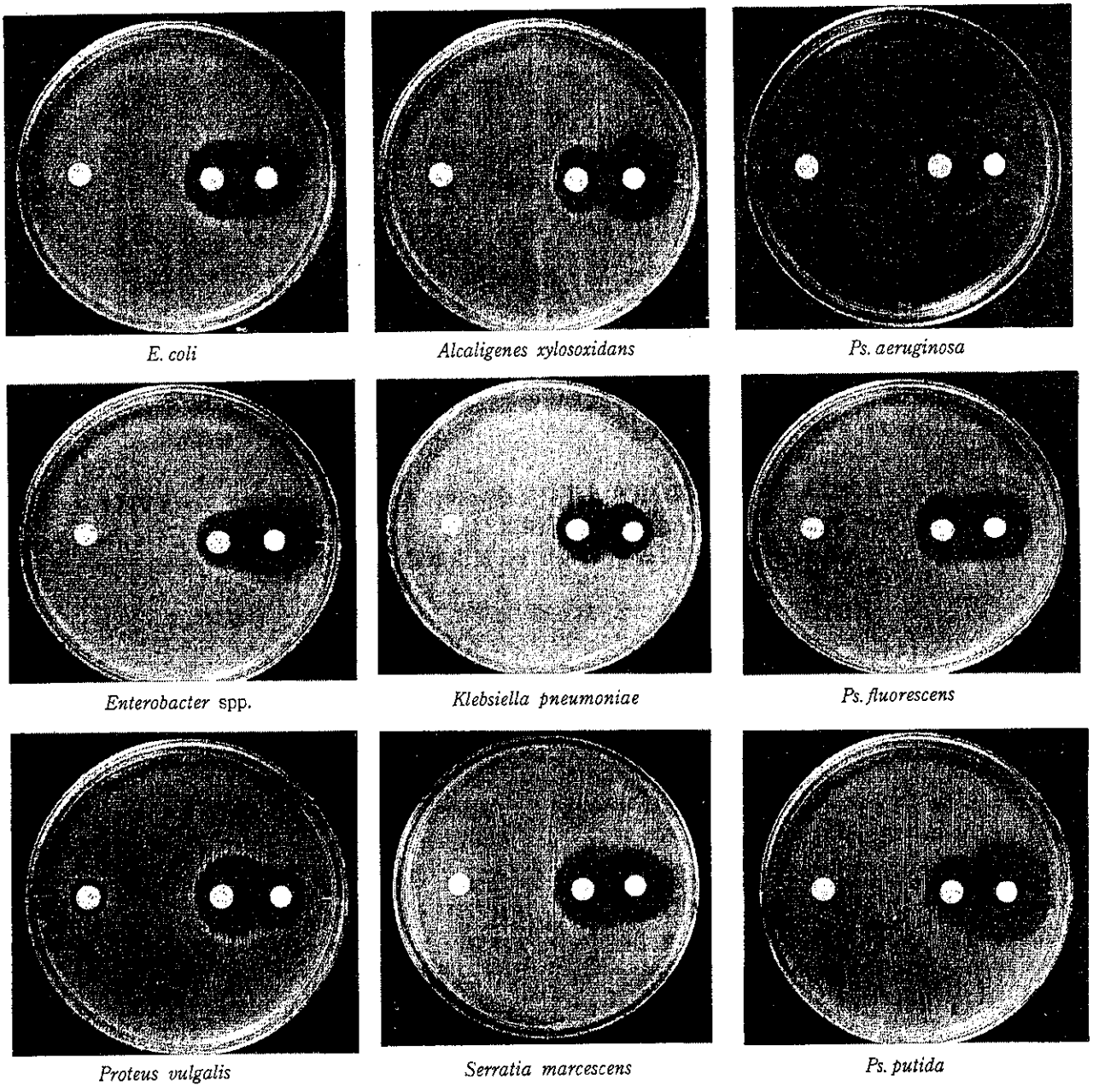


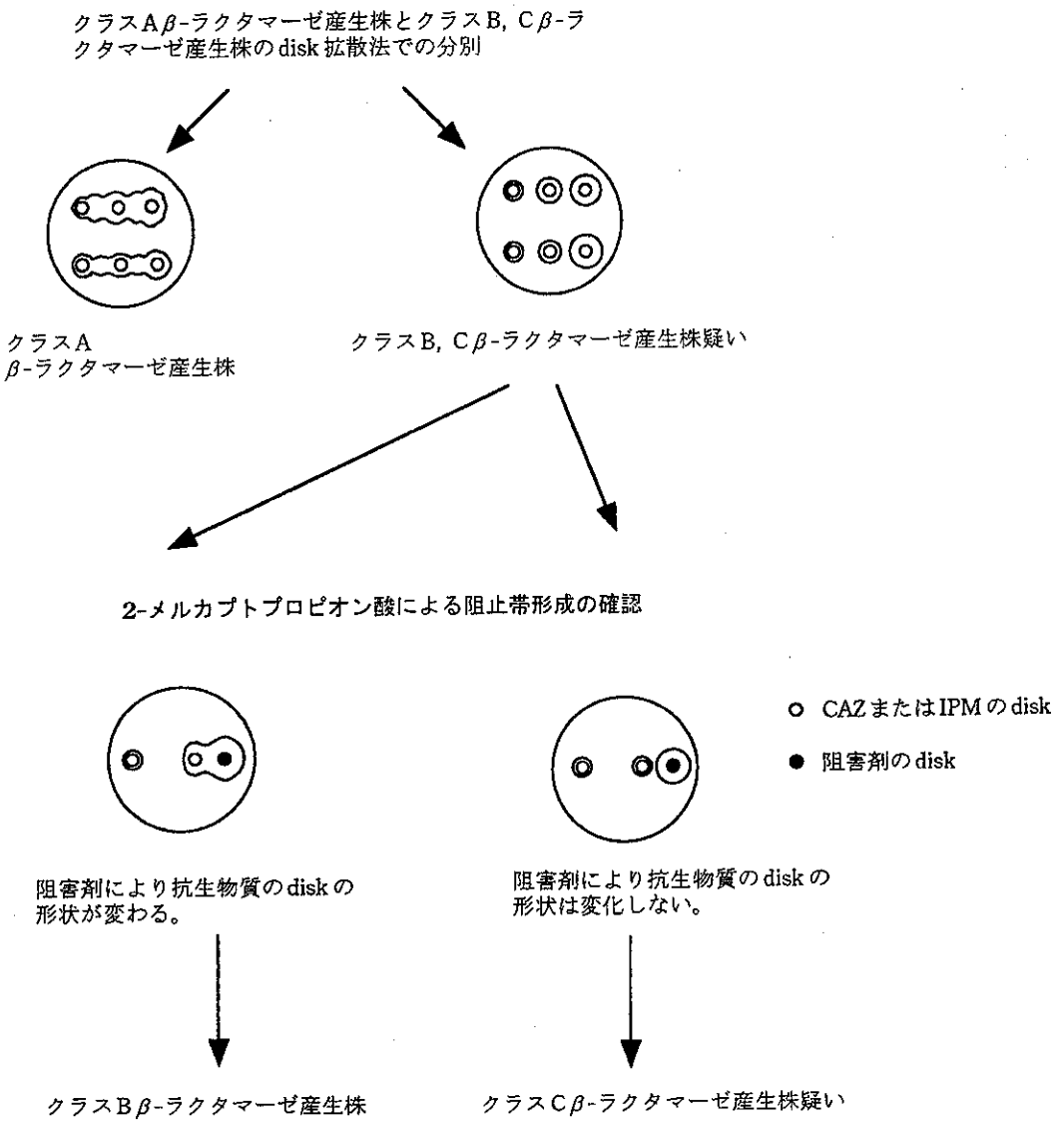
図7　各種グラム陰性桿菌における 2-メルカプトプロピオン酸による disk 拡散法の阻止帯の形成

IMP-1 型メタロ-β-ラクタマーゼ産生菌を報告している[17]。一方イタリアの Lauretti らは，最近インテグロンにやはり担われる新種のメタロ-β-ラクタマーゼ (VIM-1) を報告した[18]。こうした諸外国の状況と国内でのグラム陰性桿菌における IMP-1 遺伝子の検出状況を踏まえ，Kurokawa らは今後，メタロ-β-ラクタマーゼ産生グラム陰性桿菌の世界的な拡散を警告している[19]。

おわりに

今後，わが国では，患者の高齢化や癌，臓器移植における高度医療の推進などさまざまな問題が懸念されている。

このことは，増加が予想される免疫能低下患者，immunocompromised host を薬剤耐性菌の感染からいかに防御していけるかがますます重要な課題となっていることを示しており，その意味で迅速，正確かつ簡便な耐性菌の検出法の確立が必要となっている。



さらに確認するにはPCRを行う。

図8　クラスA，B，Cβ-ラクタマーゼ産生株の鑑別の流れ

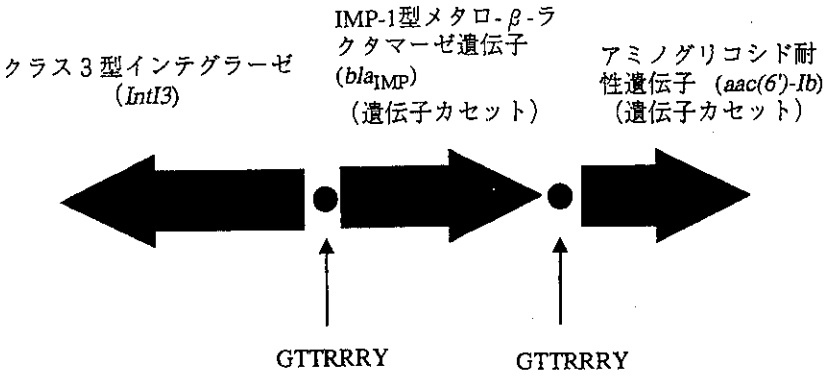


図9　IMP-1型メタロ-β-ラクタマーゼ遺伝子を担うクラス3型インテグロン構造の模式図

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特集 どうなる21世紀の化学療法
（第72回日本細菌学会総会シンポジウム「どうなる21世紀の化学療法」をもとに）

3. 薬剤耐性菌とその検出法に関わる基本的な諸注意点

荒川 宜親*

1980年代から，多種多様な薬剤耐性菌が臨床現場で蔓延しはじめ，洋の東西を問わず，大きな問題となっている。耐性菌か否かは薬剤感受性試験の結果に基づいて判定されるが，わが国での分離はいまだ稀である，バンコマイシン耐性腸球菌（VRE）や拡張型基質スペクトルβ-ラクタマーゼ（ESBL）産生グラム陰性桿菌，メタロ-β-ラクタマーゼ産生グラム陰性桿菌などの検出法は，十分に確立または普及しているとは言い難く，検出技術の一層の向上をはからねばならないものも少なくない。しかし，最近，国際的に大きな反響を巻き起こした「バンコマイシン低感受性MRSA（VISA）」，「バンコマイシンヘテロ耐性MRSA」，「バンコマイシンホモ耐性MRSA（VRSA）」などについては，その概念や定義，あるいはその生物学的本質がはっきりしておらず，また，1997年に国内で臨床分離されたMRSA 6,625株の中に，Mu50株に相当する「VRSA」は，確認されなかったことから，現時点でそれらを，日常的な細菌検査において，特殊な検査法や選択培地を用いて検出する必要性は少ないと考えられる。一方，Mu50株に相当するVCMの最小発育濃度（MIC）値が8 μg/mL以上の株や明確なバンコマイシン耐性遺伝子を保持するVRSAが，近い将来実際に出現することは十分想定され，もしそれらが出現した際には，現行の米国臨床検査標準化委員会（NCCLS）法によっても，分離・検出することは十分可能であると考えられる。

現状における臨床でのバンコマイシン（VCM）の使用状況を鑑みれば，今後とも，日常の細菌検査業務の中でグラム陽性菌におけるVCM耐性菌の出現には一層の注意を払う必要があることは云うまでもない。また，VCM耐性菌の出現を防止するためにも，VCMのみならずテイコプラニンを含むグライコペプタイド系抗菌薬の適正使用の一層の推進が強く望まれる。

Key Words: 薬剤耐性菌／薬剤感受性試験法

I はじめに

国内の医療機関で分離される黄色ブドウ球菌の過半がメチシリン耐性黄色ブドウ球菌（MRSA）と判定され、肺炎球菌においても，ペニシリン低感受性菌（PIPC）とペニシリン耐性菌（PRSP）を合わせると，半数以上を占める医療施設が多くなっている[1]。また，欧米で増加しているバンコマイシン耐性腸球菌（VRE）や拡張型基質スペクトルを示すβ-ラクタマーゼ（extended-spectrum β-lactamase：ESBL）を産生する肺炎桿菌や大腸菌もいまだ少数であるが，各地から報告されるようになった[2,3]。さらに，以前から各種の抗菌薬に耐性を示すことで臨床医を煩わせている緑膿菌も，カルバペネム薬，新しく開発されたアミノ配糖体薬，ニューキノロン薬など，「最後の命綱（last

Basic matters that demand special attention to the identification of drug-resistant bacteria.

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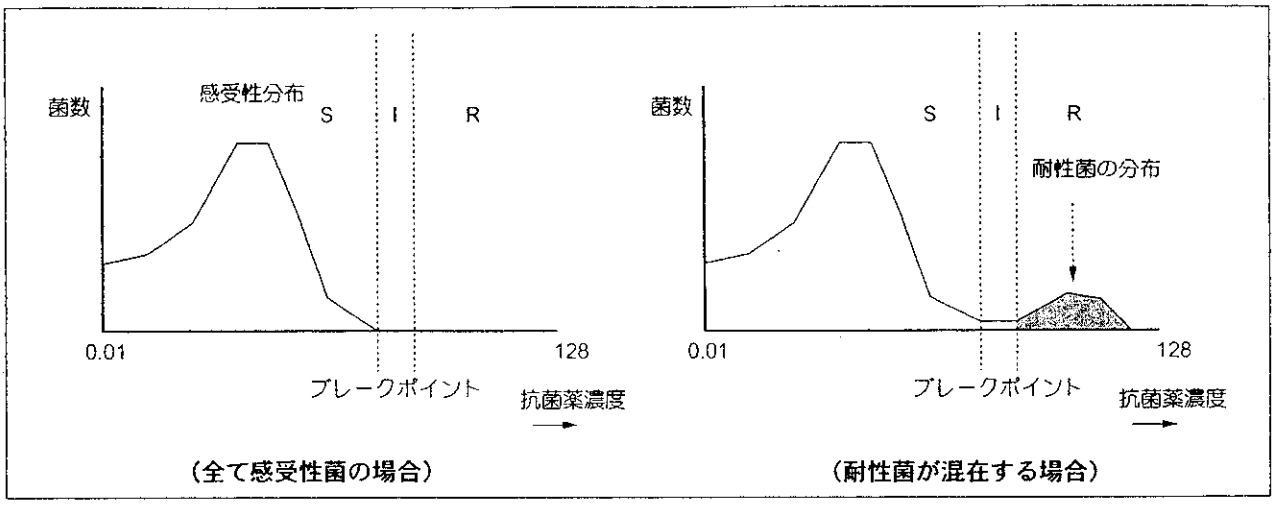

図1　臨床分離細菌に見られる一般的な感受性分布

臨床分離菌を数百株収集し，特定の抗菌薬に対する薬剤感受性を検査した場合，それらには感受性(S)，低度耐性(I)，耐性(R)などに判定される様々な性質の株が含まれ，感受性分布の表を作成することができる。
その際，感受性のみにしか存在しない場合には，左の図に示されたように(S)の領域にのみ分布が偏るが，耐性株が含まれる場合には右の図で示すように(S)と(R)の両方領域にまたがって二峰性の分布を示すことが多い。(R)の領域に分布する株は，薬剤に耐性を獲得するための遺伝子の突然変異や新たな耐性遺伝子を獲得している場合が多く，それらは安定な耐性度を示す。

resort)的抗菌薬」に耐性を獲得した臨床分離株が徐々に増加傾向にある[4]。

本稿では，これらの薬剤耐性菌の現状を踏まえ，薬剤耐性菌の検査法における初歩的および基本的な諸注意点を概括的に整理してみたい。

Ⅱ　「薬剤耐性菌」とは

あらためて述べるまでもないが，臨床分離菌のみならず，自然環境中に存在する細菌も，通常，何らかの抗菌薬や消毒薬などに耐性を示す場合が多い。したがって，ある意味では細菌はほぼ全て「薬剤耐性菌」ということになる。しかし，「薬剤耐性菌」が問題とされるのは，主に臨床現場において感染症治療を実施する際に化学療法に抵抗し，回復を遅らせたり，場合によっては患者を感染死させる場合である。細菌がどの程度の薬剤耐性レベルを獲得した場合，化学療法に抵抗するようになるかの境界を定めるのは，多くの因子を考慮しなければならず大変困難な作業であるが，一応の目安として「ブレークポイント」が設定されている。

Ⅲ　薬剤耐性菌の判定のための「ブレークポイント」

臨床分離される特定の菌種のある抗菌薬に対する感受性分布を調べた場合，感受性から耐性まで広く連続的に分布する場合が一般的であり，どの値を境界とするかは議論の分かれるところである。現在，感受性か耐性かを判定する目安として，「ブレークポイント」が日本化学療法学会や米国臨床検査標準化委員会(NCCLS)などにより設定されている[5]。この値は，血中または感染部位で維持できる抗菌薬濃度などを参考にして設定されているが，MRSAにおけるVCMや肺炎球菌におけるペニシリンなど，値の見直しが検討されているものもある。また，日本化学療法学会とNCCLSでブレークポイントの値が異なっているものや，NCCLSではブレークポイントが定められていない抗菌薬も少なくない。

Ⅳ　「薬剤耐性」のメカニズム

臨床分離菌を数百株集め，その薬剤感受性分布の表を作成した場合，感受性菌のみの場合はSの領域に分布するが，耐性菌が含まれる場合には，

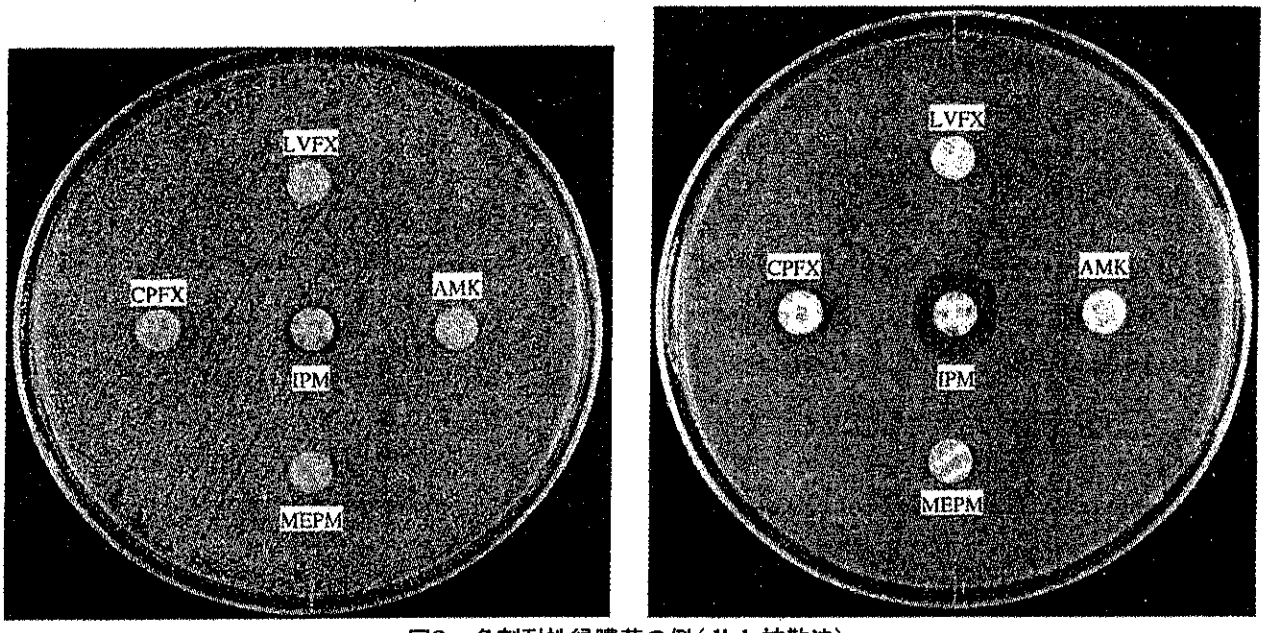

図2　多剤耐性緑膿菌の例(disk 拡散法)

左：IMP-1産生多剤耐性緑膿菌　IMP-1 型メタロ-β-ラクタマーゼを産生しカルバペネムに高度耐性を示す。その他，フルオロキノロン，アミノ配糖体にも広範な耐性を獲得している。

右：IMP-1非産生多剤耐性緑膿菌　IMP-1 型メタロ-β-ラクタマーゼは産生しないが，D2 ポリン蛋白の減少などによりカルバペネムに耐性を示すと考えられる。このような株では，IPM よりMEPM に耐性度が高くなる傾向が見られる。その他，本株はフルオロキノロン，アミノ配糖体にも広範な耐性を獲得している。

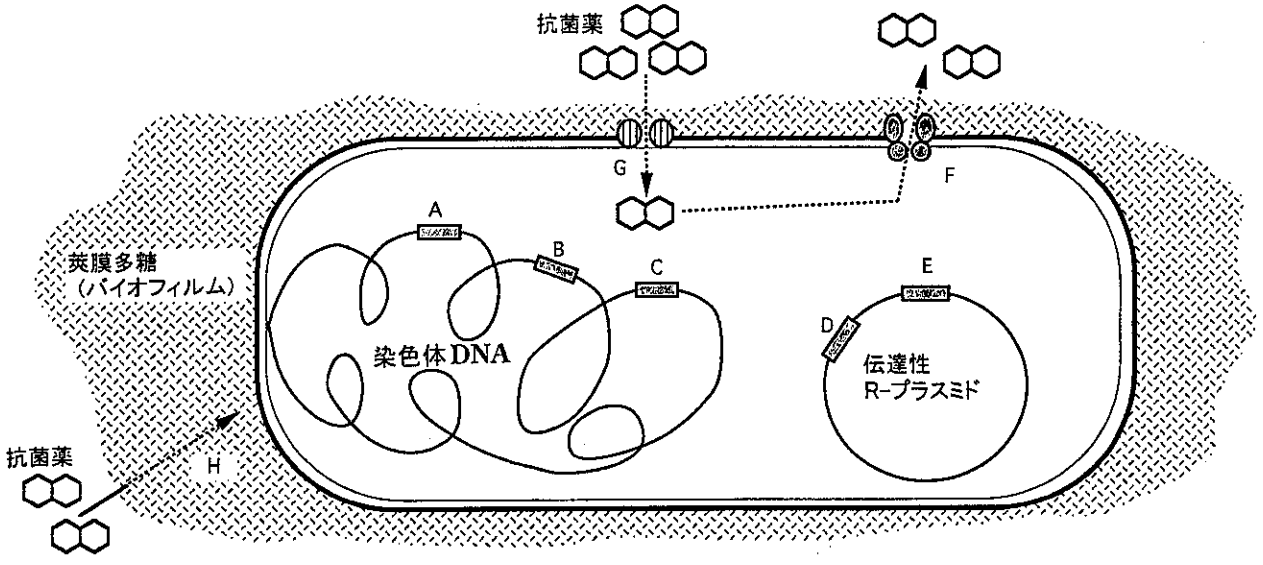

図3　緑膿菌における多剤耐性化に関与する主な因子

A：染色耐性の DNA ジャイレース遺伝子の変異による各種キノロン耐性の獲得，**B**：染色耐性のトポイソメラーゼ IV 遺伝子の変異による各種フルオロキノロン耐性の獲得，**C**：染色耐性の AmpC 型セファロスポリナーゼ遺伝子の過剰発現による各種セフェム薬耐性の獲得，**D**：プラスミド性のβ-ラクタマーゼ遺伝子による(IMP-1 メタロ-β-ラクタマーゼ遺伝子，オキサシリナーゼ遺伝子など)によるオキサシリン，セフェム，セファマイシン，カルバペネム耐性の獲得，**E**：プラスミド性のアミノグリコシド耐性遺伝子(*aac*, *aph* など)によるアミカシンなどのアミノ配糖体耐性の獲得，**F**：薬剤汲み出しポンプ(active efflux system)の活性化による抗菌薬や消毒薬などの菌体外への排出の促進，**G**：外膜ポリン蛋白の質的・量的変化による薬剤の菌体内への浸透の減少，**H**：バイオフィルム形成により，薬剤の菌体への到達率が低下。