に3つのディスクを1列に配置してある。ストリップ状の基体の形状や寸法には特に制限はない。このキットを使用する固体培地の大きさ等を考慮して適宜決定できる。尚、ストリップ状の基体は、阻止円の判読を容易にするため、透明性の高い素材で形成することもできる。各ディスクの間隔やβ－ラクタム薬のディスク中の含有量等は、上記本発明の方法において説明したと同様の点を考慮して適宜決定できる。尚、図中に記載してある薬剤名や寸法は例示として記載したものであり、本発明のキットはこれに限定されるものではない。また、本発明のキットを培地上に置いた後に、メタロ－β－ラクタマーゼ阻害剤を含有させるための１つのディスクに、メタロ－β－ラクタマーゼ阻害剤を添加することができるように、ストリップ状の基体のメタロ－β－ラクタマーゼ阻害剤を含有させるための１つのディスクに通じる部分に小孔を設けることもできる。この小孔を介して、ディスクにメタロ－β－ラクタマーゼ阻害剤を添加することができる。例えば、この小孔を介して、ディスクに液状のメタロ－β－ラクタマーゼ阻害剤を滴下することができる。

【００２２】

上記本発明のキットを用いる判別するメタロ－β－ラクタマーゼ産生菌の方法は、このキットのメタロ－β－ラクタマーゼ阻害剤を含有させるためのディスクに、メタロ－β－ラクタマーゼ阻害剤を含有させ、このキットを検出対象である菌が塗布された固体培地の表面に置き（図３の上図参照）、培養を行い、培養後、2箇所あるβ－ラクタム薬のディスクの周囲に形成される阻止円の違いにより、検出対象である菌がメタロ－β－ラクタマーゼ産生菌か否かを判別することからなる。上記キットを用いること以外は、上記本発明の方法をそのまま用いることができる。例えば、図３の左下に示すように、培養後、メタロ－β－ラクタマーゼ阻害剤を含有させたディスクに隣接するβ－ラクタム薬のディスクの周囲には阻止円が形成され、メタロ－β－ラクタマーゼ阻害剤を含有させたディスクと反対側の端にあるβ－ラクタム薬のディスクの周囲には阻止円が形成されないか、形成されても、ディスクに近接した小さな阻止円である場合、検査対象の菌は、メタロ－β－ラクタマーゼ産生菌であると判別できる。また、図３の右下に示すように、検査対象の菌がメタロ－β－ラクタマーゼ産生菌でない場合、培養後

、いずれのβ－ラクタム薬含有ディスクの周囲にも阻止円が形成されないか、または形成されても、ディスクに近接した小さな阻止円である。

【００２３】

メタロ－β－ラクタマーゼ阻害剤は、固体培地表面及びその内部での拡散性を考慮すると、比較的低分子量かつ低沸点の化合物から選ばれることがある。そのため、本発明のキットでは、メタロ－β－ラクタマーゼ阻害剤を含有させるためのディスクには、使用直前にメタロ－β－ラクタマーゼ阻害剤を含有させることとした。しかし、ディスクに予め不揮発性のメタロ－β－ラクタマーゼ阻害剤を含有させ、このディスクを密封しておくことで、メタロ－β－ラクタマーゼ阻害剤の散逸を防止したキットとすることもできる。

【００２４】

【実施例】

以下、本発明の試験方法を実施例によりさらに説明する。

検査対象となる菌として、メタロ－β－ラクタマーゼ産生菌として（１）IMP-1(プラスミド性メタロ－β－ラクタマーゼ)産生セラチア・マルセセンス(S.marcescens)、（２）IMP-1産生クレブシエラ・ニューモニアエ(K.pneumoniae)、及び（３）IMP-1産生緑膿菌）、並びにメタロ－β－ラクタマーゼ産生菌ではない菌として（４）AmpC過剰産生セラチア・マルセセンス(S.marcescens)、（５）SHV-5a産生クレブシエラ・ニューモニアエ(K.pneumoniae)、及び（６）AmpC過剰産生緑膿菌を選び、以下の試験を行った。

日本化学療法学会標準法に従い、ミューラーヒントン液体培地でMacFarland 0.5に調整した被検菌の菌液を、感受性試験用の綿棒で取り、試験管の管壁に押し当てて絞った後、２回、薬剤感受性試験用の寒天培地（ミューラーヒントン寒天培地）に塗布し、短時間表面を軽く乾燥させる。

セフタジジム(CAZ)など、市販の第３世代セフェム薬の感受性ディスクと抗菌薬を含まない「ろ紙（厚みが0.5 ～1.0 mm, 直径が約6.3 mm）」を、約20 mm間隔をあけて置く。その中央より直角方向に40 mm隔てたところに、同様の３世代セフェム薬の感受性ディスクを置く。

寒天培地上に置いた、抗菌薬を含まない「ろ紙」に、阻害薬（メルカプトプロ

ピオン酸、メルカプト酢酸などのチオール化合物や含硫化合物の原液、重金属塩溶液、金属キレート剤等）を、3 μl、マイクロピペット等を用いて吸収させる。

37℃で一夜、培養し、感受性ディスクの周囲の、発育阻止円の形状の差を観察し、メタロ-β-ラクタマーゼを産生する菌か否かを判定する。結果を図1に示す。

２つの第３世代セフェム薬の感受性ディスクの周囲の阻止円の形状を比較し、

ａ．阻止円の形状に差が見られる場合〔（１）～（３）の菌〕は、メタロ-β-ラクタマーゼ産生株

ｂ．阻止円の形状に差が見られない場合〔（４）～（６）の菌〕は、メタロ-β-ラクタマーゼ非産生株

と判定される。

【００２５】

【発明の効果】

本発明のによれば、メタロ－β－ラクタマーゼ産生菌を、ＰＣＲ法など特殊な方法を用いることなく、病院の検査室においても実施することが可能な程簡便な方法で判別することができる。

さらに本発明によれば、より簡便にメタロ－β－ラクタマーゼ産生菌を判別する方法を実施できるキット及びこのキットを用いたメタロ－β－ラクタマーゼ産生菌を判別する方法を提供することがある。

【図面の簡単な説明】

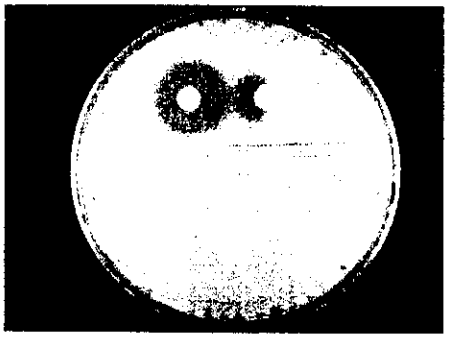
【図１】　実施例で得られた固体培地上の阻止円の状態を示す図面に代わる写真。

【図２】　本発明のキットの説明図。

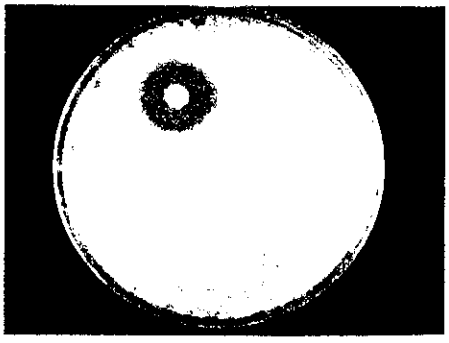
【図３】　本発明のキットを用いた方法の説明図。

【書類名】　図面

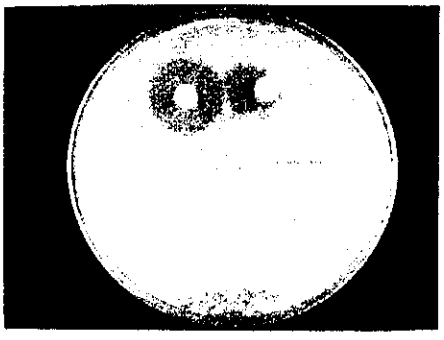
【図１】



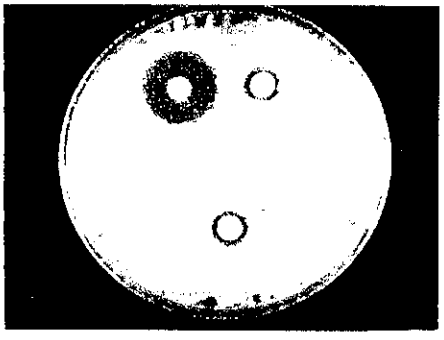
(1)



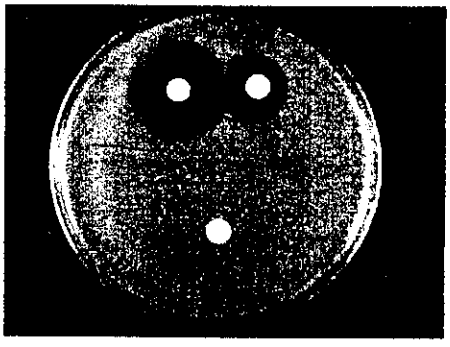
(4)



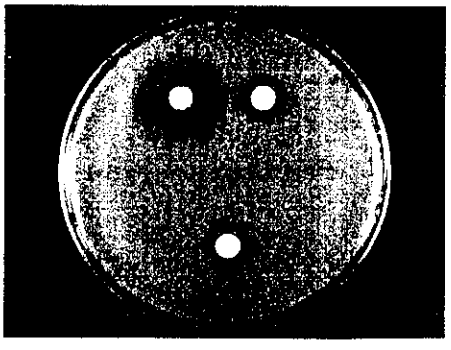
(2)



(5)



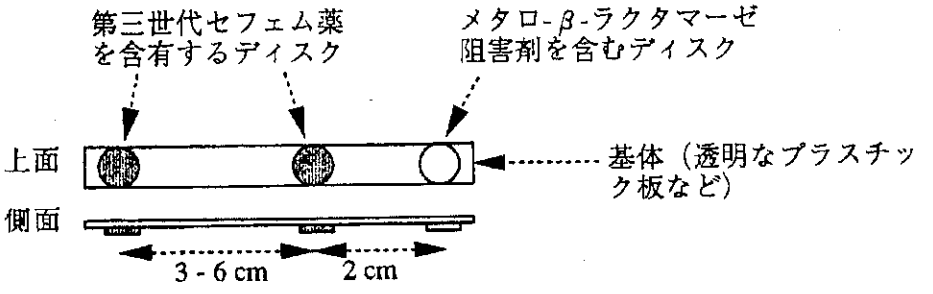
(3)



(6)

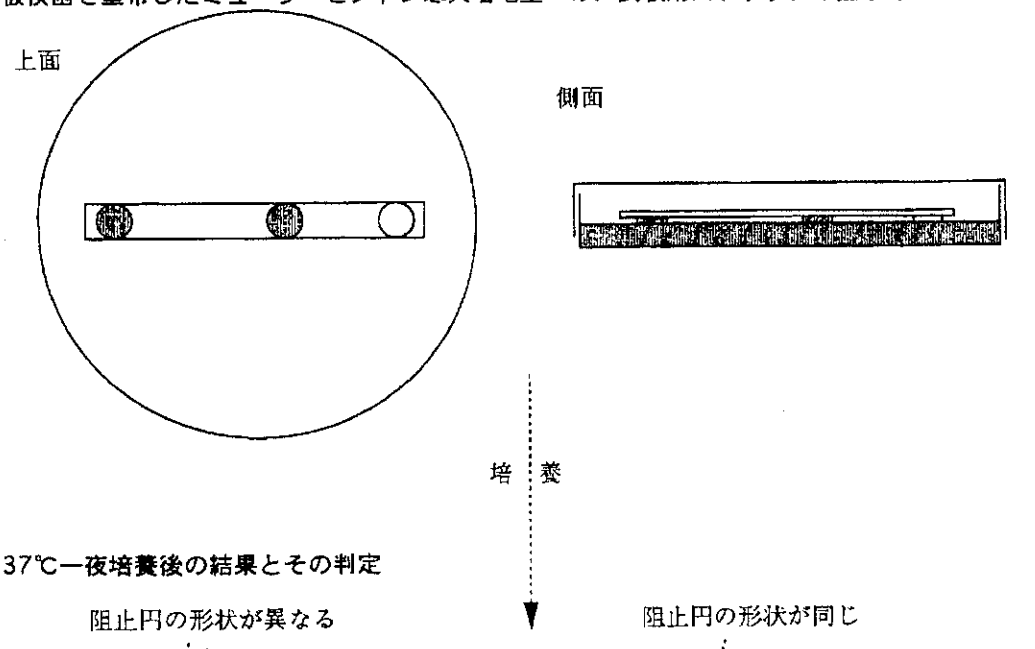
【図２】

試験用ストリップの形状

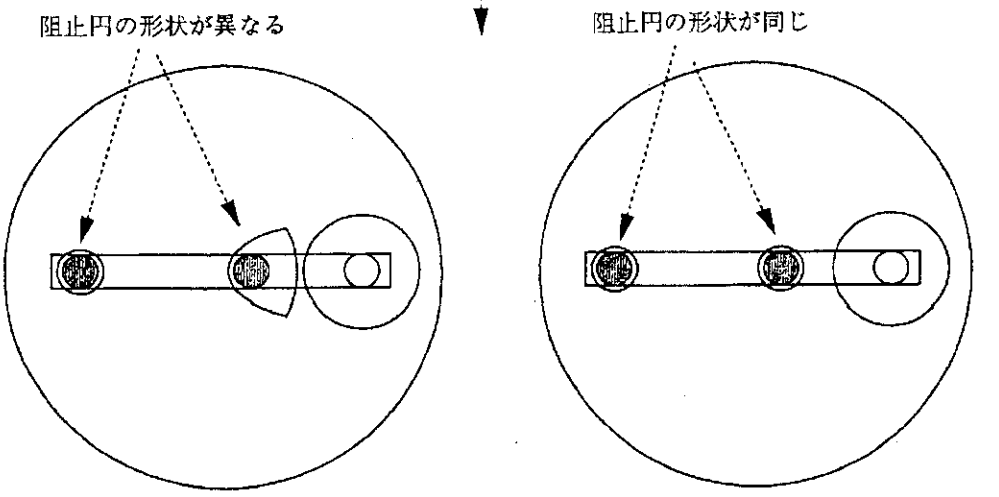


【図３】

被検菌を塗布したミューラーヒントン寒天培地上への、試験用ストリップの置き方



37℃一夜培養後の結果とその判定



メタロ-β-ラクタマーゼを産生する
第三世代セフェム耐性菌

メタロ-β-ラクタマーゼを産生しない
第三世代セフェム耐性菌

【書類名】　要約書

【要約】

【課題】　メタロ－β－ラクタマーゼ産生菌を判別する方法であって、病院の検査室においても実施することが可能な程に簡便な方法及びこの方法を利用するキットの提供。

【解決手段】　検出対象である菌が塗布された固体培地の表面に、メタロ－β－ラクタマーゼ阻害剤を点在させ、さらに、この阻害剤からの距離が異なる2箇所にβ－ラクタム薬を点在させ、上記固体培地を培養し、培養後、上記2箇所のβ－ラクタム薬の周囲に形成される阻止円の違いにより、検出対象である菌がメタロ－β－ラクタマーゼ産生菌か否かを判別する方法。この判別方法に使用するキット。

【選択図】　図３

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X. 主要な論文別冊

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A New SHV-Derived Extended-Spectrum β-Lactamase (SHV-24) That Hydrolyzes Ceftazidime through a Single-Amino-Acid Substitution (D179G) in the Ω-Loop

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A new SHV-derived extended spectrum β-lactamase (SHV-24) conferring high-level resistance to ceftazidime but not cefotaxime and cefazolin was identified in Japan. This enzyme was encoded by a transferable 150-kb plasmid from an *Escherichia coli* clinical isolate. The pI and K_m for CAZ of this enzyme were 7.5 and 30 μM, respectively. SHV-24 was found to have a D179G substitution in the Ω-loop of the enzyme.

TEM- and SHV-derived extended-spectrum β-lactamases (ESBLs) that give resistance to the oxyimino-β-lactams such as ceftazidime (CAZ) and/or cefotaxime (CTX) have become a general concern (5, 9), and at least 12 SHV-derived ESBLs and more than 70 TEM-derived ESBLs have been enrolled in the EMBL-GenBank and SWISS-PROT databases at present. Several CTX-resistant *Escherichia coli* isolates producing CTX-M-related β-lactamases such as Toho-1 have been reported so far in Japan (8, 15); however, the presence of TEM- or SHV-derived ESBLs has not been demonstrated. Thus, we made a preliminary survey on ESBL-producing gram-negative rods in Japan (16). Prior to this survey, we isolated an unique *E. coli* strain that shows a high-level resistance to CAZ (MIC, >128 μg/ml). In this study, we report the character of a new SHV-derived ESBL that has a single-amino-acid substitution in the Ω-loop of SHV-type β-lactamases (7, 10).

CAZ-resistant *E. coli* strains were isolated from urine samples of an inpatient in Chiba Prefecture in June 1996. *E. coli* strain HKY453 showed high-level resistance to CAZ (MIC, >128 μg/ml); however, this strain was susceptible to CTX (MIC, 4 μg/ml) and cefazolin (MIC, 4 μg/ml). Strain HKY453 showed intermediate resistance to chloramphenicol (MIC, 16 μg/ml), while the same strain was susceptible to minocycline, streptomycin, gentamicin, nalidixic acid, fluoroquinolones, trimethoprim, and rifampin.

Conjugal transfer of CAZ resistance from *E. coli* strain HKY453 to *E. coli* CSH2 (F^- *metB*, resistant to both nalidixic acid and rifampin) was done by a method described elsewhere (14). *E. coli* CSH2 was provided by T. Sawai, Chiba University School of Medicine, Japan. The *E. coli* strain HKY453 and an *E. coli* XL1-Blue clone that harbors recombinant plasmid pSHV001 carrying a 3.1-kb *Bam*HI insert were subjected to a double-disk diffusion test using three disks containing CAZ, CTX, and amoxicillin plus clavulanate, respectively. Strain HKY453, an *E. coli* CSH2 transconjugant that received a large plasmid pCAZR001 (approximately 150 kb in size) carrying a CAZ resistance gene (bla_{SHV-24}), and the clone *E. coli* XL1-Blue(pSHV001) were also subjected to antibiotic susceptibility testing by the agar dilution method according to the protocol recommended by NCCLS (11), and the results are shown in Table 1, as are those obtained with *E. coli* CSH2 and *E. coli* XL1-Blue. *E. coli* CSH2(pCAZR001) showed intermediate resistance to chloramphenicol (MIC, 16 μg/ml). Both the transconjugant and the clone were susceptible to CTX and cefazolin, and resistance levels to CAZ in these strains were lower than that of the parental strain HKY453 (Table 1). Since CAZ resistance was blocked by the presence of 4 μg of clavulanate per ml in both the parental strain and the clone, it was suggested that HKY453 produces, not class C or class B β-lactamases, but a class A β-lactamase belonging to Bush's class 2be.

PCR analyses using several sets of primers for class A β-lactamases, including TEM-derived ESBLs, Toho-1 type β-lactamase, and SHV-derived ESBLs were done as described elsewhere (16), and only the PCR using SHV-specific primers yielded a band of amplicon. Thus, it was suggested HKY453 produces plasmid-dependent SHV-derived ESBL. The total DNA preparation from HKY453 was digested with *Bam*HI and ligated with *Bam*HI cleaved pBCSK+. Then *E. coli* XL1-Blue cells were transformed with the resultant recombinant DNA mixture by an electroporation method, and several CAZ-resistant colonies were isolated. Cloning vector pBCSK+ and *E. coli* strain XL1-Blue were purchased from Stratagene (La Jolla, Calif.). The clone *E. coli* XL1-Blue(pSHV001) showed CAZ resistance, and a PCR product was detected when an SHV-specific primer was used. For confirmation of the location of the bla_{SHV-24} gene in pSHV001, the DNA insert was digested from both sides with mung bean nuclease and exonuclease III with a deletion kit (Nippon Gene Co. Ltd., Toyama, Japan), and the bla_{SHV-24} gene was localized in the central region of the insert of pSHV001. Nucleotide sequencing was performed on PCR products from parental strain HKY453 and plasmid DNA carrying the bla_{SHV-24} gene by dye-terminator methods, using six primers for the SHV-derived ESBL gene (16) on both DNA strands. Mutations found in the nucleotide sequence of the bla_{SHV-24} gene were again checked on these deletion mutants. As a result, the *E. coli* HKY453 and a clone were found to produce a new β-lactamase. A single point mutation was found in the coding region of the β-lactamase gene compared with that encoding SHV-6 (3), and this mutation resulted in a single amino acid substitution at amino acid residue 179 (D179G) based on the consensus sequence of SHV-derived ESBLs (1). Two nucleotide mutations were also observed in the genes for SHV-24 and SHV-8 (13). The en-

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TABLE 1. Result of antibiotic susceptibility testing

Strain	MIC (μg/ml)														
	AMP[a]	PIP	CER	CFZ	CFP	CAZ	CAZ+CVA	CTX	CPM	CMZ	CMX	MOX	ATM	SUL+CFP	IPM
E. coli HKY453	>128	>128	16	4	8	>128	4	4	16	1	1	8	4	1	<0.5
E. coli CSH2(pCAZR001)	>128	128	8	2	4	64	1	1	4	1	0.5	2	1	<0.5	<0.5
E. coli CSH2	2	1	1	1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
E. coli XL1-Blue(pSHV001)	>128	>128	8	4	4	128	1	2	8	1	0.5	2	1	0.5	<0.5
E. coli XL1-Blue	2	2	1	1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5

[a] Abbreviations: AMP, ampicillin; PIP, piperacillin; CER, cephaloridine; CFZ, cefazolin; CFP, cefoperazone; CAZ, ceftazidime; CAZ+CVA, ceftazidime and clavulanic acid (4 μg/ml); CTX, cefotaxime; CPM, cefpirome; CMZ, cefmetazole, CMX, cefminox; MOX, moxalactam; ATM, aztreonam; SUL+CFP, sulbactam and cefoperazone (1:1); IPM, imipenem.

zymes used for gene manipulation were purchased from Nippon Gene Co. Ltd. or TAKARA Co. Ltd. (Kyoto, Japan). The primers for PCR and sequencing analyses were designed by using the GENETYX system, version 5.0.0 (SDC, Tokyo, Japan) and made by TAKARA Co. Ltd. The nucleotide sequences and amino acid sequences of SHV-derived ESBLs were downloaded from the EMBL-GenBank and SWISS-PROT databases, respectively. The nucleotide sequence determined in this study was analyzed by using the GENETYX system, version 10.0. The consensus sequence of SHV-derived ESBLs was referred to a research organization for inclusion in a standard numbering scheme by Ambler et al. (1) and Jacoby (9) (more information can be found elsewhere [http://www.lahey.org/studies/webt.html]).

An *E. coli* clone carrying bla_{SHV-24} was cultured in 2 liters of Luria-Bertani broth at 37°C overnight, and then bacterial cells were harvested by centrifugation (15,000 × *g* for 20 min). SHV-24 was purified according to a method described previously (15). Purified SHV-24 was subjected to isoelectric focusing by using IPGphor (Amersham Pharmacia Biotech, Uppsala, Sweden). Determination of kinetic parameters was done according to a method described previously (2) using purified SHV-24. The molar extinction coefficients ($\Delta\varepsilon$) used were as follows: for ampicillin (235 nm), $\Delta\varepsilon = 1.2\ mM^{-1}\ cm^{-1}$; for aztreonam (315 nm), $\Delta\varepsilon = 0.68\ mM^{-1}\ cm^{-1}$; for cephaloridine (295 nm), $\Delta\varepsilon = 1.0\ mM^{-1}\ cm^{-1}$; for CTX (264 nm), $\Delta\varepsilon = 7.25\ mM^{-1}\ cm^{-1}$; and for CAZ (272 nm), $\Delta\varepsilon = 7.6\ mM^{-1}\ cm^{-1}$. The K_i values of clavulanic acid were determined with ABPC and CAZ, respectively.

The kinetic parameters of SHV-24 are shown in Table 2. These data suggest that SHV-24 can hydrolyze ABPC like SHV-1. It is also suggested that the affinity of CAZ to SHV-24 was similar to that of ABPC. This enzyme, however, hydrolyzes CAZ with a very low catalytic rate. Cephaloridine and aztreonam were also hydrolyzed detectably, but hydrolysis of CTX by SHV-24 was too poor to calculate the kinetic parameters. Four micrograms of clavulanic acid per milliliter blocked SHV-24, an effect that was observed for the other ESBLs. According to the kinetic parameters of SHV-24, this enzyme is able to hydrolyze CAZ with a low catalytic rate, but the parental strain *E. coli* HKY453 demonstrated a relatively high level of resistance to CAZ, as shown in Table 1. A similar observation was reported for SHV-6, and this finding may implicate other factors, such as alterations in the bacterial cell membrane, in the high-level CAZ resistance in HKY453. This speculation was supported by the fact that the MIC of CAZ for the *E. coli* XL1-Blue clone producing SHV-24 was lower than that for parental strain HKY453 despite multicopy expression of *bla* gene in the clone. Hence, both production of a large amount of SHV-24 and decreased permeation of CAZ through the bacterial outer membrane might be implicated in the high level of CAZ resistance in *E. coli* strain HKY453.

TABLE 2. Kinetic parameters of SHV-24

β-lactam antibiotic[a]	V_{max}[b] (μM/min)	K_m (μM)	V_{max}/K_m	Relative V_{max}/K_m	K_i[c] (μM)
AMP	2.00	32	0.0625	100	57
CER	2.37	210	0.0113	18.1	ND[d]
AZT	0.735	500	0.00147	2.35	ND
CAZ	0.043	30	0.000143	0.23	37

[a] AMP, ampicillin; CER, cephaloridine; AZT, aztreonam; CAZ, ceftazidime.
[b] Calculated with 5.46 μg of purified SHV-24.
[c] K_i was calculated using clavulanic acid.
[d] ND, not done.

A single-amino-acid substitution (D179G) was found in the newly identified SHV-24. Since Asp179 makes a salt bridge to Arg164 across the neck of the Ω-loop (10, 13), an amino acid substitution from Asp to Gly that has no anionic R-side chain will cancel the salt bridge and cause the distortion of the Ω-loop at the active site of this enzyme, and this may well result in a change in the enzyme substrate specificity. The same amino acid substitution (D179G) has been documented in an OHIO-type β-lactamase that also hydrolyzes CAZ (4). Similar single-amino-acid substitutions at residue 179 are observed in SHV-6 (D179A) (3) and SHV-8 (D179N) (13). SHV-24 had an isoelectric point (pI = 7.5) similar to that of SHV-6 (pI = 7.6). Hence, from the viewpoint of enzyme kinetics and molecular features, it seems reasonable to separate the group of SHV-6, SHV-8, and SHV-24 from the group of SHV-2, SHV-3, SHV-4, and SHV-5, which have a common amino acid substitution (G238S) between two β-strands, B3 and B4 (7, 10).

SHV-24 has not been found so far in Europe or the United States, where many SHV-derived ESBLs have been identified. Much clinical use of CAZ in Japan might lead to the emergence of this kind of new enzyme that enables bacteria to survive in a high-level concentration of CAZ such as was found in the urine sample of an inpatient. Strain HKY453 was isolated from urine sample and demonstrates high-level CAZ resistance (MIC, >128 μg/ml), but it was apparently susceptible to cefazolin (MIC, 4 μg/ml) as shown Table 1. Emergence of this kind of enzyme may reflect the trend of antibiotic use in Japan, where the broad-spectrum cephems tend to be preferentially used rather than the first-generation cephems and penicillins.

Various *Klebsiella pneumoniae* and *E. coli* strains producing plasmid-mediated TEM- or SHV-derived ESBLs have been found worldwide. In Japan, however, no TEM- or SHV-derived ESBL producer had been recognized until our recent survey on ESBL producers (16), although several outbreaks of *E. coli* that produce Toho-1 type class A enzyme have been reported (8, 15). It has been speculated that this phenomenon

may have something to do with the widespread use of cephamycins and carbapenems as first-line drugs in Japan. Recently, however, restricted use of these agents was recommended for several clinical settings to prevent further dissemination of IMP-1 type metallo-β-lactamase-producing gram-negative bacteria such as *Serratia marcescens* (12) and *Pseudomonas aeruginosa* (14), and this may contrarily induce proliferation of CAZ- or CTX-resistant bacteria that produce TEM- or SHV-derived ESBLs, as well as CTX-M-type enzymes, including Toho-1 and MEN-1, in Japan hereafter.

Nucleotide sequence accession number. The nucleotide sequence of the coding region of the β-lactamase gene has been deposited in the EMBL-GenBank nucleotide sequence data banks through the DNA Data Bank of Japan (accession number AB023477).

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A preliminary survey of extended-spectrum β-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan

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Abstract

We conducted a survey of extended-spectrum β-lactamases (ESBLs) among 16 805 *Escherichia coli* and 9794 *Klebsiella pneumoniae* clinical isolates recovered from 196 separate medical institutions during the period January 1997 to January 1998. Using the criteria for minimal inhibitory concentrations (MICs) of oxyimino-cephalosporins of ≥8 μg ml^{-1} and confirmation by double-disk test, we detected 15 *E. coli* and 34 *K. pneumoniae* isolates producing ESBLs. Genotypes of ESBLs determined by PCR with type-specific primers included one TEM-derived and 24 SHV-derived ESBLs, in addition to 24 Toho-1-type ESBLs, one of the major types of ESBLs reported in Japan. Nucleotide sequence analysis of SHV-specific PCR products revealed that SHV-12 was the dominant type of SHV-derived ESBL. In addition, we also identified TEM-26 and SHV-2. This is the first report characterizing TEM- and SHV-derived ESBLs in Japan.

Keywords: Extended-spectrum β-lactamase; Toho-1-type ESBL; TEM-derived ESBL; SHV-derived ESBL; SHV-12

1. Introduction

Since the 1980s, extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria have been isolated in many countries [1]. Many outbreaks caused by such bacteria, especially *Klebsiella pneumoniae*, have been reported [2–4]. The predominant ESBLs reported in Western and Asian countries are TEM- and SHV-derived ESBLs [4–6]. These β-lactamases possess one or more amino acid substitutions which correspond to the narrow-spectrum prototype, TEM-1 and SHV-1, and these amino acid substitutions confer resistance to oxyimino β-lactam antibiotics [7].

In Japan, ESBLs are rarely isolated [8–10]. This suggests differences in the distribution of ESBLs between Japan and Western countries. However, since no systematic survey of ESBL-producing bacteria was conducted in Japan, it remains unclear whether or not TEM- and SHV-derived ESBLs are present in Japan. To investigate the prevalence of ESBLs in Japan, we examined *Escherichia coli* and *K. pneumoniae* clinical isolates recovered from 196 separate medical institutions.

2. Materials and methods

2.1. Bacterial stains and conjugation

A total of 16 805 *E. coli* and 9794 *K. pneumoniae* clinical isolates were collected from 152 hospitals and 44 outpatient clinics from January 1997 to January 1998. The bed capacity of most of these hospitals is less than 300. They were located in five distinct Prefectures and Metropolitan Tokyo. Strains isolated from the same patient were included only when recovered from different sites or showing evidently different susceptibility profiles. Bacteria were grown in Luria–Bertani broth with the appropriate antibiotics. *E. coli* CSH2 (*metB* F$^-$ NA^r RFP^r) was used as a recipient of a conjugation experiment. Conjugation was performed by the broth method as described previously [9].

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