表1 SSI防止のためのガイドライン（抜粋）

ランク付け	適切に企画された実験的、臨床的、疫学的検討により証明されていて強力に推奨される項目（IA）	いくつかの実験的、臨床的、疫学的検討と強い理論的論拠により証明されていて強力に推奨される項目（IB）	理論的根拠があってその実施が支持される項目（II）	有効性に関して合意に達していない項目（NR、not recommended）
術前準備に関して	待機手術では手術部位以外の感染でもあらかじめ治療しておく 切開部位の体毛が邪魔にならなければ除毛しない 除毛する場合は電気バリカンを用い、手術直前に行う	糖尿病患者は血糖値を適切に管理する 待機手術前は30日間の禁煙を勧める SSI防止のために血液製剤の術前使用を控える必要はない	皮膚消毒は同心円状に広い範囲を行う 手術前の入院期間は最小限とする	手術前にステロイドの投与量を減らす SSI防止のために栄養補給を増強する 術前に鼻腔にムピロシン軟膏を塗布する SSI防止のために創への酸素供給を増加させる
手術室の清掃と消毒		床などが汚れた場合には汚染範囲のみを消毒する 感染性患者の術後に特別な消毒は行わない SSI防止のために粘着マットは使用しない	当日の最後の手術終了後は手術室の床の湿式清掃を行う	手術と手術の間に床の消毒を行うこと
手術時の服装と覆布		口と鼻を完全に覆う手術用マスクを着用する 頭髪を完全に覆うために帽子を着用する 手洗い後滅菌ガウンを着用した後に手袋をつける 手術用ガウンおよび覆布は液体バリア効果のある材質を用いる 手術着が汚染したら着替える SSI防止のために靴カバーは着けない		手術着の洗濯方法や場所、手術室外に出るときの対応について
予防的抗菌薬投与	適応があるときのみ予防的抗菌薬投与を行い、各特定の手術のSSI惹起に最も一般的な病原菌に対する効果や出版された勧告に基づいて薬剤を選択する	バンコマイシンを予防的抗菌薬投与に日常的に使用してはならない		
無菌操作や手術手技	血管内カテーテル留置、脊椎麻酔、硬膜外麻酔、静脈注射などは無菌操作で行う	手術部位の組織は丁寧に扱い、十分に止血し、壊死組織や異物はできる限り除去し、死腔が残らないように工夫する 手術創の汚染が著しい場合には創の二次閉鎖を考慮する ドレーンは閉鎖吸引ドレーンを使用する、ドレーンは手術創以外から挿入し、できる限り早期に抜去する	滅菌物への薬液注入は使用直前に行う	
手術後の対応		創は滅菌した被覆材で術後24～48時間保護する 包帯交換および手術部位へ触れる前後には手を洗う	包帯交換は無菌操作で行う 適切な手術創管理について患者に十分説明する	一次閉鎖した切開創の48時間以降の被覆の必要性についてや創部を被覆せずに入浴可能な時期については保留

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2005, p. 600–605
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.2.600–605.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.
Vol. 49, No. 2

Novel SHV-Derived Extended-Spectrum β-Lactamase, SHV-57, That Confers Resistance to Ceftazidime but Not Cefazolin

Ling Ma,[1] Jimena Alba,[2] Feng-Yee Chang,[3] Masaji Ishiguro,[4] Keizo Yamaguchi,[2] L. K. Siu,[1]* and Yoshikazu Ishii[2]

Division of Clinical Research, National Health Research Institutes,[1] and Division of Infectious Disease and Tropical Medicine, Department of Internal Medicine, Tri-Service General Hospital, National Defense Medical Center,[3] Taipei, Taiwan, and Department of Microbiology, Toho University School of Medicine, Tokyo,[2] and Suntory Institute for Bioorganic Research, Shimamoto, Osaka,[4] Japan

Received 14 April 2004/Returned for modification 19 June 2004/Accepted 3 October 2004

A new SHV-derived extended-spectrum β-lactamase, SHV-57, that confers high-level resistance to ceftazidime but not cefotaxime or cefazolin was identified from a national surveillance study conducted in Taiwan in 1998. An *Escherichia coli* isolate resistant to ampicillin, cephalothin, and ceftazidime but sensitive to cefoxitin, ceftriaxone, cefotaxime, imipenem, and a narrow-spectrum cephem (cefazolin) was isolated from the urine of a patient treated with β-lactam antibiotics. Resistance to β-lactams was conjugatively transferred with a plasmid of about 50 kbp. The pI of this enzyme was 8.3. The sequence of the gene was determined, and the open reading frame of the gene was found to consist of 861 bases (GenBank accession number AY223863). Kinetic parameters showed that SHV-57 had a poor affinity to cefazolin. The K_m value toward cefazolin (5.57×10^3 μM) was extremely high in comparison to those toward ceftazidime (30.9 μM) and penicillin G (67 μM), indicating its low affinity to cefazolin. Although the K_m value of the β-lactamase inhibitor was too high for the study of catalytic activity (k_{cat}), indicating the low k_{cat} of SHV-57, the SHV-57 carrier was highly susceptible to a β-lactam–β-lactamase inhibitor combination. Comparison of the three-dimensional molecular model of SHV-57 with that of the SHV-1 β-lactamase suggests that the substitution of arginine for leucine-169 in the Ω loop is important for the substrate specificity.

Since the first extended-spectrum β-lactamase (ESBL) was isolated in Germany in 1983 (11), TEM-, SHV-, CTX-, and OXA-type ESBLs have been described in various members of the family *Enterobacteriaceae* (G. A. Jacoby and K. Bush, http://www.lahey.org/studies/webt.htm). Most of the ESBLs have altered hydrolytic activities compared with those of the classical enzymes TEM-1, TEM-2, and SHV-1 as a result of amino acid changes in different specific positions (10, 17). SHV-1 is a narrow-spectrum β-lactamase with activity against penicillins. The first extended-spectrum SHV enzyme was described in 1985 and was named SHV-2 (10). The serine at amino acid position 238 was found to be replaced by glycine in SHV-1 and was found to cause resistance to extended-spectrum β-lactams. Since then, many SHV-type ESBLs have been reported. Most of the substitutions are at Ambler position 179 or 238, alone or in combination with alterations at positions 35 and 240, which are important for substrate extension (G. A. Jacoby and K. Bush, http://www.lahey.org/studies/webt.htm). X-ray crystallography shows that mutations which cause amino acid changes on or close to the Ω loop of the enzyme are highly correlated to resistance to extended-spectrum β-lactams (12). The mutation at Gly238 has frequently been reported in SHV-type ESBLs. It causes resistance to various antibiotics, ranging from narrow-spectrum cephalosporins (cefazolin) to extended-spectrum cephalosporins (ceftazidime and cefotaxime). However, resistance only to extended-spectrum cephalosporins with susceptibility to narrow-spectrum cephalosporins is rarely encountered. In this report we delineate the mechanism of resistance of an *Escherichia coli* isolate recovered during an island-wide survey in Taiwan (7). This isolate is highly resistant only to ceftazidime but is susceptible to cefazolin.

MATERIALS AND METHODS

Bacterial strains. *E. coli* 981223 was collected during an island-wide study of antibiotic resistance in Taiwan in 1998 (7). It was isolated in September 1998 from the urine of an 18-month-old boy with pneumonia. He had a history of failure to thrive, multiple abnormalities, intussusceptions, intestinal resection, and recurrent pneumonia that had resulted in 10 hospital admissions since birth. Before strain isolation, the patient had received empirical antibiotic treatment, including treatment with penicillin, oxacillin, cefotaxime, cefuroxime, ceftriaxone, amikacin, ceftazidime, vancomycin, erythromycin, and gentamicin, during the 10 hospitalizations. The strain was identified as *E. coli* with the Vitek system (bioMerieux Vitek, Inc., Hazelwood, Mo.).

Conjugation. The transfer of resistance was carried out by conjugation. A rifampin-resistant strain of *E. coli* (strain JP-995) (18) was used as the recipient. Recipients and donors were separately inoculated into brain heart infusion broth (Oxoid Ltd., Basingstoke, England) and incubated at 37°C for 4 h. They were then mixed at a ratio of 1:10 (by volume) for overnight incubation at 37°C. A 0.1-ml volume of the overnight broth mixture was then spread onto a MacConkey agar plate containing rifampin (100 μg/ml) and ceftazidime (2 μg/ml).

Susceptibility testing. Antimicrobial susceptibility was determined by the broth microdilution test, according to the guidelines of the National Committee for Clinical Laboratory Standards (16). The following antimicrobial agents were used: ampicillin, cephalothin, cefazolin, cefoxitin, cefotaxime, cefotaxime-clavulanic acid, ceftriaxone, ceftriaxone-clavulanic acid, ceftazidime, ceftazidime-clavulanic acid, imipenem, amikacin, aztreonam, and ciprofloxacin. All drugs except ciprofloxacin were incorporated into Mueller-Hinton broth (TREK Diagnostic System Ltd., Chichester, West Sussex, United Kingdom) in serial twofold concentrations from 0.25 to 32 μg/ml; a lower concentration of 0.03 μg/ml was used for ciprofloxacin. Two control strains, *E. coli* ATCC 35218 and ATCC 25922, were included in each test run. The inoculated plates were incubated at 35°C for

* Corresponding author. Mailing address: Division of Clinical Research, National Health Research Institutes (99), 128, Yen-Chiu-Yuan Rd., Sec. 2., Taipei, 11529, Taiwan. Phone: 886 2 26524094. Fax: 886 2 27890254. E-mail: lksiu@nhri.org.tw.

TABLE 1. Oligonucleotide primers used for amplification and/or sequencing reactions

Primer	Sequence	Description or position[a]
M4	5'-GTTTTCCAGTCACGAC-3'	From M13 vector sequence
RV	5'-CAGGAAACAGCTATGAC-3'	From M13 vector sequence
SHV-F	5'-GGTTCATATGCGTTATATTCGCCTGTGT-3'	For overproduction system
SHV-R	5'-TCCTTCTCGAGTTAGCGTTGCCAGTG-3'	For overproduction system
SHV-F1	5'-TTGTGAATCAGCAAAACGCC-3'	38–57
SHV-F2	5'-ATGCGTTATATTCGCCTGTG-3'	125–144
SHV-R1	5'-TAAAGGTGCTCATCATGGGA-3'	329–310
SHV-F3	5'-TCAGCGAAAAACACCTTG-3'	435–452
SHV-R2	5'-CCGTTTCCCAGCGGTCAAGG-3'	614–595
SHV-R3	5'-GTTAGCGTTGCCAGTGCTCG-3'	989–970

[a] The sequence position was designated according to the numbering of Mercier et al. (15).

16 to 18 h. The MIC of each antimicrobial agent was defined as the lowest concentration that inhibited visible growth of the organism.

Isoelectric focusing. After 20 h of culture in brain heart infusion broth, the bacterial cells were harvested by centrifugation and the pellet was resuspended in 1 ml of phosphate buffer (10 mM; pH 7). Enzymes were released by two cycles of freezing at −70°C and thawing at room temperature and sonication for 5 min in a sonicator in ice-cold water. Isoelectric focusing was performed in an ampholine gel (pH 3.0 to 10.0; Pharmacia, Uppsala, Sweden). Preparations from standard strains known to harbor SHV-5 and CTX-M-14 were used as standards. After isoelectric focusing, β-lactamases were detected by spreading nitrocefin (50 μg/ml) on the gel surface (14).

Cloning of SHV-57 gene. Plasmid DNA from the transconjugant was isolated with a plasmid mini kit (Qiagen, Inc., Mississauga, Ontario, Canada) and was partially digested with Sau3AI. The fragments were ligated into the BamHI site of pHSG298 by using T4 DNA ligase (Invitrogen, Carlsbad, Calif.) and electroporated into *E. coli* DH5α. Clones were selected on Luria-Bertani agar plates containing 25 μg of kanamycin/ml and 5 μg of ceftazidime/ml.

DNA sequencing analysis. The plasmid containing the cloned bla_{SHV-57} gene was prepared with a Concert Rapid Plasmid Miniprep system (GibcoBRL, Grand Island, N.Y.). The cloned gene was sequenced with the primers listed in Table 1. The N-terminal sequence was obtained by analyzing purified SHV-57 with a protein sequencer (PPSQ-23; Shimadzu, Kyoto, Japan) by the Edman degradation method (1). Mass spectrum analysis was done with an AXIMA-CFR plus mass spectrometer (Shimadzu).

Construction of overproduction system. XhoI and NdeI restriction sites were inserted into the plasmid DNA of the original SHV-57 enzyme. This was done during PCR amplification with the sense and antisense primers listed in Table 1. PCR amplification conditions were denaturation at 94°C for 5 min, followed by 25 cycles of amplification at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 7 min. The resulting PCR product was purified with a QIAquick PCR purification kit (Qiagen, Inc.). The amplified product was then inserted into plasmid vector pCR2 Blunt II TOPO (Invitrogen), and the plasmid was transformed into *E. coli* MV1184 [*ara* Δ(*lac-proAB*) *rpsL thi*(φ80*lacZ*ΔM15) Δ(*srl-recA*)*306*::Tn*10*(*tet'*) F' *traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 (Takara Shuzo Co. Ltd., Shiga, Japan). Sequencing was done with an automatic sequencer (ABI Prism 310 genetic analyzer; Perkin-Elmer Biosystems, Norwalk, Conn.). After the sequence was confirmed, XhoI and NdeI (Takara Shuzo Co. Ltd., Tokyo, Japan) were used to digest the PCR product. The product was then cloned into the vector pET 28a (Novagen, Madison, Wis.).

Purification of β-lactamase. *E. coli* BL21(DE3)pLysS, F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)pLysS (Novagen), was used to produce the enzyme as a soluble protein. Since bacteria grown at 35 and 30°C produced inclusion bodies, a growth temperature of 25°C was used for the bacteria, which were incubated in 1 liter of Super broth on a rotating shaker. When the optical density at 600 nm of the culture reached an absorbance of 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, Steinheim, Germany) was added at concentrations of 0, 10, 50,100, 150, 200, 500, and 1 mM. A final concentration of 50 μM IPTG gave an appropriate level of enzyme expression. The maximum activity of the enzyme was reached 7 h after induction. The bacteria were incubated on ice for 10 min and then harvested by centrifugation at 5,000 × *g* for 10 min at 4°C. The cells were then suspended in 100 ml of 30 mM Tris-HCl buffer (pH 8.0) containing 27% sucrose. Liberation of the periplasmic content was achieved by addition of lysozyme (final concentration, 0.4 mg/ml) and EDTA (final concentration, 5 mM) to the cooled solution. After 50 min of incubation on ice, the reaction was stopped by adding $CaCl_2$ (final concentration, 2 mM). The sample was then centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was dialyzed overnight at 4°C in 5 liters of 10 mM sodium acetate buffer (pH 5.0). Purification was done with a HiPrep 16/10 SP XL system (Amersham Biosciences AB, Uppsala, Sweden) equilibrated with 10 mM sodium acetate buffer (pH 5.0). The initial rate of hydrolysis of 100 μM nitrocefin ($\Delta\varepsilon_{482}$ = +10,000 $M^{-1}cm^{-1}$; Oxoid Ltd.) was measured, and all fractions containing β-lactamase activity were pooled. The purity of the β-lactamase preparation was controlled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels stained with Coomassie brilliant blue. The purification process was done with an AKTA purifier (Amersham Biosciences AB).

Determination of kinetic parameters. The activity of the highly purified β-lactamase was measured by spectrophotometric assay with a UV-2550 spectrophotometer (Shimadzu) connected to a personal computer. The rate of hydrolysis of the antibiotics by SHV-57 and SHV-1 was determined by monitoring the variation in the absorbance of each β-lactam. Steady-state kinetic parameters were determined for the following β-lactam compounds diluted in 50 mM phosphate buffer (pH 7.0). Benzylpenicillin ($\Delta\varepsilon_{233}$ = −780 $M^{-1}cm^{-1}$) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Ceftazidime ($\Delta\varepsilon_{265}$ = −10,300 $M^{-1}cm^{-1}$) and clavulanic acid were gifts from Glaxo SmithKline (Tokyo, Japan). Cefazolin was a gift from Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan), and tazobactam was a gift from Taiho Pharmaceutical Co. (Tokyo, Japan). Four to seven different substrate concentrations were used to determine the kinetic parameters for each substrate, and the values for the parameters reported are averages of three independent measurements. The reactions were performed in a total volume of 500 μl at 30°C. Bovine serum albumin (20 mg/ml) was added to the enzyme to prevent denaturation. The values for all kinetic parameters were determined by measuring the initial rate of hydrolysis of the selected antibiotic and using Hanes-Wolf linearization of the Michaelis-Menten equation. In the case of poor substrates, the K_m was determined as the competitive inhibition constant (K_i) with nitrocefin as the reporter substrate.

Structural model of SHV-57. A structural model of SHV-57 was constructed by mutating Leu169 to arginine with the Biopolymer module installed in the Insight II program (version 2000; Accelrys Inc., San Diego, Calif.). The structure of the model was minimized with the Discover 3 program (version 2000; Accelrys Inc.) until the final root mean square deviation became less than 0.1 kcal/mol/Å. Ceftazidime and cefazolin were manually docked into the binding site of SHV-1 and SHV-57 by placing the carbonyl oxygen atom of the β-lactam at the oxyanion hole formed by the amide groups of Ser70 and Ala237. The energies of the complex structures were minimized with the Discover 3 program, and the binding sites of the minimized structures were then covered with a sphere of water molecules of 20 Å in diameter centered at the Ser70 residue. The optimized complex structure was selected from 100 energy-minimized structures sampled by molecular dynamics calculations, which were performed at 300 K on residues within 12 Å from the β-lactam compound with a cutoff distance of 10 Å, a distance-dependent dielectric constant, and a time step of 1 fs for 100 ps by sampling the conformation every 1 ps by using the Consistent Valence Force Field parameters in the Discover 3 program (version 98; Accelrys Inc.) at 298 K for 100 ps.

Nucleotide sequence accession number. The nucleotide sequence data for the SHV-57 gene were submitted to the National Center for Biotechnology Information Data Libraries (GenBank), and the sequence has been given accession number AY223863.

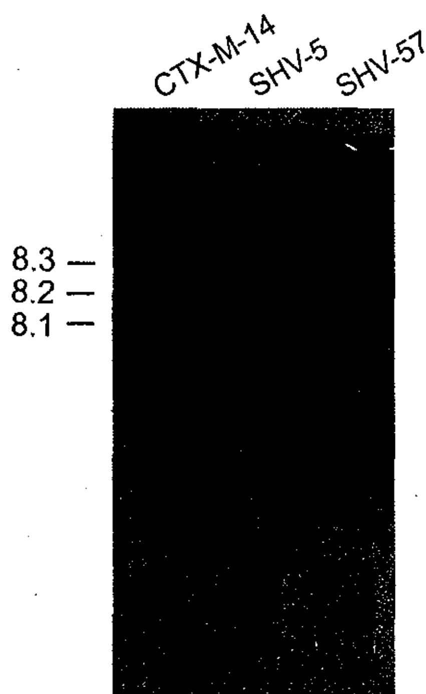

FIG. 1. Isoelectric focusing of the new β-lactamase variant and reference β-lactamases. The numbers on the left are in kilobases.

TABLE 2. MICs of various antibiotics for strains producing the SHV-57 β-lactamase

Antibiotic[a]	MIC (μg/ml)			
	E. coli isolate	Transconjugant	Cloned strains	JP-995
AMP	>32	>32	>32	2
LOT	>32	>32	8	4
CEZ	8	8	8	2
CFX	8	8	4	4
CTX	1	1	0.5	<0.25
CTX-CAL	<0.25	<0.25	<0.25	<0.25
CTR	8	8	4	0.5
CTR-CAL	<0.25	<0.25	<0.25	<0.25
CAZ	>32	>32	>32	<0.25
CAZ-CAL	1	≤0.25	0.5	<0.25
AZM	2	2	0.5	<0.25
IMP	<0.25	0.5	<0.25	0.5
GEN	>32	>32	1	<0.25
AMK	>32	>32	<0.25	<0.25
CIP	<0.03	<0.03	<0.03	<0.03

[a] Abbreviations: AMP, ampicillin; LOT, cephalothin; CEZ, cefazolin; CFX, cefoxitin; CTX, cefotaxime; CTX-CAL, cefotaxime and clavulanic acid; CTR, ceftriaxone; CTR-CAL, ceftriaxone and clavulanic acid; CAZ, ceftazidime; CAZ-CAL, ceftazidime and clavulanic acid; AZM, aztreonam; IMP, imipenem; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin.

RESULTS

Plasmid profile and isoelectric focusing. The β-lactam resistance of the isolate harboring the β-lactamase was found to be conjugatively transferable. Only one plasmid was found in the recipient. The plasmids from the transconjugants had molecular sizes that ranged from approximately 40 to 60 kb. Transconjugants which acquired ceftazidime resistance by conjugation appeared at a frequency of 10^{-5}. The plasmid, which was designated pMTY512 (pMTY; registered with the Plasmid Reference Center), was cleaved into 16 segments by HincII. From the sizes of the fragments obtained, the size of pMTY512 was estimated to be about 51 kb. Narrow-range ampholine gel electrophoresis revealed that, with reference to the CTX-M-14 (pI 8.1) and SHV-5 (pI 8.2) β-lactamases, the SHV-57 β-lactamase had a pI of 8.3 (Fig. 1).

Susceptibility testing. The SHV-57 carriers, transconjugants, and cloned strains were found to be ampicillin, cephalothin, and ceftazidime resistant. They were susceptible to cefazolin, cefotaxime, aztreonam, ciprofloxacin, amikacin, and imipenem. When clavulanic acid at a fixed concentration of 4 μg/ml was combined with ceftazidime, a greater than fourfold reduction in the ceftazidime MIC, a characteristic of ESBLs, was observed (Table 2).

DNA sequencing. The nucleotide sequence of 1,310 bp was determined by the strategy shown in Fig. 2. An 861-nucleotide open reading frame with a G+C content of 63.2% was present in this sequence. The sequence initiation codon (ATG) was preceded by a possible −10 region (AAAAAT) and a −35 region (TTGATT) of a putative promoter. The termination codon was TAA. From the putative open reading frame, the precursor form of SHV-57 seemed to consist of 286 amino acid residues. Consensus sequences, such as SXXK, SDN, and KTG, in class A β-lactamases were found in the amino acid sequence of the SHV-57 β-lactamase (GenBank accession number AY223863). Thus, SHV-57 is a class A β-lactamase.

Determination of kinetic parameters. The purified enzyme gave a single band on SDS-PAGE with a molecular weight of 28,904. The overproduction system and purification process yielded 1.6 mg of purified SHV-57 per ml in a total volume of 2.5 ml. The purity achieved was over 95%, as observed by SDS-PAGE. The N-terminal sequence of the mature enzyme is SPQPLEQIKLSESQLSGRVGMIEMDLASGRTLTAWRA DERFPMMSTFK. The kinetic parameters for SHV-57 and SHV-1 are summarized in Table 3. The results showed that the SHV-57 β-lactamase exhibited a narrow-spectrum activity profile, although notable differences were detected with different substrates. The K_m value toward cefazolin (5.57×10^3 μM) was extremely high compared to those toward ceftazidime (30.9 μM) and penicillin G (67 μM), indicating its low affinity to cefazolin. The K_i values of clavulanic acid and tazobactam for the inhibitors were 27 × 103 and 1.16×10^3 μM, respectively. Similarly, the concentration of cefazolin required for the study of catalytic activity (k_{cat}) was also too high and could not be detected with our equipment, indicating the low catalytic activity of SHV-57. On the other hand, SHV-57 had relatively higher catalytic activities for ceftazidime and penicillin G than for cefazolin. The hydrolytic efficiencies (k_{cat}/K_m) of the purified enzyme toward benzylpenicillin and ceftazidime were 5.67×10^{-5} and 2.78×10^{-5} μM^{-1} s^{-1}, respectively. Since the hydrolytic efficiency (k_{cat}/K_m) of cefazolin was not detectable, ceftazidime and penicillin G are relatively good substrates for SHV-57.

Structural model of SHV-57. The substitution of Arg for Leu169 induced a conformational change in the Asn170 residue, which was located at the site proximal to the moieties attached to the C-7 position of the cephalosporin skeleton.

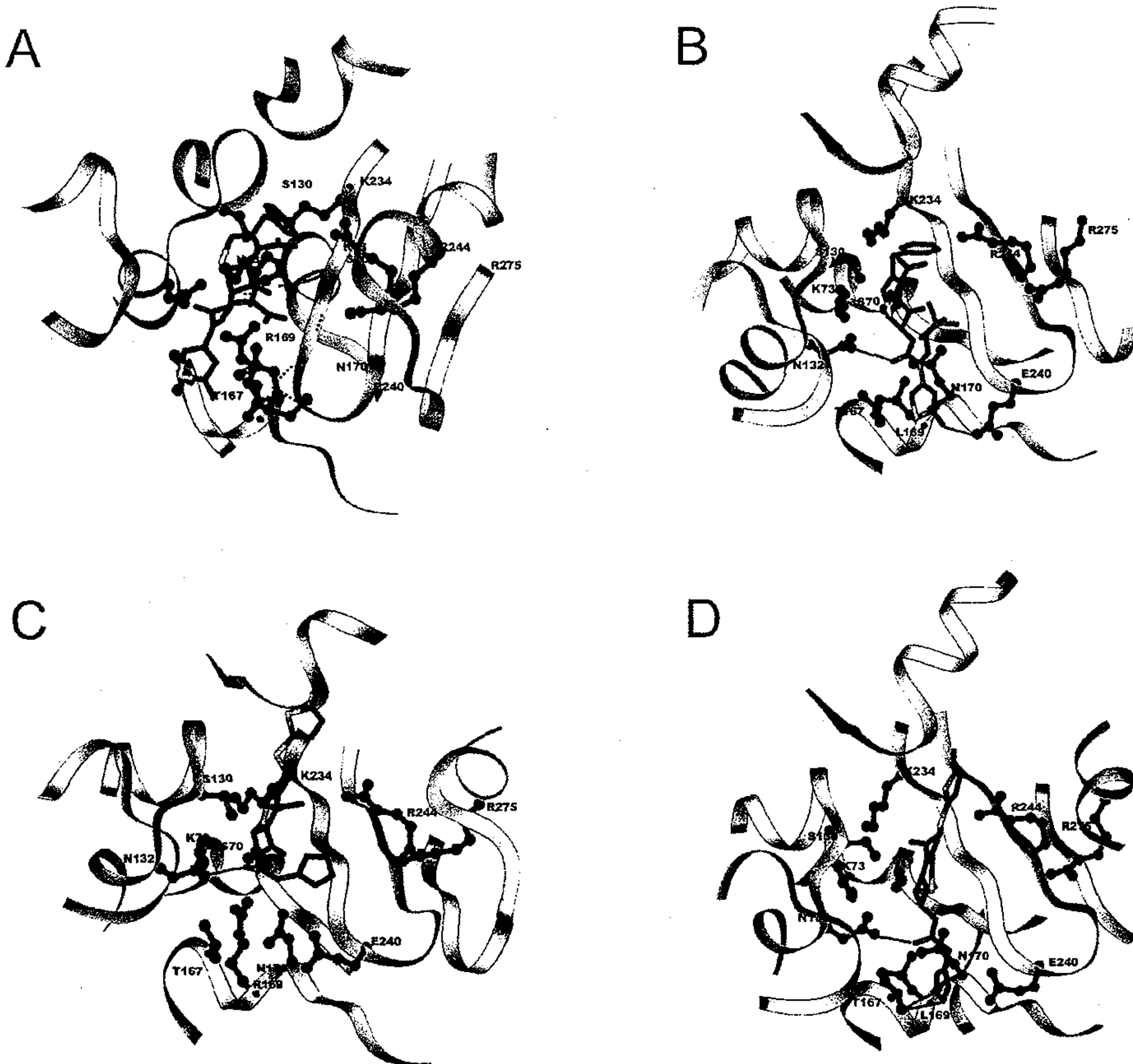

FIG. 2. (A and B) Complex structure model of ceftazidime in SHV-57 and SHV-1, respectively. Ceftazidime is shown by the stick model. The carbon atoms are in light blue; and the oxygen, nitrogen, and sulfur atoms are in red, dark blue, and yellow, respectively, unless indicated otherwise. Only the main chain of SHV-57 within 12 Å of ceftazidime is shown by the ribbon model. Major residues proximal to ceftazidime are shown by the ball-and-stick model. The carbon atoms of the residues are in green, unless indicated otherwise. The residues are indicated by one-letter code. The red dotted lines indicate the hydrogen bonds between ceftazidime and the enzyme. (C and D) Complex structure models of cefazolin in SHV-57 and SHV-1, respectively. Cefazolin is shown by the stick model. Only the main chain of SHV-57 within 12 Å of cefazolin is shown by the ribbon model. Major residues proximal to cefazolin are shown by the ball-and-stick model. The colors of the various elements are as defined for panels A and B.

Thus, the aminothiazole moiety of ceftazidime bound favorably in the pocket formed by Asn170 and Glu240, which were located within hydrogen bond distances of the positively charged amino group of the aminothiazole moiety. In addition, Asn170 formed a hydrogen bond with the carboxylate group of the dimethyl-carboxymethyloxime moiety (Fig. 2A). Since in the crystal structure of SHV-1 Asn170 has a different conformation which does not permit the formation of favorable hydrogen bonds with ceftazidime (Fig. 2B), the hydrogen bonds should play a crucial role in the binding of ceftazidime in SHV-57. Thus, ceftazidime is a good substrate for SHV-57. In contrast, the tetrazole moiety of cefazolin is electrostatically negative, and thus, interactions between the tetrazole moiety and Asn170 are unfavorable. Moreover, an unfavorable electrostatic interaction between negatively charged Glu240 and the tetrazole moiety dislocated the tetrazole moiety away from

TABLE 3. Comparison of kinetic parameters between SHV-57 and SHV-1 β-lactamases

Drug	SHV-57				SHV-1			
	K_m (μM)	K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	K_m (μM)	K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
Penicillin G	67		3.8×10^{-3}	5.67×10^{-5}	121		697	5.76
Nitrocefin	130		444	3.42	15.5		19.7	1.27
Cefazolin	5.57×10^3		UD[a]	UD	8		14.3	1.79
Ceftazidime	30.9		8.6×10^{-4}	2.78×10^{-5}	UD		UD	UD
Tazobactam		1.16×10^3				2.43		
Clavulanic acid		27×10^3				0.56		

[a] UD, undetectable.

the binding site (Fig. 2C). Asn170 in SHV-1 does not interfere with the binding of the tetrazole moiety, which formed a favorable hydrogen bond with Thr167. In addition, the tetrazole moiety had a conformation that avoided an unfavorable electrostatic interaction with the carboxylate group of Glu240. Therefore, whereas SHV-1 binds to cefazolin with favorable interactions at the tetrazole binding site (Fig. 2D), SHV-57 has unfavorable interactions with the tetrazole moiety; and thus, cefazolin is stable against SHV-57.

DISCUSSION

SHV-57 is a plasmid-encoded class A ESBL. Most SHV-type ESBLs have the Gly238Ser substitution alone or combined with alterations at position 240 or 35 (G. A. Jacoby and K. Bush, http://www.lahey.org/studies/webt.htm). The substitution at amino acid position G238S is on the β strand (13). It is the premier substitution that preserves penicillin and cephalosporin resistance (8). Another common group of SHV-type ESBLs has a single amino acid substitution at the Asn179 residue on the Ω loop. These include SHV-6 (D179A), SHV-8 (D179N), and SHV-24 (D179G), which confer resistance to ceftazidime (12). The role of Asp179 has been well studied (2, 3, 12). The X-ray crystal structure of the mutant with the D179N mutation of the PC1 β-lactamase shows a disordered Ω loop (5). Crystallographic investigations of the structures of several class A β-lactamases have shown a salt bridge between Arg164 and Asp179 that anchors the base of the Ω loop (4–6, 9, 12, 19). Vakulenko et al. (20) changed Asp179 to 19 other amino acids by site-directed mutagenesis to disrupt the salt bridge between Arg164 and Asp179. Most of the substitutions for Asp179 increased the level of resistance to ceftazidime.

In our study, MIC data indicated that in SHV ESBLs, in addition to residue 179, residue 169 on the Ω loop also plays an important role in influencing substrates. Interestingly, the susceptibility pattern showed that the enzyme caused resistance to ceftazidime but susceptibility to cefazolin and is inhibited by the β-lactamase inhibitor clavulanic acid. On the contrary, kinetic studies showed that the β-lactamase inhibitors tazobactam and clavulanic acid did not have inhibitory actions on this enzyme. A discrepancy between MIC and kinetic data has also been described for other β-lactamases, such as MOX-1 (1). Perhaps the results of experiments conducted with pure enzyme in vitro are different from those conducted in vivo, in that bacteria can have other biochemical modifications under normal conditions. This contradiction between MIC and kinetic data needs further study.

SHV-57 is a clinical variant found in Taiwan. Because of the significant role that amino acid substitution plays in β-lactamase-mediated resistance, we elucidated the substrate recognition mechanism using a model for the enzyme-substrate complex. In conclusion, the substitution of arginine for leucine-169 in the Ω loop is important for substrate specificity and causes ceftazidime resistance.

ACKNOWLEDGMENT

This work was supported by the National Health Research Institutes of Taiwan.

REFERENCES

1. **Alba, J., C. Bauvois, Y. Ishii, M. Galleni, K. Masuda, M. Ishiguro, M. Ito, J. M. Frere, and K. Yamaguchi.** 2003. A detailed kinetic study of MOX-1, a plasmid-encoded class C beta-lactamase. FEMS Microbiol. Lett. **225:**183–188.
2. **Arlet, G., M. Rouveau, D. Bengoufa, M. H. Nicolas, and A. Philippon.** 1991. Novel transferable extended-spectrum beta-lactamase (SHV-6) from *Klebsiella pneumoniae* conferring selective resistance to ceftazidime. FEMS Microbiol. Lett. **65:**57–62.
3. **Bonomo, R. A., S. D. Rudin, and D. M. Shlaes.** 1997. OHIO-1 beta-lactamase mutants: Asp179Gly mutation confers resistance to ceftazidime. FEMS Microbiol. Lett. **152:**275–278.
4. **Herzberg, O.** 1991. Refined crystal structure of beta-lactamase from *Staphylococcus aureus* PC1 at 2.0 Å resolution. J. Mol. Biol. **217:**701–719.
5. **Herzberg, O., G. Kapadia, B. Blanco, T. S. Smith, and A. Coulson.** 1991. Structural basis for the inactivation of the P54 mutant of beta-lactamase from *Staphylococcus aureus* PC1. Biochemistry **30:**9503–9509.
6. **Herzberg, O., and J. Moult.** 1987. Bacterial resistance to beta-lactam antibiotics: crystal structure of beta-lactamase from *Staphylococcus aureus* PC1 at 2.5 Å resolution. Science **236:**694–701.
7. **Ho, M., L. C. McDonald, T. L. Lauderdale, L. L. Yeh, P. C. Chen, and Y. R. Shiau.** 1999. Surveillance of antibiotic resistance in Taiwan, 1998. J. Microbiol. Immunol. Infect. **32:**239–249.
8. **Hujer, A. M., K. M. Hujer, and R. A. Bonomo.** 2001. Mutagenesis of amino acid residues in the SHV-1 beta-lactamase: the premier role of Gly238Ser in penicillin and cephalosporin resistance. Biochim. Biophys. Acta **1547:**37–50.
9. **Jelsch, C., L. Mourey, J. M. Masson, and J. P. Samama.** 1993. Crystal structure of *Escherichia coli* TEM1 beta-lactamase at 1.8 Å resolution. Proteins **16:**364–383.
10. **Kliebe, C., B. A. Nies, J. F. Meyer, R. M. Tolxdorff-Neutzling, and B. Wiedemann.** 1985. Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. Antimicrob. Agents Chemother. **28:**302–307.
11. **Knothe, H., P. Shah, V. Kremery, M. Antal, and S. Mitsuhashi.** 1983. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection **11:**315–317.
12. **Knox, J. R., and P. C. Moews.** 1991. Beta-lactamase of Bacillus licheniformis 749/C. Refinement at 2 Å resolution and analysis of hydration. J. Mol. Biol. **220:**435–455.
13. **Kuzin, A. P., M. Nukaga, Y. Nukaga, A. Hujer, R. A. Bonomo, and J. R. Knox.** 2001. Inhibition of the SHV-1 beta-lactamase by sulfones: crystallographic observation of two reaction intermediates with tazobactam. Biochemistry **40:**1861–1866.
14. **Matthew, M., and A. M. Harris.** 1976. Identification of beta-lactamases by analytical isoelectric focusing: correlation with bacterial taxonomy. J. Gen. Microbiol. **94:**55–67.

15. **Mercier, J., and R. C. Levesque.** 1990. Cloning of SHV-2, OHIO-1, and OXA-6 beta-lactamases and cloning and sequencing of SHV-1 beta-lactamase. Antimicrob. Agents Chemother. **34:**1577–1583.
16. **National Committee for Clinical Laboratory Standards.** 2002. Performance standards for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.
17. **Raquet, X., J. Lamotte-Brasseur, E. Fonze, S. Goussard, P. Courvalin, and J. M. Frere.** 1994. TEM beta-lactamase mutants hydrolysing third-generation cephalosporins. A kinetic and molecular modelling analysis. J. Mol. Biol. **244:**625–639.
18. **Siu, L. K., P. L. Ho, K. Y. Yuen, S. S. Wong, and P. Y. Chau.** 1997. Transferable hyperproduction of TEM-1 beta-lactamase in *Shigella flexneri* due to a point mutation in the Pribnow box. Antimicrob. Agents Chemother. **41:**468–470.
19. **Strynadka, N. C., H. Adachi, S. E. Jensen, K. Johns, A. Sielecki, C. Betzel, K. Sutoh, and M. N. James.** 1992. Molecular structure of the acyl-enzyme intermediate in beta-lactam hydrolysis at 1.7 Å resolution. Nature **359:**700–705.
20. **Vakulenko, S. B., M. Toth, P. Taibi, S. Mobashery, and S. A. Lerner.** 1995. Effects of Asp-179 mutations in TEMpUC19 beta-lactamase on susceptibility to beta-lactams. Antimicrob. Agents Chemother. **39:**1878–1880.

JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2005, p. 458–461 Vol. 43, No. 1
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.1.458–461.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

Clonal Diversity of Metallo-β-Lactamase-Possessing *Pseudomonas aeruginosa* in Geographically Diverse Regions of Japan

Soichiro Kimura,[1] Jimena Alba,[1] Katsuaki Shiroto,[1] Reiko Sano,[2] Yoshihito Niki,[3] Shigefumi Maesaki,[4] Koji Akizawa,[5] Mitsuo Kaku,[6] Yuji Watanuki,[7] Yoshikazu Ishii,[1]* and Keizo Yamaguchi[1]

Department of Microbiology, Toho University School of Medicine, Tokyo[1]; Nara Medical University, Nara[2]; Kawasaki Medical School, Okayama[3]; Saitama Medical School, Saitama[4]; Hokkaido University Hospital, Sapporo[5]; Tohoku University Graduate School of Medicine, Miyagi[6]; and Kanagawa Cardiovascular and Respiratory Diseases Center, Kanagawa,[7] Japan

Received 24 March 2004/Returned for modification 30 April 2004/Accepted 1 June 2004

The aim of this study was to determine the distribution of metallo-β-lactamase-producing *Pseudomonas aeruginosa* in Japan and to investigate the molecular characteristics of resistance gene cassettes including the gene encoding this enzyme. A total of 594 nonduplicate strains of *P. aeruginosa* isolated from 60 hospitals throughout Japan in 2002 were evaluated. This study indicated that although the prevalence of imipenem-resistant *P. aeruginosa* has not increased compared to that found in previous studies, clonal distribution of the same strain across Japan is evident.

Class A, B, and D β-lactamases, as defined by Ambler et al., can hydrolyze carbapenems (1, 9). In particular, class B β-lactamases, termed metallo-β-lactamases, are an increasingly serious clinical problem because they have a very broad substrate profile that includes penicillins, expanded-spectrum cephalosporins, and carbapenems and excludes only monobactams, such as aztreonam. It has been reported that IMP-1 metallo-β-lactamase-producing *Serratia marcescens* was first isolated in Japan in 1991 (10). Recently, metallo-β-lactamase-producing *Pseudomonas aeruginosa* and *S. marcescens* probably have the highest incidence of isolation in Japan (7).

Most metallo-β-lactamase genes are located on integrons, which are genetic elements containing gene cassettes that can facilitate their spread and mobilize the genes to other integrons or to other sites. The gene cassettes often encode clinically important antibiotic resistance genes, including those encoding β-lactamases such as extended-spectrum β-lactamases and carbapenemases, and also aminoglycoside-modifying enzymes (12).

Little is known about the distribution of the clone(s) that produces metallo-β-lactamases in Japan. Therefore, we conducted a surveillance study covering a wide geographic area with the aim of determining the distribution of metallo-β-lactamase producers in Japan and to investigate the molecular characteristics of the resistance gene cassettes that included the gene encoding a metallo-β-lactamase.

A total of 594 nonduplicate strains of *P. aeruginosa* isolated from 60 hospitals throughout Japan in the year 2002 were evaluated. The susceptibility of *P. aeruginosa* to several antibiotics was measured with the Etest strip, and the strains were stored on Casitone medium (Eiken Chemical Co. Ltd., Tokyo, Japan) (data not shown). After 6 months, the antibiotic susceptibility of these isolates was reassessed by the National Committee for Clinical Laboratory Standards broth microdilution method with cation-adjusted Mueller-Hinton broth (Difco, Detroit, Mich.). The isolates were screened for the presence of metallo-β-lactamase by a double-disk synergy test reported by Arakawa et al. (2). Integron analysis was performed by PCR mapping (5′-conserved segment *intI* to 3′-conserved segment *qacEΔ1*) of the typical antibiotic resistance genes and integron with specific primer sets (Table 1). The specificity of the primer sets for bla_{IMP-1}-like and bla_{VIM-2}-like gene was confirmed with positive-control strains producing IMP-1 or VIM-2 metallo-β-lactamase. The specificity of amplicons obtained by specific primer sets (*aacA4*, *aadA1*, *aadA2*, and bla_{OXA-2}) was also partially verified with the automatic sequencer ABI Prism 310 genetic analyzer (Applied Biosystems/Perkin-Elmer Biosystems). PCR with Ex *Taq* polymerase (Takara Bio, Inc., Tokyo, Japan) were carried out by standard methodology (13). pulsed-field gel electrophoresis analysis was performed by a modified method of the standard protocol (6). The restriction enzyme used was SpeI (15). By use of the dendrogram, isolates with a genetic relatedness of >80% were

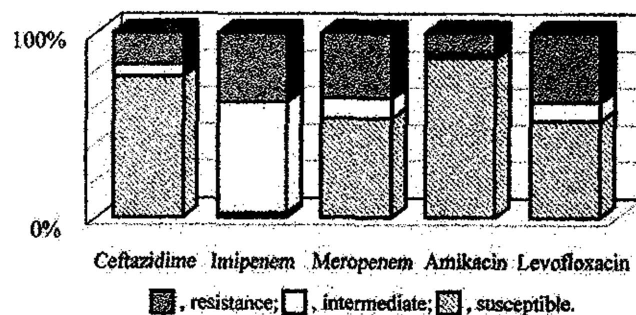

FIG. 1. Antimicrobial susceptibilities of imipenem-nonsusceptible *P. aeruginosa* isolates.

* Corresponding author. Mailing address: Department of Microbiology, Toho University School of Medicine, 5-21-16 Omori-nishi, Ota-ku, Tokyo 1438540, Japan. Phone: 81-3-3762-4151, ext. 2396. Fax: 81-3-5493-5415. address: E-mail: yoishii@med.toho-u.ac.jp.

TABLE 1. Nucleotide sequences of PCR primers used in this study

Gene[a]	Primer sequence (5′ to 3′)	T_m (°C)	Reference
*int*A(S)	ATC ATC GTC GTA GAG ACG TCG G	67.4	11
intB(AS)	GTC AAG GTT CTG GAC CAG TTG C	66.9	11
bla_{IMP-1}(S)	CTA CCG CAG CAG AGT CTT TG	62.7	This study
bla_{IMP-1}(AS)	AAC CAG TTT TGC CTT ACC AT	59.9	This study
bla_{VIM-2}(S)	AAA GTT ATG CCG CAC TCA CC	63.9	This study
bla_{VIM-2}(AS)	TGC AAC TTC ATG TTA TGC CG	64.5	This study
aacA4(S)	GAC CTT GCG ATG CTC TAT GAG TGG CTA AAT	73.0	This study
aacA4(AS)	TTC GCT CGA ATG CCT GGC GTG TT	76.9	This study
aadA1(S)	TGA TCG CCG AAG TAT CGA CTC	66.3	This study
aadA1(AS)	CCT TGG TGA TCT CGC CTT TC	65.8	This study
aadA2(S)	TTC GAA CCA ACT ATC AGA GGT GCT AA	67.4	This study
aadA2(AS)	AAA GCG AAT AAA TTC TTC CAA GTG ATC T	66.4	This study
bla_{OXA-2}(S)	CAA TCC GAA TCT TCG CGA TAC TT	66.9	This study
bla_{OXA-2}(AS)	AAG TAT CGC GAA GAT TCG GAT TG	66.9	This study
*qacE*Δ*1*	CTC TCT AGA TTT TAA TGC GGA TG	60.6	This study

[a] (S), sense; (AS), antisense.

considered to represent the same pulsed-field gel electrophoresis type (4).

Eighty-eight (15%) of 594 isolates were not susceptible (MIC ≥ 8 mg/ml) to imipenem. Among 88 isolates, 88 (100%), 21 (24%), 41 (47%), 12 (14%), and 42 (48%) were not susceptible to imipenem, ceftazidime, meropenem, amikacin, and levofloxacin, respectively (Fig. 1). Screening of metallo-β-lactamase producers was carried out for these isolates by the double-disk synergy test. Eleven (1.9%) of 594 isolates were found to produce metallo-β-lactamase. Ten of these isolates were IMP-1-like, and the other was a VIM-2-like metallo-β-lactamase producer.

The type of metallo-β-lactamase gene was also confirmed by PCR. The genetic relatedness of these isolates was also evaluated by pulsed-field gel electrophoresis as described above (Fig. 2, Table 2). Strains TUM1683, TUM1708, TUM1709, TUM1710, and TUM1732 had related electrophoresis chromosomal DNA banding patterns, whereas other strains (TUM1672, TUM1673, TUM1682, TUM1721, TUM1733, and TUM1757) showed different banding patterns. Strain TUM1708, TUM1709, and TUM1710 were isolated from same hospital, suggesting nosocomial spread. Interestingly, although strains TUM1683, TUM1708 (or TUM1709 and TUM1710), and TUM1732 has been isolated in different hospitals, Kawasaki, Saitama, and Nara, respectively, these isolates had related patterns. Since the distance from Okayama to Saitama and from Saitama to Nara is about 800 and 400 km, respectively, the results observed suggested clonal spread of metallo-β-lactamase-producing strains.

Several researchers have reported an incidence of metallo-β-lactamase-producing *P. aeruginosa* of between 0.4 and 1.3% in Japan from 1992 to 2002 (5, 7, 14, 16). In this study, we isolated 1.9% metallo-β-lactamase-producing *P. aeruginosa* strains from geographically diverse regions in Japan. We suggest that the incidence of metallo-β-lactamase-possessing *P. aeruginosa* has not increased during the past decade. However, the same clone of metallo-β-lactamase-carrying *P. aeruginosa* has now spread throughout Japan.

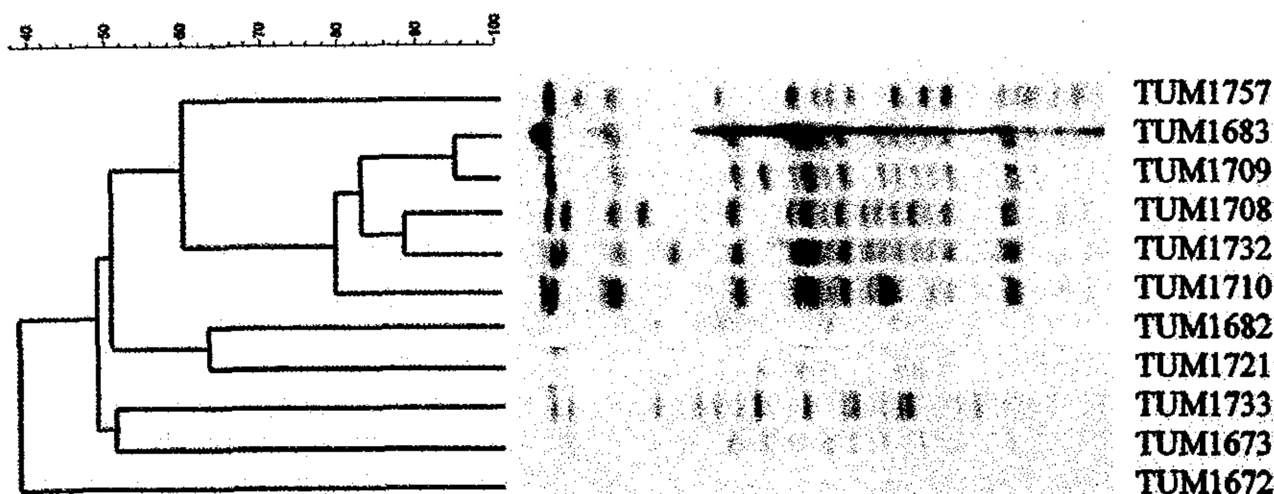

FIG. 2. Pulsed-field gel electrophoresis profiles obtained with SpeI chromosomal digestion of metallo-β-lactamase-carrying *P. aeruginosa*. The second through sixth lanes contained related strains TUM1683, TUM1709, TUM1708, TUM1732, and TUM1710, respectively. Lanes first and seventh to eleventh lanes contained unrelated strains TUM1757, TUM1682, TUM1721, TUM1733, TUM1673, and TUM1672, respectively.

TABLE 2. Characteristics of bla_{IMP}-containing non-imipenem-susceptible *P. aeruginosa* isolates

Strain	Hospital no.	Material	Type of enzyme	Pattern[b]	Integron structure[c]	MIC (μg/ml)[a]									
						CAZ	IPM	MEM	LVX	AZT	AMK	NET	GEN	KAN	ABK
TUM1672	1	Urine	VIM-2-like	A	I	64	>128	>128	16	32	0.06	0.5	0.5	8	0.06
TUM1673	1	Sputum	IMP-1-like	B	II	>128	8	32	16	8	64	>128	4	>128	16
TUM1682	2	Sputum	IMP-1-like	C	III	>128	64	>128	32	32	32	>128	2	>128	2
TUM1683	2	Sputum	IMP-1-like	D	IV	>128	64	>128	32	64	16	>128	2	>128	2
TUM1708	3	Urine	IMP-1-like	D	IV	>128	64	>128	32	32	32	>128	4	>128	4
TUM1709	3	Urine	IMP-1-like	D	IV	>128	64	>128	32	32	32	>128	4	>128	2
TUM1710	3	Urine	IMP-1-like	D	IV	>128	64	>128	32	64	32	>128	2	>128	4
TUM1721	4	Urine	IMP-1-like	E	V	>128	64	>128	32	32	32	>128	>128	>128	64
TUM1732	5	Urine	IMP-1-like	D	IV	>128	64	>128	32	128	32	>128	4	>128	2
TUM1733	5	Pus	IMP-1-like	F	VI	>128	64	>128	64	32	2	>128	>128	>128	1
TUM1757	6	Sputum	IMP-1-like	G	VII	>128	64	>128	16	16	32	>128	1	>128	16

[a] CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; LVX, levofloxacin; AZT, aztreonam; AMK, amikacin; NET, netilmicin; GEN, gentamicin; KAN, kanamycin; ABK, arbekacin.

[b] PEGE profiles obtained with SpeI chromosomal digestion of *P. aeruginosa* carrying a metallo-β-lactamase gene as recommended by Tenover et al. (15).

[c] Integron structures possessed by each gene as mentioned in the text. I, bla_{VIM-2}-like, *aacA4* and *aadA2*; II, bla_{IMP-1}-like, *aadA1* and *orfG*; III, bla_{IMP-1}-like, *aadA1* and unknown gene; IV, bla_{IMP-1}-like, *aadA1* and unknown gene; V, bla_{IMP-1}-like, *aacA4*, *aadA1* and bla_{OXA-2}; VI, bla_{IMP-1}-like, *aacA4*; VII, only bla_{IMP-1}-like gene.

It has been reported that genetic analysis of bla_{IMP-1} revealed features typical of an integron-located gene (9). The detection of a type 1 integron was confirmed in 11 strains. In these strains, bla_{IMP-1}-like or bla_{VIM-2}-like genes were located immediately downstream of the *IntI*1 integrase gene. However, these isolates possessed a variety of gene cassettes, such as the *aacA4* aminoglycoside 6′-*N*-acetyltransferase gene and *aadA1* and *aadA2* aminoglycoside adenyltransferase genes between the metallo-β-lactamase gene and *qacΔE1*. Therefore, these isolates are likely resistant not only to β-lactams but also to aminoglycosides. Interestingly, strain TUM1721 possessed not only the bla_{IMP-1}-like genes *aacA4* and *aadA1* but also an OXA-type β-lactamase gene on the integron gene cassette.

Little is known about optimal chemotherapy for infection due to metallo-β-lactamase-producing *P. aeruginosa*. To detail the antibiotic susceptibility of *P. aeruginosa* possessing a metallo-β-lactamase, the MICs of several antibiotics were evaluated (Table 2). All of the isolates were resistant to ceftazidime, meropenem, and levofloxacin. Ten of the 11 were resistant to imipenem and netilmicin, nine were resistant to aztreonam, and eight were not susceptible to amikacin. Bellais et al. reported that chemotherapy with high aztreonam doses effectively reduced viable cells of a metallo-β-lactamase-producing strain of *P. aeruginosa* in a rat pneumonia model (3). In general, although metallo-β-lactamases do not hydrolyze aztreonam, 9 of 11 isolates were resistant to aztreonam in this study (MIC $\geq$ 32 μg/ml). On the other hand, arbekacin was found to suppress the growth of some isolates in this study. In Japan, arbekacin, which has fewer side effects than vancomycin, has been used against methicillin-resistant *Staphylococcus aureus* (8). Recently, arbekacin-resistant *P. aeruginosa* possessing the 16S rRNA methylase gene *rmtA* was isolated in Japan (17). However, the incidence of these isolates is still low (0.8%, 9 of 1,113 clinical isolates). Therefore, arbekacin could be used as treatment against metallo-β-lactamase-possessing *P. aeruginosa*.

In conclusion, this study indicates that although the prevalence of metallo-β-lactamase-producing *P. aeruginosa* has not increased, this pathogen has spread from a single source to a wide geographic area of Japan. Further surveillance and monitoring of multidrug-resistant *P. aeruginosa* should be a high priority.

This study was supported by grants from the Ministry of Health, Labor and Welfare of Japan during 2003 (H15-Iyaku-003 and H15-shinkou-009). S.K. was supported by a grant from the Society of Japanese Pharmacopoeia.

We thank Kenneth S. Thomson, Creighton University School of Medicine, for useful advice. We also thank Kunimoto Hotta, National Institute of Infectious Diseases, for helpful discussions.

REFERENCES

1. **Ambler, R. P.** 1980. The structure of β-lactamases. Phil. Trans R. Soc. Lond. B Biol. Sci. **289**:321–331.
2. **Arakawa, Y., N. Shibata, K. Shibayama, H. Kurokawa, T. Yagi, H. Fujiwara, and M. Goto.** 2000. Convenient test for screening metallo-β-lactamase-producing gram-negative bacteria by using thiol compounds. J. Clin. Microbiol. **38**:40–43.
3. **Bellais, S., O. Mimoz, S. Leotard, A. Jacolot, O. Petitjean, and P. Nordmann.** 2002. Efficacy of β-lactams for treating experimentally induced pneumonia due to a carbapenem-hydrolyzing metallo-β-lactamase-producing strain of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **46**:2032–2034.
4. **Duck, W. M., C. D. Steward, S. N. Banerjee, J. E. McGowan, Jr., and F. C. Tenover.** 2003. Optimization of computer software settings improves accuracy of pulsed-field gel electrophoresis macrorestriction fragment pattern analysis. J. Clin. Microbiol. **41**:3035–3042.
5. **Hirakata, Y., K. Izumikawa, T. Yamaguchi, H. Takemura, H. Tanaka, R. Yoshida, J. Matsuda, M. Nakano, K. Tomono, S. Maesaki, M. Kaku, Y. Yamada, S. Kamihira, and S. Kohno.** 1998. Rapid detection and evaluation of clinical characteristics of emerging multiple-drug-resistant gram-negative rods carrying the metallo-β-lactamase gene bla_{IMP}. Antimicrob. Agents Chemother. **42**:2006–2011.
6. **Ishii, Y., J. Alba, S. Kimura, K. Nakashima, Y. Abe, and K. Yamaguchi.** 2002. Rapid pulsed-field gel electrophoresis technique for determination of genetic diversity of *Serratia marcescens*. J. Infect. Chemother. **8**:368–370.
7. **Kurokawa, H., T. Yagi, N. Shibata, K. Shibayama, and Y. Arakawa.** 1999. Worldwide proliferation of carbapenem-resistant gram-negative bacteria. Lancet **354**:955.
8. **Matsuo, H., M. Kobayashi, T. Kumagai, M. Kuwabara, and M. Sugiyama.** 2003. Molecular mechanism for the enhancement of arbekacin resistance in a methicillin-resistant *Staphylococcus aureus*. FEBS Lett. **546**:401–406.
9. **Nordmann, P., and L. Poirel.** 2002. Emerging carbapenemases in Gram-negative aerobes. Clin. Microbiol. Infect. **8**:321–331.
10. **Osano, E., Y. Arakawa, R. Wacharotayankun, M. Ohta, T. Horii, H. Ito, F. Yoshimura, and N. Kato.** 1994. Molecular characterization of an enterobacterial metallo β-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob. Agents Chemother. **38**:71–78.
11. **Rosser, S. J., and H. K. Young.** 1999. Identification and characterization of class 1 integrons in bacteria from an aquatic environment. J. Antimicrob. Chemother. **44**:11–18.
12. **Rowe-Magnus, D. A., and D. Mazel.** 2002. The role of integrons in antibiotic resistance gene capture. Int. J. Med. Microbiol. **292**:115–125.
13. **Sambrook, J., and D. W. Russell.** 2001. In vitro amplification of DNA by the polymerase chain reaction, p. 8.1–8.126. In J. Sambrook and D. W. Russell (ed.), Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

14. **Senda, K., Y. Arakawa, K. Nakashima, H. Ito, S. Ichiyama, K. Shimokata, N. Kato, and M. Ohta.** 1996. Multifocal outbreaks of metallo-β-lactamase-producing *Pseudomonas aeruginosa* resistant to broad-spectrum β-lactams, including carbapenems. Antimicrob. Agents Chemother. **40:** 349–353.
15. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
16. **Yamasaki, K., M. Komatsu, T. Yamashita, K. Shimakawa, T. Ura, H. Nishio, K. Satoh, R. Washidu, S. Kinoshita, and M. Aihara.** 2003. Production of CTX-M-3 extended-spectrum β-lactamase and IMP-1 metallo β-lactamase by five Gram-negative bacilli: survey of clinical isolates from seven laboratories collected in 1998 and 2000, in the Kinki region of Japan. J. Antimicrob. Chemother. **51:**631–638.
17. **Yokoyama, K., Y. Doi, K. Yamane, H. Kurokawa, N. Shibata, K. Shibayama, T. Yagi, H. Kato, and Y. Arakawa.** 2003. Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. Lancet **362:**1888–1893.

Antimicrobial susceptibility testing of vancomycin-resistant *Enterococcus* by the VITEK 2 system, and comparison with two NCCLS reference methods

Intetsu Kobayashi,[1] Hiroe Muraoka,[1] Takako Iyoda,[1] Minoru Nishida,[1] Miyuki Hasegawa[1] and Keizou Yamaguchi[2]

Correspondence
Intetsu Kobayashi
mbc-ka@sa2.so-net.ne.jp

[1]Chemotherapy Division, Mitsubishi Kagaku Bio-Clinical Laboratories Inc., 3-30-1 Shimura, Itabashi-ku, Tokyo 174-8555, Japan

[2]Department of Microbiology, Toho University of Medicine, Tokyo, Japan

Received 3 June 2004
Accepted 17 August 2004

We evaluated the automated VITEK 2 system (bioMérieux) for antimicrobial susceptibility testing of vancomycin-resistant *Enterococcus* (VRE). The results obtained with the VITEK 2 system were compared to those obtained using two NCCLS reference methods. The VITEK 2 system produced MICs for penicillin G, erythromycin and vancomycin that were very similar to those of the reference agar-dilution test with all results being within a twofold dilution. When MICs of teicoplanin for these isolates were measured by the agar-dilution method and VITEK 2 system, there was one 'very major' error and seven 'minor' errors. There were no 'major' errors for any of the antibiotics tested. When the results obtained by the micro broth-dilution method were compared with those obtained by the VITEK 2 system, there was one 'very major' error for teicoplanin by the VITEK 2 system, as was the case with the agar-dilution method. There were two 'minor' errors for erythromycin and seven 'minor' errors for teicoplanin. There were no 'major' errors for any of the antibiotics tested. The 35 VRE strains identified phenotypically by the VITEK 2 Advanced Expert System included nine of *Enterococcus faecalis* and 23 of *Enterococcus faecium*. Neither *Enterococcus avium* nor *Enterococcus hirae* were identified. A total of 32 phenotypes were classified into 22 VanA and 10 VanB strains. PCR genotyping demonstrated 23 *vanA*$^+$ and nine *vanB*$^+$ strains. There were differences between the VITEK 2 system results and those of PCR. Overall, 54·3 % of the test results were obtained within 7 h. All MIC values for the 35 VRE isolates were determined within 13 h of completing incubation. The VITEK 2 system is a simple method for accurately detecting vancomycin-resistant strains of *Enterococcus* and can be used to rapidly determine MICs.

INTRODUCTION

Vancomycin-resistant *Enterococcus* (VRE) has increasingly been implicated as a causative pathogen in nosocomial infections (CDC, 1993; Murray, 1995; Tokars *et al.*, 1999). Vancomycin-resistant strains of *Enterococcus* are generally resistant not only to vancomycin but also to various types of commercially available antibiotics, and can cause severe infections in compromised hosts (French, 1998). Rapid identification of the infectious bacterial strain and its susceptibility to antibiotics are necessary from both the clinical and the economic viewpoint.

The VITEK 2 system is a recently developed automated method for rapid bacterial identification and antimicrobial susceptibility testing. The usefulness of the VITEK 2 system for identifying *Enterococcus* species has already been reported (van den Braak *et al.*, 2001; Garcia-Garrote *et al.*, 2000). However, different genotypes (*vanA*, *vanB*) were found to encode either high, intermediate or low levels of resistance glycopeptides, mainly in *Enterococcus faecium* and *Enterococcus faecalis* (Perichon *et al.*, 1997; Fines *et al.*, 1999). The difficulties encountered by several automated susceptibility tests in accurately detecting bacterial resistance to vancomycin have also been described (van den Braak *et al.*, 2001; Sahm & Olsen, 1990).

The present study was designed to evaluate the ability of the VITEK 2 system to determine VRE susceptibility.

Abbreviation: VRE, vancomycin-resistant *Enterococcus*.

METHODS

Test strains. Thirty-five isolates of VRE species, including nine *Enterococcus faecalis*, 23 *Enterococcus faecium*, two *Enterococcus avium* and one *Enterococcus hirae*, obtained from clinical specimens (urine, faeces and blood) of patients with infections in several Japanese

hospitals were identified by the VITEK 2 system between March 1999 and September 2000.

Antimicrobial agents. The antimicrobial agents used in the reference micro broth-dilution panels and agar-dilution method were penicillin G, erythromycin, vancomycin and teicoplanin. All antibiotics were purchased from Sigma except for teicoplanin which was supplied by a pharmaceutical company (Aventis Pharma) as a standard powder with a known potency.

These antimicrobial agents were selected on the basis of antimicrobial agents which can be measured by the VITEK 2 system card according to NCCLS guideline M7-A5 (NCCLS, 2000a).

VITEK 2 system susceptibility tests. Antimicrobial susceptibilities of the test organisms were determined using the VITEK 2 system (software version 1.02) (bioMérieux) according to the manufacturer's recommendations.

The test organisms from colonies grown on Trypticase Soy agar (Becton Dickinson) after 18 h incubation were suspended in sterilized physiological saline to 0·5 McFarland standards. The bacterial suspension was used to fill the Antimicrobial Susceptibility Testing P516 card, which was then inserted into the incubator-reader of the VITEK 2 system.

Reference susceptibility tests. Susceptibility tests were also performed using two reference methods: the micro broth-dilution and agar-dilution with Mueller–Hinton broth and agar. These tests were performed according to NCCLS M7-A5 guidelines (NCCLS, 2000a) and M100-S10 guidelines (NCCLS, 2000b), respectively. The MICs were interpreted using the recommended NCCLS thresholds.

Discrepant MIC values. The interpretive category errors were estimated for each drug based on the following definitions: 'very major' error, susceptible by the VITEK 2 system but resistant by the agar and/or broth reference method; 'major' error, resistant by the VITEK 2 system but susceptible by the agar and/or broth reference method; 'minor' error, intermediately resistant by either the VITEK 2 system or the agar and/or broth reference method and either susceptible or resistant by the other method.

Amplification of *vanA* and *vanB* genes by PCR. PCR was used to detect *vanA* and *vanB* genes as previously described by Clark *et al.* (1993). The control organisms used for PCR were *E. faecalis* ATCC 51299 (*vanB*-positive), *E. faecium* C.I. (clinical isolate; *vanA*-positive) and *E. faecalis* ATCC 29212 (negative control).

RESULTS AND DISCUSSION

The MICs of the four antibiotics for 35 vancomycin-resistant isolates of *Enterococcus* were determined using the VITEK 2 system and the two reference methods (Table 1).

The MICs of penicillin G for these isolates were measured by the VITEK 2 system, micro broth-dilution or agar-dilution, allowing comparisons of the results obtained by these three methods. The MICs of penicillin G by the three methods were $\geq 16\ \mu g\ ml^{-1}$ (resistant region) for 28 strains and $\leq 8\ \mu g\ ml^{-1}$ (susceptible region) for seven strains. The results obtained by the three methods were the same for all strains. The majority of MICs for erythromycin in the resistance region (MIC $\geq 8\ \mu g\ ml^{-1}$) determined using the VITEK 2 system were the same as those determined by the reference methods. All 35 VRE were clearly demonstrated to be

Table 1. Comparison of MICs for 35 VRE isolates determined using the VITEK 2 system and two reference methods

Method	No. isolates with the following MIC ($\mu g\ ml^{-1}$)																											
	Penicillin G						Erythromycin								Vancomycin						Teicoplanin							
	S				R		S				I			R	S			I		R	S						I	R
	≤1	2	4	8	16	≥32	0·06	0·12	0·25	0·5	1	2	4	≥8	1	2	4	8	16	≥32	0·25	0·5	1	2	4	8	16	≥32
VITEK 2 system	0	1	1	5	3	25	0	0	2	0	1	0	1	31	0	0	0	0	0	35	0	7	4	0	0	0	6	18
Micro broth-dilution	2	4	0	1	3	25	0	1	0	1	0	1	1	31	0	0	0	0	0	35	0	2	3	3	2	0	1	24
Agar-dilution	0	2	4	1	3	25	0	1	0	1	0	1	1	31	0	0	0	0	0	35	5	2	2	0	0	1	1	24

resistant to vancomycin using the three methods, and no differences were observed between the VITEK 2 system and the reference method in MIC distribution patterns.

Teicoplanin MICs in the resistance region (MIC $\geqslant$32 $\mu g\ ml^{-1}$) were demonstrated in 24 of the 35 strains using the reference methods, whereas 18 strains were determined to be resistant to teicoplanin using the VITEK 2 system. Furthermore, one and six of the 35 strains were intermediately resistant when MICs were determined using the reference methods and the VITEK 2 system, respectively. The teicoplanin MICs for the other 11 strains (31·4 % of the 35 strains) were 0·5–1 $\mu g\ ml^{-1}$, in the susceptibility region when determined by the VITEK 2 system, but these MICs showed a broad distribution between concentrations of 0·5 and 4 $\mu g\ ml^{-1}$ when determined by the micro broth-dilution method and between 0·25 and 8 $\mu g\ ml^{-1}$ when determined by the agar-dilution method. Although MICs obtained by the VITEK 2 system and the two reference methods generally agreed, the prevalences of 'very major', 'major' and 'minor' errors in the MICs obtained by the VITEK 2 system were determined (Table 2). With the agar-dilution method there was one 'very major' error and seven 'minor' errors for teicoplanin while there were no major errors with the VITEK 2 system. There were no errors for any of the other antibiotics.

When the MICs obtained by the micro broth-dilution method were compared with those obtained by the VITEK 2 system, there was one 'very major' error with the VITEK 2 system for teicoplanin, as was the case for the agar-dilution method, but there were no 'major' errors. However, there were seven 'minor' errors for teicoplanin and two 'minor' errors for erythromycin.

The *vanA*- and *vanB*-mediated resistance of the 35 vancomycin-resistant isolates of *Enterococcus* was analysed according to the method of Clark *et al.* (1993) after PCR amplification; the results obtained were then compared with the phenotypes of the isolates determined by the VITEK 2 system. The strain determined to be neither *vanA*$^{+}$ nor *vanB*$^{+}$ by both methods was an *E. hirae*, and two *E. avium* strains could not be identified by the VITEK 2 system alone. Thirty-two strains were identifiable by both methods. Of these, the strains identified by VITEK 2 system phenotyping included 22 VanA and 10 VanB, while PCR genotyping identified 23 *vanA*$^{+}$ and nine *vanB*$^{+}$ strains. The MIC of teicoplanin against the *E. faecium* strain (no. 14) was 1 $\mu g\ ml^{-1}$ by the VITEK 2 system and the phenotype of this strain was determined to be VanB. The MICs of teicoplanin against this strain were 32 $\mu g\ ml^{-1}$ by the agar- and broth-dilution methods. PCR showed the genotype of this strain to be *vanA*$^{+}$.

The times required to obtain the final MICs of vancomycin for the 35 vancomycin-resistant isolates of *Enterococcus* are shown in Table 3. Overall, 54·3 % of the test results were obtained within 7 h. MIC values for all 35 isolates were determined within 13 h of incubation.

VITEK 2 systems have recently been adopted by many microbiological laboratories for rapid identification and the determination of antimicrobial susceptibilities of various types of pathogens, including VRE (van den Braak *et al.*, 2001; Funke *et al.*, 1998; Garcia-Garrote *et al.*, 2000). The prevalence of VRE infections in compromised hosts has become a serious problem (Jochimsen *et al.*, 1999), and treatment options are limited (Saraiva *et al.*, 1997). To control the transmission of VRE and outbreaks of cross-infection within hospitals and the community, several technical problems involving laboratory tests must be overcome. Given the current situation with VRE infections, it is necessary to confirm the speed and accuracy of the VITEK 2 system in determining MICs for VRE. Furthermore, rapid detection of resistant phenotypes in VRE isolates from patients is essential for prompt and effective antibiotic treatment.

Table 3. Times required to obtain the final results of susceptibility testing in 35 VRE isolates using the VITEK 2 system

Time (h)	No. isolates (%)*
< 6	5 (14·3)
6–7	14 (40·0)
8–9	13 (37·1)
10–11	2 (5·7)
12–13	1 (2·9)

*Number and percentage of isolates whose testing results were available at the indicated times of incubation.

Table 2. Interpretive category errors of the VITEK 2 system in susceptibility testing of 35 VRE isolates compared to that of the reference methods

Antibiotic	No. errors/agar-dilution			No. errors/broth-dilution		
	'Very major'	'Major'	'Minor'	'Very major'	'Major'	'Minor'
Penicillin G	0	0	No criteria	0	0	No criteria
Erythromycin	0	0	0	0	0	2
Vancomycin	0	0	0	0	0	0
Teicoplanin	1	0	7	1	0	7

We evaluated the ability of the VITEK 2 system to determine the antimicrobial susceptibility of recent VRE isolates using penicillin G, erythromycin, vancomycin and teicoplanin as test antibiotics. The strains which were determined to be resistant to penicillin G, erythromycin and vancomycin by the VITEK 2 system were also resistant according to both reference methods employed. There were differences between the MICs of these antibiotics by the VITEK 2 system and the reference methods, but susceptible strains were not identified as being resistant and resistant strains were not indicated to be susceptible.

There was one 'very major' error and seven 'minor' errors when the MICs of teicoplanin were calculated using the VITEK 2 system, as compared with the reference method results. The failure of VITEK 2 to determine resistance to teicoplanin has already been reported (van den Braak *et al.*, 2001; Garcia-Garrote *et al.*, 2000). Some vancomycin-resistant strains of *Enterococcus* were phenotypically classified as VanB by the VITEK 2 system in the study by van den Braak *et al.* (2001).

In the present study, there was one 'very major' error in the teicoplanin MIC for one strain (VRE no. 14), i.e. the MIC was 1 $\mu g\ ml^{-1}$ using the VITEK 2 system but 32 $\mu g\ ml^{-1}$ by the two reference methods. This error was considered to be the same as that reported by van den Braak *et al.* (2001). However, the VITEK 2 system detected VRE accurately and distinguished them from the 11 teicoplanin-susceptible strains; the VITEK 2 system results corresponded well with those obtained using the two reference methods.

The VITEK 2 system is easy to use and provides accurate results in detecting resistance of *Enterococcus* species to penicillin G, erythromycin and glycopeptides; this system can also be used to determine the antimicrobial susceptibility of *Enterococcus* including vancomycin-resistant isolates. One of the major advantages of the VITEK 2 system is the significant reduction in handling time as compared with conventional test procedures.

A 24 h incubation period is needed to determine the MICs of vancomycin or teicoplanin for VRE by either the micro broth-dilution or the agar-dilution method according to NCCLS guidelines (2000b). However, the MICs of these antibiotics for 35 VRE isolates were determined within 13 h by the VITEK 2 system, with the phenotypes of all 35 isolates being simultaneously determined during this period. The VITEK 2 system promises to expedite work in clinical microbiological laboratories. Furthermore, the VITEK 2 system may play an important role in the investigation of nosocomial VRE infections.

REFERENCES

CDC (1993). Nosocomial enterococci resistant to vancomycin – United States, 1989–1993. *Morb Mortal Wkly Rep* 42, 597–599.

Clark, N. C., Cooksey, R. C., Hill, B. C., Swenson, J. M. & Tenover, F. C. (1993). Characterization of glycopeptide-resistant enterococci from U.S. hospitals. *Antimicrob Agents Chemother* 37, 2311–2317.

Fines, M., Perichon, B., Reynolds, P., Sahm, D. F. & Courvalin, P. (1999). VanE, a new type of acquired glycopeptide resistance in *Enterococcus faecalis* BM4405. *Antimicrob Agents Chemother* 43, 2161–2164.

French, G. L. (1998). Enterococci and vancomycin resistance. *Clin Infect Dis* 27 (Suppl. 1), S75–S83.

Funke, G., Monnet, D., deBernardis, C., von Graevenitz, A. & Freney, J. (1998). Evaluation of the VITEK 2 system for rapid identification of medically relevant gram-negative rods. *J Clin Microbiol* 36, 1948–1952.

Garcia-Garrote, F., Cercenado, E. & Bouza, E. (2000). Evaluation of a new system, VITEK 2, for identification and antimicrobial susceptibility testing of enterococci. *J Clin Microbiol* 38, 2108–2111.

Jochimsen, E. M., Fish, L., Manning, K., Young, S., Singer, D. A., Baker, R., Jarvis, W. R. (1999). Control of vancomycin-resistant enterococci at a community hospital: efficacy of patient and staff cohorting. *Infect Control Hosp Epidemiol* 20, 106–109.

Murray, B. E. (1995). What can we do about vancomycin-resistant enterococci? *Clin Infect Dis* 20, 1134–1136.

NCCLS (2000a). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 5th edn. NCCLS document M7-A5. Villanova, PA: National Committee for Clinical Laboratory Standards.

NCCLS (2000b). Performance standards for antimicrobial susceptibility testing. Tenth informational supplement (aerobic dilution), M100-S10. Villanova, PA: National Committee for Clinical Laboratory Standards.

Perichon, B., Reynolds, P. & Courvalin, P. (1997). VanD-type glycopeptide-resistant *Enterococcus faecium* BM4339. *Antimicrob Agents Chemother* 41, 2016–2018.

Sahm, D. F. & Olsen, L. (1990). In vitro detection of enterococcal vancomycin resistance. *Antimicrob Agents Chemother* 34, 1846–1848.

Saraiva, I. H., Jones, R. N., Erwin, M. & Sader, H. S. (1997). Evaluation of antimicrobial sensitivity of 87 clinical isolates of vancomycin-resistant enterococci. *Rev Assoc Med Bras* 43, 217–222 (in Portuguese).

Tokars, J. I., Satake, S., Rimland, D. & 8 other authors (1999). The prevalence of colonization with vancomycin-resistant *Enterococcus* at a Veterans' Affairs institution. *Infect Control Hosp Epidemiol* 20, 171–175.

van den Braak, N., Goessens, W., van Belkum, A., Verbrugh, H. A. & Endtz, H. P. (2001). Accuracy of the VITEK 2 system to detect glycopeptide resistance in enterococci. *J Clin Microbiol* 39, 351–353.

International Journal of Infectious Diseases (2005) 9, 27–36

ELSEVIER

http://intl.elsevierhealth.com/journals/ijid

PROTEKT 1999–2000: a multicentre study of the antimicrobial susceptibility of respiratory tract pathogens in Japan

Matsuhisa Inoue[a,*], Shigeru Kohno[b], Mitsuo Kaku[c], Keizo Yamaguchi[d], Jun Igari[e], Kiyoharu Yamanaka[f]

[a]*Department of Microbiology, Kitasato University School of Medicine, Kanagawa, Japan*
[b]*Second Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, Japan*
[c]*Department of Molecular Diagnostics, Tohoku University Graduate School of Medicine, Sendai, Japan*
[d]*Department of Microbiology, Toho University School of Medicine, Tokyo, Japan*
[e]*Department of Clinical Pathology, Juntendo University School of Medicine, Tokyo, Japan*
[f]*Division of Clinical Laboratory, Otemae Hospital, Osaka, Japan*

Received 15 August 2003; received in revised form 17 February 2004; accepted 3 March 2004

Corresponding Editor: Michael Whitby, Brisbane, Australia

KEYWORDS
Respiratory pathogens;
Antimicrobial susceptibility;
Macrolide antibiotics;
Japan

Summary

Design: A six-centre study in Japan during the winter of 1999–2000 assessed the in vitro activity of >20 antimicrobial agents against the common respiratory pathogens *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. The minimum inhibitory concentrations (MIC) of each antimicrobial was determined against these isolates using National Committee for Clinical Laboratory Standards (NCCLS) methodology.
Results: Among *S. pneumoniae* isolates, 44.5% were penicillin resistant. The macrolide resistance rate was 77.9% with 90.5% of penicillin-resistant strains also being macrolide resistant. Resistance mechanisms in macrolide-resistant isolates were identified as *mef*(A) or *erm*(B) in 42.5% and 52.5%, respectively. Of the fluoroquinolone-resistant isolates (1.3%), most were also penicillin and macrolide resistant. All strains were inhibited by telithromycin at ≤1 mg/L. Among *S. pyogenes* isolates, erythromycin resistance was 17.5% overall but showed considerable variation among the six centres. For *H. influenzae*, 8.5% produced β-lactamase and a single β-lactamase-negative, ampicillin-resistant isolate (0.36%) was obtained, and there was no fluoroquinolone resistance. All isolates were susceptible to telithromycin.

* Corresponding author. Tel.: +81 42 778 9355; fax: +81 42 778 9350.
E-mail address: matsu@kitasato-u.ac.jp (M. Inoue).

1201-9712/$30.00 © 2004 International Society for Infectious Diseases. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijid.2004.03.008

Most antimicrobials showed good activity against M. catarrhalis, although 96.7% were β-lactamase positive.
Conclusion: The prevalence of antimicrobial resistance to macrolides, penicillin and the fluoroquinolones among the common respiratory pathogens is high in Japan.
© 2004 International Society for Infectious Diseases. Published by Elsevier Ltd. All rights reserved.

Introduction

The prevalence of resistant isolates of common bacterial respiratory tract pathogens is increasing, and nowhere more so than in Asia. In some Asian countries, penicillin resistance may be as high as 70%.[1–3] In the last decade, macrolide resistance has also increased dramatically, exceeding penicillin resistance in some areas,[2] and growing resistance to chloramphenicol, co-trimoxazole and tetracycline continues relentlessly.[4]

Most respiratory tract infections are viral in origin but are frequently followed by secondary infections resulting from opportunistic invasion by commensal respiratory bacteria. The four most important bacterial pathogens associated with community-acquired upper and lower respiratory tract infections (RTIs – acute/chronic sinusitis, acute/chronic otitis media, acute/chronic pharyngitis, community-acquired pneumonia, acute bacterial exacerbation of chronic bronchitis and acute bacterial exacerbation of chronic obstructive airways disease) are *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. Less commonly, atypical and intracellular pathogens including *Legionella pneumophila*, *Mycoplasma pneumoniae*, and *Chlamydophila (Chlamydia) pneumoniae* are also found as causes of community-acquired RTIs.[5–7]

S. pneumoniae in particular has acquired resistance to several classes of antimicrobial compounds, including penicillins, macrolides and fluoroquinolones, by a variety of mechanisms.[8] For *Haemophilus* species and *M. catarrhalis*, β-lactamase production is the principal mechanism of resistance to penicillins and cephalosporins. The choice of antimicrobial therapy in community-acquired RTIs is generally empirical and complicated by increasing bacterial resistance. Effective strategies for ensuring adequate antimicrobial therapy are therefore necessary but may only be achieved through an understanding of the geographic variation in resistance and by monitoring trends in resistance development.

Established in 1999, PROTEKT (Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin) is an international surveillance study to chart the prevalence of important resistance phenotypes and examine the susceptibility of community-acquired RTI pathogens to a range of antimicrobial compounds. Telithromycin is the first ketolide antibacterial to be approved for clinical use for the treatment of upper and lower RTIs. With over 35 countries and 500 centres now participating, PROTEKT is able to concentrate on defining trends in specific regions and countries. Detailed data from the examination of isolates of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. pyogenes* collected during the 1999–2000 winter season in Japan are now presented and, where possible, related to trends seen in previous studies.[2,9,10]

Materials and methods

Participating centres

During the 1999–2000 winter season, six centres took part in the study: Kanagawa, Sendai, Tokyo (two centres), Nagasaki and Osaka.

Bacterial isolates

Centres were asked to collect the following isolates from patients with community-acquired upper and lower RTIs: ≥40 isolates each of *S. pneumoniae* and *H. influenzae*, ≥25 of *S. pyogenes*, and ≥20 of *M. catarrhalis*. Sources for isolates were cultures from blood, sputum, bronchoalveolar lavage, middle ear fluid, nasopharyngeal swab or aspirate, and sinus aspirate. Duplicate strains or strains originating from previous collections were not accepted.

Identification and antimicrobial susceptibility testing

Isolates were identified at source and re-identified at the central laboratory by methods previously described in detail.[11] Minimum inhibitory concentrations (MICs) were determined using previously described broth microdilution methods,[11] according to the National Committee for Clinical Laboratory Standards (NCCLS) of the USA guidelines, for the following antimicrobial agents: amoxicillin–clavulanate, cefaclor, cefcapene, cefdinir, cefditoren,

cefixime, cefpodoxime, cefuroxime, telithromycin, erythromycin, roxithromycin, clarithromycin, azithromycin, rokitamycin, minocycline, tetracycline, ciprofloxacin, levofloxacin, sparfloxacin and tosufloxacin. MICs were also determined for penicillin and clindamycin against *S. pneumoniae* and *S. pyogenes* isolates and for ampicillin and amoxicillin against *H. influenzae* and *M. catarrhalis* isolates. Test results were acceptable only if the MICs for the control strains were within performance range. The following control strains were used: *S. aureus* ATCC 29213, *E. coli* ATCC 25922 and ATCC 35218, *H. influenzae* ATCC 49766, *H. influenzae* ATCC 49247, and *S. pneumoniae* ATCC 49619.

Breakpoint concentrations used to interpret MIC data qualitatively were based upon those published by the NCCLS of the USA,[12] where available. For telithromycin, NCCLS approved (SAST 2003) breakpoints were applied: *S. pneumoniae*: susceptible ≤1 mg/L, intermediate 2 mg/L, resistant ≥4 mg/L; and for *H. influenzae*: susceptible ≤4 mg/L, intermediate 8 mg/L, resistant ≥16 mg/L. No NCCLS breakpoints are available for *S. pyogenes* or *M. catarrhalis*.

β-lactamase detection

β-lactamase activity was detected using the chromogenic cephalosporin (nitrocefin) test (Unipath Ltd. Basingstoke, UK).

Macrolide resistance mechanism detection

For *S. pneumoniae*, the presence of resistance mechanisms for both MLS_B (*erm*) and M-resistance (*mef*) was analysed using a rapid-cycle multiplex PCR method with probe detection. This method detects *erm*(A), *erm*(A) subclass *erm*(TR), *erm*(B), *erm*(C), and *mef*(A) genes.[13]

Results

Streptococcus pneumoniae

A total of 308 *S. pneumoniae* isolates from the six participating centres were tested. The prevalence of penicillin resistance (MIC ≥2 mg/L) was 44.5% overall and ranged narrowly between 44.2% and 48.4% for five of the six centres, with Osaka lower at 36.4%. Penicillin-intermediate (MIC 0.12–1 mg/L) isolates (19.8% overall) were less evenly distributed, with centres reporting between 7.7% (Kanagawa) and 31.6% (Sendai) (Table 1). The prevalence of macrolide resistance (erythromycin MIC ≥1 mg/L) was 77.9%, far exceeding that of penicillin resistance, and ranged from 67.3% (Kanagawa) to 86.4% (Osaka) (Table 1). Only one strain was of the intermediate type (erythromycin MIC 0.5 mg/L). Almost half of all *S. pneumoniae* isolates (40.3%) were co-resistant to penicillin and erythromycin (macrolide) (Table 1).

Of the 239 macrolide-resistant isolates of *S. pneumoniae* analysed for their resistance mechanism, 52.7% carried *erm*(B) (MLS_B resistance) and 42.7% carried *mef*(A) (efflux resistance), with 3.3% ($n = 8$) of isolates carrying both mechanisms (*mef*(A)+*erm*(B)) (Table 2). *erm*B isolates were evenly distributed across the three penicillin resistance phenotypes, whereas *mef*(A) resistance was associated predominantly with penicillin-resistant (70.6%) rather than penicillin-susceptible (16.7%) isolates.

Among the β-lactams, the most active were cefditoren (MIC_{90} 1 mg/L, 98.4% of all isolates susceptible) and amoxicillin–clavulanate (MIC_{90} 2 mg/L, 96.4% of all isolates susceptible). Both retained >90% activity among the penicillin- and macro-

Table 1 Penicillin and macrolide susceptibility and cross-resistance of *Streptococcus pneumoniae* isolates from Japan.

Centre	No. of isolates	Pen-I[a]		Pen-R[b]		Mac-R[c]		Pen-R/Mac-R	
		n	%	*n*	%	*n*	%	*n*	%
Kanagawa	52	4	7.7	23	44.2	35	67.3	18	34.6
Sendai	38	12	31.6	18	47.4	32	84.2	18	47.4
Tokyo 1	54	11	20.4	24	44.4	43	79.6	22	40.7
Tokyo 2	62	10	16.1	30	48.4	48	77.4	27	43.5
Nagasaki	58	14	24.1	26	44.8	44	75.9	23	39.7
Osaka	44	10	22.7	16	36.4	38	86.4	16	36.4
Total	308	61	19.8	137	44.5	240	77.9	124	40.3

[a] Penicillin-intermediate: MIC 0.12–1 mg/L.
[b] Penicillin-resistant: MIC ≥ 2 mg/L.
[c] Erythromycin-resistant: MIC ≥ 1 mg/L.

Table 2 Effect of specific macrolide-resistance mutations for 239 macrolide-resistant isolates of *Streptococcus pneumoniae* from Japan and classified by penicillin susceptibility phenotype.

Genotype	MAC-R[a]		MIC range (mg/L)	PEN-S[b]		PEN-I[c]		PEN-R[d]	
	n	%		*n*	%	*n*	%	*n*	%
mef(a)	102	42.7	1–≥128	17	16.7	13	12.7	72	70.6
erm(b)	126	52.7	32–≥128	44	34.9	34	27.0	48	38.1
mef(a) + *erm*(b)	8	3.3	64–≥128	2	25	2	25	4	50
None specified	3	1.3	64–≥128	3	100	0	0	0	0

[a] Macrolide-resistant (erythromycin MIC ≥1 mg/L).
[b] Penicillin-susceptible: MIC ≤ 0.06 mg/L.
[c] Penicillin-intermediate: MIC 0.12–1 mg/L.

lide-resistant isolates (Table 3). With the exception of telithromycin and the fluoroquinolones, susceptibility to non-β-lactams was low (Table 3). Among the penicillin-resistant isolates, <10% were susceptible to macrolides and tetracycline.

Erythromycin, roxithromycin, azithromycin and clarithromycin gave typical trimodal MIC distributions with clusters of isolates inhibited by 0.06–0.12 mg/L, 2–4 mg/L and >32–>64 mg/L (Figure 1). Small numbers of isolates were inhibited

Table 3 Comparative in vitro activity and percentage susceptibility of various antimicrobials against penicillin-intermediate, penicillin-resistant and erythromycin-resistant isolates of *Streptococcus pneumoniae* from Japan using NCCLS (2002) interpretative breakpoints.

Antimicrobial	All isolates (*n* = 308)			PEN-I[a] (*n* = 61)			PEN-R[b] (*n* = 137)			MAC-R[c] (*n* = 240)		
	MIC_{50} (mg/L)	MIC_{90} (mg/L)	%S[d]	MIC_{50} (mg/L)	MIC_{90} (mg/L)	%S[d]	MIC_{50} (mg/L)	MIC_{90} (mg/L)	%S[d]	MIC_{50} (mg/L)	MIC_{90} (mg/L)	%S[d]
Penicillin	0.5	4	35.7	0.25	1	0	2	4	0	2	4	27.9
Amoxicillin–clavulanate[e]	0.5	2	96.4	0.25	0.5	100	2	2	92.0	0.5	2	95.8
Cefaclor	16	>64	20.8	16	64	8.2	64	>64	0	2	4	11.3
Cefcapene	2	4	NA	2	4	NA	4	4	NA	2	4	NA
Cefdinir	4	8	44.5	2	4	41.0	8	8	2.2	4	8	35.8
Cefditoren	0.5	1	98.4	0.5	1	96.7	1	1	97.8	0.5	1	97.9
Cefixime	16	32	–[f]	16	64	–[f]	32	64	–[f]	32	64	–[f]
Cefpodoxime	2	4	40.3	2	4	36.1	2	4	0	2	4	32.1
Cefuroxime	4	8	41.2	4	8	37.7	8	8	0	4	8	33.3
Telithromycin	0.06	0.25	100[g]	0.06	0.5	100[g]	0.06	0.12	100[g]	0.06	0.25	100[g]
Erythromycin	8	>64	21.8	64	>64	19.7	4	>64	9.5	64	>64	0
Roxithromycin	8	>32	NA	>32	>32	NA	8	>32	NA	64	64	NA
Clarithromycin	4	>32	22.1	32	>32	19.7	4	>32	9.5	32	>32	0
Azithromycin	8	>64	21.8	64	>64	18.0	8	>64	9.5	64	>64	0
Rokitamycin	0.12	>32	NA	1	>32	NA	0.12	>32	NA	1	64	NA
Clindamycin	0.12	>4	54.9	4	>4	41.0	0.12	>4	62.0	4	>4	42.1
Minocycline	8	16	NA	16	16	NA	8	16	NA	16	16	NA
Tetracycline	>16	>16	20.8	16	>16	18.0	>16	>16	9.5	>16	>16	4.2
Ciprofloxacin	1	2	NA	1	2	NA	1	2	NA	1	2	NA
Levofloxacin	1	1	96.4	0.5	1	100	1	1	97.1	1	1	96.3
Sparfloxacin	0.25	0.5	96.1	0.25	0.25	100	0.25	0.25	96.4	0.25	0.5	95.8
Tosufloxacin	0.12	0.12	NA	0.06	0.12	NA	0.06	0.12	NA	0.12	0.12	NA

[a] Penicillin-intermediate: MIC 0.12–1 mg/L.
[b] Penicillin-resistant: MIC ≥2 mg/L.
[c] Erythromycin-resistant: MIC ≥1 mg/L.
[d] % of isolates susceptible.
[e] Also applies to amoxicillin.
[f] Susceptibility predicted from penicillin.
[g] NCCLS (SAST Jan 2003) approved breakpoint for telithromycin: susceptible ≤1 mg/L; NA = NCCLS breakpoints not available.

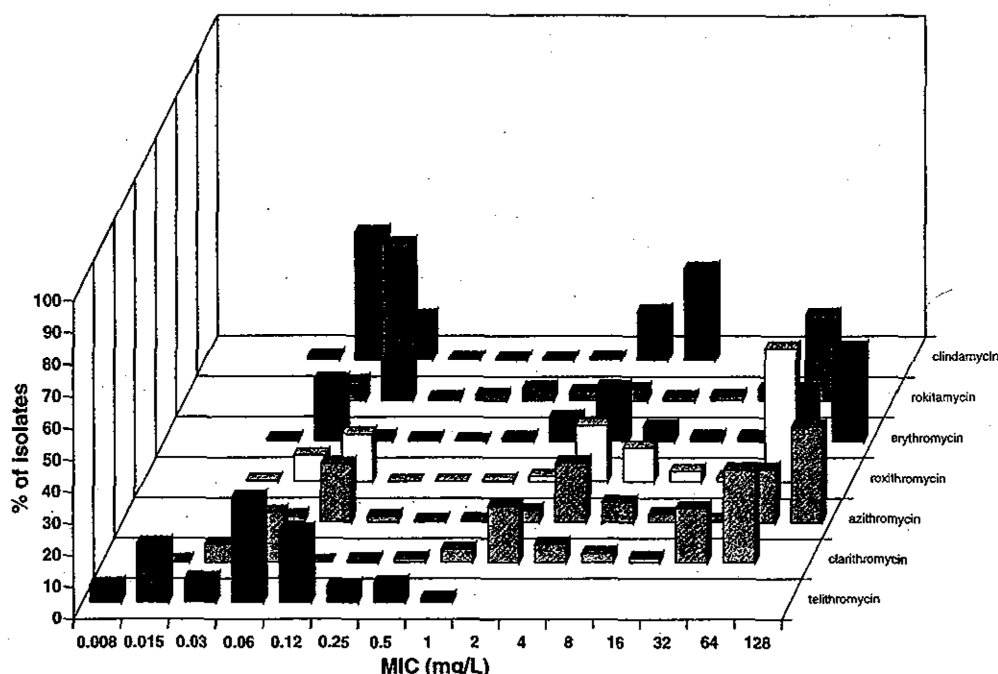

Figure 1 MIC distribution for macrolide-lincosamide-streptogramin (MLS) class antimicrobials against *Streptococcus pneumoniae* from Japan.

by each inter-mode concentration of each antimicrobial. Rokitamycin and clindamycin showed two obvious clusters in their MIC distributions, with just under half the isolates inhibited within the lowest concentration cluster. This was reflected in the MIC_{50} (0.12 mg/L) for rokitamycin and clindamycin, which differed considerably from the four macrolides with typical trimodal MIC distributions (MIC_{50} 4–8 mg/L).

Telithromycin showed much lower mode MIC (0.06 mg/L) and MIC_{90} (0.25 mg/L) than the macrolides (Figure 1). Among the macrolide-resistant isolates, the telithromycin MIC_{90} value was markedly higher for the *erm*(B) genotype (0.5 mg/L) than the *mef*(A) genotype (0.12 mg/L). Despite a shift upwards in the distribution of telithromycin MIC values among the macrolide-resistant isolates (particularly among the eight *erm*(B)+*mef*(A) strains (Figure 2)) compared with macrolide-susceptible isolates (telithromycin MIC_{90} 0.015 mg/L), all isolates were susceptible to telithromycin at $\leq$1 mg/L.

Fluoroquinolone resistance (levofloxacin MIC $\geq$8 mg/L) was 1.3% overall, with little variation among centres. Of the four fluoroquinolone-resistant isolates, three were penicillin-resistant and one was penicillin-susceptible. All four fluoroquinolone-resistant isolates were also macrolide- and tetracycline-resistant. Susceptibility to telithromycin was unaffected by fluoroquinolone resistance. Overall, of those antibacterial agents tested, the most active against *S. pneumoniae* in the winter season 1999–2000 in Japan (in terms of potency and susceptibility percentage) were telithromycin, sparfloxacin, levofloxacin, cefditoren and amoxicillin–clavulanate.

Streptococcus pyogenes

The most potent antimicrobial against *S. pyogenes* isolates was penicillin (MIC_{90} 0.008 mg/L) against which all 120 isolates were susceptible. Macrolide resistance showed considerable variation among the six centres, with the highest prevalence (42.1%) in Sendai and 0% in Nagasaki (although this centre collected only three isolates). Overall, 82.5% of isolates were erythromycin-susceptible. Among the 21 (17.5%) erythromycin-resistant isolates, the mechanisms of resistance detected were *mef*(A) in 15 isolates, *erm*(A) subclass *erm*(TR) in five isolates and *erm*(B) in one isolate. Telithromycin had mode MIC (0.015 mg/L) and MIC_{90} (0.25 mg/L), values which were 16- to 32-fold lower than those of the tested macrolides.

Haemophilus influenzae

β-lactamase production amongst *H. influenzae* isolates (n = 281) had an overall incidence of 8.5% and variation among centres of 5.1% to 11.5%. A single β-lactamase-negative, ampicillin-resistant (MIC $\geq$4 mg/L) strain (BLNAR) was identified (from Sendai). A further nine β-lactamase-negative isolates,