手術部位感染(SSI)サーベイランスの意義

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◇手術部位感染(SSI)の発生は、患者の身体的・精神的負担を増加させるとともに、病院経営の面からも将来マイナスとなることが予想される。
◇SSIの発生率低減のためにはSSIの発生状況を常に監視し、SSIに関するデータを客観的な方法で集計・評価することが必要である。
◇日本では1999年から全国規模のSSIサーベイランスが開始され、現在もその規模は順次拡大しつつあり、データの信頼度も高まっている。
◇分析されたSSIサーベイランスのデータを利用することによって、SSIに対する認識を高め、有効な感染制御策を構築し、ひいてはSSI発生率そのものを低減することが可能と考えられる。

KeyWords
手術部位感染
SSI
サーベイランス
感染制御
NNIS
JNIS

はじめに

近年、本邦では急速に人口構成の高齢化が進んでおり、医療現場においては、今後さまざまな合併症を抱えた患者の増加が予想される。そのため、入院患者における感染症発生の現状を正確に把握し、感染症発生率の抑制・感染拡大の防止などの対策を講じることは、今後のあらゆる医療分野において必須であると考えられる。

手術部位感染(Surgical Site Infection：SSI)は、外科領域において頻度の多い感染症のひとつである。SSIの発生は、患者の身体的負担や精神的負担を増加させるとともに、創傷処置の増加や入院期間の延長をもたらす。さらに、将来導入される予定の医療報酬の診断群別定額支払い制度(diagnosis related groups/prospective payment system：DRG/PPS)のもとでは、SSIに関連する医療費は基本的に病院側の負担になることが予想されるため、健全な病院経営の観点からもSSIの発生は可能な限り防がなければならない。そのためには、SSIの発生状況を常に監視し、SSIに関するデータを客観的な方法で集計・評価するシステムが必要となる。

病院感染サーベイランス

米国では、患者リスクの異なる施設間・年度間でも、病院感染の発生率や疾病比を客観的に評価できるよう、CDCが中心となりNational Nosocomial Infection Surveillance(NNIS) systemが構築され、1970年からこのNNISシステムにのっとって全国規模の病院感染サーベイランスが、現在に至るまで継続的に運用されている。2000年には、315施設におけるSSIを含む病院感染のデータがデータベー

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スとして構築され、インターネットを介して誰でも閲覧できるようになっている[1]。さらに現在では、このデータを管理・分析するためのソフトウエアがCDCによって開発され、NNIS加盟病院で使用されている[1]。

このような豊富で信頼性の高い病院感染サーベイランスのデータを臨床現場で利用し、また新しいデータをサーベイランスにフィードバックすることにより、感染対策においても慣習や憶測によるものではなく、科学的根拠に基づいた医療(EBM)が可能になると考えられる。米国には、サーベイランスという行為そのものが、SSIをはじめとする病院感染の発生を低下させる効果があるとする報告がある[2]。

日本における感染サーベイランス

一方、本邦においては全国規模の病院感染に関する疫学調査は近年まで非常に限られていた。そこで、旧厚生省の働きかけにより病院感染対策に関する研究班が1997年から設けられ、集中治療部門(ICU)・検査部門・入院患者・手術部位感染(SSI)・新生児集中治療部門(NICU)の5部門において、段階的に感染サーベイランスが開始されるようになった[1]。これと並んで1998年には、日本病院感染サーベイランス(Japanese Nosocomial Infection Surveillance：JNIS)委員会が日本環境感染学会の学会事業として構築され、1999年1月から全国9病院の協力を得て、NNISシステムに準じて主にSSIに関する疫学調査を施行している[1]。

SSIサーベイランスの目的

JNIS委員会が行うSSIサーベイランスの目的は、米国NNISと同じく、病院感染に関する質の高い即時性のデータを全国の病院に提供することにある。すなわち、

a)正確なSSIの発生状況を把握するために、サーベイランス参加病院からSSIサーベイランスデータを集める

b)サーベイランスデータを分析することによって、感染率の動向や抗生物質耐性菌などの状況を明らかにする

c)各病院に分析データを提供し、これを利用する各病院のSSIに対する認識を高め、有効な感染制御の方策を立てる

ということにある[3]。

手術部位感染(SSI)の定義

手術部位感染(SSI)とは、外科手術において縫合閉鎖した切開創の感染を指す。米国CDCは1992年、SSIを以下の3つに分類・定義した[2]。本邦でもこの定義に準じている。

a)切開創表層SSI(superficial incisional SSI)：術後30日以内に発生した、切開創の皮膚もしくは皮下組織に限局した感染

b)切開創深層SSI(deep incisional SSI)：術後30日以内に発生した、切開創の深部軟部組織(筋膜や筋肉)の感染

c)臓器・体内腔SSI(organ/space SSI)：術後30日以内に発生した、手術に関連した臓器・体内腔の感染

感染リスク別による分類

観察期間や施設の異なるSSI発生率は、単純にその数値だけでは比較することはできない。SSIの発生は、手術を受ける患者の状態に大きく左右されると考えられる。また、単開腹から汎発性細菌性腹膜炎まで、手術の清潔度も疾患・術式により大きく異なり、これら手術清潔度の違いもSSI発生頻度に影響すると考えられる。

そこで、客観的評価に耐え得るSSIサーベイランスを行うには、手術患者のリスク・手術の清潔度を適切に分類し、リスク調整を行うことが必要となる。

1．患者全身状態による分類

手術患者の全身状態を分類・評価する際、米国麻酔科医学会(American Society of Anesthesiologists：ASA)による分類、いわゆるASAスコアが頻繁に引用されており、本邦においてもASAスコアを利用することが一般的である(表1)。

2．創分類

上述のように、SSIの発生頻度は手術の清潔度・汚染度と密接な関係があると考えられる。米国NNISは、手術の清潔度を4つに分類している(表2)。

Class Ⅰは単開腹術や脾摘術など、Class Ⅱは通常の胃切除術や結腸切除術など、Class Ⅲは消化管

表1　米国麻酔科医学会（ASA）の全身状態による分類

ASAスコア	
PS1	健康な患者
PS2	軽い全身疾患を持った患者（高血圧など）
PS3	重い全身疾患を持った患者（陳旧性心筋梗塞など）
PS4	常に生命にかかわるような重い全身疾患を持った患者（心不全など）
PS5	手術しなければ救命し得ない瀕死の患者（大動脈瘤破裂など）
PS6	臓器提供のために摘出手術を受ける脳死患者

表2　手術の清潔度（創分類）

分　類	
Class Ⅰ　clean	清潔手術（脾摘術など）
Class Ⅱ　clean-contaminated	準清潔手術（胃切除術など）
Class Ⅲ　contaminated	汚染手術（清潔操作の破綻）
Class Ⅳ　dirty and infected	感染手術（腹膜炎の手術など）

手術操作が破綻した場合（消化液の腹腔内への漏出）や急性胆嚢炎に対する胆摘術など、Class Ⅳは汎発性細菌性腹膜炎に対する手術などに相当する。JNISのサーベイランスでも、このNNISによる創分類が利用されている。

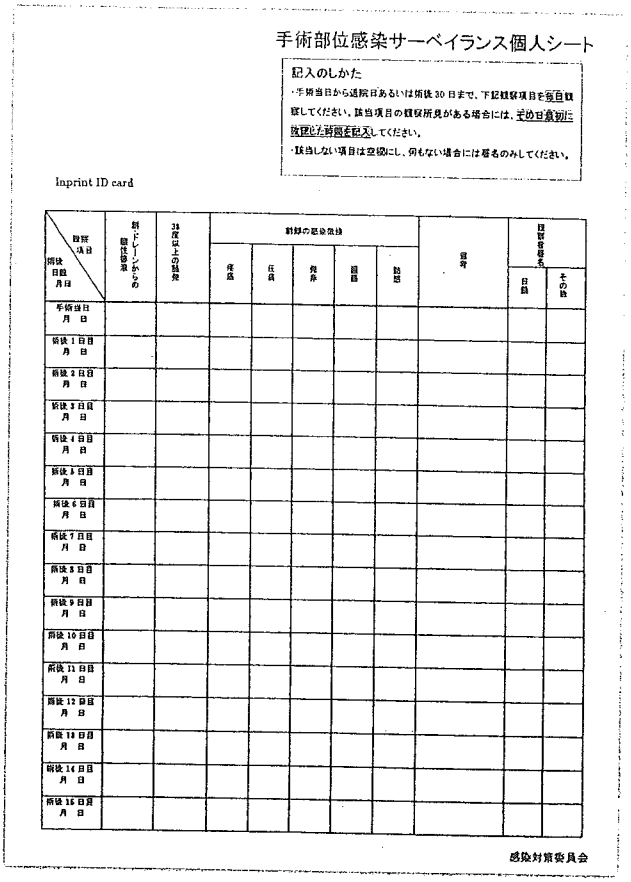
SSIサーベイランスの実際

前述のとおり、JNIS委員会設立とともに全国規模のSSIサーベイランスが1999年1月から開始されている。ここではサーベイランスの具体的方法を、当初からのサーベイランス参加施設であり、JNIS事務局でもあるNTT東日本関東病院で行っているSSIサーベイランスを例にとって説明する。

院内に感染コントロールチーム（Infection Control Team：ICT）を組織し、院内で施行された消化器外科手術の全症例を対象として、JNISシステムに沿ってSSIサーベイランスを実施した。ICTは、感染対策を主導する専任の医師（infection control doctor：ICD）および看護師（infection control nurse：ICN）が中心となり、外科医師、看護師、薬剤師、臨床検査技師から構成されている。また、疫学的・公衆衛生学的観点から、院内の感染発生状況を分析・検討する専任の保健学士（infection control practitioner：ICP）も参加している。

SSIの判定はNNISの判定基準[3]に準じ、①化膿性排液の流出、②病因菌の証明、③感染兆候の出現（疼痛・発赤・腫脹・熱感など）、④再手術・病理学的検査・放射線検査などによる感染の証明、⑤担当医によるSSIの診断、のいずれかに相当する場合をSSIと認定した。

図1　SSIサーベイランス記入シート


手術部位感染サーベイランス個人シート

記入のしかた
・手術当日から退院日あるいは術後30日まで、下記観察項目を毎日観察してください。該当項目の観察所見がある場合には、その日の欄に該当した時間を記入してください。
・該当しない項目は空欄にし、何もない場合には署名のみしてください。

Inprint ID card

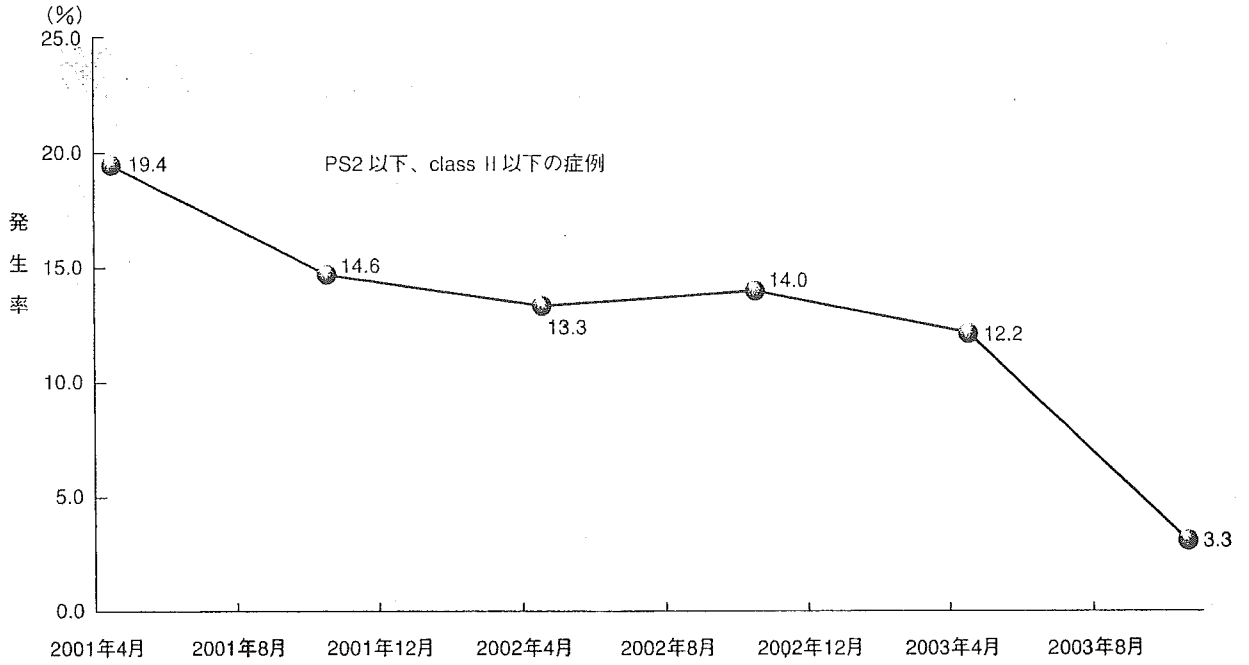
感染対策委員会

術後の創観察は、入院中および退院後の外来を含め術後30日間継続して行った。担当医と担当看護師が創の状態を毎日観察し、創の状態を細かくチェックできるSSIサーベイランスシート（図1）に記入し、創状態の変化や感染兆候をいち早く認知できるようにした。なお、このシートは電子カルテの導入に伴って現在では電子化され、記入に要する時間は大幅に短縮された。

ICTは、外科医師および外科病棟看護師全員とともに、週1回、外科入院患者全員の症例を検討し、疾患・術式・ASAスコア・創分類などの基礎情報を収集するとともに、SSI発生の有無と感染部位（表層、深層、臓器・体内腔SSI）を調査した。

ASAスコアによる患者の全身状態評価・創の清潔度・手術時間によるrisk index scoreを算出して、適切なリスク調整を行い、これらを加味した詳細なSSIに関するデータをひと月ごとにJNIS事務局に提出した。それと同時に、院内でもICTおよび外科医師・看護師全員で1か月に1回、SSIサーベ

図2　SSI発生率の推移（NTT東日本関東病院）



イランスカンファランスを開き、1か月間の消化器外科手術患者全症例を検討し、SSI発生の有無を1例ごとにチェックした。SSI発生例については、感染原因の推測や経過などを詳細に検討し、SSI発生率低減のための有効な感染予防策を随時討議した。

SSIサーベイランスの効果

NTT東日本関東病院では、SSIサーベイランスおよびスタッフ全員でのカンファランスを継続して施行することによって、

a）抗生物質の術前投与を徹底し、標準投与日数を設定
b）電子化クリニカルパス導入による治療・処置の標準化
c）創処置時のマスク・手袋の装着を義務付け
d）閉腹時の皮下洗浄の導入

などを順次施行していき、さらに外科医・看護師ともに、手術および術後創処置の際、SSIの発生防止を常に意識して行うようになった。その結果、ASAスコアPS2以下、創分類class II以下の消化器外科症例におけるSSI発生率は、現在までほぼ一貫した減少傾向を示している（図2）。

おわりに

このように、SSIに対するサーベイランスを継続して施行・検討することにより、SSI発生率自体を低下させることが可能と考えられる。近年のSSIに関する関心の高まりとともに、当初9施設で開始されたJNISによるSSIサーベイランスも、現在では全国50施設を超え、集積されたデータの客観性・信頼性はいっそう高まっている。また2002年には、このJNISが行うSSIサーベイランスが厚生労働省管轄の国家事業へと昇格した。

SSIサーベイランスを継続して行うことは、確かに人手と時間を要する作業であるが、病院感染の発生率低減は今や国家的急務であるといっても過言ではない。全国各施設でSSIサーベイランスが施行され、またJNISの感染に関するデータを有効に活用して、実効性のあるSSI感染予防策を行うことが期待される。

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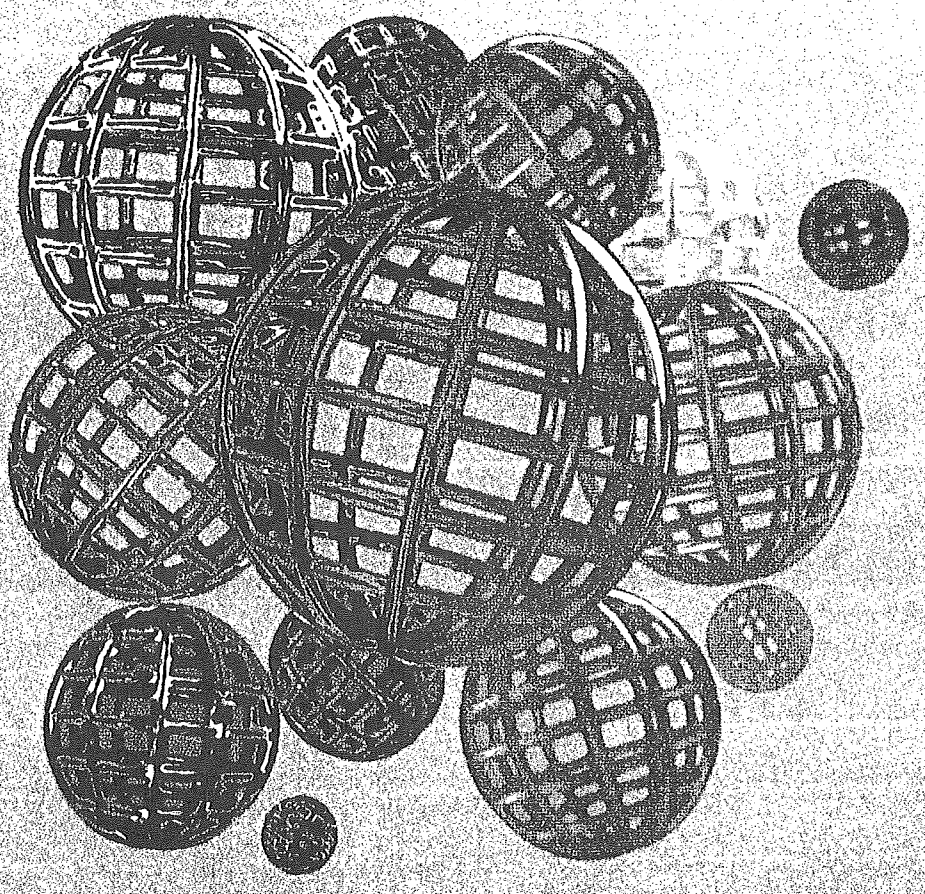
Q&Aシリーズ

ムダ・ムリ・ムラをなくす感染対策

病院感染対策 Q&A

責任編集●小林寛伊 関東病院院長



照林社

Q SSI（手術部位感染）対策って何？ 12

A 手術部位感染を防ぐための術前準備、手術手技、術後処置などの工夫のことです。

1．SSI（手術部位感染）とは

●SSI（手術部位感染）とはsurgical site infectionの略語で、手術操作を直接加えた部位の感染のことである。
●これには術後30日以内の切開創部の感染（表層、深部）や手術した臓器や腹腔、胸腔などの感染が含まれる。
●3種類の分類として、表層切開部位、深部切開部位、器官／体腔があげられる。

2．SSIの起炎菌は

●SSIの起炎菌は手術患者自身のもっている菌によることが多い。
●不十分な消毒により残存した細菌や術中の落下細菌などの関与もありうる。
●消化器外科手術の場合には、手術中に開放された消化管や胆管内などの常在菌が、大きく関与していると考えられている。

3．手術の汚染度とSSI発生率

●手術はその清浄度により、clean（清潔）、clean-contaminated（準清潔）、contaminated（不潔）、dirty/ infected（汚染または感染）に分類される。
●手術の汚染度が高まるにつれて、SSI発生率が高くなることが知られている。

4．SSI防止のためのガイドライン

●1999年4月に米国CDC（Centers for Disease Control and Prevention）はSSI防止のためのガイドラインを発表している。
●信頼性のある研究により証明されていて強力に推奨される項目（IA、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）とランク付けされていて、いわゆるEBMの考え方に基づいたガイドラインとなっている（表1）。

（針原　康／小西敏郎）

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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

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

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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.

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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

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.

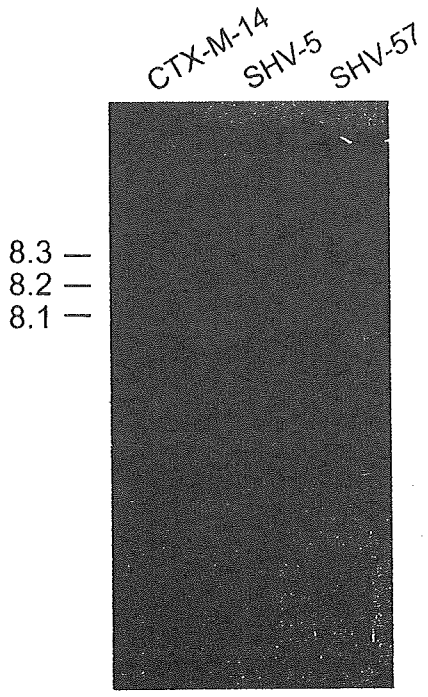

FIG. 1. Isoelectric focusing of the new β-lactamase variant and reference β-lactamases. The numbers on the left are in kilobases.

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.

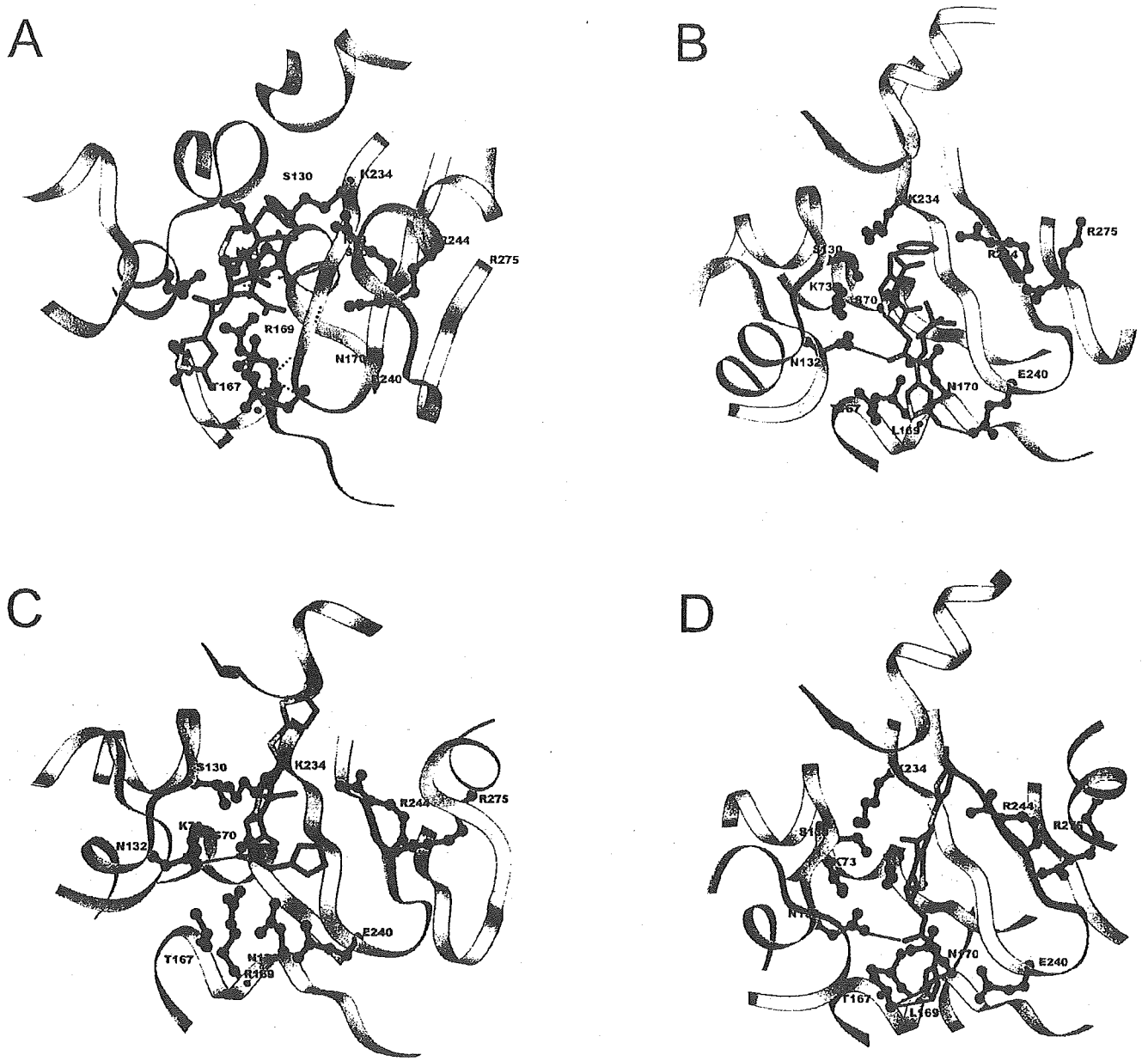

FIG. 2. (A and B) Complex structure model of ceftazidime in SHV-57 and SHV-1, respectively. Ceftazidime is shown by the stick model. The carbon atoms are in light blue; and the oxygen, nitrogen, and sulfur atoms are in red, dark blue, and yellow, respectively, unless indicated otherwise. Only the main 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.

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

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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 *int*I 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

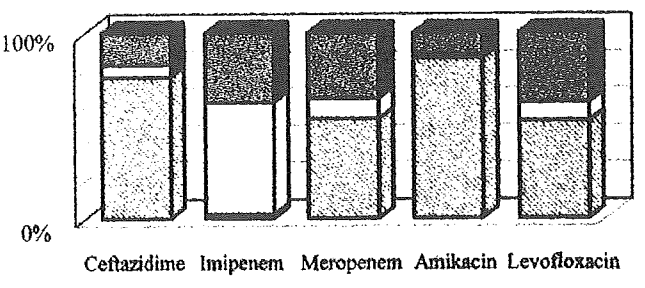

FIG. 1. Antimicrobial susceptibilities of imipenem-nonsusceptible *P. aeruginosa* isolates.

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

Gene[a]	Primer sequence (5′ to 3′)	T_m (°C)	Reference
intA(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
qacEΔ1	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.

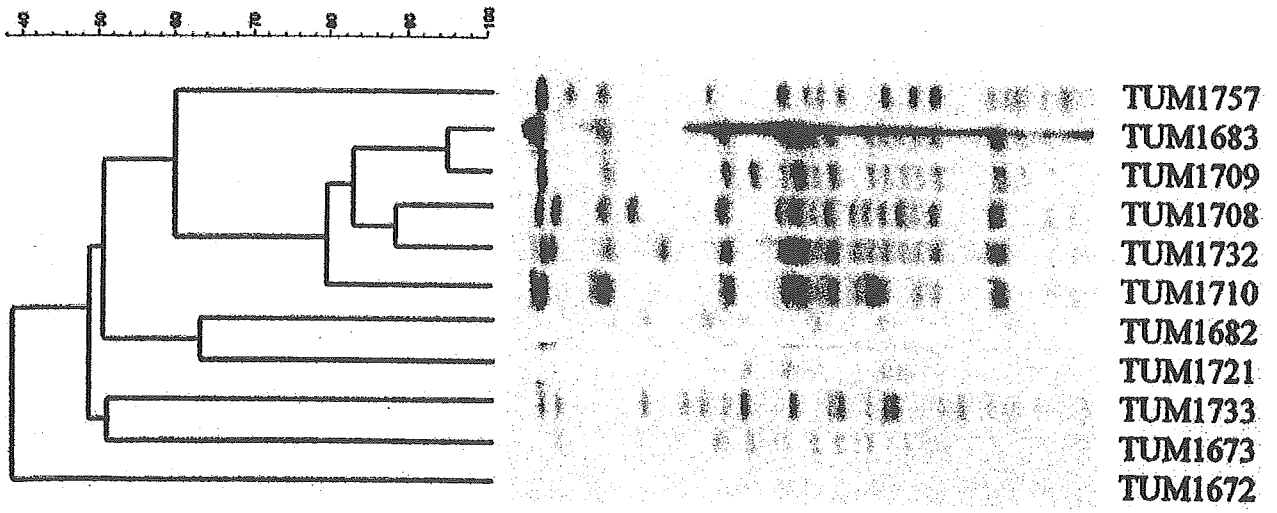

FIG. 2. Pulsed-field gel electrophoresis profiles obtained with SpeI chromosomal digestion of metallo-β-lactamase-carrying *P. aeruginosa*. The second through sixth lanes contained related strains TUM1683, TUM1709, TUM1708, TUM1732, and TUM1710, respectively. Lanes first and seventh to eleventh lanes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

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

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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.

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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 ≥ 16 μg ml^{-1} (resistant region) for 28 strains and ≤ 8 μg 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 ≥ 8 μg 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 (μg 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 $\geq$32 μg 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 μg 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 μg ml^{-1} when determined by the micro broth-dilution method and between 0·25 and 8 μg 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 μg 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 μg 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