PCR-RFLP 法を利用したニューキノロン低感受性チフス菌・パラチフス A 菌の *gyrA* 変異のスクリーニング法の検討

渡邊治雄　廣瀬健二（協力研究者）
国立感染症研究所細菌第一部

腸チフス・パラチフスは、日本を除く東アジア、東南アジア、インド亜大陸、中東、東欧、中南米、アフリカなどに蔓延し、現在もなお流行を繰り返している。わが国でも昭和初期から終戦直後までは腸チフスが年間約4万人、パラチフスが約5000人の発生がみられていた。そして、1970年代までに、環境衛生状態の改善によって年間約300例の発生まで減少した。その後さらに減少し、1990年代に入ってからは腸チフス・パラチフスを併せて年間約100例程度で推移している。そのほとんどは海外からの輸入事例で、海外旅行が日常化したことによる。

現在、腸チフス・パラチフスの治療には、ニューキノロン系抗菌剤が第一選択薬として使われている。ニューキノロン系抗菌剤(LVFX, SPFX, TFLX)を 14 日間経口投与が一般的な腸チフス・パラチフスの治療である。ところが、腸チフスの治療の第一選択薬であるニューキノロン系抗菌剤に耐性または低感受性を示し、治療にニューキノロン系抗菌剤の効果がみられない症例が論文や学会で数多く報告されている。日本にもニューキノロン系抗菌剤に低感受性を示すチフス菌・パラチフス A 菌が、海外からの輸入事例として入ってきている。これらは NCCLS のブレイクポイントから判定すると、ニューキノロン系抗菌剤に耐性ではない。しかし、ニューキノロン低感受性株ではニューキノロン系抗菌剤に対する MIC が感受性株の約 10 倍またはそれ以上高い。また、ニューキノロン低感受性株はナリジクス酸に耐性で、第 3 世代セフェム系抗菌剤には感受性を示す。ニューキノロン低感受性菌による腸チフス・パラチフスでは、ニューキノロン系抗菌剤による治療には反応せず、速やかに解熱しない。ニューキノロン系抗菌剤の効果が望めない症例では第 3 世代セフェム系抗菌剤（CTX,CTRX など）が使用される。ニューキノロン系抗菌剤に低感受性を示すチフス菌・パラチフス A 菌は *gyrA* 遺伝子に突然変異を持っていることが私たちのいままでの研究で明らかになっている。ニューキノロン低感受性菌の低感受性の原因は、GyrA の 83 番または 87 番のアミノ酸が突然変異により置換されていることである。私たちは、GyrA の 83 番または 87 番の点突然変異を PCR-RFLP(Restriction fragment length polymorphism)法によりスクリーニングする方法を開発した。PCR 法により *gyrA* 遺伝子のキノロン耐性決定領域を増幅し *HinfI* で切断しポリアクリルアミド電気泳動で切断パターンを比較する方法である。この方法により *gyrA* 遺伝子のキノロン耐性決定領域の DNA 配列決定作業をすることなく迅速に変異の入っている場所を知ることができる。この方法を用いてニューキノロン低感受性チフス菌・パラチフス A 菌臨床分離株で変異のパターンを調べたところ、試験したすべての株で変異の入っている場所を特定することができた。また、切断パターンの比較によってニューキノロン耐性株、ニューキノロン低感受性株、ニューキノロン感受性株との区別もすることができた。

分担研究課題：サルモネラの多剤耐性菌のレファレンスと分子疫学

分担研究者：山本友子（千葉大学大学院薬学研究院）
研究協力者：内村眞佐子、依田清江（千葉県衛生研究所）
渡辺治雄、泉谷秀昌（国立感染症研究所）

非チフス性サルモネラ症は世界的に最も頻度の高い食中毒であるが、我が国においては 1992 年に発生件数、患者数ともに第一位を占めて以来、発生状況は現在も大型化の傾向を示している。これらの原因となるサルモネラの血清型は様々であるが、近年わが国を含む先進諸国において特に問題となっているのは、*Salmonella enterica* serovar Enteritidis (SE)とそれに次いで 2 番目に多く検出される *Salmonella enterica* serovar Typhimurium (ST)である。特に多くの薬剤に耐性を示す多剤耐性の ST、中でも definitive type 104 (DT104)が急増していることである。多剤耐性 DT104 の複数の耐性遺伝子は、染色体上の *Salmonella* Genomic Island (SGI)1 と名付けられた領域にクラスターをなして存在することから、薬剤の使用を止めても感受性に戻りにくく、食中毒発症時の治療を困難にしている。かかる現状において本研究は、我が国における DT104 をはじめとする *Salmonella* 多剤耐性菌の出現状況を明らかにし、さらに耐性機構並びに多剤耐性獲得機構の解明を目的として行われる。

本年度は、1999 年から 2002 年に千葉県下で発生した食中毒散発事例より分離された ST37 株を対象に分子疫学的解析を行い、以下の事柄を明らかにした。

（１）37 株中 23 株が多剤耐性 DT104 にみられる Ampicillin (Ap), Streptomycin (Sm), Sulfonamide (Su), Chloramphenicol (Cm), Tetracycline (Tc)耐性を有し、さらに、Kanamycin, Trimethoprim, Nalidixic acid (Nx) 耐性が加わったものが存在した。又、Cm, Sm, Su 耐性株 1 株および Sm, Su 耐性株が 12 株存在した。

（２）全塩基配列が公開された DT104 strain 96-5227 の耐性遺伝子(MDR)領域は、2 つの class I インテグロンすなわち *IntI1-aadA2-qacEΔ1-sulΔ1* と *intI1Δ-pse1-qacE Δ1-sul* により挟まれて構築されている。この構造を参考にして上記 Ap,Sm,Su,Cm,Tc 耐性 23 株について、β-lactamase Typing、PCR Mapping、Southern Hybridization、PFGE、ファージ型別を行い以下のことを明らかにした。

15 株が PSE1 type、5 株が OXA1 type、3 株が TEM type のβ-lactamase をコードしていた。PSE1 type のうち 14 株が *IntI1-aadA2-qacEΔ1-sulΔ1* と *intI1Δ-pse1-qacEΔ1-sul* を有し、MDR は strain 96-5227 と同様の構造であると考えられたが、PFGE pattern は 5 タイプに分かれた。この中には DT104 以外のファージ型が 2 株存在したことから、SGI1 の水平伝播が起きていると考えられた。1 株は *intI1Δ-pse1-qacEΔ1-sul* と他の class I インテグロンを有していたが MDR 領域全体の構造は異なっており、MDR の再配列が起きていると考えられた。

OXA1 typeは、すべてが *intI1-oxa1-aadA1* のインテグロンを有していたが、PFGE patternは4タイプに分かれた。これらのファージ型を検討したところすべてDT104以外のものであったことから、他のDTサルモネラにおいても新たなMDRによる多剤耐性化が進行していることが懸念される。

（3）同時期に分離されたSE68株の薬剤耐性を検討したところ、Tc耐性1株、Nx耐性1株、Sm耐性45株であり、現在の所SEでの多剤耐性化は進んでいないと考えられた。

Characteristics of the 20 Typhimurium isolates harboring the class 1 integron

Year	Phage type	Integron	Designation
1998	Not DT104 related	[*IntI1-oxa1-aadA1-qacEΔ1-sul1*]	CK653
1999	DT104	[*intI1Δ-pse1-qacEΔ1-sul*] and [*IntI1-aadA2-qacEΔ1-sulΔ1*]	CK2 CK4 CK7 CK8 CK9 CK10 CK11
	Not DT104 related	[*intI1Δ-pse1-qacEΔ1-sul*] and unidentified class 1 integron	CK657
	Not DT104 related	[*IntI1-oxa1-aadA1-qacEΔ1-sul1*]	CK3 CK6 CK658
2000	DT104	[*intI1Δ-pse1-qacEΔ1-sul*] and [*IntI1-aadA2-qacEΔ1-sulΔ1*]	CK15 CK16 CK17
	Not DT104 related	[*IntI1-oxa1-aadA1-qacEΔ1-sul1*]	CK23
2001	DT104	[*intI1Δ-pse1-qacEΔ1-sul*] and [*IntI1-aadA2-qacEΔ1-sulΔ1*]	CK26 CK28
	DT104B	[*intI1Δ-pse1-qacEΔ1-sul*] and [*IntI1-aadA2-qacEΔ1-sulΔ1*]	CK25
	U302	[*intI1Δ-pse1-qacEΔ1-sul*] and [*IntI1-aadA2-qacEΔ1-sulΔ1*]	CK30

[*intI1Δ-pse1-qacEΔ1-sul*] and [*IntI1-aadA2-qacEΔ1-sulΔ1*]: 14/23 isolates

[*intI1Δ-pse1-qacEΔ1-sul*] and unidentified class 1 integron: 1/23 isolates

[*IntI1-oxa1-aadA1-qacEΔ1-sul1*]: 5/23 isolates

定量的 RT-PCR 法を利用した緑膿菌多剤排出システムの発現検出

後藤直正、○村田 健、西野武志
京都薬科大学・微生物

[目的] 感染症治療において使用される抗菌薬は、起因菌の感受性を加味して適切に選択されることが望まれる。現在行われている分離培養後の抗菌薬感受性測定に変わる新しい方法として、
感染症患者より得られた検体から抗菌薬耐性だけでなく、病原性などの情報を短期間に得られるシステム開発の一端として、本研究では臨床分離緑膿菌株を対象に抗菌薬排出システムおよび外膜透過孔に起因する抗菌薬耐性機構の発現を定量的 RT-PCR によって mRNA レベルで観察し、その結果と各種抗菌薬感受性とを比較することによって分離された緑膿菌株の抗菌薬感受性を mRNA レベルで推定可能かどうかを調べた。
[方法] 使用菌株として実験室株 PAO1 と人工的に構築した抗菌薬耐性機構発現株および欠損株を用いた。更に、2002 年 6 月から 2003 年 6 月に分離された緑膿菌株の内、MIC 値が IMP ≧12.5 μg/ml を 1 株、LVFX ≧12.5 μg/ml を 4 株、AMK ≧25 μg/ml を 2 株、LVFX, AMK 交差耐性を示す 2 株、計 9 株を選択した。1 晩培養後の各菌体から Total RNA を精製し、抗菌薬排出タンパク質をコードする *mexB*、*mexY* およびカルバペネム系薬の透過孔をコードする *oprD* の発現を定量的 RT-PCR および特異抗体を用いたイムノブロットで観察し、抗菌薬感受性測定の結果と比較した。
[結果および考察] 実験室株 PAO1 と MexAB-OprM, MexXY, OprD それぞれの発現株および欠損株の定量的 RT-PCR およびイムノブロットによる発現量と抗菌薬感受性とを比較した結果、それぞれに良好な相関性が観察された。臨床分離株については、*mexB* および *mexY* の発現量は定量的 RT-PCR の結果とイムノブロットの結果とでほぼ相関していたが、mRNA 検出量が蛋白質検出量よりも若干高い株が数株存在していた。このことから、翻訳または翻訳後に何らかの制御が存在することが示唆された。*oprD* については、mRNA 検出量と蛋白質検出量間での相関性が乏しい結果となったが、現在、定量的 RT-PCR での検出プライマー配列が適切かどうかを調べるために今回用いた臨床分離株の *oprD* の塩基配列の解析を行っている。更に耐性遺伝子の mRNA の検出量と抗菌薬感受性を比較した結果、検出量が高いにもかかわらず、その耐性機構に起因する抗菌薬耐性が観察されない株が存在したが、逆に検出量が PAO1 と同等またはそれより低下している株では、耐性化を示す株は存在しなかった。このことは、臨床分離株の mRNA から抗菌薬耐性に影響を与える遺伝子の発現量を測定することによって、高発現していない耐性機構を推定し、それに応じた治療薬を選択できる可能性を示唆している。

Staphylococcus aureus における penicillin-binding protein の分布

○ 和田昭仁 （国立感染症研究所 細菌第一部）

methicillin-sensitive *Staphylococcus aureus* (MSSA)、および methicillin-resistant *S. aureus* (MRSA) の増殖に、*S. aureus* のもつ penicillin-binding protein (PBP) 1 が必須であることは、遺伝学的手法によりすでに示されている (J. Bacteriol. 180:2759-2765, 1998; 第 1 回薬剤耐性菌研究会抄録 [群馬県 2003])。しかし、PBP 1 は、MRSA においても MSSA における場合と同様にβラクタム剤感受性であり、PBP アッセイにて低濃度のβラクタム剤との反応性を観察することができる。この二者の矛盾点（遺伝学的に示された PBP1 の必須性と、PBP アッセイで観察されるβラクタム剤感受性)を説明するために、蛍光 penicillin による PBP アッセイ、ならびに *S. aureus* 菌体表面と菌体内 PBP の観察をおこなった。

【方法と対象】MSSA として NCTC8325 由来の BB255、MRSA として COL をもちいた。これらの菌を直径 0.1 mm のマイクロビーズをもちいて破砕し、膜各分を調整した。PBP アッセイには 2 種類の蛍光 penicillin (Bocillin FL and Bocillin 650/665, Molecular Probe 社) をもちい、これらと反応させた膜画分をミニゲルで分離後、蛍光スキャナー(Typhoon 9400, Amersham Biosciences 社)でイメージを取りこみ、蛍光シグナルの定量を行った。菌体の染色には、上記蛍光 penicillin をもちい、共焦点レーザー顕微鏡 (LSM510 META, Carl Zeiss 社) による観察をおこなった。

【結果と考察】菌体を破砕し、調整した膜各分に未標識 penicillin G を暴露させ、その後に蛍光 penicillin を反応させる古典的な “競合” PBP アッセイでは、100 μg/ml の未標識 penicillin G にて、99%の PBP 1 および PBP 2 が蛍光 penicillin との反応性を失っていたが、菌体破砕前の全菌体に未標識 penicillinG を暴露させ、その後、菌体を破砕し調整した膜各分と蛍光 penicillin との反応性をみる PBP アッセイでは、約 10%の PBP 1 および PBP 2 を蛍光 penicillin にて検出することができた。この結果は、BB255 においても COL においても同じであった。これより、菌体に penicillin G を反応させた場合、本来は、βラクタム剤に感受性のある PBP がそれとの反応を免れるというモデルを考えることができた。penicillin V に、それと分子量がほぼ等しく疎水性の高い蛍光物質を結合させた Bocillin 650/665 を、BB255 および COL の菌体に反応させたとき、菌体の内部に Bocillin 650/665 由来の赤色のシグナルを検出することができた。このシグナルが、上記の penicillin G との反応を免れている PBP に由来するものであるかどうかを知るためには、各 PBP の特異的蛍光検出、proteinA の影響を受けないような金粒子免疫染色が必要である。

平成 15 年度：厚生労働科学研究費補助金　新興・再興感染症研究事業
「新型の薬剤耐性菌レファレンス並びに耐性機構の解析及び迅速簡便検出法に関する研究」

呼吸器感染症からの検査材料を用いた直接 PCR による原因菌の迅速検索法の確立

分担研究者：北里大学北里生命科学研究所，大学院感染制御科学府感染情報学　生方　公子

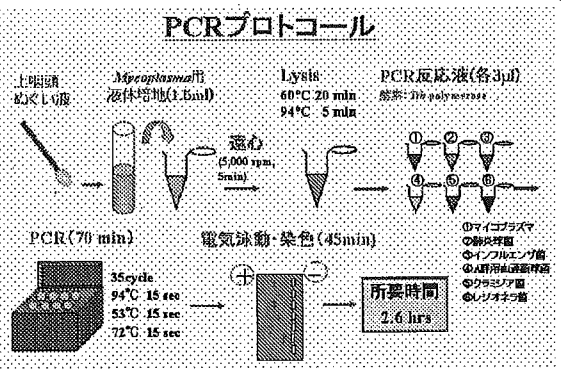
§ 目的

呼吸器感染症の起炎菌となり得る菌種において，急速に耐性化が進行し，難治症例が目立つようになってきている。このような背景には，外来患者に対して細菌検索することなく empiric therapy の行われていることが適正とは言えない抗菌薬の使用に繋がり，耐性菌増加のひとつの要因になっている。耐性菌の増加を抑えるには，empiric therapy から evidence に基づいた化学療法(Evidence Based　Chemotherapy: EBC)へレベルアップすることが必要で，結果的には医療費抑制にも貢献すると考えた。

そのためには感染の初期段階に起炎菌となりうる微生物を網羅的に同時検索する手法の確立が必要である。当面の目標として，2 - 2.5 時間で主要原因菌を確定できる「呼吸器感染症・起炎菌検索キット」のシステム構築を目的にした。なお，本方法の最終目標は，入院患者に対しては少なくとも入院当日に，外来患者に対しては再診時に抗菌薬使用の是非も含めて最も適切な抗菌薬投与へと切り替えられるようにすることにある。

§迅速検索方法

市中で発症する吸器感染症の起炎菌検索の対象としたのは，① 肺炎球菌，② インフルエンザ菌，③ A群溶血レンサ球菌，④ *M. pneumoniae*，⑤ *C. pneumoniae*，⑥ *L. pneumophila*である。これらは同一条件でPCRが実行できること，感度を同一レベルに保つこと，増幅DNAの長さに差をつけていることが特徴である。それぞれのprimerの感度は1-7 CFU／reaction tubeで，検体採取用綿棒の先に10^3 CFUの目的菌が付着していれば，PCR陽性と判定される感度とした。



§ 小児由来・上咽頭ぬぐい液への応用

上述した目的の遂行のために，「小児急性呼吸器感染症研究会(ARD)研究会」で収集した検査材料について検討を行った。

肺炎球菌やインフルエンザ菌，A群溶血レンサ球菌の陽性率は培養法よりもやや優れ，*M. pneumoniae*では培養法よりもはるかに優れ，*C .pneumoniae*に対しても確実に優れていると考えられた。小児での*Legionella*検出は1例のみであったが，別途依頼された成人例では陽性例を2例経験した。

§ 考察

ここに述べた起炎菌の迅速検索法は，網羅的検索を指向する方法のひとつに過ぎないと考えている。将来の迅速診断のあり方は，呼吸器感染症が疑われた症例に対し，抗菌薬投与前に菌検索と同時に耐性遺伝子検索までが高い精度で実施できることが必要である。

分担研究課題：セフェム系薬耐性に関わる遺伝子の迅速診断法の確立

北里大学医学部
井上松久（分担研究者）、
岡本了一、中野竜一、中野百実子、兼子謙一（研究協力者）

グラム陰性菌におけるセフェム系薬の耐性化は、それてぞれの菌種の染色体上に普遍的に座位するclass C型β-ラクタマーゼAmpCの構造遺伝子発現を調節する複数の遺伝子変異によって調節していることが明らかとなっている。先ず *E.cloacae* 由来のAmpCをコードする遺伝子セットをクローニングし、これを *E.coli*K12株に形質転換させ、代表的なセフェム系薬の *in vitro* 選択により耐性変異菌を分離し、これを詳細に調べた。その結果、得られた変異菌は、調べた限り全て *ampD* 変異であり、変異部位はDNA塩基配列の結果、*ampD* の各部位に点在していた。中には、*E.coli*K12由来にISの挿入変異株も検出された。また、*ampD* 変異に伴ってAmpC量も増加していた。次いで、相補試験の結果少なくとも野生型 $ampD^{+}$ および $ampR^{+}$ 産物は、変異型遺伝子（$ampD^{-}$、$ampR^{-}$）に対してそれぞれ優性であり、得られた変異株は $ampD^{+}$ の共存によってセフェム系薬に対するMIC、AmpC量共に低下した。

次に、実験変異株によりセフェム系薬耐性菌の遺伝子変異を特定できることが判ったことから、臨床分離セフェム系薬耐性 *E.cloacae* について遺伝子変異の特定と遺伝子迅速検出について調べた。その結果、臨床分離菌の多くが、*ampD* 変異によるセフェム系薬高度耐性化の要因となっていることが判明した。しかし、一部の耐性菌の中には、*ampR* 変異菌も検出された。

カルバペネム耐性菌のレファレンスと研究：ダンシル基とチオール基をもつ蛍光剤によるメタロ-β-ラクタマーゼ(IMP-1)の検出

熊本大学大学院医学薬学研究部　　後藤正文，○黒崎博雅，山口佳宏

β-ラクタム剤は感染症治療において頻繁に用いられている抗菌剤であり、とくに広域抗菌スペクトルを示す第三セフェム系やカルバペネム薬は近年急速に使用量が伸びている。しかし、耐性を獲得した病原菌が院内感染の要因ともなり大きな社会的問題となっている。その原因の 1 つにメタロ-β-ラクタマーゼの産生が挙げられる。これは、新しいβ-ラクタム加水分解機構をもつので既存のβ-ラクタマーゼ阻害剤には無効であり、カルバペネムを含むほとんど全てのβ-ラクタム剤を不活化する。中でも IMP-1 はその遺伝子が伝達性プラスミド上に存在することから菌種を超えた伝播が可能であり、その世界的な蔓延が危惧されている。そのため、感染菌のメタロ-β-ラクタマーゼ産生の有無を初期段階で確認することは化学療法において極めて重要である。そこで、蛍光プローブ法による簡易検出を目指し、メタロ-β-ラクタマーゼ(IMP-1)の活性中心に存在する亜鉛(II)に特異的に結合するチオール基と蛍光発色団のダンシル基を有する DansylCnSH(n = 2−6)を合成した(図 1)。そして IMP-1 との相互作用を蛍光スペクトルにより検討を行い、さらに IMP-1 との複合体の X 線結晶構造解析を行った。以下に本研究で得られた知見を要約する。

(1) DansylCnSH (n = 2−6) は 340 nm で励起すると 535 nm 付近で発光した。IMP-1 が共存すると蛍光強度は増大し、極大波長は低波長側へシフトした。亜鉛含有酵素，カーボニックアンヒドラーゼ存在下では蛍光強度の増大は観測されず IMP-1 に結合することが示唆された。

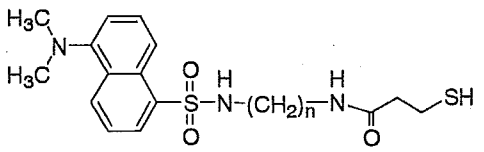


Fig. 1. Structure of DansylCnSH (n = 2−6)

(2) DansylCnSH (n = 2−6)の蛍光強度に及ぼす IMP-1 の濃度の効果から、蛍光剤と IMP-1 は 1 対 1 で結合し、n = 4 のときに最も強く結合する。

(3) DansylCnSH (n = 2−6) は IMP-1 の基質加水分解活性を拮抗阻害し、その阻害定数 K_i は、蛍光スペクトルにより求めた解離定数 K_d とほぼ一致した。

(4) IMP-1 の活性中心には 2 個の亜鉛 (II) が存在しており、DansylCnSH (n = 2−6) のチオール基がこの亜鉛 (II) に結合することによって IMP-1 中にとりこまれ、ダンシル基が IMP-1 の疎水的環境に置かれたため、IMP-1 存在下で蛍光強度は増大することが分かった。

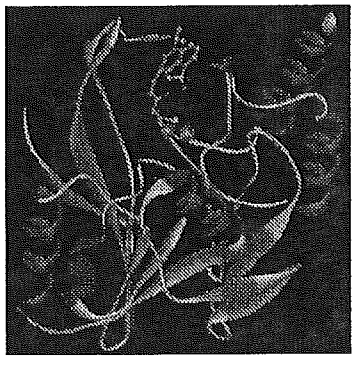


Fig. 2. X-ray crystal structure of the complex of IMP-1 with DansylC4SH

(5) 最も強く IMP-1 に結合する DansylC4SH と IMP-1 との複合体の X 線結晶構造解析を行った(図 2)。DansylC4SH のチオール基は IMP-1 活性中心の 2 個の亜鉛(II)に架橋して、ダンシル基は基質と結合する際に重要と考えられているフラップ部位のトリプトファン残基と疎水的な相互作用をしていることが分かった。

以上のことより、DansylCnSH(n = 2−6)はメタロ-β-ラクタマーゼ(IMP-1)と特異的に結合しタンパク質側鎖との相互作用により蛍光強度が増大することが示唆された。

臨床分離病原菌における薬剤耐性の分子機構ならびに遺伝子型別に関する研究

柴田尚宏、山根一和、土井洋平、鈴木里和、柴山恵吾、蒲地一成、和知野純一、
金井京子、加藤はる、八木哲也、荒川宜親 （国立感染症研究所　細菌第二部）

【背景】昨今、臨床分離される細菌における薬剤耐性の獲得状況は、軽視できない全地球規模の緊急的事態となっている。これまで、MRSA や VRE などのグラム陽性菌で先行していた多剤耐性の獲得が、現在、緑膿菌などのグラム陰性菌でも急速に進んでおり、カルバペネムやフルオロキノロン耐性を獲得した緑膿菌は既に２０％を超える状況にある。さらに、半合成アミノグリコシドへの耐性も数％に達している。
【方法】国内の医療機関で臨床分離された様々な病原細菌について、それらにおける薬剤耐性の分子機構を解析するとともに、それに関与する遺伝子の型別、さらに、保有状況等を調査・分析した。
【結果】主な研究成果を以下に示す。

1．臨床的に有用なほぼ全てのアミノグリコシドに耐性を付与する全く新しい分子機構として、16S rRNA メチラーゼ遺伝子を緑膿菌やセラチアより発見。
2．国内で分離された広域β-ラクタム薬耐性株について、メタロ-β-ラクタマーゼの遺伝子型別の分離状況を調査した。
3．セファマイシンを分解する変異型 AmpC、および GES-4 と命名した新しいクラス Aβ-ラクタマーゼを発見した。
4．牛や院内感染事例より分離されたセフォタキシム耐性菌について解析を行い、CTX-M-2 型β-ラクタマーゼの産生がその原因である事を確認した。
5．AAC(6’)-Iad と命名した新たなアミノグリコシドアセチル化酵素を Acinetobacter より発見した。

【考察】我が国の臨床現場においては、上記したように多種多様な薬剤耐性遺伝子を保有したり新たに獲得した病原細菌が患者材料より分離されている。医療の高度化が推進される中で、それらの耐性菌の存在を念頭におき、日常的な医療行為の中で、抗菌薬の適正使用ならびに院内感染対策が一層推進される必要がある。また、日常の検査業務の中で、それらを分離検出し識別できる能力、技量を高める事が急務となっている。

VanD 型バンコマイシン耐性 *Enterococcus raffinosus*

分担研究者 ： 池 康嘉[1,2]

研究協力者 ： 野村隆浩[1]、谷本弘一[2]、小澤良之[1]、藤本修平[1]、丸山英行[3]、荒川宜親[4]

群馬大院・医・細菌感染制御学[1]、同 薬剤耐性菌実験施設[2]、済生会習志野病院・細菌検査[3]、国立感染症研究所・細菌第二部[4]

現在までのところ 6 種類(A、B、C、D、E、F、G)のバンコマイシン耐性遺伝子群が知られている。そのうち臨床上問題になるのは高度バンコマイシン耐性を示す A、B、D 型である。今回、世界的にも数株しか報告のない VanD 型 VRE が初めて日本で分離されたため解析を行った。

分離された菌株は同定の結果 *E. raffinosus* であった。MIC 測定の結果は VCM (1024 μ g/ml) 、 TEIC(256 μ g/ml) 、 GM(2048 μ g/ml) 、 KM(2048 μ g/ml) 、 SM(1024 μ g/ml)、EM(2048 μ g/ml)、TC(256 μ g/ml)、ABPC(32 μ g/ml) であった。CP には感受性だった。

バンコマイシン耐性の型別を行うために *vanA*、*vanB*、*vanC*、*vanD1*、*vanE* に特異的なプライマーを用いた PCR を行ったが陰性であった。そこで大腸菌の *ddlA*、*ddlB* と VRE の *van*A 型 ligase の間で保存されているアミノ酸配列から設計されたプライマーを用いて PCR を行ったところ、増幅された DNA 断片を検出することができた。PCR に用いたプライマーで直接シークエンスをしたところ vanD4 と高い相同性が確認された。VanD4 型の VRE は 1 株報告があり、その塩基配列を参考に作成したプライマーを用いて PCR やシークエンスを行いリガーゼ遺伝子の残りの塩基配列を決定した。その結果、1032 塩基中 2 塩基(362 番目の G が T に、930 番目の C が T)が変化していた。コードされるアミノ酸は 121 番目の Gly が Val に変化したのみで、2 番目の変異によってアミノ酸は変化しなかった。この結果から、この *E. raffinosus* が VanD4 型の VRE である事がわかった。

接合伝達実験を行ったがバンコマイシン耐性は伝達しなかった。また、プラスミドの分離を試みたが明らかなプラスミドの存在が確認できなかった。このことからバンコマイシン耐性遺伝子群は染色体上に存在している事が考えられた。バンコマイシン耐性遺伝群の発現はバンコマイシンによって誘導される事が知られているので Northern Hybridization を行い VanD 遺伝子の転写を調べたところ。この VanD4 遺伝子群はバンコマイシン非存在下でも発現しており、恒常的に発現している事がわかった。

Ⅳ．研究成果の刊行物･別刷

Escherichia coli Producing CTX-M-2 β-Lactamase in Cattle, Japan

Yutaka Shiraki,* Naohiro Shibata,† Yohei Doi,† and Yoshichika Arakawa†

From November 2000 to June 2001, *Escherichia coli* strains producing CTX-M-2 β-lactamase were isolated from 6 (1.5%) of 396 cattle fecal samples and 2 (0.7%) of 270 surface swabs of cattle carcasses in Japan. The $bla_{CTX-M-2}$ gene responsible for CTX-M-2 production was encoded on transferable plasmids, and the gene was transferred to *E. coli* CSH2 with a very high frequency (2×10^{-4} to 6×10^{-1} per donor cells) by conjugation. Random amplified polymorphic DNA analysis of nine isolates showed at least five different patterns. These findings suggest that CTX-M-2 producers might have originated from cattle through the use of cephalosporins such as ceftiofur and that cattle could be a reservoir of CTX-M-2–producing *E. coli*. Continuous and strategic surveillance of antimicrobial-resistant bacteria in livestock is essential to suppress further dissemination of these bacteria into society at large.

Shortly after a variety of expanded-spectrum cephalosporins were introduced in the 1980s, bacterial strains producing extended-spectrum β-lactamases (ESBLs), such as TEM- or SHV-derived ESBLs, emerged in Europe (1), and since then, their variants have been proliferating around the world (2,3). More recently, CTX-M-type β-lactamases, a small but growing family of broad-spectrum class A β-lactamases, were initially discovered as MEN-1 (EMBL accession no. X92506) and also later found as Toho-1 in Japan in 1993 (4). Since the early 1990s, these β-lactamases have been identified in various bacterial species belonging to the family *Enterobacteriaceae* (5). Several questions regarding the origin and mode of proliferation of the CTX-M-type β-lactamases are unclear. Unlike TEM- and SHV-derived ESBL producers, the CTX-M-type β-lactamase producers have been incidentally and sporadically detected as single clinical isolates from patients with urinary tract infections and the like (6,7) over an extensive geographic area, including Europe, South America, and the Middle and Far East. The cause of this global distribution is not well known (5,8). Moreover, derivation of the CTX-M-type enzymes or the prototype of this enzyme with its narrow spectrum remains unknown (9–12).

In Japan, clinical isolation of the TEM- or SHV-derived ESBL producers is still rare (13,14); *Escherichia coli* strains producing CTX-M-2 β-lactamase, one of the CTX-M family, have been predominantly isolated to date (13). On the other hand, clinical isolates producing IMP-1 type metallo-β-lactamase, which show resistance to carbapenems and cephamycins as well as various expanded-spectrum cephalosporins, have been identified in Japan (15), and the proliferation of these strains has become a clinical concern (16). As for the disproportionately low isolation rate of the TEM- or SHV-derived ESBL producers in Japan, carbapenems and cephamycins, whose use has been restricted in many Western countries, have been preferentially used as first-line drugs in Japan (13,15). This practice makes it more plausible that TEM- or SHV-derived ESBL producers would be rarely isolated and that metallo-β-lactamases would be isolated often in Japan. However, it is not easy to explain the predominant isolation of *E. coli*-producing CTX-M-2 β-lactamase that is usually susceptible to carbapenems and cephamycins like TEM- or SHV-derived ESBL producers. In addition, since CTX-M-2 β-lactamase producers tend to be isolated from patients who have neither received antimicrobial drugs nor been hospitalized, the existence of healthy carriers of CTX-M-2 producers was suspected (17,18). Still, one cannot assume healthy carriers exist on the basis of the low isolation rate of strains producing broad-spectrum class A β-lactamases in Japan.

One hypothesis to address these issues is that CTX-M-2 might have emerged elsewhere than in humans and that the enzyme might have originated in livestock. Recently, a global threat developed because certain antimicrobial-resistant bacteria, such as vancomycin-resistant enterococci (19), *Salmonella enterica* Typhimurium DT104 (20) and fluoroquinolone-resistant *Campylobacter jejuni* and *C. coli* (21) emerged in food animals possibly through the use of antimicrobial drugs for growth promotion or disease treatment. However, few reports have been published

*Gifu Prefectural Office of Meat Inspection, Gifu, Japan; and †National Institute of Infectious Diseases, Tokyo, Japan

about strains in animals producing ESBLs or CMY-type cephamycinases, which confer resistance to expanded-spectrum cephalosporins (22,23), and no CTX-M-type β-lactamase producer has been isolated from animals. Therefore, to examine this hypothesis, we conducted a study to isolate any strains producing extended-spectrum class A β-lactamases from cattle at Japanese slaughterhouses.

Materials and Methods

Sampling and Bacterial Culture

From November 2000 to June 2001, a total of 396 fecal samples of cattle and surface swabs of 270 cattle carcasses were collected at two slaughterhouses in Gifu Prefecture, Japan. ESBL screening agar plates (17), which were prepared using BTB Lactose agar (Nissui Pharmaceutical Co., Tokyo, Ltd., Japan) containing 2 μg/mL of cefotaxime (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) and 8 μg/mL of vancomycin (Shionogi & Co., Ltd., Osaka, Japan), were used to isolate gram-negative enterobacteria that produce broad-spectrum class A β-lactamases. One swab was used to sample each cattle feces, and two swabs were used for each cattle carcass. For sampling of the cattle feces, a swab was inserted into the core of a lump of feces. When several cattle were kept in the same enclosure, a direct rectal swab was sampled from each of the cattle. Shoulder and rump were swabbed separately in each cattle carcass; the size of the swabbed area was approximately 20 x 20-square centimeters for each swab. Swabs of feces were plated directly on the screening agar. Swabs of carcasses were suspended in a 10-mL Trypticase soy broth (Nissui Pharmaceutical Co.) containing 2 μg/mL of cefotaxime and 8 μg/mL of vancomycin, and then plated on the screening agar. The remaining Trypticase soy broth with bacteria was further incubated overnight. A swab of bacterial culture was then plated on the screening agar. Colonies suspected to be enterobacteria were isolated and identified by using the API 20E system (bioMérieux, Marcy l'Etoile, France). *E. coli* isolates were serotyped with a slide agglutination kit (Denka Seiken Co., Ltd., Tokyo, Japan) and were screened for genes of virulence factors, including Shiga toxins and *E. coli* attaching and effacing factor by polymerase chain reaction (PCR) (24).

Detection of β-Lactamases

The acidmetric β-lactamase test was performed by using P/Case TEST (Showa Yakuhin Kako Co., Ltd., Tokyo, Japan) to detect β-lactamase production in the isolates. According to the manufacturer's instructions, the colonies were spread on two indicator disks, containing benzylpenicillin and cephaloridine with clavulanic acid, respectively. When the strain produces class A β-lactamases, including TEM- or SHV-derived ESBLs, or CTX-M-type enzymes, the color of a disk containing benzylpenicillin turns yellow. The other disk, containing cephaloridine with clavulanic acid, remains purple because hydrolysis of cephaloridine by the class A β-lactamases is blocked in the presence of clavulanic acid. If the strain produces class C or class B β-lactamases, both disks turn yellow because these enzymes are no longer blocked by clavulanic acid. The isolates suggested to produce extended-spectrum class A β-lactamase were further investigated to determine whether they produced ESBLs by the double-disk diffusion test (25), using two Kirby-Bauer disks (Eiken Chemical Co., Ltd., Tokyo, Japan). A swab of bacterial culture (approximately 10^6 CFU/mL) to be tested was spread on a Mueller-Hinton agar plate (Eiken Chemical Co.), and one disk containing cefotaxime, ceftazidime, ceftriaxone, cefpodoxime, aztreonam, or cefepime was put on the plate. The other disk, containing amoxicillin+clavulanic acid, was also placed alongside the first disk (center-to-center distance of approximately 3 cm), and the agar plate was then incubated for 18 hours. When an expansion of the inhibitory zone between the two disks was observed, the isolates were speculated to produce ESBL.

Conjugation and Plasmid Profiles

Conjugation experiments were performed by using *E. coli* CSH2 as a recipient, as previously described (17). A mixture of donor and recipient strains was incubated in Luria-Bertani broth (Difco Laboratories, Detroit, MI) at 37°C for 18 hours. Transconjugants were selected by using BTB Lactose agar plates supplemented with 100 μg/mL of rifampicin (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan) and 2 μg/mL of cefotaxime to inhibit the growth of the donor strain and the recipient strain, respectively. Frequency of transfer was calculated by dividing the number of transconjugants by the number of donors. Plasmid DNA was prepared from the isolates and their transconjugants by using Quantum Prep Plasmid Miniprep Kit (Bio-Rad Laboratories, Richmond, CA), according to the manufacturer's instructions. After agarose gel electrophoresis, the sizes of the plasmids were determined by comparing their migration distances with those of plasmids of known sizes.

Susceptibility Testing

MICs were determined by overnight broth-microdilution method using MicroScan ESBL Confirmation Panel (Dade Behring, Sacramento, CA). This panel was designed to detect ESBL producers in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) document M100-S9 (26). The MIC of ceftiofur (Pharmacia

Co., Kalamazoo, MI), an expanded-spectrum cephalosporin often used in veterinary medicine, was also determined by the broth-microdilution method in accordance with NCCLS document M7-A4 (27). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality-control strains.

PCR and DNA Sequencing

To determine the genotype of strains producing broad-spectrum class A β-lactamases, PCR was performed by using primers specific to TEM, SHV (13), CTX-M-1 (MEN-1) (28), CTX-M-2 (29), and CTX-M-9 (8) genes. The PCR products were sequenced by using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) with the same primers for PCR. The DNA sequences were analyzed in an ABI PRISM 377 XL Sequencer Analyzer (Applied Biosystems).

RAPD Analysis

Random amplified polymorphic DNA (RAPD) analysis was performed by using Ready-To-Go RAPD analysis beads (Amersham Pharmacia Biotech, Piscataway, NJ), according to the manufacturer's instructions. DNA was prepared from the isolates using InstaGene DNA Purification Matrix (Bio-Rad Laboratories), also according to the manufacturer's instructions. The reaction mixture contained 25 pmol of one of six RAPD analysis primers (Amersham Pharmacia Biotech) and 10 μL of DNA preparation in a final volume of 25 μL. Amplification was performed with initial denaturation at 95°C for 5 minutes, followed by 45 cycles of 1 minute at 95°C, 1 minute at 36°C, and 2 minutes at 72°C. The amplified products were separated by electrophoresis in 1.5% agarose gel. The fingerprints were compared visually, and patterns were considered different when they differed by at least one amplification band.

Results

Identification of β-Lactamases

Of 396 fecal samples of cattle, 104 (26.3%) samples gave colonies on the ESBL screening agar. Among the strains grown on the screening agar, 32 strains of *E. coli* and 2 strains of *Citrobacter koseri* were positive through the P/Case TEST for production of penicillinase, cephalosporinase, or both (Table 1). The double-disk diffusion test was performed on 28 strains that were speculated to produce penicillinase; 7 strains isolated from 6 (1.5%) of 396 fecal samples were positive. However, two strains, GS553 and GS554, which produced cephalosporinase and penicillinase, showed a clear expansion of the inhibitory zone only when a disk of cefepime, a better detection agent for ESBLs in the presence of an AmpC β-lactamase (30), was used. By a PCR analysis with a set of PCR primers specific for $bla_{CTX\text{-}M\text{-}2}$, a 900-bp fragment was amplified from the seven strains that were positive in the double-disk diffusion test (Table 1). However, since CTX-M-2 and Toho-1 have only one amino acid substitution, genes for $bla_{CTX\text{-}M\text{-}2}$ and $bla_{Toho\text{-}1}$ were indistinguishable by the PCR. DNA sequencing of the PCR products subsequently showed that all were 100% identical with the $bla_{CTX\text{-}M\text{-}2}$ reported (31). Similarly, two strains isolated from 2 (0.7%) of 270 surface swab samples of cattle carcasses were positive in the double-disk diffusion test and possessed $bla_{CTX\text{-}M\text{-}2}$ (Table 1).

Although all 9 isolates producing CTX-M-2 β-lactamase were *E. coli*, their serotype of O antigen could not be defined with 43 commercially available antisera that were representative serotypes of pathogenic *E. coli*. Moreover, genes of virulence factors described previously were not detected from the strains by PCR.

Antimicrobial Susceptibility Testing

The susceptibilities of two representative isolates, GS528 and GS554, and their transconjugants are shown in Table 2. All the isolates were resistant to piperacillin, cefotaxime, ceftriaxone, cefpodoxime, cefepime, and aztreonam, and more resistant to cefotaxime than to ceftazidime. Except for strains GS553 and GS554, the β-lactamase inhibitor clavulanic acid (fixed concentration of 4 μg/mL) reduced MICs of cefotaxime and ceftazidime by $>2^{10}$- and $\geq 2^4$-fold, respectively. These susceptibility profiles of the isolates were similar to those observed for strains that produced CTX-M-2 β-lactamase (31). Both GS553 and GS554 strains, which produced cephalosporinase as well as penicillinase, were resistant to cefotetan, cefmetazole, and cefoxitin as well as piperacillin, cefotaxime, ceftriax-

Table 1. Number of β-lactamase producers isolated from cattle[a]

Sample (no.)	Species	Acidmetric β-lactamase test				PCR typing
		Total	PC	PC and CS	CS	
Feces (396)	*Escherichia coli*	32	7	19	6	7 (CTX-M-2)
	Citrobacter koseri	2	2	0	0	0
Swab[b] (270)	*E. coli*	5	2	2	1	2 (CTX-M-2)
	C. freundii	1	0	1	0	0

[a]PC, penicillinase; CS, cephalosporinase; PCR, polymerase chain reaction.
[b]Swab, surface swab of cattle carcass.

Table 2. MICs of β-lactams for *Escherichia coli* strains isolated from cattle, transconjugants, and recipients[a]

Antimicrobial drug	MIC (μg/mL) for *E. coli* strain:				
	GS528	CSH2 trGS528	GS554	CSH2 trGS554	CSH2
Piperacillin	>64	>64	>64	>64	≤16
Cefotaxime	>128	>128	>128	>128	≤0.5
Cefotaxime + CLA[b]	≤0.12	≤0.12	32	32	≤0.12
Ceftazidime	2	4	32	32	≤0.5
Ceftazidime + CLA	≤0.12	≤0.12	16	32	≤0.12
Aztreonam	>64	64	>64	64	≤0.5
Ceftriaxone	>64	>64	>64	>64	≤0.5
Cefpodoxime	>64	>64	>64	>64	≤0.5
Cefepime	>32	>32	>32	>32	≤1
Cefotetan	≤0.5	≤0.5	>32	>32	≤0.5
Cefmetazole	1	1	>16	>16	1
Cefoxitin	≤2	≤2	>32	>32	≤2
Meropenem	≤0.5	≤0.5	8	4	≤0.5
Ceftiofur	>1,024	>1,024	>1,024	>1,024	≤0.25

[a]*E. coli* CSH2 trGS528 and trGS554 were transconjugants of *E. coli* GS528 and GS554, respectively.
[b]CLA, clavulanic acid at a fixed concentration of 4 μg/mL.

one, cefpodoxime, cefepime, and aztreonam. In addition, clavulanic acid hardly reduced the resistance levels of these two strains to cefotaxime and ceftazidime. These results, together with those obtained through the double-disk diffusion test, suggested that both GS553 and GS554 strains produced putative AmpC β-lactamase at high levels as well as the CTX-M-2 β-lactamase. All the isolates producing CTX-M-2 β-lactamase were highly resistant to ceftiofur (MIC >1,024 μg/mL).

Plasmid and RAPD Analysis

Conjugation experiments indicated that all the isolates were able to transfer their cefotaxime resistance to the recipient and that the resistance to cephamycins observed in both strains GS553 and GS554 was also transferred to the transconjugant. All transconjugants produced the same β-lactamase(s) of their donor strains, and susceptibility profiles of the transconjugants were also similar to those of donor strains (Table 2). These results demonstrated that $bla_{CTX\text{-}M\text{-}2}$ genes of the isolates might be encoded on transferable plasmids. The frequency of transfer was very high (2×10^{-4} to 6×10^{-1} per donor cells) (Table 3). Plasmid profiles of the isolates showed one to three large plasmids with five different patterns in each strain, while an approximately 33-MDa plasmid was common among all the strains. Approximately 33-MDa and 50-MDa plasmids were both transferred to recipient cells in all the strains (Table 3). RAPD analysis of a total of nine isolates gave at least five different patterns (Figure, Table 3). Although strains GS553 and GS554 were isolated from the same fecal sample, they differed in RAPD pattern and plasmid profile.

Discussion

We investigated the cause of the disproportionate emergence of CTX-M-2 β-lactamase and so-called ESBLs, including TEM- or SHV-derived enzymes, in Japan. We isolated *E. coli* strains producing CTX-M-2 β-lactamase from 6 (1.5%) of 396 fecal samples from cattle and 2 (0.7%) of 270 surface swabs of cattle carcasses. Negative results, however, do not necessarily mean the organisms are absent on the slaughterhouse carcasses because of the limited size of the overall swabbed surface area.

Table 3. Characteristics of CTX-M-2 β-lactamase–producing *Escherichia coli* isolated from cattle[a]

Strain	Source	β-lactamase[b]	Plasmid profile (MDa)	Transferred plasmid (MDa)	Frequency of transfer	RAPD pattern
GS528	Feces 1	PC	33, 50, 86	33, 50	6×10^{-4}	A
GS542	Feces 2	PC	33, 50, 86	33, 50	2×10^{-4}	A
GS547	Feces 3	PC	33, 50, 86	33, 50	3×10^{-4}	A
GS553	Feces 4	PC and CS	33, 50, 61	33, 50	3×10^{-1}	B
GS554	Feces 4	PC and CS	33, 50	33, 50	2×10^{-1}	C
GS721	Feces 5	PC	33	33	9×10^{-2}	D
GS733	Feces 6	PC	33	33	2×10^{-1}	D
GS631	Swab[c] 1	PC	33, 86	33	5×10^{-1}	E
GS671	Swab 2	PC	33, 86	33	6×10^{-1}	E

[a]PC, penicillinase; CS, cephalosporinase; RAPD, random amplified polymorphic DNA.
[b]β-lactamases were detected by acidmetric β-lactamase test.
[c]Swab, surface swab of cattle carcass.

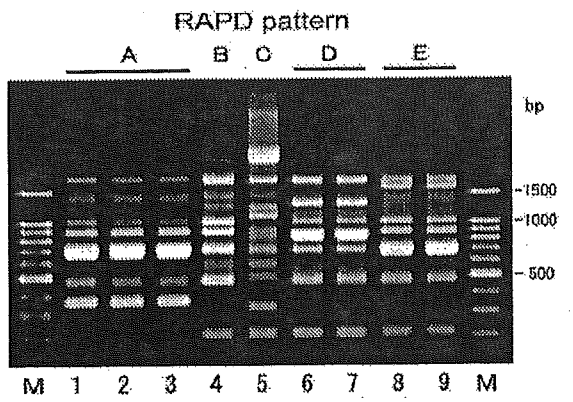


Figure. Random amplified polymorphic DNA (RAPD) patterns of CTX-M-2 β-lactamase–producing *Escherichia coli* isolated from cattle. Lanes M, 100-bp DNA ladder; lanes 1–9, strains GS528, GS542, GS547, GS553, GS554, GS721, GS733, GS631, and GS671, respectively. Five RAPD patterns, A to E, were produced with RAPD analysis primer 4 (Amersham Pharmacia Biotech, Piscataway, NJ).

Our findings raised a complex question: Did CTX-M-2 β-lactamase producers initially emerge in cattle or humans? We assume they emerged from cattle. Indeed, we found no direct evidence of transmission of *E. coli* strains producing CTX-M-2 β-lactamase from cattle to humans, but our results strongly suggested that transmission of the CTX-M-2–producing microorganism might have occurred between cattle and humans. This speculation is supported by the fact that CTX-M-2 β-lactamase-producers isolated from humans in Japan are identified predominantly as *E. coli,* as was observed in Japanese cattle. According to the survey of ESBLs in human clinical isolates in Japan, Toho-1-type β-lactamase was the most prevalent, and half of the Toho-1-type β-lactamase producers were *E. coli* (13). Moreover, the Toho-1-type β-lactamases reported in several studies in Japan were later found to be CTX-M-2 by PCR and sequencing analyses. Furthermore, according to the survey of ESBL producers in human stool specimens reported recently in Japan, Toho-1-type β-lactamase–producing enterobacteria were isolated from 2 (0.5%) of 366 specimens (17). Since the survey samples were from 231 inpatients and 135 outpatients with diarrhea, the rate of CTX-M-2 producers in healthy humans in Japan is estimated to be <0.5%. Indeed, by chi-square analysis, the isolation rate (1.5%) of CTX-M-2 producers in cattle feces obtained in our study showed no statistically significant difference from that of ESBL producers in human cases reported previously in Japan (17). However, we speculate that CTX-M-2 producers found in cattle have something to do with those from humans. Many reports substantiate that bacteria can be transmitted from food-producing animals to humans through the food chain, and we found that the surface of cattle carcasses was stained with the CTX-M-2–producing bacteria. Our speculation is also supported by the fact that TEM- or SHV-derived ESBLs have not been detected from livestock so far even in Western countries, where they have been widely detected with a high frequency in various medical institutions. In other words, if transmission of ESBL producers from human to cattle can occur with some frequency, several TEM- or SHV-derived ESBL producers would be isolated also from cattle. However, no such finding has been reported even in Western countries. Thus, prospective investigations should be conducted to understand the current status of *E. coli* strains that produce CTX-M-enzymes in livestock, especially in those countries where CTX-M-enzymes have been found in humans.

Recently, SHV-12 β-lactamase–producing *E. coli* was isolated from a dog with recurrent urinary tract infections (22). The origin of the isolate, however, was not known since the treatment with expanded-spectrum cephalosporins was not been recorded. In livestock, although penicillinases such as TEM-1 and TEM-2 have been identified from cattle (23,32–34), pigs (35), and poultry (36), isolation of ESBL producers has not been reported. On the other hand, ceftriaxone-resistant *Salmonella* isolates, which produce plasmid-mediated AmpC-type β-lactamase such as CMY-2, are proliferating globally (37). Ceftriaxone-resistant *Salmonella* and *E. coli* strains have been also isolated from cattle recently in the United States (23,32,33,35). These findings suggest that cattle can serve as an incubator or reservoir of these antimicrobial drug-resistant bacteria. The authors of the U.S. studies suggested that the emergence of the AmpC-mediated cephalosporin resistance may have been a consequence of the use of ceftiofur, the only cephalosporin approved for systemic use in food animals in the United States (23,32,35). Dunne et al. support this hypothesis, indicating that the use of ceftiofur in cattle may have contributed to the emergence of the ceftriaxone-resistant *Salmonella* because the isolate shows cross-resistance between ceftiofur and ceftriaxone (33). In our study, all the isolates producing CTX-M-2 β-lactamase were also highly resistant to ceftiofur. What antimicrobial agents had been used at Japanese cattle farms where the CTX-M-2 producers were isolated is not well known, since the samples were collected at slaughterhouses. However, ceftiofur was the only expanded-spectrum cephalosporin approved for livestock in Japan when our study was conducted. In addition, the MIC (>1,024 μg/mL) of ceftiofur for CTX-M-2 producers isolated in this study was relatively higher than those (2 to >32 μg/mL) for TEM- or SHV-derived ESBL producers (38) that have been emerging in so many humans. Thus, the emergence of CTX-M-2 β-lactamase–producing *E. coli* in Japan might also be a consequence of the use of cef-

tiofur for livestock. However, why CMY-2 type class C β-lactamase is predominantly found in livestock in the United States is not clear. The types of antimicrobial agents and their use for livestock in that country may have contributed to its high prevalence of CMY-2 producers, although no statistical data are available about the differences in usage of antimicrobial agents between the United States and Japan. Continuous and prospective investigations of veterinary usage of the antimicrobial agents as well as surveillance of antimicrobial-resistance seem necessary for preventing the emergence and further proliferation of antimicrobial-resistant bacteria in livestock.

The CTX-M-2 producers were not considered to reflect a clonal expansion of an *E. coli* strain carrying $bla_{CTX-M-2}$ because five distinct RAPD patterns and plasmid profiles were identified in the nine isolates. These findings suggest that stealthy plasmid-mediated dissemination of $bla_{CTX-M-2}$ gene among *E. coli* strains might be under way with the continuous consumption of the third-generation cephalosporin for veterinary use. Conjugal transfer of R-plasmid might occur in the intestinal tract, which is the main habitat of ESBL producers (17,39). Both strains GS553 and GS554 were isolated from the same fecal sample and produced the same β-lactamase, but they were different in terms of RAPD analysis and plasmid profile. Frequencies of transfer of the isolates were high (Table 3). These results suggested that conjugal transfer of the R-plasmids also occurred in the intestinal tract of cattle. Therefore, the possibility of further transfer of the resistance profile of *E. coli* to expanded-spectrum cephalosporins to other pathogenic bacteria such as *Salmonella* spp. and diarrheagenic *E. coli* should not be ignored.

The isolates in this study did not correspond to the serotypes of pathogenic *E. coli*, and they did not possess the virulence factors assayed. However, lack of virulence factors might contribute to subclinical increase of healthy carriers of these strains and might promote their dissemination among both cattle and human. Especially in livestock, environmental contamination and transmission among individual animals by these strains could expand rapidly because of their breeding system. Therefore, CTX-M-2 producers may well be disseminated even further in cattle farms hereafter. Although nosocomial bacteria that produce extended-spectrum class A β-lactamases have thus far been considered to emerge only among in humans, our study suggested that CTX-M-2 producers could potentially emerge in livestock and that cattle might be an original reservoir of CTX-M-2 producers. Therefore, active and continuous surveillance and strategic countermeasures are necessary for antimicrobial-resistant bacteria, including those strains producing such β-lactamases as CTX-M-type, CMY-type (37,40) and metalloenzymes (16) in livestock, especially in countries where these producers have emerged in human populations.

This work was supported by grant nos. H12-Shinko-19 and H12-Shinko-20 from the Ministry of Health, Labor and Welfare, Japan.

Mr. Shiraki is a microbiological specialist in the isolation and identification of pathogenic microorganisms from foods, environments, and clinical samples. His major research interests are epidemiology and the mechanisms of antimicrobial resistance in life-threatening bacteria. He conducts microbiological diagnostic tests of microbial pathogens isolated from clinical institutions and works in the Department of Infectious Diseases, Gifu Prefectural Institute of Health and Environmental Sciences.

References

1. Knothe H, Shah P, Kromery V, Antal M, Mitsuhashi S. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection 1983;11:315–7.
2. Rasheed JK, Anderson GJ, Queenan AM, Biddle JW, Oliver A, Jacoby GA, et al. TEM-71, a novel plasmid-encoded, extended-spectrum β-lactamase produced by a clinical isolate of *Klebsiella pneumoniae*. Antimicrob Agents Chemother 2002;46:2000–3.
3. Rasheed JK, Anderson GJ, Yigit H, Queenan AM, Domenech-Sanchez A, Swenson JM, et al. Characterization of the extended-spectrum β-lactamase reference strain, *Klebsiella pneumoniae* K6 (ATCC 700603), which produces the novel enzyme SHV-18. Antimicrob Agents Chemother 2000;44:2382–8.
4. Ishii Y, Ohno A, Taguchi H, Imajo S, Ishiguro M, Matsuzawa H. Cloning and sequence of the gene encoding a cefotaxime-hydrolyzing class A β-lactamase isolated from *Escherichia coli*. Antimicrob Agents Chemother 1995;39:2269–75.
5. Tzouvelekis LS, Tzelepi E, Tassios PT, Legakis NJ. CTX-M-type β-lactamases: an emerging group of extended-spectrum enzymes. Int J Antimicrob Agents 2000;14:137–42.
6. Gniadkowski M, Schneider I, Palucha A, Jungwirth R, Mikiewicz B, Bauernfeind A. Cefotaxime-resistant *Enterobacteriaceae* isolates from a hospital in Warsaw, Poland: identification of a new CTX-M-3 cefotaxime-hydrolyzing β-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme. Antimicrob Agents Chemother 1998;42:827–32.
7. Bonnet R, Sampaio JL, Labia R, De Champs C, Sirot D, Chanal C, et al. A novel CTX-M β-lactamase (CTX-M-8) in cefotaxime-resistant *Enterobacteriaceae* isolated in Brazil. Antimicrob Agents Chemother 2000;44:1936–42.
8. Sabaté M, Tarragó R, Navarro F, Miró E, Vergés C, Barbé J, et al. Cloning and sequence of the gene encoding a novel cefotaxime-hydrolyzing β-lactamase (CTX-M-9) from *Escherichia coli* in Spain. Antimicrob Agents Chemother 2000;44:1970–3.
9. Simarro E, Navarro F, Ruiz J, Miró E, Gómez J, Mirelis B. *Salmonella enterica* serovar Virchow with CTX-M-like β-lactamase in Spain. J Clin Microbiol 2000;38:4676–8.
10. Bradford PA, Yang Y, Sahm D, Grope I, Gardovska D, Storch G. CTX-M-5, a novel cefotaxime-hydrolyzing β-lactamase from an outbreak of *Salmonella typhimurium* in Latvia. Antimicrob Agents Chemother 1998;42:1980–4.
11. Nordmann P. Trends in β-lactam resistance among Enterobacteriaceae. Clin Infect Dis 1998;27(Suppl 1):S100–6.

12. Hirakata Y. Extended-spectrum β-lactamases (ESBLs) producing bacteria [in Japanese]. Nippon Rinsho 2001;59:694–700.
13. Yagi T, Kurokawa H, Shibata N, Shibayama K, Arakawa Y. A preliminary survey of extended-spectrum β-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan. FEMS Microbiol Lett 2000;184:53–6.
14. Kurokawa H, Yagi T, Shibata N, Shibayama K, Kamachi K, Arakawa Y. A new SHV-derived extended-spectrum β-lactamase (SHV-24) that hydrolyzes ceftazidime through a single-amino-acid substitution (D179G) in the omega-loop. Antimicrob Agents Chemother 2000;44:1725–7.
15. Arakawa Y, Ike Y, Nagasawa M, Shibata N, Doi Y, Shibayama K, et al. Trends in antimicrobial-drug resistance in Japan. Emerg Infect Dis 2000;6:572–5.
16. Senda K, Arakawa Y, Nakashima K, Ito H, Ichiyama S, Shimokata K, et al. Multifocal outbreaks of metallo-β-lactamase-producing *Pseudomonas aeruginosa* resistant to broad-spectrum β-lactams, including carbapenems. Antimicrob Agents Chemother 1996;40:349–53.
17. Komatsu M, Aihara M, Shimakawa K, Yamanaka T, Matsuo S. Detection of extended-spectrum β-lactamases producing *Enterobacteriaceae* in feces [in Japanese]. Kansenshogaku Zasshi 2000;74:250–8.
18. Kawakami S, Ono Y, Yamamoto M, Matumura M, Okamoto R, Inoue M, et al. Extended-spectrum β-lactamase (ESBL) produced by *Escherichia coli* and *Klebsiella pneumoniae* isolated from Teikyo University Hospital—the second report. [in Japanese] Kansenshogaku Zasshi 2000;74:24–9.
19. Bates J, Jordens Z, Selkon JB. Evidence for an animal origin of vancomycin-resistant enterococci. Lancet 1993;342:490–1.
20. Fone DL, Barker RM. Associations between human and farm animal infections with *Salmonella typhimurium* DT104 in Herefordshire. Commun Dis Rep CDR Rev 1994;4:R136–40.
21. Endtz HP, Mouton RP, van der Reyden T, Ruijs GJ, Biever M, van Klingeren B. Fluoroquinolone resistance in *Campylobacter* spp isolated from human stools and poultry products. Lancet 1990;335:787.
22. Teshager T, Domínguez L, Moreno MA, Saénz Y, Torres C, Cardeñosa S. Isolation of an SHV-12 β-lactamase-producing *Escherichia coli* strain from a dog with recurrent urinary tract infections. Antimicrob Agents Chemother 2000;44:3483–4.
23. Bradford PA, Petersen PJ, Fingerman IM, White DG. Characterization of expanded-spectrum cephalosporin resistance in *E. coli* isolates associated with bovine calf diarrhoeal disease. J Antimicrob Chemother 1999;44:607–10.
24. Gannon VPJ, D'souza S, Graham T, King RK, Rahn K, Read S. Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. J Clin Microbiol 1997;35:656–62.
25. Jarlier V, Nicolas MH, Fournier G, Philippon A. Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev Infect Dis 1988;10:867–78.
26. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing. Ninth informational supplement, M100-S9. Wayne (PA): The Committee; 1999.
27. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 4th ed. Approved standard M7-A4. Wayne (PA): The Committee; 1997.
28. Nakamura T, Uchida S, Heijyo H, Masuda M, Takahashi H, Komatsu M, et al. A SHV-derived extended-spectrum β-lactamase (SHV-12) produced by an *Escherichia coli* recovered from wound abscess in post operative case with rectal carcinoma [in Japanese]. Kansenshogaku Zasshi 2000;74:112–9.
29. Radice M, González C, Power P, Vidal MC, Gutkind G. Third-generation cephalosporin resistance in *Shigella sonnei*, Argentina. Emerg Infect Dis 2001;7:442–3.
30. Thomson KS. Controversies about extended-spectrum and AmpC β-lactamases. Emerg Infect Dis 2001;7:333–6.
31. Bauernfeind A, Stemplinger I, Jungwirth R, Ernst S, Casellas JM. Sequences of β-lactamase genes encoding CTX-M-1 (MEN-1) and CTX-M-2 and relationship of their amino acid sequences with those of other β-lactamases. Antimicrob Agents Chemother 1996;40:509–13.
32. Fey PD, Safranek TJ, Rupp ME, Dunne EF, Ribot E, Iwen PC, et al. Ceftriaxone-resistant salmonella infection acquired by a child from cattle. N Engl J Med 2000;342:1242–9.
33. Dunne EF, Fey PD, Kludt P, Reporter R, Mostashari F, Shillam P, et al. Emergence of domestically acquired ceftriaxone-resistant *Salmonella* infections associated with AmpC β-lactamase. JAMA 2000;284:3151–6.
34. Casin I, Breuil J, Brisabois A, Moury F, Grimont F, Collatz E. Multidrug-resistant human and animal *Salmonella typhimurium* isolates in France belong predominantly to a DT104 clone with the chromosome- and integron-encoded β-lactamase PSE-1. J Infect Dis 1999;179:1173–82.
35. Winokur PL, Brueggemann A, DeSalvo DL, Hoffmann L, Apley MD, Uhlenhopp EK, et al. Animal and human multidrug-resistant, cephalosporin-resistant *Salmonella* isolates expressing a plasmid-mediated CMY-2 AmpC β-lactamase. Antimicrob Agents Chemother 2000;44:2777–83.
36. Kariuki S, Gilks CF, Kimari J, Muyodi J, Waiyaki P, Hart CA. Plasmid diversity of multi-drug-resistant *Escherichia coli* isolated from children with diarrhoea in a poultry-farming area in Kenya. Ann Trop Med Parasitol 1997;91:87–94.
37. Yan JJ, Ko WC, Chiu CH, Tsai SH, Wu HM, Wu JJ. Emergence of ceftriaxone-resistant *Salmonella* isolates and rapid spread of plasmid-encoded CMY-2-like cephalosporinase, Taiwan. Emerg Infect Dis 2003;9:323–8.
38. Deshpande L, Pfaller MA, Jones RN. In vitro activity of ceftiofur tested against clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* including extended spectrum β-lactamase producing strains. Int J Antimicrob Agents 2000;15:271–5.
39. Ohkawa T, Yoshinaga M, Ikarimoto N, Miyanohara H, Miyata K, Doi Y, et al. Characterization of *Klebsiella pneumoniae* and *Escherichia coli* strains that produce CTX-M-2-type broad spectrum β-lactamase isolated from a child with leukemia. Pediatr Infect Dis J 2002;21:260–2.
40. Doi Y, Shibata N, Shibayama K, Kamachi K, Kurokawa H, Yokoyama K, et al. Characterization of a novel plasmid-mediated cephalosporinase (CMY-9) and its genetic environment in an *Escherichia coli* clinical isolate. Antimicrob Agents Chemother 2002;46:2427–34.

Address for correspondence: Yutaka Shiraki, Department of Infectious Diseases, Gifu Prefectural Institute of Health and Environmental Sciences, 1-1 Naka-Fudogaoka, Kakamigahara, Gifu 504-0838, Japan; fax: +81-583-80-2131; email: shiraki-yutaka@pref.gifu.lg.jp

JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2003, p. 5530–5536 Vol. 41, No. 12
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.12.5530–5536.2003

Nosocomial Outbreak of Infections by *Proteus mirabilis* That Produces Extended-Spectrum CTX-M-2 Type β-Lactamase

Noriyuki Nagano,[1]* Naohiro Shibata,[2] Yuko Saitou,[1] Yukiko Nagano,[1] and Yoshichika Arakawa[2]

Medical Microbiology Laboratory, Funabashi Medical Center, 1-21-1 Kanasugi, Funabashi, Chiba,[1] and Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo,[2] Japan

Received 9 May 2003/Returned for modification 25 August 2003/Accepted 18 September 2003

Nineteen multidrug-resistant *Proteus mirabilis* strains were isolated from 19 patients suffering from infections probably caused by *P. mirabilis*. These strains were recovered from urine or other urogenital specimens of 16 inpatients and three outpatients with a hospitalization history in a urology ward of Funabashi Medical Center, from July 2001 to August 2002. These strains demonstrated resistance to cefotaxime, ceftriaxone, cefpodoxime, and aztreonam, while they were highly susceptible to ceftazidime (MIC, ≤0.5 μg/ml). The resistance level of these strains to cefotaxime was decreased by the presence of clavulanic acid. Therefore, the strains were speculated to produce extended-spectrum class A β-lactamases. These strains were later found to carry $bla_{CTX\text{-}M\text{-}2}$ genes by both PCR and sequencing analyses. The profiles of *Sma*I-digested genomic DNA of 19 isolates were distinguished into five different clusters by biased sinusoidal field gel electrophoresis. Four of them, consisting of 18 isolates, were suggested to be a clonal expansion. These findings suggested that a nosocomial outbreak of infections by CTX-M-2-producing *P. mirabilis* had occurred in our medical center. Most patients suffered from urogenital malignancies with long-term catheterization. Cefazolin, cefoperazone-sulbactam, and/or levofloxacin were mostly administered to the patients, but these agents seemed ineffective for eradication of CTX-M-2 producers. Early recognition and rapid identification of colonizing antimicrobial-resistant bacteria, including CTX-M-2-producing *P. mirabilis*, would be the most effective measures to cope with further spread of this kind of hazardous microorganism in clinical environments.

The increasing prevalence of plasmid-mediated extended-spectrum β-lactamases (ESBLs) in members of the family *Enterobacteriaceae* has become a serious clinical problem on a worldwide scale (8). ESBLs of Ambler's molecular class A (1) belonging to Bush's functional group 2be (10) are capable of hydrolyzing a wide range of β-lactams, including oxyimino-β-lactams and monobactam, but usually remain ineffective against cephamycins such as cefoxitin, cefmetazole, and cefotetan as well as carbapenems. These class A β-lactamases tend to be blocked by β-lactamase inhibitors such as clavulanic acid (10). The majority of ESBLs are derivatives of TEM-1, TEM-2, or SHV-1 enzymes, resulting from a few amino acid substitutions (10). In contrast to these TEM- and SHV-derived ESBLs, CTX-M type β-lactamases, which constitute a new family of class A enzymes, are exclusively active against cefotaxime compared to other oxyimino-cephalosporins, including ceftazidime (39).

More than 30 CTX-M-type β-lactamases have so far been described in various species of *Enterobacteriaceae* but mostly in *Salmonella enterica* serovar Typhimurium, *Escherichia coli*, and *Klebsiella pneumoniae* since the initial reports of Toho-1-producing *E. coli* in Japan (18) and CTX-M-1/MEN-1 in 1989 in Germany and France (2, 3). Strains producing other CTX-M enzymes have been isolated in separate geographic areas, including Europe (9, 15, 16, 35, 40), South America (4, 7, 30), and the Middle and Far East (4, 18, 23, 41). CTX-M-type β-lactamases can be classified into four clusters according to their amino acid sequences: CTX-M-1-group, with CTX-M-1 (4), -M-3 (16), -M-10 (27), -M-11 (GenBank accession no. AY005110), -M-12 (21), -M-15 (20), -M-22 (GenBank accession no. AY080894), and -M-23 (GenBank accession no. AF488377); CTX-M-2-group, with CTX-M-2 (4), -M-4 (15), -M-5 (9), -M-6 (14), -M-7 (14), -M-20 (36), -M-24 (GenBank accession no. AY143430), and Toho-1 (18); CTX-M-8 group (7); and CTX-M-9 group, with CTX-M-9 (35), -M-13 (12), -M-14/18 (29), -M-16 (6), -M-19 (32), -M-21 (36), and Toho-2 (23).

In this paper, we report a nosocomial outbreak of infections caused by CTX-M-2 β-lactamase-producing *Proteus mirabilis* in a urology ward. *P. mirabilis* is one of the most common causes of urinary tract infections. Because of the difficulty in eradicating *P. mirabilis* species from immunocompromised hosts (13), this bacterial species is usually considered an important cause of nosocomial infections (34). Although the most predominant plasmid-mediated β-lactamases found in clinical isolates of *P. mirabilis* are TEM-derived ESBLs (5, 11, 22, 24, 28, 33), the emergence of CTX-M-type enzymes with extended substrate specificity has been a serious concern (7, 36). In the present study, we investigated the CTX-M-2-producing *P. mirabilis* strains that caused a nosocomial outbreak in a urology ward in our medical center.

MATERIALS AND METHODS

Patients and bacterial strains. From July 2001 to August 2002, 19 nonduplicated multiresistant *P. mirabilis* clinical strains were isolated from 19 patients suffering from infections probably caused by *P. mirabilis*. These strains were recovered from urine or other urogenital specimens of 16 inpatients and three outpatients with a hospitalization history in a urology ward of Funabashi Medical Center. This hospital has 426 beds and serves as an acute-care municipal hospital

* Corresponding author. Mailing address: Medical Microbiology Laboratory, Funabashi Medical Center, 1-21-1 Kanasugi, Funabashi, Chiba 273-8588, Japan. Phone: 81-47- 438-3321. Fax: 81-47-438-7323. E-mail: naganoyn@d3.dion.ne.jp.

TABLE 1. Origins of CTX-M-2-producing *P. mirabilis* isolates and medical records of patients

Patient no.	Strain no.[a]	Date of isolation (day/mo/yr)	Source	Age (yr)/sex	Underlying disease	Antibiotic used within 30 days before detection[b]
1	1	9/7/01	Operative wound	77/M	Bladder cancer	KAN, CFZ, CFP-SUL
2	2	24/7/01	Indwelling catheter urine	83/F	Bladder stone	CDR, CFZ, CFP-SUL
3	3	15/8/01	Indwelling catheter urine	66/M	Prostatic cancer	LVX, CFZ, CFP-SUL
4	4	30/8/01	Midstream urine	62/M	Retroperitoneal fibrosis, renal failure	LVX, CDR, CFZ, IPM/CS, CTM-HE
5	5	18/10/01	Indwelling catheter urine	83/M	Bladder cancer	LVX, CFZ, CFP-SUL, IPM/CS
6	6	22/10/01	Indwelling catheter urine	59/M	Bladder cancer	LVX, CFZ, CFP-SUL
7	7	29/10/01	Indwelling catheter urine	79/F	Postrenal failure, hydronephrosis	LVX, CFZ
8	8	8/11/01	Midstream urine	77/M	Bladder cancer	LVX, CFZ, CFP-SUL, IPM/CS
9	9	12/11/01	Midstream urine	73/M	Prostatic cancer	LVX, CFZ, CFP-SUL, CDR
10	10	12/11/01	Catheter urine	70/M	Renal failure, diabetes mellitus	IPM/CS, GEN, LVX, MIN
11	11	19/11/01	Midstream urine	72/M	Bladder cancer	CFZ, CFP-SUL
12	12	29/11/01	Midstream urine	62/M	Bladder cancer	KAN, LVX, CDR, CFZ
13	13	30/11/01	Indwelling catheter urine	44/M	Stomach cancer, hydronephrosis, pyelonephritis	CFP-SUL, ISP
14	14	6/12/01	Indwelling catheter urine	83/M	Prostatic cancer	CFZ, AMP, CAZ
15	15	30/4/02	Sputum	56/M	Rectal cancer, bladder cancer	FMOX, CAZ
16	16	19/6/01	Catheter urine	80/M	Bladder stone	LVX
17	17	13/12/01	Indwelling catheter urine	80/F	Bladder cancer	CFZ, CDR, IPM/CS, CFP-SUL
18	18	2/8/02	Midstream urine	65/M	Prostatic cancer	CFZ, CFP-SUL
19	19	29/8/02	Indwelling catheter urine	59/M	Bladder cancer	LVX, CFZ, CFP-SUL

[a] Strain no. 16, 17, and 18 were derived from outpatients with a hospitalization history in a urology ward.
[b] KAN, kanamycin; CFZ, cefazolin; CFP-SUL, cefoperazone-sulbactam; CDR, cefdinir; LVX, levofloxacin; IPM/CS, imipenem/cilastatin; CTM-HE, cefotiam-hexetil; GEN, gentamicin; MIN, minocycline; ISP, isepamicin; AMP, ampicillin; CAZ, ceftazidime; FMOX, flomoxef.

for a population of 560,000 in Funabashi City, Chiba, Japan. Tables 1 and 2 show the clinical background of patients for each isolate and their respective treatment outcomes. All 19 isolates were suggested to produce inhibitor-susceptible class A β-lactamase based on the double-disk synergy test results. Biochemical identification of isolates was performed with an NEG Combo 5J panel and WalkAway-96 SI System (Dade Behring, Sacramento, Calif.) according to the manufacturer's instructions. β-Lactamase testing was performed based on microacidimetry with a commercial product (P/Case Test; Nissui Pharmaceutical, Tokyo, Japan). Bacterial strains were stored before use in Casitone medium (Eiken Chemical, Tokyo, Japan) at room temperature.

Antimicrobial susceptibility testing. MICs were determined by a microdilution broth method with a WalkAway-96 SI System (NEG Combo 5J and NEG MIC 5J panels; Dade Behring) with an inoculum of 10^4 CFU per well. Susceptibility categories were determined according to the National Committee for Clinical Laboratory Standards (NCCLS) criteria (26).

ESBL plus Panel (Dade Behring) with an inoculum of 10^4 CFU per well was used complementarily for MIC measurements, with incubation for 18 h at 35°C, and then assessed visually.

Double-disk synergy test. For screening ESBL-producing strains, the double-disk synergy test was used. Antimicrobial disks for Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) tests, cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), and amoxicillin-clavulanic acid (20 μg and 10 μg) were obtained from Nissui Pharmaceutical. The distance between disks was adjusted so that synergy could be detected accurately (38).

PCR analysis. A search for bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, and $bla_{CTX-M-9}$ genes in clinical isolates was performed by PCR amplification with the following sets of primers: 5′-CCGTGTCGCCCTTATTCC-3′ and 5′-AGG CACCTATCTCAGCGA-3′ for bla_{TEM}, 5′-ATTTGTCGCTTCTTTACTCG C-3′ and 5′-TTTATGGCGTTACCTTTGACC-3′ for bla_{SHV}, 5′-CGGTGCTG AAGAAAAGTG-3′ and 5′-TACCCAGCGTCAGATTAC-3′ for $bla_{CTX-M-1}$, 5′-ACGCTACCCCTGCTATTT-3′ and 5′-CCTTTCCGCCTTCTGCTC-3′ and for $bla_{CTX-M-2}$, and 5′-GCAGATAATACGCAGGTG-3′ and 5′-CGCCGTGG TGGTGTCTCT-3′ for $bla_{CTX-M-9}$. Freshly isolated colonies were suspended in distilled water and adjusted to a 0.5 MacFarland, which was boiled for 10 min. Supernatant obtained after centrifugation at 13,000 rpm for 5 min was used as template DNA.

PCRs were carried out in 50-μl volumes containing 5 μl of DNA, 0.5 μM each primer, 200 μM deoxynucleoside triphosphates, 1.25 U of TaKaRa Ex *Taq* (Takara), and PCR buffer (Takara) with the following parameters: initial denaturation at 94°C for 2 min, denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1.5 min, repeated for 30 cycles; and a final extension at 72°C for 5 min.

CTX-M-2-specific PCR and DNA sequencing. Amplification of the $bla_{CTX-M-2}$ gene and flanking regions was carried out with the oligonucleotide primers M-2-F (5′-TTCGCCGCTCAATGTTA-3′) and M-2-R (5′-GCATCAGAAACC GTGGG-3′), corresponding to nucleotides 22 to 38 and 852 to 868, respectively, of the structural gene. Plasmid DNA was prepared from each isolate by the Kado and Liu method (19) and used as templates for PCR analyses. PCRs were performed as described above. Cycling conditions were denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min, repeated for 30 cycles. PCR-generated amplicons were purified with a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, Calif.), and sequenced directly on both strands with a BigDye terminator cycle sequencing ready reaction kit and ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.).

Genomic typing. Chromosomal DNAs from clinical isolates embedded in agarose gel plugs (InCert; Bio-Whittaker Molecular Applications, Rockland, Maine) were subjected to treatments with lysozyme and sodium dodecyl sulfate containing proteinase K, then incubated overnight at 30°C with 12.5 U of *Sma*I (Takara Shuzo Co., Kyoto, Japan). Plugs were mounted into the wells of a 1% SeaKem GTG Agarose (Bio-Whittaker) in 50 mM Tris-borate-EDTA buffer (pH 8.4). The biased sinusoidal field gel electrophoresis system (Atto Corp., Tokyo, Japan) (25), a modified pulsed-field gel electrophoresis technique utilizing a biased sinusoidal electric field for separation of large DNA molecules, was employed at 12°C with the field parameters Eb = 1.2 V/cm and Es = 7.3 V/cm. Lambda DNA ladders (48.5 kb to 1 Mb; Takara) were used as molecular size markers.

RESULTS

Bacterial strains and clinical features. Multiresistant *P. mirabilis* isolates were obtained from 16 inpatients in a urology ward and three outpatients with a hospitalization history in