dime through a single amino acid substitution in the omega loop. Antimicrob. Agents Chemother. **45**:3355–3361.
33. **Prilli, M., E. Dell'Amico, B. Segatore, M. R. de Massis, C. Bianchi, F. Luzzaro, G. M. Rossolini, A. Toniolo, G. Nicoletti, and G. Amicosante.** 2002. Molecular characterization of extended-spectrum β-lactamases produced by nosocomial isolates of *Enterobacteriaceae* from an Italian nationwide survey. J. Clin. Microbiol. **40**:611–614.
34. **Rozalski, A., Z. Sidorezyk, and K. Kotelko.** 1997. Potential virulence factors of *Proteus* bacilli. Microbiol. Mol. Biol. Rev. **61**:65–89.
35. **Sabate, M., R. Tarrago, F. Navarro, E. Miro, C. Verges, J. Barbe, and G. Prats.** 2000. Cloning and sequence of the gene encoding a novel cefotaxime-hydrolyzing β-lactamase (CTX-M-9) from *Escherichia coli* in Spain. Antimicrob. Agents Chemother. **44**:1970–1973.
36. **Saladin, M., V. Bao Cao, T. Lambert, J. L. Donay, J. L. Herrmann, Z. Ould-Hocine, C. Verdet, F. Deleisle, A. Philippon, and G. Arlet.** 2002. Diversity of CTX-M β-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. FEMS Microbiol. Lett. **209**: 161–168.
37. **Saurina, G., J. M. Quale, V. M. Manikal, E. Oydna, and D. Landman.** 2000. Antimicrobial resistance in *Enterobacteriaceae* in Brooklyn, N. Y.: epidemiology and relation to antibiotic usage patterns. J. Antimicrob. Chemother. **45**:895–898.
38. **Sirot, J.** 1996. Detection of extended-spectrum plasmid-mediated β-lactamases by disk diffusion. Clin. Microbiol. Infect. **2**:S35–S39.
39. **Tzouvelekis, L. S., E. Tzelepi, P. T. Tassios, and N. J. Legakis.** 2000. CTX-M-type β-lactamases: an emerging group of extended-spectrum enzymes. Int. J. Antimicrob. Agents **14**:137–142.
40. **Tzouvelekis, L. S., M. Gazouli, A. Markogiannakis, E. Paraskaki, N. J. Legakis, and E. Tzelepi.** 1998. Emergence of resistance to third-generation cephalosporins amongst *Salmonella typhimurium* isolates in Greece: report of the first three cases. J. Antimicrob. Chemother. **42**:273–275.
41. **Yagi, T., H. Kurokawa, K. Senda, S. Ichiyama, H. Ito, S. Ohsuka, K. Shibayama, K. Shimokata, N. Kato, M. Ohta, and Y. Arakawa.** 1997. Nosocomial spread of cephem-resistant *Escherichia coli* strains carrying multiple Toho-1-like β-lactamase genes. Antimicrob. Agents Chemother. **41**:2606–2611.

厚生労働省のサーベイランス事業（NICU）

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厚生労働省のサーベイランス事業（NICUサーベイランス）について「どのように行うものか」を具体的に教えてください．

現在10施設でサーベイランス入力が開始されています．ソフトの改善中のために，現在一般参加はできませんが，今後検討中です．

はじめに

2001年，厚生科学研究の一環で，極低出生体重児（VLBW）の院内感染（病院感染）のアンケート調査を行い，各NICUの院内感染予防対策内容とMRSA感染発症率との関係を解析しました．ここでMRSA保菌患児の予防的治療や保育器の終末消毒等のMRSA感染症発症率と関係する項目が見い出され，感染症例情報のみならず，予防対策項目を含めた施設情報のサーベイランスを継続的に行う必要性が生じました．

入力システムの概要

既存の厚生労働省院内感染対策サーベイランス事業（ICU・全入院患者・検査）に一部準拠した形で，周産期医療体制に関する研究（平成12年度厚生科学研究中村班2001年2月作成分）のハイリスク入院基本情報を基礎にして，「ファイルメーカープロVer.5.0」により入力システムを構築しました．

入力項目内容は，①患者基本情報70項目（うち新規33項目），②感染症・細菌情報20項目，③予防対策（施設）情報55項目の3頁です．①の基本情報には，各種ディバイスデイや3歳時の発育データ・発達検査を盛り込み，②では各

NICU感染症サーベイランス用感染情報　施設コード [47001]

基本情報入力
施設情報入力
報告データ出力
充足度表示

＜感染症関連データ＞　患児ID－No [　　]　患児氏名 [　　]

発症日 [　　]　発症日齢 [　]　治療開始日齢 [　]　治療終了日齢 [　]

手術による感染の有無 [　]　その手術日 [　　]　その手術内容 [　　]

感染症入力

敗血症 | 肺炎 | 髄膜/脳室炎 | 尿路感染 | 血流感染 | NEC | 腸炎 | カンジダ | NTED | SSSS | 肝炎 | その他

条件1のすべておよび条件2の1つ以上に該当する場合に血流感染と診断する

条件1
- □1回もしくは複数の血液培養から微生物が確認される
- □培養された微生物は他の部位の感染に関係がない
- □血管内留置装置での感染が疑わしい

条件2
- □カテーテルなどの早期抜去により急速な改善を認める
- □疑ったカテーテル先端培養が陽性
- □三方活栓内培養やラインからの逆血培養で陽性

※ただし，感染発症前48時間以内に以下のディバイスを留置している場合は選択すること
- □中心静脈ライン
- □末梢（動静脈）ライン
- □臍（動静脈）カテーテル

CRP(最高値) [　]　白血球(最高値) [　]　血小板(最低値) [　]

原因菌種名 [　]　[　　]

MRSAの検査 [　] [　] [　] [　]

緑膿菌の検査 [　] [　] [　] [　]

陽性検体材料 [　]　使用抗菌剤1 [　]　使用抗菌剤3 [　]

検体番号 [　]　使用抗菌剤2 [　]　使用抗菌剤4 [　]

検体保存 [　]

抗生物質感受性リスト（SIR）　耐性の有無 [　]

薬剤名	検査結果	薬剤名	検査結果	薬剤名	検査結果	薬剤名	検査結果	薬剤名	検査結果

併用治療

□γグログリン投与　□G－CSF投与　□外科的処置（穿刺・排膿）　□その他
□血液製剤投与　□交換輸血　□顆粒球輸注

図1　感染症関連データの入力パターン
（「血流感染」の項目を例としてあげた）

INFECTION CONTROL　2003増刊

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NICU感染症サーベイランス用感染情報　　施設コード

＜施設の形態と規模および感染対策内容＞

基本情報入力
感染症入力
報告データ出力
充足度表示

年間人工換気症例数　例　　病院の形態

NICU病床数/面積 / m^2　病棟勤務医師数　　病棟勤務看護師数

GCU病床数/面積 / m^2　医師当直回数(月)　　看護師の夜勤担当患児数

感染対策委員会　　感染対策マニュアル　　カンジダ

ICT　　MRSA　　その他菌名

NICU感染係　　緑膿菌

院内感染対策施行

院内感染対策施行項目

□ガウンテクニック　□手袋　□隔離室入室　□保菌患児検査・治療　□その他
□マスク　□保育器隔離　□独立看護単位　□保菌職員検査・治療

＜手洗い＞

手洗い水　　よく使う手洗い用消毒剤

次によく使う手洗い用消毒剤

＜手袋＞

手袋の使用　　手袋の使用：その他

手袋使用対象児　　手袋の使用対象児：その他

＜保育器消毒＞

保育器使用後の終末消毒有無　　終末消毒法　　回数　1/　日

（終末消毒とはホルマリンガスなどで滅菌すること）

毎日の保育器清拭消毒剤　　保育器清拭消毒剤の濃度　%

＜器具の個別化＞

□聴診器　□体温計　□メジャー　□はさみ　□テープ　□オリーブ油　□筆記用具　□ワセリン

＜MRSA＞

NICUからMRSAが消えた期間　　MRSAが消失していた期間　ヵ月以上

職員のMRSA保菌率　%　最新実施年月　　保菌職員のMRSA除菌

患児のMRSA保菌率　%　最新実施年月　　保菌患児のMRSA除菌

保菌患児の消毒方法

他病棟におけるMRSA定着　　MRSA陽性病棟とNICU職員交代

＜MRSA＞

超早期授乳　　抗真菌剤経口予防投与

ビフィズス菌製剤予防投与　　気管内抗生物質予防投与

予防的IgG投与

図2　施設情報の入力画面

（施設の内容と感染対策項目を入力できる）

感染症の診断基準をチェックしながら入力する形に整えました（図1）．③の施設情報には，2002年のアンケート調査から感染率に影響を一部与えている項目を主に盛り込みました(図2)．

①，②はNICU退院患者（月単位）を入力対象とし，1～6月，7～12月分のデータ報告として出力します．③は変更した予防対策項目だけを入力し，研究班参加施設を対象として解析を行います．研究班としては参加各施設へのデータ報告とともに，各出力項目で自施設の全体の中における位置を示す自己評価の可能なシステムを作ります．

現在の参加施設

現在の参加施設は，横浜市立大学医学部，聖マリアンナ医科大学西部病院，長野県立こども病院，順天堂伊豆長岡病院，名古屋大学医学部附属病院，名古屋第二赤十字病院，大阪府立母子保健総合医療センター，国立病院九州医療センター,福岡市立こども病院・感染症センター，鹿児島市立病院の10施設です．

入力システムの詳細

○入力項目と入力ソフトについて

・院内感染症の定義は，入院時に保菌していなかった菌による感染症のことを指します．新生児では72時間以内の発症は主に母体由来の菌によるものと考えます．しかし分娩時の蘇生などにより担当した医療者からの菌によることが明らかな場合には,院内感染としてよいでしょう．

・入力項目は中村班による「ハイリスク新生児基本情報」に感染情報を加えたものにします．

・入力ソフトは，「ファイルメーカーVer.5」とします．

・データ入力は患者がNICUを退院した時点で行い，厚生労働省へのデータの送付は今後その頻度を決めて定期的に行います．6月（前期）と12月（後期）の年2回です．

・感染症の診断基準はこの研究班で確定し，表としました．

・この診断基準は病名診断と連携させて画面上でみることができます．

・感受性試験の対象抗菌薬と感受性評価（SIR法），薬剤耐性菌の判断基準は荒川宜親研究班で作成したものとします．

○入力規則について

・基本的には当該児が退院した時点で入力を完全に行います．

・6ヵ月以上の入院期間にあるものは，6ヵ月時点での報告時に新規分を入力します（入院データの更新）．この時に基本情報の1枚目の退院時情報の「転帰」の「入院中」をクリックします．

・入力データは6ヵ月ごとに報告します．

○解析方法

以下のような基本戦略で行います．

症例データの全国的な蓄積は，ランダムなデータの集まりでしかなく感染症流行の地域的分

ます．この死亡率の出力も入院数を母数として(1) に準じます．

(4) 感染症診断別発症数

母数を入院数として (1) に準じて出力します．

(5) 原因菌種別感染症数

母数を入院数として (1) に準じて出力します．

(6) MRSA感染症発症数および率

母数を入院数として (1) に準じて出力します．

(7) MRSA感染症／総感染症

MRSA感染症率を明示します．(1) に準じて出力します．

(8) 器具（ディバイス）使用率（出生体重別に）

・臍動・静脈カテーテル挿入率＝臍動・静脈カテーテル挿入日数／のべ入院患者数．

・中心静脈ライン挿入率＝中心静脈ライン挿入日数／のべ入院患者数．

・人工呼吸器装着率＝人工呼吸器装着日数／のべ入院患者数．

(9) 敗血症・その他の血流感染／ディバイス日数×1,000

各種ライン類の設置による血流感染症発症率を出力します．

(10) 肺炎発症数／人工呼吸器装着日数×1,000

人工換気療法依存性肺炎発症率を出力します．またこの肺炎は挿管中あるいは抜管後2日以内を対象とします．

(11) 外科的処置による感染数／外科的処置を受けたのべ患者数×1,000

SSIとしてその感染症発生率を出力します．

(12) NEC発症数と発症率（＜1,500g）

母数を入院数（＜1,500g）として出力します．

(13) 腸管原性敗血症数と発症率（＜1,500g）

腸管に定着している菌による敗血症で，母数を入院数（＜1,500g）として出力します．

(14) カンジダ腸炎発症数（＜1,500g）

母数を入院数（＜1,500g）として出力します．

(15) 原因不明肝炎発症数と発症率（＜1,000g）

母数を入院数（＜1,000g）として出力します．

(16) 死亡・後遺症例と原因菌株の内容検索リスト

＊（13）～（16）は，研究班の研究事業としてのものです．

○データマネジメントの確定

まずもっとも問題となっているMRSA関連の項目から解析する予定です.

MRSA感染における予防対策の各項目の評価付けに向けて

①MRSA感染／総感染率を各病院で求めます,
②高率の病院において頻度の高い項目の意味を評価します.
③実施されやすい順に予防対策項目の改善を勧めます.
④半年後, 院内感染率の変化を考察し項目を再度評価します.

MRSA特異菌株と後遺症の関係について

①特異菌株の特定.
②特異菌株の収集.
③特異菌株の解析.
④再度, 特異菌株の収集と臨床データの解析.

おわりに

現在まだサーベイランスシステムは十分に稼動できていない状況ですが, 施設情報としての感染対策項目の違いによるデータ集計が十分に集まれば, 対策項目の更なる改善につなげられると信じます. これに基づき今後のサーベイランス施設を拡大してゆく予定です.

参考文献

1) 平成13年度厚生科学研究費補助金／新興・再興感染症研究事業.「薬剤耐性菌の発生動向のネットワークに関する研究」厚生労働省院内感染対策サーベイランス事業, 新生児集中治療室（NICU）における院内感染対策サーベイランスに関する研究. 分担研究者：北島博之. 2002, 44-7.
2) 平成13年度厚生科学研究費補助金（医薬安全総合研究事業).「新生児及び乳幼児のMRSA感染等の院内感染のリスク評価及び対策に関する研究」新生児集中治療室（NICU）における院内感染対策サーベイランスシステム構築. 分担研究者：北島博之. 2002, 35-9.
3) 平成14年度厚生科学研究費補助金／新興・再興感染症研究事業.「薬剤耐性菌の発生動向のネットワークに関する研究」厚生労働省病院感染対策サーベイランス事業, 新生児集中治療室（NICU）における病院感染対策サーベイランスに関する研究. 分担研究者：北島博之. 2003, 75-82.
4) Hiroyuki Kitajima. Prevention of Methicillin-resistant *Staphylococcus aureus* Infections in Neonates. Pediatrics. International. 45, 2003, 238-45.

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

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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

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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}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, and $bla_{CTX\text{-}M\text{-}9}$ 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 $bla_{CTX\text{-}M\text{-}1}$, 5′-ACGCTACCCCTGCTATTT-3′ and 5′-CCTTTCCGCCTTCTGCTC-3′ and for $bla_{CTX\text{-}M\text{-}2}$, and 5′-GCAGATAATACGCAGGTG-3′ and 5′-CGCCGTGG TGGTGTCTCT-3′ for $bla_{CTX\text{-}M\text{-}9}$. 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 $bla_{CTX\text{-}M\text{-}2}$ 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

TABLE 2. Treatment outcome for patients with respect to detection of CTX-M-2-producing *P. mirabilis* isolates

Patient no.	Antibiotic used after isolation of bacteria[a]		Treatment outcome[b]	Coisolates[c]
	During persistent detection	Upon eradication		
1	CFP-SUL, FMOX, CFZ		Failure	*S. aureus*, *Streptococcus agalactiae*
2	CDR		Failure	*K. pneumoniae*, *E. faecalis*
3	CFP-SUL, LVX		Indeterminate	ND
4	LVX, CDR, CFP-SUL		Failure	*P. aeruginosa*
5	LVX, CFZ, CFP-SUL, IPM/CS, ISP, CDR		Failure[d]	MRSA, *E. faecalis*, *P. aeruginosa*
6	CFP-SUL, FMOX, IPM/CS		Indeterminate[d]	*E. faecalis*
7	LVX	TMP, FMOX	Eradication	ND
8	LVX, TMP, CFPN-PI, GAT		Failure	ND
9	CFP-SUL, CDR		Failure	ND
10	LVX		Failure	MRSA, *S. agalactiae*
11	IPM/CS, MIN		Indeterminate[d]	ND
12		FMOX, LVX	Eradication[d]	*Tatumella ptyseos*
13	ISP, CFP-SUL	CFP-SUL, CAZ	Eradication[d]	ND
14	CAZ		Failure[d]	*E. faecalis*, *S. haemolyticus*, *E. cloacae*, MRSA
15	FMOX		Indeterminate[d]	*P. aeruginosa*, *S. aureus*, *K. oxytoca*
16	LVX		Indeterminate	*E. faecalis*
17			Indeterminate	MRSA, *Morganella morgannii*
18	LVX		Indeterminate	ND
19		KAN, MTZ, LVX	Eradication	ND

[a] CFP-SUL, cefoperazone-sulbactam; FMOX, flomoxef; CFZ, cefazolin; CDR, cefdinir; LVX, levofloxacin; IPM/CS, imipenem/cilastatin; ISP, isepamicin; TMP, trimethoprim-sulfamethoxazole; CFPN-PI, cefcapene-pivoxil; GAT, gatifloxacin; MIN, minocycline; CAZ, ceftazidime; KAN, kanamycin; MTZ, metronidazole.
[b] Indeterminate, lack of necessary information.
[c] ND, not detected; MRSA, methicillin-resistant *S. aureus*.
[d] Died due to underlying disease.

that ward, 44 to 83 years old, 16 males and 3 females, from July 2001 to August 2002 (Table 1). Of 19 nonduplicated isolates, 9 isolates (strains 2, 3, 5, 6, 7, 13, 14, 17, and 19) were recovered from indwelling catheter urine samples, six isolates (strains 4, 8, 9, 11, 12, and 18) were from midstream urine samples, two isolates (strains 10 and 16) from catheter urine samples, one (strain 1) from an operative wound, and one (strain 15) from sputum. The common underlying disease was urogenital malignancies, including bladder cancer in nine patients, prostatic cancer in four patients, followed in order by renal failure in three, bladder stone in two, and stomach cancer as well as pyelonephritis in one patient. Cefazolin and cefoperazone-sulbactam had been administered most frequently in 12 patients, and levofloxacin had been prescribed to 11 patients within 30 days before isolation of the multiresistant *P. mirabilis*.

Table 2 shows the outcomes of antibiotic therapy for 19 patients after detection of the isolates, in which 12 patients were traceable for evaluation. Eradication was achieved in four patients with sulfamethoxazole-trimethoprim and flomoxef for patient 7, flomoxef and levofloxacin for patient 12, cefoperazone-sulbactam and ceftazidime for patient 13, and kanamycin, metronidazole, and levofloxacin for patient 19. In eight patients, *P. mirabilis* isolates were persistently detected despite the therapy with cefoperazone-sulbactam (patients 1, 4, 5, and 9), flomoxef (patient 1), imipenem/cilastatin (patient 5), and ceftazidime (patient 14). Treatment outcome could not be evaluated in seven patients due to lack of bacteriological follow-up in four and death from underlying disease in three patients. In 11 of 19 patients, other bacterial species were isolated besides *P. mirabilis*; *Enterococcus faecalis*, methicillin-resistant *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were predominant. Moreover, no ESBL producers were detected among eight bacterial strains belonging to the family *Enterobacteriaceae* or *P. aeruginosa* isolated from 19 patients.

Determination of antibiotic susceptibility. MICs of antimicrobial agents for 19 clinical isolates are shown in Table 3. These isolates showed very similar susceptibility profiles, characterized by elevated MICs of cefotaxime (MICs, >128 μg/ml), ceftriaxone (MICs, >64 μg/ml), cefpodoxime (MICs, >64 μg/ml), aztreonam (MICs, 8 to >16 μg/ml), while they were susceptible to ceftazidime (MICs, ≤0.5 μg/ml). For all isolates, the MICs of cefotaxime were decreased drastically to ≤0.12 μg/ml in the presence of 4 μg of clavulanic acid per ml, whereas the MICs of ceftazidime for these strains were not obviously influenced by the presence of clavulanic acid. The MICs of cefoperazone-sulbactam were 8 to 32 μg/ml. MICs of other β-lactams, imipenem and melopenem, for the same strains were 2 μg/ml and ≤0.5 μg/ml, respectively. There was a trend towards resistance to gentamicin (MICs, 2 to >8 μg/ml), minocycline (MICs, >8 μg/ml), and levofloxacin (MICs, 2 to >4 μg/ml) among the isolates.

β-Lactamase study. The production of β-lactamase was detected by the P/Case test, which can distinguish between penicillinase (benzylpenicillin as the substrate) and cephalosporinase (cephaloridine and clavulanic acid as the substrate). Penicillinase production was detected in all 19 strains tested. With the double-disk synergy test, expanded growth-inhibitory zones indicative of class A β-lactamase production were observed with cefotaxime, ceftazidime, and aztreonam disks among all 19 strains (data not shown). MICs of cefotaxime (>128 μg/ml) decreased dramatically to ≤0.12 μg/ml in the

TABLE 3. Antibiotic susceptibilities of clinical *P. mirabilis* isolates presented in Table 1

Antibiotic(s)	MIC (μg/ml)[b] distribution (no. of isolates tested)
Ampicillin	>16 (19)
Amoxicillin/CLA	4/2 (19)
Piperacillin	>64 (19)
Cefazolin	>16 (19)
Cefotiam	>16 (19)
Cefoperazone/SUL	8/4 (2), 16/8 (14), 32/16 (3)
Cefotaxime	>128 (19)
Cefotaxime/CLA	≤0.12/4 (19)
Ceftazidime	≤0.5 (19)
Ceftazidime/CLA	≤0.12/4 (19)
Ceftriaxone	>64 (19)
Cefpirome	>16 (19)
Cefepime	>32 (19)
Cefozopran	>16 (19)
Cefaclor	>16 (19)
Cefpodoxime	>64 (19)
Cefoxitin	4 (10), 8 (8), 32 (1)
Cefmetazole	≤4 (18), 32 (1)
Cefotetan	≤0.5 (19)
Flomoxef	≤1 (17), 2 (2)
Imipenem	2 (19)
Meropenem	≤0.5 (19)
Aztreonam	8 (2), >16 (17)
Gentamicin	2 (4), 8 (1), >8 (14)
Amikacin	4 (5), 8 (14)
Minocycline	>8 (19)
Levofloxacin	2 (1), >4 (18)
Fosfomycin	>16 (19)

[a] NEG Combo 5J and NEG MIC 5J panels, and ESBL plus Panal were used for MIC determination.

presence of 4 μg of clavulanic acid per ml, suggesting the production of a CTX-M type class A β-lactamase (Table 2).

PCR and sequencing of $bla_{CTX-M-2}$ gene. The preliminary PCR search revealed that all 19 *P. mirabilis* isolates showed 780-bp amplification products for $bla_{CTX-M-2}$ genes, whereas no amplicons were observed for bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1}$, or $bla_{CTX-M-9}$. The entire coding sequences of the $bla_{CTX-M-2}$ gene and flanking regions were subsequently amplified with more specific primers for $bla_{CTX-M-2}$, M-2-F and M-2-R, and sequenced on both strands. The BLAST analysis of the nucleotide sequences and the deduced protein sequences showed that *P. mirabilis* isolates produced CTX-M-2 group β-lactamase (4).

***Sma*I-digested genomic DNA profiles.** The *Sma*I-digested genomic DNAs of 19 clinical isolates were classified into five different clusters (Fig. 1). Thirteen isolates of strains 1, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 17 had the same restriction profiles (pattern I), while the patterns of strains 15, 18, and 19 differed from pattern I in only one band (pattern II). The patterns of strains 4 (pattern III) and 16 (pattern IV) were different from each other in three bands and differed in two bands from pattern I. Therefore, these 18 isolates were clonally related. The pattern of strain 2 (pattern V) was completely distinguishable from those of 18 isolates (Fig. 1).

DISCUSSION

ESBL production in *P. mirabilis* was first documented in 1993 (17), and the increase in clinical prevalence of ESBL-producing strains has recently been noted in survey studies in separate geographic areas, including the United States, Europe, and Asia. The proportion of ESBL-positive isolates has increased from 0.8% of *P. mirabilis* isolates in 1991 (17) to 6.9% in 1998 (13) in France. Surveillance studies conducted in the United States and Italy showed 9.5% and 8.8% ESBL prevalence among *P. mirabilis* isolates, respectively (22, 37). TEM-derived ESBLs showing a wide diversity were the most predominant among ESBLs (5, 11, 22, 24, 28, 33), but other enzymes belonging to group 2be (7, 36) have also been observed in *P. mirabilis*. Because of the production of such diverse class A β-lactamases in *P. mirabilis*, as well as its predilection for the urinary tract, the emergence and proliferation of multidrug resistant *P. mirabilis* could pose a threat, especially in catheterized patients with malignancy as a cause of subsequent nosocomial infections.

To our knowledge, this is the first report of a nosocomial outbreak of infections caused by CTX-M-2-producing *P. mirabilis* strains in a Japanese medical institution. Nineteen isolates found in its urology ward were initially speculated to produce CTX-M-type class A β-lactamase, since consistent high MICs

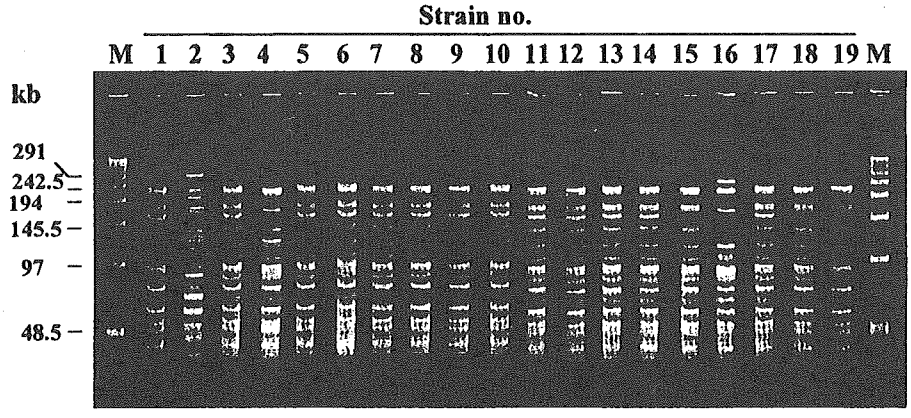

FIG. 1. Profiles of the genomic DNAs of 19 *P. mirabilis* isolates producing CTX-M-2 β-lactamase after digestion with *Sma*I. See Table 1 for the origins and backgrounds of the isolates. Lane M, lambda DNA ladder as molecular size markers.

of cefotaxime, ceftriaxone, cefpodoxime, and aztreonam were observed for these strains. The drastic reduction in the MICs of cefotaxime in the presence of clavulanic acid supported this speculation. Guidelines for screening and confirmatory tests for ESBL producers have been established by the National Committee for Clinical Laboratory Standards (26). These guidelines apply specifically to *K. pneumoniae*, *E. coli*, and *Klebsiella oxytoca*. However, the incidence of ESBLs producers has also been increasing in many other genera belonging to the family *Enterobacteriaceae* such as *Citrobacter*, *Enterobacter*, *Morganella*, *Proteus*, *Providencia*, *Salmonella*, *Serratia*, and other gram-negative bacilli (8). This is also the case in our medical center, where nosocomial infection due to ESBL-producing *Acinetobacter baumannii* has recently been identified (unpublished data).

In our experience, the NCCLS guidelines could be applicable for detection of CTX-M-2 producers among *P. mirabilis* that produce no intrinsic AmpC cephalosporinases, although some modification in the NCCLS criteria might be needed. This would be of critical importance to be able to detect ESBL and CTX-M-type β-lactamase producers for effective clinical management of patients with infections by reliable and appropriate therapeutic options. Thus, accurate monitoring of ESBL prevalence would be mandatory to promote hospital infection control procedures.

The production of ESBL is alternatively confirmed by the double-disk synergy test, by which a synergistic effect on growth inhibition is observed with the coexistence of clavulanic acid and broad-spectrum cephalosporins, including cefotaxime, ceftazidime, or aztreonam. This test was also useful to detect CTX-M-2 production in *P. mirabilis* strains. The NCCLS recommends, however, reporting that *Klebsiella* spp. and *E. coli* strains producing ESBLs may be clinically resistant to therapy with penicillins, cephalosporins, or aztreonam, despite apparent in vitro susceptibility to some of these agents. This recommendation might cause confusion, implying that ceftazidime may be ineffective for treatment of infections caused by cefotaxime-resistant strains that produce CTX-M-type β-lactamase despite the fact that strains producing only CTX-M-type β-lactamase seem highly susceptible to ceftazidime.

As a practical matter, eradication of CTX-M-2-producing *P. mirabilis* isolates was successfully achieved by therapy with ceftazidime in patient 13, although combination therapy with cefoperazone-sulbactam was employed in this case. However, ceftazidime therapy alone failed to eradicate the organism in patient 14 (Table 2). There was a difference in treatment regimens between these two cases. Continuous administration of ceftazidime (1 g/day intravenously) for 8 days was used for patient 13, while two courses of 3-day-repeated administration of the same dosage at a 19-day interval was administered to patient 14. Furthermore, while patient 13 was infected only with *P. mirabilis*, patient 14 had polymicrobial infection with *E. faecalis*, *Staphylococcus haemolyticus*, *Enterobacter cloacae*, and methicillin-resistant *S. aureus* in addition to CTX-M-2-producing *P. mirabilis*. This, together with the incomplete antibiotic therapy, might explain the poor therapeutic response to ceftazidime in patient 14. To our knowledge, no clinical evidence obtained by double-blind clinical trials supporting the ineffectiveness of ceftazidime for infections with CTX-M-type producers has been reported. Clinical studies to address this issue should be conducted immediately to either corroborate or call into question the NCCLS recommendation.

Most patients from whom CTX-M-2-producing *P. mirabilis* strains were isolated had urological malignancies and long-term catheterization. All patients had received antibiotic therapy for 30 days before isolation of *P. mirabilis*, in which cefazolin, cefoperazone-sulbactam, and/or levofloxacin had been most frequently administered. Among the antibiotics used after detection of the organisms, cefoperazone-sulbactam, imipenem/cilastatin, and flomoxef showed low MICs. However, eradication with cefoperazone-sulbactam was noted in only one (patient 13) of five patients, and it was ineffective in combination with isepamicin. Since CTX-M-type enzymes can hydrolyze cefoperazone and tend to be hardly blocked by sulbactam, CTX-M producers usually demonstrate insusceptibility or resistance to the combination of cefoperazone-sulbactam (41). Thus, random or uniform prescription of cefoperazone-sulbactam may induce nosocomial spread of gram-negative bacilli which produce CTX-M-type enzymes.

Actually, 12 of 19 patients were prescribed cefoperazone-sulbactam prior to the isolation of CTX-M-2-producing *P. mirabilis*. The imipenem/cilastatin used in one patient (patient 5) proved in ineffective. Flomoxef was used in three patients (patients 1, 7, and 12), and eradication was achieved in the latter two cases. Levofloxacin, which tends to be preferentially used in urinary tract infections in Japan, showed high MICs for *P. mirabilis* isolates and seemed ineffective for most of the eight patients for whom it was used. These findings suggest difficulties in eradication of CTX-M-2-producing *P. mirabilis* strains even with antibiotics with low MICs. The biofilm-forming ability of bacteria, including *P. mirabilis*, on urinary catheters may be one reason for the failure to eradicate the organisms.

The restriction profiles of genomic DNAs from 19 *P. mirabilis* isolates shared concordant patterns (pattern I) among 13 isolates (strains 1, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 17), suggesting a clonal expansion of CTX-M-2 producers. Moreover, a clonal relatedness among five isolates (strains 15, 18, 19, 4, and 16 corresponding to patterns II, III, and IV) was also indicated. These five strains presumably relate to the previous 13 strains with pattern I. However, the pattern of strain number 2 (pattern V) was apparently distinct from those of the other 18 isolates classified as patterns I, II, III, and IV. These findings suggested that a nosocomial outbreak of 18 clonally related isolates and one isolate subjected to a different genetic lineage had occurred in a certain ward carrying the same CTX-M-2 β-lactamase determinant.

The Dienes test, which visualizes a unique feature of the swarming ability of *P. mirabilis*, has been utilized for an epidemiologic typing method (31), which we adopted in the present study for initial epidemiologic characterization of isolates. The 16 isolates demonstrating pattern I or II were indistinguishable and formed one Dienes type, whereas one isolate with patterns III and one isolate with pattern V formed independent Dienes type. Furthermore, one isolate subjected to pattern IV showed less than detectable swarming ability (data not shown). While the Dienes test was indeed partially applicable for typing of *P. mirabilis*, the biased sinusoidal field gel electrophoresis employed in this study was much more useful

for the epidemiological analyses, although it requires skill and involves somewhat complicated procedures.

Infection control at the initial stage of the outbreak was difficult due to frequent patient transfers within the urology ward. Eventually, the outbreak was successfully brought under control by intensive surveillance, improvement of facilities including disinfection equipment, prudent use of antibiotics, and due precautions to prevent contact transmission of microorganisms. The most effective measures to prevent the further spread of CTX-M-2 β-lactamase-producing *P. mirabilis* were rapid identification of colonization status of such bacteria among all immunocompromised patients with severe urological disorders by periodic urine culturing at admission and once-per-week follow-up testing with informed consent.

In our medical center, first- and third-generation cephalosporins have been preferentially used as first-line drugs. Carbapenems and penicillins as well as first- and third-generation cephalosporins have accounted for the great majority. These antibiotics might well provide selective pressure for proliferation of the CTX-M-2 producers. Early recognition of bacterial strains possessing antimicrobial resistance would contribute to an appropriate antibiotic treatment regimen that would be essential for prevention of nosocomial infections.

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REFERENCES

1. **Ambler, R. P.** 1980. The structure of β-lactamases. Phil. Trans. R. Soc. London Biol. **289**:321–331.
2. **Barthelemy, M., J. Peduzzi, H. Bernard, C. Tancrede, and R. Labia.** 1992. Close amino acid sequence relationship between the new plasmid-mediated extended-spectrum β-lactamase MEN-1 and chromosomally encoded enzymes of *Klebsiella oxytoca*. Biochem. Biophys. Acta **1122**:15–22.
3. **Bauernfeind, A., H. Grimm, and S. Schweighart.** 1990. A new plasmidic cefotaximase in a clinical isolate of *Escherichia coli*. Infection **18**:294–298.
4. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, S. Ernst, and J. M. Casellas.** 1996. 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. **40**:509–513.
5. **Bonnet, R., C. De Champs, D. Sirot, C. Chanal, R. Labia, and J. Sirot.** 1999. Diversity of TEM mutants in *Proteus mirabilis*. Antimicrob. Agents Chemother. **43**:2671–2677.
6. **Bonnet, R., C. Dutour, J. L. Samapaio, C. Chanal, D. Sirot, R. Labia, C. D. Champs, and J. Sirot.** 2001. Novel cefotaximase (CTX-M-16) with increased catalytic efficiency due to substitution Asp-240->Gly. Antimicrob. Agents Chemother. **45**:2269–2275.
7. **Bonnet, R., J. L. Samapaio, R. Labia, C. De Champs, D. Sirot, C. Chanal, and J. Sirot.** 2000. A novel CTX-M β-lactamase (CTX-M-8) in cefotaxime-resistant *Enterobacteriaceae* isolated in Brazil. Antimicrob. Agents Chemother. **44**:1936–1942.
8. **Bradford, P. A.** 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. **14**:933–951.
9. **Bradford, P. A., Y. Yang, D. Sahm, I. Grope, D. Gardovska, and G. Storch.** 1998. CTX-M-5, a novel cefotaxime-hydrolyzing β-lactamase from an outbreak of *Salmonella typhimurium* in Latvia. Antimicrob. Agents Chemother. **42**:1980–1984.
10. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39**:1211–1233.
11. **Chanal, C., R. Bonnet, C. De Champs, D. Sirot, R. Labia, and J. Sirot.** 2000. Prevalence of β-lactamases among 1, 072 clinical strains of *Proteus mirabilis*: a 2-year survey in a French hospital. Antimicrob. Agents Chemother. **44**: 1930–1935.
12. **Chanawong, A., F. H. M'Zali, J. Heritage, J. H. Xiong, and P. M. Hawkey.** 2002. Three cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among *Enterobacteriaceae* in the People's Republic of China. Antimicrob. Agents Chemother. **46**:630–637.
13. **De Champs, C., R. Bonnet, D. Sirot, C. Chanal, and J. Sirot.** 2000. Clinical relevance of *Proteus mirabilis* in hospital patients: a two year survey. J. Antimicrob. Chemother. **45**:537–539.
14. **Gazouli, M., E. Tzelepi, A. Markogiannakis, N. J. Legakis, and L. S. Tzouvelekis.** 1998. Two novel plasmid-mediated cefotaxime-hydrolyzing β-lactamases (CTX-M-5 and CTX-M-6) from *Salmonella typhimurium*. FEMS Microbiol. Lett. **165**:289–293.
15. **Gazouli, M., E. Tzelepi, S. V. Sidorenko, and L. S. Tzouvelekis.** 1998. Sequence of the gene encoding a plasmid-mediated cefotaxime-hydrolyzing class A β-lactamase (CTX-M-4): involvement of serine 237 in cephalosporin hydrolysis. Antimicrob. Agents Chemother. **42**:1259–1262.
16. **Gniadkowski, M., I. Schneider, A. Palucha, R. Jungwirth, B. Mikiewiez, and A. Bauernfeind.** 1998. 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. **42**:827–832.
17. **Goldstein, F. W., Y. Pean, A. Rosato, J. Gertner, and L. Gutmann.** 1993. Characterization of ceftriaxone-resistant *Enterobacteriaceae*: a multicentre study in 26 French hospitals. Vigil'Roc Study Group. J. Antimicrob. Chemother. **32**:595–603.
18. **Ishii, Y., A. Ohno, H. Taguchi, S. Imajo, M. Ishiguro, and H. Matsuzawa.** 1995. Cloning and sequence of the gene encoding a cefotaxime-hydrolyzing class A β-lactamase isolated from *Escherichia coli*. Antimicrob. Agents Chemother. **39**:2269–2275.
19. **Kado, C. I. And S. T. Liu.** 1981. A rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
20. **Karim, A., L. Poirel, S. Nagarajan, and P. Nordmann.** 2001. Plasmid-mediated extended-spectrum β-lactamase (CTX-M-3 like) from India and gene association with insertion sequence *ISEcp1*. FEMS Microbiol. Lett. **201**:237–241.
21. **Kariruki, S., J. E. Corkill, G. Revathi, R. Musoke, and C. A. Hart.** 2001. Molecular characterization of a novel plasmid-encoded cefotaximase (CTX-M-12) found in clinical *Klebsiella pneumoniae* isolates from Kenya. Antimicrob. Agents Chemother. **45**:2141–2143.
22. **Luzzaro, F., M. Perilli, G. Amicosante, G. Lombardi, R. Belloni, Z. Alessanro, C. Bianchi, and A. Toniolo.** 2001. Properties of multidrug-resistant, ESBL-producing *Proteus mirabilis* isolates and possible role of β-lactam/β-lactamase inhibitor combinations. Int. J. Antimicrob. Agents **17**:131–135.
23. **Ma, L., Y. Ishii, M. Ishiguro, H. Matzuzawa, and K. Yamaguchi.** 1998. Cloning and sequencing of the gene encoding Toho-2, a class A beta-lactamase preferentially inhibited by tazobactam. Antimicrob. Agents Chemother. **42**:1181–1186.
24. **Mariotte, S., P. Nordmann, and M. H. Nicolas.** 1994. Extended-spectrum β-lactamase in *Proteus mirabilis*. J. Antimicrob. Chemother. **33**:925–935.
25. **Masubuchi, Y., H. Oana, M. Matsumoto, and M. Doi.** 1997. Brownian dynamics simulation of biased sinusoidal field gel electrophoresis. Macromolecules **30**:912–918.
26. **National Committee for Clinical Laboratory Standards.** 2002. Performance standards for antimicrobial susceptibility testing. Twelfth informational supplement. Approved standard M100-S12. National Committee for Clinical Laboratory Standards, Wayne, Pa.
27. **Oliver, A., J. C. Perez-Diaz, T. M. Coque, F. Baquero, and R. Canton.** 2001. Nucleotide sequence and characterization of a novel cefotaxime-hydrolyzing β-lactamase (CTX-M-10) isolated in Spain. Antimicrob. Agents Chemother. **45**:616–620.
28. **Pagani, L., R. Migliavacca, L. Pallecchi, C. Matti, E. Giacobone, G. Amicosante, E. Romero, and G. M. Rossolini.** 2002. Emerging extended-spectrum β-lactamases in *Proteus mirabilis*. J. Clin. Microbiol. **40**:1549–1552.
29. **Pai, H., E. H. Choi, H. J. Lee, J. Y. Hong, and G. A. Jacoby.** 2001. Identification of CTX-M-14 extended-spectrum β-lactamase in clinical isolates of *Shigella sonnei*, Escherichia coli, and *Klebsiella pneumoniae* in Korea. J. Clin. Microbiol. **39**:3747–3749.
30. **Petroni, A., A. Corso, R. Melano, M. L. Cacace, A. M. Bru, A. Rossi, and M. Galas.** 2002. Plasmidic extended-spectrum β-lactamases in *Vibrio cholerae* O1 El Tor isolates in Argentina. Antimicrob. Agents Chemother. **46**:1462–1468.
31. **Pfaller, M. A., I. Mujeeb, R. J. Hollis, R. N. Jones, and G. V. Doern.** 2000. Evaluation of the discriminatory powers of the Dienes test and ribotyping as typing methods for *Proteus mirabilis*. J. Clin. Microbiol. **38**:1077–1080.
32. **Poirel, L., T. Naas, I. Le Thomas, A. Karim, E. Bingen, and P. Nordmann.** 2001. CTX-M-type extended-spectrum β-lactamase that hydrolyzed ceftazi-

dime through a single amino acid substitution in the omega loop. Antimicrob. Agents Chemother. **45**:3355–3361.
33. **Prilli, M., E. Dell'Amico, B. Segatore, M. R. de Massis, C. Bianchi, F. Luzzaro, G. M. Rossolini, A. Toniolo, G. Nicoletti, and G. Amicosante.** 2002. Molecular characterization of extended-spectrum β-lactamases produced by nosocomial isolates of *Enterobacteriaceae* from an Italian nationwide survey. J. Clin. Microbiol. **40**:611–614.
34. **Rozalski, A., Z. Sidorezyk, and K. Kotelko.** 1997. Potential virulence factors of *Proteus* bacilli. Microbiol. Mol. Biol. Rev. **61**:65–89.
35. **Sabate, M., R. Tarrago, F. Navarro, E. Miro, C. Verges, J. Barbe, and G. Prats.** 2000. Cloning and sequence of the gene encoding a novel cefotaxime-hydrolyzing β-lactamase (CTX-M-9) from *Escherichia coli* in Spain. Antimicrob. Agents Chemother. **44**:1970–1973.
36. **Saladin, M., V. Bao Cao, T. Lambert, J. L. Donay, J. L. Herrmann, Z. Ould-Hocine, C. Verdet, F. Deleisle, A. Philippon, and G. Arlet.** 2002. Diversity of CTX-M β-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. FEMS Microbiol. Lett. **209**: 161–168.
37. **Saurina, G., J. M. Quale, V. M. Manikal, E. Oydna, and D. Landman.** 2000. Antimicrobial resistance in *Enterobacteriaceae* in Brooklyn, N. Y.: epidemiology and relation to antibiotic usage patterns. J. Antimicrob. Chemother. **45**:895–898.
38. **Sirot, J.** 1996. Detection of extended-spectrum plasmid-mediated β-lactamases by disk diffusion. Clin. Microbiol. Infect. **2**:S35–S39.
39. **Tzouvelekis, L. S., E. Tzelepi, P. T. Tassios, and N. J. Legakis.** 2000. CTX-M-type β-lactamases: an emerging group of extended-spectrum enzymes. Int. J. Antimicrob. Agents **14**:137–142.
40. **Tzouvelekis, L. S., M. Gazouli, A. Markogiannakis, E. Paraskaki, N. J. Legakis, and E. Tzelepi.** 1998. Emergence of resistance to third-generation cephalosporins amongst *Salmonella typhimurium* isolates in Greece: report of the first three cases. J. Antimicrob. Chemother. **42**:273–275.
41. **Yagi, T., H. Kurokawa, K. Senda, S. Ichiyama, H. Ito, S. Ohsuka, K. Shibayama, K. Shimokata, N. Kato, M. Ohta, and Y. Arakawa.** 1997. Nosocomial spread of cephem-resistant *Escherichia coli* strains carrying multiple Toho-1-like β-lactamase genes. Antimicrob. Agents Chemother. **41**:2606–2611.

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

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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

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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.

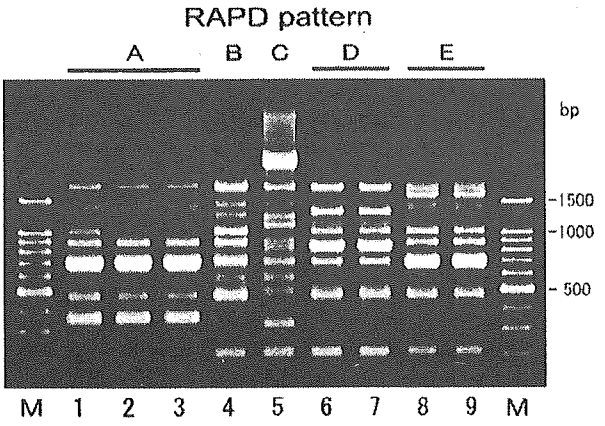

Figure. Random amplified polymorphic DNA (RAPD) patterns of CTX-M-2 β-lactamase–producing *Escherichia coli* isolated from cattle. Lanes M, 100-bp DNA ladder; lanes 1–9, strains GS528, GS542, GS547, GS553, GS554, GS721, GS733, GS631, and GS671, respectively. Five RAPD patterns, A to E, were produced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