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 Arakawa²

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 $\mu\text{g/ml}$). 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-M-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 oxymino- β -lactams and monobactam, but usually remain ineffective against cephamycins such as cefoxitin, cefmetazole, and ceftotetan 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 oxymino-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: naganoynd@3.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 Walk-Away-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 μ g), ceftazidime (30 μ g), aztreonam (30 μ g), and amoxicillin-clavulanic acid (20 μ g and 10 μ g) 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'-CCGTGTCGCCCTTATCC-3' and 5'-AGG CACATATCTCAGCGA-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'-CCTTCCGCTTCTGCTC-3' and for *bla*_{CTX-M-2}, and 5'-GCAGATAATACGAGGTG-3' and 5'-CGCCGTGG TGGTGCTCT-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- μ l volumes containing 5 μ l of DNA, 0.5 μ M each

primer, 200 μ M 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'-TTGCGCGCTCAATGTTA-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 *E*_b = 1.2 V/cm and *E*_s = 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	<i>S. aureus</i> , <i>Streptococcus agalactiae</i>
2	CDR		Failure	<i>K. pneumoniae</i> , <i>E. faecalis</i>
3	CFP-SUL, LVX		Indeterminate	ND
4	LVX, CDR, CFP-SUL		Failure	<i>P. aeruginosa</i>
5	LVX, CFZ, CFP-SUL, IPM/CS, ISP, CDR		Failure ^d	MRSA, <i>E. faecalis</i> , <i>P. aeruginosa</i>
6	CFP-SUL, FMOX, IPM/CS		Indeterminate ^d	<i>E. faecalis</i>
7	LVX	TMP, FMOX	Eradication	ND
8	LVX, TMP, CFPN-PI, GAT		Failure	ND
9	CFP-SUL, CDR		Failure	ND
10	LVX		Failure	MRSA, <i>S. agalactiae</i>
11	IPM/CS, MIN		Indeterminate ^d	ND
12		FMOX, LVX	Eradication ^d	<i>Tatumella pyseos</i>
13	ISP, CFP-SUL	CFP-SUL, CAZ	Eradication ^d	ND
14	CAZ		Failure ^d	<i>E. faecalis</i> , <i>S. haemolyticus</i> , <i>E. cloacae</i> , MRSA
15	FMOX		Indeterminate ^d	<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>K. oxytoca</i>
16	LVX		Indeterminate	<i>E. faecalis</i>
17			Indeterminate	MRSA, <i>Morganella morganii</i>
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 (μ g/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	$\leq$ 0.12/4 (19)
Ceftazidime	$\leq$ 0.5 (19)
Ceftazidime/CLA	$\leq$ 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	$\leq$ 4 (18), 32 (1)
Cefotetan	$\leq$ 0.5 (19)
Flomoxef	$\leq$ 1 (17), 2 (2)
Imipenem	2 (19)
Meropenem	$\leq$ 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 μ g 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 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.

ACKNOWLEDGMENTS

We are grateful to N. Sato and Y. Watanabe, Department of Urology; Y. Suzuki, H. Tabeta, H. Kamii, M. Shoji, and K. Iwata, Infection Control Team; and N. Suwa, and M. Toyama, Medical Microbiology Laboratory, Funabashi Medical Center, for their careful infection control.

This work was supported in part by two grants from the Ministry of Health, Labor and Welfare of Japan: the Research Project for Emerging and Reemerging Infectious Diseases (2001-2003): Molecular Analyses of Drug-Resistant Bacteria and Establishment of Rapid Identification Methods (grant no. H12-Shinkou-19), and Surveillance of Infectious Diseases Caused by Drug-Resistant Bacteria (grant no. H12-Shinkou-20).

REFERENCES

- Ambler, R. P. 1980. The structure of β -lactamases. *Phil. Trans. R. Soc. London Biol.* **289**:321-331.
- 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.
- Bauernfeind, A., H. Grimm, and S. Schweighart. 1990. A new plasmidic cefotaximase in a clinical isolate of *Escherichia coli*. *Infection* **18**:294-298.
- 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.
- 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.
- Bonnet, R., C. Dutour, J. L. Samapao, 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.
- Bonnet, R., J. L. Samapao, 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- Gazouli, M., E. Tzelepi, A. Markogiannakis, N. J. Legakis, and L. S. Tzouveleakis. 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.
- Gazouli, M., E. Tzelepi, S. V. Sidorenko, and L. S. Tzouveleakis. 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.
- Gniadkowski, M., I. Schneider, A. Palucha, R. Jungwirth, B. Mikiwicz, 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- Luzzaro, F., M. Perilli, G. Amicosante, G. Lombardi, R. Belloni, Z. Alesnaro, 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.
- Ma, L., Y. Ishii, M. Ishiguro, H. Matuzawa, and K. Yamaguchi. 1998. Cloning and sequencing of the gene encoding Toho-2, a class A β -lactamase preferentially inhibited by tazobactam. *Antimicrob. Agents Chemother.* **42**:1181-1186.
- Mariotte, S., P. Nordmann, and M. H. Nicolas. 1994. Extended-spectrum β -lactamase in *Proteus mirabilis*. *J. Antimicrob. Chemother.* **33**:925-935.
- Masubuchi, Y., H. Oana, M. Matsumoto, and M. Doi. 1997. Brownian dynamics simulation of biased sinusoidal field gel electrophoresis. *Macromolecules* **30**:912-918.
- 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.
- 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.
- 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.
- 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.
- 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.
- Pfaller, M. A., I. Mujeib, 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.
- 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.