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Ⅲ. 研究成果の刊行物・別冊

（平成１５～１７年度）

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2006, p. 791–795
0066-4804/06/$08.00+0 doi:10.1128/AAC.50.2.791–795.2006

Vol. 50, No. 2

PCR Classification of CTX-M-Type β-Lactamase Genes Identified in Clinically Isolated Gram-Negative Bacilli in Japan

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Received 26 March 2005/Returned for modification 15 June 2005/Accepted 1 November 2005

Of 1,456 strains isolated from 2001 to 2003 demonstrating resistance to either oxyimino-cephalosporin, 317 strains, isolated in 57 of 132 clinical facilities, were found to harbor bla_{CTX-M} genes by PCR. Fifty-seven, 161, and 99 strains harbored bla_{CTX-M} genes belonging to the $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, and $bla_{CTX-M-9}$ clusters, respectively.

In recent years, CTX-M-type β-lactamases have been recognized as a growing family possessing a high level of hydrolyzing activities, especially against cefotaxime (CTX) and ceftriaxone. Nearly 40 variants of the CTX-M-type enzymes have been identified (2, 4, 13, 25) and registered to date (http://www.lahey.org/studies/other.asp#table_1). Further proliferation of CTX-M-type β-lactamase-producing gram-negative bacteria has become a great concern (6), since a large number of nosocomial outbreaks caused by such bacteria have so far been recognized and reported in various medical facilities in many countries (1, 3, 5, 7–9, 19, 21).

In Japan, FEC-1 and Toho-1 were initially identified (12, 15) and were later included in CTX-M-type enzymes. Since then, various strains that produce a Toho-1-like β-lactamase have been identified in Japanese clinical settings (26, 28). Almost all of them, however, were found to be CTX-M-2 by sequence analyses (N. Shibata, et al. Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2-2235, 2001). However, the trends for several CTX-M-type β-lactamases other than CTX-M-2 have remained unclear. In the present study, we investigated the molecular types of CTX-M-type β-lactamases produced by nosocomial gram-negative bacilli isolated in Japanese clinical facilities using PCR methods.

From January 2001 to December 2003, 1,456 gram-negative bacterial isolates demonstrating resistance to oxyimino-cephalosporins were submitted from 132 hospitals to the reference laboratory at our institute. These strains were then subjected to screening for β-lactamases, including TEM- and SHV-derived extended-spectrum β-lactamases (ESBLs), CTX-M-type β-lactamases, AmpC- and CMY-type class C cephalosporinases and cephamycinases, and class B metallo-β-lactamases (MBLs). The strains were checked for ESBL production by the double-disk diffusion synergy test recommended by the CLSI (formerly the NCCLS) (18). The MICs of ceftazidime (CAZ) and CTX for the clinical isolates were determined by the agar dilution method recommended by the CLSI guidelines. When a clinical isolate demonstrated resistance to either oxyimino-cephalosporin, the strain was then subjected to PCR analyses for detection of bla_{CTX-M} genes. PCR analysis was performed by the method reported previously (27). The four sets of PCR primers used for detection of bla_{CTX-M} genes in the present study were as follows: primers CTX-M-1-F (5′-GCT GTT GTT AGG AAG TGT GC-3′) and CTX-M-1-R (5′-CCA TTG CCC GAG GTG AAG-3′), primers CTX-M-2-F (5′-ACG CTA CCC CTG CTA TTT-3′) and CTX-M-2-R (5′-CCT TTC CGC CTT CTG CTC-3′), primers CTX-M-8-F (5′-CGG ATG ATG CTA ATG ACA AC-3′) and CTX-M-8-R (5′-GTC AGA TTG CGA AGC GTC-3′), and primers CTX-M-9-F (5′-GCA GAT AAT ACG CAG GTG-3′) and CTX-M-9-R (5′-CGG CGT GGT GGT GTC TCT-3′). Only one strain was selected from an individual patient and subjected to the PCR test.

As shown in Table 1, the inhibition patterns by combination of the double-disk diffusion synergy test for ESBL detection and the sodium mercaptoacetic acid (SMA) disk test for MBL detection were classified into four groups. Of 1,456 strains tested, 59 were resistant only to CAZ and susceptible to clavulanic acid. It was speculated that these strains produce mainly SHV- or TEM-derived ESBLs, because SHV-12-producing strains have been prevalent in Japan (27). On the other hand, 276 strains showed resistance to CTX but were susceptible to CAZ. The MIC of CTX was significantly decreased in the presence of clavulanic acid. It was speculated that these strains chiefly produce CTX-M-type β-lactamases. Five hundred forty-eight isolates demonstrated resistance to both CAZ and CTX; but the inhibitory effect of clavulanic acid was not clear in these strains, and the production of MBL was suggested, because the MICs of CAZ and CTX were reduced in the presence of SMA, which is a specific inhibitor of metallo-β-lactamase (23). The remaining 573 strains, which demonstrated resistance to either of the oxyimino-cephalosporins, did not become susceptible to these agents in the presence of SMA, suggesting the production of some AmpC-type enzymes, including plasmid-mediated CMY-type enzymes.

Of 1,397 strains subjected to the PCR analyses, 317 strains were suggested to harbor bla_{CTX-M} genes. Of these strains, 57 appeared to carry genes of the $bla_{CTX-M-1}$ group, including

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TABLE 1. Results of screening by double-disk diffusion synergy tests

Bacterial species	Pattern of double-disk diffusion synergy test				Total no. of strains tested
	Resistant to CAZ and susceptible to clavulanic acid (no. of strains)	Resistant to CTX and susceptible to clavulanic acid[a]	Resistant to CAZ and CTX and susceptible to SMA[a]	Resistant to either oxyimino-cephalosporin and not susceptible to SMA	
Escherichia coli	33	157/157	7/24	4/4	218
Proteus mirabilis	0	71/71	0/1	0/0	72
Klebsiella pneumoniae	15	42/42	7/31	1/2	90
Klebsiella oxytoca	4	5/5	1/3	0/2	14
Serratia marcescens	7	0/0	0/65	10/77	149
Enterobacter cloacae	0	0/0	2/11	1/20	31
Enterobacter aerogenes	0	0/0	0/2	1/8	10
Citrobacter freundii	0	0/0	0/4	2/15	19
Citrobacter koseri	0	0/0	0/0	1/1	1
Providencia rettgeri	0	1/1	0/2	0/0	3
Acinetobacter baumannii	0	0/0	1/49	3/40	89
Other bacterial species[d]	0	0/0	0/356	0/404	760
Total[e]	59	276/276	18/548[b]	23/573[c]	1,456

[a] The data represent the number of bla_{CTX-M}-positive strains by PCR/total number of strains demonstrating each inhibition pattern and subjected to PCR.
[b] Strains that produce metallo-β-lactamase are included.
[c] Strains that produce plasmid-mediated CMY-type cephalosporinase or chromosomal AmpC hyperproducers are included.
[d] *Pseudomonas* spp., *Alcaligenes* spp., *Achromobacter* spp., and *Burkholderia* spp. demonstrating resistance to ceftazidime or cefotaxime were included; but *Stenotrophomonas* spp. and *Chryseobacterium* spp. that produce intrinsic metallo-β-lactamase were excluded.
[e] Out of the total number of strains being subjected to PCR analysis (1,397; represented in columns 2, 3, and 4), 317 were found to be bla_{CTX-M} positive.

$bla_{CTX-M-1}$, $bla_{CTX-M-3}$, and $bla_{CTX-M-15}$, as shown in Table 2. Moreover, 161 strains were suggested to harbor the genes encoding the CTX-M-2 group of enzymes, such as CTX-M-2, CTX-M-20, and CTX-M-31. Furthermore, 99 strains appeared to carry the genes for the CTX-M-9 group of enzymes, such as CTX-M-9, CTX-M-14, and CTX-M-16. No strain harboring genes for the CTX-M-8 or the CTX-M-25 group of enzymes was found among the strains tested.

As shown in Table 3, strains that harbored genes for the CTX-M-type enzymes were isolated from 57 of 132 hospitals across Japan, except for the Hokkaido region, throughout the 3-year investigation period. Fourteen and 24 strains that harbored genes for the CTX-M-1 group of enzymes were identified in 7 and 10 hospitals located in the Kanto and Chubu regions, respectively (Table 3). However, no strain harboring genes for the CTX-M-1 group of enzymes were found in the Chugoku and Shikoku regions (Table 3). In 22 of 57 hospitals, genes for multiple CTX-M-type β-lactamases belonging to different groups were identified during the investigation period (Fig. 1). Interestingly, genes for all three groups of CTX-M-type enzymes were identified in 7 of 57 hospitals (Fig. 1; Table 3).

TABLE 2. Number of strains that produce CTX-M-type β-lactamases as detected by PCR

Bacterial species	No. of strains by the following PCR type:			Total
	CTX-M-1 group[a]	CTX-M-2 group[b]	CTX-M-9 group[c]	
Escherichia coli	33	46	89	168
Proteus mirabilis	0	71	0	71
Klebsiella pneumoniae	10	31	9	50
Klebsiella oxytoca	2	3	1	6
Serratia marcescens	9	1	0	10
Enterobacter cloacae	0	3	0	3
Enterobacter aerogenes	1	0	0	1
Citrobacter freundii	2	0	0	2
Citrobacter koseri	0	1	0	1
Providencia rettgeri	0	1	0	1
Acinetobacter baumannii	0	4	0	4
Total	57	161	99	317

[a] The PCR primers used can detect genes for CTX-M-1 and several variants, such as CTX-M-3 and CTX-M-15.
[b] The PCR primers used can detect genes for CTX-M-2 and several variants, such as CTX-M-20 and CTX-M-31.
[c] The PCR primers used can detect genes for CTX-M-9 and several variants, such as CTX-M-14 and CTX-M-16.

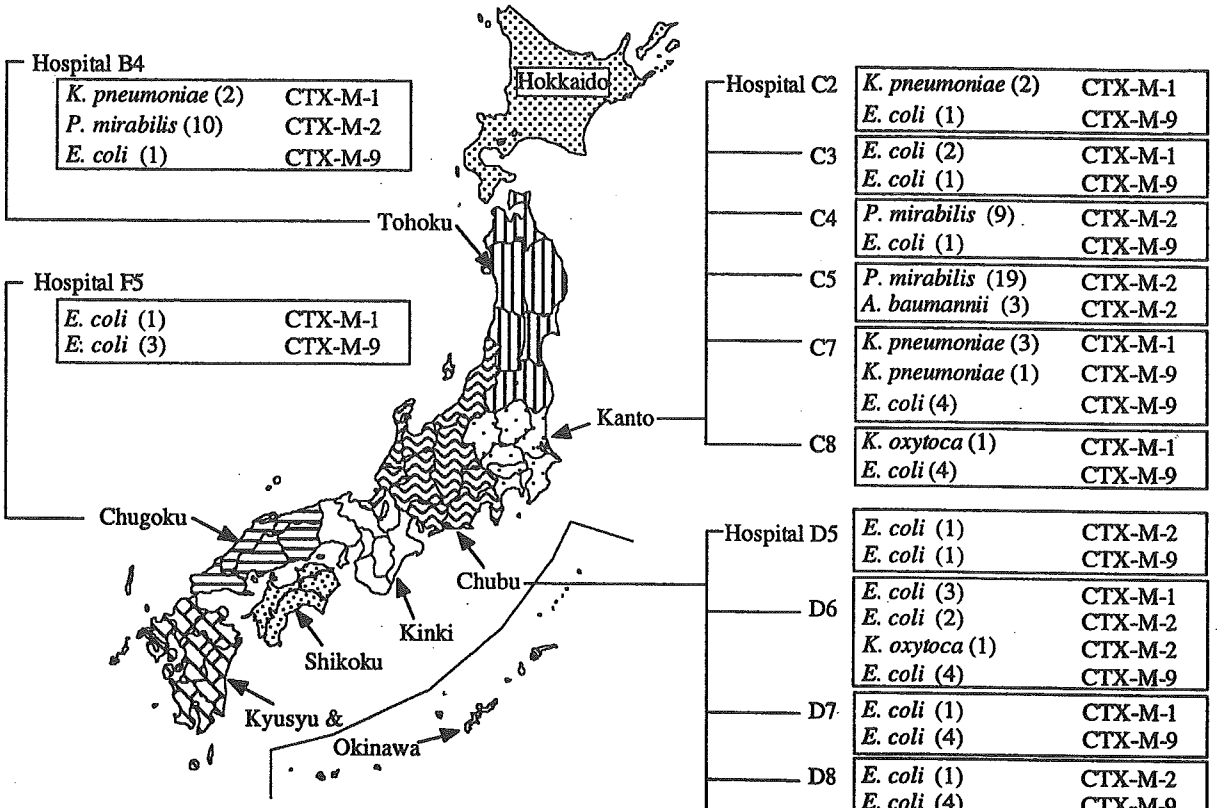
After the first description of Toho-1 in Japan in 1995, several outbreaks caused by CTX-M-type β-lactamase producers have been reported in there (17, 26, 28). In the present investigation, it became clear that gram-negative nosocomial bacilli producing the CTX-M-1, CTX-M-2, or CTX-M-9 group of enzymes have already been dispersed in various clinical settings in Japan, although strains that produce TEM- or SHV-derived ESBLs are not predominant to date.

Recently, the CTX-M-1 group of enzymes, such as CTX-M-3 and CTX-M-15, have emerged in Europe and Asia (3, 8–10, 14, 22, 28). In the present study, we also identified the genes for the CTX-M-1 group of enzymes in various bacterial species, including *Escherichia coli*, *Serratia marcescens*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca*, in addition to *Providencia rettgeri*, *Citrobacter freundii*, *Citrobacter koseri*, and *Enterobacter cloacae*. This finding may be suggestive of the lateral transfer of very similar plasmids bearing bla_{CTX-M} genes among different bacterial species. Actually, probable nosocomial transmissions of CTX-M-producing bacterial strains were suspected in several medical facilities, as shown in Fig. 1 and Table 3. Especially in hospitals D18, D20, and E5, all three groups of genes for CTX-M enzymes were identified; and genes for CTX-M-type enzymes were detected in various gram-negative bacterial species, suggesting the horizontal transfer of the bla_{CTX-M} genes among different bacterial species. Interestingly,

TABLE 3. Bacterial species that produce each group of CTX-type β-lactamases

Region	PCR type	Bacterial species (no. of isolates)	Hospital (no. of isolates)
Hokkaido (0[a]/7[b])		None	None
Tohoku (4/17)	CTX-M-1	*K. pneumoniae* (2[c])	B4 (2[c])
	CTX-M-2	*E. coli* (1)	B1 (1)
		P. mirabilis (10)	B4 (10)
	CTX-M-9	*E. coli* (6)	B2 (1), B3 (4), B4 (1)
Kanto (9/26)	CTX-M-1	*E. coli* (7)	C1 (1), C3 (2), C9 (4)
		K. pneumoniae (6)	C2 (2), C6 (1), C7 (3)
		K. oxytoca (1)	C8 (1)
	CTX-M-2	*P. mirabilis* (28)	C4 (9), C5 (19)
		A. baumannii (3)	C5 (3)
	CTX-M-9	*K. pneumoniae* (1)	C7 (1)
		E. coli (11)	C2 (1), C3 (1), C4 (1), C7 (4), C8 (4)
Chubu (22/37)	CTX-M-1	*E. coli* (12)	D2 (1), D3 (5), D6 (3), D7 (1), D20 (1), D22 (1)
		K. pneumoniae (2)	D1 (1), D20 (1)
		C. freundii (2)	D18 (2)
		E. aerogenes (1)	D19 (1)
		S. marcescens (5)	D18 (5)
	CTX-M-2	*E. coli* (29)	D5 (1), D6 (2), D8 (1), D13 (4), D14 (1), D15 (5), D18 (1), D20 (14)
		K. pneumoniae (21)	D20 (20), D22 (1)
		K. oxytoca (3)	D6 (1), D15 (1), D20 (1)
		P. mirabilis (17)	D14 (4), D16 (11), D17 (1), D18 (1)
		S. marcescens (1)	D20 (1)
		E. cloacae (3)	D18 (1), D20 (2)
		A. baumannii (1)	D20 (1)
	CTX-M-9	*E. coli* (34)	D4 (1), D5 (1), D6 (4), D7 (4), D8 (4), D9 (3), D10 (1), D11 (1), D12 (1), D14 (1), D16 (4), D18 (1), D20 (3), D21 (5)
		K. pneumoniae (4)	D12 (4)
		K. oxytoca (1)	D12 (1)
Kinki (10/19)	CTX-M-1	*E. coli* (6)	E5 (4), E7 (1), E10 (1)
		K. oxytoca (1)	E4 (1)
		S. marcescens (4)	E1 (4)
	CTX-M-2	*E. coli* (8)	E3 (1), E5 (6), E8 (1)
		K. pneumoniae (6)	E5 (6)
		P. mirabilis (15)	E2 (1), E5 (14)
		P. rettgeri (1)	E8 (1)
	CTX-M-9	*E. coli* (11)	E2 (2), E3 (1), E5 (6), E6 (1), E9 (1)
		K. pneumoniae (2)	E2 (1), E5 (1)
Chugoku (5/13)	CTX-M-2	*E. coli* (3)	F2 (2), F5 (1)
		K. pneumoniae (2)	F3 (2)
	CTX-M-9	*E. coli* (8)	F1 (4), F4 (1), F5 (3)
Shikoku (3/5)	CTX-M-2	*E. coli* (1)	G2 (1)
		C. koseri (1)	G3 (1)
	CTX-M-9	*E. coli* (15)	G2 (15)
		K. pneumoniae (2)	G1 (1), G2 (1)
Kyushu and Okinawa (4/8)	CTX-M-1	*E. coli* (8)	H1 (1), H2 (6), H3 (1)
	CTX-M-2	*E. coli* (4)	H1 (1), H4 (3)
		K. pneumoniae (2)	H4 (2)
		P. mirabilis (1)	H2 (1)
	CTX-M-9	*E. coli* (4)	H1 (3), H2 (1)
Total (57/132)			

[a] Number of medical facilities where bla_{CTX-M}-harboring strains were detected.
[b] Number of medical facilities that submitted strains to our laboratory.
[c] Number of clinical isolates harboring bla_{CTX-M} gene.



Hospital B4	*K. pneumoniae* (2)	CTX-M-1	
	P. mirabilis (10)	CTX-M-2	
	E. coli (1)	CTX-M-9	
Hospital F5	*E. coli* (1)	CTX-M-1	
	E. coli (3)	CTX-M-9	
Hospital C2	*K. pneumoniae* (2)	CTX-M-1	
	E. coli (1)	CTX-M-9	
C3	*E. coli* (2)	CTX-M-1	
	E. coli (1)	CTX-M-9	
C4	*P. mirabilis* (9)	CTX-M-2	
	E. coli (1)	CTX-M-9	
C5	*P. mirabilis* (19)	CTX-M-2	
	A. baumannii (3)	CTX-M-2	
C7	*K. pneumoniae* (3)	CTX-M-1	
	K. pneumoniae (1)	CTX-M-9	
	E. coli (4)	CTX-M-9	
C8	*K. oxytoca* (1)	CTX-M-1	
	E. coli (4)	CTX-M-9	
Hospital D5	*E. coli* (1)	CTX-M-2	
	E. coli (1)	CTX-M-9	
D6	*E. coli* (3)	CTX-M-1	
	E. coli (2)	CTX-M-2	
	K. oxytoca (1)	CTX-M-2	
	E. coli (4)	CTX-M-9	
D7	*E. coli* (1)	CTX-M-1	
	E. coli (4)	CTX-M-9	
D8	*E. coli* (1)	CTX-M-2	
	E. coli (4)	CTX-M-9	
D12	*E. coli* (1)	CTX-M-9	
	K. pneumoniae (4)	CTX-M-9	
	K. oxytoca (1)	CTX-M-9	
D14	*E. coli* (1)	CTX-M-2	
	P. mirabilis (4)	CTX-M-2	
	E. coli (1)	CTX-M-9	
D15	*E. coli* (5)	CTX-M-2	
	K. oxytoca (1)	CTX-M-2	
D16	*P. mirabilis* (11)	CTX-M-2	
	E. coli (4)	CTX-M-9	
D18	*S. marcescens* (5)	CTX-M-1	
	C. freundii (2)	CTX-M-1	
	E. coli (1)	CTX-M-2	
	P. mirabilis (1)	CTX-M-2	
	E. cloacae (1)	CTX-M-2	
	E. coli (1)	CTX-M-9	
D20	*E. coli* (1)	CTX-M-1	
	K. pneumoniae (1)	CTX-M-1	
	E. coli (14)	CTX-M-2	
	K. pneumoniae (20)	CTX-M-2	
	K. oxytoca (1)	CTX-M-2	
	E. cloacae (2)	CTX-M-2	
	A. baumannii (1)	CTX-M-2	
	S. marcescens (1)	CTX-M-2	
	E. coli (3)	CTX-M-9	
D22	*E. coli* (1)	CTX-M-1	
	K. pneumoniae (1)	CTX-M-2	
Kinki — Hospital E2	*P. mirabilis* (1)	CTX-M-2	
	E. coli (2)	CTX-M-9	
	K. pneumoniae (1)	CTX-M-9	
E3	*E. coli* (1)	CTX-M-2	
	E. coli (1)	CTX-M-9	
E5	*E. coli* (4)	CTX-M-1	
	E. coli (6)	CTX-M-2	
	P. mirabilis (14)	CTX-M-2	
	K. pneumoniae (6)	CTX-M-2	
	E. coli (6)	CTX-M-9	
	K. pneumoniae (1)	CTX-M-9	
E8	*E. coli* (1)	CTX-M-2	
	P. rettgeri (1)	CTX-M-2	
Shikoku — Hospital G2	*E. coli* (1)	CTX-M-2	
	E. coli (15)	CTX-M-9	
	K. pneumoniae (1)	CTX-M-9	
Kyusyu & Okinawa — Hospital H1	*E. coli* (1)	CTX-M-1	
	E. coli (1)	CTX-M-2	
	E. coli (3)	CTX-M-9	
H2	*E. coli* (6)	CTX-M-1	
	P. mirabilis (1)	CTX-M-2	
	E. coli (1)	CTX-M-9	
H4	*E. coli* (3)	CTX-M-2	
	K. pneumoniae (2)	CTX-M-2	

FIG. 1. Clinical facilities where multiple bla_{CTX-M} genes belonging to different genetic clusters were identified. Facilities where multiple bacterial species that bear bla_{CTX-M} genes were isolated are also added. The numbers in parentheses demonstrate the number of clinical isolates of each bacterial species.

all 71 *Proteus mirabilis* strains were identified as CTX-M-2 producers, and they were isolated in widely separate medical facilities located far apart in Japan, implying a close relatedness between CTX-M-2 and *P. mirabilis* in Japanese clinical environments. The plasmids carrying $bla_{CTX\text{-}M\text{-}2}$ may be very adaptive for *P. mirabilis*, which may either serve as a reservoir for plasmids carrying $bla_{CTX\text{-}M\text{-}2}$ gene (16, 17) or have preferentially accepted $bla_{CTX\text{-}M\text{-}2}$ genes from some environmental *Kluyvera* spp. (11, 20). Comparative analyses of plasmids that bear the $bla_{CTX\text{-}M\text{-}2}$ gene would provide a clue to elucidate the relatedness and origins of the plasmids.

The CTX-M-9 group of enzymes, including CTX-M-14, have so far been found worldwide in the species belonging to the family *Enterobacteriaceae* (7–9). However, almost all of the CTX-M-9 group of enzymes were found in *E. coli* in the present study, and some of them were suggested to be CTX-M-14. Precise analysis of the genetic environments mediating the $bla_{CTX\text{-}M\text{-}9}$ group of genes among these strains as well as their genome profiles would explain the presence of CTX-M-producing pandemic strains in Japan.

In conclusion, the aim of the present study was to make a rough estimate of the current status of CTX-M-type β-lactamases produced by nosocomial gram-negative bacilli isolated from Japanese medical facilities. The findings obtained imply that various plasmid-mediated genetic determinants for CTX-M-type β-lactamases have already been disseminated in Japanese clinical environments. Since CTX-M-2 was also identified in livestock (24), we must take special precautions against the further proliferation of gram-negative bacterial strains that harbor plasmids carrying genes for CTX-M-type β-lactamases, together with the other classes of plasmid-mediated β-lactamases, such as CMY-type cephamycinases and MBLs.

We thank all medical institutions for submitting bacterial strains to the national reference laboratory for performance of this study.

This work was mainly supported by two grants (grants H15-Shinko-9 and H15-Shinko-10) from the Ministry of Health, Labor and Welfare of Japan. PCR typing of *bla* genes was supported by grants (grants 1017221 and 13770141) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2006, p. 534–541
0066-4804/06/$08.00+0 doi:10.1128/AAC.50.2.534–541.2006

Vol. 50, No. 2

Horizontal Transfer of bla_{CMY}-Bearing Plasmids among Clinical *Escherichia coli* and *Klebsiella pneumoniae* Isolates and Emergence of Cefepime-Hydrolyzing CMY-19

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Received 30 June 2005/Returned for modification 17 August 2005/Accepted 4 November 2005

Nine *Escherichia coli* and 5 *Klebsiella pneumoniae* clinical isolates resistant to various cephalosporins and cephamycins were identified in a Japanese general hospital between 1995 and 1997. All nine *E. coli* isolates and one *K. pneumoniae* isolate carried bla_{CMY-9}, while the other four *K. pneumoniae* isolates harbored a variant of bla_{CMY-9}, namely, bla_{CMY-19}. The pulsed-field gel electrophoresis patterns of the nine CMY-9-producing *E. coli* isolates were almost identical, suggesting their clonal relatedness, while those of the five *K. pneumoniae* isolates were divergent. Plasmid profiles, Southern hybridization, and conjugation assays revealed that the genes for the CMY-9 and the CMY-19 β-lactamases were located on very similar conjugative plasmids in *E. coli* and *K. pneumoniae*. The genetic environment of bla_{CMY-19} was identical to that of bla_{CMY-9}. A single amino acid substitution, I292S, adjacent to the H-10 helix region was observed between CMY-9 and CMY-19. This substitution was suggested to be responsible for the expansion of the hydrolyzing activity against several broad-spectrum cephalosporins, and this finding was consistent with the kinetic parameters determined with purified enzymes. These findings suggest that the bla_{CMY-19} genes found in the four *K. pneumoniae* isolates might have originated from bla_{CMY-9} gene following a point mutation and dispersed among genetically different *K. pneumoniae* isolates via a large transferable plasmid.

Resistance to β-lactam antibiotics in gram-negative bacilli is mainly mediated by the production of β-lactamases, which are divided into four major molecular classes, classes A, B, C, and D (1, 10). Genes for AmpC (class C) β-lactamases are generally encoded on the chromosomes in many gram-negative microbes, including *Enterobacter* spp., *Citrobacter freundii*, *Serratia marcescens*, *Morganella morganii*, and *Pseudomonas aeruginosa* (27). Chromosomal AmpC enzymes are usually inducible and are often responsible for resistance to cephalosporins (27) as well as to penicillins. Plasmid-mediated class C β-lactamases have mainly been described in *Klebsiella* spp., *Escherichia coli*, and *Salmonella* spp. throughout the world (25). A cephamycin-resistant *Klebsiella pneumoniae* strain producing a plasmid-mediated class C β-lactamase, CMY-1, was first reported in 1989 in Korea (7, 8). Plasmid-mediated class C enzymes are currently divided into at least five clusters (25) on the basis of amino acid sequence similarities, together with their putative progenitor chromosomal AmpC enzymes. In Japan, MOX-1 (16), CMY-8 (unpublished data), CMY-9 (12), CMY-2 (unpublished data), CFE-1 (23), and DHA-1 (unpublished data) have so far been found as plasmid-mediated AmpC β-lactamases, mainly in nosocomial isolates of the family *Enterobacteriaceae*.

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Between 1995 and 1997, eight additional *E. coli* isolates and five *K. pneumoniae* isolates resistant to both oximino-cephalosporins and cephamycins were isolated in the same hospital where the first CMY-9-producing *E. coli* strain (strain HKYM68) was isolated in 1995 (12). In the present study, the molecular and biochemical mechanisms underlying the multiple-cephalosporin resistance among these 14 isolates as well as their genetic relatedness were elucidated.

MATERIALS AND METHODS

Bacterial strains. Nine *E. coli* isolates and five *K. pneumoniae* isolates displaying a high level of resistance to cephalosporins and cephamycins were isolated between 1995 and 1997 in a general hospital in Yamaguchi Prefecture, Japan, and stored in our laboratory. Among these isolates, *E. coli* strain HKYM68 was previously found to produce CMY-9 (12). Phenotypic identification of each isolate was performed by using a commercial identification system (API 20E system; bioMerieux, Marcy l'Etoile, France), according to the instructions of the manufacturer.

Phenotypic test for β-lactamase types. A simple initial screening test for the presumptive identification of the β-lactamase types in clinical isolates was performed by use of the double-disk synergy test with Kirby-Bauer disks. Two disks which contained ceftazidime (30 μg per disk) or cefotaxime (30 μg per disk) were used in combination with three different disks containing either amoxicillin-clavulanate (20 μg per disk/10 μg per disk), sodium mercaptoacetic acid (3 mg per disk), or 3-aminophenyl boronic acid (APB) (300 μg per disk), which are specific inhibitors of class A, class B, and class C β-lactamases, respectively (2, 32).

Identification of β-lactamase genes by PCR and sequencing analyses. The samples were screened by PCR with 12 sets of primers for the detection of TEM- and SHV-derived extended-spectrum β-lactamases; GES-type, CTX-M-2-type, CTX-M-3-type, and CTX-M-9-type class A β-lactamases; CMY-1-, CMY-2-, and DHA-1-type class C β-lactamases; and IMP-1-, IMP-2-, and VIM-2-type class B β-lactamases. The sets of PCR primers and the amplification conditions used to detect various plasmid-mediated β-lactamase genes found thus far in Japan have been reported previously (28, 31). The PCR amplicons were electrophoresed on

a 2% agarose gel and purified with a MinElute gel extraction kit (QIAGEN K. K., Tokyo, Japan), and both strands were sequenced.

Transfer of β-lactam resistance. A conjugation experiment was performed by the broth mating method with *E. coli* strain CSH-2 (*metB* F^- Rif^r Nal^r) as the recipient. The donor-to-recipient ratio was 1:4, and the mating time was 3 h. Transconjugants were selected on Luria-Bertani (LB) agar plates supplemented with both rifampin (100 μg/ml) and nalidixic acid (50 μg/ml), together with cefotaxime (10 μg/ml) or ceftazidime (10 μg/ml).

Antibiotic susceptibility tests. Susceptibilities to antibiotics were tested by the agar dilution method according to the procedure recommended by the CLSI (formerly the National Committee for Clinical Laboratory Standards) document M7-A5 (24). *E. coli* ATCC 25922 was used as the control strain for the antimicrobial susceptibility testing.

Isoelectric focusing of β-lactamases. Bacterial cells were grown in 10 ml of LB broth supplemented with cephalothin (50 μg/ml) and were harvested by centrifugation (4,000 × *g* for 15 min) The cell pellet was resuspended in 1 ml of 50 mM sodium phosphate buffer. The pI of β-lactamase was determined as described previously (31).

Pulsed-field gel electrophoresis (PFGE). Total DNA preparations containing both chromosomal and plasmid DNAs were extracted from each isolate and digested overnight with XbaI (New England Biolabs, Beverly, MA) in agarose gel plugs. The digested DNAs were subjected to electrophoresis with a CHEF-DRII drive module (Bio-Rad Laboratory, Hercules, CA), with pulses ranging from 12.5 to 40 s at 6 V/cm for 24 h at 16°C.

Plasmid analysis and Southern hybridization. Large plasmids mediating bla_{CMY} genes were prepared from clinical isolates and their transconjugants according to the procedure described by Kado and Liu (17) and electrophoresed on a 0.8% agarose gel. The plasmid DNAs of the transconjugants were also prepared by using a QIAGEN midi-prep kit (QIAGEN K. K.), digested with SacI, and then transferred to a nylon membrane (Bio-Rad Laboratories). The 999-bp digoxigenin (DIG)-labeled DNA probes were prepared by using a PCR DIG Probe Synthesis kit (Roche Diagnostics, Tokyo, Japan); and the DNA template was prepared from a bla_{MOX}-positive *E. coli* strain HKYM68 (12), together with two PCR primers, primers MOX-F (5′-AAC AAC GAC AAT CCA TCC-3′) and MOX-R (5′-TGT TGA AGA GCA CCT GGC-3′).

PCR and sequencing analyses of flanking regions of bla_{CMY}. To determine the genetic environments of the bla_{CMY} genes, standard PCR amplification experiments and sequencing analyses were performed with an Expand High-Fidelity PCR system (Roche) and several sets of primers, which were designed on the basis of the nucleotide sequences deposited in the EMBL/GenBank/DDBJ databases under accession number AB061794. The resultant PCR products were purified by using a MinElute gel extraction kit (QIAGEN) and were subsequently sequenced with the appropriate primers.

Cloning of bla_{CMY-9} and bla_{CMY-19} for purification of enzymes. To amplify bla_{CMY-9} and bla_{CMY-19}, conjugative plasmids pK209 and pK466 were used as the template DNA, respectively. A highly reliable PCR amplification was performed with primers CMY-S1 (5′-CAG GGC GTG AGG ATA AAG-3′) and CMY-S2 (5′-GGG ACG AGA TAG AGA AAT-3′) by using the Expand High-Fidelity PCR system (Roche). Each amplicon was ligated to the pGEM-T vector (Promega, Madison, WI) and subjected to confirmatory sequencing. Selected plasmids with no amplification error, pGEM-CMY-9 and pGEM-CMY-19, which carry bla_{CMY-9} and bla_{CMY-19}, respectively, were digested with XhoI and EcoRI. The resultant fragments were ligated to pBCSK+ (Stratagene, La Jolla, Calif.) restricted with the same enzymes; and competent cells of *E. coli* strain DH5α [*supE44 ΔlacU169* (*ϕ80 lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1 acrAB*$^+$], purchased from TOYOBO, Co., Ltd, Tokyo, Japan, were transformed by electroporation with the mixture of the constructed plasmids.

Purification of CMY-9 and CMY-19 β-lactamases. *E. coli* strain DH5α, which harbored pBC-CMY-9 carrying the bla_{CMY-9} gene or pBC-CMY-19 carrying the bla_{CMY-19} gene, was separately cultured overnight in 2 liters of LB broth containing cephalothin (50 μg/ml) and chloramphenicol (30 μg/ml). The cells were harvested by centrifugation and washed in 50 mM sodium phosphate buffer (pH 7.0). The pellets were resuspended with 10 ml of 20 mM Tris-HCl buffer (pH 7.5) and destroyed with a French press. After low-speed centrifugation (3,300 × *g* for 15 min) to remove the cellular debris and unbroken cells, the supernatant was again centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant containing β-lactamase was chromatographed through a HiTrap Q HP column (Amersham Biosciences) that had been preequilibrated with 20 mM Tris-HCl buffer (pH 7.5). β-Lactamase activity was detected in the flowthrough fraction, which was then dialyzed against 50 mM sodium phosphate buffer (pH 6.0). This partially purified fraction was again applied to a HiTrap SP HP column (Amersham Biosciences) that had been preequilibrated with 50 mM sodium phosphate buffer (pH 6.0). The enzymes were eluted with a linear gradient of NaCl in the same buffer. Fractions with β-lactamase activity were dialyzed against 50 mM sodium phosphate buffer (pH 7.0) and condensed by use of an Ultrafree-15 centrifuge filter device (Millipore Corporation, Bedford, MA). The production of CMY-19 was not enough in the *E. coli* transformant, so the following method was used. The bla_{CMY-19} gene was amplified with primers CMY-F2 (5′-CAT ATG CAA CAA CGA CAA TCC ATC C-3′), which has an NdeI linker (underlined), and CMY-R2 (5′-GAA TTC TCA ACC GGC CAA CTG CGC CA-3′), which has an EcoRI linker (underlined), and the Expand High-Fidelity PCR system (Roche). The amplicon was ligated with a pGEM-T vector (Promega), subjected to confirmatory sequencing, and then excised by digestion with NdeI and EcoRI and subcloned into the expression vector pET29a(+) (Novagen, Madison, WI), which was cleaved with the same enzymes. The constructed expression vector, named pET-CMY-19, was introduced into *E. coli* BL21(DE3)pLysS [F^- *ompT hsdSB* ($r_B^-m_B^-$) *gal dcm* (DE3) pLysS], which was obtained from Novagen through TAKARA BIO Inc., Kyoto, Japan. The transformant was cultured in 1 liter of LB broth containing kanamycin (50 μg/ml) and chloramphenicol (30 mg/ml) at 37°C. Isopropyl-β-D-thiogalactopyranoside was added when the culture reached an optical density at 600 nm of 0.55, and the culture was incubated for an additional 6 h at 25°C. CMY-19 was purified by the same methods used for the purification of CMY-9. The purity of the β-lactamases was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue (CBB) staining. The purified CMY-9 and CMY-19 β-lactamases were also subjected to isoelectric focusing analysis with an Ampholine PAG plate (Amersham Biosciences) and stained with CBB.

Assay of kinetic parameters. The kinetic parameters of CMY-9 and CMY-19 against various β-lactam substrates were assayed at 30°C in 50 mM sodium phosphate buffer (pH 7.0) by using an autospectrophotometer (V-550; Nihon Bunko Ltd., Tokyo, Japan). The absorption maxima of the substrates used were as follows: ampicillin, 235 nm; piperacillin, 232 nm; cephalothin, 262 nm; cephaloridine, 297 nm; ceftizoxime, 257 nm; ceftazidime, 274 nm; cefotaxime, 264 nm; cefpirome, 267 nm; cefepime, 275 nm; cefoxitin, 270 nm; cefmetazole, 259 nm; moxalactam, 274 nm; imipenem, 298 nm. K_m and k_{cat} values were obtained by a Michaelis-Menten plot of the initial steady-state velocities at different substrate concentrations. K_i was determined by the procedure described in our previous study (13), with cephalothin used as a reporter substrate.

Nucleotide sequence accession number. The open reading frame of bla_{CMY-19} was deposited in the EMBL/GenBank databases through DDBJ and assigned accession number AB194410.

RESULTS

Properties of nine *E. coli* and five *K. pneumoniae* clinical isolates. The MICs of six β-lactams for the 14 clinical isolates are shown in Table 1. The *K. pneumoniae* and *E. coli* clinical isolates exhibited resistance to oximino-cephalosporins and cephamycins but were susceptible to carbapenems, although *E. coli* HKYM68 also showed resistance to imipenem. In a double-disk synergy test, no synergistic effect of clavulanic acid on the activities of ceftazidime and cefotaxime was detectable in any of the 14 isolates. A lack of metallo-β-lactamase production was also suggested by the results of the sodium mercaptoacetic acid disk tests. An apparent expansion of the growth inhibitory zone was observed with the 14 clinical isolates only between a disk containing 300 μg of 3-aminophenyl-boronic acid and a disk containing ceftazidime or cefotaxime, suggesting the production of a class C β-lactamase. These findings indicate that the property of resistance to oxyimino-cephalosporins and cephamycins was likely due to the production of a class C β-lactamase.

PCR detection of various β-lactamase genes and sequencing revealed that a *K. pneumoniae* isolate (HKY209) carried bla_{CMY-9}, while the other four *K. pneumoniae* isolates carried bla_{CMY-19}, a variant gene of bla_{CMY-9} (Table 1). A single nucleotide mutation at position 944 was found between bla_{CMY-9} and the newly identified bla_{CMY-19} gene, and this point mutation resulted in the I292S substitution near the H-10 helix domain in CMY-19, as shown in Fig. 1. All nine *E. coli* clinical isolates carried both the bla_{CMY-9} and the bla_{TEM} genes (Table 1).

TABLE 1. MICs for parent strains and their transconjugants

Strain	Date of isolation (mo and yr)	Patient	Source	β-Lactamase	MIC (μg/ml)[a]							Transconjugant (*E. coli* CSH-2)	β-Lactamase	MIC (μg/ml)[a]						
					PIP	CAZ	CAZ + APB[b]	CTX	FEP	CMZ	IPM			PIP	CAZ	CAZ + APB[b]	CTX	FEP	CMZ	IPM
K. pneumoniae												*E. coli* CSH-2		1	0.13	0.13	≤0.06	≤0.06	0.5	0.25
HKY209	Jul. 95	A	Sputum	CMY-9	32	>128	1	>128	0.25	>128	0.25	*E. coli*(pK209)	CMY-9	4	64	0.25	128	≤0.06	128	0.25
HKY327	Apr. 95	B	Sputum	CMY-19	128	>128	32	128	4	64	0.25	*E. coli*(pK327)	CMY-19	64	>128	8	128	4	32	0.5
HKY363	Jun. 96	C	Sputum	CMY-19	128	>128	16	64	4	64	0.25	*E. coli*(pK363)	CMY-19	32	>128	8	64	2	16	0.25
HKY466	Oct. 96	D	Sputum	CMY-19	128	>128	16	64	4	64	0.25	*E. coli*(pK466)	CMY-19	32	>128	8	64	2	16	0.25
HKY474	Jan. 97	E	Sputum	CMY-19	64	>128	16	64	4	64	0.13	*E. coli*(pK474)	CMY-19	32	>128	4	64	4	16	0.25
E. coli																				
HKY154	Mar. 95	F	Sputum	CMY-9 and TEM-1-like[c]	32	>128	1	>128	0.5	>128	0.13	*E. coli*(pE154)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY191	Jun. 95	G	Pus	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.13	*E. coli*(pE191)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY200	Jun. 95	H	Throat swab	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.25	*E. coli*(pE200)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY215	Jul. 95	H	Sputum	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.25	*E. coli*(pE215)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY224	Aug. 95	I	Stool	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.25	*E. coli*(pE224)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKYM68	Nov. 95	J	Sputum	CMY-9 and TEM-1-like	32	>128	2	>128	2	>128	32[d]	*E. coli*(pEM68)	CMY-9	8	64	0.5	128	≤0.06	128	0.25
HKY297	Mar. 96	K	Sputum	CMY-9 and TEM-1-like	32	>128	1	>128	0.25	>128	0.13	*E. coli*(pE297)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY315	Apr. 96	L	Throat swab	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.25	*E. coli*(pE315)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY334	Apr. 96	K	Sputum	CMY-9 and TEM-1-like	64	>128	1	>128	0.5	>128	0.13	*E. coli*(pE334)	CMY-9	4	64	0.5	128	≤0.06	128	0.25

[a] Abbreviations: PIP, piperacillin; CAZ, ceftazidime; APB, 3-aminophenyl-boronic acid; CTX, cefotaxime; FEP, cefepime; CMZ, cefmetazole; IPM, imipenem.
[b] APB was used at a concentration of 300 μg/ml.
[c] The nucleotide sequence of the PCR amplicon derived from the *bla* gene was identical to that of the bla_{TEM-1} gene, although the total nucleotide sequence of the *bla* gene was not determined.
[d] Imipenem resistance may be due to alteration in bacterial membrane as reported previously (3, 9, 29).

292

CMY-19 (*K. pneumoniae* HKY466)	YPVTEQTLLAGNSAKVSLEANPTAA---PRESGSQVLFNKTGSTNGFGAYVAFVPARGIG
CMY-9 (*K. pneumoniae* HKY209)	YPVTEQTLLAGNSAKVILEANPTAA---PRESGSQVLFNKTGSTNGFGAYVAFVPARGIG
CMY-11 (*E. coli* K983802)	YPVTEQTLLAGNSAKVSLEANPTAA---PRESGSQVLFNKTGSTNGFGAYVAFVPARGIG
FOX-1 (*K. pneumoniae* BA32)	YPLTEQALLAGNSPAVSFQANPVTRFAVPKAMGEQRLYNKTGSTGGFGAYVAFVPARGIA
AmpC (*E. aerogenes* Ear1)	WPVSPEVLINGSDNKVALAATPVTAVKPPAPPVKASWVHKTGSTGGFGSYVAFIPQQDLG
AmpC (*E. aerogenes* Ear2)	WPVSPEVLINGSDNKVAPAATPVTAVKPPAPPVKASWVHKTGSTGGFGSYVAFIPQQDLG
AmpC (*E. cloacae* P99)	LDAQANTVVEGSDSKVALAPLPVAEVNPPAPPVKASWVHKTGSTGGFGSYVAFIPEKQIG
AmpC (*E. cloacae* CHE)	LDAQANTVVEGSD------PLPVVEVNPPAPPVKASWVHKTGSTGGFGSYVAFIPEKQIG
AmpC (*E. coli* K-12)	WPVNPDSIINGSDNKIALAARPVKAITPPTPAVRASWVHKTGATGGFGSYVAFIPEKELG
AmpC (*E. coli* HKY28)	WPVNPDIIIN---NKIALAARPVKPITPPTPAVRASWVHKTGATGGFGSYVAFIPEKELG
AmpC (*S. marcescens* S3)	LDAELSRLIEGNNAGMIMNGTPATAITPPQPELRAGWYNKTGSTGGFSTYAVFIPAKNIA
AmpC (*S. marcescens* HD)	LDAELSRLIEGNNAGMI----PATAITPPQPELRAGWYNKTGSTGGFSTYAVFIPAKNIA

H-10 helix

FIG. 1. Alignments of amino acid residues near the H-10 helix. A partial amino acid sequence alignment of CMY-9 (12), CMY-19 (this study), CMY-11 (21), FOX-1 (15), AmpC of *E. cloacae* Ear1 and Ear2 (5), AmpC of *E. cloacae* P99 and HD (6), AmpC of *E. coli* K-12 and HKY28 (13), and AmpC of *S. marcescens* S3 and HD (22) is shown. Square boxes show the amino acid substitutions or deletions that are predicted to affect the hydrolyzing activity of cefepime. The conserved motif KTG is underlined. Dashes indicate deletions of amino acid residues. CMY-11- and FOX-type enzymes have a serine residue at amino acid position 292, but no observation about their property against cefepime was described in the articles. The numbering of the amino acid residues is in reference to that of the mature CMY-1 reported by Bauernfeind et al. (7).

The mechanism of imipenem resistance of HKYM68 was not characterized in this work.

Transferability of β-lactam resistance. The oximino-cephalosporin and cephamycin resistance trait of the five *K. pneumoniae* was transferred to a recipient *E. coli* strain (strain CSH-2) at a frequency of 10^{-4} to 10^{-5} cells per recipient cell by broth mating. Conjugal transfer of the resistance trait from the nine *E. coli* isolates was also observed at a frequency of about 10^{-3} to 10^{-4} cells per recipient cell.

PCR analyses confirmed the presence of bla_{CMY-9} or bla_{CMY-19} in each transconjugant, indicating that these genes are located on transferable plasmids. PCR analysis of the transconjugants also revealed no cotransmission of the bla_{TEM} gene to the *E. coli* transconjugants that harbored the bla_{CMY-9} gene.

PFGE analysis. The PFGE patterns of the five *K. pneumoniae* isolates after XbaI digestion were highly variable (Fig. 2A), which

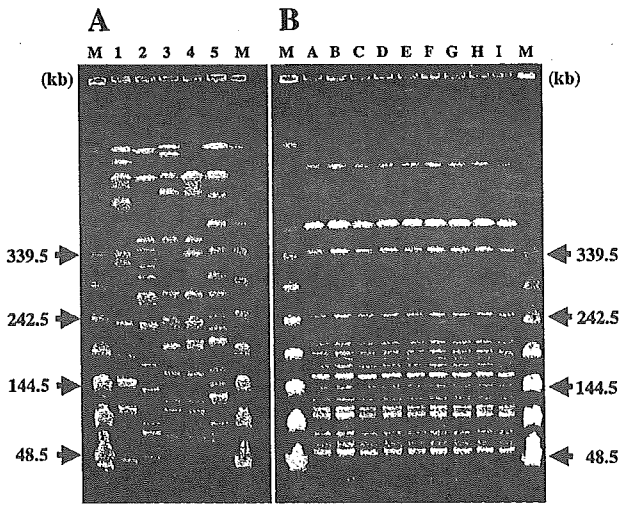

FIG. 2. PFGE analysis of *K. pneumoniae* and *E. coli* isolates. (A) Lanes: M, PFGE marker; 1, *K. pneumoniae* HKY209; 2, *K. pneumoniae* HKY327; 3, *K. pneumoniae* HKY363; 4, *K. pneumoniae* HKY466; 5, *K. pneumoniae* HKY474. (B) Lanes: M, PFGE marker; A, *E. coli* HKY154; B, *E. coli* HKY191; C, *E. coli* HKY200; D, *E. coli* HKY215; E, *E. coli* HKY224; F, *E. coli* HKY297; G, *E. coli* HKY315; H, *E. coli* HKY334; and I, *E. coli* HKYM68.

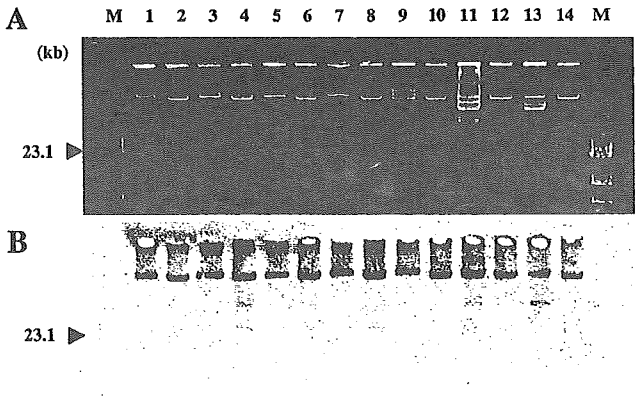

FIG. 3. Plasmid profiles and Southern hybridization. (A) Plasmid profiles of clinical isolates and their tranconjugants; (B) hybridization with the probe specific for the CMY-1- and MOX-1-type β-lactamase gene. Lanes: M, HindIII-digested DNA marker; 1, *K. pneumoniae* HKY209; 2, *E. coli* CSH-2/pK209; 3, *K. pneumoniae* HKY327; 4, *E. coli* CSH-2/pK327; 5, *K. pneumoniae* HKY363; 6, *E. coli* CSH-2/pK363; 7, *K. pneumoniae* HKY466; 8, *E. coli* CSH-2/pK466; 9, *K. pneumoniae* HKY474; 10, *E. coli* CSH-2/pK474; 11, *E. coli* HKY154; 12, *E. coli* CSH-2/pE154; 13, *E. coli* HKYM68; and 14, *E. coli* CSH-2/pEM68.

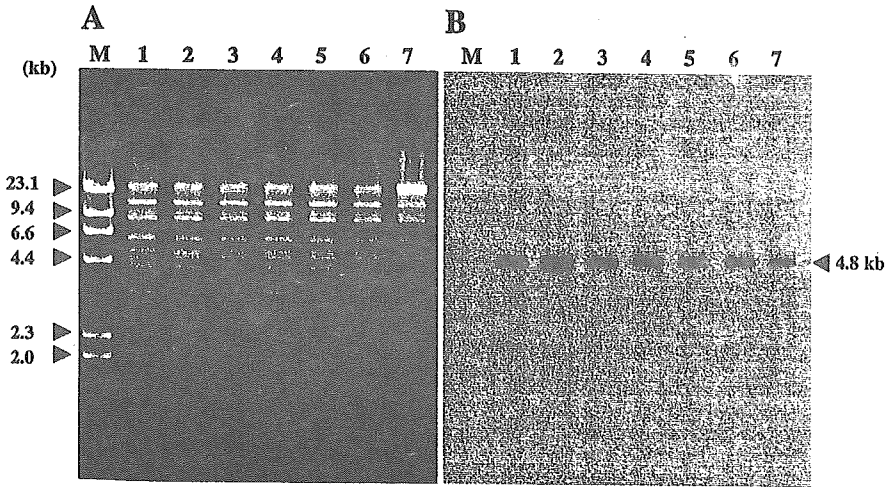

FIG. 4. Plasmid patterns after restriction enzyme digestion and Southern hybridization. (A) SacI-digested plasmid DNAs prepared from the representative transconjugants; (B) hybridization patterns with the probe specific for CMY-1- and MOX-1-type β-lactamase gene. Lanes: M, HindIII-digested DNA marker; 1, *E. coli* CSH-2/pK209; 2, *E. coli* CSH-2/pK327; 3, *E. coli* CSH-2/pK363; 4, *E. coli* CSH-2/pK466; 5, *E. coli* CSH-2/pK474; 6, *E. coli* CSH-2/pE154; and 7, *E. coli* CSH-2/pEM68.

revealed their clonal diversity. In contrast, the PFGE patterns of the *E. coli* isolates were very similar to one another (Fig. 2B), which revealed their clonal relatedness.

Plasmid analyses and Southern hybridization. The plasmid DNA profiles prepared from the five *K. pneumoniae* isolates and their transconjugants and from two representative *E. coli* isolates (isolates HKY154 and HKYM68) and their transconjugants are shown in Fig. 3A. One to three large plasmids were apparently present in the five *K. pneumoniae* isolates, and one large plasmid was transferred to *E. coli* from each strain in the conjugation experiment. *E. coli* isolate HKY154 had four plasmids, and identical plasmid profiles were found in seven other *E. coli* isolates (isolates HKY191, HKY200, HKY215, HKY224, HKY297, HKY315, and HKY334) (data not shown). *E. coli* HKYM68 harbored three plasmids that were similar to three of the four plasmids found in HKY154. All the *E. coli* transconjugants carried a single plasmid apparently identical to that transferred from the *K. pneumoniae* isolates (Fig. 3A and data not shown).

A plasmid of similar size hybridized with the DNA probe specific for the CMY-1-type β-lactamase genes in all the *K. pneumoniae* and *E. coli* isolates and their transconjugants (Fig. 3B). The SacI restriction profiles of the plasmid DNAs from representative transconjugants were very similar to each other (Fig. 4A), and the DNA probe specific for bla_{CMY-1}-group genes hybridized with a band of about 4.8 kb in size in all cases (Fig. 4B).

Isoelectric focusing of β-lactamases. A β-lactamase band with a pI of >8.45 was detected in all *K. pneumoniae* and *E. coli* clinical isolates and their transconjugants but not in *E. coli* CSH-2 (data not shown). This band likely corresponded to the CMY-9 or CMY-19 β-lactamase. No band with an acidic pI value was detected in the nine *E. coli* clinical isolates carrying the bla_{TEM} gene, likely because of a low level of expression of that gene.

Genetic environments of bla_{CMY-9} and bla_{CMY-19} genes. The structure of the flanking regions of the bla_{CMY-9} gene in *E. coli* HKYM68 was already reported in a previous study (12). The structure surrounding the bla_{CMY} genes in the other 13 isolates was identical to that found in HKYM68. Both bla_{CMY-9} and bla_{CMY-19} were located at the 3′ end of a putative transposase gene, *orf513*. A *sul1*-type class 1 integron structure consisting of *intI1* (an integrase gene), a fused *aacA1-orfG* gene cassette (responsible for aminoglycoside resistance), *qacEΔ1*, and *sul1* (responsible for trimethoprim-sulfamethoxazole resistance) were found at the 5′ end of *orf513* (Fig. 5).

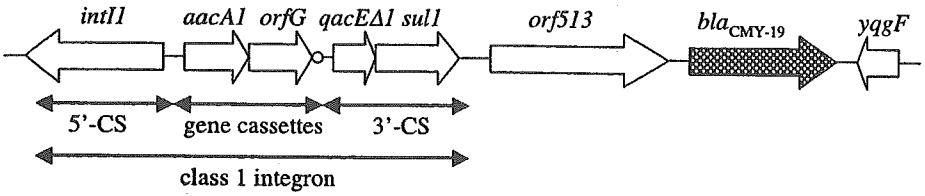

FIG. 5. Gene organization around bla_{CMY} genes. The bla_{CMY} gene on the conjugative plasmid found in the *K. pneumoniae* and *E. coli* clinical isolates located just downstream of *orf513* is shown as it was found in our previous study on a CMY-9 producing *E. coli* HKHM68 (12). Open circle, position of the 59-base element; CS, conserved segment of a class 1 integron. *orf513* is speculated to encode a putative transposase, and various antimicrobial resistance genes tend to be integrated just downstream the *orf513*. The product from the *yqgF* gene encodes a hypothetical protein very similar to the YqgF identified in *Aeromonas hydrophila* (EMBL accession no. AJ276030), but the function is unknown.

TABLE 2. MICs of β-lactams for CMY-9-producing and CMY-19-producing *E. coli* transformants

β-Lactam	MIC (μg/ml)		
	Transformant		Recipient, *E. coli* DH5α (pBCSK+)
	E. coli DH5α (pBC-CMY-9) CMY-9	*E. coli* DH5α (pBC-CMY-19) CMY-19	
Ampicillin	64	>128	2
Piperacillin	8	64	0.5
Piperacillin + TAZ[a]	4	32	0.5
Cephalothin	>128	>128	2
Cephaloridine	64	128	2
Ceftizoxime	64	16	≤0.06
Ceftazidime	64	>128	≤0.06
Ceftazidime + APB[b]	0.5	8	≤0.06
Cefotaxime	>128	128	≤0.06
Cefotaxime + APB[b]	2	1	≤0.06
Cefpirome	8	16	≤0.06
Cefepime	0.13	4	≤0.06
Cefoxitin	>128	128	2
Cefmetazole	128	32	0.5
Cefminox	128	32	0.5
Moxalactam	8	8	≤0.06
Aztreonam	4	16	≤0.06
Imipenem	0.25	0.25	0.13
Meropenem	≤0.06	≤0.06	≤0.06

[a] TAZ, tazobactam, which was used at a concentration of 4 μg/ml.
[b] APB, 3-Aminophenyl boronic acid, which was used at a concentration of 300 μg/ml.

MICs for CMY-9- or CMY-19-producing *E. coli* transformants. The MICs of various β-lactams for CMY-9- or CMY-19-producing *E. coli* transformants are shown in Table 2. Some notable differences were observed between the MICs of the two strains. The MICs of ampicillin and piperacillin for the CMY-19 producer were higher than those for the CMY-9 producer. Concerning ceftizoxime and cefotaxime, the MICs for the CMY-9 producer were higher than those for the CMY-19 producer, but in the case of ceftazidime, the level of resistance was reversed. The CMY-19 producer showed higher levels of resistance to cefpirome and cefepime than the CMY-9 producer. The MICs of cephamycins, such as cefoxitin, cefmetazole, and cefminox, were higher for the CMY-9 producer than for the CMY-19 producer. A remarkable reduction in the MICs by the addition of a class C β-lactamase specific inhibitor, 3-aminophenyl boronic acid, was observed with both the CMY-9 and the CMY-19 producers.

Kinetic parameters. To purify the CMY-9 and the CMY-19 β-lactamases, initially, *E. coli* DH5α(pBC-CMY-9) and *E. coli* DH5α(pBC-CMY-19) were cultured in 2 liters of LB broth. However, the yield of purified CMY-19 β-lactamase was insufficient for the assay of kinetic parameters. Therefore, a pET29a(+) expression vector and an *E. coli* BL21(DE3) pLysS strain were used for overproduction and purification of that enzyme. The purified enzymes gave a single band on SDS-PAGE with CBB staining that suggested >95% purity (data not shown).

The kinetic parameters of CMY-9 and CMY-19 against selected β-lactams are shown in Table 3. The hydrolyzing activity (k_{cat}/K_m) of CMY-19 for penicillins, including ampicillin and piperacillin, were higher than those of CMY-9. Although CMY-9 and CMY-19 had similar k_{cat} values for cefotaxime, CMY-19 had a 100-fold-higher K_m than CMY-9, resulting in a lower catalytic efficiency for this substrate. Ceftazidime, cefpirome, and cefepime behaved as poor substrates for CMY-9 due to the high K_m values for these agents, while CMY-19 showed different behaviors against these compounds. CMY-19 had a 140-fold-lower K_m against ceftazidime than CMY-9. The k_{cat} value of CMY-9 for cefepime could not be determined, but CMY-19 measurably hydrolyzed this compound. The hydrolyzing efficiencies (k_{cat}/K_m values) of CMY-19 against cephamycins such as cefoxitin and cefmetazole were lower than those of CMY-9. Although CMY-19 had a lower K_m against cephamycins than CMY-9, it showed a much lower k_{cat} against these compounds.

DISCUSSION

A plasmid-mediated class C β-lactamase (CMY-1) was first reported in 1989 in a *K. pneumoniae* isolated in South Korea (8). Subsequently, several variants of that enzyme, such as MOX-1 (16), CMY-8 (33), CMY-9 (12), CMY-10 (20), and CMY-11 (21), have been identified, mainly in East Asian countries, including Taiwan and Japan. The dissemination of CMY-

TABLE 3. Kinetic parameters of CMY-9 and CMY-19

Substrate	CMY-9			CMY-19		
	K_m or K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m or K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Ampicillin	91 ± 28	1.0 ± 0.1	1.1×10^4	16 ± 1	0.35 ± 0.01	2.2×10^4
Piperacillin	97 ± 21	0.14 ± 0.01	1.4×10^3	8.9 ± 0.5	0.031 ± 0.001	3.5×10^3
Cephalothin	120 ± 10	630 ± 10	5.3×10^6	230 ± 10	380 ± 10	1.7×10^6
Cephaloridine	1200 ± 100	99 ± 2	8.3×10^4	1500 ± 100	240 ± 10	1.6×10^5
Ceftizoxime	5.5 ± 0.2	1.3 ± 0.1	2.4×10^5	11 ± 1	0.71 ± 0.03	6.5×10^4
Ceftazidime	560 ± 110	1.8 ± 0.3	3.2×10^3	3.7 ± 0.1	0.085 ± 0.002	2.3×10^4
Cefotaxime	0.28 ± 0.01	0.27 ± 0.01	9.6×10^5	31 ± 2	0.33 ± 0.01	1.1×10^4
Cefpirome	390 ± 50	3.6 ± 0.3	9.2×10^3	25 ± 2	0.58 ± 0.02	2.3×10^4
Cefepime	950 ± 50	NH[a]	ND[b]	630 ± 170	1.8 ± 0.4	2.9×10^3
Cefoxitin	60 ± 2	50 ± 1	8.3×10^5	0.90 ± 0.03	0.12 ± 0.01	1.3×10^5
Cefmetazole	5.1 ± 0.2	1.7 ± 0.1	3.3×10^5	0.26 ± 0.01	0.045 ± 0.001	1.7×10^5
Moxalactam	0.22 ± 0.01	NH	ND	0.40 ± 0.03	NH	ND
Imipenem	4.6 ± 0.3	NH	ND	4.3 ± 0.1	NH	ND

[a] NH, not hydrolyzed.
[b] ND, not determined.

10- and CMY-11-producing isolates of the family *Enterobacteriaceae* was also reported in Korea (18, 19). In the present study, CMY-type β-lactamase-producing *K. pneumoniae* and *E. coli* clinical isolates from a Japanese general hospital were investigated. Through PFGE analysis, it was found that the five *K. pneumoniae* isolates had little genetic relatedness to each other, while the nine *E. coli* isolates belonged to the same clonal lineage. Interestingly, plasmid analysis showed that all 14 isolates harbored a very similar conjugal plasmid that encodes a CMY-type β-lactamase which was either CMY-9 or CMY-19, a variant that differs from CMY-9 by a single amino acid substitution (I292S). Since the flanking structures of the bla_{CMY} genes were identical in all plasmids, it is probable that one conjugal plasmid carrying bla_{CMY-9} was horizontally transferred to *E. coli*, *K. pneumoniae*, and then an *E. coli* clone and various *K. pneumoniae* strains harboring the bla_{CMY} gene-bearing plasmids might have spread in the hospital.

β-Lactamases can modify their substrate specificity through a single amino acid substitution (30). CMY-19 had a single amino acid substitution, I292S, near the H-10 helix domain, compared with the sequence of CMY-9 (Fig. 1). Indeed, a serine residue at the same amino acid position was found in all the FOX-type enzymes (15, 26), including CAV-1 (14), and also in CMY-11 (21), as shown Fig. 1; but no peculiar behavior against cefepime was documented with those enzymes. Through the I292S substitution, CMY-19 would have developed extended substrate specificity against cefepime and cefpirome, as well as ampicillin, piperacillin, cephaloridine, and ceftazidime, compared with that of CMY-9, although the hydrolyzing activities against ceftizoxime, cefotaxime, and cephamycins were impaired. The expansion of hydrolyzing activity against cefepime found in CMY-19 was a most remarkable property because cefepime is generally stable against AmpC β-lactamases (11). Similar developments of extended hydrolyzing activity against cefepime through amino acid substitutions or deletions adjacent to the H-10 helix have been observed in several chromosomally encoded AmpC β-lactamases, such as the AmpC of *Serratia marcescens* that lacks four amino acids at positions 293 to 296 (22), an AmpC of *E. coli* that lacks three amino acids at positions 286 to 288 (13), an AmpC of *Enterobacter cloacae* that lacks six amino acids at positions 289 to 294 (6), and an AmpC of *Enteroacter aerogenes* that acquired an L293P substitution (5). Furthermore, Barlow and Hall reported on the in vitro selection of CMY-2 β-lactamase variants with several amino acid substitutions, including replacements at positions 292, 293, 294, 296, and 298, which demonstrated increased resistance to cefepime (4). From our previous molecular modeling analyses (13), it was found that the expansion of an open space in the vicinity of the R-2 side chain of ceftazidime or cefepime through the deletion of tripeptides in the H-10 helix of *E. coli* AmpC (Fig. 1) played a crucial role in the acquisition of the greater hydrolyzing activity against those agents possessing a bulky R side chain. Although the actual mechanism for the higher cefepime-hydrolyzing activity in CMY-19 compared to that in CMY-9 has not been elucidated, a similar molecular distortion at the active center of the enzyme might well have occurred in CMY-19. This speculation would be substantiated by molecular modeling and X-ray crystallographic analyses.

ACKNOWLEDGMENTS

We are grateful to Kumiko Kai and Fusako Yokokawa for technical assistance.

This work was supported by the Ministry of Health, Labor and Welfare of Japan (grants H15-Shinkou-9 and H15-Shinkou-10). The research activity of J. Wachino was supported by a Scholarship for Young Scientists provided by the Japan Society for the Promotion of Science.

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Jpn. J. Infect. Dis., 59, 2006

Laboratory and Epidemiology Communications

A Nosocomial Outbreak Due to Novel CTX-M-2-Producing Strains of *Citrobacter koseri* in a Hematological Ward

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Communicated by Kiyosu Taniguchi

(Accepted January 10, 2006)

Citrobacter koseri is a member of the family *Enterobacteriaceae*. Urinary tract infections caused by *C. koseri* have been observed in as many as 12% of all isolates in adults (1). In compromised hosts, *Citrobacter* spp. could cause pneumonitis, empyema (2), biliary infection (3), and bacteremia (4). *Citrobacter* spp. were formerly susceptible to oximinocephalosporins including cefotaxime (3), but recently, *C. koseri* has been reported to have developed resistance to some cephalosporins and cephamycins through the production of an inducible chromosomally-encoded cephalosporinase that can inactivate these agents (5). Most clinically isolated *C. koseri* are susceptible to oximinocephalosporins and carbapenems. Recently, oximinocephalosporin resistance among Gram-negative bacteria has been developed due to the hydrolysis of beta-lactams by beta-lactamases including extended-spectrum beta-lactamases (ESBLs). ESBLs show variable levels of resistance to cefotaxime, ceftazidime, and other broad-spectrum cephalosporins and monobactams. Nosocomial outbreaks due to SHV-4-type ESBL-producing strains and TEM-type ESBL-producing strains of *C. koseri* have already been reported (6,7). We have identified a novel CTX-M-2-type of ESBL among nosocomially isolated *C. koseri* strains, causing a probable outbreak in the hematological ward.

Sixty-eight strains of *C. koseri* were isolated from the blood, urine, feces, sputum, ascites, and pharynx of 31 patients with a hematological malignancy that had lasted over 18 months (Figure 1). *C. koseri* not only colonized but also caused bacteremia, urinary tract infection, enteritis, and peritonitis. These strains showed similar antibiotic susceptibility profiles (Table 1). We collected 5 strains of *C. koseri* from 4 patients (Table 2) and used the double-disk synergy test and plasmid profiling to screen for ESBL-producing strains as reported previously (8,9). All of the 5 strains harbored a plasmid mediating the CTX-M-2 type beta-lactamase gene. Epidemiological study using pulsed-field gel electrophoresis (PFGE) of total DNA prepared from the 5 strains revealed patterns that were indistinguishable from each other (Figure 2). The results suggested that the 5 strains characterized belong to a single epidemic strain.

In general, multiple factors may help to decrease the immunity of patients with hematological malignancies, including impairment of phagocytosis, impaired cellular immunity, and defective production of antibodies. Moreover, intensive chemotherapies usually induce severe granulocytopenia. Thus, bacterial infections are a major cause of complications and death in patients with hematological malignancies. Recently, two studies (10,11) revealed the efficacy of the prophylactic use of quinolon by neutropenic patients. As for febrile neutropenia, empirical antibiotic therapy using cefepime or cefotaxime has been emphasized (12,13). All 31 patients in this study had hematological malignancy and underwent intensive chemoradiotherapy. After that, most of the patients in our ward were administered prophylactic and therapeutic systemic antibiotics such as quinolon, cefepime, and cefotaxime, which might well be associated with the selection of antibiotic-resistant microorganisms. Unlike other members of the family *Enterobateriaceae*, CTX-M-2-producing *C. koseri* might survive in a patient's bowel flora, because of its resist-

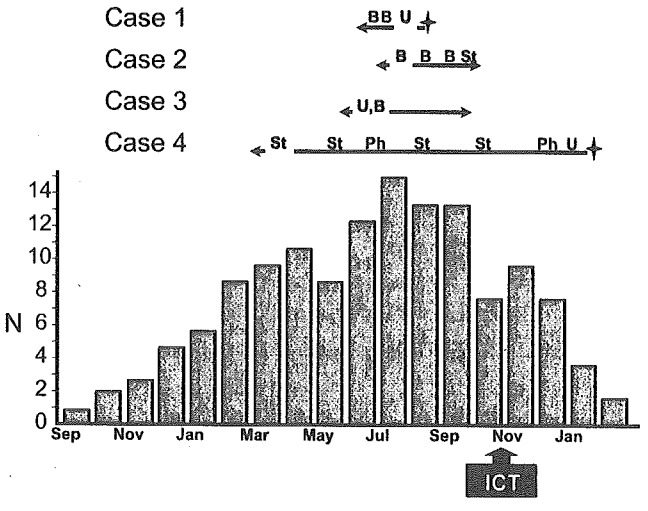

Fig. 1. The number of patients infected and/or colonized with *C. koseri*. Bars indicate the number of patients infected and/or colonized per month with *C. koseri*, the antibiotic susceptibility of which showed the same pattern. Case numbers are identical to those in Table 1. Arrows indicate the duration of each patient's hospitalization in the ward. The bald signs on each arrow indicate the samples, from which *C. koseri* was isolated. The network-breaking characters indicate the samples, from which genetically identical strains were isolated in our study. An infection control team (ICT) intervened in the ward to resolve the outbreak (see article). B, blood; U, urine; St, stool; Ph, pharynx.

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Table 1. Antibiotics susceptibility profile of *C. koseri* isolated in this outbreak

ampicillin	>16
ampicillin/ clavulanate	16
piperacillin	>64
cefazolin	>16
cefotaxime	>16
cefotiam	>32
ceftazidime	>16
cefpirome	>16
cefpodoxime proxetil	>4
cefcapene pivoxil	>1
cefmetazole	>32
flomoxef	32
sulbactam/cefoperazone	>32
aztoreonam	>16
imipenem	<1
gentamicin	<1
amikacin	<1
minomicin	2
levofloxacin	>4

MICs were determined by microdilution method recommended by NCCLS (currently CLSI) guideline with Muller-Hinton broth (Difco, Detroit, Mich., USA) using MicroScan-kit (Dade Behring, West Sacramento, Calif., USA).

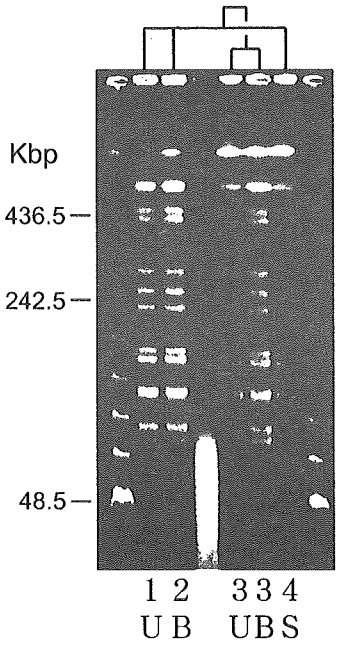

Fig. 2. PFGE analysis of *C. koseri* strains. Bacterial DNA was extracted, digested and subjected to PFGE, as previously described (8,9). Lanes 1, 2, 3, and 4, were sampled from patients Nos. 1, 2, 3, and 4, respectively. U, B, and S indicate urine, blood, and stool, respectively.

Table 2. Profiles of cases involved in the outbreak

No.	Age/Sex	Underlying disease	Therapy/Outcome	Infection	Sources of *C. koseri*
1	71/M	malignant lymphoma	chemotherapy/refractory	sepsis	Urine
2	61/M	adult T-cell leukemia	chemotherapy/partial response	sepsis	Blood
3	25/F	acute lymphoblastic leukemia	bone marrow transplantation	sepsis	Urine, Blood
4	63/F	acute lymphoblastic leukemia	chemotherapy/complete remission	enteritis	Stool

ance to quinolon, cefepime, and cefotaxime. In addition, urinary tract infections tended to be easily associated with urinary catheterization in our cases. We speculated that the situation was as follows. Once *C. koseri* colonizes in the bladder or intestine, it will then disseminate into the blood stream causing severe bacteremia during intensive chemotherapy. The symptoms of sepsis caused by *C. koseri* were often very serious, and could only be cured by appropriate and immediate administration of carbapenem. However, the use of carbapenem in high amounts and at high frequency in our ward could create a grave epidemiological problem.

The number of *C. koseri* infections increased significantly, and standard infection control measures were not effective to stop this outbreak. Therefore, we began to enforce the following precautions. We introduced barrier precautions against not only infected patients but also colonized patients, using disposable gloves and drapes. Mandatory hand washing was done immediately before and after any manipulation involved in the nursing care. Hand hygiene using commercial alcoholic disinfectant (Welpas; Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) was promoted not only for medical workers but also for patients. As for the environment, the water taps were converted to the hands-free types, and all doorknobs and bars for drip injection were sterilized using 70% alcohol twice daily. We also tried to restrict the prophylactic use of quinolon for high-risk patients with neutropenia decreasing under 100/μL which was keeping for more than 1 week. After these procedures, the incidence of *C. koseri* isolation decreased, but this type of infection has not yet been eradicated, as shown in Figure 1. We continue to make an effort to prevent nosocomial transmission of *C. koseri*.

In this report, we emphasize the appearance of *C. koseri* and its new type of drug resistance. We also warn that it is quite difficult to control the outbreak of such antimicrobial-resistant microorganisms in a hematological ward. In the future, we must pay close attention to the nosocomial spread of this type of *C. koseri*, which has demonstrated resistance to a broad spectrum of cephalosporins, cephamycins, and carbapenems.

Genetic characterization of beta-lactamase genes were supported by the grants (H15-Shinko-9 and -10) from the Ministry of Health, Labour and Welfare, Japan.

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