Prevalence and molecular characterization of CTX-M extended-spectrum *β*-lactamase-producing *Escherichia coli* from 2000 to 2010 in Japan

Takafumi Hara, Takafumi Sato, Tsukasa Horiyama, Sachi Kanazawa, Takahiro Yamaguchi and Hideki Maki

Infectious Diseases, Discovery Research Laboratory for Core Therapeutic Areas, Shionogi & Co., Ltd., Toyonaka, Osaka 561–0825, Japan

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The prevalence of extended-spectrum *β*-lactamase (ESBL) in Enterobacteriaceae has been increasing worldwide. The aims of this study were to determine the prevalence of ESBLs among clinical isolates of *Escherichia coli* obtained from 2000 to 2010 in Japan, and to characterize the sequence type (ST) and antimicrobial susceptibility of the $bla_{\text{CTX-M}}$-carrying strains. The genes for *β*-lactamases were determined by conventional PCR and sequencing, and the antimicrobial susceptibility test was performed by the broth microdilution method. Among the 948 strains, 35 were judged as ESBL-positive strains. The positive rates ranged from 0.6% to 3.9% until 2008, but surged to 10.3% in 2010. Thirty-three of them carried $bla_{\text{CTX-M}}$, but all were negative for ESBL-type bla_{TEM} and bla_{SHV}. $bla_{\text{CTX-M-14}}$ was the most prevalent (18/33) among $bla_{\text{CTX-M}}$-carrying strains, followed by $bla_{\text{CTX-M-15}}$ (7/33) of which five were isolated in 2008 and 2010. Additionally, $bla_{\text{CTX-M-27}}$ appeared in 2010 for the first time in this study and accounted for more than a third of the $bla_{\text{CTX-M}}$-carrying strains. From the MLST analysis, ST131 known as a world pandemic clone, has been predominantly isolated since 2006. The major types of ESBLs carried by ST131 strains clearly shifted from $bla_{\text{CTX-M-14}}$ to $bla_{\text{CTX-M-15}}$ and/or $bla_{\text{CTX-M-27}}$ between 2006 and 2010. Most of these isolates were still susceptible to doripenem, latamoxef (moxalactam), flomoxef and cefmetazole. Our results suggest that a change of the dominant type of ESBL among Enterobacteriaceae is currently in progress in Japan, and therefore further periodic surveillance is needed.

Introduction

Recently, there has been an explosive increase in the prevalence of Enterobacteriaceae with high-level resistance to various *β*-lactams due to the presence of extended-spectrum *β*-lactamase (ESBL) enzymes worldwide[1]. ESBLs can hydrolyze penicillins as well as oxyimino-cephalosporins, such as ceftazidime and cefotaxime, which have played a valuable role in the treatment of Gram-negative infections. Some researchers have reported that the ESBL-producing Enterobacteriaceae are associated with infections that result in poor clinical outcomes, delayed initiation of appropriate antibacterial therapy, longer hospital stays and greater hospital expenses[2, 3]. Indeed, the infections caused by these bacteria are an emerging public health concern worldwide.

Most ESBLs belonging to Ambler Class A can be classified into three types: TEM, SHV, and CTX-M[4]. During the 1990s, ESBLs corresponded approximately to the derivatives of TEM- and SHV-type *β*-lactamases in *Escherichia coli* worldwide[5]. However, since the late 1990s, CTX-M-type ESBLs have dramatically increased and become predominant. These have been found in both hospital-acquired and community-acquired infections. The CTX-M-type of ESBL can be further classified into five groups according to their amino acid sequences: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 groups. Some of the CTX-M enzymes have specific geographical distributions: CTX-M-9 and CTX-M-14 are found in Spain, and CTX-M-14 in South East Asia and North America. Both of these enzymes belong to the CTX-M-9 group. As for the CTX-M-1 group, CTX-M-1 is found in Italy and CTX-M-3 in Poland, while CTX-M-15 is currently the most common variant distributed worldwide[6]. Additionally, molecular epidemiology studies reveal that one specific *E. coli* pandemic clone, ST131, has been closely associated with the spread of CTX-M enzymes including CTX-M-15[7]. In Japan, the dominant CTX-M-group carried by *E. coli* underwent a shift from CTX-M-2 to CTX-M-14 in the early 2000s[8]. Several studies have recently reported the isolation of global pandemic CTX-M-15-carrying strains in Japan, suggesting that an epidemiological change might be starting[9, 10]. However, there have been few reports of long-term nationwide surveillance in Japan about the changes in dominance of CTX-M enzymes.

In this study, we investigated the prevalence and molecular characteristics of ESBLs among *E. coli* isolates obtained from 20 Japanese hospitals between 2000 and 2010, including their antimicrobial susceptibility to commonly used antimicrobials.

Materials and Methods

Bacterial strains

A total of 948 non-duplicate clinical isolates of *E. coli* collected from 20 hospitals in various regions in Japan every two years between 2000 and 2010 were examined.

Screening for ESBL

ESBL production was confirmed by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[11]. All isolates with MIC of either cefotaxime or ceftazidime of ≥2 μg/mL and with a ≥8-fold MIC reduction of either cefotaxime or ceftazidime occurring in the presence of clavulanic acid (4 μg/mL) were identified as potential ESBL producers. These isolates were subjected to polymerase chain reaction (PCR) analyses for the detection of ESBL genes.

Detection of ESBL genes and DNA sequencing

PCR was performed using a single conventional PCR assay with GoTaq® Master Mix (Promega) to identify ESBL genes, including bla_{TEM}, bla_{SHV}, $bla_{\text{OXA-1 group}}$, $bla_{\text{CTX-M-1 group}}$, $bla_{\text{CTX-M-2 group}}$, $bla_{\text{CTX-M-9 group}}$, $bla_{\text{CTX-M-8 group}}$, $bla_{\text{CTX-M-25 group}}$ and bla_{CMY}. Crude genomic DNA was extracted from the isolates by heat lysis. Briefly, colonies were suspended in 100 μL of distilled water, and the cells were lysed by heating at 95℃ for 10 min. Cellular debris was removed by centrifugation, and then supernatant containing DNA was subjected to PCR. The PCR amplification consisted of a pre-PCR stage at 94℃ for 5 min, and then 25 cycles at 94℃ for 30 s, at 55℃ for 30 s and at 74℃ for 1 min, and a final extension stage at 74℃ for 7 min using C1000™ Thermal Cycler (Bio-Rad). The nine sets of primers used for amplification and sequencing are shown in Table 1. Direct sequencing of PCR products was performed by Eurofins Genomics (Tokyo, Japan), and the obtained sequences that were truncated approximately 30 base of both flanking region was used to classify consistent sub-types.

Antimicrobial susceptibility testing

The susceptibilities to 12 antimicrobials such as ampicillin-sulbactam, tazobactam-piperacillin, cefepime, cefmetazole, cefotaxime, ceftazidime, ceftriaxone, flomoxef, latamoxef (moxalactam), doripenem, levofloxacin, and sulfamethoxazole-trimethoprim were determined by the broth microdilution method with cation-adjusted Mueller-Hinton broth (Difco, USA), according to the CLSI guidelines[11]. MIC interpretation of susceptibility was determined based on the CLSI breakpoint (as the MIC breakpoints of flomoxef and cefozopran were unavailable, those of latamoxef and cefepime, respectively, were applied instead)[12].

Multilocus sequence typing (MLST)

MLST was carried out on all $bla_{\text{CTX-M}}$-carrying strains according to the protocol and primer sets specified on the *E. coli* MLST web site (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). After performing the gene amplification and sequencing of 7 standard housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*), the allelic profile and ST determinations were identified.

Table 1. Primers used in this study

Target[a]	Sequence (5' - 3')	Reference
CTX-M-1 group-F	CCGACATATGGTTAAAAAATCACTGCGTCA	This study
CTX-M-1 group-R	CCGTGAATTCTTACAAACCGTTGGTGACGATTT	This study
CTX-M-2 group-F	CCGACATATGATGACTCAGAGCATTCGCC	This study
CTX-M-2 group-R	CCGTGAATTCTCAGAAACCGTGGGTTACGATT	This study
CTX-M-8 group-F	CCGACATATGATGATGCTAATGACAACGGCC	This study
CTX-M-8 group-R	CCGTGAATTCATAACCGTCGGTGACGATTTT	This study
CTX-M-9 group-F	CCGACATATGGTGACAAAGAGAGTGCAAC	This study
CTX-M-9 group-R	CCGTGAATTCTTACAGCCCTTCGGCGATGAT	This study
CTX-M-25 group-F	CCGACATATGATGATGAGAAAAAGCGTAAGG	This study
CTX-M-25 group-R	CCGTGAATTCTTAATAACCGTCGGTGACAAT	This study
TEM-F	CCGACATATGAGTATTCAACATTTTCGTGTC	This study
TEM-R	CCGTGAATTCTTACCAATGCTTAATCAGTGAGG	This study
SHV-F	CCGACATATGCGTTATATTCGCCTGTGTAT	This study
SHV-R	CCGTGAATTCTTAGCGTTGCCAGTGCTCGAT	This study
OXA-1-F	CCGACATATGAAAAACACAATACATATCAACTT	This study
OXA-1-R	CCGTGAATTCTTATAAATTTAGTGTGTTTAGAATGGT	This study
CMY-F	CACCATCACCCCGTTGATG	This study
CMY-R	GTTCAGCATCTCCCAGCC	This study

[a] CTX-M-1 group includes CTX-M-15. CTX-M-9 group includes CTX-M-14 and CTX-M-27.

Results

Detection rates of ESBL-positive strains and distribution of ESBL genes

Among the 948 *E. coli* strains isolated between 2000 and 2010, 35 were identified as ESBL-positive strains. The detection rates each year ranged from 0.6% (1/164) to 3.9% (6/155) until 2008, however, the rate in 2010 surged to 10.3% (16/156) (Fig. 1). Determining the ESBL genes by PCR and sequencing showed that all the strains except two were positive for at least one $bla_{\text{CTX-M}}$ gene. One of the $bla_{\text{CTX-M}}$-negative strains carried $bla_{\text{TEM-1}}$ (non-ESBL gene), while the other did not carry any of the β-lactamase genes tested in this study. Figure 1 also shows the distribution of $bla_{\text{CTX-M}}$ each year, indicating that the most frequently detected ESBL gene was $bla_{\text{CTX-M-14}}$, followed by $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-2}}$ throughout the observation period, with the exception of $bla_{\text{CTX-M-27}}$. In 2010, strains carrying $bla_{\text{CTX-M-27}}$ were detected for the first time in this study and accounted for 43.8% (7/16), of which one strain concurrently carried $bla_{\text{CTX-M-15}}$. The

Fig. 1. Number of extended-spectrum *β*-lactamase (ESBL)-producing *Escherichia coli* and distribution of bla_{CTX-M} genes among ESBL-positive isolates between 2000 and 2010
Numbers in parentheses represent the total number of isolates examined.

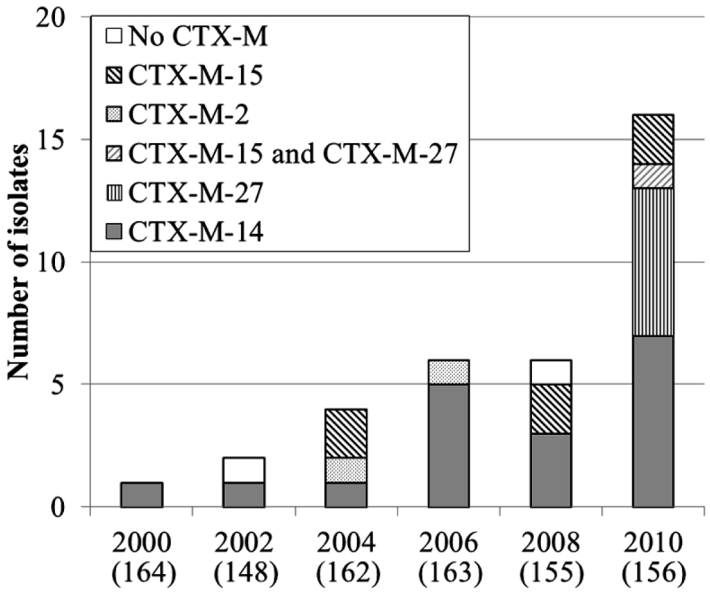

Table 2. Number of ST131 strains carrying bla_{CTX-M} genes

year	n	Number of ST131 isolates			
		CTX-M-14	CTX-M-15	CTX-M-27	CTX-M-15 and 27
2006	5	5			
2008	2	1	1		
2010	10	1	2	6	1

other types of ESBL genes (bla_{TEM}, $bla_{SHV,}$ $bla_{CTX-M-8\ group}$ and $bla_{CTX-M-25\ group}$) were not detected in any strains tested in this study.

MLST

Among the 33 bla_{CTX-M}-carrying *E. coli*, 17 were identified as the global pandemic clone ST131. The rest strains were belonged to several ST types including ST38, ST354 and ST648. In this study, the ST131 strains were first isolated in 2006, and became predominant (10/16) in 2010 among bla_{CTX-M}-carrying strains (Table 2). All five ST131 types isolated in 2006 were equivalent to all $bla_{CTX-M-14}$-carrying strains isolated in 2006. However, the number of ST131 among $bla_{CTX-M-14}$-carrying strains decreased to one in three in 2008 and one in seven in 2010. In 2008

and 2010, ST131 was dominated by $bla_{\text{CTX-M-15}}$- and/or $bla_{\text{CTX-M-27}}$-carrying strains; all the $bla_{\text{CTX-M-15}}$- and/or $bla_{\text{CTX-M-27}}$-carrying strains isolated in 2010 belonged to ST131.

Susceptibility for antimicrobials

As for the injectable antimicrobials, almost all the $bla_{\text{CTX-M}}$-carrying *E. coli* were susceptible to doripenem (carbapenem), flomoxef, latamoxef (oxacephem), and cefmetazole (cephamycin). They were also comparatively susceptible to tazobactam-piperacillin, but not to some cephalosporins such as ceftriaxone, cefotaxime, ceftazidime and cefepime (Fig. 2). The numbers of susceptible strains were 8 out of 33 strains for levofloxacin (Table 3). Focusing on each $bla_{\text{CTX-M}}$ type, the numbers of strains carrying $bla_{\text{CTX-M-15}}$ which were susceptible to cephalosporins as well as β-lactam-β-lactamase inhibitor combinations were relatively lower than those of $bla_{\text{CTX-M-14}}$- and $bla_{\text{CTX-M-27}}$-carrying strains. None of the $bla_{\text{CTX-M-27}}$-carrying strains were susceptible to ceftazidime, and the susceptibility rate was much lower than that of $bla_{\text{CTX-M-14}}$-carrying strains (13 of the 18 strains). One $bla_{\text{CTX-M-14}}$-carrying strain showing low susceptibility to some antimicrobials, including cefmetazole and flomoxef, carried the $bla_{\text{CMY-2}}$ Class C β-lactamase gene, known to be responsible for resistance to them.

Fig. 2. Cumulative percentage of isolates inhibited against $bla_{\text{CTX-M}}$-carrying *Escherichia coli* (n=33) for 10 injectable antimicrobials

[a] fixed tazobactam concentration of 4 μg/mL; [b] fixed ratio of 2 : 1.

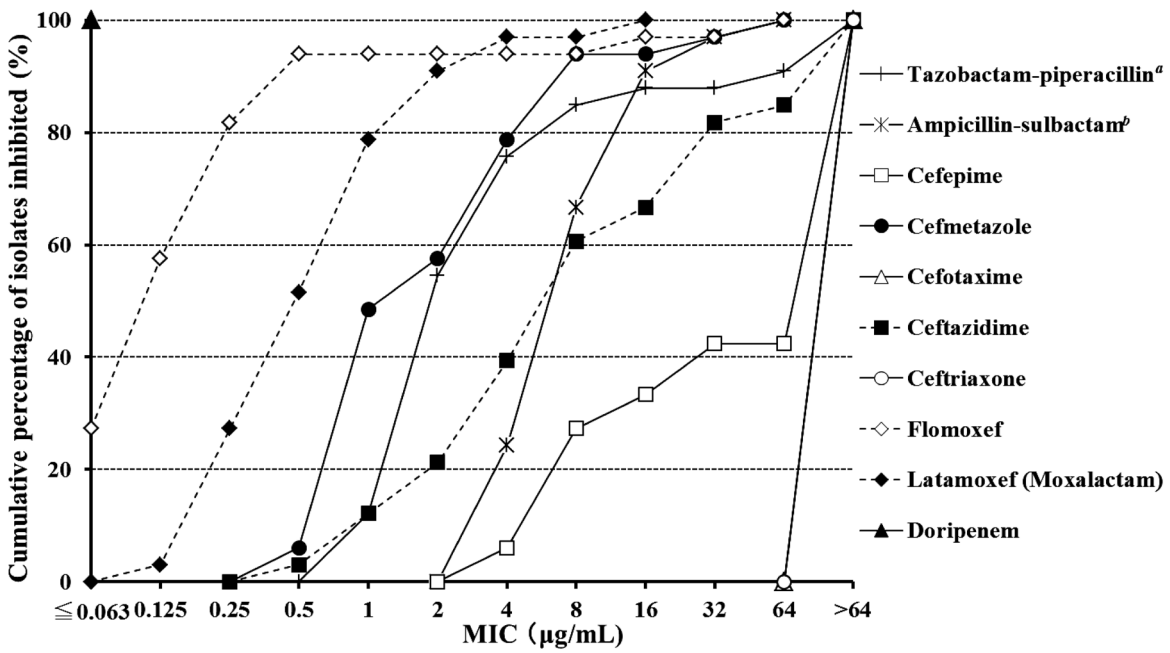

Table 3. Antimicrobial susceptibility of *bla*$_{CTX-M}$-carrying *Escherichia coli*

Antimicrobials	Number of susceptible isolates					
	All CTX-Ms (n=33)	CTX-M-14[d] (n=18)	CTX-M-27 (n=6)	CTX-M-15 (n=6)	CTX-M-15 and CTX-M-27 (n=1)	CTX-M-2 (n=2)
Tazobactam-piperacillin[a]	29	16	6	4	1	2
Ampicillin-sulbactam[b]	7	3	4	0	0	0
Cefepime	9	5	4	0	0	0
Cefmetazole	31	17	5	6	1	2
Cefotaxime	0	0	0	0	0	0
Ceftazidime	13	13	0	0	0	0
Ceftriaxone	0	0	0	0	0	0
Flomoxef	31	17	5	6	1	2
Latamoxef (Moxalactam)	32	18	5	6	1	2
Doripenem	33	18	6	6	1	2
Levofloxacin	8	6	0	1	0	1
Sulfamethoxazole-trimethoprim[c]	10	5	2	3	0	0

[a] fixed tazobactam concentration of 4 μg/mL; [b] fixed ratio of 2:1; [c] fixed ratio of 19:1; [d] one of these strains carried the *bla*$_{CMY-2}$ Class C β-lactamase gene.

Discussion

Since the late 1990s, CTX-M-type *β*-lactamase have become the predominant ESBL among clinical isolates of *E. coli* worldwide, and infections caused by these pathogens have become an emerging public health concern[5]. Many epidemiological studies of CTX-M have been performed in different countries. In this study, we investigated the prevalence of Japanese clinical isolates and compared the results with those of past reports.

Our date revealed the detection rates of ESBL-carrying *E. coli* gradually increased and surged to 10.3% in 2010. A similar observations were reported by Chong, *et al.* and Nakamura, *et al.*[13, 14]. These results, together with our findings, indicate that there is no longer any doubt about the increase of ESBL-carrying *E. coli* in Japan in recent years. Both previous reports also showed that the most frequently identified ESBL genes were $bla_{\text{CTX-M}}$-type, with the $bla_{\text{CTX-M-9 group}}$ predominating, followed by the $bla_{\text{CTX-M-1 group}}$, thus showing the same tendency as our result. Although these two studies did not determine the detailed sub-types of $bla_{\text{CTX-M}}$ in ESBL-carriers, we classified them by sequence analysis.

We found that $bla_{\text{CTX-M-27}}$ appeared in 2010 for the first time in this study and the numbers of isolation reached almost the same levels as $bla_{\text{CTX-M-14}}$. Both $bla_{\text{CTX-M}}$ genes belong to the $bla_{\text{CTX-M-9 group}}$. On the other hand, as for the $bla_{\text{CTX-M-1 group}}$, only $bla_{\text{CTX-M-15}}$ was detected in 2008 and 2010. Chong, *et al.* and Nakamura, *et al.* also deduced the existence of $bla_{\text{CTX-M-15}}$ in *E. coli* isolates carrying the $bla_{\text{CTX-M-1 group}}$, but they did not refer to the emergence of $bla_{\text{CTX-M-27}}$-carrying strains among the identified $bla_{\text{CTX-M-9 group}}$-carrying strains because it is well-known that $bla_{\text{CTX-M-14}}$ was dominant in Japan in 2000s[13–15]. It has been reported that the clonal outbreaks of $bla_{\text{CTX-M-27}}$-carrying *E. coli* ST131 isolates were occurred in Japan even though the area was limited[9, 10]. Yano, *et al.* reported that as many as 21 out of 71 *E. coli* isolates collected from tertiary hospitals in the Tohoku district between 2008 and 2011 were positive for $bla_{\text{CTX-M-27}}$[9]. Kuroda, *et al.* also identified 22 $bla_{\text{CTX-M-27}}$-carrying strains collected between 2008 and 2009, though these isolates were restricted to the Tohoku and Kanto districts in Japan[10]. Thus, clonal outbreaks of $bla_{\text{CTX-M-27}}$-carrying *E. coli* ST131 have definitely occurred centering around eastern Japan, and our findings demonstrate the emergence of $bla_{\text{CTX-M-27}}$ found in various regions in Japan.

The emerging CTX-M-27-producers are less susceptible to ceftazidime compared to CTX-M-14-producers according to the previous report[16]. Our result also demonstrated that 3rd generation cephalosporins including ceftazidime as well as levofloxacin were completely inactive against $bla_{\text{CTX-M-27}}$-carrying strains. Also, the *β*-lactam-*β*-lactamase inhibitor combinations in addition to ampicillin-sulbactam was less active against $bla_{\text{CTX-M-15}}$-carrying strains. As these antimicrobials are frequently used for treatment in many countries including Japan, there is a concern that these drugs will apply selection pressure to these isolates. Under such circumstances, it is noteworthy that most of these $bla_{\text{CTX-M}}$-carrying strains were still susceptible to doripenem, oxa-

cephems and cefmetazole.

In conclusion, our study reveals a sharp rise of $bla_{CTX\text{-}M}$-carrying *E. coli* in recent years in Japan and the spread of not only $bla_{CTX\text{-}M\text{-}15}$ (the same as in other countries) but also $bla_{CTX\text{-}M\text{-}27}$ (differing from other countries) among clinical isolates of *E. coli* in Japan. The spread of $bla_{CTX\text{-}M\text{-}27}$ is closely associated with ST131. In fact, a change of the dominant type of ESBL in Japan may be currently in progress. Further periodic surveillance is needed.

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Conflict of interest

The authors declare no conflict of interest.

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