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Clinical and Molecular Epidemiology of Extended-Spectrum β-lactamase-Producing *Klebsiella pneumoniae* and *Escherichia Coli* in a Japanese Tertiary Hospital

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Abstract

The increase in the incidence of extended-spectrum β-lactamase (ESBL)-producing bacteria has become a serious problem worldwide, but the distribution of ESBL-producing bacteria can vary according to geographical area or institution. The aim of this study was to analyze epidemiologic data on ESBL-producing bacteria and their genotypes in our hospital. The hospital microbiology laboratory databases were reviewed for ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* from 2006 to 2010. The ESBL-producers were also molecularly analyzed and included the CTX-M, TEM and SHV genes. In a 5-year study, there were 1359 isolates of *E. coli* and 725 isolates of *K. pneumoniae*. Incidence of ESBL-producing *E. coli* and *K. pneumoniae* increased from 5.5% and 0.5% in 2006 to 20.4% and 4.7% in 2010. One half of the ESBL-producing *E. coli* was positive for at least 2 ESBL genes. The most common genotype was TEM+CTX-M (48.8%) for *E. coli* and TEM+SHV (30.0%) for *K. pneumoniae*. The MIC_{50} of ceftriaxone and cefepime in the CTX-M or TEM/SHV+CTX-M type ESBL-producing *E. coli* were higher than those in the TEM/SHV type isolates. The increase of bacteria with multiple ESBL genes may be an emergent problem. Therefore, ESBL genotyping is needed for monitoring the important ESBLs that can lead to treatment failure and contribute to the appropriate use of antimicrobial agents and infection control.

Keywords: Extended-spectrum β-lactamase; *Escherichia coli*; *Klebsiella pneumonia*; TEM; SHV; CTX-M

Introduction

The emergence of extended-spectrum β-lactamase (ESBL)-producing bacteria, particularly *Escherichia coli* and *Klebsiella pneumoniae*, is now a critical concern for the development of therapies against bacterial infection. Since the early 1980s, the number of nosocomial infections by ESBL-producing, gram-negative bacteria has been increasing worldwide, and β-lactamase production has become a major causative factor for increasing resistance to antibiotics [1-3]. The ESBL genes are mostly plasmid encoded [4], and most ESBLs can be divided into 3 genotypes: TEM, SHV, and CTX-M [5]. The major ESBL producer was *K. pneumoniae* before 2000, and the predominant ESBL genotypes were TEM and SHV [1]. *E. coli* has now become an important ESBL carrier in Western countries. In addition, a genotype CTX-M has become more prevalent worldwide compared to the TEM and SHV genotypes [1]. During the 1990s, ESBL-producing organisms were described mainly as members of the TEM- and SHV-β-lactamase families in *E. coli* and *K. pneumoniae* causing nosocomial outbreaks [6]. In the 1990s, a novel type of ESBL, the CTX-M enzyme, emerged worldwide [6]. The CTX-M types, now exceeding 50 different types, can be divided into 5 groups based on their amino acid identities: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 [7]. It is clear that various CTX-M type ESBLs have spread worldwide and that specific CTX-M subgroups have been localized to different geographic areas [1,8,9]. Thus, in Japan, it is known that the CTX-M-8 and CTX-M-25 groups of enzymes are rarely found [10]. In Japan, since the first isolate of ESBL-producing bacterium in 1993 [11], the detection rates of ESBL-producers have been much lower than those in the rest of Asia; however, the increase in the incidence of ESBL producers remains a common issue [12,13]. Because the current trend of both bacteria in Japan is unknown and few studies with longitudinal observations of the nosocomial spread of these bacteria have been reported [14], we investigated epidemiologic data on the ESBL-producing *E. coli* and *K. pneumoniae* in our hospital and the ESBL genotypes. In addition, the antimicrobial susceptibilities of the ESBL producers were examined.

Materials and Methods

Study design

The present study was conducted at Nagasaki University Hospital located in southwestern Japan, which has 861 beds. Hospital microbiology laboratory databases from January 2006 to December 2010 were reviewed, and clinical isolates of *E. coli* and *K. pneumoniae* from specimens, except for feces, were analyzed for bacteriological and molecular epidemiology. The bacteria were identified using the Vitek-2 system (bioMerieux Japan Ltd., Tokyo, Japan) or the BD Phoenix™ Automated Microbiology System (BD Diagnostic Systems, Sparks, MD). For the isolates identified by Vitek-2, additional susceptibility testing was performed. When several strains with ESBL were detected from the same patient, only 1 sample was counted as an ESBL isolate.

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Information on the isolated strains, including etiology and susceptibility to antibiotics, was also obtained.

Antimicrobial susceptibility testing

Information on the susceptibility of the bacterial strains that had been performed according to the Clinical and Laboratory Standard Institute (CLSI) was obtained from the hospital microbiology laboratory databases [15]. The analyzed drugs were the following 11 agents: penicillin, ceftazidime, ceftriaxone, cefepime, imipenem, meropenem, aztreonam, gentamicin, minocycline, ciprofloxacin, and levofloxacin.

Detection of ESBL-producing *E. coli* and *K. pneumonia*

The isolates identified by BD Phoenix™ were analyzed with the integrated BDXpert™ System [16]. These processes were performed according to the original algorithm [17]. For the isolates identified by Vitek-2, the confirmatory testing for ESBL was performed by cephalosporin plus clavulanic acid (CA). Confirmation of ESBL isolates was based on 8-fold reduction with CA when combined with cefpodoxime, cefotaxime and ceftazidime. The proportional reduction in growth in the wells containing cephalosporin plus CA compared with those containing cephalosporin alone was considered indicative of ESBL production.

Extraction of plasmids

A few colonies were suspended in 700 μL of Tris-EDTA buffer (pH 8.0). The suspensions were boiled for 10 min and subsequently centrifuged for 5 min at 13000 rpm. The supernatant, containing DNA, was transferred to new tubes and stored at 4°C for subsequent PCR analysis.

Genotyping of ESBL

PCR was performed using 5 sets of previous published primers to amplify type-specific ESBL genes, including CTX-M-1, CTX-M-2, CTX-M-9, TEM and SHV [18]. For detecting TEM or SHV genes, initial denaturation at 95°C for 10 min, denaturation at 95°C for 1 min, primer annealing at 56°C for 1 min, and extension at 72°C for 1 min, was repeated for 40 cycles; with a final extension at 72°C for 7 min. For CTX-M gene, initial denaturation at 95°C for 10 min, denaturation at 95°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 1 min, was repeated for 40 cycles; a final extension at 72°C for 7 min was carried out. The PCR products were analyzed using 2% agarose gel electrophoresis and visualized by staining with ethidium bromide.

Results

Identification of ESBL-producing *E. coli* strains

ESBL-producing *E. coli* accounted for 160 of 1359 isolates (11.8%) and ESBL-producing *K. pneumoniae* accounted for 20 of 725 isolates (2.8%). The number of ESBL-producing *E. coli* markedly increased from 17 isolates (6.8%) in 2006 to 68 isolates (22.7%) in 2010 (Figure 1). ESBL-producing *K. pneumoniae* was not detected in 2006, but appeared after 2007 and peaked in 2009 with 9 isolates (6.4%). The majority of ESBL-producing *E. coli* were isolated from urine (59%), followed by respiratory specimens (14%), pus (12%), blood (9%) and others (6%) (Figure 2).

Molecular characterization of ESBL-producing strains

The phenotypically identified ESBL-producing *E. coli* and *K. pneumoniae* were molecularly analyzed. Of the 160 ESBL-producing *E. coli* isolates, 143 (89.4%) were positive for ESBL genes (Table 1). Fifty

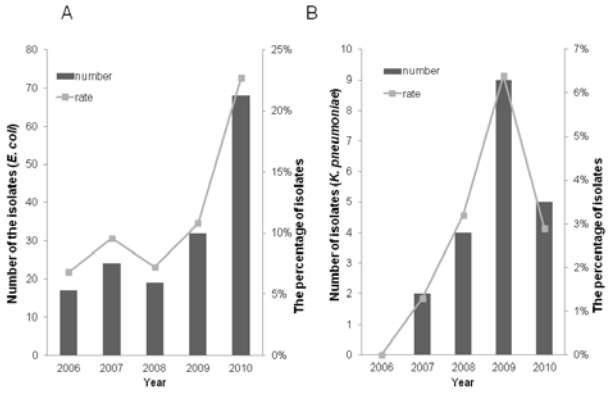

Figure 1: Frequencies of ESBL-producing organisms.
The number and the rate of ESBL-producing *E. coli* (A) and *K. pneumoniae* (B) in our hospital

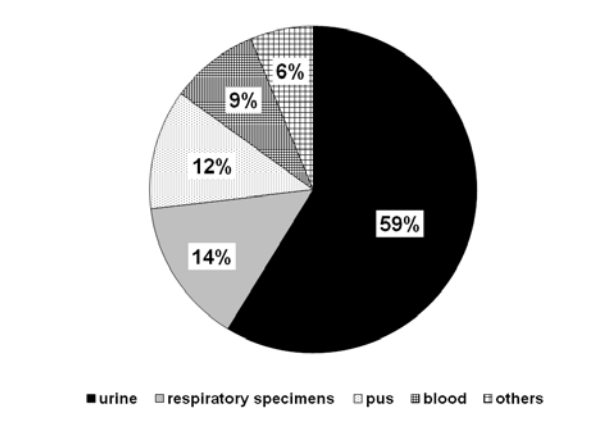

Figure 2: The origins of ESBL-producing *E. coli.*
The isolates were obtained from urine (n=94, 59%), respiratory specimens (n=23, 14%), pus (n=19, 12%), blood (n=14, 9%), and others (n=10, 6%).

percent of the isolates had at least 2 ESBL genes. The number of ESBL-producing *E. coli* with TEM, SHV, CTX-M-1, CTX-M-2 and CTX-M-9 was 13 (8.1%), 5 (3.1%), 7 (4.4%), 3 (1.9%) and 35 (21.9%), respectively (Table 1). The number of ESBL-producing *E. coli* with TEM+SHV, TEM+CTX-M-1, TEM+CTX-M-2, TEM+CTX-M-9, TEM+CTX-M-1+CTX-M-9, TEM+CTX-M-1+CTX-M-2 and SHV+CTX-M-9 was 2 (1.3%), 10 (6.3%), 3 (1.9%), 61 (38.1%), 2 (1.3%), 1 (0.6%) and 1 (0.6%), respectively. In addition, the detection number of ESBL-producing *E. coli* of the CTX-M-1 group increased from 1 to 11 from 2006 to 2010. For *K. pneumoniae*, 17 (85.0%) of the 20 isolates were positive for ESBL genes (Table 2). The number of ESBL-producing *K. pneumoniae* with TEM, SHV, TEM+SHV, SHV+CTX-M-1, SHV+CTX-M-2, SHV+CTX-M-9 and TEM+SHV+CTX-M-1 was 1 (5%), 5 (25%), 6 (30%), 1 (5%), 1 (5%), 2 (10%), and 1 (5%), respectively. ESBL-producing *K. pneumoniae* isolates carrying CTX-M alone were not detected.

Antimicrobial susceptibilities of ESBL-producing *E. coli*

We analyzed the antimicrobial susceptibilities of the ESBL-producing *E. coli* between genotypes (Table 3). The isolates were categorized according to the following genotypes: TEM/SHV (TEM and/or SHV), CTX-M (CTX-M-1, CTX-M-2, and CTX-M-9), and

	Year					
Genotype(s)	**2006**	**2007**	**2008**	**2009**	**2010**	**Total n (%)**
TEM	0	1	0	4	8	13 (8.1)
SHV	2	0	0	1	2	5 (3.1)
TEM+SHV	0	0	0	0	2	2 (1.3)
CTX-M-1	0	0	1	1	5	7 (4.4)
CTX-M-1+TEM	1	1	1	1	6	10 (6.3)
CTX-M-2	0	0	1	0	2	3 (1.9)
CTX-M-2+TEM	0	2	0	0	1	3 (1.9)
CTX-M-9	6	8	7	6	8	35 (21.9)
CTX-M-9+TEM	7	10	9	11	24	61 (38.1)
CTX-M-1+CTX-M-2+TEM	0	1	0	0	0	1 (0.6)
CTX-M-1+CTX-M-9+TEM	0	0	0	2	0	2 (1.3)
CTX-M-9+SHV	1	0	0	0	0	1 (0.6)
Undetected	0	1	0	6	10	17 (10.6)
Total	17	24	19	32	68	160

Table 1: Genotypes of ESBL-producing *E. coli* from 2006 to 2010.

	Year					
Genotype(s)	**2006**	**2007**	**2008**	**2009**	**2010**	**Total n (%)**
TEM	0	0	0	1	0	1 (5)
SHV	0	1	1	2	1	5 (25)
TEM+SHV	0	1	2	2	1	6 (30)
CTX-M-1+SHV	0	0	0	1	0	1 (5)
CTX-M-2+SHV	0	0	0	0	1	1 (5)
CTX-M-9+SHV	0	0	1	1	0	2 (10)
CTX-M-1+TEM+SHV	0	0	0	1	0	1 (5)
Undetected	0	0	0	1	2	3 (15)
Total	0	2	4	9	5	20

Table 2: Genotypes of ESBL-producing *K. pneumoniae* from 2006 to 2010.

Antibiotic	**TEM/SHV**		**CTX-M**		**TEM/SHV+CTX-M**	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Penicillin	≥ 32	≥ 32	≥ 32	≥ 32	≥ 32	≥ 32
Ceftazidime	2	≥ 32	2	≥ 32	2	16
Ceftriaxone	2	≥ 32	≥ 32	≥ 32	≥ 32	≥ 32
Cefepime	1	≥ 32	8	≥ 32	8	≥ 32
Imipenem	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤0.5
Meropenem	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5
Aztreonam	2	≥ 32	4	≥ 32	8	≥ 32
Gentamicin	2	16	1	16	4	16
Minocycline	2	8	2	16	2	16
Ciprofloxacin	8	8	8	8	8	8
Levofloxacin	8	8	8	8	8	8

Table 3: Antimicrobial susceptibilities of ESBL-producing *E. coli*.

TEM/SHV+CTX-M (TEM/SHV and CTX-M), as previously reported [8]. The value of MIC_{50} of TEM/SHV in ceftriaxone and cefepime were 2 μg/mL and 1 μg/mL, respectively. The values of MIC_{50} of CTX-M or TEM/SHV+CTX-M in ceftriaxone and cefepime were ≥ 32 μg/mL and 8 μg/mL respectively. All of the TEM/SHV- and CTX-M-producing isolates retained favorable susceptibility to carbapenems. In all genotypes, the value of MIC_{50} in fluoroquinolones was 8 μg/mL.

Discussion

In this study, we examined the current trend of ESBL-producing *E. coli* and *K. pneumoniae* in our hospital, as well as the ESBL genotypes and the antimicrobial susceptibilities. Our data, based on the clinical isolates collected over 5 years, suggested that the incidence of ESBL-producing *E. coli* and *K. pneumoniae* have increased. The prevalence of ESBL-producing bacteria has been on the rise, particularly in Asia compared to other regions. A study conducted in 2007 reported that the frequencies of ESBL-producing *K. pneumoniae* and *E. coli* isolates exceeded 30% in both bacterial populations [11]. The proportion of ESBL-producing isolates in Japan was 4.3% in *E. coli* and 3.1% in *K. pneumoniae* in 2006 [13]. Thus, the geographical distribution can vary according to countries and institutions, although the prevalence of ESBL-producing bacteria is a global problem [19]. The prevalence of ESBL-producers in our study was lower than that reported by global surveillances [20] and in other Asian countries [21,22] but it was found to increase, implying that ESBL producers could become common drug-resistant bacteria in the near future. In addition, our study suggested that urine can be an important source of ESBL-producing *E. coli* as previously reported [23]. Worldwide, the predominant genotype of ESBL-producing *E. coli* has changed from TEM and/or SHV (TEM/SHV) to CTX-M [1,24], and the detection rate of CTX-M has increased dramatically [25]. In this study, CTX-M was found most frequently in ESBL-producing *E. coli* isolates, in particular, the CTX-M-9 group whose prevalence increased during the observation period. Our results were similar to previously reported Japanese trends on the increase of CTX-M type ESBL [10,26], but different in that the increase of prevalence of CTX-M-9 was in combination with an increase in the prevalence of the TEM genotype. An ESBL-producing *E. coli* clone O25:H4-ST131 that often carries CTX-M-1-type ESBL has been spreading worldwide. Because of its resistance to fluoroquinolones and aminoglycosides, in addition to β-lactams, the increase of *E. coli* clone O25:H4-ST131 has become a serious problem [23]. We did not investigate any specific clones including *E. coli* clone O25:H4-ST131 in this study; however, the mild increase of CTX-M-1-type ESBL may imply the spreading of O25:H4-ST131. In fact, this clone has previously been found in Japan [26]; therefore, further examination will be required. In the present study, about one-half of the ESBL-producing *E. coli* isolates were molecularly confirmed to have 2 or more ESBL genes. The incidence of TEM+CTX-M-9 has increased remarkably in these last 2 years. These findings suggest that *E. coli* carrying multiple ESBL genes may be increasing. In contrast, those isolates from which these specific genes were not detected likely produce other types of enzymes that have yet to be investigated. Alternatively, some identification errors may be involved in our results because a high false-positivity rate of Phoenix identification system had been reported [27]. The most common ESBL genotype among the *K. pneumoniae* isolates was TEM+SHV, as opposed to a previous report from Japan [8] which showed that SHV+CTX-M was the most common. Because of the limited number of isolates in our hospital, it is difficult to place TEM+SHV as the predominant genotype of ESBL-producing *K. pneumoniae*. The potential for hydrolysis of β-lactams can vary according to the type of ESBL enzymes. CTX-M β-lactamase can elevate the MIC values of ceftriaxone, cefepime, and ceftazidime [28,29]. In the present study, the MIC_{50} of ceftriaxone and cefepime in *E. coli* with CTX-M or TEM/SHV+CTX-M was higher than in those *E. coli* with TEM/SHV. Among the *E. coli* with CTX-M, the MIC_{90} and MIC_{50} of ceftazidime in *E. coli* with TEM+CTX-M-9, the predominant ESBL in this study, were lower than other *E. coli* with TEM/SHV+CTX-M (data not shown). CTX-M ESBLs can be also resistant to fluoroquinolones [30], but there were no significant differences in the MICs of fluoroquinolones between ESBL genotypes. Thus, ESBLs showed different hydrolysis potentials and some plasmids can possess other drug-resistant genes in addition to a β-lactamase gene. Therefore, ESBL genotyping can help in monitoring the important ESBLs that can lead to treatment failure and contribute to the appropriate use of antimicrobial agents and the infection control. In conclusion, ESBL-producing *E. coli* and *K. pneumoniae* were less frequent compared to

the global trend but were found to be increasing in a tertiary hospital. Two or more ESBLs were detected in many isolates by molecular analysis, and MIC of some cephalosporin's was elevated in the isolates with CTX-M-type ESBL. Considering regional variations and some easy-expanding clones, constant and careful surveillance is needed.

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