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

Yosuke Harada, Yoshitomo Morinaga, Koichi Yamada, Yohei Migiyama, Kentaro Nagaoka, Naoki Uno, Shigeki Nakamura, Yoshifumi Imamura, Taiga Miyazaki, Hiroo Hasegawa, Koichi Izumikawa, Hiroshi Kakeya, Katsunori Yanagihara and Shigeru Kohno

*1 Department of Laboratory Medicine, Japan*  
*2 Second Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan*  
*3 Global COE Program, Nagasaki University, Nagasaki, Japan*

**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-producing isolates 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\_\_p of ceftriaxone and ceftazime 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 pneumoniae*; 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.

*Corresponding author: Yoshitomo Morinaga, MD, PhD, Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan, Tel: +81-95-819-7574; Fax: +81-95-819-7422; E-mail: y-morina@nagasaki-u.ac.jp*

Received June 28, 2013; Accepted September 03, 2013; Published September 06, 2013


Copyright: © 2013 Harada Y, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
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, cefazidime, ceftriaxone, ampicillin, 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 cefalosporins plus clavulanic acid (CA). Confirmation of ESBL isolates was based on 8-fold reduction with CA when combined with cefpodoxime, cefotaxime and cefazidime. The proportionate reduction in growth in the wells containing cefalosporin plus CA compared with those containing cefalosporin 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 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-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
all genotypes, the value of MIC50 in fluoroquinolones was 8 µg/mL. Producing isolates retained favorable susceptibility to carbapenems. In our study, the MIC50 of CTX-M were 8 µg/mL and 8 µg/mL respectively. All of the TEM/SHV- and TEM/SHV+CTX-M in ceftriaxone and cefepime were ≥ 32 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 increased number of ESBL ESBL-producing isolates 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 cefotaxime [28,29]. In the present study, the MIC50 of ceftriaxone and cefepime in *E. coli* with CTX-M or TEM/SHV+CTX-M was higher than those in TEM/SHV+CTX-M. Among the *E. coli* with CTX-M, the predominant ESBL in this study, were lower than other *E. coli* with TEM/SHV+CTX-M (data not shown). CTX-M ESBLs can also be 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

<table>
<thead>
<tr>
<th>Genotype(s)</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3 (15)</td>
</tr>
<tr>
<td>SHV</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>5 (25)</td>
</tr>
<tr>
<td>TEM+SHV</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>7 (35)</td>
</tr>
<tr>
<td>CTX-M-1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>5 (25)</td>
</tr>
<tr>
<td>CTX-M-2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2 (10)</td>
</tr>
<tr>
<td>CTX-M-9</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>5 (25)</td>
</tr>
<tr>
<td>TEM+CTX-M</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>9</td>
<td>5</td>
<td>20 (100)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>TEM/SHV</th>
<th>CTX-M</th>
<th>TEM/SHV+CTX-M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC50</td>
<td>MIC90</td>
<td>MIC50</td>
</tr>
<tr>
<td>Penicillin</td>
<td>≥ 0.5</td>
<td>≥ 0.5</td>
<td>≥ 0.5</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>≥ 2</td>
<td>≥ 2</td>
<td>≥ 2</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>≥ 2</td>
<td>≥ 2</td>
<td>≥ 2</td>
</tr>
<tr>
<td>Cefepine</td>
<td>≥ 2</td>
<td>≥ 2</td>
<td>≥ 2</td>
</tr>
<tr>
<td>Imipenem</td>
<td>≤ 0.5</td>
<td>≤ 0.5</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>Meropenem</td>
<td>≤ 0.5</td>
<td>≤ 0.5</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>≥ 2</td>
<td>≥ 2</td>
<td>≥ 2</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Minocycline</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 2: Antimicrobial susceptibilities of ESBL-producing *E. coli*.
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 cephalosporins was elevated in the isolates with CTX-M-type ESBL. Considering regional variations and some easy-expanding clones, constant and careful surveillance is needed.

References


Submit your next manuscript and get advantages of OMICS Group submissions

Unique features:
• User friendly/feasible website-translation of your paper to 50 world’s leading languages
• Audio Version of published paper
• Digital articles to share and explore

Special features:
• 250 Open Access Journals
• 20,000 editorial team
• 21 days rapid review process
• Quality and quick editorial, review and publication processing
• Indexing at PubMed (portfolio), Scopus, B&CCO, Index Copernicus and Google Scholar etc
• Sharing Option, Social Networking Enabled
• Authors, Reviewers and Editors rewarded with online Scientific Credits
• Better discount for your subsequent articles

Submit your manuscript at: http://www.omicsonline.org/submission