Microbiology and Infectious Disease / Epidemiology of ESBLs in the Kinki Region of Japan

Epidemiology of Escherichia coli, Klebsiella Species, and Proteus mirabilis Strains Producing Extended-Spectrum β-Lactamases From Clinical Samples in the Kinki Region of Japan

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Key Words: Extended-spectrum β-lactamases; Polymerase chain reaction; Surveillance; Replicon type

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Abstract

In the present study, nonduplicate, clinical isolates of extended-spectrum β-lactamase (ESBL)-producing Escherichia coli, Klebsiella spp, and Proteus mirabilis were collected during a 10-year period from 2000 to 2009 at several hospitals in the Kinki region, Japan. The detection rate of E coli markedly increased from 0.24% to 7.25%. The detection rate of Klebsiella pneumoniae increased from 0% to 2.44% and that of P mirabilis from 6.97% to 12.85%. The most frequently detected genotypes were the CTX-M9 group for E coli, the CTX-M2 group for K pneumoniae, and the CTX-M2 group for P mirabilis. E coli clone O25:H4-ST131 producing CTX-M-15, which is spreading worldwide, was first detected in 2007. The most common replicon type of E coli was the IncF type, particularly FIB, detected in 466 strains (69.7%). Of the K pneumoniae strains, 47 (55.3%) were of the IncN type; 77 P mirabilis strains (96.3%) were of the IncT type. In the future, the surveillance of various resistant bacteria, mainly ESBL-producing Enterobacteriaceae, should be expanded to prevent their spread.

Extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae have been increasingly reported worldwide since their first description in 1983.1 Moreover, they have emerged worldwide as a significant cause of community and health care–associated infections.2 ESBLs are the major cause of resistance to oxyimino-cephalosporins in Enterobacteriaceae. ESBLs are mostly plasmid-mediated bacterial enzymes that can hydrolyze a wide variety of penicillins and cephalosporins.

Most ESBLs have evolved by genetic mutation from native β-lactamases, particularly TEM-1, TEM-2, and SHV-1. These parent enzymes are commonly found in gram-negative bacteria, particularly in Enterobacteriaceae.3 Until the 2000s, most of the ESBLs were structurally related to the narrow-spectrum TEM- and SHV-type β-lactamases, with one to several amino acid substitutions surrounding their active site. During the 1990s, they were described mainly as members of the TEM- and SHV-β-lactamase families in Escherichia coli and Klebsiella pneumoniae causing nosocomial outbreaks.3,4 Furthermore, in the late 1990s, a novel type of ESBL, the CTX-M enzymes, emerged worldwide, mostly from E coli.3,4 ESBL-producing E coli of the Toho-1-type were reported first in Japan in 1988.5 Nowadays, they are mostly found in E coli that cause community-acquired infections and, with increasing frequency, contain CTX-M enzymes. Moreover, E coli producing a CTX-M-type ESBL is an emerging cause of community-acquired urinary tract infection in young women in the United States,6 Europe,7 Hong Kong,8 and elsewhere. Increased community-acquired infection by ESBL-producing bacteria is complicating the selection of therapeutic drugs.

More than 50 CTX-M enzymes reported thus far can be grouped into 5 main subgroups according to the similarity of their amino acid sequence (CTX-M-1, CTX-M-2, CTX-M-8,
CTX-M-9, and CTX-M-25). In particular, E coli clone O25:H4-ST131 producing CTX-M-15, which is resistant to many antibacterial agents, is spreading worldwide and causing serious problems. In the present study, nonduplicate clinical isolates of ESBL-positive E coli, Klebsiella spp, and Proteus mirabilis were collected during a 10-year period from 1999 to 2009 at several hospitals in the Kinki region, Japan. Our study examined the prevalence and type of β-lactamase genes and plasmid replicon type among the isolates. Moreover, susceptibility to oral antimicrobial agents were determined.

### Materials and Methods

#### Bacterial Isolates

This laboratory surveillance was conducted with the cooperation of 18 institutions (17 clinical laboratories of various hospitals and 1 commercial laboratory) in the Kinki region, which is located in midwestern Japan. Specimens were collected from 2000 to 2009. A total of 40,522 isolates of gram-negative bacilli including E coli, K pneumoniae, and P mirabilis were collected from 2000 to 2009. P mirabilis isolates, K pneumoniae isolates, and Proteus mirabilis were investigated by the double-disk synergy (DDS) test screening isolates. Strains that met the cefpodoxime MIC criterion of more than 2 μg/mL were determined by the broth diffusion method using CLSI criteria for broth dilution. Throughout this study, the results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The quality control strains used in this study were E coli ATCC 25922 and E coli ATCC 621.

#### Screening for ESBL

The cefpodoxime minimum inhibitory concentration (MIC) criterion of more than 2 μg/mL was used to initially screen isolates. Strains that met the cefpodoxime MIC criterion were investigated by the double-disk synergy (DDS) test with amoxicillin–clavulanic acid (20 μg per disk/10 μg per disk), cefotaxime (30 μg per disk), according to methods published previously. DDDS-positive strains were subjected to polymerase chain reaction (PCR) analyses for the detection of ESBL genes.

#### PCR Amplification for the Detection of ESBL Genes

All DDS-positive strains were screened for the resistance genes SHV, TEM, and CTX-M by using a single PCR assay. Genetic detection and genotyping of TEM, SHV, and CTX-M were performed by using PCR with bacterial DNA, which was extracted from the isolates by boiling the bacterial suspensions. A solution with an extracted DNA concentration of 0.1 ng/mL was used as the template for PCR analysis. In the case of genotyping of CTX-M genes, 4 primer sets that amplify group-specific CTX-M genes were used, as described previously: the CTX-M1 group includes CTX-M-1, CTX-M-3, CTX-M-10 to CTX-M-12, CTX-M-15, CTX-M-22, CTX-M-23, and CTX-M-28 to CTX-M-30; the CTX-M2 group, CTX-M-2, CTX-M-4 to CTX-M-7, CTX-M-20, and Toho-1; the CTX-M8 group, CTX-M-8; and the CTX-M9 group, CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16 to CTX-M-19, CTX-M-21, CTX-M-27, and Toho-2. The PCR products were analyzed by using 2% agarose gel electrophoresis and visualized by staining with ethidium bromide.

#### Detection of CTX-M15 O25:H4-ST131

The serotyping of the CTX-M1 group was carried out by using E coli O and H antisera purchased from Denka Seiken (Tokyo, Japan), according to the manufacturer’s instructions. The complete nucleotide sequences of CTX-M1 group O25:H4 E coli genes were determined on both strands by direct sequencing of the PCR products. Multilocus sequence typing (MLST) was performed on 17 strains of CTX-M15 O25:H4 E coli by following the recommended procedure at the E coli MLST Web site (http://mlst.ucc.ie/dbs/Ecoli).

#### Plasmid Replicon Type Determination

PCR-based replicon typing was performed on 837 strains as described by Carattoli et al. Eighteen primer pairs targeting the FIA, FIB, FIC, HI1, HI2, I1-Ic, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FII replicons were used in separate PCR reactions.

#### Susceptibility to Oral Antimicrobial Agents

The susceptibilities to oral antimicrobial agents, amoxicillin-clavulanic acid (AMC), minocycline, levofloxacin, fosfomycin, colistin, and trimethoprim-sulfamethoxazole (SXT), were determined by the broth diffusion method on Mueller-Hinton agar (Eiken Chemical, Tokyo, Japan), according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The quality control strains used in this study were E coli ATCC 25922 and E coli ATCC 35218. Throughout this study, the results were interpreted using CLSI criteria for broth dilution.

#### Results

**ESBL Detection**

Between 2000 and 2009, a total of 40,522 strains, including 25,320 E coli, 11,582 K pneumoniae, 2,933 K oxytoca, and 1,187 P mirabilis strains, were analyzed. ESBL isolation rates are shown in Figure 1 and Table 1. The detection of ESBL-producing E coli markedly increased...
from 2 strains (0.24%) in 2000 to 224 strains (7.25%) in 2009. The detection of ESBL-producing \textit{K pneumoniae} increased from 0 (0.00%) to 30 strains (2.44%) and that of ESBL-producing \textit{K oxytoca} increased from 0 (0.00%) to 3 strains (1.18%). The detection of ESBL-producing \textit{P mirabilis} increased from 14 (6.97%) to 23 strains (12.85%) in a survey conducted since 2004. In the whole of the Kinki region, the detection rate increased from 0.42% (between 2000 and 2004) to 3.4% (between 2005 and 2009).

**Molecular Detection of ESBL**

The changes in the genotypes detected during the survey period are shown in **Figure 2** for \textit{E coli} and in **Figure 3** for \textit{K pneumoniae}. The most frequently detected genotypes in the 10 years were the CTX-M9 group for \textit{E coli} (374 strains [55.9%]), the CTX-M2 group for \textit{K pneumoniae} (38 strains [44.7%]), and the CTX-M2 group for \textit{P mirabilis} (79 strains [98.8%]). The detection number of \textit{E coli} of the CTX-M9 group increased from 25 strains to 144 strains from 2005. In addition, the detection number of \textit{E coli} of the CTX-M9 group increased from 11 strains to 73 strains from 2005. The detection number of \textit{K pneumoniae} of the CTX-M9 group increased from 2006. The detection rates

<table>
<thead>
<tr>
<th>Organism</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Escherichia coli}</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Collected strains</td>
<td>820</td>
<td>1,297</td>
<td>3,013</td>
<td>3,095</td>
<td>2,813</td>
<td>2,429</td>
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<td>2,806</td>
<td>2,897</td>
<td>3,088</td>
<td>25,320</td>
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<td>ESBL</td>
<td>2 (0.24)</td>
<td>7 (0.54)</td>
<td>15 (0.50)</td>
<td>12 (0.39)</td>
<td>15 (0.53)</td>
<td>62 (2.55)</td>
<td>89 (2.91)</td>
<td>101 (3.60)</td>
<td>142 (4.90)</td>
<td>224 (7.25)</td>
<td>669 (2.64)</td>
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<tr>
<td>Collected strains</td>
<td>542</td>
<td>906</td>
<td>1,487</td>
<td>1,468</td>
<td>1,144</td>
<td>1,146</td>
<td>718</td>
<td>1,205</td>
<td>1,237</td>
<td>1,229</td>
<td>11,082</td>
</tr>
<tr>
<td>ESBL</td>
<td>0 (0.00)</td>
<td>1 (0.11)</td>
<td>7 (0.47)</td>
<td>4 (0.27)</td>
<td>0 (0.00)</td>
<td>10 (0.87)</td>
<td>5 (0.70)</td>
<td>8 (0.66)</td>
<td>30 (2.44)</td>
<td>85 (0.77)</td>
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<tr>
<td>\textit{Klebsiella oxytoca}</td>
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<td></td>
</tr>
<tr>
<td>Collected strains</td>
<td>154</td>
<td>228</td>
<td>400</td>
<td>366</td>
<td>383</td>
<td>319</td>
<td>201</td>
<td>341</td>
<td>297</td>
<td>254</td>
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<td>ESBL</td>
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<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
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<td>0 (0.00)</td>
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<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>3 (0.10)</td>
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<td>\textit{Proteus mirabilis}</td>
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</tr>
<tr>
<td>Collected strains</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>201</td>
<td>214</td>
<td>118</td>
<td>251</td>
<td>224</td>
<td>179</td>
</tr>
<tr>
<td>ESBL</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>14 (6.97)</td>
<td>6 (2.80)</td>
<td>3 (2.54)</td>
<td>10 (3.98)</td>
<td>24 (10.71)</td>
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<tr>
<td>Total</td>
<td>1,516</td>
<td>2,431</td>
<td>4,900</td>
<td>4,919</td>
<td>4,541</td>
<td>4,108</td>
<td>4,099</td>
<td>4,603</td>
<td>4,655</td>
<td>4,750</td>
<td>40,522</td>
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<table>
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<tr>
<th>Organism</th>
<th>2000</th>
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<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{E coli}</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collected strains</td>
<td>2 (0.13)</td>
<td>8 (0.33)</td>
<td>22 (0.45)</td>
<td>16 (0.33)</td>
<td>29 (0.64)</td>
<td>78 (1.90)</td>
<td>97 (2.37)</td>
<td>119 (2.59)</td>
<td>186 (4.00)</td>
<td>280 (5.89)</td>
<td>837 (2.07)</td>
</tr>
</tbody>
</table>

ESBL, extended-spectrum β-lactamase; ND, not done.

* Data are given as number or number (percentage).
Prevalence of Plasmid Replicons

The most common replicon type of *E coli* was the IncF type, particularly FIB, detected in 466 strains (69.7%), followed by FIA in 336 strains (50.2%), II-1 in 83 strains (12.4%), and N in 65 strains (9.7%) Table 2. Of the *K pneumoniae* strains, 47 (55.3%) were of the N type. Most of the bacteria carried a single plasmid, but some bacteria carried multiple plasmid types. The average number of plasmids carried by the bacteria was from 1 to 1.05. Of the *P mirabilis* strains, 77 (96.3%) were of the IncT type.

Susceptibility for Oral Drug

Oral drug susceptibility rates Table 3 for *E coli* were 96.0% for colistin, 93.7% for fosfomycin, 62.6% for minocycline, 47.2% for AMC, 44.3% for SXT, and 32.2% for levofloxacin. The levofloxacin susceptibility rate was the highest for the CTX-M2 group genotype. The drug susceptibility rates for *K pneumoniae* were 86.9% for levofloxacin, 86.9% for colistin, 83.3% for fosfomycin, 51.2% for AMC, 46.4% for SXT, and 21.4% for minocycline. The SXT susceptibility rate was the lowest (0%) for the TEM/SHV group genotype. Drug susceptibility rates for *P mirabilis* were 100% for AMC, 64.6% for SXT, 50.6% for fosfomycin, 15.2% for levofloxacin, 0.0% for colistin, and 0.0% for minocycline. The AMC susceptibility rate was 100%.

Discussion

In this study, long-term surveillance between 2000 and 2009 demonstrated that ESBL-producing *E coli* increased about 30 times from 0.24% to 7.25% in 10 years. According
to Fang et al.,22 ESBL-producing *E. coli* increased by about 10 times between 2001 and 2006 in Sweden, a result comparable with that from a different survey conducted in the same period. Recently, many reports have been made on intestinal bacteria that acquired genes such as *KPC*23 and *NDM-1*.24 Until now, ESBL-producing *E. coli* have rarely been detected in Japan. This study may be useful for the prediction of resistant bacteria in the future.

Our study revealed the changes in the ESBL genotypes of ESBL-producing *E. coli*, *Klebsiella* spp, and *P. mirabilis* in the Kinki region of Japan. Shibata et al.25 investigated ESBL-producing intestinal bacteria of the CTX-M group in Japan. They reported that 89 of 168 *E. coli* strains belonged to the CTX-M9 group. In particular, the CTX-M9 group, frequently detected in *E. coli*, is presumably the most common genotype in Japan. Similarly, in this study, 374 of 669 *E. coli* strains were of the CTX-M9 group. In particular, contrary to the decreased CTX-M2 group, the CTX-M9 group has increased since 2007. The CTX-M2 group is frequently detected in food products such as meat.26 It was thought that usage restrictions of the antimicrobial agent to domestic animals had influenced a decrease of CTX-M-2. On the other hand, the CTX-M1 group has slightly increased. In Japan, the trend of the *E. coli* clone O25:H4-ST131 producing CTX-M-15, which causes problems worldwide, is unknown. Hawkey10 reported trends of increase in Asia. In addition, detection in India and Pakistan were reported. The problem is that this strain is detected in hospital- and community-acquired urinary tract infections and develops multidrug resistance. This study demonstrated that the *E. coli* clone O25:H4-ST131 producing CTX-M-15 was detected in 2007 or later, suggesting the continuing existence of this strain in Japan. At present, community-acquired infection with this strain is uncommon. However, attention should be given to future trends.

The study of plasmid replichon types provides information about the spread and risks of ESBL-producing bacteria. The genes responsible for CTX-M β-lactamases are encoded by plasmids belonging to the narrow host-range incompatibility types (ie, IncFI, IncFII, IncHI2, and IncI) or the broad host-range incompatibility types (ie, IncN, IncP-1-a, IncL/M, and IncA/C).27 In this study, the IncF group predominated among the *E. coli* strains, regardless of their genotypes. Many strains acquired multiple plasmids. Similar results have been obtained in other studies.28 About 10% of the strains were of the I1-1- and N-types, suggesting the existence of various *E. coli* clones. The spread of community-acquired infection indicates the potential spread of these various clones in different forms. On the other hand, the N-type predominated among *K. pneumoniae* strains and the T-type among the *P. mirabilis* strains, suggesting the spread of a single clone or plasmid. Many reports have been published particularly on these 2 strains in hospital-acquired infection.29-31 In addition, in this study, both species were occasionally detected in the same facility and ward, suggesting an epidemiology different from that of *E. coli*. Bacterial properties vary with species. Thus, measurements should be done carefully, according to the species.

Recently, the spread of community-acquired infection by ESBL-producing strains of the CTX-M type is causing problems. Reportedly, more ESBL-producing strains have been detected in females with urinary tract infection.6-9 The spread of community-acquired infection complicates the selection of antibacterial agents for outpatient care. Few oral antibacterial agents effective against ESBL-producing bacteria are available. According to recent reports, quinolone resistance is regarded as a serious problem.32 In addition, in this study, quinolone-resistance rates were high among the *E. coli* strains: about 70% of the strains were resistant. In particular, the resistance rate of the CTX-M1 group was the highest (about 85%). Thus, quinolones cannot be used for the treatment of those infectious diseases. The susceptibility of *E. coli* to fosfomycin is being maintained. The reason for it will be that 3 g/d is recommended, as described in the Sanford guidelines.33 On the other hand, 80% of the *K. pneumoniae* strains are susceptible

**Table 3** Susceptibility of Oral Antibiotics in Each ESBL

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th><em>Escherichia coli</em></th>
<th><em>Klebsiella pneumoniae</em></th>
<th><em>Proteus mirabilis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTX-M1</td>
<td>CTX-M2</td>
<td>CTX-M9</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>75</td>
<td>89.5</td>
<td>64.3</td>
</tr>
<tr>
<td>Minocycline</td>
<td>31.3</td>
<td>18.4</td>
<td>0</td>
</tr>
<tr>
<td>SXT</td>
<td>68.8</td>
<td>42.1</td>
<td>64.3</td>
</tr>
<tr>
<td>Fusfomycin</td>
<td>56.3</td>
<td>89.5</td>
<td>100</td>
</tr>
<tr>
<td>AMC</td>
<td>43.8</td>
<td>57.9</td>
<td>35.7</td>
</tr>
<tr>
<td>Colistin</td>
<td>87.5</td>
<td>88.6</td>
<td>64.3</td>
</tr>
</tbody>
</table>

AMC, amoxicillin–clavulanic acid; ESBL, extended-spectrum β-lactamase; SXT, trimethoprim-sulfamethoxazole.
to quinolones. Thus, quinolones should be effective for *K. pneumoniae*. Antibacterial susceptibility varies with strains and genotypes. Thus, antibacterial agents for areas should be selected on the basis of the results obtained by studies on the epidemiologic backgrounds of those areas.

The detection rate of the ESBL-producing *E. coli* in our institutions was low, in comparison with that in Western countries and other areas in Asia. One of the reasons for this may be the difference in the type of antibiotics used in these countries: carbapenems and oxacephems, in particular, have often been used in Japan. Because ESBL-producing bacteria are susceptible to these drugs, they might have suppressed the diffusion of these bacteria. However, recently, reduced use of these drugs is recommended in clinical settings to avoid the overall resistance to these antibiotics. This may lead to an increase of the ESBL-producing bacteria in the near future in Japan.

This report described the epidemiologic trends of ESBL-producing *E. coli*, *Klebsiella* spp, and *P. mirabilis* in Japan. Various causative genes are known for β-lactam–resistant intestinal bacteria. Many reports have been published on serious resistant bacteria (eg, KPC, NDM-1, CTX-M-15) worldwide. In the future, the surveillance of various resistant bacteria, mainly ESBL-producing bacteria, should be expanded to prevent their spread.

**References**


