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Title: Emergence and spread of B2-ST131-O25b, B2-ST131-O16, and D-ST405 clonal groups among extended-spectrum β-lactamase-producing Escherichia coli in Japan

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Running Title: ST131-O25b and -O16 ESBL E. coli clonal groups

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Synopsis

Objectives: The increasing prevalence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* has been associated with the emergence of the CTX-M-producing sequence type 131 (ST131) pandemic clonal group, a member of the O25b serogroup and the B2 phylogenetic group. To assess the clonal spread of ESBL-producing *E. coli* in Japan, a regional surveillance program was conducted.

Methods: A total of 581 ESBL-producing clinical specimen *E. coli* isolates were collected between 2001 and 2010. Clonal groups, including ST131, D-ST405, D-ST393, and D-ST69, were determined using the PCR O-type, phylogenetic grouping by triplex PCR, allele-specific PCR, and multilocus sequence typing (MLST). A subset of clonal groups underwent PFGE.

Results: Among clonal strains, 215 isolates (37%) were identified as belonging to the ST131 group, 185 as B2-ST131-O25b (32%), 26 as B2-ST131-O16 (4%), three as B1-ST131-O25b, and one as B2-ST131-O-non-typeable. Forty-one isolates (7%) were identified as D-ST405 clonal group, seven (1%) as D-ST69, and two (0.3%) as D-ST393. B2-ST131-O16 clonal group was characterised by CTX-M-14 and a significantly lower ciprofloxacin-resistant rate than B2-ST131-O25b clonal group. The B2-ST131-O16 and B2-ST131-O25b clonal groups each made up a single PFGE cluster, with 65% similarity. The rate of ESBL-producing *E. coli* increased over the years (0.2% in 2001 to 9.7% in 2010) and corresponded to increases in the numbers of B2-ST131-O25b, B2-ST131-O16, and D-ST405 clonal groups.

Conclusions: B2-ST131-O25b, B2-ST131-O16, and D-ST405 clonal groups have contributed to the spread of ESBL-producing *E. coli* in Japan.
Introduction

In recent years, the prevalence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* has increased dramatically worldwide. Clonal group detection by multilocus sequence typing (MLST) has suggested the reason for this pandemic. The emergence of an international pandemic clonal group, CTX-M-type ESBL-producing *E. coli* with sequence type 131 (ST131) belonging to the O25b serogroup and the B2 phylogenetic group, has contributed greatly to the pandemic. The success of the ST131 clonal group is explained by its acquisition of fluoroquinolone resistance and additional virulence factors. In addition to the ST131 clonal group, a CTX-M-15-producing ST405 clonal group belonging to phylogenetic group D has been detected worldwide. However, the prevalence and evolution of this clonal group has not been well investigated. Detailed studies on the ST131 clonal group among ESBL-producing *E. coli* in Japan are lacking.

Other clonal groups disseminated worldwide include D-ST393-O15 and D-ST69. The D-ST393-O15 clonal group is characterised by K52:H1 serotypes and fluoroquinolone resistance. CTX-M-14 producers have also been identified. The D-ST69 clonal group is known as “clonal group A” and is frequently found among trimethoprim-sulfamethoxazole-resistant uropathogenic *E. coli*. A study conducted in 2009 in Spain indicated that these two clonal groups and the ST131 clonal group accounted for 38% of fluoroquinolone-resistant *E. coli* isolates and 32% of trimethoprim-sulfamethoxazole-resistant isolates.

In this study, we analysed the genetic relatedness of ESBL-producing *E. coli* isolates in 2010 in the Kyoto and Shiga regions of Japan using random amplified polymorphic DNA (RAPD) fingerprinting and found that the B2-ST131-O16 clonal group was closely related to the B2-ST131-O25b clonal group. Then, we investigate the contribution and characteristics of the clonal groups, including ST131, ST405, ST393, and ST69. We further investigated ST131 variants that were non-B2 or non-O25b isolates, which mostly consisted of B2-ST131-O16 isolates.
Materials and methods

Bacterial isolates. This study was conducted at seven acute care hospitals in the Kyoto and Shiga regions of Japan. Between April 2001 and December 2010, 12,607 non-duplicate E. coli isolates were obtained from inpatients and outpatients. Of those, 643 isolates that tested positive in an ESBL confirmation test were sent to a reference laboratory (Kyoto University) and were further investigated. The collection was conducted every year, and the period of collection was different depending on the year. Isolates were collected and saved anonymously, without accompanying demographic data.

Identification and susceptibility testing. At each hospital, microbiological identification and susceptibility testing were performed using the Vitek2 system (bioMérieux, Marcy l’Etoile, France) or the MicroScan system (Siemens Healthcare diagnostics, Tokyo, Japan). Subsequently, the ESBL screening test was performed according to the CLSI microdilution methodology (cefotaxime, ceftriaxone, ceftazidime, cefpodoxime, and aztreonam), and the ESBL confirmation test was performed using the double-disk synergy test following the CLSI guidelines. In a reference laboratory, the antibiotic susceptibility was re-evaluated by microdilution using Dry Plate Eiken (Eiken, Tokyo, Japan) and included testing with ampicillin-sulbactam, piperacillin-tazobactam, ciprofloxacin, gentamicin, tobramycin, amikacin, imipenem, meropenem, minocycline, and trimethoprim-sulfamethoxazole. The results were interpreted using the 2012 CLSI breakpoints. Intermediate susceptibility to each antibiotic was considered to be resistance.

β-lactamase identification. The presence of ESBL or plasmid-mediated AmpC β-lactamase (pAmpC) genes was detected by PCR amplification and sequencing of the CTX-M, TEM, SHV, OXA-1 genes, and the 6 main groups of pAmpC-type genes as described previously. The isolates that were resistant to imipenem or meropenem (MIC > 1 mg/L) were analysed to determine the
presence of the carbapenemases GES, OXA-48-like, IMP, VIM, KPC, and NDM.\textsuperscript{12, 13}

**Detection of clonal groups.** ESBL-producing isolates were analysed to determine their phylogenetic groups (A, B1, B2, and D) using the triplex PCR technique of Clermont et al.\textsuperscript{14} In addition, they were analysed to determine their PCR O type using PCR amplification of rfb variants (O1, O2, O4, O6, O7, O12, O15, O16, O18, O25a, O75, O157, and O25b).\textsuperscript{15, 16}

For the detection of the B2-ST131-O25b clonal group, the B2-ST131-O25b pabB allele-specific PCR (Clermont-pabB PCR) was performed as described by Clermont et al.\textsuperscript{17} The Clermont-pabB PCR targets a different region of the pabB gene from that used in the Pasteur MLST scheme. Isolates that belonged to phylogenetic group B2 and were positive for the O25b rfb allele and the Clermont-pabB PCR were classified into the B2-ST131-O25b clonal group. Twelve selected B2-ST131-O25b isolates identified by these presumptive methods were confirmed by MLST.

Isolates that were positive for Clermont-pabB PCR but non-B2 or non-O25b were also subjected to MLST.

For the detection of the B2-ST131-O16 clonal group, all of the phylogenetic B2 and O16 rfb-positive isolates were subjected to MLST. Isolates displaying a single-locus variant (SLV) of ST131 were also included in the ST131 clonal group.

For the detection of the ST405 clonal group, adk35 allele-specific PCR and sequencing of the mdh allele were performed. Isolates that belonged to phylogenetic group D and were positive for the adk35 allele and mdh4 allele were classified as D-ST405 clonal group. Eight selected D-ST405 isolates were confirmed by MLST. The primers adk35f (5’-TGGCAAACTGCGTC ACT-3’) and adk35r (5’-CGTTGACGCTATCGTC-3’) were designed for the detection of ST405-associated single-nucleotide polymorphisms in adk35 (i.e., C148T, T316C, T322C, and A331C). Amplification was performed with 1X PCR buffer, 2 mM MgCl\textsubscript{2}, 0.2 mM deoxynucleoside triphosphates, 0.4 μM of each primer, 1 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), and 1 μL boiled cell lysate in a total volume of 20 μL. The cycling protocol was as follows: 95°C for 5 min,
then 30 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 20 s, and then a final extension at 72°C for 5 min.

For the detection of the D-ST393-O15 clonal group, isolates that were positive for the O15 rfb allele were sequenced to determine the fumC allele profile. All phylogenetic group D and fumC106-positive isolates were confirmed as ST393 clonal group by MLST.

For the detection of the D-ST69 clonal group, fumC35 allele-specific PCR was performed as described previously. Phylogenetic group D and fumC35 PCR-positive isolates were sequenced to determine the fumC and gyrB allele profiles. The fumC35 and gyrB27 isolates were considered to comprise the D-ST69 clonal group. One randomly selected D-ST69 isolate was confirmed by MLST.

MLST. MLST was performed according to the Achtman scheme (http://mlst.ucc.ie/mlst/dbs/Ecoli) using seven housekeeping genes (adk, fumC, gyrB, icd, mdh, purA, and recA). The ST131 isolates determined by the Achtman scheme were further characterised by the Pasteur MLST scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html) using eight housekeeping genes (dinB, icdA, pabB, polB, putP, trpA, trpB, and uidA).

PCR genomic fingerprinting and PFGE. RAPD fingerprinting using a DAF4 primer was performed for the ESBL-producing isolates obtained in 2010 to analyse the genetic relatedness of the B2-ST131-O25b clonal group. Genomic DNA from all of the isolates subjected to the Achtman MLST underwent XbaI PFGE. The profiles obtained from RAPD or PFGE were analysed with GelCompar II, version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was applied using the unweighted pair-group method based on Dice coefficients to quantify the similarities.

Phylogenetic grouping of B1-ST131-O25b isolates.

To confirm the B1 phylogenetic grouping of the B1-ST131-O25b isolates, these isolates were tested for the chuA gene amplification using primer pairs only for chuA in triplicate because a failure to detect the chuA gene would classify an isolate into B1 phylogenetic group. In addition, other
primer pairs\textsuperscript{22} for the \textit{chuA} gene were used. A phylogenetic tree was built using neighbour-joining method in the CLUSTAL X program for the ST131 isolates found in this study and the reference strains for the B1 and B2 phylogenetic groups from the ECOR collection.\textsuperscript{23} This analysis was based on the nucleotide sequence data for the seven genes used in the Achtman scheme and the eight genes used in Pasteur scheme, which were obtained from each MLST website.

\textbf{Plasmid-mediated quinolone resistance determinants.} All of the ESBL-producing isolates were characterised based on their plasmid-mediated quinolone resistance (PMQR) determinants (\textit{qnrA}, \textit{qnrB}, \textit{qnrC}, \textit{qnrS}, and \textit{aac(6\textsuperscript{'})-Ib-cr}).\textsuperscript{24}

\section*{Statistical analysis.} All categorical variables were compared using Fisher’s exact test. A $P$ value <0.05 was considered statistically significant. We conducted our statistical analysis using Stata, version 11.2 (StataCorp, College Station, TX, USA).

\section*{Results}

\textbf{Recognition of the B2-ST131-O16 clonal group.} The PCR analysis detected 185 ESBL-producing \textit{E. coli} isolates in 2010. PCR O-typing identified 77 O25b isolates, 15 O1 isolates, and nine O16 isolates that represented three major PCR O-types. The Clermont-\textit{pabB} PCR-positive isolates included 75 B2-ST131-O25b isolates, two B1-ST131-O25b isolates, and one B2-ST131-ONT (O-non-typeable) isolate. RAPD analysis indicated two large clusters (comprised of 86 and 76 isolates) and 10 small clusters that included less than 7 isolates with 50\% similarity (data not shown). The largest cluster of 86 isolates was made up of 74 B2-ST131-O25b isolates, two B1-ST131-O25b isolates, one B2-ST131-ONT isolate, seven B2-O16 isolates, one B2-O6 isolate, and one B2-ONT isolate. These results prompted us to perform MLST analysis of the B2-O16 isolates. All of the nine B2-O16 isolates, including seven isolates in the largest cluster and two isolates in the second largest cluster, belonged to the ST131 group but were negative by the Clermont-\textit{pabB} PCR.
Annual rate of ESBL-producing *E. coli*. Between 2001 and 2010, a total of 581 ESBL-producing *E. coli* of the 643 isolates that were positive in the ESBL confirmation test were confirmed by PCR analysis and further characterised. Five hundred fifty-one isolates (94.8%) were positive for CTX-M, 28 isolates were positive for TEM- or SHV-type ESBL, and the other two isolates were positive for both CTX-M and TEM or SHV. Five isolates that were resistant to imipenem or meropenem did not harbour a carbapenemase. Ten CTX-M-producing isolates were co-producers of pAmpC (CMY-2, n=9; DHA-1, n=1). The rate of ESBL-producing *E. coli* has increased from 0.2% in 2001 to 9.7% in 2010 (Figure 1).

**Characteristics of the clonal groups.** PCR O-typing of 581 ESBL-producing *E. coli* indicated 190 O25b isolates and 26 O16 isolates. Two O25b isolates belonged to phylogenetic group D and were negative by the Clermont-*pabB* PCR. All of the B2-O16 isolates except one and all of the isolates that were positive by the Clermont-*pabB* PCR but non-B2 (n=3) or non-O25b (n=1) were classified as ST131 by MLST analysis. The other B2-O16 isolate belonged to a novel ST, ST2784, a SLV of ST131. ST2784 had a single nucleotide polymorphism in the *fumC* gene when compared with ST131. Therefore, 215 isolates (37%) belonged to the ST131 group, which included 185 B2-ST131-O25b isolates, 26 B2-ST131-O16 isolates, three B1-ST131-O25b isolates, and one B2-ST131-ONT isolate. Forty-one isolates (7%) were identified as D-ST405 clonal group, seven isolates (1%) as D-ST69, and two isolates (0.3%) as D-ST393. Figure 1 presents the increasing trends in the rates of the B2-ST131-O25b, B2-ST131-O16, and D-ST405 clonal groups, although some annual variation exists.

Table 1 lists the antimicrobial susceptibilities and resistance genes of the clonal groups, including others group, which comprised all of the isolates not belonging to any clonal groups. The ciprofloxacin resistance rate of the B2-ST131-O25b clonal group (91%) was higher than others group (47%) and B2-ST131-O16 clonal group (19%). The minocycline resistance rate of the B2-ST131-O25b clonal group (12%) was lower than the B2-ST131-O16 clonal group (58%).
B2-ST131-O25b clonal group (6%) was less frequently resistant to piperacillin-tazobactam than the D-ST405 clonal group (17%). CTX-M-14 (44%), CTX-M-27 (24%), and CTX-M-15 (18%) were the most frequent ESBLs noted in the B2-ST131-O25b clonal group. However, the B2-ST131-O16 and D-ST405 clonal groups more frequently produced CTX-M-14 and did not produce CTX-M-27. CTX-M-2 and SHV were less frequently found in B2-ST131-O25b than in others group. All of the 41 D-ST405 isolates were resistant to ciprofloxacin. In addition, they were more frequently resistant to ampicillin-sulbactam and tobramycin than the B2-ST131-O25b or others group. All of the D-ST405 isolates, except one, produced CTX-M-14 or CTX-M-15. CTX-M-2 was not produced by any of the isolates. All of the seven D-ST69 isolates were susceptible to ciprofloxacin. The resistance rate to trimethoprim-sulfamethoxazole (57%) was similar to other isolates. CTX-M-2 was the most prevalent ESBL type (57%). Both D-ST393 isolates were resistant to ciprofloxacin and tested positive for CTX-M-15- or SHV-type ESBL.

**Pasteur MLST analysis of ST131 clonal group.** All of the ST131 isolates identified by the Achtman MLST scheme were subjected to a Pasteur MLST analysis (Table 2). Nine of 12 B2-ST131-O25b isolates, three B1-ST131-O25b isolates, and one B2-ST131-ONT isolate belonged to PST43, where PST indicates the ST under the Pasteur scheme. The other two B2-ST131-O25b isolates belonged to a novel PST, PST568, a SLV of PST43, and the other B2-ST131-O25b isolate belonged to PST527, a double-locus variant of PST43. Twenty-one of 26 B2-ST131-O16 isolates belonged to PST506. The other five B2-ST131-O16 isolates belonged to novel PSTs, PST566 or PST567, both of which were SLVs of PST506.

**PFGE analysis.**

All of the B2-ST131-O25b, B1-ST131-O25b, and B2-ST131-ONT isolates made up a cluster with 67% similarity (Figure 2). All of the B2-ST131-O16 isolates made up a cluster with 67% similarity. All of these ST131 isolates made up a cluster with 65% similarity. D-ST405 and D-ST69 isolates had less than 55% similarity to ST131 isolates.
Phylogenetic grouping of B1-ST131-O25b isolates.

The absence of the chuA gene in all of the three B1-ST131-O25b isolates was confirmed by retesting and performing PCR using different primers. The phylogenetic tree for the B1 and B2 reference strains and the nine ST131 variants, with regard to ST and PST (Table 2), showed that B1-ST131-O25b and the other ST131 variants belonged to the B2 phylogenetic group cluster (Figure 3).

Discussion

This study investigated the clonal groups present among ESBL-producing E. coli isolates collected by regional surveillance in Japan from 2001 to 2010, the era of the CTX-M-producing ST131 pandemic clonal group. The rate of ESBL-producing E. coli increased along with the rates of the ST131 and ST405 clonal groups. We found that both the B2-ST131-O25b and B2-ST131-O16 clonal groups contributed to this situation.

The B2-ST131-O25b is well recognised as an international pandemic clonal group. However, B2-ST131-O16 has not been previously described as either a pandemic or a major clonal group. We have found that 4% of ESBL-producing E. coli were in the B2-ST131-O16 clonal group, and are characterised by fluoroquinolone susceptibility and minocycline resistance. The B2-ST131-O16 clonal group differs from B2-ST131-O25b clonal group by more than two loci in the Pasteur MLST scheme and also differs when compared by PFGE analysis. The Clermont-pabB PCR for the B2-ST131-O25b clonal group correctly identified the B2-ST131-O25b clonal group. However, the B2-ST131-O25b clonal group carried the pabB15 or pabB74 alleles, and the B2-ST131-O16 clonal group also carried the pabB74 allele. The allele could be carried by B2-ST131-O25b and B2-ST131-O16 because the targeted pabB gene segment differs in the Clermont-pabB PCR and Pasteur MLST scheme. A recent Australian study described 211 B2-ST131-O25b and two B2-ST131-O16 isolates along with one B2-ST131-O157 isolate that were identified among...
fluoroquinolone-resistant extraintestinal *E. coli* infections from humans and companion animals.\textsuperscript{25} Peirano et al. investigated bloodstream ESBL-producing *E. coli* in Canada and found 113 ST131 isolates that tested positive when subjected to Clermont-*pabB* PCR and had > 60% similarity in their PFGE profiles.\textsuperscript{26} The researchers also found 4 ST131 isolates that tested negative when subjected to Clermont-*pabB* PCR and had < 60% similarity in their PFGE profiles with those 113 ST131 isolates. These 4 isolates were possibly B2-ST131-O16 isolates. These two studies suggest the B2-ST131-O16 may be a candidate for an international clonal group.

B2-ST131-O25b variants other than B2-ST131-O16 were also identified: B1-ST131-O25b and B2-ST131-ONT. The same PST was observed among all B1-ST131-O25b and some B2-ST131-O25b isolates, and these isolates had > 85% similarity in the PFGE analysis. According to the population structure of *E. coli*, it is impossible that ST131 belongs to the B1 and B2 phylogenetic branches of the *E. coli* population. We confirmed that three B1-ST131-O25b isolates found in this study were classified into the B1 phylogenetic group by the widely used triplex PCR method.\textsuperscript{14} However, this method is known to be less reliable than the MLST-based method.\textsuperscript{27} The phylogenetic tree in Figure 3 shows that the B1-ST131-O25b isolates should be classified into the B2 phylogenetic group. B2-ST131-ONT was also close to B2-ST131-O25b by the Pasteur MLST scheme and PFGE analysis. In addition, both B1-ST131-O25b and B2-ST131-ONT can be detected using Clermont-*pabB* PCR. When investigating the ST131 clonal group, these ST131 variants should be taken into consideration. In addition to the testing for O25b *rfb*, testing for O16 *rfb* is recommended. Allele-specific PCR targeting *gyrB* and *mdh* of ST131\textsuperscript{7} may be an alternative method to correctly identify the ST131 clonal group.

CTX-M-15 is most closely associated with the ST131 clonal group and thus is the most widely distributed CTX-M subtype. CTX-M-14 was the most prevalent among our B2-ST131-O25b isolates, followed by CTX-M-27 and CTX-M-15. In a Japanese nationwide surveillance study conducted between 2002 and 2003, most of the ST131 clonal group harboured CTX-M-14\textsuperscript{28}, which was
consistent with our results. However, none of the isolates belonging to the previous ST131 clonal group harboured CTX-M-27 or CTX-M-15. Therefore, CTX-M-27 and CTX-M-15 emerged as new ESBL types. In other studies, CTX-M-14-producing ST131 was the most prevalent isolate in Spain and the second-most prevalent in Korea and Canada. The ST131 clonal group frequently harbours genes for TEM-1, OXA-1, and aac(6′)-Ib-cr. In our study, these associations were not observed.

D-ST405 was the second-most prevalent clonal group (7%) in our study. All of the D-ST405 isolates were resistant to ciprofloxacin and predominantly harboured CTX-M-14 and CTX-M-15. In Korea (21%) and Canada (7%), D-ST405 was also the second-most prevalent ESBL-producing clonal group. Both studies reported CTX-M-15 and CTX-M-14 was the most prevalent ESBL. As far as we know, the only study which investigated the epidemiology and ciprofloxacin susceptibility of the ESBL-producing D-ST405 clonal group is a Canadian one. This study reported that all 14 D-ST405 isolates were resistant to ciprofloxacin. These results suggest that the ciprofloxacin-resistant, CTX-M-14- and CTX-M-15-producing D-ST405 isolates compose another pandemic clonal group.

D-ST69 has never been reported as an ESBL producer. We have identified seven ESBL-producing D-ST69 isolates but the rate was only 1%. Only two D-ST393 isolates were identified. These clonal groups were of little importance in terms of prevalence among the ESBL producers in our study.

In conclusion, the increasing rate of ESBL-producing *E. coli* in the Kyoto and Shiga regions of Japan is associated with increases in the rates of the B2-ST131-O25b, B2-ST131-O16, and D-ST405 clonal groups. The importance of these clonal groups, especially ST131 and ST405, appears to be underscored by the fact that collectively B2-ST131-O25b, B2-ST131-O16, and D-ST405 clonal groups comprised 43% of the ESBL-producing *E. coli* in our study. The rates of the already world-wide pandemic clonal groups, B2-ST131-O25b and D-ST405, are not striking. However, the B2-ST131-O16 clonal group may be worth special attention. This clonal group should be investigated to clarify its spread in other geographical areas, clinical significance, and microbiological characteristics, as it might have been overlooked in previous studies.
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Members of the Kyoto-Shiga Clinical Microbiology Study Group


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Transparency Declaration

None to declare.
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Table 1. Characteristics of B2-ST131-O25b, B2-ST131-O16, D-ST405, D-ST69, and D-ST393 clonal groups in ESBL-producing *E. coli*.

<table>
<thead>
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Antimicrobial resistance

| Drug                | B2-ST131       | B2-ST131 | Other | ST131<sup>a</sup> | D-ST405 | D-ST69 | D-ST393 | ‘others’ | Overall | B2-ST131-O25b vs. | ST405 | 5 vs. |
|---------------------|----------------|----------|-------|-------------------|---------|--------|---------|----------|---------| B2-ST1 | D-ST | ‘others’ | ‘other |
| Ampicillin-sulbactam| 131 (71%)      | 23 (88%) | 4 (100%) | 39 (95%)          | 7 (100%) | 1 (50%) | 245 (78%) |          | 0.003   | 0.062 | 0.001 | 0.109 | 0.007 |
| Piperacillin-tazobactam| 11 (6%)       | 3 (12%) | 2 (50%) | 7 (17%)           | 0 (0%)  | 0 (0%) | 31 (10%) |          | 0.048   | 0.389 | 0.026 | 0.181 | 0.176 |
| Imipenem            | 1 (1%)         | 0 (0%)   | 0 (0%) | 0 (0%)            | 0 (0%)  | 0 (0%) | 2 (1%)   |          | 1.000   | 1.000 | 1.000 | 1.000 | 1.000 |
| Meropenem           | 0 (0%)         | 0 (0%)   | 0 (0%) | 0 (0%)            | 0 (0%)  | 0 (0%) | 4 (1%)   |          | 0.615   | 0.127 | 1.000 | 1.000 | 1.000 |
| Ciprofloxacin       | 168 (91%)      | 5 (19%)  | 4 (100%) | 41 (100%)         | 0 (0%)  | 2 (100%) | 148 (47%) | 76 (24%) | <0.001  | <0.001 | 0.047 | <0.001 | <0.001 |
| Gentamicin          | 50 (27%)       | 3 (12%)  | 2 (50%) | 17 (41%)          | 2 (29%) | 0 (0%) | 76 (24%) |          | 0.085   | 0.097 | 0.088 | 0.458 | 0.023 |
| Tobramycin          | 46 (25%)       | 3 (12%)  | 0 (0%) | 21 (51%)          | 2 (29%) | 0 (0%) | 57 (18%) |          | <0.001  | 0.213 | 0.001 | 0.085 | <0.001 |
| Amikacin            | 1 (1%)         | 0 (0%)   | 0 (0%) | 2 (5%)            | 0 (0%)  | 0 (0%) | 4 (1%)   |          | 0.318   | 1.000 | 0.086 | 0.656 | 0.143 |
| Minocycline         | 22 (12%)       | 15 (58%) | 1 (25%) | 9 (22%)           | 2 (29%) | 2 (100%) | 115 (36%) |          | <0.001  | <0.001 | 0.128 | <0.001 | 0.081 |
| Trimethoprim        | 95 (51%)       | 13 (50%) | 1 (25%) | 28 (68%)          | 4 (57%) | 2 (100%) | 186 (59%) |          | 0.195   | 1.000 | 0.057 | 0.113 | 0.310 |
sulfamethoxazole

**ESBL Type**

<table>
<thead>
<tr>
<th>Type</th>
<th>Count</th>
<th>%</th>
<th>Count</th>
<th>%</th>
<th>Count</th>
<th>%</th>
<th>Count</th>
<th>%</th>
<th>Count</th>
<th>%</th>
<th>Count</th>
<th>%</th>
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</thead>
<tbody>
<tr>
<td><strong>CTX-M-14</strong></td>
<td>81\textsuperscript{a,c}</td>
<td>(44%)</td>
<td>19 (73%)</td>
<td>2 (50%)</td>
<td>30\textsuperscript{b} (73%)</td>
<td>2 (29%)</td>
<td>0 (0%)</td>
<td>150\textsuperscript{b}</td>
<td>(47%)</td>
<td>0.001</td>
<td>0.006</td>
<td>0.001</td>
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<td><strong>CTX-M-15</strong></td>
<td>33\textsuperscript{b} (18%)</td>
<td>2 (8%)</td>
<td>0 (0%)</td>
<td>12\textsuperscript{b} (29%)</td>
<td>0 (0%)</td>
<td>1 (50%)</td>
<td>45\textsuperscript{b}</td>
<td>(14%)</td>
<td>0.089</td>
<td>0.265</td>
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<tr>
<td><strong>CTX-M-2</strong></td>
<td>17 (9%)</td>
<td>1 (4%)</td>
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<td>0 (0%)</td>
<td>4 (57%)</td>
<td>0 (0%)</td>
<td>58 (18%)</td>
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<td>0.006</td>
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<td><strong>CTX-M-27</strong></td>
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<td>0 (0%)</td>
<td>0 (0%)</td>
<td>6 (2%)</td>
<td>&lt;0.001</td>
<td>0.020</td>
<td>&lt;0.00</td>
<td>&lt;0.00</td>
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<tr>
<td><strong>CTX-M-3</strong></td>
<td>4\textsuperscript{c} (2%)</td>
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<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (14%)</td>
<td>0 (0%)</td>
<td>11 (3%)</td>
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<td>0.588</td>
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<td><strong>CTX-M-24</strong></td>
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<td>0 (0%)</td>
<td>1 (2%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4 (1%)</td>
<td>0.715</td>
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<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>9 (3%)</td>
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<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>17 (5%)</td>
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<td>0.123</td>
<td>1.000</td>
<td>&lt;0.001</td>
<td>0.237</td>
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<td><strong>TEM-type ESBL</strong></td>
<td>2 (1%)</td>
<td>1 (4%)</td>
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<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>3 (1%)</td>
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<td><strong>SHV-type ESBL</strong></td>
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<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (50%)</td>
<td>18 (6%)</td>
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<td><strong>CMY-2</strong></td>
<td>2 (1%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>7 (2%)</td>
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<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (0%)</td>
<td>1.000</td>
<td>0.127</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>TEM-1</strong></td>
<td>85 (46%)</td>
<td>10 (38%)</td>
<td>4 (100%)</td>
<td>16 (39%)</td>
<td>3 (43%)</td>
<td>1 (50%)</td>
<td>127 (40%)</td>
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<td><strong>OXA-1</strong></td>
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<td>0 (0%)</td>
<td>3 (7%)</td>
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<td>0 (0%)</td>
<td>7 (2%)</td>
<td>0.425</td>
<td>1.000</td>
<td>0.394</td>
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PMQR determinants

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<th>Gene</th>
<th>% Positive</th>
<th>PMQR 1</th>
<th>PMQR 2</th>
<th>PMQR 3</th>
<th>PMQR 4</th>
<th>PMQR 5</th>
<th>PMQR 6</th>
<th>EPMQR</th>
<th>PMQR 7</th>
<th>PMQR 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>qnr</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>5* (2%)</td>
<td>0.465</td>
<td>0.127</td>
<td>1.000</td>
<td>0.163</td>
</tr>
<tr>
<td>aac(6')-Ib-cr</td>
<td>16 (9%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4 (10%)</td>
<td>0 (0%)</td>
<td>16 (5%)</td>
<td>0.419</td>
<td>0.703</td>
<td>0.766</td>
<td>0.131</td>
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</tbody>
</table>

* Three B1-ST131-O25b isolates and one B2-ST131-ONT isolate were included.

b One B2-ST131-O25b isolate, two D-ST405 isolates, and three isolates in the Others group were positive for both CTX-M-14 and CTX-M-15.

c Two isolates were positive for both CTX-M-14 and CTX-M-3.

d CTX-M-55 was found in the B2-ST131-O16 group and CTX-M-55 (n=6), CTX-M-1 (n=5), CTX-M-65 (n=2), CTX-M-19 (n=1), CTX-M-30 (n=1).

e CTX-M-44 (n=1), and CTX-M-126 (n=1) were identified in the Others group.

f Three isolates were positive for qnrS, and two isolates were positive for qnrB.
Table 2. Allele profiles of the ST131 clonal group among the ESBL-producing *E. coli*

| Number of isolates | Phylogenetic group | PCR O type | Achtman ST | ST | dinB | icdA | pabB | polB | putP | trpA | trpB | uidA |
|-------------------|------------------|-------------|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 9                 | B2               | 25b         | 131         | 43  | 9   | 1   | 15  | 7   | 4   | 9   | 6   | 9   |
| 2                 | B2               | 25b         | 131         | 568 | 9   | 1   | 74  | 7   | 4   | 9   | 6   | 9   |
| 1                 | B2               | 25b         | 131         | 527 | 9   | 20  | 15  | 7   | 4   | 9   | 6   | 129 |
| 3                 | B1               | 25b         | 131         | 43  | 9   | 1   | 15  | 7   | 4   | 9   | 6   | 9   |
| 1                 | B2               | Non-typeable| 131         | 43  | 9   | 1   | 15  | 7   | 4   | 9   | 6   | 9   |
| 20                | B2               | 16          | 131         | 506 | 9   | 134 | 74  | 134 | 4   | 72  | 1   | 9   |
| 4                 | B2               | 16          | 131         | 567 | 9   | 20  | 74  | 134 | 4   | 72  | 1   | 9   |
| 1                 | B2               | 16          | 2784        | 506 | 9   | 134 | 74  | 134 | 4   | 72  | 1   | 9   |
| 1                 | B2               | 16          | 131         | 566 | 9   | 1   | 74  | 134 | 4   | 72  | 1   | 9   |

Of 185 putative ESBL-producing B2-ST131-O25b isolates, 12 randomly selected isolates were subjected to MLST analysis. All of the 26 B2-O16, 3 B1-ST131-O25b, and 1 B2-ST131-O-non-typeable isolates were subjected to MLST analysis.
Figure 1. Rates of the B2-ST131-O25b, B2-ST131-O16, and D-ST405 clonal groups among ESBL-producing *E. coli* from 2001 to 2010.
Figure 2. PFGE of *Xba*1-digested DNA from the ST131, D-ST405, and D-ST69 clonal groups. In addition to all of the B2-ST131-O16, B1-ST131-O25b, and B2-ST131-O-non-typeable isolates, 12 B2-ST131-25b, eight D-ST405, and one D-ST69 isolate identified using the Achtman MLST scheme were included. Two D-ST393 isolates were non-typeable. ST indicate the STs under the Achtman scheme, whereas PST indicates the STs under the Pasteur scheme.
Figure 3. Phylogenetic tree of the ECOR reference strains of B1 and B2 phylogenetic groups and the nine variants of ST131 clonal group detailed in Table 2. This tree was constructed from the concatenated nucleotide sequence of the seven genes used in the Achtman scheme and the eight genes used in the Pasteur scheme with the neighbour-joining method.