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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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 programme 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%), 3 as B1-ST131-O25b (0.5%) and 1 as B2-ST131-O16 non-typeable (0.1%). Forty-one isolates (7%) were identified as belonging to the D-ST405 clonal group, seven (1%) as D-ST69 and two (0.3%) as D-ST393. The B2-ST131-O16 clonal group was characterized by CTX-M-14 and a significantly lower ciprofloxacin resistance rate than the 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 the B2-ST131-O25b, B2-ST131-O16 and D-ST405 clonal groups.

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

Keywords: ESBLs, clonal genotypes, sequence types, CTX-M-14

Introduction

In recent years, the prevalence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* has increased dramatically worldwide.1 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.2 In addition to the ST131 clonal group, a CTX-M-15-producing ST405 clonal group belonging to phylogenetic group D has been detected worldwide.3 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-ST69.4–5 The D-ST393-O15 clonal group is characterized by K52:H1 serotypes and fluoroquinolone resistance.7 CTX-M-14 producers have also been identified.6 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 investigated 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, 12607 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 Vitek 2 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, ceftepime and aztreonam) and the ESBL confirmation test was performed using the double disc synergy test following the CLSI guidelines. In a reference laboratory, antibiotic susceptibility was re-evaluated by microdilution using Dry Plate Eiken (Eiken Chemical, Tokyo, Japan), and this 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 and OXA-1 genes, and of the six 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.

**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. 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).

For the detection of the B2-ST131-O25b clonal group, B2-ST131-O25b pabB allele-specific PCR (Clermont-pabB PCR) was performed as described by Clermont et al. 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 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 mdt gene were performed. Isolates that belonged to phylogenetic group D and were positive for the adk35 allele and mdh4 were classified as D-ST405 clonal group. Eight selected D-ST405 isolates were confirmed by MLST. The primers adk35f (5’-TGGCAAAC TGGTCTACT-3’) and adk35r (5’-CGGTCACGCTATGGTCGTC-3’) were designed for the detection of ST405-associated single-nucleotide polymorphisms in adk35 (i.e. C1487T, T316C, T322C and A331C). Amplification was performed with 1× PCR buffer, 2 mM MgCl2, 0.2 mM deoxynucleoside triphosphates, 0.4 μM primers, 1 μM of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) and 1 μL of 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 and 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 fumC016-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, mdt, purA and recA). The ST131 isolates determined by the Achtman scheme were further characterized by the Pasteur MLST scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html) using eight housekeeping genes (dnaB, icdA, pabB, pabL, 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 by RAPD and 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 by amplification using primer pairs only for chuA, in triplicate, because failure to detect the chuA gene would classify an isolate into B1 phylogenetic group. In addition, other primer pairs for the chuA gene were used. A phylogenetic tree was built using the 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. 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 web site.

**Plasmid-mediated quinolone resistance (PMQR) determinants**

All of the ESBL-producing isolates were characterized based on their PMQR determinants [\textit{qnrA}, \textit{qnrB}, \textit{qnrC}, \textit{qnrS} and \textit{aac(6\textquoteright)Ib-cr}].

**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).

**Results**

**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 9 O16 isolates, which represented three major PCR O types. The Clermont-pabB PCR-positive isolates included 75 B2-ST131-O25b isolates, 2 B1-ST131-O25b isolates and 1 B2-ST131-ONT isolate (where ONT stands for O-non-typeable). RAPD analysis indicated two large clusters (comprising 86 and 76 isolates) and 10 small clusters that included less than 7 isolates with \( \geq 50\% \) similarity (data not shown). The largest cluster (86 isolates) was made up of 74 B2-ST131-O25b isolates, 2 B1-ST131-O25b isolates, 1 B2-ST131-ONT isolate, 7 B2-O16 isolates, 1 B2-O6 isolate and 1 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 in the Clermont-pabB PCR.

**Annual rate of ESBL-producing \textit{E. coli}**

Between 2001 and 2010, of the 643 isolates that were positive in the ESBL confirmation test, 581 ESBL-producing \textit{E. coli} were confirmed by PCR analysis and further characterized. Of these, 551 isolates (94.8\%) were positive for CTX-M, 28 isolates were positive for TEM- or SHV-type ESBL, and the other 2 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 \textit{E. coli} 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 \textit{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 test. All but one of the B2-O16 isolates and all of the isolates that were positive by 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, an SLV of ST131. ST2784 had a single nucleotide polymorphism in the \textit{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, 3 B1-ST131-O25b isolates and 1 B2-ST131-ONT isolate; 41 isolates (7\%) were identified as D-ST405 clonal group, 7 isolates (1\%) were identified as D-ST69 and two isolates (0.3\%) were identified 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 the ‘others’ group, which
Table 1. Characteristics of the B2-ST131-O25b, B2-ST131-O16, D-ST405, D-ST69 and D-ST393 clonal groups in ESBL-producing E. coli

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>B2-ST131-O25b (n=185)</th>
<th>B2-ST131-O16 (n=26)</th>
<th>other ST131&lt;sup&gt;a&lt;/sup&gt; (n=4)</th>
<th>D-ST405 (n=41)</th>
<th>D-ST69 (n=7)</th>
<th>D-ST393 (n=2)</th>
<th>‘others’ (n=316)</th>
<th>overall</th>
<th>B2-ST131-O25b versus B2-ST131-O16</th>
<th>ST405 versus ‘others’</th>
<th>‘others’ versus ‘others’</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antimicrobial resistance</strong></td>
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</tr>
<tr>
<td>Ampicillin/sulbactam</td>
<td>131 (71)</td>
<td>23 (88)</td>
<td>4 (100)</td>
<td>39 (95)</td>
<td>7 (100)</td>
<td>1 (50)</td>
<td>245 (78)</td>
<td>0.003</td>
<td>0.062</td>
<td>0.001</td>
<td>0.109</td>
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<tr>
<td>Piperacillin/tazobactam</td>
<td>11 (6)</td>
<td>3 (12)</td>
<td>2 (50)</td>
<td>7 (17)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>31 (10)</td>
<td>0.048</td>
<td>0.389</td>
<td>0.026</td>
<td>0.181</td>
</tr>
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<td>Imipenem</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (1)</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
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<tr>
<td>Meropenem</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (1)</td>
<td>0.615</td>
<td>0.127</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>168 (91)</td>
<td>5 (19)</td>
<td>4 (100)</td>
<td>41 (100)</td>
<td>2 (100)</td>
<td>148 (47)</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>50 (27)</td>
<td>5 (19)</td>
<td>21 (51)</td>
<td>5 (100)</td>
<td>0 (0)</td>
<td>76 (24)</td>
<td>0.085</td>
<td>0.097</td>
<td>0.088</td>
<td>0.458</td>
<td>0.023</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>46 (25)</td>
<td>3 (12)</td>
<td>57 (18)</td>
<td>57 (18)</td>
<td>0 (0)</td>
<td>115 (36)</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
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<td>0.001</td>
</tr>
<tr>
<td>Minocycline</td>
<td>22 (12)</td>
<td>15 (58)</td>
<td>4 (100)</td>
<td>41 (100)</td>
<td>0 (0)</td>
<td>148 (47)</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
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<tr>
<td>Trimethoprim/sulfamethoxazole</td>
<td>95 (51)</td>
<td>13 (50)</td>
<td>28 (68)</td>
<td>4 (100)</td>
<td>2 (100)</td>
<td>186 (59)</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
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</tr>
<tr>
<td><strong>ESBL type</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>CTX-M-14</td>
<td>81&lt;sup&gt;b,c&lt;/sup&gt; (44)</td>
<td>19 (73)</td>
<td>2 (50)</td>
<td>30&lt;sup&gt;b&lt;/sup&gt; (73)</td>
<td>2 (29)</td>
<td>0 (0)</td>
<td>150&lt;sup&gt;b&lt;/sup&gt; (47)</td>
<td>0.001</td>
<td>0.006</td>
<td>0.001</td>
<td>0.458</td>
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<tr>
<td>CTX-M-15</td>
<td>33&lt;sup&gt;b&lt;/sup&gt; (18)</td>
<td>2 (8)</td>
<td>0 (0)</td>
<td>12&lt;sup&gt;b&lt;/sup&gt; (29)</td>
<td>0 (0)</td>
<td>1 (50)</td>
<td>45&lt;sup&gt;b&lt;/sup&gt; (14)</td>
<td>0.089</td>
<td>0.265</td>
<td>0.128</td>
<td>0.308</td>
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<td>CTX-M-2</td>
<td>17 (9)</td>
<td>1 (4)</td>
<td>2 (50)</td>
<td>0 (0)</td>
<td>4 (57)</td>
<td>58 (18)</td>
<td>&lt;0.001</td>
<td>0.705</td>
<td>0.047</td>
<td>0.006</td>
<td>0.043</td>
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<td>CTX-M-27</td>
<td>44 (24)</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>6 (2)</td>
<td>&lt;0.001</td>
<td>0.020</td>
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<td>&lt;0.001</td>
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<tr>
<td>CTX-M-3</td>
<td>4&lt;sup&gt;c&lt;/sup&gt; (2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (1)</td>
<td>0.715</td>
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<td>4 (2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (1)</td>
<td>0.049</td>
<td>0.327</td>
<td>1.000</td>
<td>1.000</td>
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<tr>
<td>CTX-M-9</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
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<td>0.049</td>
<td>0.327</td>
<td>1.000</td>
<td>1.000</td>
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<tr>
<td>Other CTX-M&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>1 (4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>17 (5)</td>
<td>0.013</td>
<td>0.123</td>
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<tr>
<td>TEM-type ESBL</td>
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<td>1 (4)</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (1)</td>
<td>0.049</td>
<td>0.327</td>
<td>1.000</td>
<td>1.000</td>
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<tr>
<td>SHV-type ESBL</td>
<td>3 (2)</td>
<td>2 (8)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (50)</td>
<td>18 (6)</td>
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<td>0.116</td>
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<td><strong>Other β-lactamase</strong></td>
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<tr>
<td>CMY-2</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
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<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>
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<tr>
<td>TEM-1</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>
<td>0.266</td>
<td>0.532</td>
<td>0.489</td>
<td>0.224</td>
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<tr>
<td>OXA-1</td>
<td>7 (4)</td>
<td>0 (0)</td>
<td>3 (7)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>7 (2)</td>
<td>0.425</td>
<td>1.000</td>
<td>0.394</td>
<td>0.400</td>
<td>0.095</td>
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<td><strong>PMQR determinants</strong></td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>16 (5)</td>
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<td>0.703</td>
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<sup>a</sup>Three B1-ST131-O25b isolates and one B2-ST131-ONT isolate were included.<br><sup>b</sup>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.<br><sup>c</sup>Two isolates were positive for both CTX-M-14 and CTX-M-3.<br><sup>d</sup>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), CTX-M-44 (n=1) and CTX-M-126 (n=1) were identified in the ‘others’ group.<br><sup>e</sup>Three isolates were positive for qnrS and two isolates were positive for qnrB.
comprises 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 that of the ‘others’ group (47%) and the B2-ST131-O16 clonal group (19%). The minocycline resistance rate of the B2-ST131-O25b clonal group (12%) was lower than that of the B2-ST131-O16 clonal group (58%). The 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 the ‘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 those of the B2-ST131-O25b group or the ‘others’ group. All but one of the D-ST405 isolates produced CTX-M-14 or CTX-M-2. 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 that of the isolates in the ‘others’ group. 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.

**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. Together, all of these ST131 isolates made up a cluster with 65% similarity. D-ST405 and D-ST69 isolates had <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 recognized 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 found that 4% of ESBL-producing *E. coli* were in the B2-ST131-O16 clonal group, and characterized by fluoroquinolone susceptibility and minocycline resistance. The B2-ST131-O25b clonal group carried *pabB* alleles, and the

**Pasteur MLST analysis of ST131 clonal group**

All of the ST131 isolates identified by the Achtman MLST scheme were subjected to Pasteur MLST analysis (Table 2). Nine of 12 B2-ST131-O25b isolates, 3 B1-ST131-O25b isolates and 1 B2-ST131-ONT isolate belonged to PST43 (PST indicates the ST under the Pasteur scheme). The other two B2-ST131-O25b isolates belonged to a novel PST, PST568 (an SLV of PST43), and the other B2-ST131-O25b isolate belonged to PST527 (a double-locus variant of PST43). Of the 26 B2-ST131-O16 isolates, 21 belonged to PST506, and the other 5 belonged to novel PSTs: PST566 or PST567, both of which are SLVs of PST506.
that were identified among fluoroquinolone-resistant extraintestinal E. coli infections from humans and companion animals. 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. The researchers also found four ST131 isolates that tested negative when subjected to Clermont-pabB PCR and had 60% similarity in their PFGE profiles with the 113 ST131 isolates. These four isolates were possibly B2-ST131-O16. 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 >67% 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. However, this method is known to be less reliable than the MLST-based method. 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 testing for O25b rfb, testing for O16 rfb is recommended. Allele-specific PCR targeting gyrB and mdh of ST131 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, 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

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**Figure 3.** Phylogenetic tree of the ECOR reference strains of the B1 and B2 phylogenetic groups and the nine variants of the 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.
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 and Canada, D-ST405 was also the second most prevalent ESBL-producing clonal group (21% and 7%, respectively). Both studies reported that CTX-M-15 and CTX-M-14 were the most prevalent ESBLs. 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 comprise 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 worldwide pandemic clonal groups, B2-ST131-O25b and D-ST405, were 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 declarations
None to declare.

References


