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1 **Original article**

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

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

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18

19 **Synopsis**

20 **Objectives:** The increasing prevalence of extended-spectrum β -lactamase (ESBL)-producing
21 *Escherichia coli* has been associated with the emergence of the CTX-M-producing sequence type
22 131 (ST131) pandemic clonal group, a member of the O25b serogroup and the B2 phylogenetic
23 group. To assess the clonal spread of ESBL-producing *E. coli* in Japan, a regional surveillance
24 program was conducted.

25 **Methods:** A total of 581 ESBL-producing clinical specimen *E. coli* isolates were collected between
26 2001 and 2010. Clonal groups, including ST131, D-ST405, D-ST393, and D-ST69, were determined
27 using the PCR O-type, phylogenetic grouping by triplex PCR, allele-specific PCR, and multilocus
28 sequence typing (MLST). A subset of clonal groups underwent PFGE.

29 **Results:** Among clonal strains, 215 isolates (37%) were identified as belonging to the ST131 group,
30 185 as B2-ST131-O25b (32%), 26 as B2-ST131-O16 (4%), three as B1-ST131-O25b, and one as
31 B2-ST131-O-non-typeable. Forty-one isolates (7%) were identified as D-ST405 clonal group, seven
32 (1%) as D-ST69, and two (0.3%) as D-ST393. B2-ST131-O16 clonal group was characterised by
33 CTX-M-14 and a significantly lower ciprofloxacin-resistant rate than B2-ST131-O25b clonal group.
34 The B2-ST131-O16 and B2-ST131-O25b clonal groups each made up a single PFGE cluster, with
35 65% similarity. The rate of ESBL-producing *E. coli* increased over the years (0.2% in 2001 to 9.7%
36 in 2010) and corresponded to increases in the numbers of B2-ST131-O25b, B2-ST131-O16, and
37 D-ST405 clonal groups.

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

40

41 **Introduction**

42 In recent years, the prevalence of extended-spectrum β -lactamase (ESBL)-producing
43 *Escherichia coli* has increased dramatically worldwide.¹ Clonal group detection by multilocus
44 sequence typing (MLST) has suggested the reason for this pandemic. The emergence of an
45 international pandemic clonal group, CTX-M-type ESBL-producing *E. coli* with sequence type 131
46 (ST131) belonging to the O25b serogroup and the B2 phylogenetic group, has contributed greatly to
47 the pandemic.² The success of the ST131 clonal group is explained by its acquisition of
48 fluoroquinolone resistance and additional virulence factors.² In addition to the ST131 clonal group, a
49 CTX-M-15-producing ST405 clonal group belonging to phylogenetic group D has been detected
50 worldwide.³ However, the prevalence and evolution of this clonal group has not been well
51 investigated. Detailed studies on the ST131 clonal group among ESBL-producing *E. coli* in Japan are
52 lacking.

53 Other clonal groups disseminated worldwide include D-ST393-O15 and D-ST69.⁴⁻⁶ The
54 D-ST393-O15 clonal group is characterised by K52:H1 serotypes and fluoroquinolone resistance.⁷
55 CTX-M-14 producers have also been identified.⁸ The D-ST69 clonal group is known as “clonal
56 group A” and is frequently found among trimethoprim-sulfamethoxazole-resistant uropathogenic *E.*
57 *coli*.⁹ A study conducted in 2009 in Spain indicated that these two clonal groups and the ST131
58 clonal group accounted for 38% of fluoroquinolone-resistant *E. coli* isolates and 32% of
59 trimethoprim-sulfamethoxazole-resistant isolates.⁶

60 In this study, we analysed the genetic relatedness of ESBL-producing *E. coli* isolates in
61 2010 in the Kyoto and Shiga regions of Japan using random amplified polymorphic DNA (RAPD)
62 fingerprinting and found that the B2-ST131-O16 clonal group was closely related to the
63 B2-ST131-O25b clonal group. Then, we investigate the contribution and characteristics of the clonal
64 groups, including ST131, ST405, ST393, and ST69. We further investigated ST131 variants that
65 were non-B2 or non-O25b isolates, which mostly consisted of B2-ST131-O16 isolates.

66

67

68 **Materials and methods**

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

76 **Identification and susceptibility testing.** At each hospital, microbiological identification and
77 susceptibility testing were performed using the Vitek2 system (bioMérieux, Marcy l'Etoile, France)
78 or the MicroScan system (Siemens Healthcare diagnostics, Tokyo, Japan). Subsequently, the ESBL
79 screening test was performed according to the CLSI microdilution methodology (cefotaxime,
80 ceftriaxone, ceftazidime, cefpodoxime, and aztreonam), and the ESBL confirmation test was
81 performed using the double-disk synergy test following the CLSI guidelines.¹⁰ In a reference
82 laboratory, the antibiotic susceptibility was re-evaluated by microdilution using Dry Plate Eiken
83 (Eiken, Tokyo, Japan) and included testing with ampicillin-sulbactam, piperacillin-tazobactam,
84 ciprofloxacin, gentamicin, tobramycin, amikacin, imipenem, meropenem, minocycline, and
85 trimethoprim-sulfamethoxazole. The results were interpreted using the 2012 CLSI breakpoints.¹⁰
86 Intermediate susceptibility to each antibiotic was considered to be resistance.

87 **β -lactamase identification.** The presence of ESBL or plasmid-mediated AmpC β -lactamase
88 (pAmpC) genes was detected by PCR amplification and sequencing of the CTX-M, TEM, SHV,
89 OXA-1 genes, and the 6 main groups of pAmpC-type genes as described previously.¹¹ The isolates
90 that were resistant to imipenem or meropenem (MIC > 1 mg/L) were analysed to determine the

91 presence of the carbapenemases GES, OXA-48-like, IMP, VIM, KPC, and NDM.^{12, 13}

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

96 For the detection of the B2-ST131-O25b clonal group, the B2-ST131-O25b *pabB* allele-specific
97 PCR (Clermont-*pabB* PCR) was performed as described by Clermont et al.¹⁷ The Clermont-*pabB*
98 PCR targets a different region of the *pabB* gene from that used in the Pasteur MLST scheme. Isolates
99 that belonged to phylogenetic group B2 and were positive for the O25b *rfb* allele and the
100 Clermont-*pabB* PCR were classified into the B2-ST131-O25b clonal group. Twelve selected
101 B2-ST131-O25b isolates identified by these presumptive methods were confirmed by MLST.
102 Isolates that were positive for Clermont-*pabB* PCR but non-B2 or non-O25b were also subjected to
103 MLST.

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

107 For the detection of the ST405 clonal group, *adk35* allele-specific PCR and sequencing of the *mdh*
108 allele were performed. Isolates that belonged to phylogenetic group D and were positive for the
109 *adk35* allele and *mdh4* allele were classified as D-ST405 clonal group. Eight selected D-ST405
110 isolates were confirmed by MLST. The primers *adk35f* (5'-TGGCAAACCTGGTCACT-3') and
111 *adk35r* (5'-CGTTGACCGTATCGTC-3') were designed for the detection of ST405-associated
112 single-nucleotide polymorphisms in *adk35* (i.e., C148T, T316C, T322C, and A331C). Amplification
113 was performed with 1X PCR buffer, 2 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates, 0.4 μM
114 of each primer, 1 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), and 1 μL
115 boiled cell lysate in a total volume of 20 μL. The cycling protocol was as follows: 95°C for 5 min,

116 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
117 min.

118 For the detection of the D-ST393-O15 clonal group, isolates that were positive for the O15 *rfb* allele
119 were sequenced to determine the *fumC* allele profile. All phylogenetic group D and
120 *fumC*106-positive isolates were confirmed as ST393 clonal group by MLST.

121 For the detection of the D-ST69 clonal group, *fumC*35 allele-specific PCR was performed as
122 described previously.¹⁸ Phylogenetic group D and *fumC*35 PCR-positive isolates were sequenced to
123 determine the *fumC* and *gyrB* allele profiles. The *fumC*35 and *gyrB*27 isolates were considered to
124 comprise the D-ST69 clonal group. One randomly selected D-ST69 isolate was confirmed by MLST.

125 **MLST.** MLST was performed according to the Achtman scheme (<http://mlst.ucc.ie/mlst/dbs/Ecoli>)
126 using seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*).¹⁹ The ST131 isolates
127 determined by the Achtman scheme were further characterised by the Pasteur MLST scheme
128 (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html>) using eight housekeeping genes
129 (*dinB*, *icdA*, *pabB*, *polB*, *putP*, *trpA*, *trpB*, and *uidA*).

130 **PCR genomic fingerprinting and PFGE.** RAPD fingerprinting using a DAF4 primer²⁰ was
131 performed for the ESBL-producing isolates obtained in 2010 to analyse the genetic relatedness of the
132 B2-ST131-O25b clonal group. Genomic DNA from all of the isolates subjected to the Achtman
133 MLST underwent *Xba*I PFGE.²¹ The profiles obtained from RAPD or PFGE were analysed with
134 GelCompar II, version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was
135 applied using the unweighted pair-group method based on Dice coefficients to quantify the
136 similarities.

137 **Phylogenetic grouping of B1-ST131-O25b isolates.**

138 To confirm the B1 phylogenetic grouping of the B1-ST131-O25b isolates, these isolates were tested
139 for the *chuA* gene amplification using primer pairs only for *chuA* in triplicate because a failure to
140 detect the *chuA* gene would classify an isolate into B1 phylogenetic group.¹⁴ In addition, other

141 primer pairs²² for the *chuA* gene were used. A phylogenetic tree was built using neighbour-joining
142 method in the CLUSTAL X program for the ST131 isolates found in this study and the reference
143 strains for the B1 and B2 phylogenetic groups from the ECOR collection.²³ This analysis was based
144 on the nucleotide sequence data for the seven genes used in the Achtman scheme and the eight genes
145 used in Pasteur scheme, which were obtained from each MLST website.

146 **Plasmid-mediated quinolone resistance determinants.** All of the ESBL-producing isolates were
147 characterised based on their plasmid-mediated quinolone resistance (PMQR) determinants (*qnrA*,
148 *qnrB*, *qnrC*, *qnrS*, and *aac(6')-Ib-cr*).²⁴

149 **Statistical analysis.** All categorical variables were compared using Fisher's exact test. A *P* value
150 <0.05 was considered statistically significant. We conducted our statistical analysis using Stata,
151 version 11.2 (StataCorp, College Station, TX, USA).

152

153

154 **Results**

155 **Recognition of the B2-ST131-O16 clonal group.** The PCR analysis detected 185 ESBL-producing
156 *E. coli* isolates in 2010. PCR O-typing identified 77 O25b isolates, 15 O1 isolates, and nine O16
157 isolates that represented three major PCR O-types. The Clermont-*pabB* PCR-positive isolates
158 included 75 B2-ST131-O25b isolates, two B1-ST131-O25b isolates, and one B2-ST131-ONT
159 (O-non-typeable) isolate. RAPD analysis indicated two large clusters (comprised of 86 and 76
160 isolates) and 10 small clusters that included less than 7 isolates with 50% similarity (data not shown).
161 The largest cluster of 86 isolates was made up of 74 B2-ST131-O25b isolates, two B1-ST131-O25b
162 isolates, one B2-ST131-ONT isolate, seven B2-O16 isolates, one B2-O6 isolate, and one B2-ONT
163 isolate. These results prompted us to perform MLST analysis of the B2-O16 isolates. All of the nine
164 B2-O16 isolates, including seven isolates in the largest cluster and two isolates in the second largest
165 cluster, belonged to the ST131 group but were negative by the Clermont-*pabB* PCR.

166 **Annual rate of ESBL-producing *E. coli*.** Annual rate of ESBL-producing *E. coli*. Between 2001
167 and 2010, a total of 581 ESBL-producing *E. coli* of the 643 isolates that were positive in the ESBL
168 confirmation test were confirmed by PCR analysis and further characterised. Five hundred fifty-one
169 isolates (94.8%) were positive for CTX-M, 28 isolates were positive for TEM- or SHV-type ESBL,
170 and the other two isolates were positive for both CTX-M and TEM or SHV. Five isolates that were
171 resistant to imipenem or meropenem did not harbour a carbapenemase. Ten CTX-M-producing
172 isolates were co-producers of pAmpC (CMY-2, n=9; DHA-1, n=1). The rate of ESBL-producing *E.*
173 *coli* has increased from 0.2% in 2001 to 9.7% in 2010 (Figure 1).

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

186 Table 1 lists the antimicrobial susceptibilities and resistance genes of the clonal groups, including
187 others group, which comprised all of the isolates not belonging to any clonal groups. The
188 ciprofloxacin resistance rate of the B2-ST131-O25b clonal group (91%) was higher than others
189 group (47%) and B2-ST131-O16 clonal group (19%). The minocycline resistance rate of the
190 B2-ST131-O25b clonal group (12%) was lower than the B2-ST131-O16 clonal group (58%). The

191 B2-ST131-O25b clonal group (6%) was less frequently resistant to piperacillin-tazobactam than the
192 D-ST405 clonal group (17%). CTX-M-14 (44%), CTX-M-27 (24%), and CTX-M-15 (18%) were the
193 most frequent ESBLs noted in the B2-ST131-O25b clonal group. However, the B2-ST131-O16 and
194 D-ST405 clonal groups more frequently produced CTX-M-14 and did not produce CTX-M-27.
195 CTX-M-2 and SHV were less frequently found in B2-ST131-O25b than in others group. All of the
196 41 D-ST405 isolates were resistant to ciprofloxacin. In addition, they were more frequently resistant
197 to ampicillin-sulbactam and tobramycin than the B2-ST131-O25b or others group. All of the
198 D-ST405 isolates, except one, produced CTX-M-14 or CTX-M-15. CTX-M-2 was not produced by
199 any of the isolates. All of the seven D-ST69 isolates were susceptible to ciprofloxacin. The resistance
200 rate to trimethoprim-sulfamethoxazole (57%) was similar to other isolates. CTX-M-2 was the most
201 prevalent ESBL type (57%). Both D-ST393 isolates were resistant to ciprofloxacin and tested
202 positive for CTX-M-15- or SHV-type ESBL.

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

211 **PFGE analysis.**

212 All of the B2-ST131-O25b, B1-ST131-O25b, and B2-ST131-ONT isolates made up a cluster with
213 67% similarity (Figure 2). All of the B2-ST131-O16 isolates made up a cluster with 67% similarity.
214 All of these ST131 isolates made up a cluster with 65% similarity. D-ST405 and D-ST69 isolates had
215 less than 55% similarity to ST131 isolates.

216 **Phylogenetic grouping of B1-ST131-O25b isolates.**

217 The absence of the *chuA* gene in all of the three B1-ST131-O25b isolates was confirmed by retesting
218 and performing PCR using different primers. The phylogenetic tree for the B1 and B2 reference
219 strains and the nine ST131 variants, with regard to ST and PST (Table 2), showed that
220 B1-ST131-O25b and the other ST131 variants belonged to the B2 phylogenetic group cluster (Figure
221 3).

222

223 **Discussion**

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

229 The B2-ST131-O25b is well recognised as an international pandemic clonal group.¹ However,
230 B2-ST131-O16 has not been previously described as either a pandemic or a major clonal group. We
231 have found that 4% of ESBL-producing *E. coli* were in the B2-ST131-O16 clonal group, and are
232 characterised by fluoroquinolone susceptibility and minocycline resistance. The B2-ST131-O16
233 clonal group differs from B2-ST131-O25b clonal group by more than two loci in the Pasteur MLST
234 scheme and also differs when compared by PFGE analysis. The Clermont-*pabB* PCR for the
235 B2-ST131-O25b clonal group correctly identified the B2-ST131-O25b clonal group. However, the
236 B2-ST131-O25b clonal group carried the *pabB15* or *pabB74* alleles, and the B2-ST131-O16 clonal
237 group also carried the *pabB74* allele. The allele could be carried by B2-ST131-O25b and
238 B2-ST131-O16 because the targeted *pabB* gene segment differs in the Clermont-*pabB* PCR and
239 Pasteur MLST scheme. A recent Australian study described 211 B2-ST131-O25b and two
240 B2-ST131-O16 isolates along with one B2-ST131-O157 isolate that were identified among

241 fluoroquinolone-resistant extraintestinal *E. coli* infections from humans and companion animals.²⁵
242 Peirano et al. investigated bloodstream ESBL-producing *E. coli* in Canada and found 113 ST131
243 isolates that tested positive when subjected to Clermont-*pabB* PCR and had > 60% similarity in their
244 PFGE profiles.²⁶ The researchers also found 4 ST131 isolates that tested negative when subjected to
245 Clermont-*pabB* PCR and had < 60% similarity in their PFGE profiles with those 113 ST131 isolates.
246 These 4 isolates were possibly B2-ST131-O16 isolates. These two studies suggest the
247 B2-ST131-O16 may be a candidate for an international clonal group.

248 B2-ST131-O25b variants other than B2-ST131-O16 were also identified: B1-ST131-O25b and
249 B2-ST131-ONT. The same PST was observed among all B1-ST131-O25b and some
250 B2-ST131-O25b isolates, and these isolates had > 85% similarity in the PFGE analysis. According to
251 the population structure of *E. coli*, it is impossible that ST131 belongs to the B1 and B2 phylogenetic
252 branches of the *E. coli* population. We confirmed that three B1-ST131-O25b isolates found in this
253 study were classified into the B1 phylogenetic group by the widely used triplex PCR method.¹⁴
254 However, this method is known to be less reliable than the MLST-based method.²⁷ The phylogenetic
255 tree in Figure 3 shows that the B1-ST131-O25b isolates should be classified into the B2 phylogenetic
256 group. B2-ST131-ONT was also close to B2-ST131-O25b by the Pasteur MLST scheme and PFGE
257 analysis. In addition, both B1-ST131-O25b and B2-ST131-ONT can be detected using
258 Clermont-*pabB* PCR. When investigating the ST131 clonal group, these ST131 variants should be
259 taken into consideration. In addition to the testing for O25b *rfb*, testing for O16 *rfb* is recommended.
260 Allele-specific PCR targeting *gyrB* and *mdh* of ST131⁷ may be an alternative method to correctly
261 identify the ST131 clonal group.

262 CTX-M-15 is most closely associated with the ST131 clonal group and thus is the most widely
263 distributed CTX-M subtype. CTX-M-14 was the most prevalent among our B2-ST131-O25b isolates,
264 followed by CTX-M-27 and CTX-M-15. In a Japanese nationwide surveillance study conducted
265 between 2002 and 2003, most of the ST131 clonal group harboured CTX-M-14²⁸, which was

266 consistent with our results. However, none of the isolates belonging to the previous ST131 clonal
267 group harboured CTX-M-27 or CTX-M-15. Therefore, CTX-M-27 and CTX-M-15 emerged as new
268 ESBL types. In other studies, CTX-M-14-producing ST131 was the most prevalent isolate in Spain²⁹
269 and the second-most prevalent in Korea³⁰ and Canada²⁶. The ST131 clonal group frequently harbours
270 genes for TEM-1, OXA-1, and *aac(6')-Ib-cr*.² In our study, these associations were not observed.

271 D-ST405 was the second-most prevalent clonal group (7%) in our study. All of the D-ST405 isolates
272 were resistant to ciprofloxacin and predominantly harboured CTX-M-14 and CTX-M-15. In Korea
273 (21%)³⁰ and Canada (7%)²⁶, D-ST405 was also the second-most prevalent ESBL-producing clonal
274 group. Both studies reported CTX-M-15 and CTX-M-14 was the most prevalent ESBL. As far as we
275 know, the only study which investigated the epidemiology and ciprofloxacin susceptibility of the
276 ESBL-producing D-ST405 clonal group is a Canadian one.²⁶ This study reported that all 14 D-ST405
277 isolates were resistant to ciprofloxacin. These results suggest that the ciprofloxacin-resistant,
278 CTX-M-14- and CTX-M-15-producing D-ST405 isolates compose another pandemic clonal group.

279 D-ST69 has never been reported as an ESBL producer.⁶ We have identified seven ESBL-producing
280 D-ST69 isolates but the rate was only 1%. Only two D-ST393 isolates were identified. These clonal
281 groups were of little importance in terms of prevalence among the ESBL producers in our study.

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

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304 **Transparency Declaration**

305 None to declare.

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397 **Table 1. Characteristics of B2-ST131-O25b, B2-ST131-O16, D-ST405, D-ST69, and D-ST393 clonal groups in ESBL-producing *E. coli*.**

| Characteristics | Clonal groups, numbers of isolates (%) | | | | | | | P value | | | | |
|---------------------------------|--|----------------------------|--------------------------------------|-------------------|-----------------|------------------|---------------------|------------------|-------------------|---------------|--------------|-------------------------------|
| | B2-ST131 -O25b (n=185) | B2-ST131 -O16 (n=26) | Other ST131 ^a (n=4) | D-ST405 (n=41) | D-ST69 (n=7) | D-ST393 (n=2) | 'others' (n=316) | Overall | B2-ST131-O25b vs. | | | ST40 5 vs. 'other s' |
| | | | | | | | | B2-ST1 31-O16 | D-ST 405 | 'others' ' | 'other s' | |
| Antimicrobial resistance | | | | | | | | | | | | |
| 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%) | <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

| | | | | | | | | | | | | |
|--------------------------|----------------------------|----------|----------|-----------------------|---------|---------|---------------------------|--------|-------|--------|--------|-------|
| CTX-M-14 | 81 ^{b,c} (44%) | 19 (73%) | 2 (50%) | 30 ^b (73%) | 2 (29%) | 0 (0%) | 150 ^b (47%) | 0.001 | 0.006 | 0.001 | 0.458 | 0.002 |
| CTX-M-15 | 33 ^b (18%) | 2 (8%) | 0 (0%) | 12 ^b (29%) | 0 (0%) | 1 (50%) | 45 ^b (14%) | 0.089 | 0.265 | 0.128 | 0.308 | 0.022 |
| CTX-M-2 | 17 (9%) | 1 (4%) | 2 (50%) | 0 (0%) | 4 (57%) | 0 (0%) | 58 (18%) | <0.001 | 0.705 | 0.047 | 0.006 | 0.043 |
| CTX-M-27 | 44 (24%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 6 (2%) | <0.001 | 0.020 | <0.001 | <0.001 | 1.000 |
| CTX-M-3 | 4 ^c (2%) | 0 (0%) | 0 (0%) | 0 (0%) | 1 (14%) | 0 (0%) | 11 (3%) | 0.367 | 0.496 | 1.000 | 0.588 | 0.623 |
| CTX-M-24 | 4 (2%) | 0 (0%) | 0 (0%) | 1 (2%) | 0 (0%) | 0 (0%) | 4 (1%) | 0.715 | 0.496 | 1.000 | 0.475 | 0.459 |
| CTX-M-9 | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 9 (3%) | 0.204 | 0.127 | 1.000 | 0.030 | 0.606 |
| Other CTX-M ^d | 0 (0%) | 1 (4%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 17 (5%) | 0.013 | 0.123 | 1.000 | <0.001 | 0.237 |
| TEM-type ESBL | 2 (1%) | 1 (4%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 3 (1%) | 0.498 | 0.327 | 1.000 | 1.000 | 1.000 |
| SHV-type ESBL | 3 (2%) | 2 (8%) | 0 (0%) | 0 (0%) | 0 (0%) | 1 (50%) | 18 (6%) | 0.027 | 0.116 | 1.000 | 0.036 | 0.244 |
| Other β -lactamase | | | | | | | | | | | | |
| CMY-2 | 2 (1%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 7 (2%) | 0.813 | 0.335 | 1.000 | 0.496 | 1.000 |
| DHA-1 | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 1 (0%) | 1.000 | 0.127 | 1.000 | 1.000 | 1.000 |
| TEM-1 | 85 (46%) | 10 (38%) | 4 (100%) | 16 (39%) | 3 (43%) | 1 (50%) | 127 (40%) | 0.266 | 0.532 | 0.489 | 0.224 | 1.000 |
| OXA-1 | 7 (4%) | 0 (0%) | 0 (0%) | 3 (7%) | 0 (0%) | 0 (0%) | 7 (2%) | 0.425 | 1.000 | 0.394 | 0.400 | 0.095 |

PMQR determinants

| | | | | | | | | | | | | |
|----------------------|---------|--------|--------|---------|--------|--------|---------------------|-------|-------|-------|-------|-------|
| <i>qnr</i> | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 5 ^e (2%) | 0.465 | 0.127 | 1.000 | 0.163 | 1.000 |
| <i>aac(6')-Ib-cr</i> | 16 (9%) | 0 (0%) | 0 (0%) | 4 (10%) | 0 (0%) | 0 (0%) | 16 (5%) | 0.419 | 0.703 | 0.766 | 0.131 | 0.267 |

398 ^a Three B1-ST131-O25b isolates and one B2-ST131-ONT isolate were included.

399 ^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.

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

401 ^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),
 402 CTX-M-44 (n=1), and CTX-M-126 (n=1) were identified in the Others group.

403 ^e Three isolates were positive for *qnrS*, and two isolates were positive for *qnrB*.

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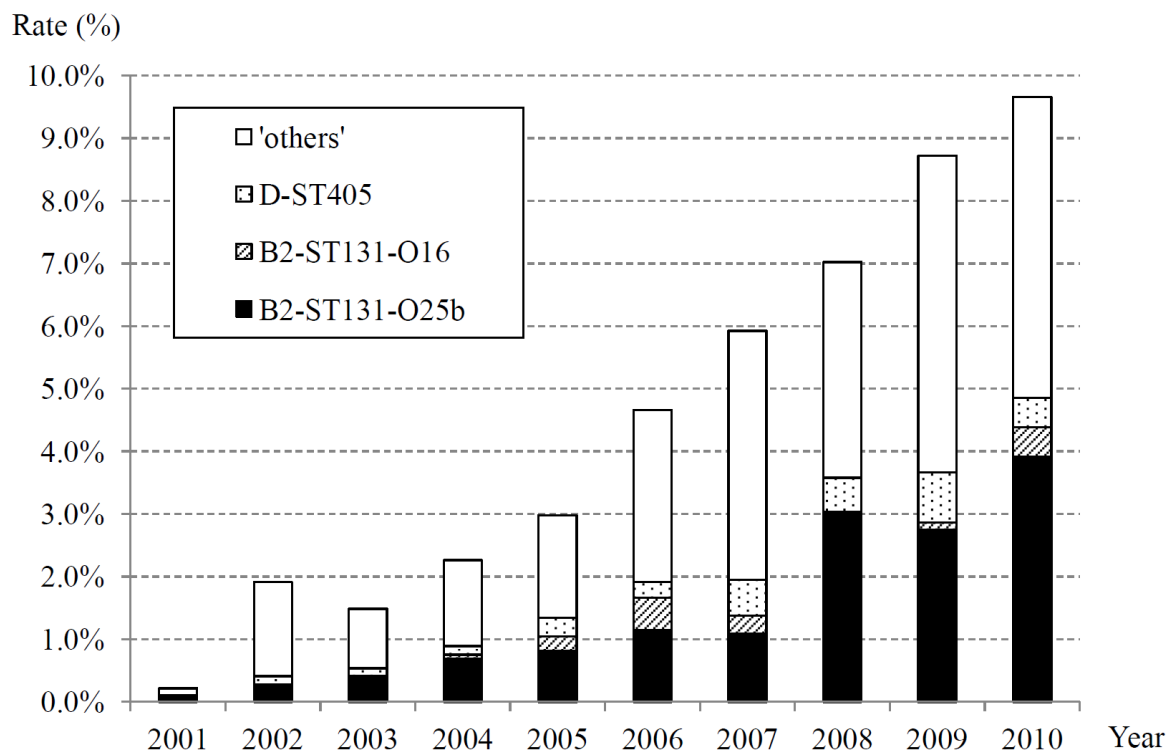
406 **Table 2. Allele profiles of the ST131 clonal group among the ESBL-producing *E. coli***

| Number of isolates | Phylogenetic group | PCR O type | Achtman ST | Pasteur MLST | | | | | | | | |
|--------------------------|-----------------------|---------------|---------------|--------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| | | | | ST | <i>dinB</i> | <i>icdA</i> | <i>pabB</i> | <i>polB</i> | <i>putP</i> | <i>trpA</i> | <i>trpB</i> | <i>uidA</i> |
| 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 |

407 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
 408 B1-ST131-O25b, and 1 B2-ST131-O-non-typeable isolates were subjected to MLST analysis.

409

410 **Figure legends**

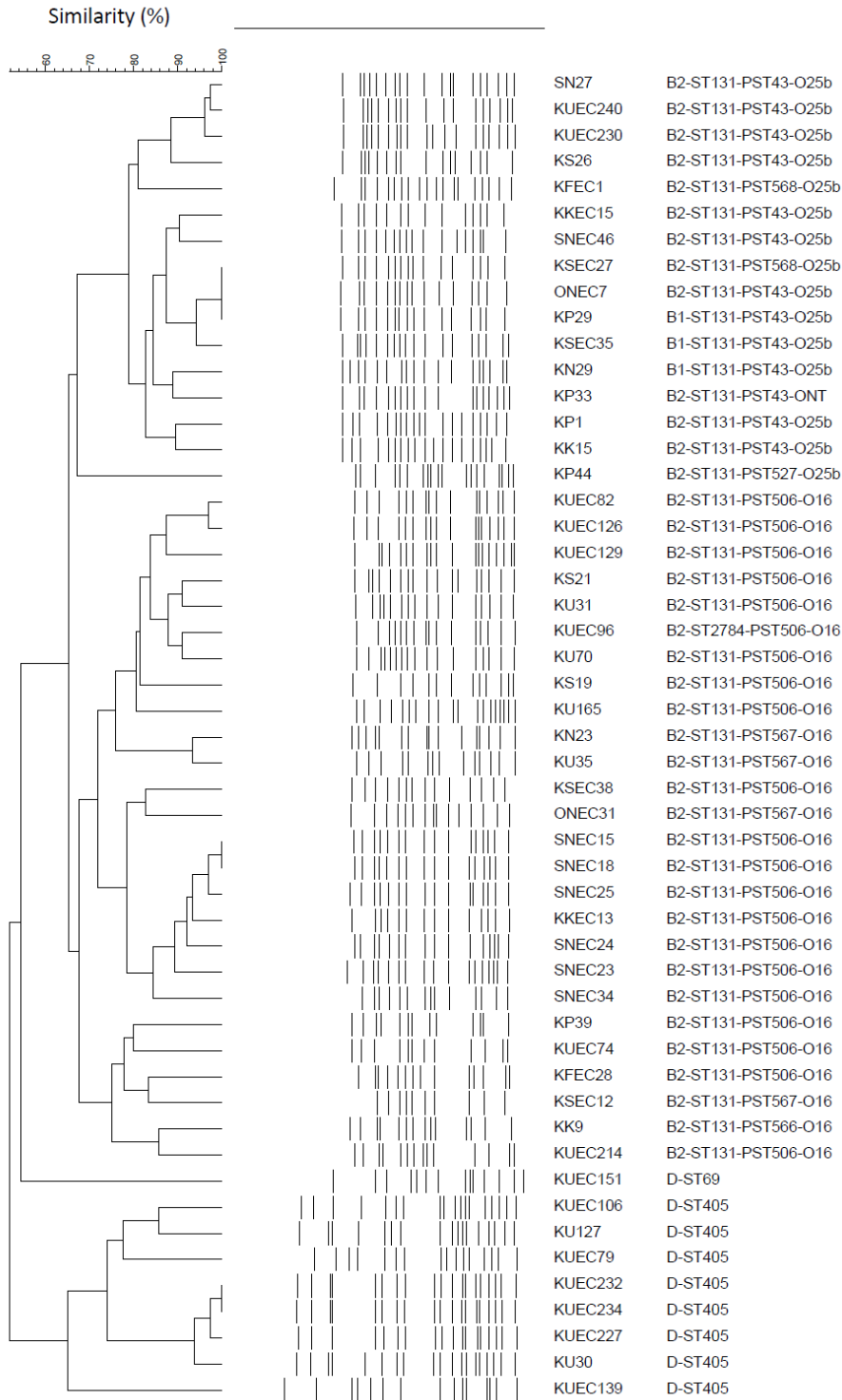


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412 Figure 1. Rates of the B2-ST131-O25b, B2-ST131-O16, and D-ST405 clonal groups among ESBL-producing

413 *E. coli* from 2001 to 2010.

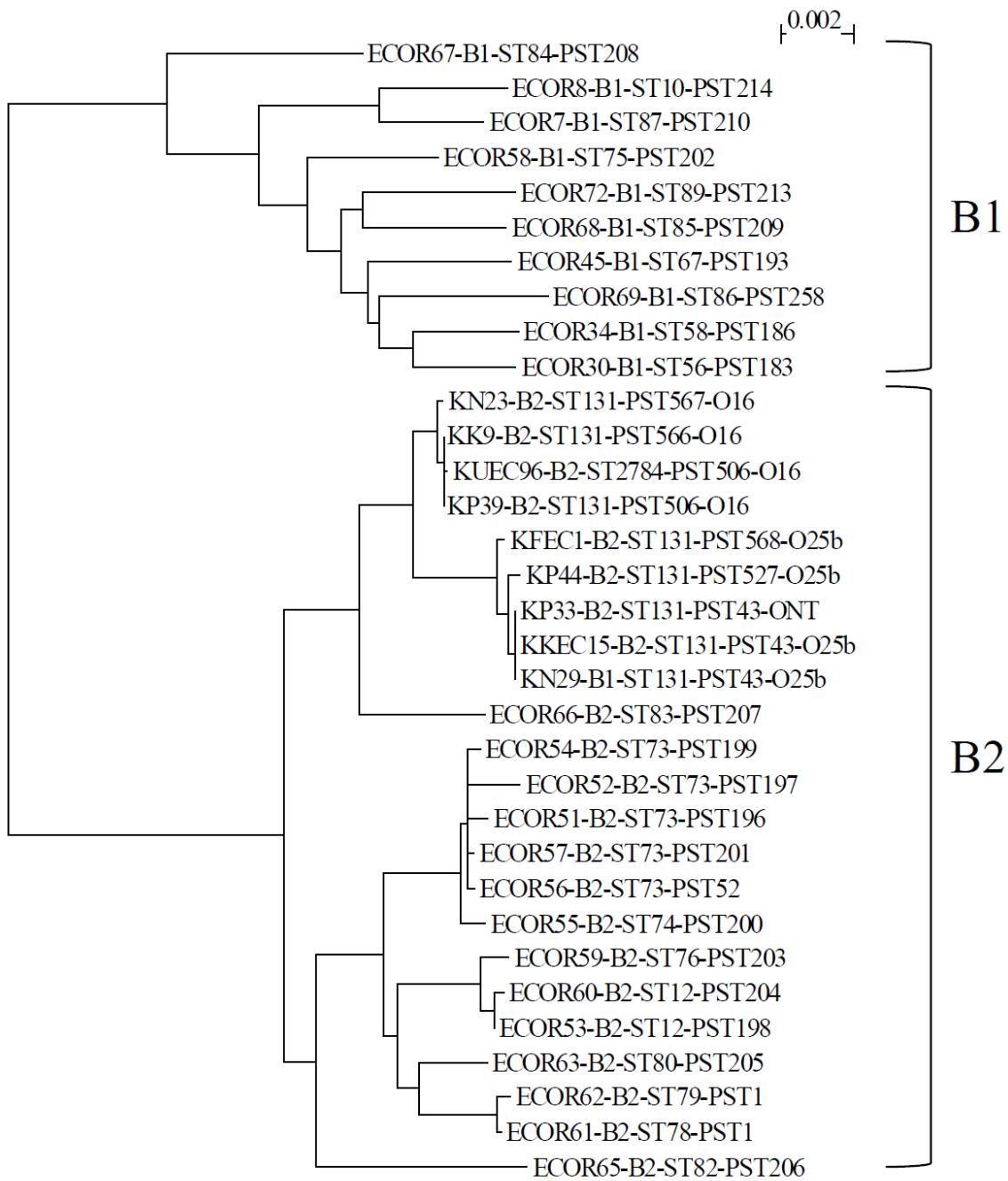
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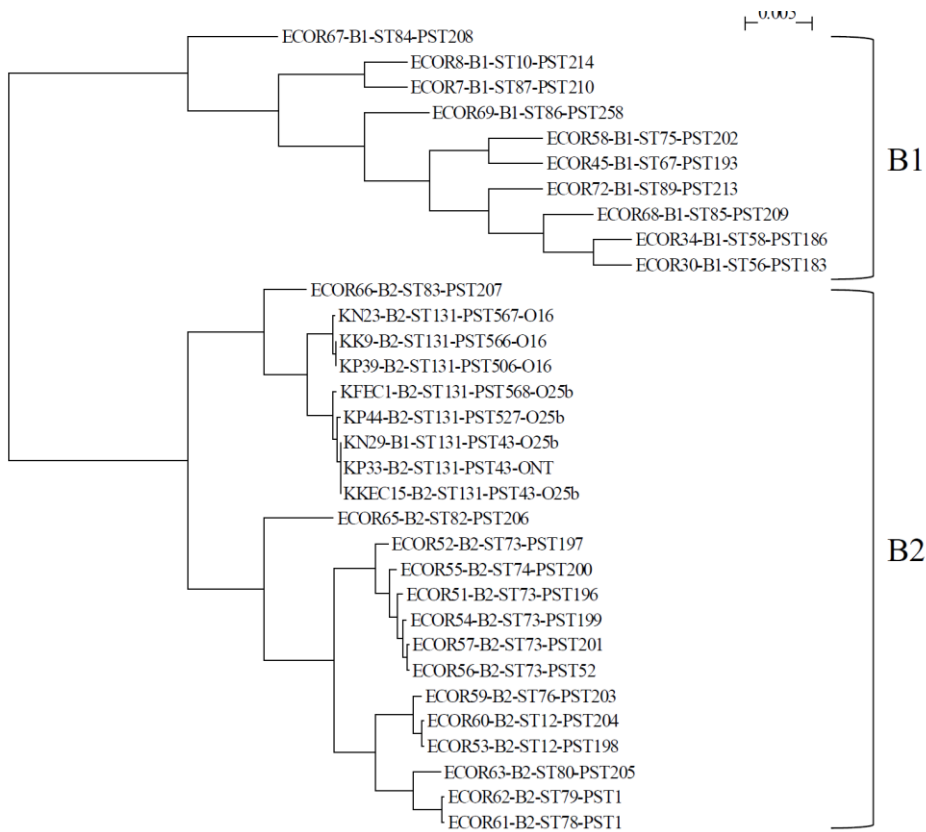
415

416 Figure 2. PFGE of *Xba*I-digested DNA from the ST131, D-ST405, and D-ST69 clonal groups. In addition to
 417 all of the B2-ST131-O16, B1-ST131-O25b, and B2-ST131-O-non-typeable isolates, 12 B2-ST131-25b, eight
 418 D-ST405, and one D-ST69 isolate identified using the Achtman MLST scheme were included. Two D-ST393
 419 isolates were non-typeable. ST indicate the STs under the Achtman scheme, whereas PST indicates the STs
 420 under the Pasteur scheme.

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425 Figure 3. Phylogenetic tree of the ECOR reference strains of B1 and B2 phylogenetic groups and the nine
 426 variants of ST131 clonal group detailed in Table 2. This tree was constructed from the concatenated nucleotide
 427 sequence of the seven genes used in the Achtman scheme and the eight genes used in the Pasteur scheme with
 428 the neighbour-joining method.

429