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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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

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.

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.

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%

in 2010) and corresponded to increases in the numbers of B2-ST131-O25b, B2-ST131-O16, and

37 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.

41 Introduction

In recent years, the prevalence of extended-spectrum β -lactamase (ESBL)-producing 42*Escherichia coli* has increased dramatically worldwide.¹ Clonal group detection by multilocus 4344 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 45(ST131) belonging to the O25b serogroup and the B2 phylogenetic group, has contributed greatly to 46 the pandemic.² The success of the ST131 clonal group is explained by its acquisition of 47fluoroquinolone resistance and additional virulence factors.² In addition to the ST131 clonal group, a 48 49 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 50investigated. Detailed studies on the ST131 clonal group among ESBL-producing E. coli in Japan are 5152lacking.

Other clonal groups disseminated worldwide include D-ST393-O15 and D-ST69.⁴⁻⁶ The D-ST393-O15 clonal group is characterised by K52:H1 serotypes and fluoroquinolone resistance.⁷ CTX-M-14 producers have also been identified.⁸ The D-ST69 clonal group is known as "clonal group A" and is frequently found among trimethoprim-sulfamethoxazole-resistant uropathogenic *E*. *coli.*⁹ 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.⁶

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.

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68 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.

76Identification and susceptibility testing. At each hospital, microbiological identification and 77susceptibility testing were performed using the Vitek2 system (bioMérieux, Marcy l'Etoile, France) or the MicroScan system (Siemens Healthcare diagnostics, Tokyo, Japan). Subsequently, the ESBL 7879 screening test was performed according to the CLSI microdilution methodology (cefotaxime, 80 ceftriaxone, ceftazidime, cefpodoxime, and aztreonam), and the ESBL confirmation test was performed using the double-disk synergy test following the CLSI guidelines.¹⁰ In a reference 81 laboratory, the antibiotic susceptibility was re-evaluated by microdilution using Dry Plate Eiken 82 (Eiken, Tokyo, Japan) and included testing with ampicillin-sulbactam, piperacillin-tazobactam, 83 ciprofloxacin, gentamicin, tobramycin, amikacin, imipenem, meropenem, minocycline, and 84 trimethoprim-sulfamethoxazole. The results were interpreted using the 2012 CLSI breakpoints.¹⁰ 85 Intermediate susceptibility to each antibiotic was considered to be resistance. 86 β-lactamase identification. The presence of ESBL or plasmid-mediated AmpC β-lactamase 87

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.
- Isolates that were positive for Clermont-*pabB* PCR but non-B2 or non-O25b were also subjected toMLST.
- 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 107 108allele 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 109 isolates were confirmed by MLST. The primers adk35f (5'-TGGCAAACTGGTCACT-3') and 110 111 adk35r (5'-CGTTGACCGTATCGTC-3') were designed for the detection of ST405-associated 112single-nucleotide polymorphisms in *adk*35 (i.e., C148T, T316C, T322C, and A331C). Amplification was performed with 1X PCR buffer, 2 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates, 0.4 µM 113114of 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, 115

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.

- 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 *fumC*106-positive isolates were confirmed as ST393 clonal group by MLST. 120For the detection of the D-ST69 clonal group, fumC35 allele-specific PCR was performed as 121described previously.¹⁸ Phylogenetic group D and *fumC*35 PCR-positive isolates were sequenced to 122determine the *fumC* and *gyrB* allele profiles. The *fumC*35 and *gyrB*27 isolates were considered to 123124comprise 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) 125using seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*).¹⁹ The ST131 isolates 126determined by the Achtman scheme were further characterised by the Pasteur MLST scheme 127(http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html) using eight housekeeping genes 128129(*dinB*, *icdA*, *pabB*, *polB*, *putP*, *trpA*, *trpB*, and *uidA*). PCR genomic fingerprinting and PFGE. RAPD fingerprinting using a DAF4 primer²⁰ was 130performed for the ESBL-producing isolates obtained in 2010 to analyse the genetic relatedness of the 131B2-ST131-O25b clonal group. Genomic DNA from all of the isolates subjected to the Achtman 132MLST underwent *Xba*I PFGE.²¹ The profiles obtained from RAPD or PFGE were analysed with 133GelCompar II, version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was 134135applied using the unweighted pair-group method based on Dice coefficients to quantify the
- 136 similarities.

137 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.¹⁴ In addition, other

141	primer pairs ²² for the <i>chuA</i> 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.

Plasmid-mediated quinolone resistance determinants. All of the ESBL-producing isolates were 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**

155Recognition of the B2-ST131-O16 clonal group. The PCR analysis detected 185 ESBL-producing E. coli isolates in 2010. PCR O-typing identified 77 O25b isolates, 15 O1 isolates, and nine O16 156isolates that represented three major PCR O-types. The Clermont-pabB PCR-positive isolates 157included 75 B2-ST131-O25b isolates, two B1-ST131-O25b isolates, and one B2-ST131-ONT 158(O-non-typeable) isolate. RAPD analysis indicated two large clusters (comprised of 86 and 76 159160isolates) 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 isolates, one B2-ST131-ONT isolate, seven B2-O16 isolates, one B2-O6 isolate, and one B2-ONT 162163 isolate. These results prompted us to perform MLST analysis of the B2-O16 isolates. All of the nine 164B2-O16 isolates, including seven isolates in the largest cluster and two isolates in the second largest 165cluster, 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

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,

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

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).

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

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

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,

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.

Table 1 lists the antimicrobial susceptibilities and resistance genes of the clonal groups, includingothers 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
- 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

191B2-ST131-O25b clonal group (6%) was less frequently resistant to piperacillin-tazobactam than the 192D-ST405 clonal group (17%). CTX-M-14 (44%), CTX-M-27 (24%), and CTX-M-15 (18%) were the 193most frequent ESBLs noted in the B2-ST131-O25b clonal group. However, the B2-ST131-O16 and 194D-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 195196 41 D-ST405 isolates were resistant to ciprofloxacin. In addition, they were more frequently resistant 197to 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 rate to trimethoprim-sulfamethoxazole (57%) was similar to other isolates. CTX-M-2 was the most 200201prevalent ESBL type (57%). Both D-ST393 isolates were resistant to ciprofloxacin and tested 202positive for CTX-M-15- or SHV-type ESBL.

Pasteur MLST analysis of ST131 clonal group. All of the ST131 isolates identified by the 203204Achtman MLST scheme were subjected to a Pasteur MLST analysis (Table 2). Nine of 12 205B2-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 206207 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 208belonged to PST506. The other five B2-ST131-O16 isolates belonged to novel PSTs, PST566 or 209 210PST567, both of which were SLVs of PST506.

211 **PFGE analysis.**

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.

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.**

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).

222

223 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.¹ However, 229230B2-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 231232characterised by fluoroquinolone susceptibility and minocycline resistance. The B2-ST131-O16 233clonal group differs from B2-ST131-O25b clonal group by more than two loci in the Pasteur MLST 234scheme 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 235B2-ST131-O25b clonal group carried the *pabB*15 or *pabB*74 alleles, and the B2-ST131-O16 clonal 236237group 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 238Pasteur MLST scheme. A recent Australian study described 211 B2-ST131-O25b and two 239B2-ST131-O16 isolates along with one B2-ST131-O157 isolate that were identified among 240

fluoroquinolone-resistant extraintestinal *E. coli* infections from humans and companion animals.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.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 248B2-ST131-ONT. The same PST was observed among all B1-ST131-O25b and some 249250B2-ST131-O25b isolates, and these isolates had > 85% similarity in the PFGE analysis. According to 251the 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 252study were classified into the B1 phylogenetic group by the widely used triplex PCR method.¹⁴ 253However, this method is known to be less reliable than the MLST-based method.²⁷ The phylogenetic 254tree in Figure 3 shows that the B1-ST131-O25b isolates should be classified into the B2 phylogenetic 255group. B2-ST131-ONT was also close to B2-ST131-O25b by the Pasteur MLST scheme and PFGE 256analysis. In addition, both B1-ST131-O25b and B2-ST131-ONT can be detected using 257Clermont-pabB PCR. When investigating the ST131 clonal group, these ST131 variants should be 258taken into consideration. In addition to the testing for O25b *rfb*, testing for O16 *rfb* is recommended. 259Allele-specific PCR targeting gyrB and mdh of ST131⁷ may be an alternative method to correctly 260261identify 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-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 Spain ²⁹
269	and the second-most prevalent in Korea ³⁰ and Canada ²⁶ . The ST131 clonal group frequently harbours
270	genes for TEM-1, OXA-1, and <i>aac(6')-Ib-cr</i> . 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

None to declare.

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Characteristics	Clonal grou	ips, numbers	of isolates (%	%)				P value				
	B2-ST131	B2-ST131	Other									ST40
	-O25b	-016	ST131 ^a	D-ST405	D-ST69	D-ST393	'others'		B2-ST131-O25b vs.			5 vs.
								Overall	B2-ST1	D-ST	[•] others	• other
	(n=185)	(n=26)	(n=4)	(n=41)	(n=7)	(n=2)	(n=316)		31-016	405	,	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%)	(78%) 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	< 0.001	< 0.001	0.047	< 0.001	< 0.00
Gentamicin	50 (27%)	3 (12%)	2 (50%)	17 (41%)	2 (29%)	0 (0%)	(47%) 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	< 0.00
Amikacin	1 (1%)	0 (0%)	0 (0%)	2 (5%)	0 (0%)	0 (0%)	4 (1%)	0.318	1.000	0.086	0.656	1 0.143
Minocycline	22 (12%)	15 (58%)	1 (25%)	9 (22%)	2 (29%)	2 (100%)	115	< 0.001	< 0.001	0.128	< 0.001	0.081
Trimethoprim-	95 (51%)	13 (50%)	1 (25%)	28 (68%)	4 (57%)	2 (100%)	(36%) 186 (59%)	0.195	1.000	0.057	0.113	0.310

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*.

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}	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%)	(14%) 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	< 0.00	< 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	0.266	0.532	0.489	0.224	1.000
OXA-1	7 (4%)	0 (0%)	0 (0%)	3 (7%)	0 (0%)	0 (0%)	(40%) 7 (2%)	0.425	1.000	0.394	0.400	0.095

PMQR determinants

qnr	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
aac(6')-Ib-cr	16 (9%)	0 (0%)	0 (0%)	4 (10%)	0 (0%)	0 (0%)	16 (5%)	0.419	0.703	0.766	0.131	0.267

^a 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.

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*.

404

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

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

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.



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*I-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.

 $\begin{array}{c} 421 \\ 422 \end{array}$







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.