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Short communication

Title: Prevalence of plasmid-mediated AmpC β-lactamase-producing *Escherichia coli* and spread of the ST131 clone among extended-spectrum β-lactamase-producing *E. coli* in Japan

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Abstract

In 2010, a total of 1327 clinical *Escherichia coli* isolates were analysed by PCR in 5 hospitals in the Kyoto and Shiga regions of Japan. The prevalence of plasmid-mediated AmpC β-lactamase (pAmpC) producers, extended-spectrum β-lactamase (ESBL) producers, and co-producers of pAmpC and ESBL were 1.7%, 9.7%, and 0.3%, respectively. Less than half of the pAmpC producers were reported to be resistant to third-generation cephalosporins, cephamycins, and β-lactam/β-lactam inhibitors with the old CLSI breakpoints in 2009. CMY-2 was the most prevalent pAmpC type (95%), and CTX-M-14 (38%), CTX-M-15 (26%), and CTX-M-27 (19%) were the most prevalent ESBL types. The worldwide O25b-ST131-B2 clone accounted for 11% of pAmpC producers and 41% of ESBL producers. The O25b-ST131-B2 clone was characterised by a CTX-M-27 or CTX-M-15 type ESBL and ciprofloxacin non-susceptibility with quadruple mutations in quinolone resistance-determining regions (S83L and D87N in GyrA and S80I and E84V in ParC). A significant proportion of pAmpC producers and the O25b-ST131-B2 clone were found in Japan by a recent regional surveillance program.

Keywords: ESBL, AmpC, ST131, CTX-M-27, prevalence

1. Introduction

In recent years, the prevalence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* has increased dramatically worldwide [1]. A CTX-M-15 ESBL-producing *E. coli* with sequence type 131 (ST131) belonging to the O25b serogroup and the B2 phylogenetic group has emerged as an international pandemic clone[2]. The prevalence of plasmid-mediated AmpC β-lactamase (pAmpC)-producing *E. coli* has likewise been increasing [3]. As standard guidelines for detecting pAmpC remain unavailable, pAmpC producers are rarely identified in routine laboratory practices. However, the current data for the ST131 clone and pAmpC-producing *E. coli* in Japan are poor. In this study, we investigated the prevalence and characteristics of the ST131 clone and pAmpC-producing *E. coli* in the Kyoto and Shiga regions of Japan.

2. Materials and methods

2.1. Bacterial isolates

This study was conducted at 5 acute care hospitals in Japan: 3 municipal hospitals and 2 university hospitals in the Kyoto and Shiga regions of Japan. All of the *E. coli* isolates collected from both in-patients and out-patients between June 2010 and December 2010 were eligible for the study. In each hospital, microbiological speciation was conducted using the Vitek2 system (bioMérieux, Marcy l'Etoile, France) or the MicroScan system (Siemens Healthcare diagnostics, Tokyo, Japan). The ESBL screening test was performed according to the CLSI microdilution methodology (cefotaxime, ceftriaxone, ceftazidime, cefpodoxime, or aztreonam) [4].

2.2. Molecular analysis

Only the first isolate from each patient that was positive in the ESBL screen was sent to a reference laboratory (Kyoto University) and subjected to PCR amplification and sequencing of the bla_{SHV}, bla_{TEM}, bla_{OXA-1}, and bla_{CTX-M} genes and the 6 main groups of pAmpC-type genes [5]. All of the isolates with ESBL or pAmpC genes were further characterised based on their plasmid-mediated

quinolone resistance determinants (*qnrA*, *qnrB*, *qnrC*, *qnrS*, and *aac(6')-Ib-cr*) [5], their phylogenetic groups using triplex PCR (A, B1, B2, D, and non-typable) [5], integrases [6], and plasmid replicon typing [7] as has been previously described. Isolates that belonged to phylogenetic group B2 and were O25b PCR positive and O25b-*pabB* PCR positive were considered to belong to the ST131 clone [8]. Five selected ST131 isolates identified by these presumptive methods were confirmed by multilocus sequence typing according to the *E. coli* MLST Web site (http://mlst.ucc.ie/mlst/dbs/Ecoli). Random amplified polymorphic DNA (RAPD) fingerprinting using a DAF4 primer was also performed [5], and the profiles were analysed by GelCompar II, version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium). Isolates with 100% similarity were designated as indistinguishable following the criteria by Tenover et al. [9]. Ciprofloxacin-non-susceptible isolates were sequenced to determine the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* [10], and the correlated amino acids were compared with the corresponding regions of *E. coli* K-12 (GenBank accession no. NC000913).

2.3. Antimicrobial susceptibility testing

The antibiotic susceptibility was evaluated by microdilution using Dry Plate Eiken (Eiken, Tokyo, Japan) following CLSI specifications and interpreted according to the 2009 and 2011 CLSI criteria [4]. The ESBL confirmation test was performed using the double-disk synergy test following the CLSI guidelines [4].

2.4. Statistical analysis

Categorical variables were compared using the Fisher's exact test. A *P* value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Prevalences of pAmpC or ESBL producers

During the study period, *E. coli* was isolated from a total of 1327 patients from 5 hospitals (Table 1). Of those isolates, 172 (13.0%) were positive in the ESBL screen. The PCR analysis identified 23

pAmpC producers and 129 ESBL producers, 4 of which were positive for both the pAmpC and the ESBL genes. The remaining 24 isolates contained neither pAmpC nor ESBL. The prevalence of pAmpC producers was 1.7%. CMY-2 was the most prevalent pAmpC type. Surveillance conducted between 2002 and 2008 in the Kinki region, which includes our study sites, showed that CMY-2 type was most prevalent [11]. However, the prevalence rate from our data (1.7%) seems to be substantially higher (0.1%) than that found previously. One possible explanation for this difference is that the prevalence has varied over time. We did not assess the yearly variation, but the prevalence of pAmpC producers has been increasing; for example, a Spanish study reported that the prevalence increased from 0.04% in 1997 to 1.1% in 2007. The prevalence of ESBL producers was 9.7%. In 2003, inpatient urine collected in 37 hospitals in Japan was studied, and the prevalence was 14% [12]. The SMART surveillance in 2009 reported a diverse prevalence within the Asia-Pacific region that ranged from 2.0% in Australia to 65.4% in China [13]. The prevalence of ESBL producers and pAmpC producers were higher in the 2 university hospitals than in the 3 municipal hospitals, which may be associated with the fact that university hospitals had less frequent community-acquired infections and had patients with more severe underlying diseases than municipal hospitals.

3.2. Antimicrobial susceptibility

Table 2 shows the characteristics of the pAmpC producers and the ESBL producers. The most frequent isolation source was urine. All of the isolates were susceptible to imipenem (minimum inhibitory concentration ≤ 1 μg/mL). Almost all of the ESBL producers were judged to be resistant to third-generation cephalosporins by the old CLSI breakpoints (prior to 2010) due to the positive results of the ESBL confirmation test. However, the revised breakpoints classified 66% of ESBL producers as susceptible to ceftazidime. This finding can be explained by the fact that 87% (42/48) of CTX-M-14-producers and 13% (4/31) of CTX-M-15-producers were susceptible to ceftazidime. This phenomenon is worth noting when implementing the revised breakpoints where CTX-M-14 is prevalent.

Less than one half of the pAmpC producers were determined to be resistant to

121 third-generation cephalosporins by old breakpoints, as all the isolates were negative in the ESBL
122 confirmation test. The revised breakpoints correctly classified more than 90% of the pAmpC
123 producers as resistant to third-generation cephalosporins. In Japan, the old breakpoints are still used,
124 and the phenotype tests for the detection of pAmpC producers are rarely conducted. Furthermore,
125 pAmpC producers are likely resistant to cefmetazole and piperacillin-tazobactam because of the
126 activity of the pAmpC enzyme [3]. However, fewer than half were judged to be resistant. When ESBL
127 screening-positive and ESBL confirmation-negative isolates are detected, the use of third-generation
128 cephalosporins, cephamycins, or β-lactam/β-lactam inhibitors requires caution irrespective of the
129 susceptibilities of the isolates because the clinical efficacies of these drugs have not yet been
130 established.
131 Twenty-four isolates without pAmpC or ESBL genes had reduced susceptibility rates to
132 β-lactam/β-lactam inhibitors (29% for ampicillin/sulbactam and 46% for piperacillin/tazobactam), and
133 elevated chromosomal AmpC was a suggested mechanism of resistance.
134
135 **3.3 Phylogenetic group, CTX-M type, and the ST131 clone isolates**
136 Virulent phylogenetic groups B2 and D were prevalent in both pAmpC and ESBL producers.
137 The CTX-M type, which is associated with the international emergence of the O25b-ST131-B2 clone,
138 is now spreading worldwide [1]. CTX-M-15 is most closely associated with the ST131 clone, and thus
139 is the most widely distributed CTX-M subtype. In our study, CTX-M-14 was the most prevalent
140 ESBL, and CTX-M-15 was the second prevalent. Among 125 ESBL producers, 51 isolates of the
141 ST131 clone (41%) were found. CTX-M-27 (41%) and CTX-M-15 (28%) were the most prevalent
142 ESBLs in the ST131 clone isolates; however, CTX-M-27 was rarely found in non-ST131 clone
143 isolates (2%). CTX-M-14 was the most frequent ESBL in non-ST131 isolates (41%). In the previous
144 Japanese nationwide surveillance study, a significant portion of ESBL producers belonged to the
145 ST131 and ST38 clones (approximately 20% each [14]. Most of the ST131 clone isolates contained
146 CTX-M-14, but none of them contained CTX-M-15 or CTX-M-27 [14]. The prevalence of the ST131
147 clone has doubled, and the distribution of CTX-M types was quite different from that found in a

previous study. RAPD analysis showed that 135 of the 148 pAmpC or ESBL producers had distinguishable patterns. All of the 13 other isolates belonged to the ST131 clone and were composed of 1 cluster of 3 isolates and 5 clusters of 2 isolates. These results suggest that the ST131 clone is a dominant and unique clone among ESBL producers in our region.

The ST131 clone frequently contained genes for TEM-1, OXA-1, *aac(6')-Ib-cr*, and ciprofloxacin resistance [2]. In our study, the ST131 clone isolates had a higher ciprofloxacin non-susceptible rate (85%) than non-ST131 clone isolates (46%). Table 3 shows all of the ciprofloxacin non-susceptible isolates that had at least 3 mutations in QRDRs. All of the 46 ciprofloxacin non-susceptible ST131 clone isolates had double mutations both in GyrA (S83L and D87N) and ParC (S80I and E84V). This genotype was rarely found in the previous study in Asia [10]. On the contrary, 30 of 43 (70%) non-ST131 clone isolates had double mutations in GyrA (S83L and D87N) and a single mutation in ParC (S80I). This genotype was found worldwide, including in Asia [10]. A significantly smaller number of the ST131 clone isolates had TEM-1, which differed from previous studies [2]. The ST131 clone isolates frequently contained OXA-1 and *aac(6')-Ib-cr*, but the difference was not statistically significant. The ST131 clone isolates more frequently contained plasmid replicons for both IncFIA and IncFIB. Associations between CTX-M-15 producers and both IncFIA and IncFIB have been reported [7].

The ST131 clone accounted for only 2 of 19 pAmpC producing-isolates. These two isolates were susceptible to ciprofloxacin. The low prevalence of pAmpC-producing ST131 is consistent with the results of studies from Europe, which found a prevalence of less than 10% [15]. IncI1 was more frequently found in pAmpC producers than in ESBL producers. A Norwegian study reported a high prevalence of IncI1 among CMY-2 producers [15].

4. Conclusion

We found that 1.7% of *E. coli* isolates from clinical specimens were pAmpC producers. Among the ESBL producers, 41% were isolates of the ST131 clone, and these isolates were characterised by ciprofloxacin non-susceptibility with quadruple mutations in QRDRs, the presence of

175 CTX-M-27, the absence of TEM-1, and the plasmid replicons for IncFIA and IncFIB.

176

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180 Competing interests: None declared.

181 Ethical approval: Not required.

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225
226

228 **Table 1. Prevalences of ESBL-producing and pAmpC-producing *E. coli* in each hospital.**

Hospital	Type of hospital	All isolates	ESBL screening test positive		ESBL confirmation test positive		ESBL[a]		pAmpC[a]		Both pAmpC and ESBL	
A	Municipal	350	35	(10.0%)	20	(5.7%)	20	(5.7%)	4	(1.1%)	1	(0.3%)
B	University	253	42	(16.6%)	33	(13.0%)	32	(12.6%)	7	(2.8%)	2	(0.8%)
C	Municipal	173	18	(10.4%)	17	(9.8%)	17	(9.8%)	1	(0.6%)	0	(0.0%)
D	Municipal	272	28	(10.3%)	23	(8.5%)	23	(8.5%)	5	(1.8%)	1	(0.4%)
E	University	279	49	(17.6%)	38	(13.6%)	37	(13.3%)	6	(2.2%)	0	(0.0%)
A, C, and D	Municipal	795	81	(10.2%)	60	(7.5%)	60	(7.5%)	10	(1.3%)	2	(0.3%)
B and E	University	532	91	(17.1%)	71	(13.3%)	69	(13.0%)	13	(2.4%)	2	(0.4%)
Total		1327	172	(13.0%)	131	(9.9%)	129	(9.7%)	23	(1.7%)	4	(0.3%)

229 All of the first isolates from each patient that were positive in the ESBL screening test were collected. The prevalences of ESBL producers and
230 pAmpC producers were higher in the 2 university hospitals than in the 3 municipal hospitals (P=0.001 and P=0.13, respectively).

231 [a] The numbers included co-producers of pAmpC and ESBL.

232

233

234 **Table 2. Sources, in-vitro susceptibilities, and molecular characteristics of pAmpC-producing and ESBL-producing *E. coli*.**

Characteristic	pAmpC only (n=19)	ESBL only (n=125)	pAmpC and ESBL (n=4)	*P* value[a]	ST131 clone (n=54)	Non-ST 131 clone (n=94)	*P* value
Source of isolates							
Urine	11 (58%)	83 (66%)	2 (50%)	0.61	40 (74%)	56 (60%)	0.11
Pus	4 (21%)	14 (11%)	0 (0%)	0.26	7 (13%)	11 (12%)	0.80
Blood	1 (5%)	9 (7%)	1 (25%)	1	1 (2%)	10 (11%)	0.06
Sputum	0 (0%)	5 (4%)	0 (0%)	1	5 (9%)	0 (0%)	0.006
Bile	0 (0%)	4 (3%)	1 (25%)	1	0 (0%)	5 (5%)	0.16
Others	3 (16%)	10 (8%)	0 (0%)	0.38	1 (2%)	12 (13%)	0.03
In-vitro susceptibility							
Cefepime	19 (100%)	86 (69%)	3 (75%)	0.002	39 (72%)	69 (73%)	1
Cefepime (old BP)	19 (100%)	0 (0%)	1 (25%)	<0.001	2 (4%)	18 (19%)	0.01
Cefotaxime	0 (0%)	2 (2%)	0 (0%)	1	1 (2%)	1 (1%)	1
Cefotaxime (old BP)	15 (79%)	0 (0%)	1 (25%)	<0.001	2 (4%)	14 (15%)	0.05

Ceftazidime	1	(5%)	82	(66%)	0	(0%)	<0.001	30	(56%)	53	(56%)	1
Ceftazidime (old BP)	9	(47%)	0	(0%)	1	(25%)	<0.001	1	(2%)	9	(10%)	0.09
Aztreonam	12	(63%)	50	(40%)	1	(25%)	0.08	21	(39%)	42	(45%)	0.61
Aztreonam (old BP)	15	(79%)	0	(0%)	1	(25%)	<0.001	2	(4%)	14	(15%)	0.05
Cefmetazole	10	(53%)	122	(98%)	2	(50%)	<0.001	52	(96%)	82	(87%)	0.08
Ampicillin-sulbactam	1	(5%)	45	(36%)	0	(0%)	0.007	25	(46%)	21	(22%)	0.003
Piperacillin-tazobactam	11	(58%)	96	(77%)	1	(25%)	0.09	47	(87%)	61	(65%)	0.004
Imipenem	19	(100%)	125	(100%)	4	(100%)	1	54	(100%)	94	(100%)	1
Amikacin	19	(100%)	125	(100%)	4	(100%)	1	54	(100%)	94	(100%)	1
Gentamicin	18	(95%)	103	(82%)	4	(100%)	0.31	48	(89%)	77	(82%)	0.35
Ciprofloxacin	14	(74%)	43	(34%)	2	(50%)	0.002	8	(15%)	51	(54%)	<0.001
Trimethoprim-sulfamethoxazole	10	(53%)	67	(54%)	2	(50%)	1	31	(57%)	48	(51%)	0.50
Minocycline	11	(58%)	93	(74%)	2	(50%)	0.17	46	(85%)	60	(64%)	0.008
Colistin	19	(100%)	125	(100%)	4	(100%)	1	54	(100%)	94	(100%)	1
ESBL confirmation test	0	(0%)	125	(100%)	3	(75%)	<0.001	52	(96%)	76	(81%)	0.01

Resistance gene											
CMY-2	18	(95%)	0	(0%)	4	(100%)	<0.001	2	(4%)	20	(21%)	0.003
DHA-1	1	(5%)	0	(0%)	0	(0%)	0.14	1	(2%)	0	(0%)	0.37
CTX-M-14[b]	0	(0%)	50	(40%)	0	(0%)	<0.001	11	(20%)	39	(41%)	0.01
CTX-M-15[b]	0	(0%)	33	(26%)	1	(25%)	0.007	15	(28%)	18	(19%)	0.31
CTX-M-27	0	(0%)	24	(19%)	0	(0%)	0.04	22	(41%)	2	(2%)	<0.001
CTX-M-2	0	(0%)	10	(8%)	1	(25%)	0.36	1	(2%)	10	(11%)	0.06
CTX-M-24	0	(0%)	4	(3%)	0	(0%)	1	2	(4%)	2	(2%)	0.62
CTX-M-1	0	(0%)	2	(2%)	2	(50%)	1	0	(0%)	2	(2%)	1
CTX-M-3	0	(0%)	0	(0%)	1	(25%)	1	0	(0%)	1	(1%)	0.37
CTX-M-9	0	(0%)	2	(2%)	0	(0%)	1	0	(0%)	1	(1%)	0.37
CTX-M-44	0	(0%)	1	(1%)	1	(25%)	1	0	(0%)	1	(1%)	0.37
CTX-M-65	0	(0%)	1	(1%)	0	(0%)	1	0	(0%)	1	(1%)	0.37
SHV type ESBL	0	(0%)	3	(2%)	0	(0%)	1	1	(2%)	2	(2%)	1
TEM-1	8	(42%)	45	(36%)	2	(50%)	0.62	12	(22%)	43	(46%)	0.005
OXA-1	0	(0%)	4	(3%)	0	(0%)	1	3	(6%)	1	(1%)	0.14

qnr[c]	1	(5%)	3	(2%)	0	(0%)	0.44	1	(2%)	3	(3%)	0.46
aac(6')-Ib-cr	0	(0%)	5	(4%)	0	(0%)	1	4	(7%)	1	(1%)	0.06
Phylogenetic group												
A	2	(11%)	4	(3%)	0	(0%)	0.18	0	(0%)	6	(6%)	0.08
B1	2	(11%)	9	(7%)	1	(25%)	0.64	0	(0%)	12	(13%)	0.004
B2	6	(32%)	66	(53%)	2	(50%)	0.14	54	(100%)	20	(21%)	<0.001
D	7	(37%)	43	(34%)	1	(25%)	0.80	0	(0%)	51	(54%)	<0.001
Non-typable	2	(11%)	3	(2%)	0	(0%)	0.13	0	(0%)	5	(5%)	0.16
ST131 clone	2	(11%)	51	(41%)	1	(25%)	0.01	54	(100%)	0	(0%)	<0.001
Class 1 integrase[d]	10	(53%)	61	(49%)	2	(50%)	0.81	25	(46%)	48	(51%)	0.61
Plasmid replicon type[e]												
IncFIA	0	(0%)	20	(16%)	1	(25%)	0.07	13	(24%)	8	(9%)	0.01
IncFIA and IncFIB	4	(21%)	51	(41%)	1	(25%)	0.13	33	(61%)	23	(24%)	<0.001
IncFIB	6	(32%)	29	(23%)	1	(25%)	0.41	5	(9%)	31	(33%)	0.001
IncI1	10	(53%)	26	(21%)	4	(100%)	0.008	7	(13%)	33	(35%)	0.004

235 The data are presented as the number (%).

236 All in vitro susceptibilities were evaluated using the revised CLSI breakpoints for 2011 except those for the antibiotics labeled "old BP"; for these

237 antibiotics, the susceptibility was evaluated using the old CLSI breakpoints for 2009 with modification of the category if the ESBL confirmation test
238 was positive. For colistin, all of the isolates in this study had minimum inhibitory concentrations of ≤ 2 μg/mL.
239 [a] *P* value for the comparison between pAmpC-only and ESBL-only isolates.
240 [b] Two ESBL-producing isolates (one ST131 clone and one non-ST131 clone) were positive for CTX-M-14 and CTX-M-15.
241 [c] *qnrB* was found in the pAmpC and ST131 group. *qnrS* was found in the ESBL and non-ST131 group.
242 [d] Class 2 and class 3 integrase genes were not found.
243 [e] The 4 most prevalent replicon types are listed. The A/C, P, B/O, K/B, N, and Y types were found, but the prevalences were less than 10%.
244

245 **Table 3. Molecular mechanism of quinolone resistance among ciprofloxacin non-susceptible ESBL-producing *E. coli*.**

		Amino acid mutations in QRDR				
	Number of	GyrA		ParC		Number of isolates
Clone type	isolates	83	87	80	84	with *aac(6')-Ib-cr*
ST131 clone	46	L	N	I	V	4
Non-ST131 clone	30	L	N	I	E	0
	5	L	N	I	G	1
	2	L	N	I	V	0
	2	L	Y	I	E	0
	1	L	N	I	A	0
	1	L	N	I	K	0
	1	L	N	I	S	0
	1	L	N	R	E	0
Wild type (*E.coli* K-12)	-	S	D	S	E	-

246 All of these isolates lacked *qnr*.