Original article

Fluoroquinolone resistance in extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in a Japanese tertiary hospital: Silent shifting to CTX-M-15-producing *K. pneumoniae*

M. Higashino*, M. Murata*, Y. Morinaga, N. Akamatsu, J. Matsuda, K. Takeda, N. Kaku, K. Kosai, N. Uno, H. Hasegawa, and K. Yanagihara

*equally contributed

Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 851-2128, Japan

Running title: CTX-M-15-producing *K. pneumoniae* in Japan

Corresponding author:

Yoshitomo Morinaga, MD, PhD

Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Tel: +81-95-819-7574; Fax: +81-95-819-7422

E-mail: y-morina@nagasaki-u.ac.jp

ABSTRACT

Purpose Fluoroquinolone resistance (FQ-r) in extended-spectrum β-lactamase (ESBL) producers is an urgent health concern in countries where ESBL-producing *K. pneumoniae* (ESBL-Kpn) is prevalent. We investigated FQ-r in Japan where ESBL-Kpn is less prevalent

Methodology Clinical ESBL-Kpn isolates from 2011 to 2013 were collected in Nagasaki University Hospital. The ESBL genotypes included CTX-M-15, and the mechanisms of FQ-r through plasmid-mediated quinolone resistance (PMQR) and mutations in quinolone resistance-determining regions (QRDRs) were examined. Clonality was analyzed by enterobacterial repetitive intergenic consensus (ERIC)-PCR and multi-locus sequence typing was performed on selected isolates.

Results Thirty ESBL-Kpn isolates, including 7 levofloxacin-resistant isolates, were obtained from different patients. An increase in CTX-M-15-producing strains was observed during the study period (0/11 in 2011, 3/8 in 2012, and 5/11 in 2013). PMQR was detected in 53.3% of the isolates and *aac-(6')-Ib-cr* was the most common (36.7%). ST15 was observed in 60.0% of the isolates, and for the most predominant ERIC-PCR profiles, 62.5% of the isolates possessed the CTX-M-15 genotype and 71.4% were levofloxacin-resistant. Levofloxacin-resistance was significantly more common in CTX-M-15 isolates (62.5%)

compared to non-CTX-M-15 isolates (9.1%). Three QRDR mutations and *aac(6')-Ib-cr*, but not *qnrB* and *qnrS*, were significantly enriched in the CTX-M-15 isolates (100.0%) compared to the non-CTX-M-15 isolates (13.6%).

Conclusion Cumulatively, these results indicate that the epidemic strain, the CTX-M-15-producing *K. pneumoniae* ST15, is covertly spreading even when ESBL-producers are not prevalent. Monitoring these epidemic strains and ESBLs in general is important for quickly identifying health crises and minimizing future risks from FQ-r ESBL-Kpn.

KEY WORDS

plasmid-mediated quinolone resistance; quinolone resistance-determining regions; CTX-M-1; ST15; ST551

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) are enzymes produced mainly by the Enterobacteriaceae family. The genes encoding ESBL can be transmitted via plasmids, and the expansion of ESBL-producing microbes is a global concern. *Klebsiella pneumoniae* is a major ESBL-producing pathogen, as well as *Escherichia coli*. *K. pneumoniae* causes severe infections, such as pneumonia, urinary tract infections, and sepsis. The worldwide prevalence of ESBL-producing *K. pneumoniae* (ESBL-Kpn) has dramatically increased since the description of this resistance in the early 1980s. However, there are some variations in both the prevalence and predominant ESBL genes found in strains of *K. pneumoniae* in different countries [1].

Specific ESBL-encoding genes and clones are involved in the global dissemination of ESBL. Classically, SHV and TEM were major ESBL-encoding genes. However, CTX-M genes, including the CTX-M-1*,* CTX-M-2*,* and CTX-M-9 groups, have become more common ESBL genes associated with *E. coli* and *K. pneumoniae* [1]. In particular, CTX-M-15, a CTX-M-1 group of ESBLs, has been recognized as an epidemic-related ESBL. Of note, most CTX-M-15-producing *E. coli* belong to a clone called ST131, and these are closely associated with fluoroquinolone resistance (FQ-r) [2]. CTX-M-15 has also been found in *K. pneumoniae,* and may be sporadically associated with multi-drug resistance and

some specific sequence types of bacteria [3, 4]. Furthermore, the relationship between CTX-M-15 and FQ-r is not well understood.

Fluoroquinolones are alternative antibiotics for patients with ESBL-Kpn infections. Fluoroquinolones target DNA gyrase and topoisomerase IV, which are encoded by *gyrA* and *parC*, respectively. Two mechanisms for quinolone resistance are the acquisition of plasmid-mediated quinolone resistance (PMQR) genes (such as *aac(6')-Ib-cr* and *qnr)* and spontaneous mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes. Acquisition of *qnr* or *aac(6')-Ib-cr* genes can reduce susceptibility to fluoroquinolone by protecting DNA gyrase from the drug's effects or by triggering N-acetylation of piperazinyl amine residues [5], respectively. *K. pneumoniae* is normally highly sensitive to quinolone antibiotics; however, quinolone-resistant ESBL producers are emerging through these aforementioned mechanisms [6-8].

In the United States and many European and Asian countries (except Japan), more than 20% of *K. pneumoniae* strains were found to be ESBL-positive [9]. In contrast, Japan had a low prevalence [10, 11], and our previous study conducted in a Japanese tertiary hospital revealed that while ESBL-Kpn constituted only 2.8% of clinical isolates, they are on the rise [12]. Similarly, 4-5% of *K. pneumoniae* identified in Canada and Australia were ESBL producers [9]. For countries with a low prevalence of ESBL-Kpn, monitoring these strains and subsequent FQ-r is critical for controlling current and future infections. Therefore, we

92 studied phenotypically-identified ESBL-Kpn in microbiology laboratory of our hospital,
93 focusing on CTX-M-15 isolates and their resistance to fluoroquinolone.
94
95 **MATERIALS AND METHODS**
96
97 Collection of clinical isolates
98 This study was conducted at Nagasaki University Hospital, which is a tertiary
99 hospital with 861 beds. Hospital microbiology laboratory databases from 2011 to 2013 were
100 reviewed, and clinical isolates of *K. pneumoniae* were analyzed for bacteriological and
101 molecular epidemiology. The identification, antimicrobial susceptibilities and ESBL
102 production were examined using BD Phoenix™ Automated Microbiology System (BD
103 Diagnostic Systems, Sparks, MD), as described previously [12]. The MIC values of
104 levofloxacin, cefotaxime and gentamicin were determined according to the Clinical and
105 Laboratory Standards Institute M100-S23 [13]. FQ-r was determined using the MIC values of
106 levofloxacin because the MIC of levofloxacin was routinely measured throughout the study
107 period. The number of phenotypically-identified ESBL-Kpn in 2011, 2012, and 2013 was 11,
108 8, and 11, respectively.
109
110

111 Extraction of plasmids and DNA

112 All isolates from patients had been preserved through a freeze-drying process. We
113 re-cultured all ESBL-Kpn isolates obtained from 2011 to 2013. Plasmids were extracted
114 using a boiling method [12]. Briefly, a few colonies were suspended in 1000 μL of Tris-EDTA
115 buffer (pH 8.0), boiled for 10 min, and subsequently centrifuged for 5 min at 12000 rpm. The
116 supernatant containing each plasmid was transferred to a new tube. For total DNA extraction,
117 the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) was used according to the
118 manufacturer's instructions.

119

120 Genotyping of ESBL

121 PCR genotyping of ESBL plasmids was performed using 6 sets of previously
122 published primers to amplify type-specific ESBL genes, including those of the CTX-M-1,
123 CTX-M-2, CTX-M-9, TEM, SHV groups, and CTX-M-15 [14, 15]. PCR thermal cycling
124 conditions were as follows: 1 cycle of 95°C for 10 min; 30 cycles of 95°C for 40 sec, 60°C for
125 40 sec, and 72°C for 1 min; and 1 cycle of 72°C for 7 min. The PCR products were run on a
126 2% agarose gel and visualized by staining with ethidium bromide.

127

128 Screening for *qnr* and *aac(6')-Ib-cr* genes

129 PMQRs including *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib-cr* were detected, as previously

reported [16]. Briefly, *qnr* genes were amplified using multiplex PCR, and *aac(6')-Ib-cr* was distinguished from *aac(6')-Ib* using pyrosequencing to discern two single-nucleotide polymorphisms [17]. PCR products of all PMQR genes were kindly obtained from Dr. K. Tateda and used as positive controls.

Pyrosequencing of QRDRs

Mutations in the QRDRs of *gyrA* and *parC* were analyzed by pyrosequencing. Primers were designed using the PyroMark Assay Design software 2.0 (Qiagen, Hilden, Germany) on the basis of sequence information available (GenBank accession number AF052258 and AF303641). The predicted amplicons contained major mutation sites in the QRDRs of *gyrA* (Ser83 and Asp87) and *parC* (Ser80 and Glu84) [18] (Table 1). The target genes were amplified by PCR using Amplitaq Gold 360 Master Mix (Applied Biosystems) with the following PCR thermal cycling conditions: 1 cycle of 95°C for 5 min; 50 cycles of 95°C for 15 sec, 67°C for 30 sec, 72°C for 15 sec; and 1 cycle of 72°C for 5 min. The PCR products were sequenced using PyroMark Q96 ID (Qiagen) and PyroMark Gold Q96 Reagents (Qiagen).

Analyses of clonality

Clonal relationships were assessed by enterobacterial repetitive intergenic

consensus-PCR (ERIC-PCR) [19]. We used previously published primers to amplify ERIC sequences. PCR thermal cycling conditions were as follows: 1 cycle of 95°C for 5 min; 40 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 2 min; and 1 cycle of 72°C for 10 min. The PCR products were analyzed using the Microchip Electrophoresis System for DNA/RNA analysis MCE®-202 MultiNA (Shimadzu, Kyoto, Japan). Fingerprints on electropherogram were compared visually and isolates having at least one different peak were classified as different ERIC profile.

Multi-locus sequence typing (MLST) was performed using seven conserved housekeeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB*) [20] for selected isolates which have specific characteristics such as CTX-M-15-positive, levofloxacin-resistant or belonging to the most dominant ERIC profile. PCR amplification and sequencing were performed by following Pasteur Institute protocol. (http://bigsdb.pasteur.fr/klebsiella/primers_used.html). The sequence type was assigned based upon the MLST database (http://bigsdb.web.pasteur.fr/index.html).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Prism Software Inc., CA). A χ^2 test was used to assess statistical differences among the frequencies of events, and differences were considered statistically significance at $p<0.05$.

RESULTS

Molecular profiles of β-lactamases in phenotypically identified ESBL-Kpn

There were 30 phenotypically identified ESBL-Kpn isolates (Table 2). Of the 30 isolates, 29 (96.7%) were positive for one or more ESBL genes. The number of isolates possessing one, two, or three ESBL genes was 11 (36.7%), 14 (46.6%), and 4 (13.3%), respectively. The most prevalent ESBL gene was SHV (n=24, 80.0%), followed by CTX-M-1 (n=11, 36.7%), TEM (n=8, 26.7%), CTX-M-2 (n=5, 16.7%), and CTX-M-9 (n=3, 10.0%). Of the CTX-M-1-positive isolates (n=11), CTX-M-15-positive isolates (n=8) accounted for 72.7%. The frequency of CTX-M-15-positive isolates gradually increased from 2011 to 2012 and 2013 (0.0%, 37.5%, and 45.5%, respectively).

Antimicrobial susceptibility test

In these phenotypically-identified ESBL-Kpn isolates, 7 (23.3%) were levofloxacin-resistant (MIC≥8 μg/mL) (Table3). The number of cefotaxime- and gentamicin-resistant isolates was 22 (73.3%), and 7 (23.3%), respectively (Table3). Only one isolate showed resistance in both gentamicin and levofloxacin.

187 Analysis of PMQRs and QRDRs

188 Fluoroquinolone-resistant genes were analyzed in all of the isolates (Table 3).

189 PMQRs were detected in 16 isolates (53.3%). The most common PMQR was
190 *aac(6')-Ib-cr* (n=11), followed by *qnrS* (n=5) and *qnrB* (n=4). Strains containing *qnrA* were
191 not obtained. Levofloxacin-resistant isolates frequently possessed *aac(6')-Ib-cr.* This was
192 also observed for levofloxacin-non-resistant isolates, but the prevalence was significantly
193 higher in the levofloxacin-resistant isolates (85.7% versus 21.7%; $p<0.01$). All nine isolates
194 carrying *qnr* (*qnrS*, n=5; *qnrB*, n=4) were levofloxacin-non-resistant.

195 Chromosomal mutations in QRDRs were observed in 12 isolates (40.0%). All seven
196 levofloxacin-resistant isolates had three mutations each in QRDRs. The most prevalent
197 amino acid substitutions were Ser83Phe and Asp87Ala in *gyrA,* in addition to Ser80Ile in
198 *parC* (n=6). The rest of the fluoroquinolone-resistant isolates displayed Ser83Phe and
199 Asp87Asn substitutions in *gyrA,* in addition to Glu84Lys in *parC*. The isolate for which the
200 levofloxacin MIC was 4.0 μg/mL displayed a Ser83Tyr change in *gyrA*. However, all five
201 isolates possessing single QRDR mutations were not phenotypically resistant to
202 levofloxacin.

203

204 Clonality analysis of the ESBL-Kpn

205 All ESBL-Kpn isolates were screened using ERIC-PCR for clonality analysis (Table

206 3 and Figure 1). ERIC-PCR categorized 30 strains into 18 groups. The most dominant
207 ERIC-PCR profile was named E1, and 10 isolates (33.3%) belonged to this profile. In the E1
208 profile, five isolates (50%) were positive for CTX-M-15 while three isolates (13.6%)
209 possessed a non-E1 profile. Of the 30 strains, a total of seven levofloxacin-resistant isolates
210 belonged to the profile E1, and no isolates with levofloxacin resistance were detected in the
211 other ERIC-PCR profiles.

212 To screen sequence types, MLST was performed on isolates that had been
213 classified as CTX-M-15-positive or levofloxacin-resistant. MLST was also performed on
214 isolates that displayed a profile E1 based upon ERIC-PCR. Among the selected isolates,
215 ST15 was most commonly observed (n=6), followed by ST551 (n=2), ST252 (n=1), and
216 ST1035 (n=1). ST15 was observed primarily in isolates that displayed an ERIC-PCR profile
217 E1 (6/10, 60%), CTX-M-15-positive genotype (5/8, 62.5%), and levofloxacin resistance (5/7,
218 71.4%).

219

220 Relationship between CTX-M-15 and quinolone-resistant genes

221 Quinolone-resistant genes were compared between isolates possessing CTX-M-15
222 versus those lacking CTX-M-15 (Table 4). Levofloxacin resistance was observed in 62.5% of
223 the isolates positive for CTX-M-15, whereas only 9.1% of the non-CTX-M-15 isolates
224 displayed levofloxacin resistance ($p<0.01$). The presence of three QRDR mutations was

also more frequent in CTX-M-15-positive isolates compared to the non-CTX-M-15 isolates (62.5% vs. 9.1%, p<0.01). For PMQRs, the percentage of isolates possessing *aac(6')-Ib-cr* was 100.0% in the CTX-M-15-positive isolates and 13.6% in the non-CTX-M-15 isolates (p<0.01). In contrast, the prevalence of *qnrB* and *qnrS* was not significantly different between the two groups.

DISCUSSION

In European countries, the predominant β-lactamase in ESBL-Kpn has dramatically shifted from SHV and TEM to the CTX-M-type. The CTX-M-1 group has been rapidly expanding, and it is now the predominant ESBL-Kpn group found in European countries [21]. In particular, CTX-M-15, a CTX-M-1 group ESBL, has been recognized as a pandemic ESBL gene in the Enterobacteriaceae family [2]. However, the number of ESBL isolates and the composition of ESBL genes can vary geographically. The CTX-M-1 group has been historically less prevalent in Japan, where the CTX-M-2 group dominated as the CTX-M-type ESBL before 2000 [21]. We previously reported on the epidemiology of clinical ESBL-Kpn isolates in our hospital from 2006 to 2010, and discovered an increase in ESBL-producers in clinical isolates of both *K. pneumoniae* and *E. coli* [12]. Thus, the present study was performed as a follow-up focusing on *K. pneumoniae*.

Compared to our previous study [12], the proportions of SHV isolates in the ESBL-Kpn population did not change (the percentages of ESBL-type SHV were 80.0% in both studies). However, the percentages of CTX-M-1-positive isolates dramatically increased from 5.0% to 36.7%. This was mainly due to the increase in CTX-M-15 isolates overall. CTX-M-15 was not previously prevalent in Japan [12, 22]. However, in other Asian countries, it reportedly constituted 59.8% of the ESBL-Kpn isolates that caused hospital-acquired pneumonia [23]. In the present study, the shift from non-CTX-M-15 to CTX-M-15 isolates was observed even though the total number of isolates was similar for each year studied. Most of the isolates possessing CTX-M-15 also had one or more other β-lactamases, implying that CTX-M-15 has the potential to coexist with other β-lactamases and it might also be acquired by ESBL strains because SHV and TEM typically dominate the population. Conversely, the expansion of CTX-M-15 in *K. pneumoniae* could be restrictive among conventional ESBL producers in Japan.

In the present study, PMQRs were observed in 53.3% of the ESBL-Kpn isolates, and *aac(6')-Ib-cr*, *qnrB*, and *qnrS* genes were detected in 36.7%, 13.3%, and 16.7% of these strains, respectively. Compared to a previous report of cephalosporin-resistant *K. pneumoniae* isolates in Japan [24], the prevalence of PMQRs was similar (66.7%), but the percentages for each PMQR gene in the total population were different (*aac(6')-Ib-cr*, 4.2%; *qnrB*, 50.0%; *qnrS*, 16.7%). These findings suggest that local factors, such as study region

and study population, can affect PMQR composition. PMQRs might be more commonly observed in *K. pneumoniae* than in *E. coli* [6, 24]. It is reported that *aac(6')-Ib-cr* has epidemiologically strong associations with CTX-M-15 [8]. *Qnr* genes are also relevant to SHV or CTX-M-9, but *qnr* genes do not lead to significant increases in fluoroquinolone MICs [6]. Thus, the present data are compatible with these earlier findings. As shown in our study, PMQRs can elevate the fluoroquinolone MIC, but their effects are mild. Importantly, these strains could still be recognized as sensitive in clinical settings. However, considering that *K. pneumoniae* is highly sensitive to fluoroquinolones (0.25 μg/mL in MIC_{90}) [25], the isolates that have a levofloxacin MIC of 1.0 μg/mL are not clinically negligible. Therefore, we should continue to monitor PMQRs in *K. pneumoniae,* and further studies are needed to assess the clinical impact of these PMQR-possessing strains.

Compared to PMQRs, QRDR mutations can dramatically elevate MIC values for fluoroquinolone. Generally, more than one double mutation in QRDRs causes high FQ-r [26]. Consistent with this report, all the isolates with three QRDR mutations were fluoroquinolone-resistant. The triplet QRDR mutations (Ser83Phe, Asp87Ala, and Ser80Ile; Ser83Phe, Asp87Asn, and Glu84Lys) in the present study have been commonly observed among fluoroquinolone-resistant *K. pneumoniae* [27, 28]. The effect of the Ser83Tyr substitution in GyrA on fluoroquinolone susceptibility is controversial. One study demonstrated that the Ser83Tyr change mildly elevated the ciprofloxacin MIC value [4],

while another report found that it did not affect fluoroquinolone susceptibility [29].

In a study conducted in nine Asian countries excluding Japan, CTX-M-15 isolates belonged to 25 different STs. However, ST11, ST15, and ST340 were the predominant STs identified [23]. In the present study, ST15 was most commonly observed in the CTX-M-15 isolates (62.5%). It is reported that the incidence of ST15 among CTX-M-15-positive *K. pneumoniae* was 57.1% in Portugal [30] and 27.3% in the U.S. [31], while ST15 was not reported in Spain [4]. In the present study, 5/7 (71.4%) fluoroquinolone-resistant isolates from different patients displayed the same molecular signature, including ST15, *aac(6')-Ib-cr*, three QRDR mutations, and CTX-M-15. The same profile has been identified in an epidemic clone in Hungary [28]. This suggests that the ST15 ESBL-Kpn clone is expanding, and might gradually spread in Japan. Although MLST was not performed for all the isolates, the ST15 strain could have some discrete characteristics that make it especially virulent, such as a tendency to obtain mutations in QRDRs and synergize with CTX-M-15 strains. It seems difficult to conclude all ST15 isolates resulted from a local outbreak because these isolates, except the isolate no.2 and no.3, have various backgrounds in isolated date and location.

There are some limitations to our study. First, our study does not include Kpn strains which have ESBL genes but are negative for phenotype-based ESBL-detection. Some SHV/TEM variants or ESBL genes co-harboring with plasmid-mediated AmpC β-lactamase can be missed by phenotype-based ESBL-detection {Canton, 2008 #1094}.

Therefore, we could not know about the relationship between these undiagnosed ESBL genes and fluoroquinolone-resistance. Second, because it focused on a single hospital, the number of specimens was small and we could not evaluate the differences among facilities. Furthermore, the clinical backgrounds of patients who had contracted the epidemic clone could not be collected. Therefore, the risk factors and the suspected routes of transmission of this strain are still unknown. Lastly, ST15 was commonly observed in fluoroquinolone-resistant isolates, but MLST was performed only in the selected isolates.

In conclusion, our study suggests that CTX-M-15-producing *K. pneumoniae* ST15 is a global pandemic clone currently emerging in Japan. This clone might be spreading even if ESBL-producers are not prevalent. Thus, it is important to monitor epidemic clones and ESBLs in countries where ESBL-producers are not prevalent, as opposed to focusing only on countries currently experiencing epidemics. To mitigate the spread of these fluoroquinolone-resistant strains, antimicrobial stewardship should be strongly encouraged in clinical settings.

ACKNOWLEDGEMENTS

This research was partly supported by a Grant-in-Aid for Scientific Research (no. 15K09572 to K.Y.), a Grant-in-Aid for Young Scientists (B) (no. 23791137 and no. 30580360 to Y.M.)

from the Japan Society for the Promotion of Science, and by the Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare, Japan (H28-Shinkou-Ippan-003).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATINOS

FQ-r, fluoroquinolone resistance; ESBL, extended-spectrum β-lactamase; Kpn, *Klebsiella pneumoniae*; PMQR, plasmid-mediated quinolone resistance; QRDR, quinolone resistance-determining region; ERIC, enterobacterial repetitive intergenic consensus; MLST, multi-locus sequence typing;

REFERENCES

1. **Paterson DL, Bonomo RA**. Extended-spectrum beta-lactamases: a clinical update. *Clinical microbiology reviews* 2005;18:657-686.
2. **Nicolas-Chanoine MH, Bertrand X, Madec JY**. Escherichia coli ST131, an intriguing clonal group. *Clinical microbiology reviews* 2014;27:543-574.
3. **Damjanova I, Toth A, Paszti J, Bauernfeind A, Fuzi M**. Nationwide spread of clonally related CTX-M-15-producing multidrug-resistant *Klebsiella pneumoniae* strains in Hungary. *European journal of clinical microbiology & infectious diseases* 2006;25:275-278.
4. **Oteo J, Cuevas O, Lopez-Rodriguez I, Banderas-Florido A, Vindel A et al.** Emergence of CTX-M-15-producing *Klebsiella pneumoniae* of multilocus sequence types 1, 11, 14, 17, 20, 35 and 36 as pathogens and colonizers in newborns and adults. *The Journal of antimicrobial chemotherapy* 2009;64:524-528.
5. **Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D et al.** Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nature medicine* 2006;12:83-88.
6. **Briales A, Rodriguez-Martinez JM, Velasco C, de Alba PD, Rodriguez-Bano J et al.** Prevalence of plasmid-mediated quinolone resistance determinants *qnr* and *aac(6')-Ib-cr* in *Escherichia coli* and *Klebsiella pneumoniae* producing extended-spectrum beta-lactamases in Spain. *International journal of antimicrobial agents* 2012;39:431-434.
7. **Zurfluh K, Abgottspon H, Hachler H, Nuesch-Inderbinen M, Stephan R**. Quinolone resistance mechanisms among extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* isolated from rivers and lakes in Switzerland. *PloS one* 2014;9:e95864.
8. **Park KS, Kim MH, Park TS, Nam YS, Lee HJ et al.** Prevalence of the plasmid-mediated quinolone resistance genes, *aac(6')-Ib-cr*, *qepA*, and *oqxAB* in clinical isolates of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* in Korea. *Annals of clinical and laboratory science* 2012;42:191-197.
9. **Center for Disease Dynamics EPC**. *State of the World's Antibiotics, 2015*. 2015.
10. **Chong Y, Shimoda S, Yakushiji H, Ito Y, Miyamoto T et al.** Community spread of extended-spectrum beta-lactamase-producing *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*: a long-term study in Japan. *Journal of medical microbiology* 2013;62:1038-1043.
11. **Canton R, Novais A, Valverde A, Machado E, Peixe L et al.** Prevalence and spread of extended-spectrum beta-lactamase-producing Enterobacteriaceae in Europe. *Clinical microbiology and infection* 2008;14 Suppl 1:144-153.
12. **Harada Y, Morinaga Y, Yamada K, Migiyama Y, Nagaoka K et al**. Clinical and molecular epidemiology of extended-spectrum ß-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli* in

a Japanese tertiary hospital. *J Med Microb Diagn* 2013;2:2161-0703.1000127.

13. **Clinical and Laboratory Standards Institute**. *Performance standards for antimicrobial susceptibility testing. M100-S23.* 2013.

14. **Doi Y, Adams-Haduch JM, Shivannavar CT, Paterson DL, Gaddad SM**. Faecal carriage of CTX-M-15-producing *Klebsiella pneumoniae* in patients with acute gastroenteritis. *The Indian journal of medical research* 2009;129:599-602.

15. **Muratani T, Kobayashi T, Matsumoto T**. Emergence and prevalence of beta-lactamase-producing *Klebsiella pneumoniae* resistant to cephems in Japan. *International journal of antimicrobial agents* 2006;27:491-499.

16. **Aoike N, Saga T, Sakata R, Yoshizumi A, Kimura S et al.** Molecular characterization of extraintestinal *Escherichia coli* isolates in Japan: relationship between sequence types and mutation patterns of quinolone resistance-determining regions analyzed by pyrosequencing. *J Clin Microbiol* 2013;51:1692-1698.

17. **Guillard T, Duval V, Moret H, Brasme L, Vernet-Garnier V et al.** Rapid detection of *aac(6')-Ib-cr* quinolone resistance gene by pyrosequencing. *J Clin Microbiol* 2010;48:286-289.

18. **Brisse S, Milatovic D, Fluit AC, Verhoef J, Martin N et al.** Comparative *in vitro* activities of ciprofloxacin, clinafloxacin, gatifloxacin, levofloxacin, moxifloxacin, and trovafloxacin against *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, and *Enterobacter aerogenes* clinical isolates with alterations in GyrA and ParC proteins. *Antimicrobial agents and chemotherapy* 1999;43:2051-2055.

19. **Versalovic J, Koeuth T, Lupski JR**. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic acids research* 1991;19:6823-6831.

20. **Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S**. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. *J Clin Microbiol* 2005;43:4178-4182.

21. **Hawkey PM**. Prevalence and clonality of extended-spectrum beta-lactamases in Asia. *Clinical microbiology and infection* 2008;14 Suppl 1:159-165.

22. **Bonnet R**. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrobial agents and chemotherapy* 2004;48:1-14.

23. **Lee MY, Ko KS, Kang CI, Chung DR, Peck KR et al.** High prevalence of CTX-M-15-producing *Klebsiella pneumoniae* isolates in Asian countries: diverse clones and clonal dissemination. *International journal of antimicrobial agents* 2011;38:160-163.

24. **Okade H, Nakagawa S, Sakagami T, Hisada H, Nomura N et al.** Characterization of plasmid-mediated quinolone resistance determinants in *Klebsiella pneumoniae* and *Escherichia coli* from Tokai, Japan. *Journal of infection and chemotherapy* 2014;20:778-783.

25. **Yanagihara K, Kadota J, Aoki N, Matsumoto T, Yoshida M et al.** Nationwide surveillance of bacterial respiratory pathogens conducted by the surveillance committee of Japanese Society of Chemotherapy, the Japanese Association for Infectious Diseases, and the Japanese Society for Clinical Microbiology in 2010: General view of the pathogens' antibacterial susceptibility. *Journal of infection and chemotherapy* 2015;21:410-420.

26. **Vila J, Ruiz J, Marco F, Barcelo A, Goni P et al.** Association between double mutation in *gyrA* gene of ciprofloxacin-resistant clinical isolates of Escherichia coli and MICs. *Antimicrobial agents and chemotherapy* 1994;38:2477-2479.

27. **Aathithan S, French GL**. Prevalence and role of efflux pump activity in ciprofloxacin resistance in clinical isolates of *Klebsiella pneumoniae*. *European journal of clinical microbiology & infectious diseases* 2011;30:745-752.

28. **Damjanova I, Toth A, Paszti J, Hajbel-Vekony G, Jakab M et al.** Expansion and countrywide dissemination of ST11, ST15 and ST147 ciprofloxacin-resistant CTX-M-15-type beta-lactamase-producing *Klebsiella pneumoniae* epidemic clones in Hungary in 2005--the new 'MRSAs'? *The Journal of antimicrobial chemotherapy* 2008;62:978-985.

29. **Bratu S, Landman D, George A, Salvani J, Quale J**. Correlation of the expression of *acrB* and the regulatory genes *marA*, *soxS* and *ramA* with antimicrobial resistance in clinical isolates of *Klebsiella pneumoniae* endemic to New York City. *The Journal of antimicrobial chemotherapy* 2009;64:278-283.

30. **Rodrigues C, Machado E, Ramos H, Peixe L, Novais A**. Expansion of ESBL-producing *Klebsiella pneumoniae* in hospitalized patients: a successful story of international clones (ST15, ST147, ST336) and epidemic plasmids (IncR, IncFIIK). *International journal of medical microbiology* 2014;304:1100-1108.

31. **Wang G, Huang T, Surendraiah PK, Wang K, Komal R et al.** CTX-M beta-lactamase-producing *Klebsiella pneumoniae* in suburban New York City, New York, USA. *Emerging infectious diseases* 2013;19:1803-1810.

Figure 1. ERIC profile of ESBL-producing *K. pneumoniae*.

Electrophoresis patterns were categorized into 18 groups after analysis of PCR products by capillary electrophoresis. Each lane numbers corresponds to the numbers in Table 3. The image was edited to be in order of indicated number using original data. M, marker. UM, upper marker. LM, lower marker.

436 Table 1. Primers for pyrosequencing

437

Primer name	Primer sequence (5'→3')
gyrA QRDR for pyrosequencing	
KPgyrAfoward	AATCAGCCCGTGTCGTTGG
KPfyrA-5biotinR	GAGAACGGCTGCGCCATA
KPgyrAseq	CCACCCGCACGGCGA
parC QRDR for pyrosequencing	
KPparC-5biotinF	TCGGCGACGTGTTGGGTA
KPparCreverse	CCAGCGGATAGCGGTAAGAGA
KPparCseq	CCATCAGCACCATCG

438

439

440 Table 2. ESBL genotypes of the 30 ESBL-producing *K. pneumoniae* isolates

441

Genotype(s)	Year			Total, n
	2011	2012	2013	
Isolates with CTX−M−15				
CTX−M−1 (CTX−M−15)	0	0	1	1
CTX−M−1 (CTX−M−15), SHV	0	0	3	3
CTX−M−1 (CTX−M−15), TEM	0	1	0	1
CTX−M−1 (CTX−M−15), TEM, SHV	0	2	1	3
Subtotal, n (% in the year(s))	0 (0.0)	3 (37.5)	5 (45.5)	8 (26.7)
Isolates without CTX−M−15				
CTX−M−1 (non−CTX−M−15), SHV	0	1	2	3
CTX−M−2, SHV	4	0	0	4
CTX−M−2, TEM, SHV	1	0	0	1
CTX−M−9	1	0	0	1
CTX−M−9, TEM	1	0	1	2
SHV	4	2	3	9
TEM, SHV	0	1	0	1
Subtotal, n (% in the year(s))	11 (100.0)	4 (50.0)	6 (54.5)	21 (70.0)
Not detected, n (% in the year(s))	0 (0.0)	1 (12.5)	0 (0.0)	1 (3.3)

442

443

Table 3. Clonal profiles and drug sensitivity-associated profiles for β−lactams and fluoroquinolones

No.	Strain	Date (MM/YY)	Department	Specimens	Sequence type*	ERIC profile	*aac(6')*	*qnr*	*gyrA*		*parC*		βlactamases	MICs (μg/mL)		
									Ser83	Asp87	Ser80	Glu84		CTX	GEN	LVX
1	EK1302	04/13	General ICU	Sputum	ST15	E1	*Ib-cr*	-	Phe	Ala	Ile	WT	CTX-M-15	≥64	≤2	≥8
2	EK1304	08/13	Cardiology	Sputum	ST15	E1	*Ib-cr*	-	Phe	Ala	Ile	WT	SHV, CTX-M-15	≥64	≤2	≥8
3	EK1305	08/13	General ICU	Tracheal aspirate	ST15	E1	*Ib-cr*	-	Phe	Ala	Ile	WT	SHV, CTX-M-15	≥64	≤2	≥8
4	EK1307	09/13	Hematology	Throat swab	ST15	E1	*Ib-cr*	-	Phe	Ala	Ile	WT	SHV, TEM, CTX-M-15	≥64	≥16	≥8
5	EK1311	11/13	Thoracic surgery	Surgical wound	ST15	E1	*Ib-cr*	-	Phe	Ala	Ile	WT	SHV, CTX-M-15	≥64	≤2	≥8
6	EK1105	04/11	Hematology	Feces	ST15	E1	-	-	WT	WT	WT	WT	CTX-M-9	≥64	≥16	≤0.5
7	EK1207	11/12	Pulmonology	Urine	ST252	E1	-	-	Phe	Asn	WT	Lys	SHV, CTX-M-1 (non-CTX-M-15)	≥64	≤2	≥8
8	EK1201	01/12	Otorhinolaryngology	Sputum	ST551	E2	*Ib-cr*	-	WT	WT	WT	WT	TEM, SHV, CTX−M−15	≥64	≥16	≤0.5
9	EK1110	10/11	Pediatrics	Urine	ST551	E1	*Ib-cr*	-	Phe	Ala	Ile	WT	SHV	≤1	≤2	≥8
10	EK1204	08/12	Digestive surgery	Surgical wound	ST1035	E1	-	-	WT	WT	WT	WT	TEM, SHV	≤1	≤2	≤0.5
11	EK1108	09/11	OB/GYN	Vaginal secretion	ST-U	E1	-	-	WT	WT	WT	WT	SHV	≤1	≤2	≤0.5
12	EK1206	11/12	Pulmonology	Feces	ST-U	E3	*Ib-cr*	*B*	WT	WT	WT	Lys	TEM,	≥64	≤2	1

													CTX-M-15			
13	EK1208	10/12	Emergency	Blood	ST-U	E4	*Ib-cr*	*B*	WT	WT	WT	WT	TEM, SHV, CTX-M-15	≥64	≤2	2
14	EK1310	11/13	Cardiac surgery	Urine		E4	-	-	WT	WT	WT	WT	SHV	2	≤2	≤0.5
15	EK1103	05/11	Digestive surgery	Feces		E4	*Ib-cr*	*B*	WT	WT	WT	WT	SHV, CTX-M-2	≥64	≤2	≤0.5
16	EK1104	04/11	Hematology	Feces		E5	*Ib-cr*	*B*	WT	WT	WT	WT	SHV	≥64	≤2	1
17	EK1107	09/11	Rheumatology	Urine		E5	-	-	WT	WT	WT	WT	SHV, CTX-M-2	≤1	≤2	≤0.5
18	EK1301	05/13	Neurology	Urine		E6	-	-	WT	WT	WT	WT	SHV	≤1	≤2	1
19	EK1303	08/13	Digestive surgery	Feces		E7	-	*S*	WT	WT	WT	WT	SHV, CTX-M-1 (non-CTX-M-15)	≥64	≤2	1
20	EK1306	09/13	OB/GYN	Vaginal secretion		E8	-	-	WT	WT	WT	WT	SHV	≤1	≤2	≤0.5
21	EK1308	10/13	Nephrology	Sputum		E9	-	*S*	WT	WT	Ile	WT	TEM, CTX-M-9	≥64	≥16	2
22	EK1309	10/13	Digestive surgery	Urine		E10	-	*S*	WT	WT	Ile	WT	SHV, CTX-M-1 (non-CTX-M-15)	≥64	≤2	1
23	EK1202	07/12	Digestive surgery	Feces		E11	*Ib*	-	WT	WT	WT	WT	SHV	32	≥16	≤0.5
24	EK1203	08/12	Anesthesiology	Feces		E12	*Ib*	-	Tyr	WT	WT	WT	SHV	≥64	8	4
25	EK1205	09/12	Pulmonology	Tracheal aspirate		E13	-	-	WT	WT	WT	WT	Not detected	≥64	≤2	≤0.5
26	EK1101	02/11	Urology	Urine		E14	-	*S*	WT	WT	Ile	WT	TEM, CTX-M-9	≥64	≥16	1
27	EK1102	03/11	Pulmonology	Urine		E15	-	*S*	WT	WT	WT	WT	TEM, SHV,	≥64	≥16	1

													CTX-M-2			
28	EK1106	09/11	Digestive surgery	Sputum	E16	-	-	WT	WT	WT	WT	SHV	≤1	≤2	≤0.5
29	EK1109	10/11	Rheumatology	Urine	E17	-	-	WT	WT	WT	WT	SHV, CTX-M-2	≥64	≤2	≤0.5
30	EK1111	12/11	Anesthesiology	Tracheal aspirate	E18	-	-	WT	WT	WT	WT	SHV, CTX-M-2	≥64	≤2	≤0.5

*: Sequence typing was performed only in selected isolates.

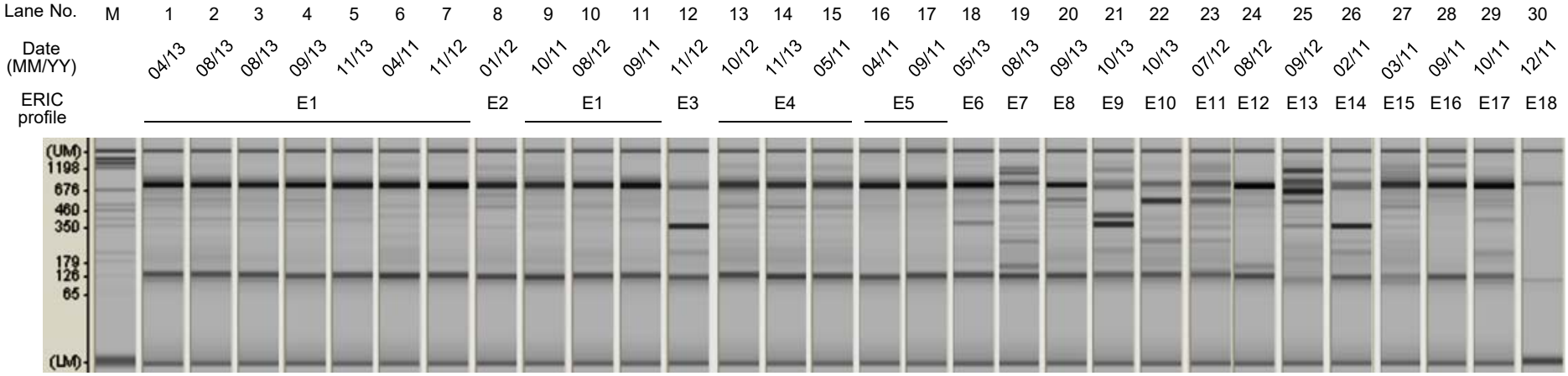
ST-U, undetermined ST. WT, wild type. OB/GYN, obstetrics and gynecology. CTX, cefotaxime. GEN, gentamicin. LVX, levofloxacin.

Table 4. Fluoroquinolone susceptibility-related factors in CTX-M-15 or non-CTX-M-15 isolates

	CTX-M-15 (n=8)	Non-CTX-M-15 (n=22)	P-value
Levofloxacin resistance	5 (62.5)	2 (9.1)	<0.01
Number of QRDR mutations			
Three	5 (62.5)	2 (9.1)	<0.01
Less than three	3 (37.5)	20 (90.9)	
PMQR carriers			
aac(6')-Ib-cr	8 (100.0)	3 (13.6)	<0.01
qnrB	2 (25.0)	2 (9.1)	ns
qnrS	0 (0.0)	5 (22.7)	ns

ns, not significant

Figure 1.


Lane No.
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Date (MM/YY)
04/13 08/13 08/13 09/13 11/13 04/11 11/12 01/12 10/11 08/12 09/11 11/12 10/12 11/13 05/11 04/11 09/11 05/13 08/13 09/13 10/13 10/13 07/12 08/12 09/12 02/11 03/11 09/11 10/11 12/11
ERIC profile
E1 E2 E1 E3 E4 E5 E6 E7 E8 E9 E10 E11 E12 E13 E14 E15 E16 E17 E18
(UM)
1198
676
460
350
179
126
65
(LM)