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**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 analysed by enterobacterial repetitive intergenic consensus (ERIC)-PCR and multi-locus sequence typing was performed on selected isolates.

**Results/Key findings.** Thirty ESBL-Kpn isolates, including seven 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 the 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.

**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 piperazine 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 studied phenotypically identified ESBL-Kpn in the microbiology laboratory of our hospital, focusing on CTX-M-15 isolates and their resistance to fluoroquinolone.

**METHODS**

**Collection of clinical isolates**

This study was conducted at Nagasaki University Hospital, which is a tertiary hospital with 861 beds. Hospital microbiology laboratory databases from 2011 to 2013 were reviewed, and clinical isolates of *K. pneumoniae* were analysed for bacteriological and molecular epidemiology. The identification, antimicrobial susceptibilities and ESBL production were examined using BD Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, MD), as described previously [12]. The MIC values of levofloxacin, cefotaxime and gentamicin were determined according to the Clinical and Laboratory Standards Institute M100-S23 [13]. FQ-r was determined using the MIC values of levofloxacin because the MIC of levofloxacin was routinely measured throughout the study period. The number of phenotypically identified ESBL-Kpn in 2011, 2012 and 2013 was 11, 8 and 11, respectively.

**Extraction of plasmids and DNA**

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

**Genotyping of ESBL**

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

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

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 analysed by pyrosequencing. Primers were designed using the PyroMark Assay Design software 2.0 (Qiagen, Hilden, Germany) on the basis of the sequence information available (GenBank accession numbers 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: one cycle of 95 °C for 5 min; 50 cycles of 95 °C for 15 s, 67 °C

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’→3’)</th>
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<td>AATCAGCCGCCTGCTGTTGG</td>
</tr>
<tr>
<td>KP*gyrAFoward</td>
<td>GAGAAGCCTGATCGGCATA</td>
</tr>
<tr>
<td>KP*gyrA-5biotinR</td>
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<td>KP*gyrAseq</td>
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<td>KP*parCseq</td>
<td>GCATCGACCGCATG</td>
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</table>
for 30 s, 72 °C for 15 s; and one 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: one 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 one cycle of 72 °C for 10 min. The PCR products were analysed using the Microchip Electrophoresis System for DNA/RNA analysis MCE–202 MultiNA (Shimadzu, Kyoto, Japan). Fingertips on the electropherogram were compared visually and isolates having at least one different peak were classified as a 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, levofoxacin-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, CA). A χ² test was used to assess statistical differences among the frequencies of events, and differences were considered statistically significant 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 four (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, seven (23.3 %) were resistant for levofoxacin (MIC ≥8 μg ml⁻¹) (Table 3). The number of cefotaxime- and gentamicin-resistant isolates was 22 (73.3 %) and seven (23.3 %), respectively

<table>
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<th>Year</th>
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<tr>
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</tr>
<tr>
<td>Not detected, n [% in the year(s)]</td>
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<td>1</td>
</tr>
</tbody>
</table>

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

(Table 3). Only one isolate showed resistance in both gentamicin and levofoxacin.

**Analysis of PMQRs and QRDRs**

Fluoroquinolone-resistant genes were analysed in all of the isolates (Table 3). PMQRs were detected in 16 isolates (53.3 %). The most common PMQR was aac(6’)-Ib-cr (n=11), followed by qnrS (n=5) and qnrB (n=4). Strains containing qnrA were not obtained. Levofoxacin-resistant isolates frequently possessed aac(6’)-Ib-cr. This was also observed for levofloxacin-non-resistant isolates, but the prevalence was significantly higher in the levofloxacin-resistant isolates (85.7 % vs 21.7 %; P<0.01). All nine isolates carrying qnr (qnrS, n=5; qnrB, n=4) were levofloxacin-non-resistant.

Chromosomal mutations in QRDRs were observed in 12 isolates (40.0 %). All seven levofloxacin-resistant isolates had three mutations each in QRDRs. The most prevalent amino acid substitutions were Ser83Phe and Asp87Ala in gyrA, in addition to Ser80Ile in parC (n=6). The rest of the fluoroquinolone-resistant isolates displayed Ser83Phe and Asp87Asn substitutions in gyrA and amino acid substitutions were Ser83Phe and Asp87Ala in gyrA, in addition to Ser80Ile in parC. The isolate for which the levofloxacin MIC was 4.0 μg ml⁻¹ displayed a Ser83Tyr change in gyrA. However, all five isolates possessing single QRDR mutations were not phenotypically resistant to levofloxacin.

**Clonality analysis of the ESBL-Kpn**

All ESBL-Kpn isolates were screened using ERIC-PCR for clonality analysis (Table 3 and Fig. 1). ERIC-PCR
Table 3. Clonal profiles and drug sensitivity-associated profiles for β-lactams and fluoroquinolones

<table>
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<th>Department</th>
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<th>Sequencetype*</th>
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<th>qnr</th>
<th>gyrA</th>
<th>parC</th>
<th>β-lactamases</th>
<th>MICs (µg ml⁻¹)</th>
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<td>Sputum</td>
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<td>-</td>
<td>Phe</td>
<td>Ala</td>
<td>Ile WT</td>
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<td>Ib-cr</td>
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<td>Ala</td>
<td>Ile WT</td>
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<td>Ib-cr</td>
<td>-</td>
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<td>Ala</td>
<td>Ile WT</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
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<td>WT</td>
<td>WT WT</td>
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<td>Asn</td>
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<td>-</td>
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<td>WT</td>
<td>WT WT</td>
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<td>WT</td>
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<td>WT</td>
<td>WT TEM</td>
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<td>E15</td>
<td>S</td>
<td>WT</td>
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ST-U, undetermined ST; WT, wild-type; OB/GYN, obstetrics and gynecology; CTX, cefotaxime; GEN, gentamicin; LVX, levofloxacin.

*Sequence typing was performed only in selected isolates.
categorized 30 strains into 18 groups. The most dominant ERIC-PCR profile was named E1, and 10 isolates (33.3 %) belonged to this profile. In the E1 profile, five isolates (50 %) were positive for CTX-M-15 while three isolates (13.6 %) possessed a non-E1 profile. Of the 30 strains, a total of seven levofloxacin-resistant isolates belonged to the profile E1, and no isolates with levofloxacin resistance were detected in the other ERIC-PCR profiles.

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

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

Quinolone-resistant genes were compared between isolates possessing CTX-M-15 versus those lacking CTX-M-15 (Table 4). Levofloxacin resistance was observed in 62.5 % of the isolates positive for CTX-M-15, whereas only 9.1 % of the non-CTX-M-15 isolates 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⁻¹ in MIC₉₀) [25], the isolates that have a levofloxacin MIC of 1.0 µg ml⁻¹ 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 Ser83Thr substitution in GyrA on fluoroquinolone susceptibility is controversial. One study demonstrated that the Ser83Thr 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 USA [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-harbouring with plasmid-mediated AmpC β-lactamase can be missed by phenotype-based ESBL-detection [32]. Therefore, we could not know about the relationship between these undiagnosed ESBL genes and fluoroquinolone-resistance. Second, because our study 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.

Funding information
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).

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study was approved by the Ethical Committees of Nagasaki University Hospital (13062425).
References


