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2 **Title Page**  
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4  
5 Original Article  
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9 **Genetic and Epidemiological Analysis of ESBL-producing *Klebsiella pneumoniae* in**  
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12 **Three Japanese University Hospitals**  
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## 26 **Authorship statement**

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29 All authors meet the ICMJE authorship criteria.  
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## 32 **Conflict of interest**

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36 The authors declare that they have no conflicts of interest.  
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## 40 **Abbreviations**

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43 ESBL, extended-spectrum beta-lactamase; EPKP, extended-spectrum beta-lactamase  
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1  
2 **Abstract** (240 words)  
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4 *Introduction:* We aimed to clarify the genetic background and molecular epidemiology of  
5 extended-spectrum beta-lactamase (ESBL)-producing *Klebsiella pneumoniae* (*K.*  
6 *pneumoniae*) at three geographically separated university hospitals in Japan.  
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11 *Methods:* From January 2014 to December 2016, 118 ESBL-producing *K. pneumoniae*  
12 (EPKP) strains that were detected and stored at three university hospitals were collected.  
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14 Molecular epidemiological analysis was performed using enterobacterial repetitive  
15 intergenic consensus (ERIC)-polymerase chain reaction (PCR) and multi-locus sequence  
16 typing (MLST). The ESBL type was determined using the PCR-sequence method. The  
17 presence of plasmid-mediated fluoroquinolone resistance (PMQR) genes was analyzed  
18 by PCR. We compared the relationships between PMQR gene possession/**quinolone**  
19 **resistance-determining region (QRDR) mutation** and levofloxacin (LVFX)/ciprofloxacin  
20 (CPFX) susceptibility.  
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34 *Results:* The detection rate of EPKP was 4.8% (144/2987 patients). MLST analysis  
35 revealed 62 distinct sequence types (STs). The distribution of STs was diverse, and only  
36 some EPKP strains had the same STs. ERIC-PCR showed discriminatory power similar  
37 to that of MLST. The major ESBL genotypes were CTX-M-15-, CTX-M-14-, and SHV-  
38 types, which were detected in 47, 30, and 27 strains, respectively. Ninety-one out of 118  
39 strains had PMQR genes and 14 out of 65 strains which were not susceptible to CPFX  
40 had QRDR mutations, and the accumulation of PMQR genes and QRDR mutations  
41 tended to lead to higher minimum inhibitory concentrations (MICs) of LVFX.  
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53 *Conclusions:* At three geographically separated university hospitals in Japan, the  
54 epidemiology of EPKP was quite diverse, and no epidemic strains were found, whereas  
55 CTX-M-14 and CTX-M-15 were predominant.  
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**Keywords**

*Klebsiella pneumoniae*, extended spectrum beta-lactamase, CTX-M, SHV, plasmid-mediated fluoroquinolone resistance, quinolone resistance-determining region

## Introduction

*Klebsiella pneumoniae*, a gram-negative, oxidase-negative bacterium of the order Enterobacterales, is a part of the gut flora. *K. pneumoniae* may cause infections such as pneumonia, urinary tract infection, biliary tract infection, and intraabdominal infection. Extended-spectrum beta-lactamase (ESBL)-producing *K. pneumoniae* (EPKP) is resistant to third-generation cephalosporins, extended-spectrum penicillins, and monobactams [1]. The incidence of EPKP is increasing worldwide, but its detection rate varies by country. Until 2000, ESBL types were predominantly TEM- and SHV-types, which were most commonly detected in *K. pneumoniae* [2]. However, in the 2000s, CTX-M-type ESBLs constituted the majority of ESBLs in the community [3]. In Latin America, from 2008 to 2010, the detection rates of ESBL-producing *Klebsiella* spp. were 60.4%, 59.2%, 49.9%, and 33.3% in Argentina, Chile, Brazil, and Mexico, respectively [4]. The rate of EPKP reported in 72 US hospitals located in the nine US census regions was 16.0%. According to the latest surveillance, the detection rate of ESBL-producing *Klebsiella* spp. has increased. In particular, a high rate of EPKP has been reported in European countries [5].

In Japan, ESBL-producing bacteria were continuously surveyed from 2004 to 2013, and the detection rate of EPKP increased from 2.1% to 6.7% [6]. The frequency of EPKP has also increased in the Kinki region of Japan [7]. These studies have shown that the CTX-M-type is the most common type in Japan.

**Japan Nosocomial Infection Surveillance** (JANIS) by the Ministry of Health, Labor, and Welfare of Japan set the criteria for third-generation cephalosporin-resistant *K. pneumoniae* [cefotaxime (CTX) minimum inhibitory concentration (MIC)  $\geq 4$   $\mu\text{g/mL}$  or ceftazidime (CAZ) MIC  $\geq 16$   $\mu\text{g/mL}$ ]. The JANIS data from 2017 showed 8.9% and

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2 5.0% of CTX-resistant and CAZ-resistant *K. pneumoniae*, respectively [8].  
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5 Some ESBL-producing strains have elevated MICs for quinolones [9]. Although  
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7 ESBL does not inactivate quinolones, some bacteria may carry additional genes or  
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9 mutations that mediate resistance to quinolones [10, 11].  
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12 Studies showed that the guidance from the Infectious Diseases Society of  
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14 America (IDSA) could not be applied to countries other than the United States due to  
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16 worldwide differences in the molecular epidemiology of resistance and availability of  
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18 anti-infective agents [12]. Compared with ESBL-producing *Escherichia coli* (*E. coli*),  
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20 EPKP bloodstream infection (BSI) has been reported to be associated with increased  
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22 admission to the intensive care unit, cardiovascular and neurological comorbidities, and  
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24 30-day mortality [13]. Therefore, it is necessary to manage BSIs caused by EPKP  
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26 separately from those caused by ESBL-producing *E. coli*, distinguished by the bacterial  
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28 species that produce ESBL. There have been no reports of **worldwide dominant STs in**  
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30 **EPKP like ST 131 in ESBL-producing *E. coli* [5]**. A study on the molecular epidemiology  
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32 of EPKP collected one strain per facility and no dominant STs were found [14]; however,  
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34 this study might have failed to show the endemicity in the facility or the region during the  
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36 particular period in question. Hence, to clarify the epidemiology of EPKPs detected in  
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38 three geographically separated university hospitals in Japan during a three-year period,  
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40 we conducted a molecular epidemiological analysis of EPKPs and the molecular  
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42 characteristics of their quinolone resistance.  
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## 53 **Material and methods**

### 54 *Bacterial isolates and antimicrobial susceptibility testing*

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58 This multicenter study involved three geographically separated university  
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2 hospitals in Japan. At each hospital, EPKP was consecutively isolated and stored from  
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4 January 2014 to December 2016; we used *K. pneumoniae* isolates obtained from various  
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6 clinical samples (such as urine, sputum, blood, bile, wound pus). **Only the first isolate**  
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8 **detected in one patient during each year was collected.** Species identification and  
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10 antimicrobial susceptibility testing were performed using Vitek 2 (bioMérieux, Marcy-  
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12 l'Étoile, France), MicroScan WalkAway (Beckman Coulter, Brea, CA, USA), and Vitek  
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14 MS (bioMérieux) according to the Clinical and Laboratory Standards Institute (CLSI)  
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16 guidelines (document M100-S22) [15]. ESBL screening was performed using the  
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18 following criteria: ceftazidime MIC  $\geq 2$   $\mu\text{g/mL}$  or cefotaxime  $\geq 2$   $\mu\text{g/mL}$ . All EPKP  
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20 strains were sent to Nagoya University Hospital, and re-identified by Vitek MS. **The**  
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22 **confirmatory test for ESBL production was performed with the disk potentiation test with**  
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24 **clavulanic acid or Cica  $\beta$ -test which was a commercially available ESBL detection kit**  
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26 **(Kanto Chemical, Tokyo, Japan). Antimicrobial susceptibility was re-tested for all EPKP**  
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28 **isolates with microdilution methods using MicroScan WalkAway according to CLSI**  
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30 **M100 Ed28 [16].**

#### 41 *Multilocus sequence typing (MLST)*

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43 The template DNA extracted using Cica Geneus DNA Extraction Reagent  
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45 (Kanto Chemical) was subjected to MLST analysis according to a previously reported  
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47 protocol [17]. The PCR products were cleaned using ExoSAP-IT (Thermo Fisher  
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49 Scientific, Waltham, MA, USA) and sent to FASMAC Co., Ltd (Kanagawa, Japan) for  
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51 sequencing. ST was determined based on the *K. pneumoniae* MLST database on the  
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53 Institut Pasteur MLST website [18]. Next-generation sequencing (NGS) was performed  
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55 on the 12 strains for which STs were difficult to determine. Genomic DNA was extracted  
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2 using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA). NGS was performed  
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4 using the MiSeq desktop sequencer (Illumina Inc., San Diego, CA, USA) and a MiSeq  
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6 Reagent Kit v3 (600 cycles) (Illumina). The sequencing library was prepared using the  
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8 Nextera XT DNA Sample Preparation Kit (Illumina). The sequence data were assembled  
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10 using the Nullarbor pipeline. The four new STs were registered in the Institut Pasteur  
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12 MLST database [18]. The phylogenetic relationships between STs were analyzed by  
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14 BioNumerics version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium). To examine  
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16 ST diversity, Simpson's diversity index was calculated as described in a previous report  
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18 [19]. The string test were performed to all EPKP strains according to the criteria described  
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20 in the previous report [20].  
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#### 29 *Enterobacterial repetitive intergenic consensus (ERIC)-PCR*

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31 Genetic relatedness was analyzed by ERIC-PCR using ERIC2 and ERIC1R  
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33 primers [21]. The cycling conditions were as follows: 3 min at 95 °C, 40 cycles of 30 s  
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35 at 92 °C, 1 min at 52 °C, 8 min at 72 °C, and a final extension of 16 min at 72 °C. The  
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37 ERIC-PCR fragments were visualized by 2.0% agarose gel electrophoresis stained with  
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39 GelRed (Biotium, Inc. Hayward, CA, USA). Fingerprints were visually compared and  
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41 patterns differing by at least one amplification band were classified as different. The  
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43 band patterns were visualized by BioNumerics version 7.1 (Applied Maths). The  
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45 dendrogram was cut off at > 90% similarity to determine whether they were genetically  
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47 related.  
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#### 56 *Detection of ESBL genes*

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58 Template DNA was extracted using the Cica Geneus DNA Extraction Reagent  
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2 (Kanto Chemical). The type of ESBL was determined using the Cica Geneus ESBL  
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4 Genotype Detection Kit (Kanto Chemical) according to the manufacturer's protocol,  
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6 which can detect six representative ESBL genotypes, namely CTX-M-1, CTX-M-2,  
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8 CTX-M-8, CTX-M-9, TEM, and SHV. For sequencing CTX-M genes, three sets of  
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10 primers were used [22]. The target genes were amplified by PCR using Premix Taq  
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12 (TaKaRa Bio, Shiga, Japan) with the following PCR thermal cycling conditions: one  
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14 cycle at 95 °C for 10 min; 32 cycles at 95 °C for 30 s (decreased by 1 °C per cycle until  
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16 the temperature reached 52 °C), 62 °C for 30 s, 72 °C for 90 s, and one cycle at 72 °C for  
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18 10 min. Sequencing of SHV-type genes and TEM-type genes was performed as described  
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20 previously [23]. The PCR products were analyzed by 2.0% agarose gel electrophoresis  
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22 and stained with GelRed (Biotium). The PCR products were cleaned using ExoSAP-IT  
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24 (Thermo Fisher Scientific), sequencing was performed at FASMAC, and the results were  
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26 analyzed using the Basic Local Alignment Search Tool (BLAST) at the National Center  
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28 for Biotechnology Information (NCBI) website [24].  
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39 *Detection of plasmid-mediated quinolone-resistant (PMQR) genes and quinolone*  
40 *resistance-determining region (QRDR) mutations*  
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43 We used the multiplex PCR primer sets of four PMQR genes [*qnrA*, *qnrB*, *qnrS*,  
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45 and *aac (6')-Ib-cr*] as described in previous reports [25, 26]. *qepA* was amplified using a  
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47 previously reported primer set [27] according to previously reported reaction conditions  
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49 [28]. The amplification products were identified by their sizes after electrophoresis on a  
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51 2.0% agarose gel at 100 V for 60 min and stained with GelRed (Biotium).  
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56 For 65 strains that were not susceptible to levofloxacin (LVFX) or ciprofloxacin  
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58 (CPFX), PCR amplification of the QRDRs of GyrA and ParC was carried out as  
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2 previously reported [29]. Sequencing of the *gyrA* and *parC* target regions was performed  
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4 at FASMAC. The data were compared with GenBank DQ673325 and AF303641 and  
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6 analyzed on the NCBI website [24].  
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### 10 11 *Statistical analysis*

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13 All p-values were calculated using Fisher's exact test. Statistical significance  
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15 was set at  $p < 0.05$ . All statistical analyses were performed using EZR version 1.53 [30].  
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17 Chi-square tests were used to compare groups. Simpson's index of diversity was  
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19 calculated for the ST distribution according to a previous report [31].  
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### 26 *Ethical approval*

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28 This study was approved by the Ethics Committee of the Nagoya University  
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30 Graduate School of Medicine on August 20, 2017 (reference number: 2017-0357).  
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## Results

### *Molecular Epidemiological Characteristics of EPKP isolates in the three facilities in Japan*

A total of 2987 *K. pneumoniae* clinical isolates were studied, of which 144 (4.8%) were EPKP. The EPKP rates were 1.2% (11/891), 6.4% (50/776), and 5.9% (83/1320) at Asahikawa Medical University Hospital, Kyushu University Hospital, and Nagoya University Hospital, respectively. One hundred eighteen EPKP isolates, which were detected as first isolate in one patient during each year, were included in this study.

MLST was performed for the 118 strains using Sanger sequencing or NGS (Table 1). Sixty-two types of STs were detected, of which 43 were detected from only a single strain. Four unregistered STs were identified in the seven isolates. Simpson's diversity index for ST was 0.967, indicating a high diversity. Figure 1 shows the results of ST cluster analysis of the isolates detected at the three facilities. ST25 (n = 12) was predominant, followed by ST45 (n = 8), ST17 (n = 7), and ST15 (n = 6). However, most STs were isolated from a single hospital, and ST14, ST15, ST17, ST25, ST35, ST37, ST39, ST45, and ST307 were detected in two different hospitals. No STs were isolated from all three hospitals.

Using a commercially available ESBL detection kit, 108 *bla*<sub>SHV</sub>, 54 *bla*<sub>TEM</sub>, 54 *bla*<sub>CTX-M-1-group</sub>, 41 *bla*<sub>CTX-M-9</sub> group, and nine *bla*<sub>CTX-M-2-group</sub> isolates were detected among the 118 isolates (Table 1 and Figure 2). The *bla*<sub>CTX-M-8</sub> group was not detected. Sequencing analysis showed that only 27 out of 108 strains possessed ESBL type *bla*<sub>SHV</sub> (9 *bla*<sub>SHV-28</sub>, 7 *bla*<sub>SHV-2</sub>, 5 *bla*<sub>SHV-12</sub>, 5 *bla*<sub>SHV-27</sub>, and 1 *bla*<sub>SHV-38</sub>), and all *bla*<sub>TEM</sub> genes were not ESBL type (54 *bla*<sub>TEM-1</sub>). The predominant *bla*<sub>CTX-M</sub> were *bla*<sub>CTX-M-15</sub> (40 isolates), *bla*<sub>CTX-M-14</sub> (32 isolates), *bla*<sub>CTX-M-3</sub> (10 isolates), and *bla*<sub>CTX-M-2</sub> (9 isolates). There were

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2 some strains in which the ESBL gene could not be detected. Nine, six, five, and four  
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4 CTX-M-15-producers belonged to ST25, ST45, ST15, and ST5455, respectively. Four  
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6 CTX-M-2-producers and CTX-M-14-producers belonged to ST1121 and ST1786,  
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8 respectively. More than half of the CTX-M-3-producers and CTX-M-14-producers  
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10 belonged to a single ST. The ERIC-PCR fingerprints and dendrogram of 118 EPKP strains  
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12 were shown in Figure 2, together with STs and ESBL types. Judging from the criterion of  
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14 genetic relatedness with 90% or more similarity in fingerprints of ERIC-PCR, STs, and  
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16 ERIC-PCR appeared to have similar discriminatory powers. String tests were performed  
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18 on all isolates, and only five strains were positive (three ST412, one ST25, and one  
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20 ST290).  
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#### 29 *Comparison of PMQR possession, QRDR mutation, and LVFX/CPFX susceptibility*

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31 The non-susceptibility rate of LVFX was 26/118 (22.0%) and that of CPFX was  
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33 65/118 (55.1%). PCR detection of PMQR genes was performed in 118 strains, and 91  
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35 strains (77.1%) had at least one PMQR gene. We detected *qnrS* in 46 strains, *aac (6')-Ib-*  
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37 *cr* in 37 strains, *qnrB* in 34 strains, and *qnrA* in three strains; *qepA* was not detected. Of  
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39 the 28 strains possessing multiple PMQR genes, 26 (92.9%) had *qnrB* and *aac (6')-Ib-*  
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41 *cr*.  
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43 The rate of non-susceptibility to LVFX was 26.4% for strains possessing the PMQR gene  
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45 and 7.4% for strains without the PMQR gene ( $p = 0.038$ ). The rate of non-susceptibility  
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47 to CPFX was 67.0% for strains with the PMQR gene and 14.8% for strains without the  
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49 PMQR gene ( $p < 0.001$ ). The rate of non-susceptibility to LVFX or CPFX tended to  
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51 increase as the number of PMQR genes increased (Figure 3a, 3b). Among the 65 strains  
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53 that were not susceptible to CPFX, 63 (96.9%) carried at least one PMQR gene and 14  
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55 (21.5%) had at least one QRDR mutation (Table 2). GyrA amino acid substitutions were  
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2 found in 13 strains (20.0%), and ParC amino acid substitutions were found in nine strains  
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4 (13.8%). Amino acid substitutions in GyrA were detected at position 83 (seven isolates  
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6 with Ser83Ile and two isolates with Ser83Phe) and position 87 (three isolates with  
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8 Asp87Tyr, two isolates with Asp87Ala, and one isolate with Asp87Asn). An amino acid  
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10 substitution of ParC was detected at position 80 (nine isolates with Ser80Ile). Only strain  
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12 N51 had no PMQR and no QRDR mutation. Of the 41 CPFY-resistant isolates, 26  
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14 (65.9 %) harbored *aac (6)-Ib-cr*. Half of the isolates resistant to both LVFX and CPFY  
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16 had PMQR in combination with *gyrA* and/or *parC* mutations. The rate of non-  
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18 susceptibility to LVFX and CPFY increased with the accumulation of PMQR genes  
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20 (Figure 3a, 3b). Moreover, as PMQR gene possessions and QRDR mutations accumulated,  
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22 the MICs of LVFX and CPFY increased (Figure 3c, 3d).  
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## Discussion

In this study, we investigated the molecular epidemiology of EPKP in three geographically separated Japanese university hospitals. The detection rate of EPKP per total *K. pneumoniae* clinical isolates was 4.8%, which was similar to the rate in a previous report from Japan [6] and lower than that reported in other countries [4]. Although the EPKP detection rate tended to increase with time in European countries [5], our detection rate in the three hospitals was stable during the study period, approximately 1% at Asahikawa Medical University Hospital and approximately 6% at Kyushu and Nagoya University Hospitals. No predominant ST appeared in the EPKP isolates examined in this study; even the most prevalent ST, ST25, which was found in 12 isolates, was detected in only two of the three hospitals. Unlike ESBL-producing *E. coli*, in which ST131 prevails worldwide [32], there have been no reports of such predominant STs in EPKP, but it is necessary to pay close attention to future trends. ST25 EPKP has been frequently detected in other studies in Japan and Argentina; this ST is associated with the hypervirulent (hypermucoviscous) type of disease. However, only one out of 12 ST25 strains was positive in the string test, which was similarly low rate to the previously report in Japan [14]. Three string test positive ST412 strains were detected at a single hospital, which suggested horizontal transmission of a strain in the hospital.

As seen in this study, MLST and ERIC-PCR appeared to have similar discriminating powers; however, MLST is more suitable for inter-hospital comparison of molecular epidemiology, whereas ERIC-PCR is more suitable for intra-hospital molecular epidemiological analysis.

For 11 of the 12 strains for which ST could not be determined by Sanger sequencing, we used NGS, which is one of the most promising applications for molecular

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2 epidemiological analysis in the future, despite its high cost and technical requirements  
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4 [33].  
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7 Our data showed that the most common CTX-M-type beta-lactamases were  
8 CTX-M-15, followed by CTX-M-14 and CTX-M-3; this result was consistent with  
9 previous reports [14, 34-37]. The CTX-M-1 group, which included CTX-M-15 and CTX-  
10 M-3, was the most predominant. Only 27 (25.0%) out of 108 isolates in which *bla*<sub>SHV</sub> was  
11 detected were ESBL producers, and all *bla*TEMs were TEM-1, not ESBL. No ESBL type  
12 was related to the specific ST of EPKP analyzed in this study.  
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22 The major mechanisms of quinolone resistance are acquisition of PMQR genes  
23 and spontaneous mutations in QRDR (such as GyrA and ParC) [38-40]. There are three  
24 types of PMQR genes: protective proteins of quinolone targets (*qnrA*, *qnrB*, and *qnrS*), a  
25 plasmid-mediated efflux pump (*qepA*), and aminoglycoside acetyltransferase (*aac* (6')-  
26 *Ib-cr*) [41, 42]. The MICs of LVFX and CPFEX were higher in the group with PMQR than  
27 in the group without PMQR. In particular, when *qnrB* and *aac* (6')-*Ib-cr* coexisted, the  
28 MIC of CPFEX tended to increase. The most common QRDR mutations found were the  
29 coexistence of Ser83Ile in GyrA and Ser87Ile in ParC substitutions, which is consistent  
30 with a previous report [38]. Compared with PMQR possession or QRDR mutations only,  
31 PMQR in combination with QRDR mutations led to a higher MIC in LVFX. PMQR itself  
32 is generally thought to confer low levels of quinolone resistance [43]. However, even a  
33 low level of increase in the MIC of quinolone would lead to an increase in the mutant  
34 selection window, increasing the possibility of giving rise to mutants with higher  
35 resistance [44]. Accumulation of PMQR genes could be an important basis for the  
36 selection of more resistant mutants, especially LVFX.  
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58 Our study has several limitations. First, we showed the molecular epidemiology  
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2 of EPKP isolates from geographically separated university hospitals; however, it was  
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4 uncertain whether this result could be generalized to the epidemiology in all of Japan.  
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6 Second, we could not determine whether the isolates were community-acquired, hospital-  
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8 acquired, colonized, or caused infections. Third, **since this study used older CLSI**  
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10 **breakpoints (M100-S28) of fluoroquinolone, some fluoroquinolone-resistant strains**  
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12 **might be missed and could be detected using newer versions of CLSI breakpoints. Fourth,**  
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16 QRDR amino acid substitutions were determined only in 65 strains not susceptible to  
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18 LVFX or CPFEX, so we could not show the entire scope of QRDR mutations in all 118  
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20 strains. In addition, amino acid substitutions other than GyrA and ParC have not been  
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22 determined; therefore, some strains may contain other amino acid substitutions (such as  
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24 GyrB or ParE). Finally, we did not analyze plasmids bearing ESBL genes, which is a  
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26 topic for future studies.  
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## 35 **Conclusions**

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37 Our study demonstrated the molecular epidemiological diversity of EPKP detected in  
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39 three geographically separated Japanese university hospitals. The most common CTX-  
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41 M-type and ST in EPKP were CTX-M-15 and ST25. The accumulation of PMQR genes  
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43 and QRDR mutations contributed to the higher MICs of LVFX and CPFEX in EPKP  
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45 isolates. Continuous surveillance of EPKP isolates in terms of resistance to both beta-  
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47 lactams and fluoroquinolones is necessary to monitor the trend of epidemiological  
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49 changes, and further molecular analyses are warranted to prevent the spread of EPKP in  
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19 **Conflict of Interest (COI)**  
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21 The authors declare that they have no conflicts of interest.  
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26 **Author contributions**  
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29 Keisuke Oka drafted the manuscript and contributed to the data analysis. Kazumitsu  
30  
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32  
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2 **Figure Legends**  
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4 **Figure 1: Cluster analysis of ESBL-producing *Klebsiella pneumoniae* distribution.**  
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9 The distributions were analyzed by BioNumerics version 7.1. The number indicates  
10 sequence type (ST). The size of the circles indicates the number of strains. The color of  
11 the circles indicates the university where they were detected: green, Asahikawa Medical  
12 University Hospital; red, Kyushu University Hospital; blue, Nagoya University Hospital.  
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14 Abbreviations: ESBL, extended spectrum beta-lactamase.  
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24 **Figure 2: ERIC-PCR dendrogram and fingerprint of 118 ESBL-producing**  
25 ***Klebsiella pneumoniae* strains.**  
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31 The dendrogram and fingerprint were analyzed by BioNumerics version 7.1. In the  
32 dendrogram, the dotted line shows 90% similarity, and when it exceeded 90%, it was  
33 judged that there was similarity. The black lines at the right show strains with 90% or  
34 more homology in the phylogenetic tree, the same ST, and the same  $\beta$ -lactamase. Most  
35 strains were independent of dendrogram/fingerprint and ST, but some strains showed  
36 similarities.  
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46 Abbreviations: ERIC-PCR, enterobacterial repetitive intergenic consensus-polymerase  
47 chain reaction; ESBL, extended-spectrum beta-lactamase; ST, sequence type; -, negative.  
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2 **Figure 3a, 3b: Relationship between levofloxacin or ciprofloxacin susceptibility and**  
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4 **PMQR gene possession (n = 118).**  
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9 As the number of PMQR genes increased, the insensitivity rates to LVFX and CPF  
10 increased.  
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17 **Figure 3c, 3d: Relationship between the MIC of levofloxacin or ciprofloxacin and**  
18 **PMQR gene possessions/QRDR mutations possession (n = 65).**  
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24 The higher the number of PMQR genes and QRDR amino acid substitutions, the higher  
25 the MIC of LVFX and CPF.  
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31 Resistance ranges according to CLSI M100 Ed28 were LVFX S  $\leq$  2  $\mu$ g/mL, I = 4  $\mu$ g/mL,  
32 R  $\geq$  8  $\mu$ g/mL; CPF S  $\leq$  1  $\mu$ g/mL, I = 2  $\mu$ g/mL, and R  $\geq$  4  $\mu$ g/mL.  
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35 Abbreviations: PMQR, plasmid-mediated quinolone resistance; QRDR, quinolone  
36 resistance-determining region; LVFX, levofloxacin; CPF, ciprofloxacin; MIC,  
37 minimum inhibitory concentration; -, negative. Susceptibility value: S, susceptible; I,  
38 intermediate; R, resistant. Amino acid substitution: A, alanine; D, aspartic acid; F,  
39 phenylalanine; I, isoleucine; N, asparagine; S, serine; Y, tyrosine.  
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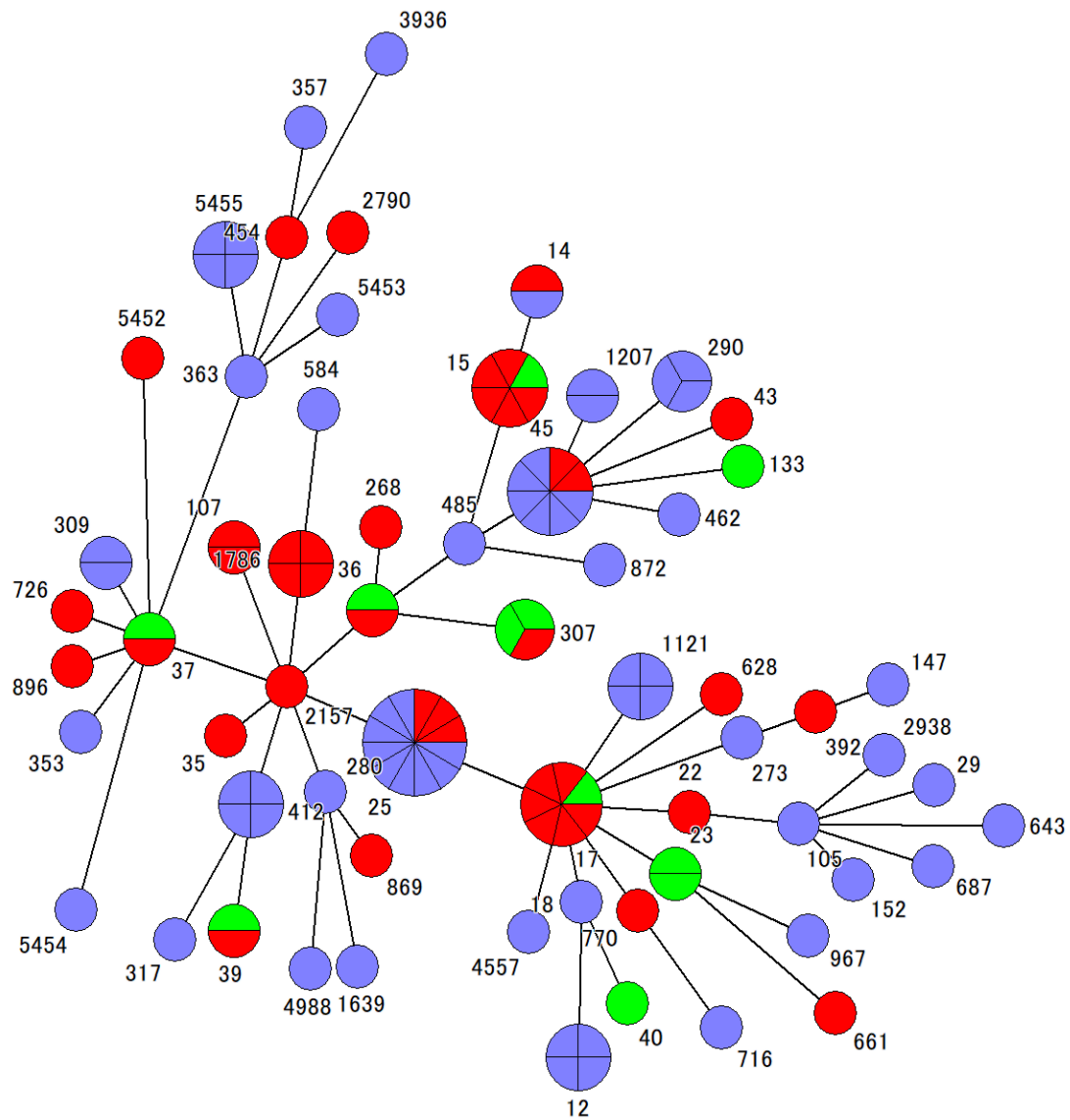
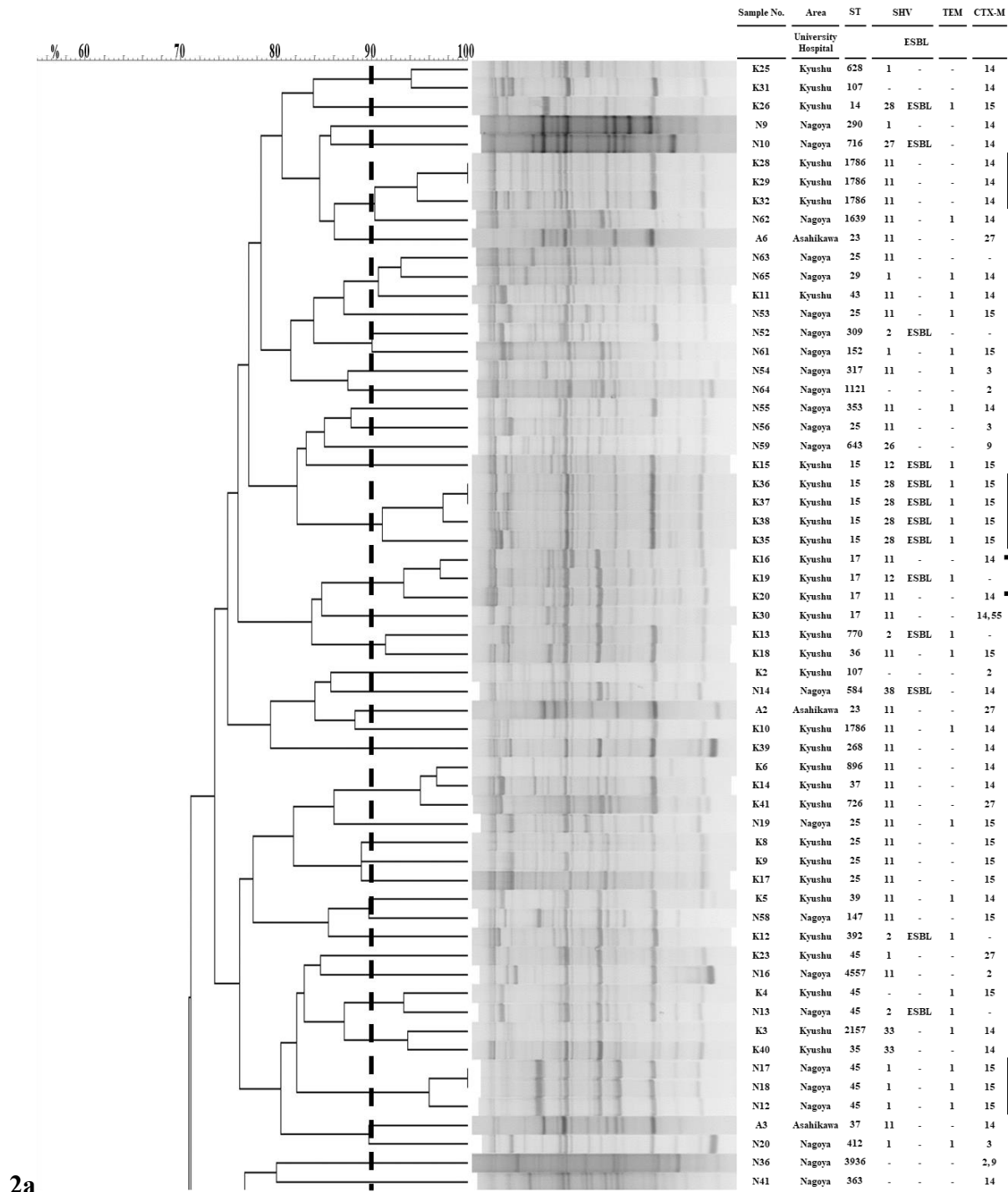


Figure 1



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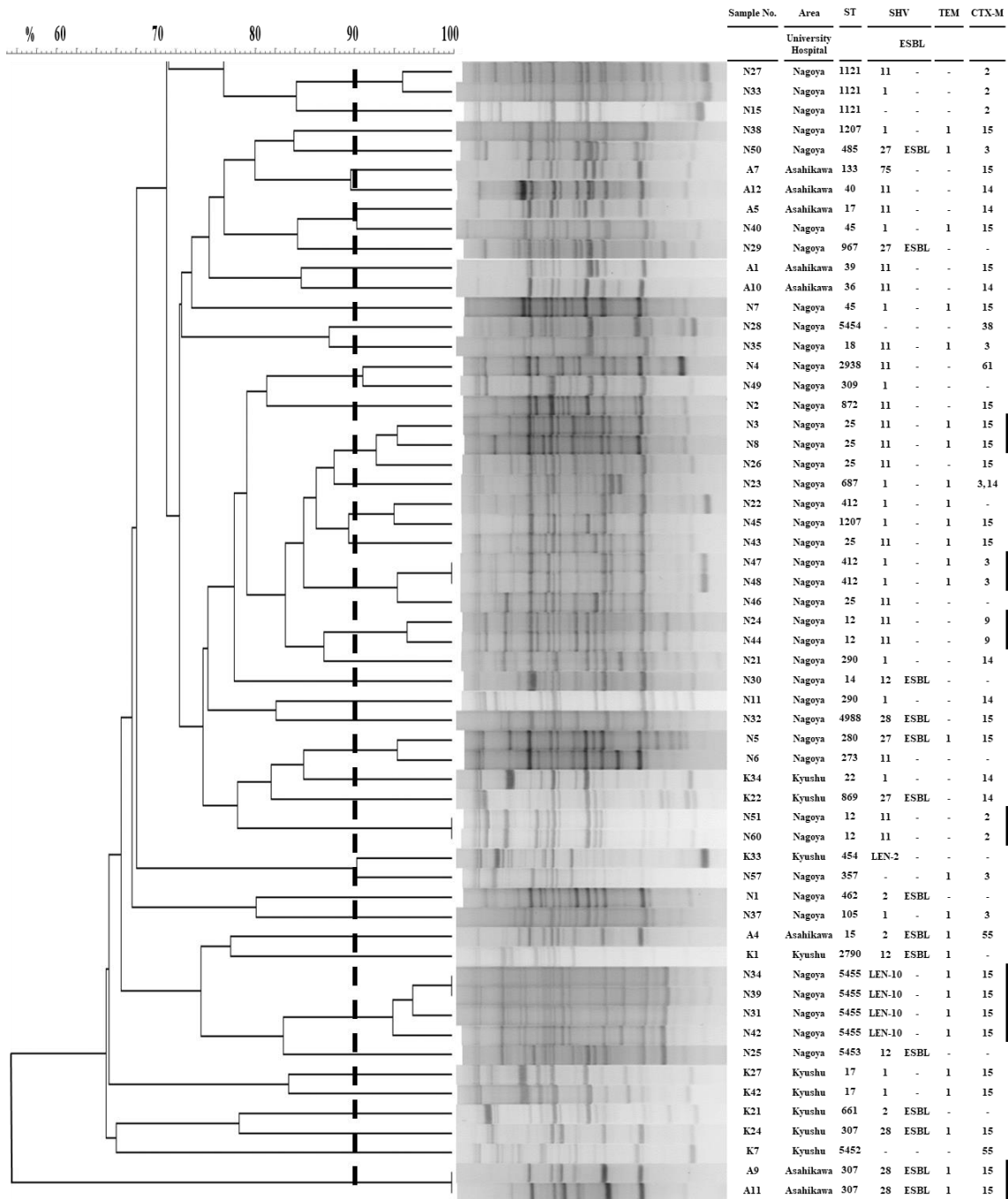
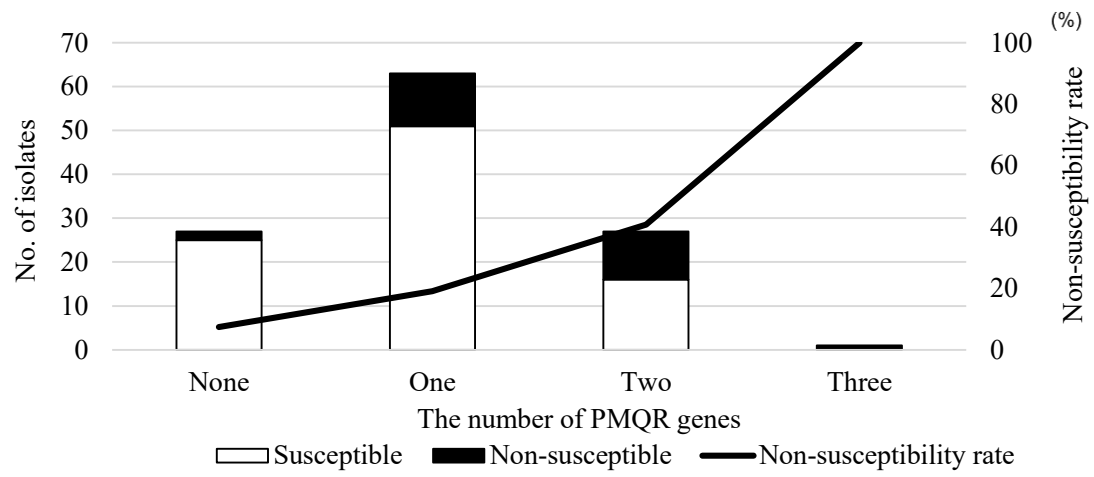
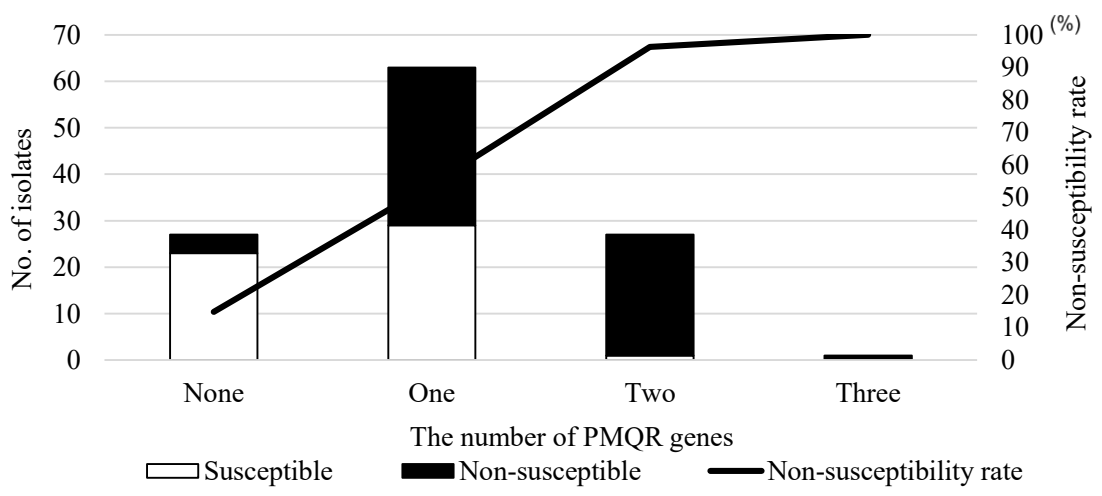


Figure 2

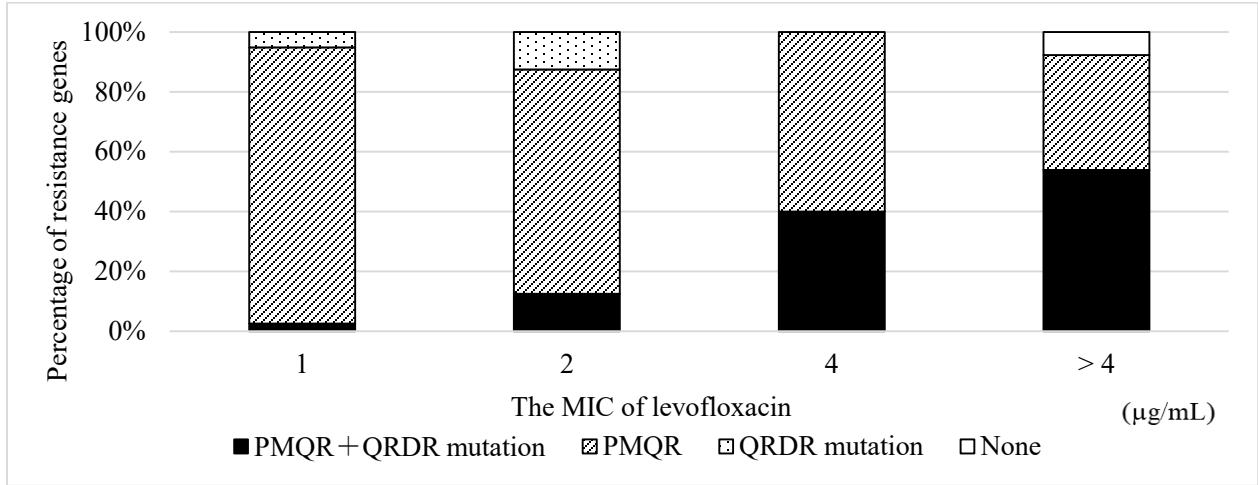
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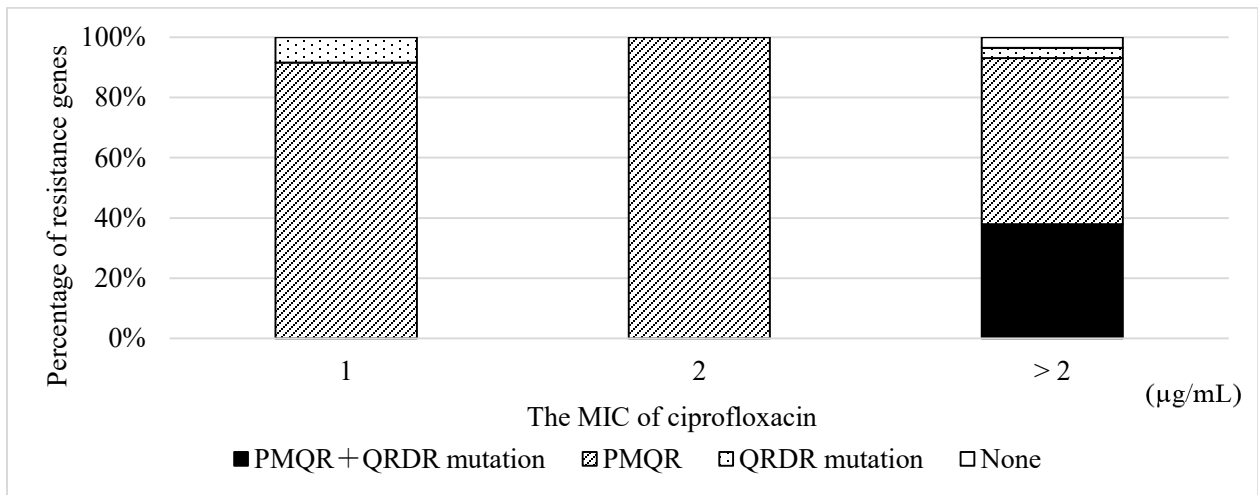


Figure 3

**Table 1: Distribution of CTX-M types and ST of ESBL-producing *Klebsiella pneumoniae* 118 isolates.**

	No. of isolates	ST																		Duplicate ST (19 STs)	Single ST (43 STs)			
		25	45	17	15	12	412	1121	1786	5455	290	307	14	23	36	37	39	107	309			1207		
<b>CTX-M-1 group</b>																								
CTX-M-15	40	9	6	2	5 (5)				4		3 (3)	1 (1)		1		1			2	34 (9)	6 (2)			
CTX-M-3	9	1				3														4	5 (1)			
CTX-M-55	2				1 (1)															1 (1)	1			
CTX-M-61	1																				1			
<b>CTX-M-2 group</b>																								
CTX-M-2	8					2		4									1			7	1			
<b>CTX-M-9 group</b>																								
CTX-M-14	30			3				4		3				1	2	1	1			15	15 (3)			
CTX-M-27	4		1										2							3	1			
CTX-M-9	3					2														2	1			
CTX-M-38	1																				1			
CTX-M-3	1																				1			
+ CTX-M-14	1																							
CTX-M-14	1			1																1				
+ CTX-M-55	1																							
CTX-M-2	1																				1			
+ CTX-M-9	1																				1			
<b>ESBL type SHVs</b>																								
SHV-28	9				4 (4)						3 (3)	1 (1)								8 (8)	1 (1)			
SHV-2	7		1		1 (1)													1		3 (1)	4			
SHV-12	5			1	1 (1)							1								3 (1)	2			
SHV-27	5																				5 (4)			
SHV-38	1																				1 (1)			
Not detected	6	2					1													1	4	2		
Overall	118	12	8	7	6	4	4	4	4	4	3	3	2	2	2	2	2	2	2	2	2	2	75	43

One hundred and eighteen strains of *Klebsiella pneumoniae* were classified into 62 types by ST. Forty-three strains of single STs were detected.

Simpson's diversity index for ST was 0.967, indicating a high degree of diversity. Abbreviations: ST; sequence type, ESBL; extended spectrum beta-lactamase.

The numbers in parentheses indicate the number of strains possessing both CTX-M and ESBL type SHV.



**Table 2: Relationship between PMQR genes/QRDR mutations and LVFX/CPFX susceptibility.**

Strain No.	PMQR				QRDR mutation			LVFX		CPFX	
	<i>qnrA</i>	<i>qnrB</i>	<i>qnrS</i>	<i>aac(6)-Ib-cr</i>	<i>GyrA</i>	<i>ParC</i>	MIC	Value	MIC	Value	
A9	-	●	-	●	S83I	-	S80I	>4	R	>2	R
A11	-	●	-	●	S83I	-	S80I	>4	R	>2	R
K12	-	●	-	●	S83I	-	S80I	>4	R	>2	R
K41	-	●	-	●	S83I	-	S80I	>4	R	>2	R
N6	-	●	-	●	S83I	-	S80I	>4	R	>2	R
A4	-	●	-	●	S83F	D87A	-	>4	R	>2	R
K24	●	●	-	-	S83I	-	S80I	>4	R	>2	R
N53	-	●	-	●	-	-	-	>4	R	>2	R
K22	-	-	●	-	-	-	-	>4	R	>2	R
N23	-	-	●	-	-	-	-	>4	R	>2	R
N48	-	-	●	-	-	-	-	>4	R	>2	R
N50	-	-	●	-	-	-	-	>4	R	>2	R
N51	-	-	-	-	-	-	-	>4	R	>2	R
N2	-	●	-	●	-	-	-	4	R	>2	R
N12	-	●	-	●	-	-	-	4	R	>2	R
K9	-	●	-	-	-	D87Y	-	4	R	>2	R
N44	●	-	-	-	-	-	-	4	R	>2	R
K15	-	-	-	●	S83F	D87A	S80I	4	R	>2	R
N7	-	●	-	●	-	-	-	2	I	>2	R
K17	-	●	-	-	-	D87Y	-	2	I	>2	R
N14	-	-	●	-	-	-	-	2	I	>2	R
N58	-	-	-	-	S83I	-	S80I	2	I	>2	R
K32	-	●	●	●	-	-	-	2	I	2	R
A10	-	-	●	-	-	-	-	2	I	2	R
N54	-	-	●	-	-	-	-	2	I	2	R
K1	-	-	-	●	-	-	-	2	I	2	R
N8	-	●	-	●	-	-	S80I	1	S	>2	R
K13	-	●	-	●	-	-	-	1	S	>2	R
K35	-	●	-	●	-	-	-	1	S	>2	R
N3	-	●	-	●	-	-	-	1	S	>2	R
N13	-	●	-	●	-	-	-	1	S	>2	R
N19	-	●	-	●	-	-	-	1	S	>2	R
A1	-	-	-	●	-	-	-	1	S	>2	R
K38	-	●	-	●	-	-	-	1	S	2	R
N1	-	●	-	●	-	-	-	1	S	2	R
N5	-	●	-	●	-	-	-	1	S	2	R
N17	-	●	-	●	-	-	-	1	S	2	R
N18	-	●	-	●	-	-	-	1	S	2	R
N40	-	●	-	●	-	-	-	1	S	2	R
N61	-	●	-	●	-	-	-	1	S	2	R
N10	-	-	●	-	-	-	-	1	S	2	R
N35	-	●	●	-	-	-	-	1	S	1	I
N26	-	●	-	●	-	-	-	1	S	1	I
K4	-	-	●	-	-	-	-	1	S	1	I
K6	-	-	●	-	-	-	-	1	S	1	I
K16	-	-	●	-	-	-	-	1	S	1	I
K20	-	-	●	-	-	-	-	1	S	1	I
K21	-	-	●	-	-	-	-	1	S	1	I
K34	-	-	●	-	-	-	-	1	S	1	I
K42	-	-	●	-	-	-	-	1	S	1	I
N20	-	-	●	-	-	-	-	1	S	1	I
N22	-	-	●	-	-	-	-	1	S	1	I
N30	-	-	●	-	-	-	-	1	S	1	I
N31	-	-	●	-	-	-	-	1	S	1	I
N34	-	-	●	-	-	-	-	1	S	1	I
N37	-	-	●	-	-	-	-	1	S	1	I
N39	-	-	●	-	-	-	-	1	S	1	I
N42	-	-	●	-	-	-	-	1	S	1	I
N46	-	-	●	-	-	-	-	1	S	1	I
N47	-	-	●	-	-	-	-	1	S	1	I
N52	-	-	●	-	-	-	-	1	S	1	I
N62	-	-	●	-	-	-	-	1	S	1	I
K23	-	-	-	●	-	-	-	1	S	1	I
A3	-	-	-	-	-	D87N	-	1	S	1	I
K8	-	-	-	-	-	D87Y	-	1	S	1	I

Sixty-five strains with either LVFX or CPFX not susceptible were included.

Resistance ranges according to CLSI M100 Ed28 were LVFX S  $\leq$  2  $\mu\text{g/mL}$ , I = 4  $\mu\text{g/mL}$ , R  $\geq$  8  $\mu\text{g/mL}$ ; CPFX S  $\leq$  1  $\mu\text{g/mL}$ , I = 2  $\mu\text{g/mL}$ , and R  $\geq$  4  $\mu\text{g/mL}$ .

Abbreviations:

PMQR, plasmid-mediated quinolone resistance; QRDR, quinolone resistance-determining region; LVFX, levofloxacin; CPFX, ciprofloxacin; MIC, minimum inhibitory concentration; -, negative.

Susceptibility value: S: susceptible, I: intermediate, R: resistant

Amino acid substitution: A, alanine; D, aspartic acid; F, phenylalanine; I, isoleucine; N, asparagine; S, serine; Y, tyrosine.