Table 3. MIC and amino acid substitutions in the QRDRs of O1-ST95 strains and O25b-ST131 strains

Strains		ID	No. of passages	MIC of CIP (µg/mL)	Amino acid substitutions in the QRDRs	
					GyrA	ParC
O1-ST95	Parent strain	O1-0	P 0	0.012	wt	wt
		O1-14	P 14	0.25	S83L	wt
	Transformant	O1T-0	P 0	0.25	wt	wt
		O1T-15	P 15	6	S83L	wt
		O1T-17	P 17	32	S83L	S80R
O25b-ST131	Parent strain	O25-0	P 0	0.012	wt	wt
		O25-4	P 4	0.19	S83L	wt
	Transformant	O25T-0	P 0	0.38	wt	wt
		O25T-13	P 13	4	S83L	wt
		O25T-17	P 17	24	S83L	S80R

wt, wild type; S83L, amino acid substitution of Serine to Leucine at position 83;
S80R, amino acid substitution of Serine to Arginine at position 80.

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Fig. 1. (A)

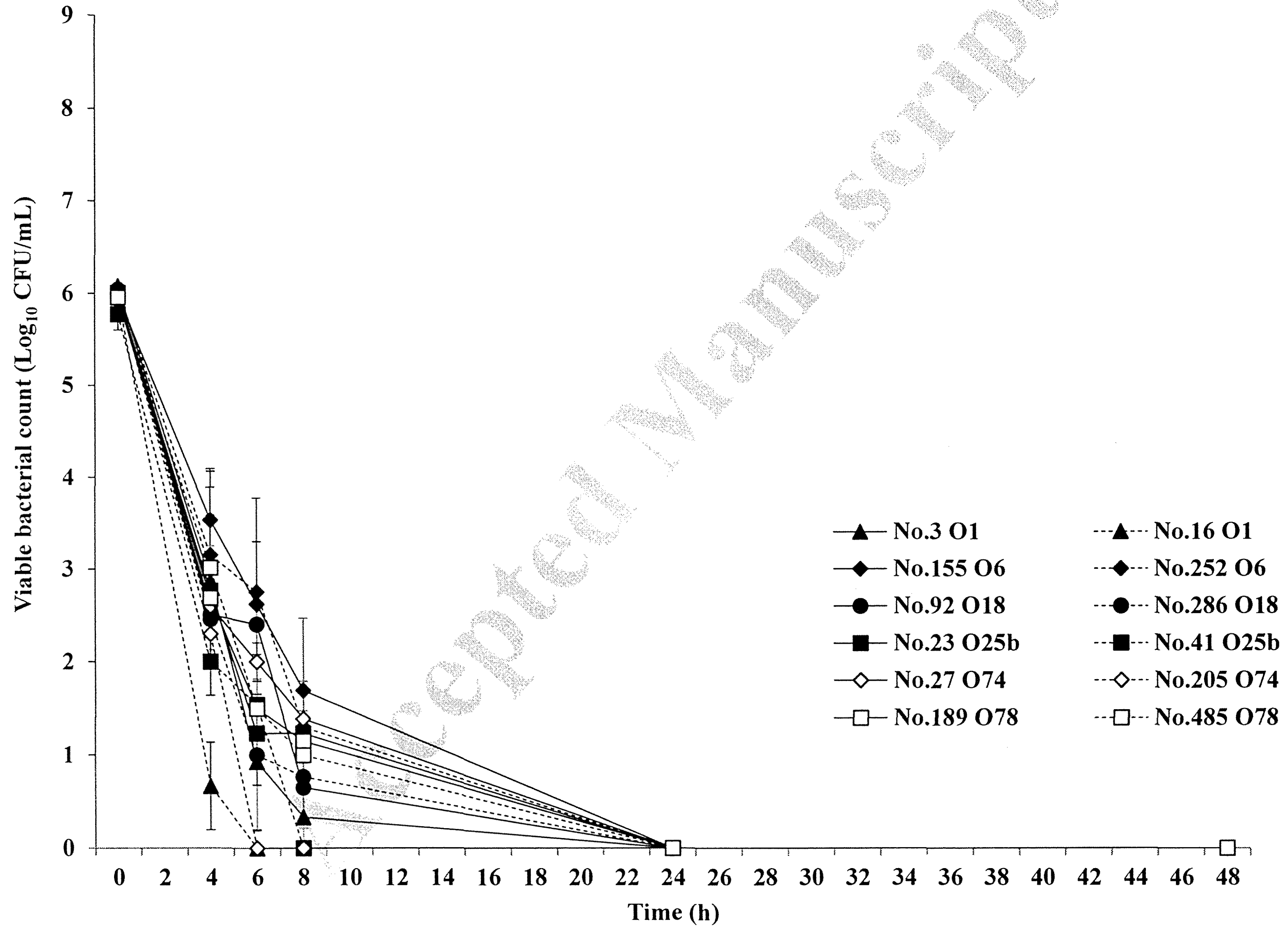

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Fig. 1. (B)

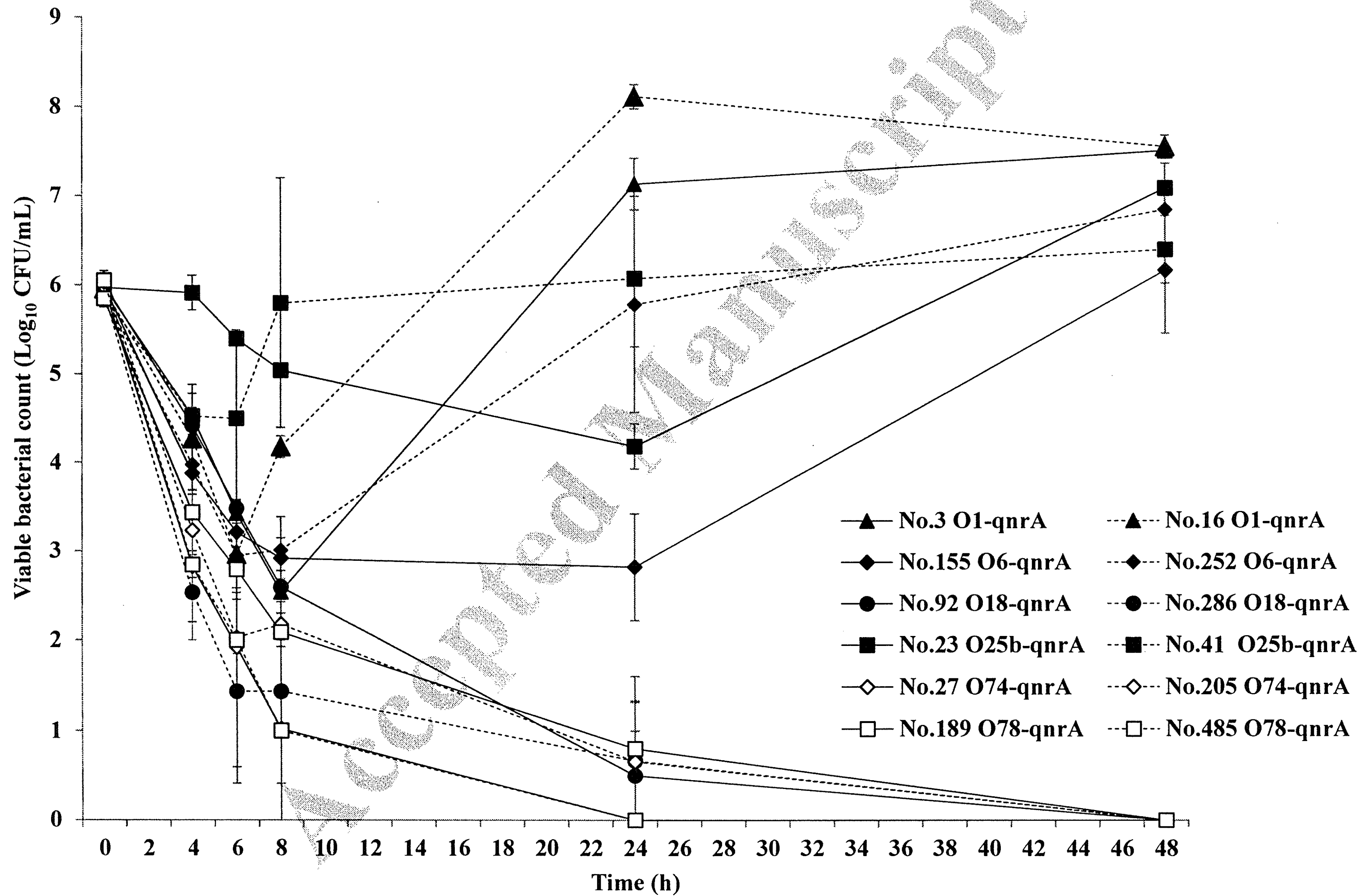

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Fig. 2

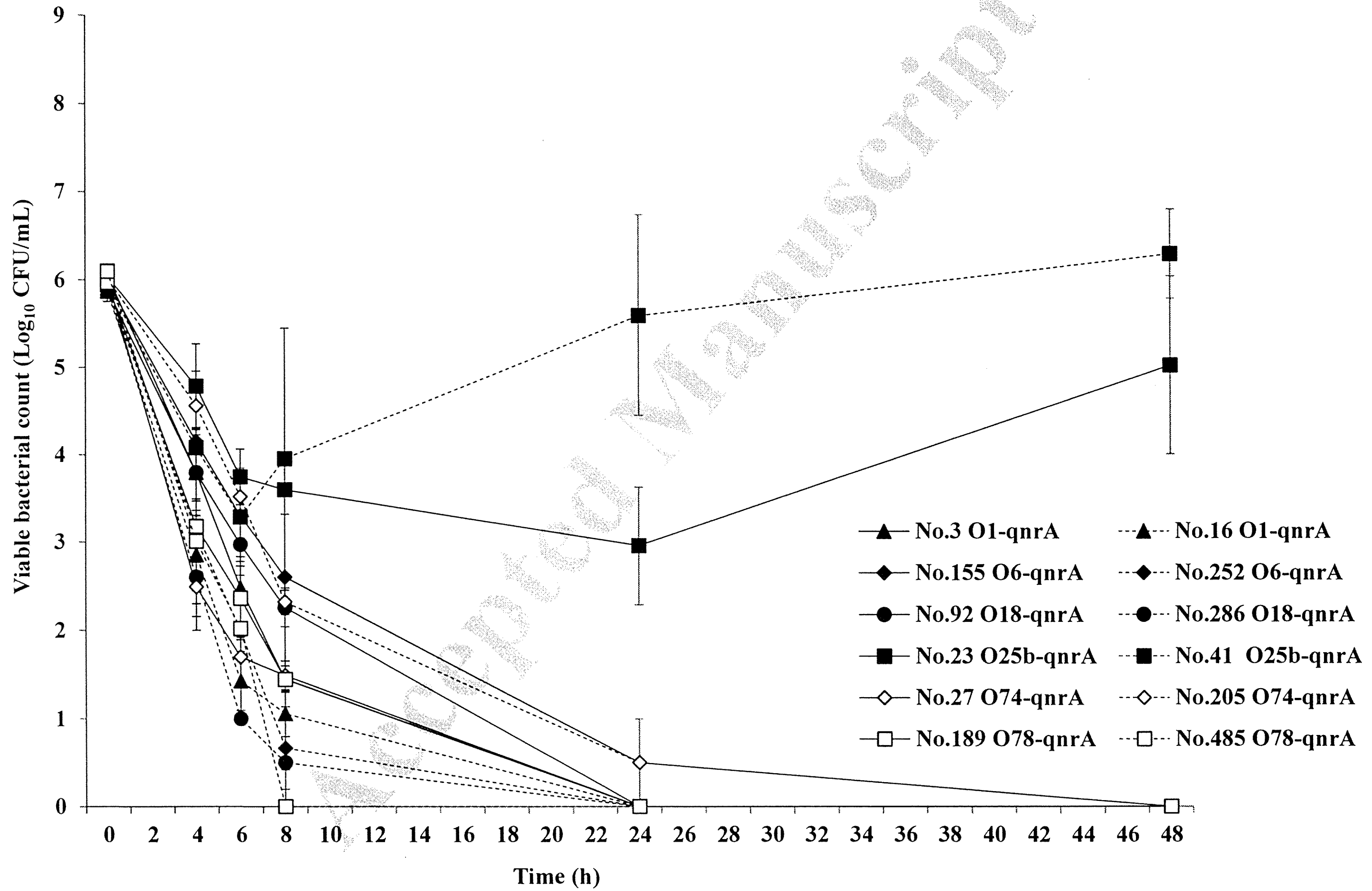

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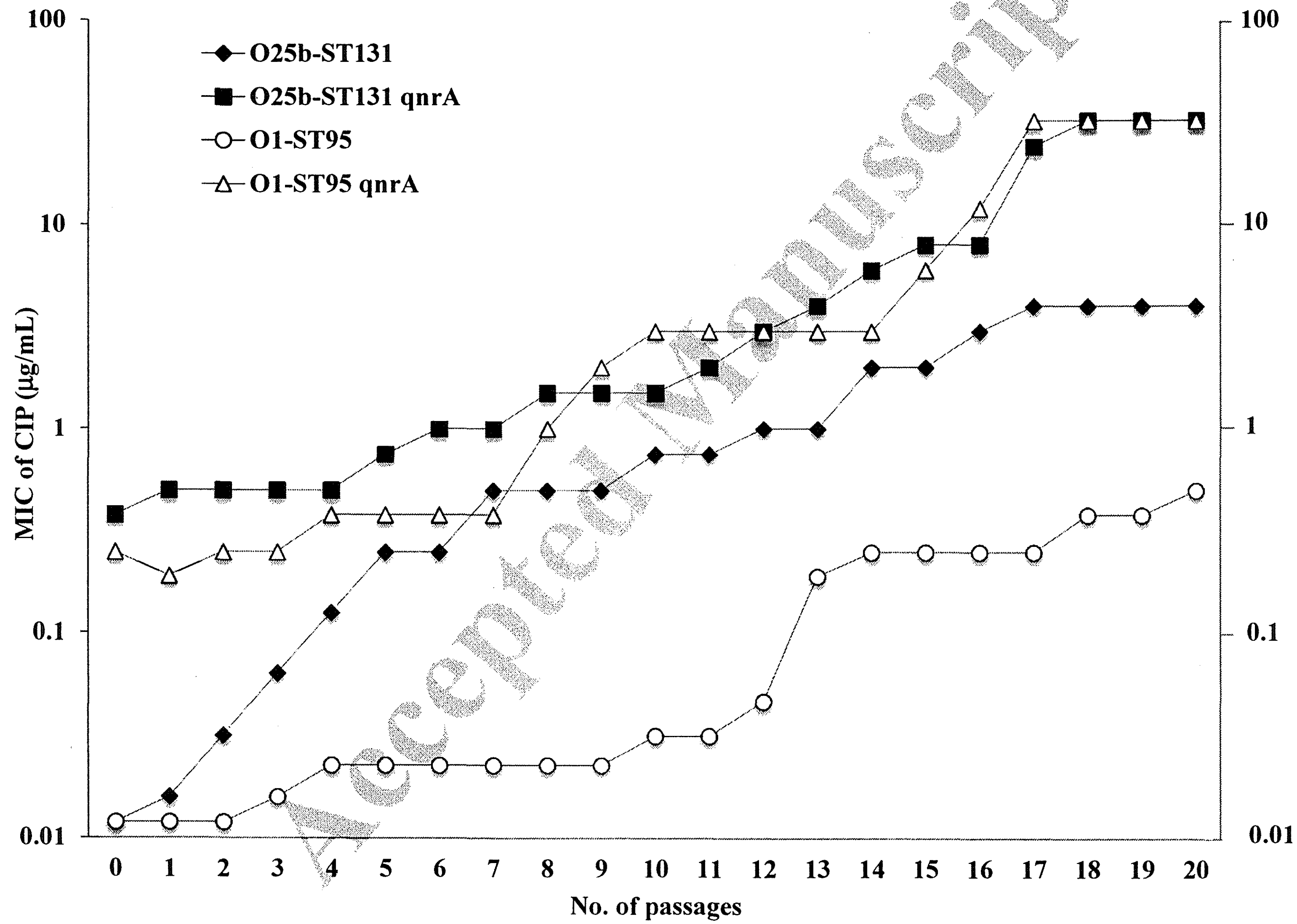
Fig. 3.



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Molecular Epidemiological Characteristics of *Klebsiella pneumoniae* Associated with Bacteremia among Patients with Pneumonia

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Some important virulence factors have been elucidated in *Klebsiella pneumoniae* infections. We investigated the relationship between virulence factors and multilocus sequence types (STs) and assessed the risk factors for bacteremia in patients with pneumonia due to *K. pneumoniae*. From April 2004 through April 2012, a total of 120 *K. pneumoniae* isolates from patients with pneumonia (23 with bacteremia and 97 without bacteremia) were collected from 10 medical institutions in Japan. Additionally, 10 strains of *K. pneumoniae* serotype K2 that were isolated >30 years ago were included in this study. These isolates were characterized using multilocus sequence typing (MLST), and the characteristics of their virulence factors, such as hypermucoviscosity phenotype and RmpA and aerobactin production between patients with and without bacteremia, were examined. MLST analysis was performed on the 120 isolates from patients with pneumonia, and some sequence type groups were defined as genetic lineages (GLs). GL65 was more prevalent among patients with bacteremia (21.7%) than in those without bacteremia (7.2%). The majority of the strains with serotype K2 were classified into GL14 or GL65, and *rmpA* and the gene for aerobactin were present in all GL65-K2 strains but absent in all GL14-K2 strains. In a multivariate analysis, the independent risk factors for bacteremia included GL65 (adjusted odds ratio [AOR], 9.46; 95% confidence interval [CI], 1.81 to 49.31), as well as neoplastic disease (AOR, 9.94; 95% CI, 2.61 to 37.92), immunosuppression (AOR, 17.85; 95% CI, 1.49 to 214.17), and hypoalbuminemia (AOR, 4.76; 95% CI, 1.29 to 17.61). GL65 was more prevalent among patients with bacteremia and was associated with the virulence factors of *K. pneumoniae*.

Klebsiella pneumoniae is a member of the family *Enterobacteriaceae* and is one of the most common pathogens causing pneumonia, abscess, bacteremia, and urinary tract infections (1, 2).

Over several decades, some virulence factors of *K. pneumoniae*, including a capsular serotype, the presence of mucoviscosity-associated gene A (*magA*), and a regulator of mucoid phenotype A (*rmpA*) gene, have been identified. The strains of serotypes K1 and K2 were found to be virulent in a mouse model (3), and the *magA* and *rmpA* genes were found to be associated with hypermucoviscosity (HV), which has an antiphagocytic effect against macrophages and neutrophils (4–6).

Clinically, *K. pneumoniae* was identified as an independent risk factor for mortality in severe community-acquired pneumonia (7). Some studies assessed the relationship between clinical findings and microbiological factors, such as capsular serotype and the presence or absence of *rmpA* and *magA*. The site of infections in these studies was mostly intra-abdominal, especially as a liver abscess (5, 8). Few studies have focused on respiratory tract infections and investigated the relationship between clinical findings and microbiological factors, including genetic characteristics (9, 10).

It is well-known that multilocus sequence typing (MLST), a nucleotide sequence-based genotyping method, is used to characterize genetic relationships among bacterial isolates and to identify and track the global spread of drug-resistant strains (11, 12). In order to predict and evaluate bacterial pathogenicity, it is important to first determine the genetic background of the bacteria causing severe infections. Recently, using MLST, some studies investigated the relationship between the pathogenicity of microbes and the specific multilocus sequence types (STs) in *Escherichia coli* and *Streptococcus pneumoniae* (13, 14). However, research on this relationship among *K. pneumoniae* infections is limited (15). Several studies have suggested that bacteremia is a major risk factor for death in patients with community-acquired pneumonia (CAP) (16, 17). However, it is still unknown which microbiological factors are involved in the onset of bacteremia in patients with pneumonia due to *K. pneumoniae*.

In the present study, we investigated the microbiological characteristics and MLST profiles of *K. pneumoniae* strains isolated from patients with and without bacteremic pneumonia, in addition to their clinical findings. The objective of the present study was to clarify the relationships between the characteristics of virulence factors and MLST profiles so as to elucidate the risk factors

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for bacteremia among patients with pneumonia due to *K. pneumoniae.*

MATERIALS AND METHODS

Study population and designs. An observational study with clinical, microbiological, and MLST data was conducted. Prospectively collected data from patients with a diagnosis of pneumonia admitted to 10 medical institutions in Japan from 15 March 2010 to 22 December 2010 were included in this study (18). In addition, consecutive patients with positive blood cultures for *K. pneumoniae* who were admitted to Nagoya University Hospital, a 1,000-bed tertiary care university hospital, between 11 April 2004 and 18 April 2012 were also included in cases meeting the inclusion criteria of pneumonia as defined previously (18). This was to consider whether or not genetic changes, such as evolutional mutations in bacterial chromosomes and amino acid substitutions, occurred with the lapse of time. This study was approved by the institutional review boards of the participating institutions.

The details regarding data collection, inclusion criteria, exclusion criteria, and definitions of comorbidities and patient status are as described previously (18). In summary, all adult patients (age ≥20 years) with pneumonia that had developed inside or outside the hospital who needed in-hospital treatment were included. Pneumonia was defined according to the international guidelines (19, 20). In this study, patients who were prescribed antimicrobials within the previous 90 days and who did not have a blood culture test performed at the time of diagnosis were excluded so as to remove the effects on the result of blood culture and to assess the microbiological factors in the progression to bacteremia. The patients whose isolates presented low-quality sequencing results in the MLST analysis were also excluded.

Bacterial isolates. The microbiological laboratories at all study institutions provided probable causative pathogens, which were cultured in a semiquantitative manner from samples of sputum, tracheobronchial aspirate, bronchoalveolar lavage fluid, pleural fluid, and blood. All isolates were subcultured and frozen at −80°C until use.

The isolates derived from a previous study (18) were reconfirmed at a central laboratory (SRL, Inc., Tokyo, Japan) to be *K. pneumoniae*, and those with positive blood cultures at Nagoya University Hospital were also reconfirmed using standard and recommended biochemical tests and the Analytical Profile Index (API) 20E system. The details of microbiological evaluation were described previously (18).

Supplementary organisms for analysis of virulence factors. The strains that were collected >30 years ago and belonged to the K2 capsular type were used for an analysis of virulence factors in order to avoid findings in which the result of MLST might be caused by local and temporal matters.

Multilocus sequence typing. MLST was performed as described previously (21) using seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*). The allele sequences and sequence types (STs) were determined using the *Klebsiella* MLST database (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html). New alleles and STs were submitted to the Institut Pasteur MLST databases. In order to define the phylogenetic relationships among the STs, we performed eBURST analysis using eBURST version 3 (http://eburst.mlst.net/).

In this study, the major primary or subgroup founders in a population snapshot determined by eBURST were considered to be central founders of a cluster of STs. Genetic lineage (GL) was defined as a group of STs whose allele profiles differed by no more than two out of a total of seven genes, in a comparison of the allele profiles of central founders.

Capsular serotyping and detection of virulence factors. The capsular serotypes of *K. pneumoniae* were determined by the double-diffusion gel precipitation (Ouchterlony) test (3). Rabbit antisera against all of the *K. pneumoniae* reference strains were prepared by M. Mori under the supervision of N. Kato (Mori et al. [22]). Polysaccharides were prepared from the culture supernatants of the isolates by the procedure described by Batshon et al. (23), and capsular polysaccharides were extracted with cetylpyridinium chloride, as described previously (3).

The presence of virulence genes was determined by PCR using previously documented primers (24, 25). The HV phenotype was defined by a positive string test, which is the formation of a viscous string >5 mm in length when bacterial colonies on an agar plate are stretched by an inoculation loop.

Statistical analyses. Pearson's chi-square test or Fisher's exact test was performed for categorical variables, and Student's *t* test and the Wilcoxon rank-sum test were performed for continuous variables. To assess the risk factors for the progression to bacteremia in patients with pneumonia due to *K. pneumoniae*, a multivariable logistic regression analysis was performed. A backward stepwise selection procedure was used, which removed variables that had a P value of >0.1 and included variables that had a P value of <0.05. In order to assess the roles of microbiological and molecular characteristics in this analysis, we used the factor that was considered to be the most important based on the findings of microbiological tests and the results from univariate analysis between bacteremia and nonbacteremia in patients with pneumonia. Clinical background factors, such as the following, were considered to be risks for bacteremia: neoplastic disease, chronic lung disease, congestive heart failure, chronic renal disease, chronic liver disease, diabetes, immunosuppression, hypoalbuminemia, nonambulatory status, and alcoholism (26, 27). Among these factors, the variables with a P value of <0.1 by univariate analysis were included in the stepwise multivariable model, in addition to age and sex (28). A multivariable analysis, including the institutional factor (medical institutions where the patients with pneumonia were admitted), was also performed to adjust for the potential confounding by including consecutive patients with positive blood cultures for *K. pneumoniae* at Nagoya University Hospital. Statistical analyses were performed using PASW Statistics 18 (SPSS, Inc., Chicago, IL, USA). All tests were two-tailed, and a P value of <0.05 was considered statistically significant.

RESULTS

Baseline characteristics. One hundred two patients from the previous cohort study (18) and 18 patients from Nagoya University Hospital made a total of 120 patients with pneumonia due to *K. pneumoniae* included in the present study. Among them, 23 (19.2%) were classified as bacteremic, and 97 (80.8%) were classified as nonbacteremic.

The median age of the 120 patients with pneumonia due to *K. pneumoniae* was 79 years (interquartile range [IQR], 71.3 to 85.0 years), 91 (75.8%) of whom were men. The baseline characteristics of all patients are described in Table 1. Nosocomial pneumonia (hospital-acquired pneumonia [HAP] and ventilator-associated pneumonia [VAP]), neoplastic disease, immunosuppression, systolic blood pressure (SBP) of <90 mm Hg, respiratory rate (RR) of ≥30/min, and albumin level of <3.0 mg/dl (hypoalbuminemia) were more frequent in patients with bacteremia than in those without bacteremia. The Sequential Organ Failure Assessment (SOFA) score was higher in patients with bacteremia than in those without.

Clinical outcome. The proportion of inappropriate initial antibiotic treatment was low, since most isolates in this study were susceptible to antimicrobial agents for pneumonia. The 30-day mortality was statistically significantly higher in patients with bacteremia (34.8%) than in those without (11.3%), and in-hospital mortality was also statistically significantly higher in those with bacteremia (43.5%) than in those without (14.4%). Also, the proportions of those needing for mechanical ventilation support and vasopressor support were significantly higher in patients with bacteremia (26.1% and 34.8%, respectively) than in those without (7.2% and 6.2%, respectively) (Table 2).

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TABLE 1 Baseline characteristics

Variable[a]	Patients with bacteremia	Patients without bacteremia	P value
n	23	97	
Males	20 (87.0)	71 (73.2)	0.166
Age (median [IQR]) (yr)	73.0 (64.0–79.0)	81.0 (74.0–86.0)	0.005
Pneumonia			
CAP	4 (17.4)	52 (53.6)	0.002
HCAP	5 (21.7)	43 (44.3)	0.047
HAP/VAP	14 (60.9)	2 (2.1)	<0.001
Comorbidities			
Neoplastic disease	13 (56.5)	16 (16.5)	<0.001
Chronic lung disease	1 (4.3)	24 (24.7)	0.042
Congestive heart failure	4 (17.4)	14 (14.4)	0.748
Chronic renal disease	4 (17.4)	8 (8.2)	0.241
Chronic liver disease	4 (17.4)	6 (6.2)	0.097
Diabetes	4 (17.4)	14 (14.4)	0.748
Immunosuppression[b]	5 (21.7)	1 (1.0)	0.001
Physical, laboratory, and radiographical findings			
Orientation disturbance (confusion)	6 (26.1)	34 (35.1)	0.412
Systolic blood pressure <90 mm Hg	7 (30.4)	7 (7.2)	0.006
Respiratory rate ≥30/min[c]	9 (47.4)	19 (19.8)	0.018
BUN ≥30 mg/dl	11 (47.8)	30 (30.9)	0.124
PaO_2/FiO_2 (median [IQR])[d]	297.1 (227.1–361.9)	258.6 (180.9–318.3)	0.158
C-reactive protein (median [IQR]) (mg/dl)	11.7 (5.4–17.3)	9.3 (4.5–16.2)	0.257
Albumin <3.0 mg/dl	19 (82.6)	36 (37.5)	<0.001
Bilateral lung involvement	9 (39.1)	58 (59.8)	0.073
Nonambulatory status[e]	11 (47.8)	35 (36.1)	0.298
Alcoholism[f]	1 (4.3)	3 (3.1)	0.578
SOFA score (median [IQR])[g]	6.0 (3.0–9.0)	3.0 (2.0–5.0)	0.015

[a] The data are presented as no. (%), unless otherwise indicated. IQR, interquartile range; CAP, community-acquired pneumonia; HCAP, health care-associated pneumonia; HAP, hospital-acquired pneumonia; VAP, ventilator-associated pneumonia; BUN, blood urea nitrogen; SOFA score, sequential organ failure assessment score.
[b] Immunosuppression included any immunosuppressive diseases, such as congenital or acquired immunodeficiency, hematological diseases, and neutropenia (<1,000/mm3), treatment with immunosuppressive drugs within the previous 30 days, or corticosteroids in daily doses of ≥10 mg/day of a prednisone equivalent for >2 weeks.
[c] Respiratory rate was evaluated in 95.8% (19 with bacteremia and 96 without bacteremia) of the study patients.
[d] Arterial blood gas analysis was performed in 89.2% (13 with bacteremia and 94 without bacteremia) of the study patients. In patients for whom arterial blood gas analyses were not performed, PaO_2 was calculated by using the prediction from SpO_2.
[e] Nonambulatory status was defined as being bedridden or using a wheelchair because of difficulty walking.
[f] Alcoholism was defined as those who drink >120 g of alcohol per day.
[g] SOFA score was evaluated in 99.2% (23 with bacteremia and 96 without bacteremia) of the study patients.

Microbiological characteristics and MLST. A microbial diagnosis in patients with pneumonia was based on the following specimens: sputum ($n = 87$), transbronchial aspirates ($n = 9$), blood ($n = 23$), and transthoracic puncture ($n = 1$) (see Table S1 in the supplemental material). To investigate the genetic relationships, MLST was performed on all 120 clinical isolates. In this study, MLST analysis revealed 88 different STs. Forty-three of them (ST1092, ST1093, ST1094, ST1095, ST1096, ST1097, ST1098, ST1099, ST1100, ST1101, ST1102, ST1108, ST1131, ST1132, ST1139, ST1140, ST1141, ST1142, ST1143, ST1144, ST1145, ST1146, ST1147, ST1148, ST1149, ST1150, ST1596, ST1597, ST1598, ST1599, ST1600, ST1605, ST1606, ST1607, ST1608, ST1609, ST1610, ST1611, ST1612, ST1613, ST1614, ST1615, and ST1616) were identified for the first time in the present study (see Table S1 in the supplemental material).

A population snapshot shows some clusters of linked STs and unlinked STs in the whole *K. pneumoniae* MLST database (as of 8 July 2014), including the isolates identified in this study, which identified four central founders (ST23, ST65, ST268, and ST347)

TABLE 2 Appropriateness of initial antibiotics and clinical outcomes

Therapy and outcome	No. (%) of patients: With bacteremia ($n = 23$)	Without bacteremia ($n = 97$)	P value
Inappropriate initial antibiotic treatment[a]	1 (4.3)	6 (6.2)	1.000
30-day mortality[b]	8 (34.8)	11 (11.3)	0.010
In-hospital mortality	10 (43.5)	14 (14.4)	0.004
Intensive care[c]			
ICU admission	5 (21.7)	9 (9.3)	0.141
Mechanical ventilation support[d]	6 (26.1)	7 (7.2)	0.018
Vasopressor support	8 (34.8)	6 (6.2)	0.001

[a] Appropriateness of initial antibiotics was assessed by susceptibility test on the basis of CLSI 2010 (42).
[b] Patients who were discharged or transferred to other hospitals in <30 days with improvement of signs and symptoms were considered alive.
[c] Intensive care included ICU admission, mechanical ventilation support, or vasopressor support within 30 days after the diagnosis of pneumonia.
[d] Noninvasive positive-pressure ventilation was included.

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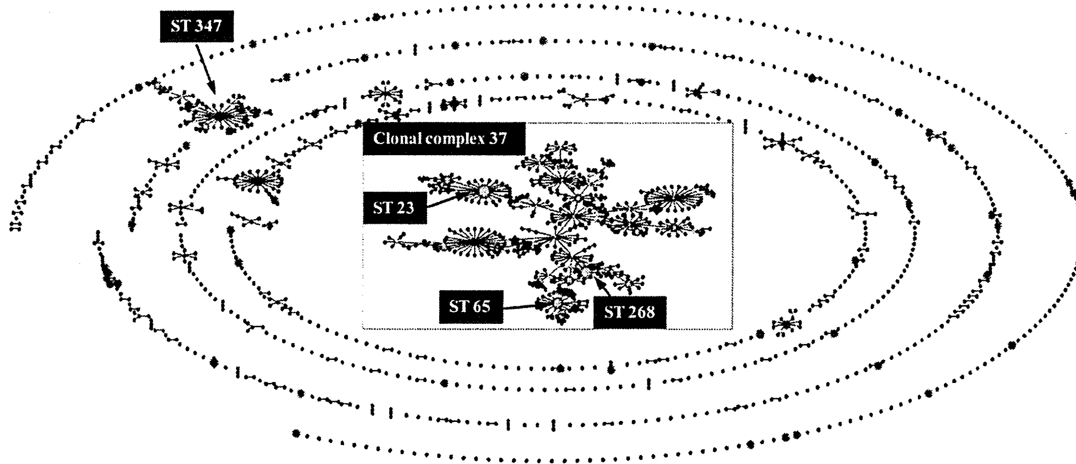

FIG 1 A population snapshot determined by eBURST analysis (http://eburst.mlst.net) showing the clusters of linked and unlinked STs in the entire *K. pneumoniae* MLST database (1,612 STs; http://www.pasteur.fr/recherche/genopole/PF8/mlst/), including the 120 isolates in this study. The size of the circles indicates the number of isolates, and large circles indicate predominant types. The color of circles indicates the type of founder (blue, primary founder; yellow, subgroup founder; black, all other STs). The pink halos surround STs that were isolated in this study. There is a large cluster of STs (clonal complex 37; green box), with ST37 as the predicted founder.

as primary or subgroup founders (Fig. 1). ST23, ST25, ST218, ST485, ST1092, and ST1373 belonged to GL23; ST65, ST25, ST35, ST60, ST66, and ST375 belonged to GL65; ST347, ST355, ST662, ST919, ST1096, ST1097, ST1132, ST1148, ST1597, ST1608, ST1611, and ST1612 belonged to GL347; and ST268, ST35, ST36, ST412, and ST485 belonged to GL268 (Table 3). The microbiological and molecular epidemiological characteristics between patients with and without bacteremia in patients with pneumonia are shown in Table 4. Of the 120 isolates of *K. pneumoniae*, 62 (51.7%) were serotyped, and the K2 serotype was most common (13/120 [10.8%]), followed by the K1 serotype (9/120 [7.5%]). The strains of serotype K2 were more common in patients with bacteremia than in those without (4/23 [17.4%] versus 9/97 [9.3%], respectively). ST23 was the most common genotype (9/120 [7.5%]) of pneumonia due to isolates of *K. pneumoniae*. The strains belonging to GL65 were more prevalent in patients with bacteremia than in those without (5/23 [21.7%] versus 7/97 [7.2%], respectively). As shown in Table S1 in the supplemental material, the serotype of the strains with GL65 was predominantly K2 (9/12 [75.0%]).

Assessment of virulence factors. An analysis of the virulence factors among strains of serotype K2 was performed in order to explore the microbiological factors related to a high proportion of bacteremia in patients with strains belonging to serotype K2 and GL65. Serotype K2 was grouped into two GLs, GL65 and GL14. GL14 comprised ST14 only (Table 5). Among strains of serotype K2, all GL65 strains, including those isolated >30 years ago, harbored *rmpA* and the gene for aerobactin, and 8 (66.7%) of the 12 GL65-K2 strains were positive for the HV phenotype by the string test. On the other hand, neither *rmpA* nor the gene for aerobactin was detected whatsoever in the GL14-K2 strains.

Risk factors for bacteremia. Table 6 shows the risk factors for bacteremia in the patients with pneumonia. The significant risk factors for bacteremia in the univariate analysis included neoplastic disease (odds ratio [OR], 6.58 [95% confidence interval {CI}, 2.46 to 17.60]), immunosuppression (OR, 26.67 [95% CI, 2.94 to 241.94]), and hypoalbuminemia (OR, 7.92 [95% CI, 2.50 to 25.12]). In the multivariate analysis, the GL65 genotype was a significant risk factor for bacteremia (adjusted odds ratio [AOR], 9.46 [95% CI, 1.81 to 49.31]), even after the clinical variables (age, sex, neoplastic disease, chronic lung disease, chronic liver disease, immunosuppression, and hypoalbuminemia) were entered in the analysis. Moreover, the GL65 genotype remained a significant risk factor for bacteremia in the multivariate analysis including the institutional factor (see Table S3 in the supplemental material).

Clinical characteristics and outcomes in patients with and without GL65. Among the patients infected by strains with GL65, pneumonia always had a community onset (CAP and health care-associated pneumonia [HCAP]), but no strains with GL65 were identified in the nosocomial pneumonia (HAP and VAP) cases. Compared with the patients infected by strains without GL65, the patients infected by strains with GL65 had odds ratios of 1.9 (95% CI, 0.5 to 7.9; $P = 0.402$), 5.5 (95% CI, 1.4 to 21.9; $P = 0.026$), and 7.9 (95% CI, 2.1 to 29.9; $P = 0.005$) with respect to 30-day mortality, mechanical ventilation, and intensive care unit (ICU) admission, respectively (see Table S2 in the supplemental material).

DISCUSSION

This multicenter study revealed that bacterial genetic lineage 65 (GL65)-K2 was associated with the presence of *rmpA* and the gene for aerobactin, as well as the expression of the HV phenotype. In multivariate analysis, the GL65 genotype was one of the probable independent risk factors of bacteremia in patients with pneumonia due to *K. pneumoniae*.

In the present study, the *rmpA* and the gene for aerobactin were positive for all the GL65-K2 strains, including those isolated >30 years ago, whereas neither *rmpA* nor the gene for aerobactin was detected whatsoever in the GL14-K2 strains (Table 5). *K. pneumoniae* strains of serotype K2 have frequently been isolated from patients with bacteremia (9, 29) and have shown strong virulence in mice (3). On the other hand, some strains of serotype K2 showed very weak virulence in mice or none at all (3, 30). The results of our study served to explain the differences in virulence among the strains of serotype K2 and were congruent with the view that the *rmpA* gene is highly positive (71%) in clonal complex

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TABLE 3 ST profile of genetic lineages

ST	No. with allele profile:							No. (%) of isolates
	gapA	*infB*	*mdh*	*pgi*	*phoE*	*rpoB*	*tonB*	(n = 120)
GL23								15 (12.5)
ST23	2	1	1	1	9	4	12	9 (7.5)
ST25[a]	2	1	1	1	10	4	13	1 (0.8)
ST218	2	3	1	1	9	4	12	2 (1.7)
ST485[a]	2	1	1	1	7	1	12	1 (0.8)
ST1092	2	1	1	1	7	4	198	1 (0.8)
ST1373	2	1	1	1	1	4	4	1 (0.8)
GL65								12 (10.0)
ST65	2	1	2	1	10	4	13	5 (4.2)
ST25[a]	2	1	1	1	10	4	13	1 (0.8)
ST35[a]	2	1	2	1	10	1	19	2 (1.7)
ST60	2	1	2	1	4	4	8	1 (0.8)
ST66	2	3	2	1	10	1	13	1 (0.8)
ST375	43	1	2	1	10	4	13	2 (1.7)
GL347								13 (10.8)
ST347	16	24	21	27	47	22	67	2 (1.7)
ST355	16	45	21	27	47	22	67	1 (0.8)
ST662	16	24	21	27	52	17	67	1 (0.8)
ST919	16	24	21	27	47	22	188	1 (0.8)
ST1096	16	24	21	27	47	22	200	1 (0.8)
ST1097	16	75	21	27	55	22	67	1 (0.8)
ST1132	16	24	21	27	106	22	204	1 (0.8)
ST1148	16	18	21	27	47	22	75	1 (0.8)
ST1597	16	24	21	27	203	107	67	1 (0.8)
ST1608	29	24	46	27	47	22	67	1 (0.8)
ST1611	16	18	21	76	47	22	67	1 (0.8)
ST1612	16	24	21	31	47	60	67	1 (0.8)
GL268								15 (12.5)
ST268	2	1	2	1	7	1	81	7 (5.8)
ST35[a]	2	1	2	1	10	1	19	2 (1.7)
ST36	2	1	2	1	7	1	7	4 (3.3)
ST412	2	1	2	1	9	1	112	1 (0.8)
ST485[a]	2	1	1	1	7	1	12	1 (0.8)

[a] ST25, ST35, and ST485 belonged to two GLs each (GL23 and GL65, GL65 and GL268, and GL23 and GL268, respectively). The allele profile of ST25 had ≥5 loci in common on the basis of the profiles of ST23 and ST65, the allele profile of ST35 had ≥5 loci in common on the basis of the profiles of ST65 and ST268, and the allele profile of ST485 had ≥5 loci in common on the basis of the profiles of ST23 and ST268.

TABLE 4 Microbiological and molecular epidemiological characteristics

Strain group[a]	No. (%) of strains from patients:		*P* Value
	With bacteremia (n = 23)	Without bacteremia (n = 97)	
Capsular serotype			0.340
K1	1 (4.3)	8 (11.1)	
K2	4 (17.4)	9 (9.3)	
K20	0 (0.0)	7 (7.2)	
K39	2 (8.7)	4 (4.1)	
K54	0 (0.0)	3 (3.1)	
K57	0 (0.0)	7 (7.2)	
Other serotype[b]	4 (17.4)	13 (13.4)	
Nontypeable	12 (52.2)	46 (47.4)	
ST			0.062
ST23	1 (4.3)	8 (8.2)	
ST36	0 (0.0)	4 (4.1)	
ST65	1 (4.3)	4 (4.1)	
ST268	0 (0.0)	7 (7.2)	
ST347	1 (4.3)	1 (1.0)	
ST872	0 (0.0)	3 (3.1)	
Other ST	20 (87.0)	70 (72.2)	
GL23[c]	3 (13.0)	12 (12.4)	1.000
GL65[c]	5 (21.7)	7 (7.2)	0.052
GL347[c]	4 (17.4)	9 (9.3)	0.271
GL268[c]	1 (4.3)	14 (14.4)	0.298
rmpA	5 (21.7)	31 (32.0)	0.336
Gene for aerobactin	5 (21.7)	32 (33.0)	0.294
Positive string test	4 (17.4)	30 (30.9)	0.195

[a] GL, genetic lineage; *rmpA*, regulator of mucoid protein A.
[b] Other serotypes included K5, K10, K16, K23, K28, K30, K41, K42, K43, K49, K55, K58, K60, and K62.
[c] GL23 included ST23, ST25, ST218, ST485, ST1092, and ST1373; GL65 included ST65, ST25, ST35, ST60, ST66, and ST 375; GL347 included ST347, ST355, ST662, ST919, ST1096, ST1097, ST1132, ST1148, ST1597, ST1608, ST1611, and ST1612; and GL268 included ST268, ST35, ST36, ST412, and ST485.

65 (CC65)-K2 strains (24). Although the definition of ST groups in the present study was different from that in the study by Brisse et al. (24), the components of CC65 (ST65, ST25, ST66, and ST243) were almost the same as those in GL65 (ST65, ST25, ST35, ST60, ST66, and ST375) in the present study. A clonal complex was defined as a group of STs differing by no more than one gene from at least one other profile of the group in the study by Brisse et al. (24). However, the MLST results showed a large genetic background diversity in 120 isolates (Fig. 1), and CC37 included ST23 and ST65. ST23 was a representative ST of K1 isolates, and ST65 was a representative ST of K2 isolates. Therefore, in the present study, genetic lineage was defined by ST groups to discern these differences. The discrepancy in the frequency of the *rmpA* gene between CC65-K2 and GL65-K2 may be explained by the housekeeping genes of MLST, which are located on a chromosome (21), unlike the *rmpA* gene, which is located on a plasmid. The string test indicated that 66.7% of the GL65-K2 strains had the HV phenotype, which was identified as an important virulence factor for *K. pneumoniae* in mouse models (31, 32). Recent genetic and phenotypic characterizations showed that the genetic background, rather than the capsular serotype, was associated with the virulence of *K. pneumoniae* (24). Thus, not only capsular serotypes but also ST groups would be applicable for predicting the prognosis of patients with pneumonia caused by *K. pneumoniae*.

In the multivariate analysis, the GL65 genotype was indicated to be one of the independent risk factors for bacteremia in patients with pneumonia due to *K. pneumoniae*, in addition to neoplastic disease, immunosuppression, and hypoalbuminemia, which were characteristics of the host. The results suggested that the factors of both bacteria and hosts are important for evaluating and predicting the clinical outcome of severe cases of pneumonia with *K. pneumoniae* (Table 6). Investigations into the relationship between the clinical findings and MLST profiles among *K. pneumoniae* isolated from pneumonia are limited. As far as we know, this is the first report suggesting that specific ST groups of microbes might be involved with bacteremia, which is related to adverse outcomes in patients with *K. pneumoniae* infections. The results are consistent with the view that HV phenotypes, which are associated with the presence of the *rmpA* gene, have more resistance to phagocytosis by macrophages and neutrophils (6).

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TABLE 5 Comparison of virulence factors present between GL65-K2 and GL14-K2

Strain by source	Virulence gene							Gene for aerobactin	String test result	ST	Reference or source
	rmpA	*magA*	*mrkD*	*kfu*	*cf29a*	*fimH*	*ureA*				
This study											
GL65-K2											
BL4	+	−	+	+	−	+	+	+	−	25	This study
BL16	+	−	+	+	−	+	+	+	+	66	This study
BL23	+	−	+	+	−	+	+	+	+	65	This study
S26	+	−	+	+	−	+	+	+	+	375	This study
S28	+	−	+	−	−	+	+	+	−	65	This study
S42	+	−	+	+	−	−	+	+	−	65	This study
W18	+	−	+	+	−	+	+	+	+	65	This study
W37	+	−	−	−	−	+	+	+	+	65	This study
W43	+	−	+	+	−	+	+	+	+	375	This study
GL14-K2											
BL2	−	−	+	+	−	+	+	−	−	14	This study
Other-K2											
S10	+	−	+	+	−	+	+	+	+	86	This study
S30	+	−	+	+	−	+	+	+	+	86	This study
S31	−	−	+	+	−	+	+	−	−	281	This study
Supplementary[a]											
GL65-K2											
K2-112	+	−	+	+	−	+	+	+	−	65	3
K2-324	+	−	+	−	−	+	+	+	+	65	3
B5055	+	−	+	+	−	+	+	+	+	66	39
GL14-K2											
K2-215	−	−	+	+	−	+	+	−	−	14	3
K2-277	−	−	+	+	−	+	+	−	−	14	3
R622	−	−	+	+	−	+	+	−	−	14	40, 41
R640	−	−	+	+	−	+	+	−	−	14	40
R642	−	−	+	+	−	+	+	−	−	14	40, 41
R761	−	−	+	+	−	+	+	−	+	14	40
Other-K2											
Chedid	+	−	+	+	−	+	+	+	+	86	3

[a] The supplementary strains were collected >30 years ago.

Thirty-day mortality was higher in patients infected by strains with GL65 than in those not infected by GL65 strains, although this was not confirmed to be significant, due to the limited number of cases. The patients infected by strains with the GL65 genotype had significantly higher proportions of ICU admission and mechanical ventilation support than did the patients infected by strains without the GL65 genotype. In this study, clinical adverse outcomes due to inappropriate initial antibiotic treatment were limited, because the proportion of inappropriate antibiotic treatment was low in both the bacteremia and nonbacteremia groups (4.3% and 6.2%, respectively). Therefore, GL65, a microbiological factor, might be relevant to clinical outcomes, and our results support the previous finding that *K. pneumoniae* was an independent risk factor for mortality in severe community-acquired pneumonia (7).

In this study, all strains with GL65 were isolated from patients

TABLE 6 Risk factors for bacteremia caused by *K. pneumoniae*

Variable	No. (%) for risk factor		OR (95% CI) in[a]:	
	Yes	No	Univariate analysis	Multivariate analysis[b]
Age > 65 yr	17/23 (73.9)	91/97 (93.8)	0.19 (0.05–0.65)	Removed
Male	20/23 (87.0)	71/97 (73.2)	2.44 (0.67–8.90)	Removed
Neoplastic disease	13/23 (56.5)	16/97 (16.5)	6.58 (2.46–17.60)	9.94 (2.61–37.92)
Chronic lung disease	1/23 (4.3)	24/97 (24.7)	0.14 (0.02–1.08)	Removed
Chronic liver disease	4/23 (17.4)	6/97 (6.2)	3.19 (0.82–12.42)	Removed
Immunosuppression	5/23 (21.7)	1/97 (1.0)	26.67 (2.94–241.94)	17.85 (1.49–214.17)
Hypoalbuminemia[c]	19/23 (82.6)	36/97 (37.5)	7.92 (2.50–25.12)	4.76 (1.29–17.61)
GL65	5/23 (21.7)	7/97 (7.2)	3.57 (1.02–12.52)	9.46 (1.81–49.31)

[a] OR, odds ratio; CI, confidence interval

[b] Stepwise procedure was performed.

[c] Hypoalbuminemia was defined as an albumin level of <3.0 mg/dl.

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with CAP or HCAP, but no strains with GL65 were isolated from patients with nosocomial pneumonia (see Table S2 in the supplemental material). The results are consistent with the previous findings that the strains having virulence factors, such as K1, K2, a mucoid phenotype, and aerobactin, were more likely to cause community-acquired infections than to cause hospital-acquired infections (5, 33). These findings might be associated with differences in the host defenses between the patients with community-acquired and hospital-acquired infections. Compared to patients with hospital-acquired infection, those with community-acquired infection tend to be more immunocompetent, and more virulence factors may be needed for bacterial invasion to cause bacteremia (34, 35). However, it is still unknown how such virulent strains arise and spread, and further studies are warranted to elucidate why such strains are highly recovered in community-acquired infections.

This study has some limitations. First, part of the data were retrospectively collected from Nagoya University Hospital, and 87% (20/23) of the patients with positive blood cultures were enrolled in the hospital. This institutional factor may be a confounder of the results of the molecular epidemiological characteristics between patients with and without bacteremia. However, GL65 strains were also found in other hospitals and existed in the isolates recovered >30 years ago, and this findings of this study suggest that the identification of GL65 is not a local and temporal matter. Second, the sample size of this study was relatively small. The results of the multivariate analysis for the risk of bacteremia showed wide 95% CIs of the ORs. Therefore, further studies with a larger sample size are needed to validate our results. However, the proportion of patients with a positive blood culture was low (5/108 [4.6%]) in those who had pneumonia due to *K. pneumoniae* in the previous cohort study (18). Therefore, it was important to assess the risk factors for bacteremia in the patients with pneumonia in the present study, even though the number was small.

In the present study, patients who previously used antibiotics were excluded in order to avoid masking the blood culture results. Resistant strains were likely to be identified in the excluded patients, which may affect the results of the MLST profile. The international spread of carbapenem-resistant *Enterobacteriaceae* (CRE), such as KPC-producing *K. pneumoniae* (KPC-Kp), has become a grave clinical concern, although KPC-Kp has not been widespread in Japan (36). It has been recognized that the predominant epidemic clone among the KPC-Kp is ST258, although the relationship between GL65 and KPC-Kp with bacteremia has not been studied. The mortality rates in the patients with bacteremia caused by KPC-Kp are 39% to 53% (37, 38). Therefore, the relationship between GL65 and KPCs is a crucial topic for future investigation.

In conclusion, the present study indicates that GL65-K2 strains were associated with the presence of *rmpA* gene and the gene for aerobactin, as well as the expression of the HV phenotype. Moreover, this study suggested that GL65, which was one of the clusters of closely related STs, as well as comorbidities, such as neoplastic disease, immunosuppression, and hypoalbuminemia, might be independent risk factors for bacteremia in those who have pneumonia caused by *K. pneumoniae*, which progresses to severe pneumonia. Clinicians should be aware of GL65 in order to evaluate and predict the severity of pneumonia caused by *K. pneumoniae*. Further basic studies on the pathogenicity of *K. pneumoniae* are warranted to elucidate why the *K. pneumoniae* GL65 tends to result in bacteremia in patients with pneumonia.

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Original Article

Molecular Epidemiology of Extended-Spectrum β-Lactamases and *Escherichia coli* Isolated from Retail Foods Including Chicken Meat in Japan

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Abstract

Contamination of retail meat with extended-spectrum β-lactamase (ESBL)–producing *Escherichia coli* has been reported, but only limited data have been documented in Japan. One hundred fifty-three retail foods including chicken meat, beef, pork, and vegetables were purchased from 29 supermarkets between January and October in 2010. ESBL producers were recovered from each food sample using McConkey agar plate supplemented with 1 mg/L of cefotaxime. ESBL type was identified by DNA sequencing analysis after polymerase chain reaction amplification. Antibiogram, O serotype, plasmid replicon type, pulsotype, and multilocus sequence type were also determined. Fifty-two epidemiologically unrelated *Escherichia coli* isolates producing ESBL were recovered from 35 (22.9%) of 153 samples, all of which were chicken meat. ESBL types were mainly CTX-M-2 group followed by CTX-M-1 group and CTX-M-8 group. The numbers of bacterial isolates (8 of 21, 38.1%) harboring $bla_{CTX\text{-}M\text{-}8}$ recovered from imported meat samples were significantly larger than those of domestic ones (one of 31, 3.2%) ($p < 0.05$). Nine O serotypes (mainly O8, O25, and O1) were found, together with O-antigen untypable (OUT). Four *E. coli* belonging to the O25b:H4-ST131 clone were recovered from domestic ($n = 1$) and imported meat samples ($n = 3$), respectively. These four isolates were susceptible to fluoroquinolones, although the *E. coli* O25b:H4-ST131 clone producing CTX-M-15, which is predominant in human isolates, is usually resistant to fluoroquinolones. By contrast, five CTX-M-15-producing *E. coli* strains were recovered only from domestic meat samples, and their serotypes were O8 or OUT instead of predominant serotype O25b. Our results showed that ESBL-producing *E. coli* isolates recovered from retail chicken meat samples in Japan are generally divergent in both genetic and serological aspects. Further comparative analyses of $bla_{CTX\text{-}M}$-mediating genetic elements would be continued in the next step to characterize the ESBL producers from retail foods in Japan.

Introduction

Production of extended-spectrum β-lactamases (ESBLs) is the most common cephalosporin resistance mechanism in bacteria belonging to the members of family *Enterobacteriaceae* (Paterson *et al.*, 2005). CTX-M-type ESBL-producing *Escherichia coli* especially have been increasing among clinical isolates recovered from community-acquired infections such as urinary tract infections and bacteremia (Cantón *et al.*, 2008). Increasing isolation rates of these microbes alert us to a probably growing health risk for public health (Pitout and Laupland, 2008). The reason for the rapid spread of CTX-M-producing *E. coli* in the community remains uncertain. Recent investigations have revealed that previous use of antimicrobials, especially oxyimino-cephalosporins and fluoroquinolones, was a risk factor for community-onset bloodstream infections caused by ESBL-producing *E. coli* (Rodríguez-Baño *et al.*, 2010). Additionally, several studies have reported a high prevalence of ESBL-producing *E. coli* isolates in fecal samples of broilers and chickens (Costa *et al.*, 2009; Li *et al.*, 2010). These findings suggested that prescriptions of oral cephalosporins for outpatients and/or extensive veterinary use of antimicrobials may well promote the rapid increase of epidemic clones of *E. coli* that produce CTX-M-type ESBL in both human and animals.

In many food animals such as chickens, pigs, and cattle, certain antimicrobials such as colistin sulfate, avilamycin, and monensin were approved to add to animal feeds for preventing infections in many countries. By contrast, the veterinary use of fluoroquinolones such as enrofloxacin and cephalosporins such as ceftiofur and cefquinome have been approved only for therapeutic purpose for livestock infected with pathogenic bacteria. Indeed, the veterinary use of these antimicrobials has been strictly restricted, but the use of such

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antimicrobials may well contribute to the emergence and spread of ESBL producers in livestock farming environments through selection of bacterial strains harboring genetic determinants responsible for cephalosporin resistance such as $bla_{CTX\text{-}M}$ and bla_{CMY}. Actually, Ma *et al.* have reported the occurrence of ESBL-producing *E. coli* in fecal samples of healthy ducks and environmental samples from a duck farm in South China (Ma *et al.*, 2012), and an increasing amount of data regarding this issue has been accumulated to date (Dhanji *et al.*, 2010; Ewers *et al.*, 2010; Ho *et al.*, 2011). Food animals and their products are now suspected to be one of the potential sources in the dissemination of the antimicrobial-resistant bacteria, especially ESBL-producing *E. coli* (Overdevest *et al.*, 2011). In Japan, Hiroi *et al.* and Asai *et al.* have reported the prevalence of ESBL-producing bacteria in food-producing animals (Asai *et al.*, 2011; Hiroi *et al.*, 2012), however, there have been few reports about the dissemination of ESBL-producing *E. coli* in samples derived from food animals and/or their products collected since 2007. The aim of our study was to assess the recent state of contamination with ESBL-producing *E. coli* in retail food samples and to characterize the types of ESBLs, together with the serotype and genotype of ESBL-producing *E. coli* isolates in Japan. Moreover, we also compared the prevalence of ESBL producers in domestic and imported meat samples, since imported meat products have recently become a commonplace in the Japanese market.

Materials and Methods

Sample collection and bacterial isolation

One hundred fifty-three retail food samples (chicken, beef, pork, and vegetables) were purchased from 29 separate supermarkets in Aichi Prefecture, Japan, between January and October 2010. As for raw chicken meat, 42 samples were domestic products and 26 samples were ones imported mainly from South America, and the remaining samples were mincemeat mixed of domestic and imported meat. Approximately 10 g of each sample was incubated in brilliant green lactose bile broth (SYSMEX bioMérieux Co., Ltd., Tokyo, Japan) at 35°C for 24 h; aliquots (100 μL) of the incubated bacterial preculture were inoculated onto McConkey agar plates (Eiken Chemical Co., Ltd., Tokyo, Japan) supplemented with 1 mg/L of cefotaxime (Jouini *et al.*, 2007), and the plates were incubated at 35°C for 24 h. The two to four colonies growing on the CTX-MacConkey agar plate were picked up and identified in terms of bacterial species by using the API-20E system (SYSMEX bioMérieux). These isolates were further subjected to screening of ESBL producers and pulsed-field gel electrophoresis (PFGE) analysis.

Screening and genetic identification of ESBL

ESBL screening was performed on the basis of the double-disk synergy test by using three different commercially available discs: ceftazidime, cefotaxime, and amoxicillin/clavulanic acid, according to the protocol recommended by the Clinical and Laboratory Standards Institute (CLSI, 2009b) and confirmed using Etest (SYSMEX bioMérieux).

The presence of CTX-M-type β-lactamase genes was determined by polymerase chain reaction (PCR) using primers specific to the CTX-M-1 group, CTX-M-2 group, CTX-M-8 group, or CTX-M-9 group as described elsewhere (Shibata *et al.*, 2006; Dallenne *et al.*, 2010). The TEM- and SHV-type β-lactamase genes were detected by PCR, and their genotypes were further determined by sequencing analysis (Yagi *et al.*, 2000). The nucleotide sequences were analyzed with BLAST software (http://blast.ddbj.nig.ac.jp/top-j.html).

Cluster analysis by PFGE

PFGE typing of the ESBL-producing isolates was performed as described elsewhere (Barrett *et al.*, 1994). Plug containing whole genomic DNA was digested with *Xba*I (Takara Bio. Inc., Tokyo, Japan), and electrophoresis was performed using a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA) with pulses ranging from 2.2 to 54.2 s at a voltage of 6 V/cm at 14°C for 19 h. A dendrogram showing genetic relatedness among the isolates was constructed with Fingerprinting II software (Bio-Rad Laboratories). In the isolates from each food sample, when ≥85% genetic similarity was observed, these were classified as a clone with a common genetic background, and one representative isolate was further studied. When <85% genetic similarity was observed, these were considered as a different clone and separately treated in this study (Carrico *et al.*, 2005).

Serotyping of ESBL-producing E. coli

Serotyping of each *E. coli* isolate was performed with the *E. coli* antisera "SEIKEN" Set 1 (Denka Seiken, Tokyo, Japan) for O-antigen and Set 2 (Denka Seiken) for H-antigen according to the manufacturer's instructions. Serotypes that could not be distinguished by this method were designated OUT (O-antigen untypable) or HUT (H-antigen untypable). Genetic serotyping of each bacterial isolate was performed according to the procedures described elsewhere (Clermont *et al.*, 2008).

Antimicrobial susceptibility testing

The antimicrobial susceptibility of each isolate was determined by the agar dilution method, according to the protocol recommended by the CLSI in document M07-A9 (CLSI, 2009a). The antimicrobial agents were obtained from the following sources: piperacillin, cefotaxime, ceftazidime, imipenem, aztreonam, gentamicin, minocycline, fosfomycin, Wako Pure Chemical Co., Inc., Tokyo, Japan; cefmetazole, amikacin, chloramphenicol, Sigma-Aldrich Japan, Tokyo, Japan; ciprofloxacin, levofloxacin, Daiichi-Sankyo Company, Tokyo, Japan; and flomoxef, Shionogi & Co., Ltd., Tokyo, Japan. Susceptibilities of ESBL-producing *E. coli* to each antimicrobial agent were categorized into susceptible, intermediate, or resistant according to the CLSI criteria. Type Culture Collection (ATCC) 25922 (Microbiologics, Inc., MN) was used as reference strain.

Multilocus sequence typing

Multilocus sequence typing (MLST) of ESBL-producing *E. coli* isolates was performed with seven conserved housekeeping genes (*adfK, fumC, gyrB, icd, mdh, purA,* and *recA*). The MLST procedure, including allelic type and sequence type (ST) assignment methods, was done according to the website (http://mlst.ucc.ie/mlst/dbs/Ecoli).

Conjugation studies and plasmid replicon typing

The conjugation experiments were performed with rifampin-resistant *E. coli* CSH-2 (*metB* F⁻, nalidixic acid resistant, rifampicin resistant) as the recipient strain by the filter mating methods. Transconjugants were selected on LB agar plates supplemented with rifampin (100 mg/L) and cefotaxime (2 mg/L) (Wako Pure Chemical Co., Inc., Tokyo). For resultant transconjugants and the parent isolates, plasmid replicon typing was performed as described elsewhere (Carattoli *et al.*, 2005).

Statistical analysis

Comparisons of proportions of ESBL producers recovered from both imported meat samples and domestic products and comparisons of proportions of susceptible isolates between imported and domestic products were made by continuity-adjusted x^2 test with SPSS software (version 20.0 for Windows; SPSS Inc., Chicago, IL); $p < 0.05$ was considered as denoting a significant difference.

Results

Isolation of ESBL producers from food samples

Thirty-five (22.9%) of 153 food samples were contaminated with ESBL-producing bacteria. The ESBL producers were only isolated from chicken meat samples, and the isolation rate (15 of 26, 57.7%) of imported chicken meat samples was slightly higher than that (19 of 42, 45.2%) of domestic chicken meat samples. Of 85 isolates recovered from 35 chicken meat samples, clonally related isolates recovered from the same food samples were excluded based on the cluster analysis by PFGE. The range of clonally different isolates was one to three per chicken sample. As a result, 53 clonally unrelated isolates (domestic products, 32; imported products including one mix product, 21) were recovered from 35 chicken meat samples as probable ESBL producers. Of the 53 isolates, 52 were identified as *E. coli* and the remaining one was *Klebsiella pneumoniae*. Fifty-two *E. coli* isolates were subjected to further studies.

Genotyping of ESBL-producing E. coli

Forty-three *E. coli* isolates produced CTX-M-type ESBLs, while the remaining nine isolates produced TEM-derived or SHV-derived ESBLs (Table 1). Of the bla_{CTX-M} genes, 22 (51.2%), nine (20.9%), five (11.6%), five (11.6%), and two (4.7%) coded for CTX-M-2, CTX-M-8, CTX-M-1, CTX-M-15, and CTX-M-3 ESBL, respectively. The number of bacterial isolates (eight of 21, 38.1%) harboring $bla_{CTX-M-8}$ recovered from imported meat samples was significantly larger than that of domestic ones (one of 31, 3.2%) ($p < 0.05$), but the number of the isolates harboring $bla_{CTX-M-2}$ in imported ones (11 of 21, 52.4%) was slightly higher than that of domestic products (11 of 31, 35.5%). Of the 52 ESBL-producing *E. coli* isolates, seven and one were SHV-12 and SHV-2 producers, respectively, and seven of eight SHV-producers were from domestic meat samples (Table 1).

Serotype of 52 ESBL-producing E. coli *isolates*

PFGE profiles of 52 ESBL-producing *E. coli* isolates were dissimilar to each other (Fig. 1). However, several strains carrying the same ESBL-genes were found to be genetically different (e.g., S7, S416), while closely related strains (e.g., S150, S424) harbored different ESBL genes. Thirty-one (59.6%) were O untypeable, and the serogroups of the remaining isolates were O8 (seven of 52, 13.5%), followed by O25 (five of 52, 9.6%) and O1 (two of 52, 3.8%), respectively. Of the five *E. coli* O25 isolates, four and one isolates were identified as O25b and O25a types, respectively, by PCR. In addition, all four O25b isolates, which harbor $bla_{CTX-M-2}$ ($n = 2$), $bla_{CTX-M-8}$ ($n = 1$), and bla_{SHV-12} ($n = 1$), belonged to ST131 by MLST analysis.

Antimicrobial susceptibility profiles of ESBL-producing E. coli

As shown in Table 2, ESBL-producing *E. coli* isolates were susceptible to cefmetazole, flomoxef, imipenem, amikacin, and fosfomycin. On the other hand, these isolates tended to be resistant to ceftazidime, aztreonam, and minocycline as well as cefotaxime. Among antimicrobial agents including β-lactams tested, susceptibility profiles to chloramphenicol and fluoroquinolones of isolates from domestic products were similar to those from imported products, although the resistance rate to gentamicin in bacterial isolates from imported products was significantly higher than those from domestic ones ($p < 0.05$) (Table 2). In addition, they showed resistance to β-lactams such as piperacillin, cefotaxime, and/or ceftazidime, whereas they were not resistant to fluoroquinolones such as ciprofloxacin and levofloxacin.

Plasmid replicon types

CTX-M-15-producing *E. coli* belonging to the O25b:H4-ST131 is a global epidemic clone, and their isolation rate has been increased recently in Japan. Therefore, conjugation experiments were performed for five isolates (non-O25) harboring $bla_{CTX-M-15}$ and four O25b:H4-ST131 isolates. The conjugal transfer of the bla_{CTX-M}-carrying plasmid to recipient cells was successful in four non-O25 and two O25b isolates.

TABLE 1. GENOTYPES OF 52 EXTENDED-SPECTRUM β-LACTAMASES PRODUCED BY *ESCHERICHIA COLI* ISOLATES

	CTX-M-1 group						*SHV*	
Products	*CTX-M-1*	*CTX-M-3*	*CTX-M-15*	*CTX-M-2*	*CTX-M-8*	*TEM-52*	*SHV-12*	*SHV-2*
Domestic products (31)	4	2	5	11	1	1	7	—
Imported products (21)	1	—	—	11[a]	8[b]	—	—	1
Total (52)	5	2	5	22	9	1	7	1

[a]One sample was an imported product mixed with domestic product, and classified into the imported products.
[b]One isolate harbored both $bla_{CTX-M-8}$ and $bla_{TEM-135}$.

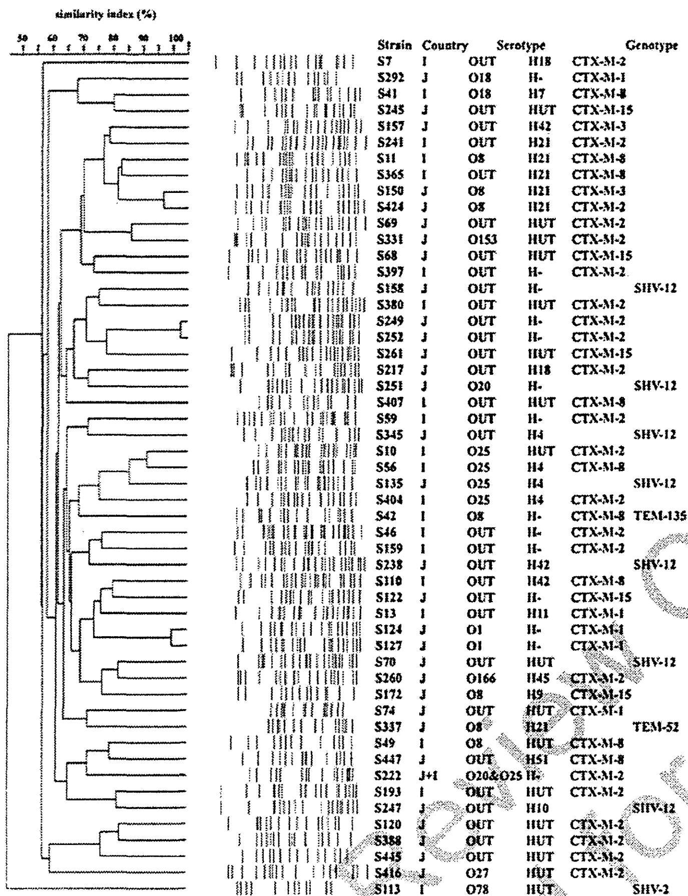

FIG. 1. Dendrogram of pulsed-field gel electrophoresis (PFGE) pattern among bla_{CTX-M}-harboring *Escherichia coli* (based on unweighted pair group method with arithmetic averages cluster analysis). Ladder patterns were analyzed by Fingerprinting II software (Bio-Rad Laboratories) to calculate the Dice similarity index. Isolates were considered to belong to a group of clonally related strains if the dice similarity index of the PFGE pattern was ≥85% (Carrico *et al.*, 2005). When two isolates were recovered from separate parts such as leg and chest purchased from the same supermarket at intervals of more than 3 weeks, the isolates were subjected to further studies as clonally unrelated isolates in the present study, even if they demonstrated 100% similarity by PFGE. J, domestic chicken meat samples; I, imported chicken meat samples; OUT, O-antigen untypeable; HUT, H-antigen untypeable.

TABLE 2. ANTIBIOTIC SUSCEPTIBILITY PROFILES OF 52 EXTENDED-SPECTRUM β-LACTAMASE (ESBL)-PRODUCING *ESCHERICHIA COLI* ISOLATES

	Domestic products (n=31)			*Imported products (n=21)*			
Agents	*S (%)*	*I (%)*	*R (%)*	*S (%)*	*I (%)*	*R (%)*	*Continuity-adjusted chi-square test (p-value)*[a]
PIPC	1 (3.2)	7 (22.6)	23 (74.2)	0 (0)	0 (0)	21 (100)	1.00
CMZ	31 (100)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	—[b]
CTX	0 (0)	2 (6.5)	29 (93.5)	0 (0)	0 (0)	21 (100)	—
CAZ	14 (45.1)	2 (6.5)	15 (48.4)	14 (66.7)	3 (14.3)	4 (19.0)	0.214
FMOX	31 (100)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	—
IPM	31 (100)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	—
AZT	12 (38.7)	6 (19.3)	13 (42.0)	12 (57.2)	2 (9.5)	7 (33.3)	0.305
GM	31 (100)	0 (0)	0 (0)	14 (66.7)	0 (0)	7 (33.3)	0.002
AMK	31 (100)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	—
MINO	12 (38.7)	0 (0)	19 (61.3)	6 (28.6)	0 (0)	15 (71.4)	0.648
CP	29 (93.5)	0 (0)	2 (6.5)	19 (90.5)	0 (0)	2 (9.5)	1.00
FOM	31 (100)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	—
CPFX	28 (90.3)	0 (0)	3 (9.3)	19 (90.5)	0 (0)	2 (9.5)	1.00
LVFX	28 (90.3)	0 (0)	3 (9.3)	19 (90.5)	1 (4.75)	1 (4.75)	1.00

Susceptibilities of 52 ESBL-producing *E. coli* to each antimicrobial agent were categorized into susceptible, intermediate, and resistant in accordance with Clinical and Laboratory Standards Institute criteria.

[a]Proportions of R+I were compared between imported meat samples and domestic meat samples.

[b]*P*-value was not obtained.

S, susceptible; I, intermediate; R, resistant; PIPC, piperacillin; CMZ, cefmetazole; CTX, cefotaxime; CAZ, ceftazidime; FMOX, flomoxef; IPM, imipenem; AZT, aztreonam; GM, gentamicin; AMK, amikacin; MINO, minocycline; CP, chloramphenicol; FOM, fosfomycin; CPFX, ciprofloxacin; LVFX, levofloxacin.

TABLE 3. REPLICON TYPES OF FOUR O25B ISOLATES AND FIVE CTX-M-15-PRODUCING ISOLATES

Sample number	*O serotype*	*Replicon types (patient)*	*Genotype of ESBL*	*Replicon types (transconjugant)*
S10	O25b	I1-γ, N, FIB, F, P	CTX-M-2	P
S56	O25b	I1-γ, FIB, F	CTX-M-8	I1-γ
S135	O25b	I1-γ, FIB, F	SHV-12	ND
S404	O25b	FIB, F	CTX-M-2	ND
S68	OUT	I1-γ, FIB, F	CTX-M-15	I1-γ
S122	OUT	I1-γ, FIB, F	CTX-M-15	I1-γ
S172	O8	FIB, F	CTX-M-15	ND
S245	OUT	F	CTX-M-15	F
S261	OUT	I1-γ, FIB, F	CTX-M-15	I1-γ

OUT, O-antigen untypeable; ND, conjugal transfer of bla_{CTX-M} to recipient cells did not succeed.

Replicon types of the plasmid were mainly I1-γ (4 of 6) followed by IncF, and IncP (Table 3).

Discussion

Contamination of foods with ESBL-producing *E. coli* has recently become a worldwide concern. ESBL-producing *E. coli* isolates were recovered from 62 (29.5%) among 210 batches in raw chicken meat imported by the United Kingdom from South America (Dhanji *et al.*, 2010). In Tunisia, the distribution of isolation rates of ESBL-producing *E. coli* were between 12.6% and 26% among the food samples including chicken and turkey (Jouini *et al.*, 2007). In the present study, the isolation rate of ESBL producers was 50.7% (35 of 69) in chicken meat samples. The high prevalence of ESBL producers in chicken meat in the present study was consistent with Japanese previous reports and was higher than reports from the United Kingdom and Tunisia (Kojima *et al.*, 2005; Jouini *et al.*, 2007; Dhanji *et al.*, 2010; Asai *et al.*, 2011; Hiroi *et al.*, 2012). In Japan, the oxyimino-cephalosporins such as ceftiofur and cefquinome are approved to treat bacterial diseases in cattle and pigs, but are not allowed for use in broilers and chickens. At present, the reason for the high prevalence of ESBL producers in chicken meat samples remains unclear.

Some investigators have reported that CTX-M-type ESBLs detected from chicken meat consisted of CTX-M-1, −2, −14, and −15 (Belgium), CTX-M-2 and −8 (United Kingdom), and CTX-M-1, −8, and −14 (Tunisia), respectively, but the CTX-M-types were somewhat different from those isolated from patients admitted to clinical settings (Jouini *et al.*, 2007; Smet *et al.*, 2008; Dhanji *et al.*, 2010). By contrast, in the Netherlands, a probable relationship between the contamination of chicken meat with drug-resistant bacteria and the appearance of ESBL-producers in humans has been reported (Leverstein-van Hall *et al.*, 2011; Overdevest *et al.*, 2011). In the present study, 43 bla_{CTX-M}-harboring *E. coli* isolates were recovered from 35 chicken meat samples, and their CTX-M-types were mainly CTX-M-2 in both domestic and imported meat samples, followed by CTX-M-8 in imported meat samples. These findings are consistent with those reported from the United Kingdom, suggesting that the genotypes of ESBLs detected from chicken meat samples were very similar to those from chicken meat in the United Kingdom, which would reflect the fact that both countries import chicken meat from South America. In Japan, the predominant CTX-M-types were CTX-M-14 and CTX-M-9 belonging to the CTX-M-9-group enzymes in clinical isolates (Suzuki *et al.*, 2009). Thus, the CTX-M-types of ESBL-producing *E. coli* recovered from chicken meat samples in the present study were inconsistent with those of clinical isolates detected more recently in Japanese hospital settings. The most probable reason for the increasing isolation of CTX-M-9-group ESBL producers from humans in Japan would be the acquisition of *E. coli* that produce CTX-M-9-group enzymes from different routes, such as human-to-human or pets-to-human transmissions (Harada *et al.*, 2012).

In the present study, several genes belonging to the $bla_{CTX-M-1}$-group, such as $bla_{CTX-M-3}$ and $bla_{CTX-M-15}$, were found from retail chicken meat samples, but the O serotypes of *E. coli* harboring the $bla_{CTX-M-1-group}$ gene were somewhat different from those of ESBL-producing *E. coli* isolated in Japanese clinical settings (Matsumura *et al.*, 2012). PFGE analysis showed that strains carrying the same ESBL genes were genetically different, while closely related strains harbored different ESBL genes, suggesting that clonal spread is less important for the distribution of antimicrobial-resistant *E. coli*. Recently, Zheng *et al.* have reported that IS*Ecp1*-like elements were found in the upstream of the $bla_{CTX-M-9-group}$ genes of ESBL producers obtained from food animals, and such genetic structures were very similar to those of humans (Zheng *et al.*, 2012). Further comparative analyses of environmental genetic elements mediating bla_{CTX-M} would be necessary to assess the probability of transmission of ESBL producers via retail foods in Japan.

The pandemic of CTX-M-15-producing *E. coli* in the past decade has been reported, especially in Europe (Woodford *et al.*, 2004; Rogers *et al.*, 2011). The epidemic *E. coli* O25b:H4-ST131 clone producing CTX-M-15 was consistently resistant to fluoroquinolones as well as various β-lactams (Nordmann and Poirel, 2005). In the present study, no CTX-M-15-producing O25b:H4 isolate was found, but four *E. coli* O25b:H4-ST131 isolates that produced CTX-M-2 or CTX-M-8 and five CTX-M-15 producers, serotypes of which were O8 or O-untypable, were recovered from retail chicken meat samples. Interestingly, these isolates were unexpectedly susceptible to fluoroquinolones. Although Inc types of plasmids encoding genes for CTX-M-15 in *E. coli* clinical isolates were usually IncFII, FIA, or FIB (Carattoli, 2009), the replicon types of three CTX-M-15-producing *E. coli* isolates were IncI1-γ plasmids in our study. Although no direct comparison between chicken meat and human isolates was performed in the present study, these results indicated that the CTX-M-15-producing *E. coli* isolates recovered from chicken meat samples have different genetic backgrounds.

Our study presents several limitations. The number of samples processed was small, and systematic surveillance was not used. However, we purchased food samples from 29 separate supermarkets between January and October 2010, and ESBL producers were excluded when the isolates were suggested to belong to the same clone by the cluster analysis of PFGE profile. This study seems to indicate the recent state of contamination with ESBL-producing microbes in retail foods. Indeed, it should be considered that plasmid and mobile genetic elements conferring resistance to third-generation cephalosporins might spread within *E. coli* via gene transfer, but the cluster analysis of PFGE profile could not exclude the possibility described above. It seems very important to

investigate horizontal gene transfer such as exchanges of plasmids or mobile genetic elements carrying genes for ESBLs between bacteria isolated from foods. In the next step of our investigation, we will carefully check the genetic structures of the mobile elements carrying ESBL genes recovered from retail chicken meat and compare them to the relevant mobile elements from human isolates accumulated in the database.

Conclusions

We found a higher-than-expected prevalence of ESBL-producing *E. coli* in retail chicken meat samples in Japan. No clonal spread of ESBL-producing isolates contaminating food samples was observed in the present study, but the high prevalence rate of ESBL producers recovered from food samples might well depend on frequent horizontal gene transfer between bacterial isolates, through exchanges of plasmids and/or mobile elements carrying ESBL genes. Further surveillance and molecular epidemiological investigations conducted in an interdisciplinary way so as to consider not only the human but also the veterinary fields would be essential to predict the future spread of ESBL producers in both humans and animals.

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Disclosure Statement

No competing financial interests exist.

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