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ORIGINAL RESEARCH

High prevalence of KPC-2-producing hypervirulent *Klebsiella pneumoniae* causing meningitis in Eastern China

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Background: *Klebsiella pneumoniae* has been the leading causative pathogen for adult bacterial meningitis in several Asian countries. The clinical and microbiological characteristics of *K. pneumoniae* meningitis in mainland China are still unknown.

Materials and methods: The clinical data of patients with *K. pneumoniae* meningitis from January 2011 to July 2017 in a tertiary hospital were retrospectively evaluated. The isolates were tested for antibiotic-resistance genes, virulence-associated genes, and molecular subtypes. Hypervirulent *K. pneumoniae* (hvKP) was defined as the presence of pLVPK-like virulence plasmid.

Results: During the study period, a total of 48 patients with meningitis caused by *K. pneumoniae* were identified, accounting for 21.2% (48/226) of Gram-negative bacilli meningitis. Of the 44 available isolates, 65.9% (29/44) were carbapenem resistant, and all except one harbored bla_{KPC-2}. K64 was the most common serotype (n=13), followed by K47 (n=11) and K1 (n=5). The pLVPK-related genetic loci were found in about half of isolates (*iutA*: 56.8%, *iucA*: 56.8%, *rmpA2*:50.0%, *rmpA*: 43.2%, and *iroN*: 40.9%). Twenty-two strains carrying pLVPK-derived virulence plasmid were defined as hvKP. Notably, the coexistence of bla_{KPC-2}-encoding plasmid and the pLVPK-derived virulence plasmid was detected in 15 strains (34.1%, 15/44), suggesting *K. pneumoniae* carbapenemase-2 (KPC-2)-producing hvKP. The proportion of KPC-2-producing hvKP by year increased remarkably from 0% (2011) to 71.4% (2017). Of the 15 KPC-2-producing hvKP strains, 80.0% (12/15) were assigned to sequence type 11 and 2 strains (13.3%) belonged to clonal complex 23. Most of the patients infected with KPC-2-producing hvKP had preceding postneurosurgical state (93.3%, 14/15) and severe pneumonia (73.3%, 11/15). All the cases (100%, 15/15) had fatal outcome.

Conclusion: The high prevalence and mortality of *K. pneumoniae*, especially KPC-2-producing hvKP meningitis, in China should be of concern. The implementation of epidemiological surveillance and identification of an effective clinical treatment are paramount.

Keywords: meningitis, hypervirulent *K. pneumonia*, bla_{KPC-2}, *rmpA2*, pLVPK-like virulence plasmid

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Introduction

Bacterial meningitis is one of the most devastating infectious diseases. It is often associated with substantial mortality and long-term neurologic complications. Gram-negative bacilli, such as *Acinetobacter baumannii* and Enterobacteriaceae, are prominent causative pathogens of meningitis in adult patients, especially postoperative meningitis.[1,2] Among the implicated Gram-negative pathogens, *Klebsiella pneumoniae* is the most common in Taiwan and several Southeast Asian countries.[3–5] In these regions, *K. pneumoniae* meningitis was associated with a new variant, designated as hypervirulent

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K. pneumoniae (hvKP) during the past few decades, with reported mortality ranging from 33.3% to 48.5%.[6,7]

Described as hvKP, this new variant commonly exhibited hypermucoviscosity (HM) phenotype and frequently belonged to specific sequence types (STs), mainly including ST23, ST65, and ST86.[8] Since first described in 1986, hvKP increasingly caused pyogenic liver abscesses (PLA) complicated by devastating metastatic infections including endophthalmitis, necrotizing fasciitis, and meningitis in young and healthy individuals.[6,9,10] However, the pathogenesis of hvKP causing metastatic infections is still not well elucidated hitherto. Over the past years, HM has been regarded as an important in vitro parameter for hvKP identification, but several controversies regarding the association of HM phenotype and virulence have been raised.[11,12] The large virulence plasmid pLVPK carrying capsular polysaccharides regulator genes (*rmpA* and *rmpA2*) and several siderophore gene clusters were recognized as essential contributors to the virulence of hvKP.[13] A correlation between carriage of pLVPK-derived virulence plasmid and pyogenic infection has been confirmed.[14] Most recently, Russo et al[15] demonstrated several pLVPK-derived locus could accurately differentiate hvKP from non-hvKP, and they might serve as potential biomarkers for hvKP.

So far, most hvKP were susceptible to commonly used antibiotics except ampicillin. In contrast, carbapenem-resistant *K. pneumoniae* (CRKP) were usually non-hvKP and resistant to almost all currently available antibiotics. At present, ~70%–90% of clinical carbapenem-resistant Enterobacteriaceae infections were attributed to CRKP in Europe and China.[16,17] Most clinical CRKP were *K. pneumoniae* carbapenemase (KPC)-producing strains[18] and belonged to clonal group 258, with ST11 the most predominant clone in Asia, especially in China.[17] As CRKP are highly transmissible and antibiotic resistant, they constitute a great threat to public health.

In most cases, CRKP and hvKP were largely nonoverlapping. Nevertheless, along with the spread of plasmids, carbapenem-resistant hvKP have been increasingly reported since first described by Zhang et al in 2015 in China.[11] For its capability of causing severe and difficult-to-treat infection, carbapenem-resistant hvKP has posed a considerable concern to the public health and will be the next "superbug" if a successful epidemic clone emerges. Up to now, most carbapenem-resistant hvKP infections generally occurred as isolated or sporadic cases,[19–21] with only two outbreaks of a small scale.[22,23] More recently, a nationwide screen revealed only 3% (11/387) of ST11 CRKP isolated from blood or sputum harbored the pLVPK-like virulence plasmid in mainland China,[22] while Ku et al[24] discovered none of the 22 hvKP strains that caused meningitis was resistant to carbapenems in Taiwan. However, information regarding the antimicrobial resistance and virulence characteristics of *K. pneumoniae* isolated from cerebrospinal fluid (CSF) in mainland China is still lacking. In the present study, we sought to investigate the epidemiology, antibiotic profiles, and representative virulence factors including pLVPK-derived genetic loci of meningitis-causing *K. pneumoniae* in a tertiary hospital over a 6-year period in China.

Materials and methods

Identification of patients

This retrospective cohort study was conducted in The First Affiliated Hospital, College of Medicine, Zhejiang University, a 2500-bed tertiary hospital having ~131,000 admissions each year in Hangzhou, East China, from January 2011 to July 2017. The hospital has three intensive care units with 73 beds, and three neurosurgery departments with 126 beds. Patients who satisfied the following three criteria were included in the analysis: 1) aged >18 years; 2) CSF culture yielded *K. pneumoniae*; 3) present with abnormal CSF examination and symptoms of meningitis. The classification of community-acquired or nosocomial acquired meningitis was according to the report by Chang et al.[4] The relevant clinical and microbiological data were extracted from electronic or paper medical records and microbiological database.

This study was conducted in accordance with the Declaration of Helsinki and approved by the hospital's Ethics Committees. Written informed consents were obtained from all patients.

Bacterial isolates and antimicrobial susceptibility testing

The VITEK-2 compact system (bioMérieux, Craponne, France) was used to establish strain identification and antimicrobial susceptibility testing. The results were interpreted according to the guideline document established by Clinical and Laboratory Standards Institute (CLSI, 2018). For tigecycline and colistin, the minimum inhibitory concentrations were determined by using broth microdilution method, and the results were categorized in accordance with the breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing criteria (version 7.1, http://www.eucast.org/clinical_breakpoints/) and CLSI, respectively. The species identification of all isolates was verified by matrix-assisted laser desorption/ionization mass spectrometry

(Bruker Daltonics Inc., Fremont, CA, USA). *K. pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 were used as the quality control strain for species identification and antimicrobial susceptibility testing, respectively.

Determination of HM phenotype

The HM phenotype was determined by "string test" as described previously.[10] Briefly, when using a bacteriology loop to stretch bacterial colony cultured on an agar plate overnight at 37°C, a formation of viscous string with >5 mm in length is considered to be positive.

Capsular serotyping and detection of resistance and virulence-associated genes

The capsular type of *K. pneumoniae* was determined by PCR and sequencing of *wzi* loci as previously described.[25] The sequences of products were compared to the *wzi* sequences deposited in the database of Institut Pasteur to identify the corresponding capsular types using BLAST program (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html). The extended-spectrum β-lactamase genes (bla_{CTX-M} and bla_{SHV}), carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48}) and 14 representative virulence genes including the pLVPK-related genetic loci (*magA*, *kfu*, *allS*, *fimH*, *wabG*, *ybtS*, *mrkD*, *uge*, *entB*, *iutA*, *rmpA*, *rmpA2*, *iucA*, and *iroN*) were amplified by PCR.[22,26] The sequences of primers are listed in Table S1. The amplicons of β-lactamase genes were purified and sequenced in an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA), and the sequences obtained compared to those in the NCBI database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Multilocus sequence typing (MLST)

MLST for all 44 isolates was done with seven housekeeping genes (*gapA*, *infB*, *mdh*, *phoE*, *pgi*, *rpoB*, and *tonB*) according to the protocol on the MLST website (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html). STs that had not been described previously were submitted to the database.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed on all of the isolates. In brief, genomic DNA was digested with XbaI (Bio-Rad Laboratories, Hercules, CA, USA), and the restriction fragments were separated in a CHEF Mapper XA System (Bio-Rad Laboratories). Cluster analysis was performed with Bionumerics software (Applied Maths NV, Sint-Martens-Latem, East Flanders, Belgium) using the Dice similarity coefficients and unweighted-pair group matching algorithm. The strains sharing >75% similarity were defined as the same PFGE pattern.

S1-PFGE and southern blot hybridization

To detect the pLVPK-like virulence plasmid and carbapenemase gene-encoding plasmid, whole chromosomal DNA of 15 strains positive for both *rmpA2* and bla_{KPC-2} were subjected to S1 nuclease (Takara, Shiga, Japan) digestion. Digested fragments were subjected to PFGE. Then, the gels were blotted onto nylon membranes (Millipore, Burlington, MA, USA) according to standard technique. The membranes were hybridized with digoxigenin-labeled *rmpA2* probe and bla_{KPC-2} probe, respectively. Consistent with the findings from Russo et al,[15] *K. pneumoniae* strains that were confirmed to contain pLVPK-like virulence plasmid by S1 nuclease PFGE and southern blot hybridization were defined as hvKP in the present study.[22]

Results

Prevalence and clinical characteristics of hvKP meningitis

From January 2011 to July 2017, a total of 355 cases of bacterial meningitis were identified in our hospital. The detailed distribution of all meningitis causing species is shown in Figure 1. Among the 48 *K. pneumoniae* causing meningitis, 44 isolates were available for further experiments (4 isolates died). Twenty-two isolates carrying the pLVPK-like plasmid were identified as hvKP. The clinical characteristics of patients with hvKP and non-hvKP meningitis are shown in Table 1. Overall, there were no significant differences between the two groups. Co-occurrence of bla_{KPC-2}-encoding plasmid and pLVPK-like virulence plasmid was detected in 68.2% (15/22) of hvKP, suggesting KPC-2-producing hvKP (Figure 2). The clinical features of 15 patients with KPC-2-producing hvKP meningitis are summarized in Table 2. In particular, most of the patients (73.3%, 11/15) suffered from severe pneumonia, and all except one patient (93.3%, 14/15) had a preceding neurosurgical state. All the cases (100%, 15/15) had fatal outcome.

As shown in Figure 3, the proportion of *K. pneumoniae* meningitis among Gram-negative bacteria causing meningitis increased continuously and obviously during the last 5 years (11.4% in 2013, 12.8% in 2014, 25.0% in 2015, 28.6% in 2016, and 50.0% in 2017, respectively), and the proportion of hvKP among *K. pneumonia* isolates increased from 2014 to 2017 (20.0% to 71.4%) as well. It was noted that among the 15 cases of KPC-2-producing hvKP meningitis, the earliest

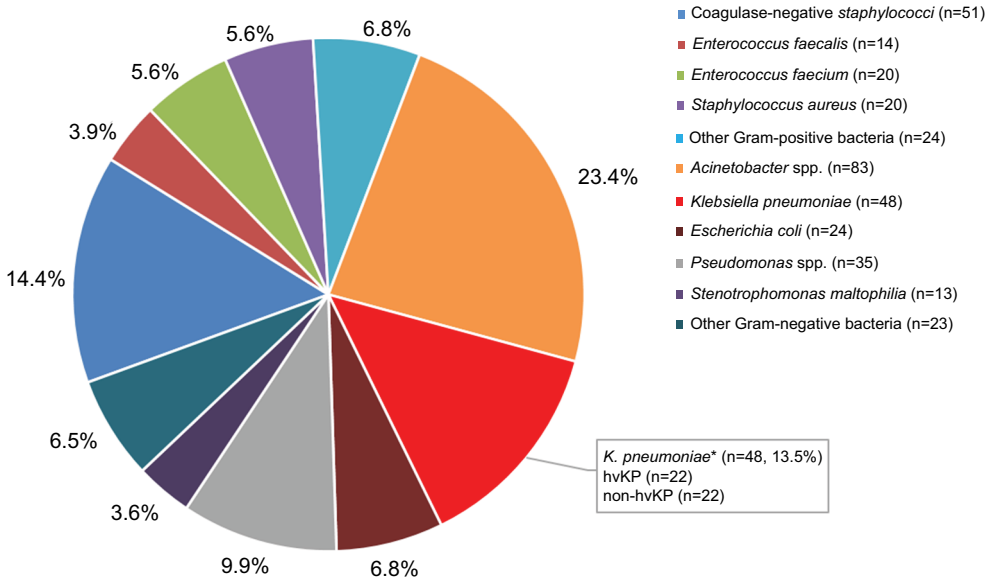


Figure 1 The distribution of all meningitis-causing species from January 2011 to July 2017.
Note: *Including four dead isolates.
Abbreviations: hvKP, hypervirulent *K. pneumonia*; non-hvKP, non-hypervirulent *K. pneumonia*.

Table 1 Demographic profiles and clinical features of 48 patients with *Klebsiella pneumoniae* meningitis

Characteristics		Available isolates (n=44)		*P*-value (hvKP vs non-hvKP)
	Total (n=48)	hvKP (n=22)	Non-hvKP (n=22)	
Demographic data				
Age (years), mean (± SD)	50.3±16.0	55.2±13.4	48.3±17.6	0.15
Male, n (%)	30 (62.5)	13 (59.1)	16 (72.2)	0.34
Acquired infection model, n (%)				
Community-acquired infection	3 (6.3)	1 (4.5)	1 (4.5)	1.00
Hospital-acquired infection	45 (93.8)	21 (95.5)	21 (95.5)	1.00
Types of infection, n (%)				
Spontaneous meningitis	3 (6.3)	1 (4.5)	1 (4.5)	1.00
Postneurosurgical/post-trauma meningitis	45 (93.8)	21 (95.5)	21 (95.5)	1.00
Comorbidity, n (%)				
Diabetes mellitus	10 (20.8)	5 (22.7)	5 (22.7)	1.00
Hypertension	14 (29.2)	6 (27.3)	8 (36.4)	0.52
Brain tumor	12 (25.0)	4 (18.2)	6 (27.3)	0.47
Head trauma	18 (37.5)	9 (40.9)	9 (40.9)	1.00
Intracerebral hemorrhage	15 (31.3)	7 (31.8)	8 (36.4)	0.75
Extrameningeal infection, n (%)				
Bacteremia	17 (35.4)	9 (40.9)	7 (31.8)	0.53
Pneumonia	18 (37.5)	11 (50.0)	7 (31.8)	0.22
Liver abscess	1 (2.1)	1 (4.5)	0 (0.0)	1.00
Brain abscess	2 (4.2)	1 (4.5)	0 (0.0)	1.00
Chronic otitis media	1 (2.1)	0 (0.0)	0 (0.0)	–
CSF profiles, median (IQR)				
RBC count (per μL)	150 (20.0–862.5)	50 (155.0–1,500.0)	10 (95.0–962.5)	0.46
WBC count (per μL)	900 (72.5–6,265.0)	60 (210–3,050.0)	140 (1,040.0–9,100.0)	0.41
Sugar (mmol/L)	0.6 (0.1–2.9)	0.1 (0.4–2.9)	0.1 (0.5–2.7)	0.88
Chlorine (mmol/L)	112 (105.5–119.8)	106.5 (112.0–120.8)	106.5 (111.5–118.5)	0.66
Protein (g/L)	2.7 (1.7–6.1)	1.6 (2.7–7.2)	1.6 (3.0–5.7)	0.98
30-day mortality, n (%)	30 (62.5)	17 (77.3)	12 (54.5)	0.11

Abbreviations: CSF, cerebrospinal fluid; hvKP, hypervirulent *K. pneumoniae*; RBC, red blood cell; WBC, while blood cell.

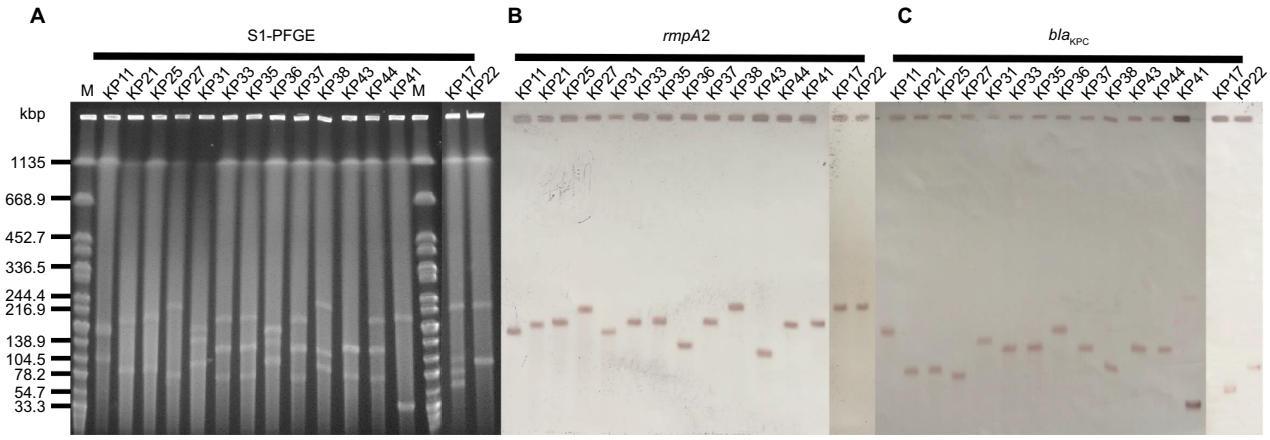


Figure 2 The S1-PFGE and Southern hybridization analysis of 15 *Klebsiella pneumoniae* strains co-harboring bla_{KPC-2} and *rmpA2*.
Notes: (**A**) S1 nuclease digestion of genomic DNA of *K. pneumoniae* strains was followed by PFGE. Plasmid bands are shown as linearized fragment on the gel. (**B**) Southern blot hybridization of the marker gene (*rmpA2*) of the virulence plasmid. (**C**) Southern blot hybridization of bla_{KPC-2}. Lane M, reference standard strain *Salmonella* serotype Braenderup H9812 restricted with XbaI.
Abbreviations: M, marker; S1-PFGE, S1 nuclease pulsed-field gel electrophoresis.

Table 2 Clinical features of 15 patients with KPC-2-producing hvKP meningitis

Patient no.	Strain ID	Age (years)	Sex	Unit	Comorbidity	Neurosurgical procedures	Treatment	Outcome
1	KP11	68	F	ICU	Severe pneumonia, bacteremia	Yes	MRP	Died
2	KP17	27	F	Surgery	ICH, pneumonia, bacteremia	Yes	MRP, MXF	Died
3	KP21	41	M	ICU	Head trauma, pneumonia, bacteremia	Yes	MRP, TGC	Died
4	KP22	63	F	Surgery	SAH, hypertension, pneumonia	Yes	MRP, LEV, AMI	Died
5	KP25	38	M	Surgery	Head trauma, pneumonia	Yes	MRP, C/S, PXE	Died
6	KP27	67	M	ICU	Meningioma, DM, heart disease	Yes	MRP	Died
7	KP31	67	M	ICU	Cerebral infarction, DM, COPD	No	C/S, TGC	Died
8	KP33	53	M	Surgery	AVM rupture, pneumonia	Yes	MRP, TGC	Died
9	KP35	58	M	Surgery	Head trauma, SDH, bacteremia	Yes	MRP, TGC, C/S	Died
10	KP36	67	F	ICU	SAH, hypertension, pneumonia	Yes	TGC, C/S	Died
11	KP37	63	M	ICU	ICH, Alzheimer disease, hypertension, pneumonia, bacteremia	Yes	MRP, TGC	Died
12	KP38	46	M	Surgery	Head trauma, renal calculus	Yes	MRP, TGC	Died
13	KP41	80	F	ICU	SAH, DM, heart disease, pneumonia	Yes	C/S	Died
14	KP43	72	M	ICU	Head trauma, hypertension, pneumonia, bacteremia	Yes	C/S, TGC, PXE	Died
15	KP44	52	F	Surgery	Hydrocephalus, pneumonia	Yes	MRP, TGC, C/S	Died

Abbreviations: AMI, amikacin; AVM, arteriovenous malformation; C/S, cefoperazone/sulbactam; DM, diabetes mellitus; F, female; hvKP, hypervirulent *Klebsiella pneumoniae*; ICH, intracerebral hemorrhage; ICU, intensive care unit; KPC-2, *K. pneumoniae* carbapenemase-2; LEV, levofloxacin; M, male; MRP, meropenem; MXF, moxifloxacin; PXE, polymyxin E; SAH, subarachnoid hemorrhage related to a brain aneurysm; SDH, subdural hemorrhage; TGC, tigecycline.

case emerged in 2013, and an increase in the proportion of these strains within *K. pneumoniae* was observed, 42.9% in 2015 (from January to December), 46.2% in 2016 (from January to December), and 71.4% in 2017 (from January to July).

Antimicrobial susceptibility and resistance mechanism among hvKP and non-hvKP isolates

The detailed antimicrobial resistance profiles of the 16 drugs are listed in Table 3. 68.2% (15/22) of hvKP strains were resistant to carbapenem. Meanwhile, resistance to all tested antimicrobials, except tigecycline and colistin, was observed in a high proportion of hvKP strains. However, the overall resistance rates of hvKP were similar to those of non-hvKP.

Twenty-eight *K. pneumoniae* strains were positive for bla_{KPC} including 15 hvKP and 13 non-hvKP. The CTX-M-9 group was detected in 13 hvKP strains ($bla_{CTX-M-14}$, n=4; $bla_{CTX-M-65}$, n=9) and 10 non-hvKP strains ($bla_{CTX-M-14}$, n=2; $bla_{CTX-M-65}$, n=8), respectively. Interestingly, the CTX-M-1 group β-lactamases $bla_{CTX-M-3}$ was exclusively detected in

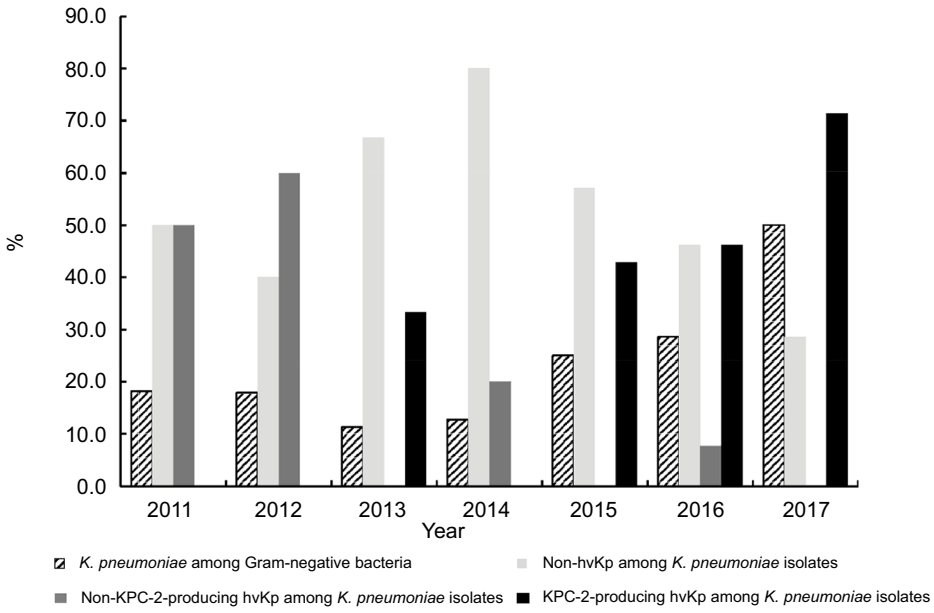


Figure 3 The prevalence of four types of *Klebsiella pneumoniae* strains from January 2011 to July 2017.
Abbreviations: KPC-2, *K. pneumoniae* carbapenemase-2; hvKP, hypervirulent *K. pneumoniae*.

Table 3 The antimicrobial resistance profile of 44 *Klebsiella pneumoniae* strains from cerebrospinal fluid

Antimicrobials	No. (%) of resistant isolates			*P*-value (hvKP vs non-hvKP)
	Total (n=44)	hvKP (n=22)	Non-hvKP (n=22)	
Ampicillin–sulbactam	35 (79.5)	17 (77.3)	18 (81.8)	1.00
Piperacillin–tazobactam	29 (65.9)	15 (68.2)	14 (63.6)	0.75
Ceftazidime	29 (65.9)	13 (59.1)	16 (72.7)	0.34
Ceftriaxone	34 (77.3)	17 (77.3)	17 (77.3)	1.00
Cefepime	27 (61.4)	13 (59.1)	14 (63.6)	0.76
Aztreonam	32 (72.7)	16 (72.7)	16 (72.7)	1.00
Ertapenem	29 (65.9)	15 (68.2)	14 (63.6)	0.75
Imipenem	28 (63.6)	15 (68.2)	13 (59.1)	0.53
Amikacin	15 (34.1)	10 (45.5)	5 (22.7)	0.11
Gentamicin	21 (47.7)	10 (45.5)	11 (50.0)	0.76
Tobramycin	17 (38.6)	10 (45.5)	7 (31.8)	0.35
Ciprofloxacin	26 (59.1)	13 (59.1)	13 (59.1)	1.00
Levofloxacin	24 (54.5)	13 (59.1)	11 (50.0)	0.55
Trimethoprim–sulfamethoxazole	17 (38.6)	9 (40.9)	8 (36.4)	0.76
Tigecycline	0 (0.0)	0 (0.0)	0 (0.0)	–
Colistin	0 (0.0)	0 (0.0)	0 (0.0)	–

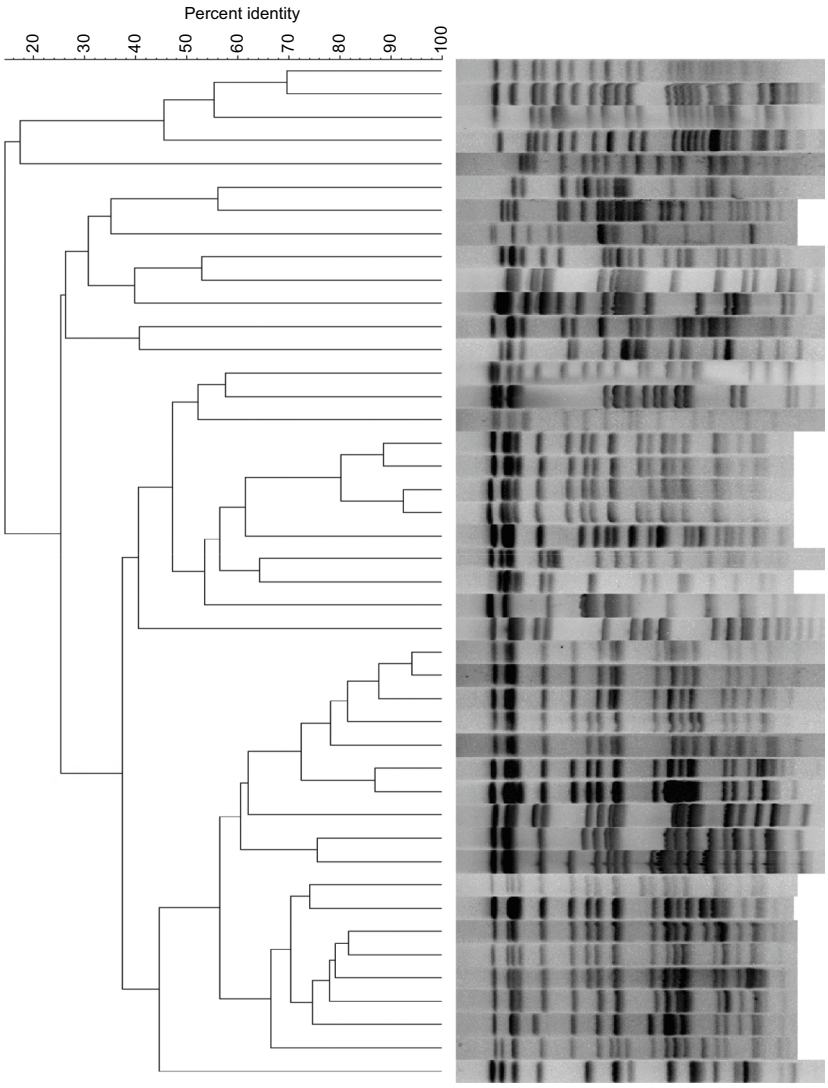
Abbreviations: hvKP, hypervirulent *K. pneumonia*; non-hvKP, non-hypervirulent *K. pneumonia*.

hvKP (13.6%, 3/22), while $bla_{\text{CTX-M-15}}$ was found only in three non-hvKP strains. Various SHV genes were detected in 20 strains (hvKP, n=10; non-hvKP, n=10). None of the strains was positive for bla_{NDM}, bla_{VIM}, bla_{IMP}, and $bla_{\text{OXA-48}}$.

Capsular serotyping and virulence-associated genes among hvKP and non-hvKP isolates

According to the results of the *wzi* typing, a total of 14 different capsular serotypes were identified (Figure 4). Overall, K64 was the most common serotype (n=13), followed by K47 (n=11), K1 (n=5), and K2 (n=4). In the hvKP group, K64 was the predominant serotype (36.4%) and K47 in non-hvKP (27.3%) group. Among the 15 KPC-2-producing hvKP strains, 7 (46.7%), 5 (33.3%), 2 (13.3), and 1 (6.7%) belonged to K64, K47, K1, and K16, respectively.

The positive rates of the 14 virulence genes in hvKP and non-hvKP isolates are shown in Figure 5. The virulence-associated genes with >50% of positive rates in hvKP and non-hvKP isolates included *wabG* (100% and 100%), *entB*



Strain ID	Isolation year	String test	Serotype	Sequence type
KP17	**2015**	**+**	**K1**	**1660**
KP40	2011	+	K1	23
KP7	2012	+	K1	23
KP22	**2015**	**+**	**K1**	**23**
KP3	2011	–	K19	29
KP18	2015	–	K37	35
KP29	2016	–	K24	15
KP30	2016	–	K18	43
KP15	2014	–	K21	290
KP42	2014	+	K2	380
KP10	2013	–	K115	3129
KP20	2015	–	K81	252
KP24	2016	–	K19	15
KP6	2012	+	K2	65
KP12	2014	+	K1	700
KP2	2011	+	K64	1764
KP35	**2016**	**–**	**K64**	**11**
KP37	**2017**	**+**	**K64**	**11**
KP43	**2017**	**–**	**K64**	**11**
KP44	**2017**	**–**	**K64**	**11**
KP36	**2016**	**+**	**K47**	**11**
KP16	2014	+	K2	86
KP34	2016	+	K2	375
KP8	2012	+	K57	412
KP41	**2017**	**–**	**K16**	**660**
KP1	2011	–	K47	11
KP4	2012	–	K47	11
KP19	2015	–	K47	11
KP13	2014	–	K64	11
KP5	2012	–	K47	11
KP21	**2015**	**–**	**K47**	**11**
KP23	2015	–	K64	11
KP39	2017	–	K47	11
KP9	2013	–	K47	11
KP11	**2013**	**+**	**K47**	**11**
KP33	**2016**	**–**	**K64**	**11**
KP38	**2017**	**–**	**K64**	**11**
KP27	**2016**	**–**	**K64**	**11**
KP32	2016	–	K64	11
KP25	**2016**	**–**	**K47**	**11**
KP26	2016	–	K64	11
KP28	2016	–	K64	11
KP31	**2016**	**+**	**K47**	**11**
KP14	2014	–	K17	768

Figure 4 PFGE dendrogram of 44 *Klebsiella pneumoniae* strains.
Notes: The 15 KPC-2-producing hvKP strains are indicated by boldface type. The similarity percentage of profiles was calculated by the Dice coefficient.
Abbreviations: hvKP, hypervirulent *K. pneumoniae*; KPC-2, *K. pneumoniae* carbapenemase-2; PFGE, pulsed-field gel electrophoresis.

(100% and 100%), *fimH* (100% and 95.5%), *uge* (95.5% and 100%), *mrkD* (90.9% and 100%), and *ybtS* (86.4% and 77.3%). The positive rates of all the five pLVPK-derived locus *iutA*, *iucA*, *rmpA*, *rmpA2*, *iroN* were significantly higher in hvKP isolates than non-hvKP isolates ($P<0.001$). Additionally, *allS* was exclusively detected in hvKP isolates (22.7%, 5/22). The HM phenotype was detected more frequently in hvKP isolates than in non-hvKP (59.1% vs 9.1%, $P<0.001$) as well.

Molecular characteristics of the *K. pneumoniae* isolates

MLST analysis revealed a total of 19 STs including one novel ST (ST3129). ST11 was the most predominant ST (52.3%, 23/44) followed by ST23 (6.8%, 3/44) and ST15 (4.5%, 2/44), while the remaining STs were found only in one strain each. In addition, the five strains with K1 serotype belonged to three STs (ST23, n=3; ST1660, n=1; and ST700, n=1) and the four strains with K2 serotype belonged to four STs (ST65, ST86, ST375, and ST380). Most strains belonged to ST11 both in hvKP and non-hvKP groups (54.5% and 50.0%, respectively). The PFGE results showed that the homology of 44 isolates was diverse (Figure 3). Only three clusters with >75% similarity were identified, and each cluster accounted for four strains.

Among the 15 KPC-2-producing hvKP, four distinct STs were identified. ST11 was the predominant ST, accounting for 80% (12/15), and ST23, ST1660, and ST660 for each

strain (Figure 6). Of the 12 ST11 KPC-2-producing hvKP, 7 strains belonged to serotype K64 and 5 strains belonged to K47. In addition to bla_{KPC-2}, all of the 12 ST11 KPC-2-producing hvKP carried $bla_{CTX-M-9}$. Four strains exhibited HM phenotype. The strains of ST23 and ST1660 belonged to K1 serotype and displayed HM phenotype. In general, ST11 KPC-2-producing hvKP strains harbored more antibiotic-resistant genes and less virulence factors when compared with the non-ST11 KPC-2-producing hvKP (Figure 5). Among the 15 KPC-2-producing hvKP strains, 4 strains (KP35, KP37, KP43, and KP44) shared >75% similarity and classified into one cluster (Figure 3), while the remaining 11 strains exhibited distinct genetic relationship.

Discussion

Up to now, although some cases of *K. pneumoniae* meningitis have been reported in mainland China,[27,28] the clinical and microbiological features of *K. pneumoniae* causing

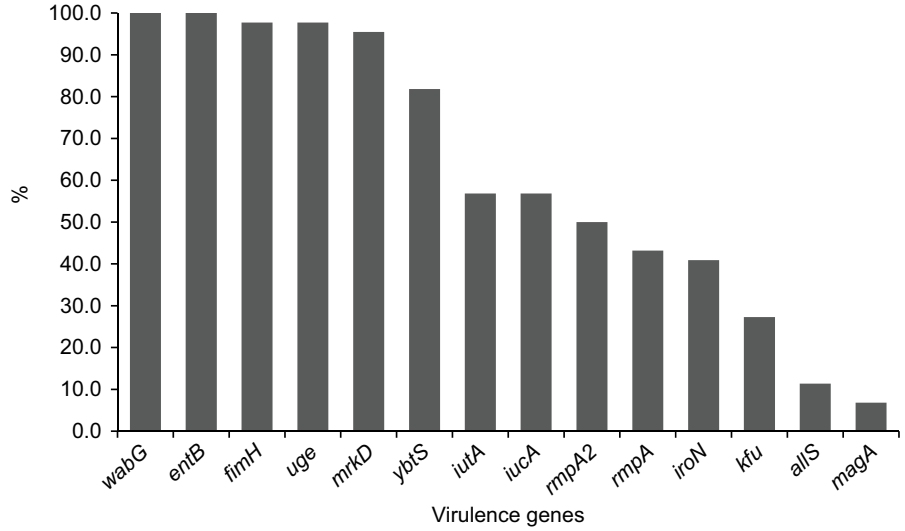


Figure 5 The distributions of virulence-associated genes among hvKP and non-hvKP strains.
Abbreviations: hvKP, hypervirulent *Klebsiella pneumonia*; non-hvKP, non-hypervirulent *K. pneumonia*.

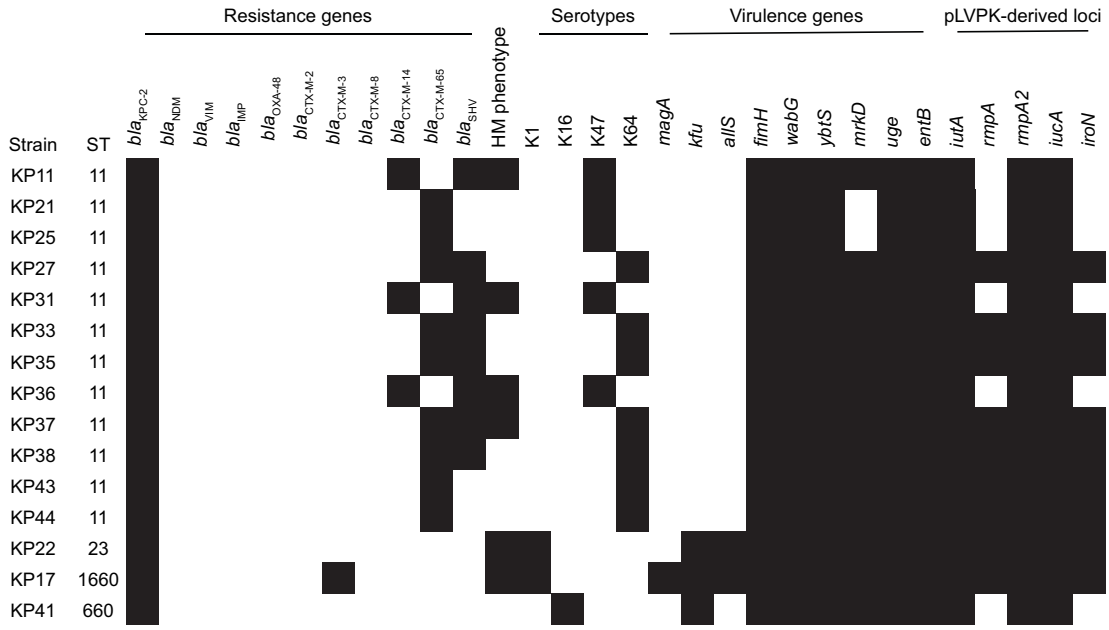


Figure 6 Antimicrobial resistance and virulence profiles of 15 KPC-2-producing hvKP strains.
Note: The black color indicated the presence of a gene in the corresponding strain.
Abbreviations: KPC-2, *Klebsiella pneumoniae* carbapenemase-2; hvKP, hypervirulent *K. pneumoniae*.

meningitis from our area remain unclear. Our study highlights a high prevalence and mortality of *K. pneumoniae*, especially KPC-2-producing hvKP causing meningitis, in a tertiary hospital in Eastern China, which posed a serious threat to public health.

Consistent with the epidemiology trend of *K. pneumoniae* meningitis in some Asian countries especially in Taiwan,[3,4] where *K. pneumoniae* is found to be the leading pathogen for meningitis, our present study shows that the proportion of *K. pneumoniae* among Gram-negative bacilli causing meningitis increased consistently from 2013 to 2017 and *K. pneumoniae* gradually becomes to be the predominant Gram-negative pathogen responsible for meningitis. However, in contrast to the reports from Taiwan, describing that most of the *K. pneumoniae* meningitis belonged to community-acquired cases,[3,4,29] only a few cases of community-acquired *K. pneumoniae* meningitis (n=3, 6.3%) were observed in our study. Sought to the possible explanations, besides the proved genetic predisposition unique to people being in different geographical locations, a higher incidence of postneurosurgical conditions as the preceding event than that reported in Taiwan (85.4% vs 56.9%) was of note. However, an inadequate infectious control program in our hospital might also contribute to the development of *K. pneumoniae* meningitis in postneurosurgical patients.

Previous studies have consistently confirmed that *K. pneumoniae* was capable of causing meningitis commonly complicated by PLA, and diabetes mellitus and liver diseases seemed to be the well-established predisposing factors for *K. pneumoniae* meningitis.[3,6,29–31] Nevertheless, diabetes was noted in 20.8% of patients and liver diseases including PLA occurred in four patients in our study. These disparities can be attributed to the fact that the previous results were obtained based on the epidemiology investigations mainly involving community-acquired cases.[3,29,31] Further exploring the detailed clinical features of the three cases of community-acquired meningitis in our study, we found that all of the patients had liver diseases with liver abscess, liver cirrhosis, and chronic hepatitis for each case, respectively, and two patients had diabetes. However, no definitive conclusions could be drawn due to the small size of the enrolled community-acquired cases.

The overall 30-day mortality was 62.5%, which was higher than that reported in Korea[5] and Taiwan.[29,30] It was believed that the interaction between host and bacterial variables contributed to the outcomes during bacterial infections. Adult patients with *K. pneumoniae* meningitis commonly suffered from severe pneumoniae and developed to bacteremia.[29] These conditions were also found in the current study. But, in contrast to the previous studies mainly from Taiwan,[3,24,29] a remarkable high prevalence of CRKP causing meningitis was observed, which implied that most of our patients did not benefit from the use of carbapenem as the initial empiric therapy. Therefore, it should be highly considered that carbapenem is indicated for meningitis empirically. hvKP was identified in 50% isolates, which was lower than that reported from Taiwan (66.7%) most recently.[24] This difference might partly be attributed to the distinct indexes for hvKP. Nine isolates designated as hvKP were determined as string negative in our study. Hence, it seemed that the prevalence of hvKP might be overestimated due to the lack of definitive diagnostic methods.

KPC has been proved to be one of the most important genetic mechanisms of carbapenem resistance for *K. pneumoniae*.[18] Since KPC-2-producing *K. pneumoniae* firstly reported in China in 2007, it has spread widely and rapidly in our region.[32] 63.6% of the strains in the present study were positive for KPC-2. This percentage is much higher than that reported in bloodstream infection samples (33.3%) by our previous study.[33] In accordance with the report by Qi et al,[34] which described ST11 was the dominant clone of KPC-2-producing *K. pneumoniae* in China, 78.6% of the KPC-2-positive isolates belonged to ST11 in this study. These results suggested the nosocomial clonal dissemination of KPC-2-producing ST11 *K. pneumoniae* played a significant role in the high detection rate of bla_{KPC-2}.

It had been common sense that the coexistence of plasmids encoding resistance genes and virulence factors in bacteria was a rare event. However, most recently, Gu et al[22] reported that there was a fatal outbreak of ST11 carbapenem-resistant hvKP in a Chinese hospital in 2016. They also demonstrated the size of the acquired pLVPK-like virulence plasmid and its intrinsic bla_{KPC}-carrying plasmid was nearly the same. In our study, both the bla_{KPC-2} and *rmpA2* probes hybridized to the ~170 kb plasmid band in KP 11 strain; so, we speculated that the genetic background of KP11 was similar to the strains described by Gu et al. However, further whole genome sequencing or plasmid curing experiment might prove the speculation. ST23 was the most common ST among hvKP, which was frequently associated with PLA. Though hvKP belonging to ST23 always displayed a wide antibiotics susceptibility profile, several cases of ST23 KPC-2-producing hvKP infection had been described in China.[35] In the present study, two out of four ST23 (including one

ST23-like variant) isolates were found to harbor the bla_{KPC-2}-bearing plasmid. Therefore, our results suggested that both ST11 and ST23 *K. pneumoniae* strains could evolve to KPC-2-producing hvKP causing meningitis, and patients with KPC-2-producing hvKP meningitis were always associated with extremely high mortality.

There were some limitations to our study. The study was a single-center study with retrospectively enrolled relatively small size of cases, which might limit its epidemiologic scope. In addition, because of the lack of a "reference standard" genotypic/phenotypic marker for hvKP up to now, *K. pneumoniae* strains harboring the pLVPK-like virulence plasmid were defined as hvKP as described by Gu et al.[22] Studies combining with cell survival tests and whole-genome framework are needed to further illustrate the virulence characteristics and key genomic elements of these isolates.

Conclusion

In summary, *K. pneumoniae* meningitis has been increasing during the last 5 years, in which a high proportion of KPC-2-producing hvKP causing meningitis was observed. Such strains were likely to cause serious infections, particularly in patients with postneurosurgical meningitis. Our study highlights the urgent need to enhance clinical awareness and management of *K. pneumoniae*, especially KPC-2-producing hvKP-causing meningitis. Effective surveillance and strict infection control strategies are of prime importance to prevent such strains from further dissemination in China.

Ethics approval and informed consent

Ethical approval was granted from the ethics committee and review board of the First Affiliated Hospital, College of Medicine, Zhejiang University, and written informed consent for publication of individual details was obtained from all patients.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Primers used to detect the resistance and virulence-associated genes in this study

Target genes	Primer name	Primer sequence (5'–3')	Product size (bp)
bla_{KPC}	KPC-F	ATGTCACTGTATCGCCGTCT	893
	KPC-R	TTTTCAGAGCCTTACTGCCC	
bla_{NDM}	NDM-F	GGTTTGGCGATCTGGTTTTC	624
	NDM-R	CGGAATGGCTCATCACGATC	
bla_{VIM}	VIM-F	GATGGTGTTTGGTCGCATA	390
	VIM-R	CGAATGCGCAGCACCAG	
bla_{IMP}	IMP-F	GGAATAGAGTGGCTTAAYTCTC	188
	IMP-R	GGTTTAAYAAAACAACCACC	
bla_{OXA-48}	OXA48-F	GCGTGGTTAAGGATGAACAC	437
	OXA48-R	CATCAAGTTCAACCCAACCG	
bla_{SHV}	SHV-F	GCCTTTATCGGCCTTCACTCAAG	898
	SHV-R	TTAGCGTTGCCAGTGCTCGATCA	
$bla_{CTX-M-1}$ group	CTX-M-1-F	CAGCGCTTTTGCCGTCTAAG	944
	CTX-M-1-R	GGCCCATGGTTAAAAAATCACTGC	
$bla_{CTX-M-2}$ group	CTX-M-2-F	GCATTCGCCGCTCAATGTTA	942
	CTX-M-2-R	GGTTCGTTGCAAGACAAGAC	
$bla_{CTX-M-8}$ group	CTX-M-8-F	ACTTCAGCCACACGGATTCA	977
	CTX-M-8-R	CGAGTACGTCACGACGACTT	
$bla_{CTX-M-9}$ group	CTX-M-9-F	GTTACAGCCCTTCGGCGATGATTC	877
	CTX-M-9-R	GCGCATGGTGACAAAGAGAATGCAA	
magA	magA-F	GGTGCTCTTTACATCATTGC	1,283
	magA-R	GCAATGGCCATTTGCGTTAG	
Kfu	kfu-F	ATAGTAGGCGAGCACCGAGA	530
	kfu-R	AGAACCTTCCTCGCTGAACA	
allS	allS-F	CCGAAACATTACGCACCTTT	508
	allS-R	ATCACGAAGAGCCAGGTCAC	
fimH	fimH-F	TGCTGCTGGGCTGGTCGATG	909
	fimH-F	GGGAGGGTGACGGTGACATC	
wabG	wabG-F	ACCATCGGCCATTTGATAGA	683
	wabG-R	CGGACTGGCAGATCCATATC	
ybtS	ybtS-F	GACGGAAACAGCACGGTAAA	782
	ybtS-R	GAGCATAATAAGGCGAAAGA	
mrkD	mrkD-F	AAGCTATCGCTGTACTTCCGGCA	340
	mrkD-R	GGCGTTGGCGCTCAGATAGG	
uge	uge-F	TCTTCACGCCTTCCTTCACT	534
	uge-R	GATCATCCGGTCTCCCTGTA	
entB	entB-F	GTCAACTGGGCCTTTGAGCCGTC	400
	entB-R	TATGGGCGTAAACGCCGGTGAT	
iutA	iutA-F	GGGAAAGGCTTCTCTGCCAT	300
	iutA-R	TTATTCGCCACCACGCTCTT	
rmpA	rmpA-F	TACATATGAAGGAGTAGTTAAT	505
	rmpA-R	GAGCCATCTTTCATCAAC	
rmpA2	rmpA2-F	TGTGCAATAAGGATGTTACATTAGT	609
	rmpA2-R	TTTGATGTGCACCATTTTTCA	
iucA	iucA-F	ATAAGGCAGGCAATCCAG	2,927
	iucA-R	TAACGGCGATAAACCTCG	
iroN	iroN-F	GTCCGGCGGTAACTTCAGCC	829
	iroN-R	TCAGAATGAAACTACCGCCC	