Infection and Drug Resistance

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ORIGINAL RESEARCH

High Prevalence of *Klebsiella pneumoniae* Infections in AnHui Province: Clinical Characteristic and Antimicrobial Resistance

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Background: *Klebsiella pneumoniae* (*K. pneumoniae*) causes community-acquired and hospital-acquired pneumonia. The mortality rates of invasive infections caused by hypervirulent *K. pneumoniae* (HvKP) are extremely high. However, the microbiological characteristics and clinical manifestations of *K. pneumoniae* in AnHui province still remain unclear.

Purpose: To show the high prevalence of HvKP infections regarding clinical characteristics and antimicrobial resistance in Anhui province.

Patients and Methods: A retrospective analysis was conducted to study the clinical data of 115 strains of *K. pneumoniae* from July 2019 to March 2020 in The First Affiliated Hospital of AnHui Medical University. The virulence genes, capsular types, carbapenemase genes, and molecular subtypes of these hypervirulent isolates were detected.

Results: Overall, 59.1% (68/115) cases were HvKP infections, mainly from the department of intensive care unit (ICU, n=14, 20.6%) and the department of respiratory and critical care (n=13, 19.1%). K2 was the most prevalent capsular serotype (n=26), followed by K1 (n=21). The results of MLST identification of 68 strains showed that ST23 (n=15, 22.1%) was the most common type of ST, followed by ST11 and ST65 (n=12, 17.6%), ST86 (n=9, 13.2%), and ST412 (n=6, 8.8%). Among 68 hvKP strains, 12 isolates were carbapenem resistant, and all except two harboured *KPC*.

Conclusion: The high incidence of carbapenemase producing HvKP in the Anhui province, especially the higher mortality of HvKP, should be paid more attention. Meanwhile, epidemiological surveillance and clinical treatment strategies should be continuously determined and implemented.

Keywords: *Klebsiella pneumoniae*, virulence genes, capsular types, antimicrobial resistance

Introduction

Klebsiella pneumoniae was first isolated in 1893 by Friedlander, from the lung tissues of patients with lobar pneumonia. It is a Gram-negative bacterium with a thick capsule, and easily causes community and nosocomial infections. After the first report of hypervirulent *K. pneumoniae* (HvKP) by Chinese researchers in Taiwan in 1986, reports on HvKP have continued to emerge. In most cases, high mucilage often indicates that the bacteria have strong virulence. Unlike common *K. pneumoniae*, HvKP can arouse severe metastatic infections such as liver abscesses, endophthalmitis, and bacteraemia in young and healthy populations. Previous studies have demonstrated that *K. pneumoniae* rates were 5% among healthy Korean adults.[1,2] Data from studies in areas where HvKP is endemic

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indicates that its prevalence can reach 12–45%.[3–5] In China, the prevalence rates of HvKP infections are about 8.33–73.9%, and the mortality rate is even as high as 60%.[6–8] Colonization of the gastrointestinal system further promotes community transmission.[9] Analyses of the Human Microbiome Project in the United States shows about 4% colonization rate for *K. pneumoniae* in faecal samples.[10] However, the rate of *K. pneumoniae* colonization in hospitalized patients was 19–38%.[11,12] At present, consistent with the string test positive for high viscosity is high appraisal main virulence *K. pneumonia* bacteria method, but it is still controversial that mucous is necessarily a high virulence. Therefore, it is inaccurate to define whether the strain is a highly virulent one only by positive "string test".[13,14] The virulence plasmid pLVPK, which carries the virulence genes *rmpA* and *rmpA2*, plays an important role in the virulence of HvKP. Therefore, when *rmpA, rmpA2*, and string test were all positive, the strain is defined as HvKP.[15] Our study also defined HvKP according to such criteria. In general, strains that are both highly virulent and resistant are rare. However, owing to the abuse of antibacterial drugs, a growing number of studies have reported the presence of multidrug-resistant (MDR) the carbapenemase producing HvKP, which is understandably detrimental.[7,16–19] In this study, we collected 115 *K. pneumoniae* strains that were "string test" positive in the First Affiliated Hospital of Anhui Medical University from July 2019 to March 2020, and screened 68 strains of HvKP to further explore the molecular biological characteristics of HvKP and antimicrobial resistance to lay a foundation for subsequent scientific research and clinical treatment.

Materials and Methods

Clinical *K. pneumoniae* Isolates and Patients' Data Collection

We collected 115 consecutive patients at The First Affiliated Hospital of Anhui Medical University who were infected *K. pneumoniae* and showed positive string test. All specimens were numbered from 1 to 115 and stored at −80°C. Some clinical data and microbiological data related to the specimens could be obtained from the electronic or paper medical records of the hospital and the microbiological database. The inpatient information included demographic characteristics, clinical manifestations, microbiological reports, post-admission treatment, outcomes, and prognosis. Each patient in this study was admitted to the hospital after signing an informed consent.

Determination of Hypervirulent Phenotype

Phenotypic identification of *K. pneumoniae* relies on the classical "string test".[8] The strain taken from the refrigerator of −80°C was inoculated on an agar plate and cultured overnight in the incubator at 37°C until colony formation. Using a bacterial inoculation ring for stretching, a positive string test that formed mucoviscous string >5 mm was considered as the hypervirulent phenotype of *K. pneumoniae.*

Capsular Serotyping and Determination of Virulence Genes *rmpA, rmpA2*

All strains were grown overnight on agar plates, the genomic DNA was extracted. Then, polymerase chain reaction (PCR) was used to amplify virulence genes *rmpA* and *rmpA2* and capsular serotype genes as previously described.[20–22] The primer sequences used are shown in I note you uploaded the file 07_Jul_2020_63.xlsx. Please advise what this is, is this to be published with your manuscript or was this requested by the reviewer? 1. The reaction mixture was prepared as follows: initial denaturation at 95°C for 3 min; followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 3 min and a final extension at 72°C for 10 min. Agarose gel electrophoresis and sequencing were used to analyse the PCR products. The capsular serotypes of 12 strains carbapenemase producing HvKP were detected by *wzi* gene sequencing. The results were submitted to http://bigsdb.pasteur.fr.

Susceptibility Testing and KPC Gene Identification

The agar dilution method was used to identify and test antimicrobial susceptibility. The antimicrobial agents included ceftazidime, ceftriaxone, cefepime, cefotaxime, piperacillin-tazobactam, cefoperazone-sulbactam, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, levofloxacin, and aztreonam. Broth microdilution method was used for drug sensitivity tests of tigecycline and colistin (since there was no MIC breakpoints of these two drugs on CLSI, we referred to it of *EScherichia coli* on EUCAST). *K. pneumoniae* 700,603 was selected as the positive control group for antimicrobial susceptibility testing, and *Escherichia coli* 25,922 was used as the negative control. The Clinical and Laboratory Standards Institute

Table 1 Primer Sequence About Virulence and Resistance Associated Genes

Prime Name		Sequence
rmpA	Forward	ACTGGGCTACCTCTGCTTCA
	Reverse	CGCACCAGTAATTCCAACAG
rmpA2	Forward	CTTTATGTGCAATAAGGATGTT
	Reverse	CCTCCTGGAGAGTAAGCATT
K1	Forward	GGTGCTCTTTACATCATTGC
	Reverse	GCAATGGCCATTTGCGTTAG
K2	Forward	GGAGCCATTTGAATTCGGTG
	Reverse	TCCCTAGCACTGGCTTAAGT
K5	Forward	GCCACCTCTAAGCATATAGC
	Reverse	CGCACCAGTAATTCCAACAG
K20	Forward	CCGATTCGGTCAACTAGCTT
	Reverse	GCACCTCTATGAACTTTCAG
K54	Forward	CATTAGCTCAGTGGTTGGCT
	Reverse	GCTTGACAAACACCATAGCAG
KPC-2	Forward	ATGTCACTGTATCGCCGTCT
	Reverse	TTTTCAGAGCCTTACTGCCC
NDM-1	Forward	GGTTTGGCGATCTGGTTTTC
	Reverse	CGGAATGGCTCATCACGATC
VIM-1	Forward	AAATTCCGGTCGGAGAGGTC
	Reverse	AATGCGCAGCACCAGGATAG
IMP-1	Forward	GGAATAGAGTGGCTTAATTCTCC
	Reverse	GGTTTAATAAAACAACCACC
OXA-48	Forward	GCGTGGTTAAGGATGAACAC
	Reverse	CATCAAGTTCAACCCAACCG

(CLSI) guidelines and CLSI breakpoints or European Committee on Antimicrobial Susceptibility Testing criteria (version 10.0, http://www.eucast.org/clinicalbreakpoints/), respectively (CLSI 2021), can be referred to for specific experimental methods and result analysis. Moreover, Table 1 showed the identification of *KPC* gene, including *KPC*-2, *NDM*-1, *VIM*-1, *IMP*-1 and *OXA*-48.

Multilocus Sequence Typing (MLST)

MLST typing was carried out for all HvKP strains screened according to the MLST website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) with seven housekeeping genes (*gapA, infB, mdh, phoE, pgi, rpoB, and tonB*). We have submitted new alleles and STs that have not been previously reported.

Pulsed-Field Gel Electrophoresis (PFGE)

All isolated strains were subjected to PFGE. *XBa* I (TaKaRa, Lot# AIF2232A) was used as the restriction endonuclease, and Salmonella H9812 as the marker for DNA size of PFGE electrophoresis. All strains were prepared by gelatinization, enzymatic digestion, and electrophoresis. Then, the PFGE images were processed by BioNumerics software and the tree diagram were drawn. The similarity coefficient of the strains in the similarity analysis matrix >80% were of the same PFGE type.[23]

Statistical Analysis

Data analysis was performed using SPSS software (version 23.0; IBM Corporation, Armonk, NY, USA). If the continuous variables followed a normal distribution, they were indicated using mean±standard deviation (SD). Chi-square or Fisher exact tests were used to analyse categorical variables. When $P<0.05$, difference between the two groups was considered statistically significant.

Results

HvKP Isolates Clinical Characteristics

Among 115 strains, 59.1% (68/115) isolates were HvKP, and 40.9% (47/115) were non-HvKP. Figure 1 shows the department source distribution of 68 HvKP isolates: 20.6% (n=14) were from the ICU and 19.1% (n=13) were from the Department of Respiratory and Critical Care. Table 2 summarizes the clinical characteristics of HvKP patients and non-HvKP patients. In the HvKP group, there were 43 (63.2%) male and 25 (36.8%) female patients. The mean age was 57.01±19.00 years. Among all 68 community-acquired HvKP isolates, 41 patients (60.33%) had pulmonary disease; 33 patients (48.5%) were attached to invasive equipment; and 21 (30.9%), 18 (26.5%), and 8 (11.8%) patients, respectively, had hypertension, diabetes, and liver abscess.

Capsular Serotyping of HvKP and Non-HvKP

Thus far, more than 100 serotypes of *K. pneumoniae* have been reported, most of which are closely associated with the types of infection and severity of disease progression.[24–26] Common clinical capsular serotypes

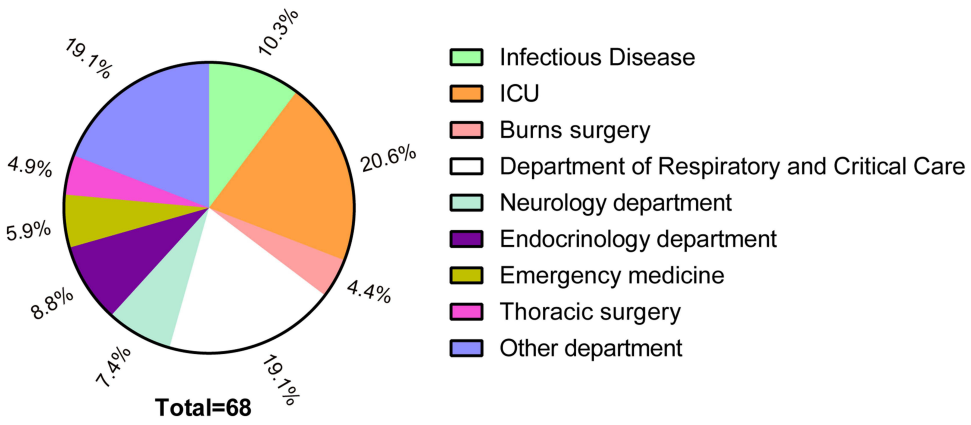

Figure 1 Distribution percentage of 68 strains of HvKP.

include K1, K2, K5, K20, and K54. We detected the capsular serotypes of the collected clinical isolates, and the results are shown in Table 2. Among HvKP isolates, the most common capsular serotype was K2 (n=26, 38.2%), followed by K1 (n=21, 30.9%). Among non-HvKP isolates too, the most common serotype was K2 (n=9, 19.1%), followed by K1 (n=2, 4.3%). Obviously, both K1 and K2 serotypes were higher in HvKP than in non-HvKP (P<0.05).

Antimicrobial Resistance Between HvKP and Non-HvKP

The sensitivity and drug resistance of 15 antimicrobial agents among HvKP and non-HvKP isolates are shown in Table 2. The resistance rate of cephalosporins in the non-HvKP group was higher than that of the HvKP group. All HvKP are sensitive to tigecycline and colistin. There were 23 carbapenemase producing isolates (12 strains in the HvKP group and 11 strains in the non-HvKP group) out of all 115 clinical strains.

Prevalence of Carbapenemase Producing HvKP

The demographic, microbiological, and clinical characteristics of the 12 carbapenemase producing HvKP patients are shown in Table 3. Nine patients developed pneumonia, and three patients experienced sepsis. Most of the 12 carbapenemase producing HvKP strains contained at least three genes encoding carbapenemase producing isolates. Consistent with domestic reports,[27,28] the results of this study showed that ST11 was the dominant serotype in carbapenemase producing HvKP isolates.

MLST Genotyping and PFGE

MLST identification of 68 strains showed that ST23 (n=15, 22.1%) was the most common type of ST, followed by ST11 and ST65 (n=12, 17.6%), ST86 (n=9, 13.2%), and ST412 (n=6, 8.8%). Homology analysis of the 68 strains of HvKP was conducted by PFGE. After *XBa*I digestion and BioNumerics software processing, a tree diagram was obtained (Figure 2). Briefly, 40 groups of different PFGE bands were detected in 68 strains of HvKP, indicating that the strain was highly polymorphic. Strain No. 32, 35, 37, and 38 of the 12 strains of carbapenemase producing HvKP were highly homologous, and most likely originated from the same strain or was caused by nosocomial infection.

Discussion

Klebsiella pneumoniae is one of the three most common causes of Gram-negative hospital-acquired infections (HAI, 10.2%), second only to *Pseudomonas aeruginosa* (11.5%) and *Escherichia coli* (10.4%).[29] *Klebsiella pneumoniae* infections can occur in any individual and thrive in different regions of the body. The mortality rate of bacteraemia caused by *Klebsiella pneumoniae* is about 20–26%.[30] This life-threatening bacterial infection has been emerging in many countries of the world and has become a major threat to host health.[31]

HvKP was defined on the basis of positive virulence genes *rmpA* and *rmpA2* and a positive string test. As is known to all, ~ 200kb virulence plasmid is an important characteristic of HvKP, which contains many virulence coding genes, such as *rmpA* and *rmpA2* mentioned above and siderophore (aerobactin and salmochelin). Nassif et al demonstrated that the aerobactin and myxoid phenotype

Table 2 Clinical and Microbiological Characteristics of HvKP Isolates

Characteristics	NO.(%) of Isolates		*P* value
	HvKP (n=68)	Non-HvKP (n=47)	
Demographic characteristics			
Male sex	43 (63.2)	35 (74.5)	0.205
Age (years) (mean ± SD)	57.01 ± 19.00	57.26 ± 16.20	0.944
K serotypes			
K1	21/68 (30.9)	2/47 (4.3)	0.000
K2	26/68 (38.2)	9/47 (19.1)	0.029
K5	12/68 (17.6)	4/47 (8.5)	0.164
K20	0/68 (0.0)	2/47 (4.3)	0.086
K57	8/68 (11.8)	1/47 (2.1)	0.059
Antimicrobial susceptibility			
Ceftazidime	13/68 (19.1)	12/47 (25.5)	0.093
Ceftriaxone	14/68 (20.6)	15/47 (32.0)	0.028
Cefepime	13/68 (19.1)	11/47 (23.4)	0.030
Cefotaxime	14/68 (20.6)	15/47 (32.0)	0.028
Piperacillin-tazobactam	12/68 (17.6)	9/47 (19.1)	0.273
Cefoperazone-sulbactam	13/68 (19.1)	10/47 (21.3)	0.254
Imipenem	12/68 (17.6)	10/47 (21.3)	0.168
Meropenem	12/68 (17.6)	10/47 (21.3)	0.168
Amikacin	12/68 (17.6)	6/47 (12.8)	0.872
Gentamicin	13/68 (19.1)	10/47 (21.3)	0.254
Ciprofloxacin	15/68 (22.1)	14/47 (29.8)	0.082
Levofloxacin	14/68 (20.6)	13/47 (27.7)	0.088
Aztreonam	15/68 (22.1)	14/47 (29.8)	0.082
Tigecycline	0/68 (0)	-	-
Colistin	0/68 (0)	-	-
Carbapenemase	12/68 (17.6)	11/47 (23.4)	0.448
Diseases			
Cancer	7 (10.3)	12 (25.5)	0.031
Liver abscess	8 (11.8)	1 (0.02)	0.059
Hypertension	21 (44.7)	15 (32.0)	0.907
Diabetes	18 (26.5)	8 (17.0)	0.234
Pulmonary disease	19 (28.0)	12 (25.5)	0.775
Invasive equipment	33 (48.5)	21 (44.7)	0.684

were associated with type K1 and K2.[32,33] Ye et al found that all detected strains contained *iuc, iro, rmpA* and *rmpA2* genes in their study of 40 pyogenic liver abscess specimens.[34] *RmpA*/*rmpA2* gene and siderophores cluster are considered to be more important in invasive infections caused by *Klebsiella pneumoniae*.[35] Some studies have found that *iroB, iucA, peg-344, rmpA*, and *rmpA2* are the most accurate molecular markers for differentiating between HvKP and classical Kp strains.[36] Indeed, in addition to *rmpA* and *rmpA2, iucA* (encoding aerobactin) has been demonstrated to be one of the most accurate genetic markers for identifying HvKP and has not been studied in the present work.

The top three units from where the 68 HvKP strains were isolated were the Department of ICU (n=14, 20.6%), Department of Respiratory Medicine and Critical Care (n=13, 19.1%), and the Department of Infectious Disease (n=7, 10.3%), poor physical quality and low immunity of the patient. Thus, these departments should be paid more attention to, to control HvKP infections. Consistent with previous studies, our results show that neither age nor sex is associated with HvKP.[37] However, *K. pneumoniae* is highly aggressive and has been linked to infections in healthy young people.[38–41] Studies have shown that HvKP infection is more prone to metastatic infection than non-HvKP infections.[42,43] But our study shows that patients with non-HvKP infection are more susceptible to tumours than those who are infected with HvKP (25.5% vs 10.3%, p=0.031). Other diseases or invasive equipment possibly have no association with HvKP.

Our study showed that HvKP was associated with K1 and K2 expression (p<0.05, Table 2), which is consistent with previous research.[8] Liu et al suggested that the resistance of K1 and K2 capsular serotypes to phagocytosis might be one of the causes, thus providing favourable conditions for the colonization and growth of bacteria.[22] Different from other literature reports,[44] our results showed that the most common capsular serotype in HvKP strain was K2 (26/68, 38.2%), followed by K1 (21/68, 30.9%). However, in general, these two serotypes are predominant.

Carbapenems including imipenem, meropenem, and ertapenem are currently considered the most effective antibiotics in the treatment of Gram-negative bacilli infection. However, owing to the abuse of antibacterial drugs, especially carbapenem antibiotics, multidrug-resistant *K. pneumoniae* strains are constantly emerging, making it difficult to control these infections.[45] The high virulence and antimicrobial resistance of *K. pneumoniae* vary to a large extent.[46] In our study, 12 of the 23 carbapenemase producing strains were HvKP isolates, and 3 of these 12 individuals eventually died. The differences in carbapenemase producing isolates between the HvKP and non-HvKP group showed no statistical significance (17.6% vs 23.4%, p=0.448). We speculate that this is because of the

Table 3 Clinical and Microbiological Characteristics of Carbapenemase Producing HvKP Isolates

	Strain4	Strain7	Strain16	Strain29	Strain32	Strain34	Strain35	Strain37	Strain38	Strain51	Strain55	Strain68
Demographic characteristics												
Gender	Male	Male	Male	Male	Female	Female	Male	Male	Male	Male	Female	Male
Age	56	52	46	54	53	33	29	81	55	50	60	44
Microbiological characteristics												
Virulence-associated features												
String test	+	+	+	+	+	+	+	+	+	+	+	+
rmpA	+	+	+	+	+	+	+	+	+	+	+	+
rmpA2	+	+	+	+	+	+	+	+	+	+	+	+
Cps genotype	K14/K64	K14/K64	K14/K64	K14/K64	K14/K64	K14/K64	K14/K64	K14/K64	K14/K64	K2	No-K-type	K14/K64
MLST	ST11	ST11	ST11	ST11	ST11	ST11	ST11	ST11	ST11	ST25	ST11	ST11
Resistance-associated features												
KPC-2	+	+	+	+	-	+	+	+	+	-	+	+
NDM-1	-	+	+	+	+	-	+	+	+	+	+	+
VIM-1	+	+	+	+	+	+	+	+	+	-	+	+
IMP-1	-	-	-	-	-	-	-	-	-	-	-	-
OXA-48	+	+	+	+	+	+	+	+	+	+	+	+
Clinical characteristics												
Specimen type	Sputum	Secretions	Sputum	Blood	Venous catheter	Sputum	Sputum	Sputum	Sputum	Sputum	Sputum	Puncture fluid
Infection type	Pneumonia	Pneumonia	Pneumonia	Pneumonia	Pneumonia	Pneumonia	Sepsis	Sepsis	Pneumonia	Pneumonia	Pneumonia	Abdominal infection
Clinical outcomes	Survived	Survived	Survived	Survived	Survived	Survived	Died	Died	Died	Survived	Survived	Survived

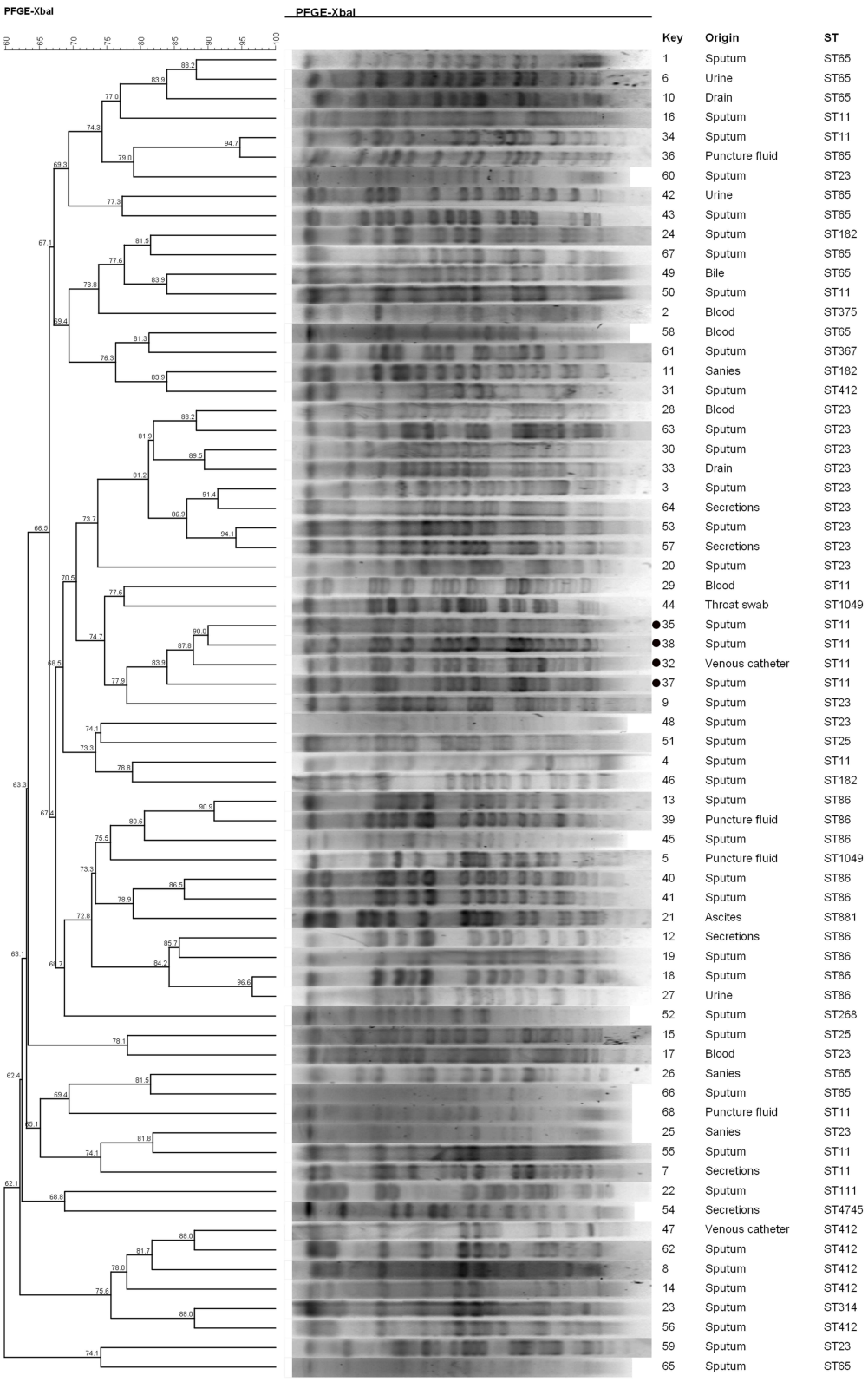

Figure 2 Pulsed field gel electrophoresis (PFGE) cluster analysis of 68 strains of HvKP from different sources. Four highly homologous isolates from carbapenemase producing HvKP have been marked with a black circle to the serial number left.

insufficient number of clinical specimens. Regarding the rare co-existence of high virulence and resistance of *K. pneumoniae*, some scholars[47] consider that HvKP has no resistance plasmids or that the resistance genes vanish when carrying virulence-related plasmids and genes. Further research studies are needed to verify these suggestions. The distribution and prevalence of ST types vary greatly from region to region. For example, ST23 is the most prevalent in Wuhan, accounting for 21.7%, while ST11 is the most prevalent in Zhejiang, accounting for 25%.[8] In our study, 11 of the 12 carbapenemase producing HvKP strains were ST11 clones, which indicated that this strain might have an epidemic phenomenon over a certain period of time. The source and collection time of these strains were variable, and the circulation of hospital personnel or airborne transmission further increased the possibility of infections in patients. Therefore, hand hygiene of medical staff and infection prevention and control in hospitals are paramount.

Carbapenemase producing HvKP has caused fatal infections,[16,19] which requires immediate action in case of multidrug-resistant infections. However, there are few studies on this strain in Anhui province. Our research highlights the high prevalence of HvKP infections in the Anhui province, including clinical characteristics and antimicrobial resistance. These severe infections caused by HvKP and the continuous occurrence of carbapenemase producing HvKP nudge us to improve clinical awareness, infection prevention, and treatment strategies.

Conclusion

Klebsiella pneumoniae (*K. pneumoniae*) causes community-acquired and hospital-acquired pneumonia. Our research shows the microbiological characteristics and clinical manifestations of *K. pneumoniae* in AnHui province, and the high incidence of carbapenemase producing HvKP, emphasizing the importance of monitoring, prevention and identifying clinical treatment strategies.

Data Sharing Statement

Data can be made available through contact with the corresponding author (Professor Jiabin Li).

Ethics Approval and Informed Consent

This study was conducted in accordance with the Declaration of Helsinki, and the protocols applied in this study were approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University, China.

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Disclosure

The authors declare no conflicts of interest in this work.

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