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Clinical and Molecular Characteristics of Emerging Hypervirulent *Klebsiella pneumoniae* Bloodstream Infections in Mainland China

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Recently, the newly emerged hypervirulent *Klebsiella pneumoniae* strain (hvKP) has caused great concern globally, but the clinical features and molecular characteristics of bacteremia caused by hvKP are rarely reported in mainland China. Seventy patients with *K. pneumoniae* bacteremia were investigated to study the clinical features of hvKP infection from 2008 till 2012 in Beijing Chao-Yang Hospital. The molecular characteristics of the hvKP strains were also studied using PCR, multilocus sequence typing, and pulsed-field gel electrophoresis (PFGE) methods. hvKP was identified in 31.4% of the patients with *K. pneumoniae* bacteremia, which displayed 4 serotypes (K1, K2, K20, and K57). Patients with hvKP infection tended to have no underlying diseases compared to those with classic *K. pneumoniae* (cKP). More hvKP-positive patients (95.5%) had community-acquired infection than did cKP-infected patients (35.4%) ($P < 0.001$). The 30-day mortality rate was lower in hvKP-infected patients than in cKP-infected patients (4.5% compared to 16.7%). Resistance to tested antimicrobials was significantly greater in cKP- than in hvKP-infected patients. Two extended-spectrum-beta-lactamase (ESBL)-producing hvKP strains were found. Seven novel sequence types (STs) and 4 new alleles of *K. pneumoniae* were revealed. A strong correlation was found between two STs (ST23, ST1265) and the K1 serotype. The hvKP isolates ($n = 22$) had 14 different PFGE patterns, and among them 10 K1 isolates shared similar PFGE patterns. The emerging hvKP strain was prevalent in patients with severe community-acquired infections in healthy individuals in China. Identification of ESBL-producing hvKP strains in hvKP-infected patients will facilitate clinical management of hvKP infection.

The classic *Klebsiella pneumoniae* strain (cKP) is one of the major pathogens causing hospital-acquired (HA) infection, particularly in immunocompromised patients. It is the second-commonest cause of Gram-negative bacteremia (1, 2). A new hypervirulent variant of *K. pneumoniae* (hvKP) was identified 20 years ago in Taiwan, and it has been reported frequently in Asia ever since (3, 4, 5). This new variant is different from the classic strain in that the appearance of colonies grown on an agar plate is hypermucoviscous. Therefore, it is also called hypermucoviscous *K. pneumoniae*. In contrast to the cKP strain, the hypervirulent variant is not only able to cause nosocomial infection in immunocompromised patients, but, more importantly, it is able to cause life-threatening community-acquired (CA) infection in healthy individuals, which has caused a great concern worldwide (6). In addition, this new strain also has the ability to spread from the primary site of infection to other parts of the body, which is an unusual feature for enteric Gram-negative bacilli in nonimmunocompromised hosts (7). Recently, hvKP infection is increasingly reported from Europe (8), South America (9), Australia (10), and North America (11). Although such cases have been extensively described worldwide, particularly in Asian patients, to date, there have been few reports on the clinical features of hvKP in mainland China. In this report, we investigate the clinical features and molecular characteristics of *K. pneumoniae* bacteremia in 70 patients over a 3-year period.

MATERIALS AND METHODS

Hospital setting and case definition. The Beijing Chao-Yang Hospital is a 1,500-bed tertiary care teaching hospital with 6 intensive-care-unit (ICU) wards and approximately 25,000 hospital admissions per year. We conducted a retrospective cohort study of patients treated in the hospital for *K. pneumoniae* bloodstream infections between June 2008 and April 2012. The patients were identified according to the records from the clinical microbiology department. Patient data were obtained from medical records, including demographic characteristics, clinical features, duration of hospital stay, antimicrobial therapy administration, mechanical ventilation, and use of invasive devices. Each case was differentiated between CA infection and HA infection. The CA bloodstream infection was defined as detection of *K. pneumoniae* in blood cultures taken within 48 h after admission. Conversely, HA bloodstream infection was defined as development of bacteremia >48 h into inpatient admission, including infections related to the presence of medical devices.

Study samples. Isolates from 70 patients who were diagnosed to have had *K. pneumoniae* bloodstream infection were available for analysis and were identified as *K. pneumoniae* by using conventional microbiologic methods and further confirmed as *K. pneumoniae* by 16S rRNA sequencing. The "string test" was performed on all isolates. The string test is positive when a bacteriology inoculation loop is able to generate a viscous string of >5 mm in length by stretching bacterial colonies on an agar plate. *K. pneumoniae* strains with a positive string test were designated hvKP.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing for isolated *K. pneumonia* was carried out by means of a Kirby-Bauer disk diffusion test on Mueller-Hinton agar, which was performed and interpreted according to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI), USA (12). A panel of 23 antimi-

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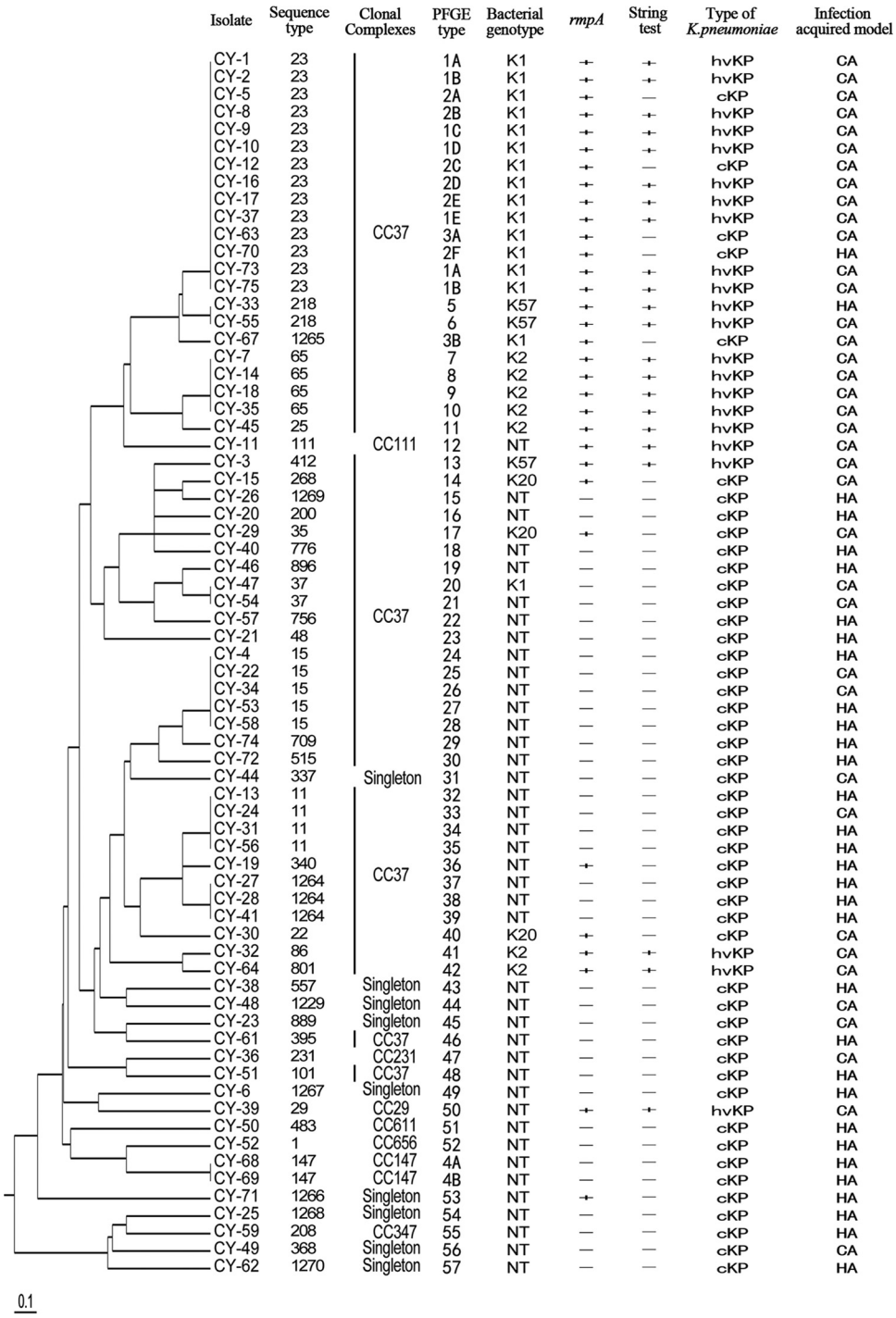

FIG 1 Graphic summary of molecular characteristic of 70 *K. pneumoniae* isolates.

crobial agents was tested, including piperacillin, ampicillin-sulbactam, amoxicillin-clavulanic acid, ticarcillin-clavulanate, cefazolin, piperacillin-tazobactam, cefoxitin, cefuroxime, ceftriaxone, ceftazidime, cefotaxime, cefepime, aztreonam, imipenem, meropenem, ciprofloxacin, levofloxacin, tetracycline, minocycline, gentamicin, tobramycin, amikacin, and trimethoprim-sulfamethoxazole. All of the *K. pneumoniae* isolates were screened and confirmed by a double-disk synergy test for produced extended-spectrum beta-lactamases (ESBLs). *K. pneumoniae* ATCC 700603 and *Staphylococcus aureus* ATCC 25923 were included in each experiment as controls.

PCR for capsular polysaccharide synthesis (CPS) genotyping. K1, K2, K5, K20, K54, and K57 serotypes were identified by detection of K serotype-specific *wzy* and *wzx* alleles using the PCR method as previously described (13). The PCR products were visualized by 1% agarose gel electrophoresis and sequenced commercially. The BLAST program at http://www.ncbi.nlm.nih.gov was used for final serotype identification.

TABLE 1 Different serotypes of *K. pneumoniae* bacteremic isolates from community-acquired or hospital-acquired infections

Serotype	No. (%) of isolates		*P* value (CA vs HA)
	Community-acquired infections ($n = 38$)	Hospital-acquired infections ($n = 32$)	
K1	15 (39.5)	1 (3.1)	<0.001[b]
K2	7 (18.4)	0	0.013[b]
K20	3 (7.9)	0	0.245
K57	2 (5.3)	1 (3.1)	1.000
K5	0	0	
K54	0	0	
NT[a]	11 (28.9)	30 (93.8)	<0.001[b]

[a] NT, not belonging to the K1, K2, K5, K20, K54, or K57 serotype.
[b] A *P* value of ≤0.05 was considered to be statistically significant.

MLST. Multilocus sequence typing (MLST) was performed according to the protocol described on the *K. pneumonia* MLST website (www.pasteur.fr/mlst) (14). Internal fragments of seven housekeeping genes for *K. pneumonia* (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) were amplified, sequenced, and analyzed. Alleles and sequence types (STs) were determined according to the MLST database (www.pasteur.fr/mlst/Kpneumoniae.html). Alleles and STs that had not been previously described were submitted to the curator of the database and were assigned as new designations. The program eBURST version 3.0 software (based upon related sequence types) was used to analyze the clustering of related STs, which were classified as clonal complexes (CCs) (15). Clonal complexes were defined as groups of two or more independent isolates that shared identical alleles at six or more loci. Each complex was named after the putative founder ST.

PFGE. Pulsed-field gel electrophoresis (PFGE) typing was conducted as previously described (16). Whole-cell genomic DNA representing each isolate was digested with the restriction enzyme XbaI (TaKaRa Biotechnology, Dalian, China) and separated by electrophoresis through 1% pulsed-field certified agarose (Bio-Rad, Richmond, CA, USA) by using a CHEF-Mapper (Bio-Rad). Electrophoretic switch times of 5 to 35 s were used with a 6-V/cm current and a switch angle of 120° under a constant temperature of 14°C. PFGE patterns were interpreted by using the criteria proposed by Tenover et al. (17).

Statistical analysis. Data were analyzed using the statistical package SPSS for windows version 17.0. For categorical data, different groups were compared using the chi-square test to analyze the quantitative variables. A *P* value of ≤0.05 was considered to be statistically significant. All susceptibility data were analyzed using WHONET, version 5.6.

TABLE 3 Demographic and clinical characteristics of patients with *K. pneumoniae* bacteremia

Characteristic	No. (%) of patients			*P* value (hvKP vs cKP)
	Total ($n = 70$)	With hvKP infection ($n = 22$)	With cKP infection ($n = 48$)	
Gender				
Male	50 (71.4)	20 (90.9)	30 (62.5)	0.021[a]
Female	20 (28.6)	2 (9.1)	18 (37.5)	0.021[a]
Age				
>60 yrs	38 (54.2)	9 (40.9)	29 (60.4)	0.196
Acquired infection model				
Community-acquired infection	38 (54.2)	21 (95.5)	17 (35.4)	<0.001[a]
Hospital-acquired infection	32 (45.7)	1 (4.5)	31 (64.6)	<0.001[a]
Underlying diseases				
None	11 (15.7)	9 (40.9)	2 (4.17)	<0.001[a]
Diabetes mellitus	16 (22.9)	6 (27.3)	10 (20.8)	0.760
Biliary tract diseases	9 (12.9)	3 (13.6)	6 (12.5)	1.000
Heart disease	4 (5.7)	0 (0)	4 (8.3)	0.301
Central nervous system diseases	5 (7.1)	0 (0)	5 (10.4)	0.173
Alcoholic cirrhosis	3 (4.3)	1 (4.5)	2 (4.17)	1.000
Hematologic diseases	3 (4.3)	0 (0)	3 (6.3)	0.547
Pulmonary infection	5 (5.7)	1 (4.5)	4 (8.3)	1.000
Cancer	14 (20)	1 (4.5)	13 (27.1)	0.050[a]
Other	9 (12.9)	1 (4.5)	8 (16.7)	0.255
Use of invasive devices	36 (51.4)	5 (22.7)	31 (64.6)	0.001[a]
Metastatic spread	2 (2.9)	2 (9.1)	0 (0)	0.096
Outcome				
Discharged	53 (75.7)	16 (72.7)	37 (77.1)	0.767
Died	9 (14.5)	1 (4.5)	8 (16.7)	0.255
Lost to follow up	8 (11.4)	5 (22.7)	3 (4.9)	0.098

[a] A *P* value of ≤0.05 was considered to be statistically significant.

Ethics statement. Permission for using the information in the medical records of the patients and the *K. pneumoniae* isolates for research purposes was granted by the ethical committee of Beijing Chao-Yang Hospital.

RESULTS

hvKP identification. The string test was used to differentiate between hvKP and cKP among 70 *K. pneumoniae* isolates. Twenty-

TABLE 2 Difference between hypervirulent and classic *K. pneumoniae*-induced infections

Infection type	No. of hvKP-induced infections ($n = 22$)					No. of cKP-induced infections ($n = 48$)					*P* value (hvKP vs cKP)
	K1	K2	K20	K57	NT[a]	K1	K2	K20	K57	NT[a]	
Liver abscess	6	4	0	0	0	0	0	0	0	0	<0.001[b]
Bacteremia	1	1	0	2	0	2	0	1	0	6	0.195
Catheter-related bloodstream infection	0	0	0	0	0	1	0	0	0	3	0.301
Hospital-acquired pneumoniae	0	0	0	0	0	0	0	0	0	13	0.004[b]
Community-acquired pneumoniae	1	0	0	0	0	2	0	0	0	1	1.000
Ventilator-associated pneumoniae	0	0	0	0	0	0	0	0	0	1	1.000
Biliary tract infection	0	0	0	1	2	1	0	1	0	7	0.741
Urological infections	2	1	0	0	0	0	0	0	0	5	0.700
Abdominal infection	0	0	0	0	0	0	0	0	0	1	1.000
Peritonitis	0	1	0	0	0	0	0	0	0	0	0.314
Acute enteritis	0	0	0	0	0	0	0	0	0	1	1.000
Pyelonephritis	0	0	0	0	0	0	0	1	0	0	1.000
Perianal abscess	0	0	0	0	0	0	0	0	0	1	1.000

[a] NT, not belonging to the K1, K2, K5, K20, K54, or K57 serotype.
[b] A *P* value of ≤0.05 was considered to be statistically significant.

TABLE 4 Differences of the antimicrobial susceptibility between hypervirulent and classic *K. pneumoniae*

Drug	No. (%) of patients susceptible			
	Total ($n = 70$)	hvKP infected ($n = 22$)	cKP infected ($n = 48$)	*P* value (hvKP vs cKP)
Piperacillin	29 (41.4)	11 (50.0)	18 (37.5)	0.434
Ampicillin-sulbactam	36 (51.4)	16 (72.7)	20 (41.7)	0.021[a]
Amoxicillin-clavulanic acid	48 (68.6)	20 (90.9)	28 (58.3)	0.011[a]
Ticarcillin-clavulanate	43 (61.4)	20 (90.9)	23 (47.9)	0.001[a]
Piperacillin-tazobactam	55 (78.6)	21 (95.5)	34 (70.8)	0.026[a]
Cefazolin	25 (35.7)	9 (40.9)	16 (33.3)	0.597
Cefoxitin	47 (67.1)	18 (81.8)	29 (60.4)	0.102
Cefuroxime	37 (52.9)	18 (81.8)	19 (39.6)	0.002[a]
Ceftazidime	45 (64.2)	20 (90.9)	25 (52.1)	0.003[a]
Ceftriaxone	45 (64.2)	20 (90.9)	25 (52.1)	0.003[a]
Cefotaxime	49 (70.0)	20 (90.9)	29 (60.4)	0.011[a]
Cefepime	61 (87.1)	22 (100)	39 (81.3)	0.049[a]
Aztreonam	49 (70.0)	21 (95.5)	28 (58.3)	0.002[a]
Meropenem	69 (98.6)	22 (100)	47 (97.9)	1.000
Imipenem	69 (98.6)	22 (100)	47 (97.9)	1.000
Ciprofloxacin	44 (62.9)	20 (90.9)	24 (50.0)	0.001[a]
Levofloxacin	46 (65.7)	20 (90.9)	26 (54.2)	0.003[a]
Tetracycline	44 (62.9)	19 (86.4)	25 (52.1)	0.007[a]
Minocycline	39 (55.7)	16 (72.7)	23 (47.9)	0.071
Gentamicin	55 (78.6)	22 (100)	33 (68.8)	0.002[a]
Tobramycin	54 (77.1)	22 (100)	32 (66.7)	0.001[a]
Amikacin	63 (90.0)	22 (100)	41 (85.4)	0.089
Trimethoprim-sulfamethoxazole	47 (67.1)	20 (90.9)	27 (56.3)	0.005[a]
ESBLs	26 (37.1)	2 (9.09)	24 (50.0)	0.001[a]

[a] A *P* value of ≤0.05 was considered to be statistically significant.

two isolates were found to be string test positive (Fig. 1, "String test" column).

Serotype identification. To identify the serotypes of *K. pneumoniae*, PCR was performed on 70 *K. pneumoniae* isolates using primers for K1, K2, K5, K20, K54, and K57 serotypes as described in Materials and Methods. A summary of the results is shown in Fig. 1. A total of 29 (41.4%) isolates tested positive for K1, K2, K20, or K57 serotypes. Sixteen (55.2%) of them displayed the K1 serotype, 7 (24.1%) isolates showed the K2 serotype, while 3 (10.3%) isolates were K20 serotype positive and 3 (10.3%) were K57 serotype positive. However, no K5 and K54 serotypes were found.

Relationship between serotypes and site of infections. K1 and K2 serotypes were more commonly found than other serotypes in patients with community-acquired infections ($P < 0.001$ and $P = 0.013$, respectively) (Table 1). We also found that patients with primary liver abscess more commonly tested positive for K1 and K2 (Table 2). In addition, K1 and K2 serotypes were also correlated with primary bacteremia, urological infection, and community-acquired pneumonia. In contrast, more classic *K. pneumoniae*-positive patients than hvKP-positive patients presented with hospital-acquired pneumonia ($P = 0.004$) (Table 2).

Comparison of demographic and clinical characteristics of patients infected with hvKP and cKP. Among the 70 *K. pneumonia* isolates analyzed, 22 (31.4%) of them were found to be hvKP and 48 (68.6%) of them were cKP. Overall, there was a trend of more male patients with *K. pneumonia* bloodstream infections. But the male predominance was more significant in hvKP-infected patients (90.9%) than in cKP-infected patients (62.5%, $P = 0.021$). There was no difference in age distribution between the two groups. More hvKP-positive patients (21/22, 95.5%) than cKP-infected patients (17/48, 35.4%) had CA infections ($P < 0.001$) (Table 3). It was also noted that 40.9% (9/22) of the hvKP-infected patients had no reported underlying diseases at the time of infection. In contrast, 95.8% (46/48) of the cKP-infected patients had one or more underlying illnesses. The 30-day mortality rate was lower in the hvKP group (4.5%) than in the cKP group (Table 3).

Antimicrobial susceptibility of *K. pneumoniae* isolates. Notably, the prevalence of cKP strains exhibiting resistance to the tested antimicrobials was higher than that of the hvKP strains (Table 4). Twenty-six (26/70, 37.1%) of the isolates produced ESBLs. The percentage of ESBL-producing cKP strains was significantly higher than that of ESBL-producing hvKP strains (50% compared to 9.09%, $P = 0.001$). Among all of the isolates, one of them (1/70, 1.4%) was resistant to carbapenem and produced *K. pneumoniae* carbapenemase 2 (KPC-2).

MLST and PFGE analysis of *K. pneumoniae* isolates and identification of clonal complexes. MLST analysis identified 42 STs among the 70 *K. pneumoniae* isolates, including 7 newly identified STs (ST1264, ST1265, ST1266, ST1267, ST1268, ST1269, ST1270) and 4 novel alleles (*rpoB88*, *tonB225*, *tonB226*, *pgi107*), as shown in Fig. 1. The most prevalent ST in *K. pneumoniae* isolates was ST23 ($n = 14$; 20%), followed by ST15 ($n = 5$; 7.1%), ST65 ($n = 4$; 5.7%), and ST11 ($n = 4$; 5.7%). These 4 STs accounted for 38.6% (27/70) of the total *K. pneumoniae* isolates. Further, 34 STs were represented by 34 isolates, respectively. Clustering analysis by eBURST showed that these 42 STs could be grouped into 8 clonal complexes (CCs) and 9 singletons with the stringent default definition of sharing the same sequences at at least 6 of 7 loci. CC37 was found to be the predominant type, because 57.1% (24/42) of the isolates belonged to this group, as shown in Fig. 2.

We found a strong correlation between ST23 and the K1 serotype. Interestingly, one of the novel STs, ST1265, was also associated with the K1 serotype. This new sequence type shares 6 alleles with ST23, indicating a new addition to the K1 serotype.

PFGE analysis showed that the 70 isolates revealed 57 different PFGE profiles. The banding patterns were designated PFGE types 1 to 57 (Fig. 1, "PFGE type" column), and a gel image is shown in Fig. 3. In addition, cKP isolates ($n = 48$) showed 43 different PFGE patterns, suggesting a polyclonal origin. The hvKP isolates ($n = 22$) had 14 different PFGE patterns, also indicating a polyclonal origin. However, among the 10 K1 serotype isolates in the hvKP group, we found two groups of isolates ($n = 7$ and $n = 3$, respectively) that shared similar PFGE patterns, suggesting a clonal origin.

Association between serotype and presence of the *rmpA* gene. *rmpA*-carrying strains are often associated with the hypermucoviscous phenotype (18). As summarized in Fig. 1, we found that all of the string test-positive isolates were also *rmpA* positive. But 10 *rmpA*-positive strains were string test negative. Two *rmpA* and string test-positive isolates were K1, K2, K5, K20, K54, and K57 serotype negative.

DISCUSSION

In this study, we identified the hypervirulent *K. pneumoniae* in 31.4% (22/70) of patients with *K. pneumoniae* bacteremia. In addition, we found that the hvKP was more commonly encountered in community-acquired infections, including liver abscess, urological infection, and pneumonia complicated with bacteremia,

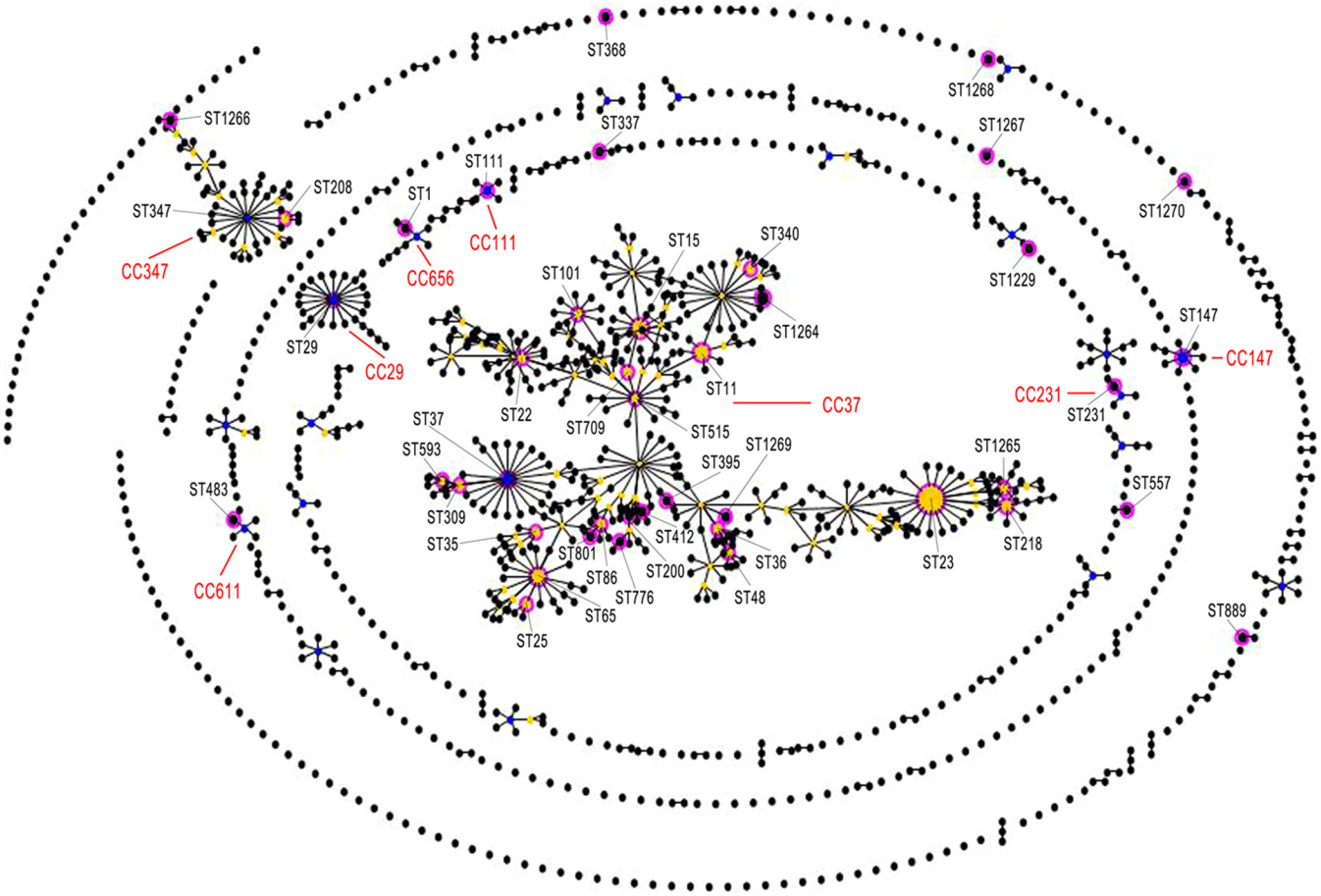

FIG 2 Comparative eBURST analyses. The population snapshot shows the clonal assignment of the STs presented in this study compared to that of the STs in the entire *K. pneumonia* MLST isolate database. Each black dot represents one ST in the database. The blue dots indicate individual founders, while yellow spots are subfounders. The pink circle highlights the matching ST type in this study with the database. The different clonal complexes are shown in red.

especially among patients without known underlying diseases. This suggests that this strain may play an important role in community-acquired infections. Furthermore, we identified seven novel STs and four new alleles of *K. pneumoniae*, indicating the uniqueness of this bacterial population in China.

To date, hvKP infection has been reported mainly in the Asian population. Although recently there was a similar study reported from China (19), unlike the present report, there was little information on the characteristics of hvKP bloodstream infection. More importantly, we found two ESBL-producing hvKP strains. To date, antimicrobial resistance in hvKP strains has been rarely reported outside mainland China. Documenting the hvKP isolates would be useful not only for clinical doctors in Asia but also for clinicians in the Western countries where hvKP infection is being reported more and more frequently.

K. pneumoniae is encapsulated, with at least 78 capsular polysaccharide serotypes existing to date (20). The capsule is an important virulence factor, and some capsular serotypes, particularly K1, K2, K5, K16, K20, K54, K57, and KN1, are recognized as hypervirulent variants of *K. pneumoniae* (20, 21, 22). In this study, we identified 4 hvKP serotypes (K1, K2, K20, and K57), which agrees with previous reports from Taiwan (20, 23). However,

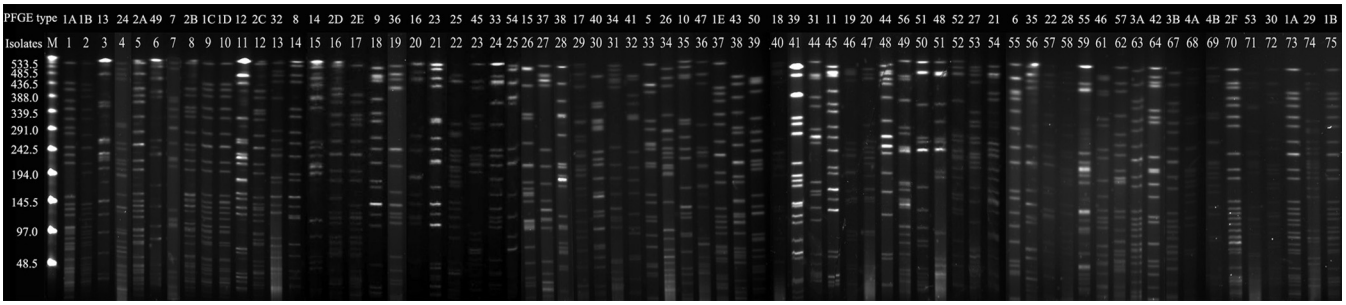

FIG 3 Gel image of PFGE result. Genomic DNA was digested using XbaI enzyme and subjected to pulsed-field gel electrophoresis.

none of our isolates was positive for K5 or K54, which has been reported previously (24, 25). In addition, we also noted that the K1 serotype was correlated with not only liver abscess but also urological infection, community-acquired pneumonia, and primary bloodstream infection, which agrees with previous studies (26, 27). Most importantly, we found that hvKP was able to cause serious infection in nonimmunocompromised hosts in the current study, because 60% (6/10) of the liver abscess patients had no underlying diseases.

Previously, it was documented that the ST23 strain was dominant in liver abscesses and clonally related in Taiwan patients (28, 29). However, some studies have shown that *K. pneumoniae*-related liver abscesses are not caused by a clonally spread strain (30). In the current study, we found that the six strains of ST23 K1 isolates that were associated with primary liver abscess also shared similar PFGE patterns, suggesting that they are clonally related, which supports previous findings from Taiwan (30). We also noticed that several isolates ($n = 4$) from patients with liver abscess had different PFGE patterns, demonstrating that genetic diversity also existed in liver abscess-causing hvKP (see Fig. 1).

In summary, we have shown clinical and molecular differences between hypervirulent *K. pneumonia* infections and classic *K. pneumonia* infections. We hope our study will draw attention from clinicians, which may lead to prompt recognition and successful management of hvKP infections.

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