Original Article

Characterization of a multidrug-resistant *Klebsiella pneumoniae* ST3330 clone responsible for a nosocomial outbreak in a neonatal intensive care unit

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Background: The incidence of *Klebsiella pneumonia* (Kp), which has often been found to produce, extended spectrum beta-lactamase (ESBL), is rising rapidly and poses a serious risk to neonates. To date, the mechanisms related to the spread of ESBL-Kp have not been fully elucidated. This study aimed to investigate the phenotypes, genotypes, and genetic relatedness of ESBL-KP that caused an outbreak of sepsis among neonates in an intensive care unit of a Beijing hospital.

Methods: Between April 2016 and May 2018, 21 non-repetitive clinical ESBL-Kp isolates were collected from a neonatal intensive care unit (NICU) in Beijing, China and were retrospectively analyzed. Pulsed-field gel electrophoresis (PFGE) was used to analyze genetic relatedness, a VITEK 2 AST test kit was used to test antimicrobial susceptibility, sequence type (ST) was analyzed through multilocus sequence typing (MLST), and resistance genes were identified by PCR. Virulence gene profiles, biofilm formation assay, and serum killing assay were used for virulence-associated determinants.

Results: All strains expressed the same antibiotype, combining ESBL production, third generation cephalosporins resistance and carbapenems sensitive. Sixteen of them produced β-lactamases (CTX-M-3 and TEM-1B), while others possessed CTX-M-15, CTX-M-24, CTX-M-66, TEM-1C, SHV-26, SHV-172, and OXA-1. PFGE confirmed 5 types (A, B, C, D and E) and MLST identified a ST3330 clone (16 strains), a ST2791 clone (2 strains), a ST37 clone (1 strain), a ST34 clone (1 strain), and a ST2740 clone (1 strain). PFGE type A strains, which belong to ST3330, were identified as the main pathogens involved in the outbreak. All isolates contained virulence genes *iutA* and *mrk*. PFGE type A carried both *mrk* (type 3 fimbriae, biofilm formation) and *fimH* (type 1 fimbriae), and other STs possessed *mrk*. Isolates belonging to the endemic ST3330 lineage produced more biofilm than other ST isolates (median OD_{590} 1.829 *vs.* 0.2280, respectively; P<0.0001). All five PFGE types isolates showed serum high sensitivity (grade 1).

Conclusions: The dissemination and outbreak of ESBL-producing *K. pneumoniae* in this study seemed to be clonal, and the outbreak was mainly caused by ST3330 *K. pneumoniae*. The detection of genes (*mrk* and *fimH*) belonging to the biofilm formation may partly explain the epidemic strain has high colonization and diffusion potential.

Keywords: Extended-spectrum-beta-lactamase (ESBL); *Klebsiella pneumoniae* (Kp); sequence type; neonatal intensive care unit (NICU); virulence

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Introduction

Neonatal septicemia, which is mainly hospital-acquired, especially in neonatal intensive care units (NICUs), is becoming more challenging for practitioners to control (1,2). Septicemia and pneumonia are the main symptoms of neonatal sepsis (3).

Klebsiella pneumoniae (Kp) is a common cause of blood infections. Multidrug-resistant (MDR) strains of Kp, notably extended-spectrum-beta-lactamase (ESBL)-producing strains, are regularly involved in nosocomial outbreaks (3-7). These strains are able to acquire additional resistance mechanisms and virulence factors via plasmids in particular, which enables them to disseminate across healthcare units and increase their invasive power in the human body (5,8). Transmission can occur either from the mother to child at birth, or acquired during nursery by person-to-person transmission, via the hands of the nursing staff and the contaminated equipment, food or the environment. Rigorous nosocomial infection prevention measures can be developed to control *klebsiella pneumoniae* in NICU. Carbapenems are the last antibiotics to control *klebsiella pneumoniae* infection. Treatment of *klebsiella pneumoniae* infection in children can draw on the experience of treatment in adults, but drug dosage needs to be adjusted. In neonates, ESBL-KP infections, which carry high morbidity and mortality (3), have become a burden and a threat to public health (5,9).

It is crucial that the source of emerging multi-drug bacteria is identified. Therefore, it is vital that strain-level discrimination of the involved pathogens is carried out and the bacteria at the core of any outbreak is characterized (3). This study aimed to conduct an examination of the phenotypes, genotypes, and genetic relatedness of ESBL-KP that caused an outbreak of sepsis among neonates in an intensive care unit of a Beijing hospital. We present the following article in accordance with the STROBE reporting checklist (available at http://dx.doi.org/10.21037/apm-20-958).

Methods

Patients' clinical and epidemiological features

Between April 2016 and May 2018, 21 strains (numbered 1–21) were collected from 21 newborns aged 1–33 days who were hospitalized in the NICU in a Beijing Hospital (*Table 1*). All 21 strains were isolated from the bloodstreams of different patients. A descriptive study based on electronic records was conducted to describe demographic data and clinical events related to ESBL-Kp infections (1,5). As the clinical samples were routinely taken for bacterial testing purposes in public hospitals, and the data were analyzed anonymously, ethical approval was not required (3).

Phenotypic characterization

Blood cultures were monitored with the Bactec Microbial Detection System (Bactec 9050, Becton-Dickinson Company, 1 Becton Drive, Franklin Lakes, New Jersey). Bacterial identification was performed by MALDI-ToF mass spectrometry (MALDI Biotyper; Bruker Daltonics, France). An automated VITEK 2 compact system (BioMérieux, Inc.) was employed to test antibiotic susceptibility. The measurement of MICs of antimicrobial agents of common clinical usage was conducted using a VITEK 2 AST-GN09 test Kit (BioMérieux, Inc.) in line with the protocol of the manufacturer. The interpretation of the results of all susceptibility testing was guided by the Clinical and Laboratory Standards Institute (CLSI) guidelines (3,10,11). In antimicrobial susceptibility testing, we chose *E. coli* ATCC 25922 as the control strain (12). Phenotypic ESBL detection was performed according to the combined antibiotic disks method (between third-generation cephalosporins and clavulanic acid) and by observing ceftriaxone susceptibility (5). Patient demographics and susceptibility data were analyzed with WHONET software (version 5.6).

Molecular detection of resistance genes

Polymerase chain reaction (PCR) assays were performed to detect Drug resistance genes, including carbapenemases (bla_{KPC}, bla_{AIM}, bla_{GIM}, bla_{SIM}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{OXA-48}), common extended-spectrum-beta-lactamase (ESBL) genes (bla_{CTX-M}, bla_{TEM}, bla_{OXA}, and bla_{SHV}). The PCR primers previously described (5,11-13) were listed in *Table 2*. The cycling parameters were as follows: 94 ℃ for 10 min followed by 36 cycles of 94 ℃ for 30 s, 52 ℃ for 40 s and 72 ℃ for 50 s, with a final extension at 72 ℃ for 5 min. Electrophoresis with a 1.5% agarose gel was carried out to screen the positive amplicons, which were then sequenced. Analysis and comparisons were then made between the DNA sequences obtained and those available on the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) via BLAST searches (13).

Table 1 characterization of 21 *K. pneumoniae* strains isolated from 21 newborns

Strain	Gender	Age (days)	Weight (kg)	Gestational age (days)	Delivery	Pregnancy complications	Ventilation	Specimen	Antibiotics	Course (days)	Length of stay (days)	Outcome	Date of isolation
1	M	9	2.37	253	CS	Negative	Yes	Blood	Meropenem	14	20	A	04/26/2016
2	F	5	1.65	224	CS	Positive	Yes	Blood	Meropenem	1	5	B	08/10/2016
3	F	16	1.68	215	CS	Positive	Yes	Blood	Sulperazone	14	24	A	12/06/2016
4	F	5	1.66	231	NVD	Negative	Yes	Blood	Meropenem	14	26	A	02/03/2017
5	M	6	1.49	219	NVD	Negative	Yes	Blood	Sulperazone	5	30	A	02/05/2017
6	M	19	1.35	203	CS	Positive	Yes	Blood	Meropenem	14	51	A	02/06/2017
7	M	4	1.89	248	CS	Positive	Yes	Blood	Meropenem	14	17	A	07/03/2017
8	F	19	1.92	229	CS	Positive	Yes	Blood	Meropenem	5	28	A	10/26/2017
9	F	1	1.04	205	CS	Positive	Yes	Blood	Meropenem	14	37	A	10/30/2017
10	F	26	1.25	203	CS	Positive	Yes	Blood	Meropenem	7	41	A	12/19/2017
11	M	23	0.87	199	CS	Positive	Yes	Blood	Meropenem	1	23	D	12/21/2017
12	M	23	0.94	199	CS	Positive	Yes	Blood	Meropenem	7	57	A	12/21/2017
13	F	7	0.82	174	NVD	Negative	Yes	Blood	Meropenem	14	24	A	01/08/2018
14	M	20	1.06	219	CS	Positive	Yes	Blood	Meropenem	14	55	A	01/30/2018
15	F	6	1.45	212	CS	Positive	Yes	Blood	Meropenem	1	5	B	03/23/2018
16	M	1	1.29	214	CS	Positive	Yes	Blood	Meropenem	3	4	A	04/01/2018
17	F	33	1.07	203	CS	Negative	Yes	Blood	Meropenem	10	58	A	04/17/2018
18	M	9	1.2	207	CS	Positive	Yes	Blood	Meropenem	10	56	A	04/18/2018
19	M	12	1.52	216	CS	Positive	Yes	Blood	Meropenem	14	30	A	04/20/2018
20	F	10	0.86	183	NVD	Positive	Yes	Blood	Sulperazone	11	68	A	05/07/2018
21	F	23	0.86	178	NVD	Positive	Yes	Blood	Meropenem	10	54	C	05/28/2018

NVD, normal vaginal delicery; CS, cesarean section; A, cured; B, transferred to surgery; C, refused further treatment; D, died of severe infection.

Table 2 Primers used in this study

Name	Pair sequence (5′→3′)	Amplicon sizes (bp)
KPC	CGTCTAGTTCTGCTGTCTTG and CTTGTCATCCTTGTTAGGCG	798
AIM	CTGAAGGTGTACGGAAACAC and GTTCGGCCACCTCGAATTG	322
GIM	TCGACACACCTTGGTCTGAA and AACTTCCAACTTTGCCATGC	477
SIM	GCTTGTCTTCGCTTGCTAACG and CGTTCGGCTGGATTGATTTG	699
NDM	GGTTTGGCGATCTGGTTTTC and CGGAATGGCTCATCACGATC	621
IMP	GGAATAGAGTGGCTTAAYTCTC and GGTTTAAYAAAACAACCACC	232
VIM	GATGGTGTTTGGTCGCATA and CGAATGCGCAGCACCAG	390
CTX-M-I gp	CCCATGGTTAAAAAATCACTGC and CAGCGCTTTTGCCGTCTAAG	346
TEM	ATTCTTGAAGACGAAAGGGC and ACGCTCAGTGGAACGAAAAC	325
OXA	GCGTGGTTAAGGATGAACAC and CATCAAGTTCAACCCAACCG	438
SHV	GGGTTATTCTTATTTGTCGC and TTAGCGTTGCCAGTGCTC	477
entB	CGCCCAGCCGAAAGAGCAGA and CATCGGCACCGAATCCAGAC	508
ybtS	CAAAAATGGGCGGTGGATTC and CCTGACGGAACATAAACGAGCG	484
kfu	ATAGTAGGCGAGCACCGAGA and AGAACCTTCCTCGCTGAACA	797
iutA	ACCTGGGTTATCGAAAACGC and GATGTCATAGCCTGATTGC	1,115
rmpA	ACTGGGCTACCTCTGCTTCA and CTTGCATGAGCCATCTTTCA	434
mrk	TAT(T/C)G(G/T)CTTAATGGCGCTGG and TAATCGTACGTCAGGTTAAAG A(C/T)C	920
fimH	GCTCTGGCCGATAC(C/T)AC(C/G)ACGG and GC(G/A)(A/T)A(G/A)TAACG(T/C)GCCTGG AACGG	423

Detection of virulence-associated genes

Genes encoding specific virulence factors involved in iron acquisition systems (*entB*, *ybtS*, *kfu*, and *iutA*), hypermucoviscous phenotype (*rmpA*), and fimbrial adhesions (*mrk* and *fimH*) were researched using a multiplex PCR, as previously described (5,14,15). The PCR primers were listed in *Table 2*. The cycling parameters were as follows: 94 ℃ for 5 min followed by 35 cycles of 94 ℃ for 1 min, 50 ℃ for 30 s and 72 ℃ for 50 s, with a final extension at 72 ℃ for 5 min. The results were analyzed by BLAST program.

Pulsed-field gel electrophoresis (PFGE) analyses

PFGE with *Xba*I was conducted for each clinical Kp isolate, as previously described (11,12,16,17). A lambda ladder pulsed-field gel marker was used as a size standard for PFGE. The identification and interpretation of the results were conducted according to the Tenover criteria. Isolates with genetic similarity of ≥95% were judged to be the same strain (PFGE type) (3).

Multilocus sequence typing (MLST)

Multilocus sequence typing was performed by PCR amplifying and sequencing seven housekeeping genes according to protocols provided on the MLST website for *K. pneumoniae* (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) (11,16,17). Sequences of seven housekeeping genes were obtained for isolates from all PFGE types. Alleles and STs were assigned accordingly. Sequences of any alleles that were not on the database were submitted to the curator and new allele numbers were obtained. Strains with a difference in two or more alleles were considered to be unrelated. A DNA analyzer (3730xl; Applied Biosystems, Life Technologies, Foster City, CA, USA) was employed to sequence each amplification product (12).

Biofilm formation assay

The standard static biofilm assay described by Naparstek *et al.* (18) was performed on the isolates. Briefly, the

isolates underwent incubation at 37 ℃ for 18 h, after which 200 μL of broth was transferred to each well of a sterile phenotypic microplate (96 cells). Next, the addition of 10 μL of bacterial suspension from the incubated isolates at an opacity 0.5 McFarland was made to the wells. The microplate was incubated at 37 ℃ for 24 h and washed with saline 3 times to keep the plate completely dry. After that, 200 μL of 0.1% crystal violet was added to the wells for 20 min and the microplate was washed 3 times with saline and dried. Finally, the addition of 200 μL of 95% ethanol was made to each well, and the plate was read with ELISA Plate Reader at 590 nm. Biofilm formation is indicated by high absorption (18-20). All experiments were repeated in triplicate. A well without bacterial suspension served as a negative control.

Serum killing assay

The blood samples of 10 healthy adults were collected for the serum killing assay. An inoculum of 25 μL (adjusted to 10^6 colony forming units/mL) prepared from the mid-log phase was diluted by 0.9% saline and added into a 10×75 mm Falcon polypropylene tube (BD Biosciences, Franklin Lakes, New Jersey) containing 75 μL of pooled human sera. The tubes underwent agitation for 0, 60, 120, or 180 min. After being exposed to serum, the number of viable bacteria were determined by removing an aliquot of each bacterial suspension at the designated time point, adding broth to dilute it 10-fold, plating it on Mueller-Hinton agar, and performing the assay, as described immediately below. Each strain was tested at least 3 times (14). The results were expressed as percentage of inoculation and the responses in relation to viable counts were graded from 1 to 6, as previously described. All isolates were classified as follows: highly sensitive (grades 1 or 2), intermediately sensitive (grades 3 or 4), or resistant (grades 5 or 6) (21).

Statistical analysis

Biofilm formation in the ST3330 group was compared with that in the other STs group, and differences were analyzed by unpaired *t*-test (18). A P value <0.05 represented statistical significance. All statistical analyses were carried out with GraphPad Prism, version 8.0 (GraphPad Software, San Diego, CA, USA).

Results

Clinical and epidemiological features

The epidemic period extended from April 2016 to May 2018. Twenty-one patients were positive for ESBL-Kp colonization and infection. One patient died of severe infection by clinical diagnosis. All patients were hospitalized on the same floor and supported by the same staff. During the study period a total of 814 neonates were admitted to the NICU including 426 (52.3%) who were isolated with gram-negative pathogens. *K. pneumoniae* accounted for 47% of these pathogens. The twenty-one patients clinical data are shown in *Table 1*.

Antimicrobial susceptibility testing and detection of resistance mechanisms

All strains had an ESBL phenotype and were therefore resistant to all third-generation cephalosporins and fully susceptible to carbapenems (*Table 3*). Among the 21 ESBL-Kp isolates, 16 produced β-lactamases (CTX-M-3 and TEM-1B), while the others possessed CTX-M-15, CTX-M-24, CTX-M-66, TEM-1C, SHV-26, SHV-172, and OXA-1 (*Table 3*).

PFGE and MLST

Pulsed-field gel electrophoresis (PFGE) analysis of the 21 ESBL-KP isolates showed 21 banding patterns classified into 5 PFGE types (A, B, C, D and E) as shown in *Figures 1* and *2*, which is confirmed by MLST (*Table 3*).

Detection of virulence-associated determinants, biofilm formation, and serum killing

Despite the reported association between the *rmpA* gene and hypermucoviscosity, which has always been associated with more virulent strains, the *rmpA* gene was not detected in any of the isolates. All the 21 strains tested negative for aerobactin, suggesting low potential for hypervirulence. Our analysis also took other virulence gene profiles into account. The virulence genes *iutA* and *mrk* were detected in all of the isolates. PFGE type A carried both *mrk* (type 3 fimbriae, biofilm formation) and *fimH* (type 1 fimbriae), which indicated stronger biofilm formation. A summary of

Table 3 Microbiological and virulence features of ESBL K. pneumoniae strains

PFGE type	A	B	C	D	E
MLST type	ST 3330	ST 2791	ST 37	ST 34	ST 2740
Number of isolate	16	2	1	1	1
MIC of antimicrobials (mg/L)					
Ceftriaxone	≥64	≥64	≥64	≥64	≥64
Cefepime	16	16	8	8	8
Piperacillin-tazobactam	32.00	16	8	≤4	8
Imipenem	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Meropenem	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Ertapenem	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12
Amikacin	≤2	≤2	≤2	≤2	≤2
Cefoxitin	≤4	≤4	≤4	≤4	≤4
Csfazolin	≥64	≥64	≥64	≥64	264
Amoxycillin/clavulanic acid	≥32	≥32	≥32	8	232
Tobramycin	8	8	≥16	≤I	8
Resistance mechanisms					
Resistance determinant	TEM-1B, CTX-M-3	TEM-1C, CTX-M-15, SHV-172	TEM-1B, CTX-M-15, SHV-40, OXA-1	CTX-M-24, SHV-26	CTX-M-66
Virulence associated features					
Virulence gene profiles					
rmpA gene	–	–	–	–	–
Iron acquisition					
Aerobactin	–	–	–	–	–
kfu	–	–	–	–	–
entB	–	–	–	–	–
ybtS	–	–	–	–	–
iutA	+	+	+	+	+
Adhesins					
fimH	+	–	–	–	–
mrk	+	+	+	+	+
Biofilm formation assays	Strong	Lack	Weak	Lack	Middle
Serum killing assay	Sensitive (grade 1)	Sensitive (grade 1)	Sensitive (grade 1)	Sensitive (grade 1)	Sensitive (grade 1)

ESBL, extended spectrum beta-lactamase; PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing.

the virulence-associated genes is given in *Table 3*.

Biofilm formation assays were performed on all 21 ESBL-Kp isolates and revealed relatively high-mass biofilms (OD_{590} range, 0.0994–2.4756). It was discovered that isolates with the endemic ST3330 lineage produced more biofilm in comparison to other ST isolates (median

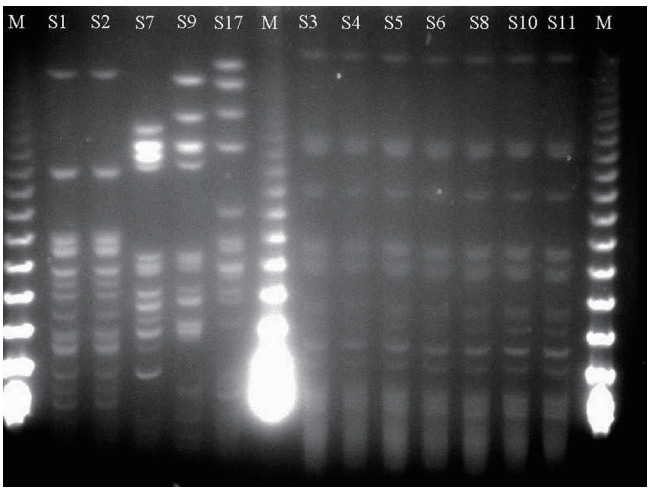

Figure 1 Pulsed-field gel electrophoresis of 12 strains. M: lambda ladder pulsed-field gel marker; 12 strains can be classified into 5 PFGE types. Type B = S1,S2, type C = S7, type D = S9, type E = S17, type A = the other 7 strains.

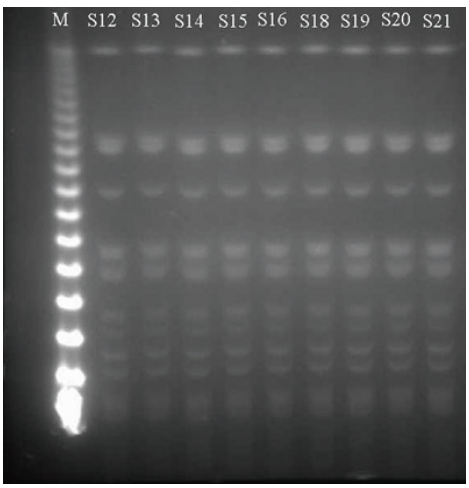

Figure 2 Pulsed-field gel electrophoresis of 9 strains. M: lambda ladder pulsed-field gel marker; 9 strains can be classified into type A.

OD_{590} 1.829 *vs.* 0.2280, respectively; P<0.0001) (*Figure 3*).

Serum killing assay found that five PFGE types of isolates showed serum high sensitivity (grade 1) (*Figure 4*).

Discussion

Premature newborns hospitalized in intensive care units are in a vulnerable and immunocompromised state. Moreover, intensive nursing is an additional risk factor for nosocomial

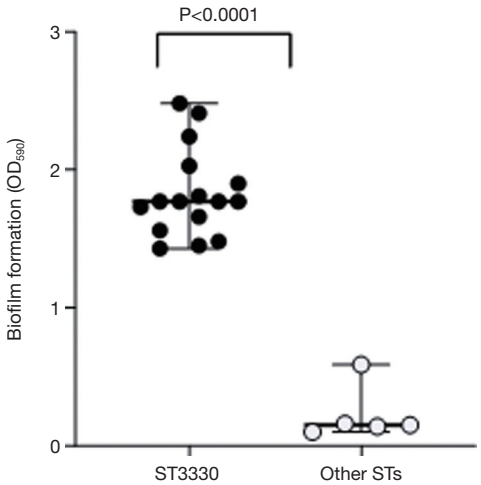

Figure 3 Biofilm formation of ESBL-*K. pneumoniae* isolates. Biofilms (expressed in OD_{590} values) were prepared from each isolate using the standard crystal violet staining method. Biofilms are presented for both ST groups. ST3330 (filled circles) and other non-epidemic STs (open circles). Isolates belonging to the ST3330 lineage formed significantly more biofilm than the other group (median OD_{590} 1.829 versus 0.2280, respectively, P<0.0001). ESBL, extended spectrum beta-lactamase; ST, sequence type.

infections (22,23). This study collected and analyzed original data from the neonatal unit at one Beijing hospital, and attests to the commonness of neonatal sepsis, as well as its association with high risk of mortality. In neonatal sepsis, the predominance of gram-negative pathogens (47% of strains were *K. pneumonia* in our hospital) indicates that infections are most likely horizontally transmitted from the environment and healthcare providers (1,5-7,24). Establishing whether a positive growth in the blood culture is a pathogen or a contaminant is paramount and a number of parameters need to be taken into account before a conclusion is reached. To date, the increased emergence of MDR organisms has limited treatment choices and affected their timeliness and efficiency. Therefore, because of the lack of suitable antibiotics for pediatric patients, the treatment of these infections presents an ever-increasing challenge for pediatricians (4,25,26).

In this investigation, PFGE type A surfaced as the most prevalent strain that disseminated within two years. The type A strain was revealed by MLST analysis to be ST3330 (*Table 3*). Therefore, there was evidence to suggest that the ESBL-*K.pneumoniae* isolates in this study had clonal dissemination. Moreover, an association was identified

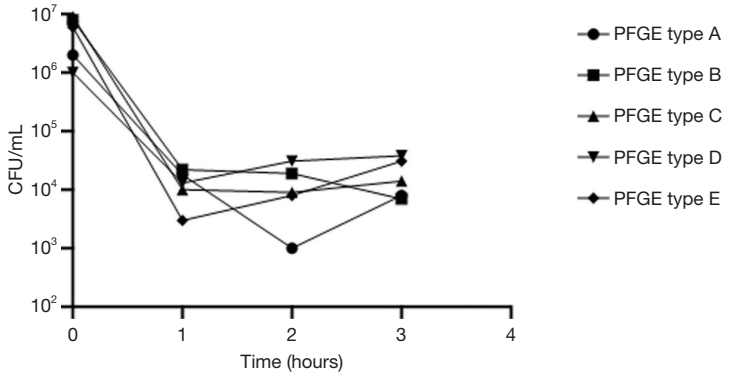

Figure 4 Serum killing assay of five PFGE types ESBI-KP strains. Survival of each type strain was assessed by enumerating viable counts at 0, 1, 2, and 3 h of incubation in the pooled human sera at 37 ℃. There was a significant decrease in the growth of all Strains. Data are mean ± SD (n=3 for each type). PFGE, pulsed-field gel electrophoresis; ESBL, extended spectrum beta-lactamase; KP, *Klebsiella pneumonia*.

between ST3330 and the spread and outbreak of CTX-M-3 and TEM-1-producing *K. pneumoniae*. This finding is at odds with a previous report that demonstrated CTX-M-15 to be the most prevalent ESBL gene in the world (5,27,28). CTX-M-3 and CTX-M-15 belong to the CTX-M 1 group. CTX-M-15 derives from the mutation of CTX-M-3 at the 240th amino acid from aspartic acid to glycine. An earlier Italian study demonstrated the existence of Kp strain co-harboring genes $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-15}}$ (5). Although type B and type C produced CTX-M-15, SHV, and OXA, they were not prevalent genes in this study. Generally, CTX-M and TEM confer resistance to third-generation cephalosporins but cannot reduce their susceptibility to carbapenems (5,9). Most of the clinical strains in the current study demonstrated high-level resistance to all third-generation cephalosporins and full susceptibility to carbapenems (*Table 3*).

Several Kp clones, such as ST23, are described as hypervirulent (hvKP) and regularly show a hypermucoviscous phenotype on agar plates. These hypermucoviscous strains are linked to production of several specific capsular serotypes (mainly K1 and K2) and the "regulator of mucoid phenotype A" (*rmpA/rmpA2*) complex (10,29). ESBL production is understood to have an association with a greater expression of virulence factors, including cell invasion proteins and fimbrial adhesins (30). Moreover, various elements may render the strain more virulent, such as biofilm formation (with fimbriae), additional iron acquisition systems (siderophores), or allantoin metabolism. The *mrkABCDF* gene cluster in our strains and the presence of the *iutA* gene that encode adhesin type 3 fimbriae and bacterial siderophores, respectively, bear a strong association with biofilm formation (30). Most of the ST3330 strains involved in this dissemination carried at least two fimbriae: *mrk* (type 3 fimbriae) and *fimH* (type 1 fimbriae). Fimbriae are adhesive structures on the surface of Kp involved in biofilm formation. Notably, type 1 (encoded by the *fim* operon) and type 3 fimbriae (encoded by the *mrk* operon) are considered to be virulence factors expressed by almost all Kp strains (5,18). Biofilm formation assays performed on all 21 ESBL-Kp isolates showed relatively high-mass biofilms. Isolates with endemic ST3330 lineage were found to produce a larger amount of biofilm than other ST isolates ($P<0.0001$) (*Figure 3*). A pediatric patient was assumed to be the source of the outbreak, but the pathogen still was being disseminated after the patient's discharge. Furthermore, the Hospital Infection Control Department did not isolate the epidemic strain during two years of environmental sampling. Strong biofilm formation ability may be a cause of unsuccessful sampling. It may partly explain the epidemic strain has high colonization and diffusion potential. The source of the infection is still unclear and needs to be traced through future prospective study.

In this study, each of the 21 strains was negative for *rmpA* and aerobactin, which indicates low potential for hypervirulence. Meanwhile, serum killing assay showed five PFGE types of isolates to present serum high sensitivity (grade 1) (*Figure 4*) (14,21). As it was impossible to collect serum from healthy newborns, human serum was obtained from 10 healthy adults, which may have led to errors. The first-line empirical treatment is Cefoperazone Sodium and Sulbactam Sodium combined with third-generation cephalosporin. In the absence of any improvement, antibiotics, carbapenems, and vancomycin are used until the

targeted blood culture results are obtained. In general, the majority outcomes of these newborns were consistent with clinical treatment excluding the case of one newborn who died of severe infection by clinical diagnosis. However, even in this case, it was difficult to establish if the infection was the only cause of death.

The present study has several limitations. First, its single-center nature and relatively small sample size mean that it might lack either the scientific precision or external validity needed to bring about widespread changes in clinical practice. Second, the samples were collected from a single hospital in Beijing. Therefore, future work including samples from a larger geographic area or a multi-center analysis is suggested.

Conclusions

Neonatal sepsis was observed and associated with elevated inpatient mortality. In neonates, sepsis can be caused by several microorganisms, among which, *K. pneumonia* is proved to be the most frequently detected pathogen. *K. pneumonia* has the capacity to develop multi-antibiotic resistance, including to third generation cephalosporins. Because of the gaps in disease records, more data on the microbiology of neonatal infection need to be collected to bring about improvements to existing practices. Hospitals should adopt approaches aimed at preventing and managing the spread of infection. Emergent ESBL-Kp infections can be traced at the earliest stage, by employing infection-control measures, such programs for stewarding antibiotics with continuous surveillance, especially in at-risk units such as NICUs.

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References

1. Gao K, Fu J, Guan X, et al. Incidence, Bacterial Profiles, And Antimicrobial Resistance Of Culture-Proven Neonatal Sepsis In South China. Infect Drug Resist 2019;12:3797-805.
2. Yin D, Zhang L, Wang A, et al. Clinical and molecular epidemiologic characteristics of carbapenem-resistant Klebsiella pneumoniae infection/colonization among neonates in China. J Hosp Infect 2018;100:21-8.
3. Ghaith DM, Zafer MM, Said HM, et al. Genetic diversity of carbapenem-resistant Klebsiella Pneumoniae causing neonatal sepsis in intensive care unit, Cairo, Egypt. Eur J Clin Microbiol Infect Dis 2020;39:583-91.
4. Bassetti M, Giacobbe DR. Reducing dissemination of carbapenem-resistant Klebsiella pneumoniae. Ann Transl Med 2019;7:S365.
5. Peltier F, Choquet M, Decroix V, et al. Characterization of a multidrug-resistant Klebsiella pneumoniae ST607-K25 clone responsible for a nosocomial outbreak in a neonatal intensive care unit. J Med Microbiol 2019;68:67-76.
6. Patil S, Chen H, Zhang X, et al. Antimicrobial Resistance and Resistance Determinant Insights into Multi-Drug Resistant Gram-Negative Bacteria Isolates from Paediatric

Patients in China. Infect Drug Resist 2019;12:3625-34.
7. Oliveira PMN, Buonora SN, Souza CLP, et al. Surveillance of multidrug-resistant bacteria in pediatric and neonatal intensive care units in Rio de Janeiro State, Brazil. Rev Soc Bras Med Trop 2019;52:e20190205.
8. Leikin-Zach V, Shany E, Yitshak-Sade M, et al. Neonatal Risk Factors for Colonization with Extended-Spectrum Beta-Lactamase-Producing Bacteria in the Neonatal Intensive Care Unit. Isr Med Assoc J 2018;20:286-90.
9. Uc-Cachón AH, Gracida-Osorno C, Luna-Chi IG, et al. High Prevalence of Antimicrobial Resistance Among Gram-Negative Isolated Bacilli in Intensive Care Units at a Tertiary-Care Hospital in Yucatan Mexico. Medicina (Kaunas) 2019;55:588.
10. An J, Lai K, Ma Y, et al. Emergence of multiple carbapenemase-producing organisms in single patients: an increasing threat to treatment of infection. J Antimicrob Chemother 2018;73:544-6.
11. Tian D, Pan F, Wang C, et al. Resistance phenotype and clinical molecular epidemiology of carbapenem-resistant Klebsiella pneumoniae among pediatric patients in Shanghai. Infect Drug Resist 2018;11:1935-43.
12. Lai K, Ma Y, Guo L, et al. Molecular characterization of clinical IMP-producing Klebsiella pneumoniae isolates from a Chinese Tertiary Hospital. Ann Clin Microbiol Antimicrob 2017;16:42.
13. Ma Y, Bao C, Liu J, et al. Microbiological characterisation of Klebsiella pneumoniae isolates causing bloodstream infections from five tertiary hospitals in Beijing, China. J Glob Antimicrob Resist 2018;12:162-6.
14. Zhang Y, Zeng J, Liu W, et al. Emergence of a hypervirulent carbapenem-resistant Klebsiella pneumoniae isolate from clinical infections in China. J Infect 2015;71:553-60.
15. Luo Y, Ma Y, Zhao Q, et al. Similarity and divergence of phylogenies, antimicrobial susceptibilities, and virulence factor profiles of Escherichia coli isolates causing recurrent urinary tract infections that persist or result from reinfection. J Clin Microbiol 2012;50:4002-7.
16. Yang J, Ye L, Guo L, et al. A nosocomial outbreak of KPC-2-producing Klebsiella pneumoniae in a Chinese hospital: dissemination of ST11 and emergence of ST37, ST392 and ST395. Clin Microbiol Infect 2013;19:E509-15.
17. Guo L, An J, Ma Y, et al. Nosocomial Outbreak of OXA-48-Producing Klebsiella pneumoniae in a Chinese Hospital: Clonal Transmission of ST147 and ST383. PLoS One 2016;11:e0160754.
18. Naparstek L, Carmeli Y, Navon-Venezia S, et al. Biofilm formation and susceptibility to gentamicin and colistin of extremely drug-resistant KPC-producing Klebsiella pneumoniae. J Antimicrob Chemother 2014;69:1027-34.
19. Huertas MG, Zarate L, Acosta IC, et al. Klebsiella pneumoniae yfiRNB operon affects biofilm formation, polysaccharide production and drug susceptibility. Microbiology 2014;160:2595-606.
20. Khodadadian R, Rahdar HA, Javadi A, et al. Detection of VIM-1 and IMP-1 genes in Klebsiella pneumoniae and relationship with biofilm formation. Microb Pathog 2018;115:25-30.
21. Abate G, Koh TH, Gardner M, et al. Clinical and bacteriological characteristics of Klebsiella pneumoniae causing liver abscess with less frequently observed multi-locus sequences type, ST163, from Singapore and Missouri, US. J Microbiol Immunol Infect 2012;45:31-6.
22. Arena F, Giani T, Becucci E, et al. Large oligoclonal outbreak due to Klebsiella pneumoniae ST14 and ST26 producing the FOX-7 AmpC beta-lactamase in a neonatal intensive care unit. J Clin Microbiol 2013;51:4067-72.
23. Cadot L, Bruguiere H, Jumas-Bilak E, et al. Extended spectrum beta-lactamase-producing Klebsiella pneumoniae outbreak reveals incubators as pathogen reservoir in neonatal care center. Eur J Pediatr 2019;178:505-13.
24. Devrim F, Serdaroglu E, Caglar I, et al. The Emerging Resistance in Nosocomial Urinary Tract Infections: From the Pediatrics Perspective. Mediterr J Hematol Infect Dis 2018;10:e2018055.
25. Zou ZH, Liu D, Li HD, et al. Prenatal and postnatal antibiotic exposure influences the gut microbiota of preterm infants in neonatal intensive care units. Ann Clin Microbiol Antimicrob 2018;17:9.
26. Corbella M, Caltagirone M, Gaiarsa S, et al. Characterization of an Outbreak of Extended-Spectrum beta-Lactamase-Producing Klebsiella pneumoniae in a Neonatal Intensive Care Unit in Italy. Microb Drug Resist 2018;24:1128-36.
27. Weissman SJ, Adler A, Qin X, et al. Emergence of extended-spectrum beta-lactam resistance among Escherichia coli at a US academic children's hospital is clonal at the sequence type level for CTX-M-15, but not for CMY-2. Int J Antimicrob Agents 2013;41:414-20.
28. Justo-da-Silva LH, De-Azeredo AN, Bueno AC, et al. Diversity of clonal types of Klebsiella pneumoniae causing infections in intensive care neonatal patients in a large urban setting. Braz J Microbiol 2019;50:935-42.

29. Lin JC, Koh TH, Lee N, et al. Genotypes and virulence in serotype K2 Klebsiella pneumoniae from liver abscess and non-infectious carriers in Hong Kong, Singapore and Taiwan. Gut Pathog 2014;6:21.

30. Nordberg V, Jonsson K, Giske CG, et al. Neonatal intestinal colonization with extended-spectrum beta-lactamase-producing Enterobacteriaceae-a 5-year follow-up study. Clin Microbiol Infect 2018;24:1004-9.

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