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# Co-outbreak of multidrug resistance and a novel ST3006 *Klebsiella pneumoniae* in a neonatal intensive care unit

## A retrospective study

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**Abstract**

The outbreak of carbapenem-resistant *Klebsiella pneumoniae* is a serious public health problem, especially in the neonatal intensive care unit (NICU).

Fifteen *K. pneumoniae* strains were isolated from 7 neonates during June 3 to 28, 2017 in an NICU. Antimicrobial susceptibility was determined by the Vitek 2 system and microbroth dilution method. Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) were used to analyze the genetic relatedness of the isolates. Whole-genome sequencing and gene function analysis were performed to investigate pathogenicity and drug resistance and screen genomic islands.

Three clones of *K. pneumoniae* were identified from 7 neonates: 7 strains of ST37, 7 of novel ST3006, and 1 of ST1224. Gene sequencing showed that the kpn1343 (ST37) strain harbored 12 resistance genes (*OXA-33*, *TEM-1*, *SHV-11*, *AAC (6')-Iid*, *AAC (3)-IIa*, *AAC (6')-Ib-cr*, *catB3*, *arr-3*, *sul1*, *oqxB*, *oqxA*, *CRP*, and *catB3*) and included 15 genomic islands and 205 reduced virulence genes. The kpn1344 (ST3006) strain harbored 4 antibiotic-resistant genes (TEM-1, CTX-M-3, vgaC, and CRP) and included 19 genomic islands and 209 reduced virulence genes. MLST and PFGE showed that 15 strains of *K. pneumoniae* were divided into 3 groups with a high level of homology. ST1224 (kpn1362) was isolated on June 28, 2017, which was 10 days after the last isolate (kpn1359, June 18, 2017); thus, we speculated that ST1224 was not the clone that caused the outbreak.

This co-outbreak of *K. pneumoniae* involved 2 clones: ST37 and ST3006. ST37 carried the multidrug-resistant genes, such as *OXA-33*, *TEM-1*, and *SHV-11*, and ST3006 was a novel *K. pneumoniae* ST typing. Whole-genome sequencing may be an effective method for screening bacterial-resistant genes and their functions.

**Abbreviations:** CARD = Comprehensive Antibiotic Resistance Database, MLST = multilocus sequence typing, NICU = neonatal intensive care unit, PCR = polymerase chain reaction, PFGE = pulsed-field gel electrophoresis, PHI = pathogen–host interaction.

**Keywords:** co-outbreak, *Klebsiella pneumoniae*, multidrug resistance, neonatal intensive care unit, whole-genome sequencing

## 1. Introduction

*Klebsiella pneumonia* is a common and important pathogen in hospitals, and multidrug-resistant *K. pneumoniae* in particular poses a serious and urgent threat to public health.[1,2] Neonates in neonatal intensive care units (NICUs) often have underlying conditions, such as prematurity, the presence of indwelling catheters, or history of antibiotic treatment and parenteral nutrition, which are known risk factors for infection.[3] In addition, relaxed vigilance by doctors and nurses toward nosocomial infection can lead to nosocomial infection outbreaks, with considerable impact on neonatal treatment and prognosis, prolonged hospital stays, increased hospital costs, and increased mortality rates.

Nosocomial outbreaks in the NICU are frequently reported. Neonatal outbreaks have been reported from Africa (January 1, 1996 to January 1, 2016) with pathogens such as rotavirus, influenza virus, measles virus, and multidrug-resistant bacteria (*Serratia marcescens*, *Acinetobacter baumannii*, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant enterococci).[4] Johnson et al[5] reviewed the English, French, and German language literature published between 2015 and 2017, and a total of 39 outbreaks in NICUs were reported with

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Gram-negative bacteria (n = 21, 54%), with 5 viral outbreaks (respiratory syncytial virus = 3). Outbreaks caused by *Burkholderia cepacia*, *Escherichia coli*, and *Pseudomonas aeruginosa* have also been reported.[6–8] However, the reports on the outbreak of *K. pneumoniae* are rare, and it was rarer to isolate the same clonal pathogen from different sites in the same neonates.

In our study, we identified 15 strains of *K. pneumoniae* isolated from sputum specimens, blood specimens, and umbilical vein catheter tips in 7 neonates; these isolates belonged to the 3 cloned strains ST37, ST3006, and ST1224. Our objective was to characterize the outbreak strains and further remind that NICUs must be vigilant in detecting outbreaks, conducting in-depth investigations and implementing targeted strategies to prevent and control infections.

## 2. Methods

### 2.1. Patients and bacterial strains

Patient characteristics were obtained from electronic medical records. Bacterial strains were isolated from 7 neonates and stored in a refrigerator (SANYO Electric Co., Osaka, Japan) at −70°C. Strains were identified using the Vitek 2 system (BioMérieux, Craponne, France). This study was approved by the ethics committee of the hospital in which the strains were isolated (approval number K2018-01-001).

### 2.2. Multilocus sequence typing

Bacterial DNA was extracted using the Bacteria Genomic DNA Kit (CWBio, Beijing, China). Multilocus sequence typing (MLST) for *K. pneumoniae* was performed according to previously described methods.[9] The allelic profiles and sequence types were determined using online databases (https://pubmlst.org/bigsdb?db=pubmlst_mlst_seqdef). The novel allele profiles were sent to klebsiellaMLST@pasteur.fr for confirmation.

### 2.3. Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) of *XbaI*-digested genomic DNA samples of *K. pneumoniae* was performed using the CHEF MAPPER XA apparatus (Bio-Rad Laboratories, Hercules, CA) as described previously.[10] Electrophoresis was performed for 24 hours at 14°C with pulse time ranging from 5 to 35 s at 6 V/cm. PFGE profiles were analyzed and compared using the Gel Doc XR+ system, version 2.0 (Bio-Rad).

### 2.4. Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed using the Vitek 2 system (BioMérieux, France), including ampicillin-sulbactam, cefazolin, ceftriaxone, cefotetan, ceftazidime, cefepime, gentamicin, tobramycin, amikacin, levofloxacin, ciprofloxacin, aztreonam, imipenem, ertapenem, piperacillin-tazobactam, and trimethoprim-sulfamethoxazole. The MICs of tigecycline and polymyxin B were determined by the microbroth dilution method.[11] The breakpoint of all antibiotics was interpreted according to the Clinical and Laboratory Standards Institute document M100-S26,[12] with the exception of tigecycline and polymyxin B. For tigecycline and polymyxin B, the European Committee on Antimicrobial Susceptibility Testing breakpoint[13] was used. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC27853 were used for quality control.

### 2.5. Whole-genome sequencing and gene analysis

We chose KPN1343 and KPN1344 for whole-genome sequencing, which was performed using the Illumina HiSeq PE150 platform (Novogene Bioinformatics Technology Co., Ltd., Beijing, China). The Island Path-DIOMB program was used to predict the genomic islands.[14] For these pathogenic bacteria, we used the pathogen–host interaction (PHI) database and the Comprehensive Antibiotic Resistance Database (CARD) to perform pathogenicity and drug resistance analyses.[15,16]

## 3. Results

### 3.1. Clinical characteristics of patients

Patients' clinical characteristics are presented in Table 1. Subjects included 5 premature infants and 2 infants with hyperbilirubinemia. All 5 premature infants were also diagnosed as having low birth weight (2 were very low birth weight infants) and neonatal respiratory distress syndrome. Three infants were delivered by

**Table 1**

**Clinical characteristics of patients.**

| Patients | Clinical diagnosis | Mode of delivery | Age (wk) | Sex | Body weight (kg) | Antibiotics | Apgar score | Outcome |
|---|---|---|---|---|---|---|---|---|
| P1 | Premature infant<br>Low birth weight infant<br>NRDS | Caesarean delivery | 33 | F | 1.55 | TZP, MEM | 6-8-8 | Treated |
| P2 | Premature infant<br>Low birth weight infant<br>NRDS | Normal delivery | 31 | F | 1.60 | TZP | 8-8-8 | Treated |
| P3 (first of twins) | Premature infant<br>Very low birth weight infant<br>NRDS | Midwifery delivery | 29 | F | 1.45 | TZP | 8-9-9 | Treated |
| P4 (second of twins) | Premature infant<br>Very low birth weight infant<br>NRDS | Midwifery delivery | 29 | F | 1.26 | TZP | 8-9-9 | Automatic discharge |
| P5 | Neonatal hyperbilirubinemia | Forceps delivery | 40 | M | 2.76 | TZP | 10-10-10 | Treated |
| P6 | Neonatal hyperbilirubinemia | Caesarean delivery | 40 | F | 3.83 | TZP | 10-10-10 | Treated |
| P7 | Premature infant<br>Very low birth weight infant<br>NRDS | Caesarean delivery | 30 | M | 1.00 | TZP | 6-8-8 | Treated |

F = female, M = male, MEM = meropenem, NRDS = neonatal respiratory distress syndrome, P = patient, TZP = piperacillin/tazobactam.

**Table 2**

**The characteristics of *K. pneumoniae*.**

| Patient | Date of detection | Isolate no. | Isolate site | Isolates | STs | Resistance phenotype |
|---|---|---|---|---|---|---|
| P1 | 2017/6/3 AM 10:03 | kpn1340 | Blood | kpn | ND | ND |
| | | kpn1341 | UVCP | kpn | ND | ND |
| | | kpn1342 | Sputum | kpn | 37 | Carbapenemase |
| P2 | 2017/6/13 AM 07:20 | kpn1343 | Blood | kpn | 37 | Carbapenemases |
| | | kpn1344 | Blood | kpn | 3006 | ESBLs |
| | | Negative | UVCP | Negative | — | — |
| | | kpn1356 | Sputum | kpn | ND | ND |
| P3 (first of twins) | 2017/6/14 AM 09:00 | kpn1345 | Blood | kpn | 37 | Carbapenemase |
| | | kpn1346 | Blood | kpn | 3006 | ESBLs |
| | | kpn1347 | UVCP | kpn | 37 | Carbapenemase |
| | | kpn1357 | Sputum | kpn | ND | ND |
| | 2017/6/28 | kpn1362 | Sputum | kpn | 1224 | Carbapenemases |
| P4 (second of twins) | 2017/6/14 AM10:40 | kpn1348 | Blood | kpn | 3006 | ESBLs |
| | | kpn1349 | Blood | kpn | 37 | Carbapenemases |
| | | kpn1350 | UVCP | kpn | 37 | Carbapenemases |
| | | kpn1351 | UVCP | kpn | 3006 | ESBLs |
| | | kpn1358 | Sputum | kpn | ND | ND |
| P5 | 2017/6/15 AM10:56 | kpn1352 | Blood | kpn | 3006 | ESBLs |
| | | Negative | Sputum | Negative | — | — |
| P6 | 2017/6/15 PM14:46 | kpn1353 | Blood | kpn | 37 | Carbapenemases |
| | | kpn1354 | Blood | kpn | 3006 | ESBLs |
| | | Negative | Sputum | Negative | — | — |
| P7 | 2017/6/15 AM10:30 | kpn1355 | UVCP | kpn | 3006 | ESBLs |
| | 2017/6/18 AM11:08 | kpn1359 | Sputum | kpn | ND | ND |

AM = ante meridiem, kpn = *Klebsiella pneumonia*, ND = not done, P = patient, PM = post meridiem, UVCP = umbilical vein catheter tip.

cesarean section, 2 by midwives, 1 by forceps, and only 1 infant was delivered normally. The average age of the 5 premature infants was approximately 30 weeks, with an average body weight of 1.37 kg. Patient 1 was treated with piperacillin/tazobactam and meropenem, whereas the remaining patients were treated with piperacillin/tazobactam. Patients 1, 2, 3, 4, 6, and 7 had lower Apgar scores than normal (normal: 10-10-10). Patient 4, the second of a pair of twins, had poor clinical conditions but was discharged upon request of her family, whereas the remaining patients were treated and discharged.

### 3.2. Characteristics and antibiotic susceptibility of isolates

Twenty-one *K. pneumoniae* strains were identified by the VIETK 2 system in the early stage, but only 15 strains were collected and analyzed in our study. Strains were isolated from sputum, blood, and umbilical vein catheter tips. We simultaneously isolated 2 *K. pneumoniae strains* from blood and/or umbilical vein catheter tips in patients 2, 3, 4, and 6. MLST showed that there were 3 types: ST37, ST1224, and a novel ST3006 (*gapA* 69, *infB* 19, *mdh* 90, *pgi* 20, *phoE* 125, *rpoB* 18, and novel allele *tonB* 406) (Table 2).

Results of the antibiotic susceptibility testing showed that the ST37 strain was resistant to ampicillin-sulbactam, cefazolin, ceftriaxone, cefotetan, ceftazidime, cefepime, gentamicin, aztreonam, imipenem, ertapenem, and piperacillin-tazobactam. The ST3006 strain was only resistant to ampicillin-sulbactam, cefazolin, and ceftriaxone (Table 3).

### 3.3. Antibiotic-resistant genes

The CARD was used to search for the names of resistance-related genes. KPN1343 (accession numbers CP033900) was found to have 12 antibiotic resistant genes: beta-lactam resistance (*OXA-33*, *TEM-1*, *SHV-11*), aminoglycoside resistance [*AAC (6')-IId*, *AAC (3)-IIa*, *AAC (6')-Ib-cr*], phenicol resistance (*catB3*), rifamycin resistance (*arr-3*), sulfonamide resistance (*sul1*), efflux pump complex or subunit conferring antibiotic resistance (*oqxB*, *oqxA*, *CRP*), and streptogramin resistance (*catB3*). KPN1344 (accession numbers CP033901) was found to have 4 antibiotic resistant genes: beta-lactam resistance (*TEM-1*, *CTX-M-3*), and efflux pump complex or subunit conferring antibiotic resistance (*vgaC*, *CRP*). We chose the resistance gene with a best identities rate ≥0.99 (Table 4).

**Table 3**

**Antibiotic susceptibility of *K. pneumoniae* Isolates ST37 and ST3006.**

| | MIC (μg/mL) S/I/R | | | |
|---|---|---|---|---|
| Antibiotics | *K. pneumoniae* ST37 (n = 7) | | *K. pneumoniae* ST3006 (n = 7) | |
| Ampicillin-sulbactam | ≥32 | R | ≥32 | R |
| Cefazolin | ≥64 | R | ≥64 | R |
| Ceftriaxone | ≥64 | R | ≥64 | R |
| Cefotetan | ≥64 | R | ≤4 | S |
| Ceftazidime | ≥64 | R | ≤1 | S |
| Cefepime | ≥64 | R | ≤1 | S |
| Gentamicin | ≥16 | R | ≤1 | S |
| Tobramycin | 8 | I | ≤1 | S |
| Amikacin | ≤2 | S | ≤2 | S |
| Levofloxacin | ≤0.25 | S | ≤0.25 | S |
| Ciprofloxacin | ≤0.25 | S | ≤0.25 | S |
| Aztreonam | ≥64 | R | 64 | R |
| Imipenem | 4 | R | ≤1 | S |
| Ertapenem | ≥8 | R | ≤0.5 | S |
| Tigecycline* | ≤0.25 | S | ≤0.25 | S |
| Polymyxin B* | ≤0.25 | S | ≤0.25 | S |
| Piperacillin–Tazobactam | 64 | R | ≤4 | S |
| Trimethoprim–Sulfamethoxazole | ≤20 | S | ≤20 | S |

I = intermediary, MIC = minimal inhibitory concentration, R = resistance, S = sensitive.

* Tigecycline and polymyxin B antibiotic susceptibility were determined by microbroth dilution method.

**Table 4**

**Antibiotic resistance ontology annotates for antibiotic resistance genes.**

| ORF_ID | Best Hit ARO | Best identities | ARO | ARO_category |
|---|---|---|---|---|
| KPN1343_GM005273 | OXA-33 | 1 | ARO:3001781 | Antibiotic inactivation enzyme, determinant of beta-lactam resistance |
| KPN1343_GM005323 | AAC (6')-Iid | 1 | ARO:3002548 | Antibiotic inactivation enzyme, determinant of aminoglycoside resistance |
| KPN1343_GM005284 | AAC (3)-IIa | 1 | ARO:3002533 | Antibiotic inactivation enzyme, determinant of aminoglycoside resistance |
| KPN1343_GM005274 | AAC (6')-Ib-cr | 1 | ARO:3002547 | Antibiotic inactivation enzyme, determinant of aminoglycoside resistance |
| KPN1343_GM005278 | TEM-1 | 1 | ARO:3000873 | Antibiotic inactivation enzyme, determinant of beta-lactam resistance |
| KPN1343_GM002829 | SHV-11 | 1 | ARO:3001070 | Antibiotic inactivation enzyme, determinant of beta-lactam resistance |
| KPN1343_GM005272 | catB3 | 1 | ARO:3002676 | Antibiotic inactivation enzyme, determinant of phenicol resistance |
| KPN1343_GM005271 | arr-3 | 1 | ARO:3002848 | Antibiotic inactivation enzyme, determinant of rifamycin resistance |
| KPN1343_GM005269 | sul1 | 1 | ARO:3000410 | Antibiotic target replacement protein, determinant of sulfonamide resistance |
| KPN1343_GM004245 | oqxB | 1 | ARO:3003923 | Efflux pump complex or subunit conferring antibiotic resistance |
| KPN1343_GM004244 | oqxA | 1 | ARO:3003922 | Efflux pump complex or subunit conferring antibiotic resistance |
| KPN1343_GM005322 | catB3 | 0.99 | ARO:3002670 | Antibiotic inactivation enzyme, determinant of streptogramin resistance |
| KPN1343_GM000019 | CRP | 0.99 | ARO:3000518 | Efflux pump complex or subunit conferring antibiotic resistance |
| KPN1344_GM005191 | TEM-1 | 1 | ARO:3000873 | Antibiotic inactivation enzyme, determinant of beta-lactam resistance |
| KPN1344_GM005193 | CTX-M-3 | 1 | ARO:3001866 | Antibiotic inactivation enzyme, determinant of beta-lactam resistance |
| KPN1344_GM000088 | vgaC | 1 | ARO:3002831 | Efflux pump complex or subunit conferring antibiotic resistance |
| KPN1344_GM003641 | CRP | 0.99 | ARO:3000518 | Efflux pump complex or subunit conferring antibiotic resistance |

ARO = antibiotic resistance ontology, ID = identity, ORF = open reading frame.

### 3.4. PFGE

PFGE homology analysis showed that 15 *K. pneumoniae* strains were divided into 3 clusters: cluster A: 1344, 1346, 1348, 1351, 1352, 1354, 1355; cluster B: 1362; and cluster C: 1342, 1343, 1345, 1347, 1349, 1350, and 1353. MLST also divided 15 *K. pneumoniae* strains into 3 groups (Fig. 1).

### 3.5. PHIs and genomic islands

Using the BLAST software, the amino acid sequences of KPN1343 and KPN1344 were compared using the PHI database. PHI phenotype classification showed that the number of reduced virulence genes mostly matched the database mostly, that is, 205 and 209, respectively (Fig. 2). Using Island Path-DIOMB to predict genomic islands, KPN1343 had 15 genomic islands and KPN1344 had 19 genomic islands. The length and direction of the genes are shown in Figure 3 (length of only <15 kb is shown).

## 4. Discussion

*K. pneumoniae* is an important nosocomial pathogen that can cause pneumonia, urinary tract infection, digestive tract

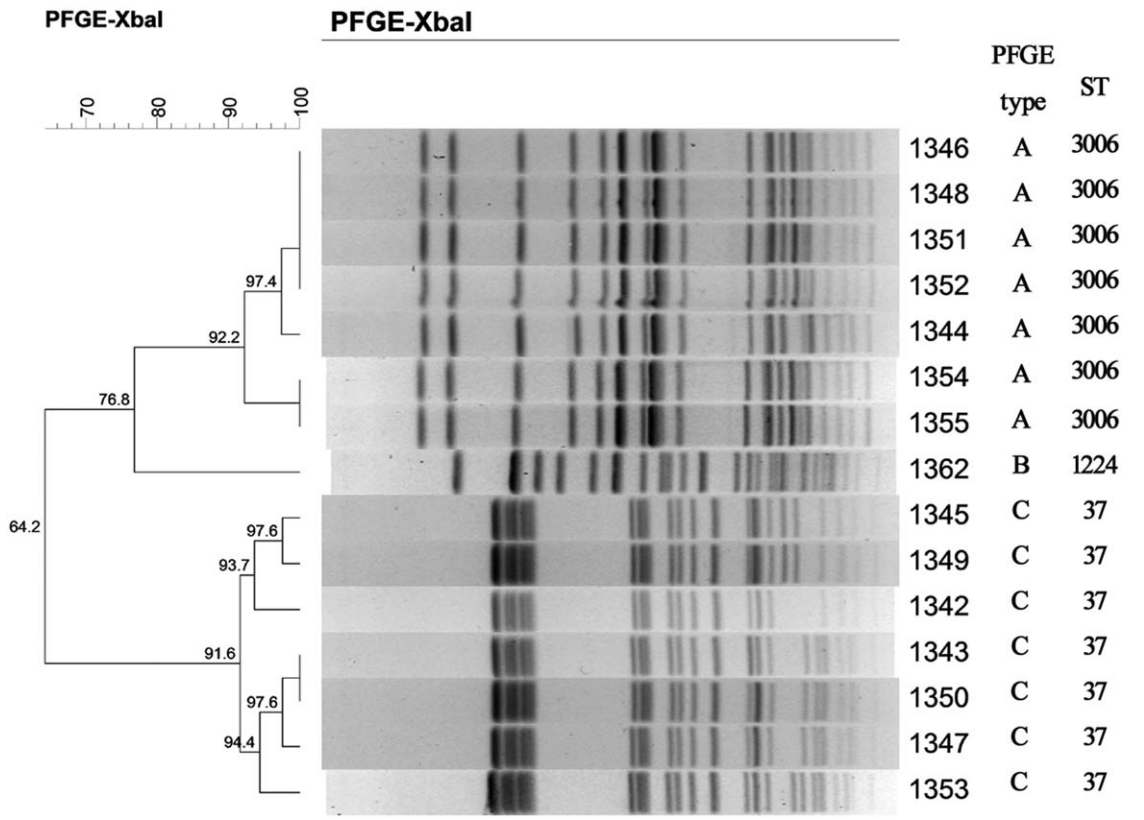


**Figure 1.** Pulsed-field gel electrophoresis (PFGE) of *K. pneumoniae*. Fifteen *K. pneumoniae* strains were divided into 3 clusters: clusters A: 1344, 1346, 1348, 1351, 1352, 1354, 1355; cluster B: 1362; and cluster C: 1342, 1343, 1345, 1347, 1349, 1350, 1353. MLST also divided 15 *K. pneumoniae* strains into 3 groups: ST37, ST1224, and ST3006. MLST = multilocus sequence typing, ST = sequence type.

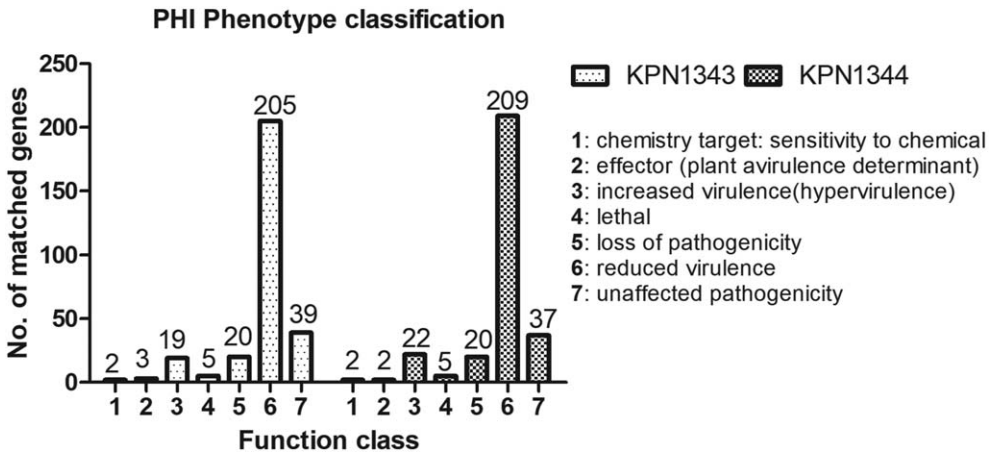


**Figure 2.** KPN1343 and KPN1344 PHI phenotype classification. PHI phenotype classification showed that the number of reduced virulence genes most matched the database, that is, 205 and 209, respectively. PHI = pathogen–host interaction.

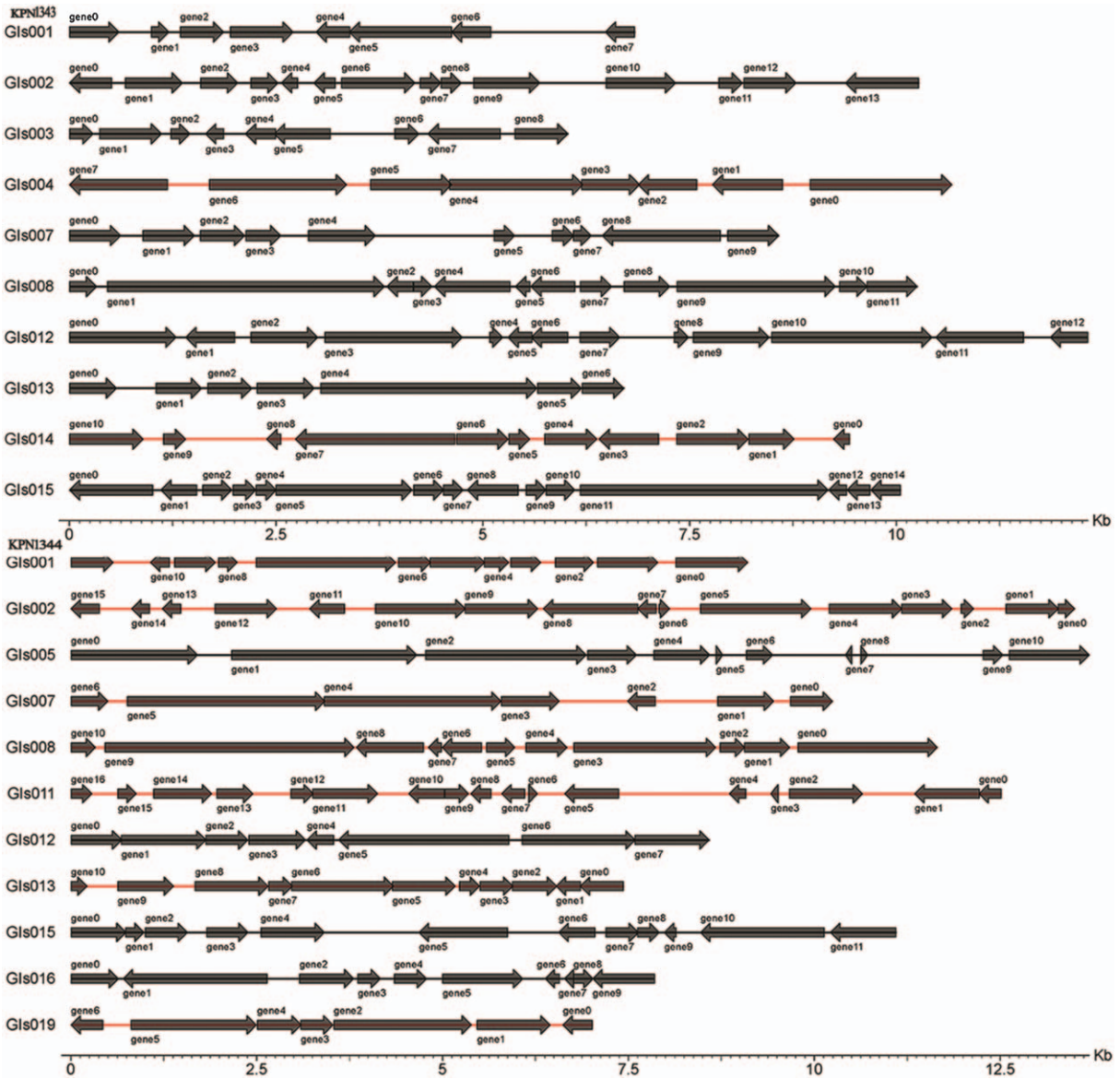


**Figure 3.** KPN1343 and KPN1344 genomic islands. KPN1343 has 15 genomic islands and KPN1344 has 19 genomic islands, and the length and direction of the genes are shown in Figure 3 (length of only <15 kb is shown).

infection, bloodstream infection, liver abscess, and meningitis.[17–20] Infection outbreaks have been frequently reported in NICUs. Extended spectrum beta-lactamase-producing and carbapenemase-producing *K. pneumoniae* can cause large outbreaks with significant morbidity and mortality effects.[21] Underlying conditions, including premature delivery, low birth weight, and neonatal respiratory distress syndrome, are risk factors for neonatal infection. Inadequate medical equipment, environmental disinfection, hand hygiene, and staffing are important factors influencing infection outbreak.[22,23] In our study, infection control practitioners took samples from incubators, air, hands of doctors and nurses, objects, and patient skin for bacterial culture. Only one *K. pneumoniae* strain was isolated from the inner surface of an incubator. However, this strain was not stored for further research.

Screening and monitoring of extended spectrum beta-lactamase-producing and carbapenemase-producing *K. pneumoniae* in hospitals are crucial.[24] However, current molecular biological techniques, such as polymerase chain reaction (PCR) and fluorescence quantitative PCR, have their limitations and cannot extensively screen drug-resistant genes. Although whole-genome sequencing may be an effective method for screening resistant genes, it is costly and time-consuming, and these factors limit its application. Homology analysis methods, such as ERIC-PCR, PFGE, and MLST, are complex and are also time consuming.[25–29] Thus, newer technologies need to be developed to effectively monitor the rapid outbreak of nosocomial infections.

Most previous reports on *K. pneumoniae* outbreaks were from single-site infections, such as respiratory specimens,[30,5] blood specimens,[31–33] or urine specimens.[34–36] However, our findings showed that pathogens were simultaneously isolated from respiratory specimens, blood specimens, and umbilical vein catheter tips of the same patient, which is relatively rare. Fortunately, after the investigation described in this study, subsequent nosocomial infection control was performed by the infection department, and no other bacteria were isolated, except kpn1362 (ST1224) from patient 3 on June 28, 2017.

Our study had some limitations. First, we did not freeze isolates (kpn1340, kpn1341, kpn1356, kpn1357, kpn1358, kpn1359) due to the weak awareness of hospital infection outbreak and insufficient scientific research consciousness at that time. Second, the third clone ST1224 has not worked more in our research because it was isolated on June 28, 2017, which was 10 days after the last isolate (kpn1359, June 18, 2017). We could saw that the similarity between ST1224 and ST3006 was 76.8%, and 64.2% between ST1224 and ST37 (Fig. 3). Thus, we speculated that ST1224 was not the clone that caused the outbreak. So, it is important for us to enhance awareness of infection outbreak and strengthen bacterial preservation. Furthermore, communication and cooperation with infection control practitioners should be strengthened to screen and prevent nosocomial transmission at an earlier stage. Hospitals should implement different strategies, such as hand-washing policies strictly enforced among staff, frequent equipment changes, and extensive cleaning of pediatric wards, to combat outbreaks.

## 5. Conclusions

In summary, we reported an outbreak of 2 clones of *K. pneumoniae*, ST37 and ST3006, in an NICU. The clones were isolated from multiple sites in the same patients, including sputum, blood, and umbilical vein catheter tips. Whole-genome sequencing showed that ST37 *K. pneumonia* harbored multidrug-resistant genes such as *OXA-33*, *TEM-1*, and *SHV-11*; thus, this method appears to be useful for detecting drug-resistant genes and analyzing gene function. Active and effective infection control measures are indispensable for preventing and controlling nosocomial infection outbreaks.

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**Writing – original draft:** Dongjie Chen, Yusheng Chen.
**Writing – review and editing:** Yusheng Chen.

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