Identification and Characterization of NDM-1-producing Hypervirulent (Hypermucoviscous) *Klebsiella pneumoniae* in China

Zhou Liu, M.D.\(^1,2\), Yi Gu, M.D.\(^1\), Xin Li, M.D.\(^2\), Yanyan Liu, M.D.\(^1,3\), Ying Ye, M.D.\(^1,3\), Shihe Guan, M.D.\(^2\), and Jiabin Li, M.D.\(^1,3,4\)

\(^1\)Department of Infectious Diseases, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China; \(^2\)Department of Laboratory Medicine, The Second Hospital of Anhui Medical University, Hefei, Anhui, China; \(^3\)Anhui Center for Surveillance of Bacterial Resistance, Hefei, Anhui, China; \(^4\)Department of Infectious Diseases, Chaohu Hospital of Anhui Medical University, Hefei, Anhui, China

**Background:** Carbapenem-resistant hypervirulent (hypermucoviscous) *Klebsiella pneumoniae* (CR-HMKP) poses a significant public health challenge. We investigated its epidemiology and molecular characteristics in a tertiary care hospital in eastern China.

**Methods:** CR-HMKP were identified among 106 non-duplicated carbapenem-resistant *K. pneumoniae* isolates (from June 2013 to September 2017) using the string test. The pulsortype (PT) and sequence type (ST) of CR-HMKP isolates were determined using pulsed-field gel electrophoresis and multilocus sequence typing. Resistance determinants, capsular serotypes, and virulence genes were detected by PCR and sequencing. Representative isolates from each PT were selected, and their virulence phenotypes were established using the serum killing and *Galleria mellonella* lethality assays.

**Results:** Of the 106 isolates, 13 (12.3%) were CR-HMKP. Seven were positive for *bla*\(^{NDM-1}\) and shared the same genotype (PT5/ST1764); the others were positive for *bla*KPC-2, belonged to ST11, and were divided into four different PTs. The serotype of all *bla*\(^{NDM-1}\)-positive isolates was K64, while that of *bla*KPC-2-positive isolates were K47 (N=4) and K64 (N=2). The NDM-1-producing HMKP isolates were positive for aerobactin, exhibited high serum resistance, and elicited significantly increased larval mortality compared with the other isolates. All patients had received invasive treatment prior to infection by NDM-1-producing HMKP. The infections occurred between July and August 2016 and were hospital-acquired.

**Conclusions:** NDM-1-producing HMKP ST1764 isolates were identified; this is the first report worldwide on an outbreak of nosocomial infection caused by these isolates. Effective surveillance and strict infection control strategies should be implemented to prevent CR-HMKP dissemination.

**Key Words:** Carbapenem-resistant, Hypervirulent, Hypermucoviscous, *Klebsiella pneumoniae*, NDM-1, Epidemiology, Molecular characteristics

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**INTRODUCTION**

*Klebsiella pneumoniae* is an important opportunistic pathogen that causes a wide range of infections and exhibits increasingly frequent acquisition of antibiotic resistance, especially carbapenem resistance [1]. In recent years, a new variant designated hypervirulent (hypermucoviscous) *K. pneumoniae* (HMKP) has raised concerns worldwide, owing to its ability to cause severe...
and life-threatening infections in young immune-competent individuals. Remarkably, these infections are often complicated by other devastating disseminated infections, including endophthalmitis and meningitis [2]. Since HMKP was first identified in East Asia, the incidence of HMKP-associated infection has increased worldwide [3]. Due to the increased production of carbapenem-resistant HMKP (CR-HMKP) isolates, in a medical center in eastern China. In addition, for the first time, we observed an outbreak of carbapenem-resistant HMKP (CR-HMKP) isolates (identified as “classic” K. pneumoniae (cKP); it was previously thought that there was no overlap between HMKP and cKP [5]. Unfortunately, virulence and resistance could converge to produce strains that are able to cause severe and untreatable invasive infections, representing a major public health concern [6]. Furthermore, carbapenem-resistant HMKP (CR-HMKP) isolates are increasingly being detected worldwide, and in particular in China [3, 7-12]. Thus, to better understand the incidence and characteristics of CR-HMKP strains, we conducted a retrospective study to investigate the clinical data, epidemiology, and molecular characteristics of CR-HMKP isolates, in a medical center in eastern China. In addition, for the first time, we observed an outbreak of nosocomial infection associated with New Delhi metallo-β-lactamase-1 (NDM-1)-producing HMKP.

**METHODS**

**Strain identification and hypermucoviscosity phenotype detection**

A total of 106 non-duplicated K. pneumoniae isolates (identified using VITEK 2 compact system [BioMérieux, Marcy-l’Étoile, France]) exhibiting a carbapenem-resistant phenotype (imipenem minimum inhibitory concentration [MIC] ≥4 μg/mL and/or ertapenem MIC ≥2 μg/mL) were consecutively collected between June 2013 and September 2017 at the Second Hospital of Anhui Medical University in Anhui, eastern China. The hypermucoviscosity phenotype of the carbapenem-resistant K. pneumoniae (CRKP) isolates was detected using the string test, as described previously [7]. Briefly, all the tested isolates were cultured overnight on Columbia blood agar plates (BioMérieux, Shanghai, China) at 37°C, and bacterial colonies were then stretched with an inoculation loop. The formation of a viscous string over five mm in length was defined as a positive result; CRKP isolates with a positive string test were defined as CR-HMKP.

**Antimicrobial susceptibility testing**

Antimicrobial susceptibility testing of CR-HMKP isolates was initially performed using the VITEK 2 compact system. Subsequently, ampicillin, amikacin, aztreonam, cefazolin, cefepime, ceftazidime, ceftriaxone, ciprofloxacin, levofloxacin, gentamicin, trimethoprim-sulfamethoxazole, tobramycin (National Institutes for Food and Drug Control, Beijing, China); ertapenem, imipenem (Merck Sharp & Dohme Corp, Hangzhou, China); meropenem (Sumitomo Pharmaceuticals, Suzhou, China); and piperacillin-tazobactam (Pfizer, New York, NY, USA). MICs were confirmed using the agar dilution method according to the recommendations and breakpoints proposed by the CLSI [13, 14]. Tigecycline (Pfizer) and colistin (Sigma Aldrich, Shanghai, China) MICs were determined by the broth microdilution method, using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines and breakpoint [15]. The standard strains Escherichia coli ATCC25922 and Pseudomonas aeruginosa ATCC27853 were used for quality control.

**Carbapenemase phenotypes and detection of resistance determinants**

Both the modified Hodge test (MHT) and imipenem-EDTA double-disk synergy test (DDST) were performed to determine the carbapenemase phenotypes as described previously [16]. Carbapenemase-encoding genes (including \( \text{bla}_{\text{KPC}} \), \( \text{bla}_{\text{NDM}} \), \( \text{bla}_{\text{IMP}} \), \( \text{bla}_{\text{NDM-1}} \), and \( \text{bla}_{\text{OXA-48}} \)) and common extended-spectrum β-lactamase (ESBL)-encoding genes (including \( \text{bla}_{\text{CTX-M}} \), \( \text{bla}_{\text{SHV}} \), and \( \text{bla}_{\text{TEM}} \)) were screened by PCR as described previously [17, 18]. All positive PCR products were sequenced and compared with the published sequences in GenBank (www.ncbi.nlm.nih.gov/blast/).

**Capsular polysaccharide serotyping and virulence genes**

The capsular polysaccharide serotypes of the CR-HMKP were determined by PCR and sequencing of the K-serotype specific \( wzi \) allele, which is closely associated with K-type [19]. The PCR products were sequenced and assigned using an online database (http://bigdadb.pasteur.fr/klebsiella/klebsiella.html), and then compared with the reference sequences in GenBank [20].
corresponding capsular types were determined according to the criteria of ≥94% DNA identity for the same types and ≤80% identity for different types [21]. Fifteen virulence-associated genes, including aerobactin, allS, entB, fimH, iroB, iucA, kfuBC, magA, mrdD, mmpA, mmpA2, ureA, wabG, ybtS, and ycfM, were detected by PCR and sequenced using previously described primers [7, 22].

Genotyping of CR-HMKP
The genotypes of the CR-HMKP isolates were determined by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Briefly, following digestion with the XbaI restriction endonuclease (Takara, Dalian, China), the genomic DNA of the tested isolates and the reference strain Salmonella H9812 were separated on agarose gels using a CHEF Mapper XA PFGE system (Bio-Rad, Hercules, CA, USA). Next, the gel was stained, and dendrograms were constructed from the PFGE data using the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) with the Dice similarity coefficient. The same pulsortype (PT) was defined when the isolates shared >90% similarity. MLST was performed as described previously [23]. The number of alleles was determined and the sequence type (ST) was then assigned using the MLST website (http://www.mlst.net/).

Serum killing and Galleria mellonella lethality assays
The virulence of the CR-HMKP isolates was assessed by serum killing and G. mellonella lethality assays. For references, the clinical hypermucoviscous K. pneumoniae strain KPN54798 (ST23, serotype K1, carbapenem-susceptible) was employed as the hypervirulent control (H-control), while the clinical non-hypermucoviscous K. pneumoniae isolate KPN49 (ST11, serotype K47, harboring blmdm.i) was employed as the low-virulence control (L-control). The clinical and microbiological characteristics of the two reference strains are listed in Supplemental Data Table S1. For the serum killing assay, human blood was collected from five healthy volunteers, and the serum was then separated and stored at -80°C. The assays were performed and the results were expressed as previously described [21, 24]. For the G. mellonella infection model, different concentrations of bacterial suspensions were diluted in 10 mM phosphate-buffered saline (PBS) and inoculated into the hemocoel of 20 larvae of G. mellonella (weighing 300±25 mg) (Kaide Ruixin Co., Ltd., Tianjin, China) at final concentrations of 1×10^3–10^7 colony-forming units (CFUs)/larva. Following injection, the larvae were incubated at 37°C and monitored every 12 hours for 72 hours. Experiments were performed three times on separate occasions. The survival rates were recorded, and the 50% lethal dose (LD50) was determined as described previously [25]. Negative control groups included larvae that were inoculated with 10 μL of PBS.

Clinical information collection and ethics
The clinical information of patients infected with CR-HMKP was collected from electronic medical records. All patients were evaluated based on the US Centers for Disease Control and Prevention (CDC) criteria to determine whether the infections were due to CR-HMKP [26]. This retrospective study was approved by the ethical committee of the Second Hospital of Anhui Medical University, with waiver of informed consent (approval number PJ-YX2018-001).

Statistical analysis
The Shapiro-Wilk method was used to test normality. Normally distributed variables were summarized as mean±SD, and non-normally distributed variables were summarized as median and range. The serum killing assays data were summarized as mean±SE. Survival data were plotted using the Kaplan-Meier method and analyzed using log-rank tests with GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). The LD50 values were calculated using the Bliss method, and the results are expressed as log10 (lg) transformed values. A one-sample t-test was used to compare the data and was performed using SPSS v. 21.0 (IBM Corp., Armonk, NY, USA). A two-sided P<0.05 was considered statistically significant.

RESULTS
Clinical and demographic characteristics of the patients
Of the 106 non-duplicated CRKP isolates, 12.3% (13/106) were positive for the string test and identified as CR-HMKP. The patients with CR-HMKP isolates were distributed across several departments, and their median length of hospitalization was 36 days (range 6–174 days). Notably, 76.9% (10/13) of the patients had a history of a stay in the intensive care unit (ICU). Of these patients, seven (53.8%) were males, and their mean age was 55.3±18.6 years. All but one patient received invasive treatment prior to infection with CR-HMKP. Eleven patients exhibited a high fever symptom following infection with CR-HMKP; four of these patients subsequently developed septic shock and eventually died because of failure of the anti-infection treatment (Table 1). Notably, seven cases occurred between July and August 2016, and all these cases were assessed as hospital-acquired (HA) infections. Five of the patients had an ICU stay history in...
Table 1. Clinical and demographic characteristics of the patients with CR-HMKP isolates

| No. Case | Sex/Age (yr) | Ward     | Admission date (month-yr) | LOS/LOS in ICU (day) | Underlying disease                           | Invasive treatment | Septic shock/ Tmax (°C) | Antimicrobial treatment | Outcome |
|----------|--------------|----------|---------------------------|----------------------|----------------------------------------------|-------------------|-------------------------|--------------------------|---------|
| KPN11    | F/63         | NEU-ICU  | 10-2013                   | 42/42                | Encephalon injury, Pneumonia                  | Surgery, MV, CVC  | No/39.8                 | TGC, FOS                 | Death   |
| KPN18    | F/80         | GS-ICU   | 11-2013                   | 36/11                | Esophageal cancer                            | MV                | No/39.3                 | TGC                      | Death   |
| KPN19    | M/42         | ES-ICU   | 11-2013                   | 39/27                | Severe acute pancreatitis                     | MV, CVC           | Yes/40.8                | MEM                      | Death   |
| KPN34    | F/69         | EICU     | 04-2015                   | 12/0                 | Pneumonia, Diabetes                          | MV                | No/37.9                 | TGC, MNO                 | Discharge|
| KPN53    | M/70         | HS-ICU   | 05-2016                   | 43/2                 | Hepatocellular carcinoma                      | Surgery           | No/38.5                 | LVX, SCF                 | Discharge|
| KPN60    | F/38         | GO-ICU   | 07-2016                   | 18/2                 | Postpartum hemorrhage                         | Surgery, MV       | No/37.9                 | LVX                      | Discharge|
| KPN63    | M/62         | GE-ICU   | 07-2016                   | 11/10                | Pneumonia                                    | Surgery, CVC      | Yes/39.0                | LVX, AMK                 | Death   |
| KPN68    | M/11         | PS       | 07-2016                   | 49/0                 | Scar contract                                | Surgery           | No/36.6                 | LVX                      | Discharge|
| KPN69    | M/49         | GS-ICU   | 07-2016                   | 26/2                 | Arterial aneurysm                             | Surgery           | No/37.3                 | LVX, FOS                 | Discharge|
| KPN72    | F/60         | EM-ICU   | 07-2016                   | 6/5                  | Thermoplegia, MOF                            | MV, CVC           | Yes/39.3                | LVX                      | Death   |
| KPN74    | F/60         | NEP-PS   | 06-2016                   | 75/0                 | CKD, Diabetes, Gangrene                      | Surgery, CVC      | No/38.6                 | LVX                      | Discharge|
| KPN100   | M/73         | GS-ICU   | 06-2017                   | 20/19                | Severe acute pancreatitis                     | MV, CVC           | Yes/39.8                | TGC                      | Death   |
| KPN104   | M/42         | ORT-ICU  | 06-2017                   | 174/7                | Pelvic fractures                             | Surgery, BC       | No/39.0                 | TGC, FOS, MEM             | Discharge|

*All cases were assessed as hospital-acquired infections; †Invasive treatment prior infection with CR-HMKP; ‡Antimicrobial treatment after CR-HMKP was identified.

Abbreviations: AMK, amikacin; BC, bladder catheter; CKD, chronic kidney diseases; CR-HMKP, carbapenem-resistant hypervirulent (hypermucoviscous) Klebsiella pneumoniae; CVC, central venous catheter; EICU, emergency intensive care unit; EM, emergency medicine department; ES, emergency surgery department; F, female; FOS, fosfomycin; GE, gastroenterology department; GO, gynecology and obstetrics department; GS, general surgery department; HS, hepatobiliary surgery department; ICU, intensive care unit; LOS, length of stay; LVX, levofloxacin; M, male; MEM, meropenem; MV, mechanical ventilation; NEP, nephrology department; NEU, neurosurgery department; ORT, orthopedics department; PS, plastic surgery department; SCF, cefoperazone-sulbactam; TGC, tigecycline; Tmx, maximal body temperature.

Table 2. Antimicrobial susceptibility testing result of CR-HMKP isolates

| Isolate | SPE | MIC of antimicrobial agents (μg/mL) |
|---------|-----|------------------------------------|
|         | AMP | AMK | ATM | CAZ | CIP | COL | CRO | CZO | ETP | FEP | GEN | IPM | LVX | MEM | SXT | TGC | TOB | TZA |
| KPN11   | SP  | >256 | 64  | >128 | >32 | 1   | >32 | >256 | >32 | 64  | 16  | >32 | >64 | >32 | 2/38 | 0.50 | 32 | 512 |
| KPN18   | SP  | >256 | 32  | >128 | >128| >32 | 1   | >32 | >256 | >32 | 64  | 8   | >32 | >64 | >32 | 2/38 | 0.50 | 8  | 512 |
| KPN19   | BL  | >256 | 32  | >128 | >128| >32 | 2   | >32 | >256 | >32 | >128| 8   | >32 | >64 | >32 | 2/38 | 0.50 | 16 | >512|
| KPN34   | SP  | >256 | 256 | >128 | >32 | >32 | 2   | >32 | >256 | >32 | 64  | >32 | >64 | >32 | 2/38 | 0.25 | 64 | 256 |
| KPN53   | PU  | >256 | 1   | 1   | >128| 1   | 1   | >32 | >256 | >32 | 16  | 0.5 | >32 | >32 | 1/19 | 0.50 | 1  | 256 |
| KPN60   | SE  | >256 | 1   | 2   | >128| 2   | 2   | >32 | >256 | >32 | 16  | 0.5 | >32 | >32 | 1/19 | 0.50 | 1  | 256 |
| KPN63   | SP  | >256 | 1   | 2   | >128| 1   | 1   | >32 | >256 | >32 | 16  | 0.5 | >32 | >32 | 1/19 | 0.50 | 1  | 256 |
| KPN68   | SE  | >256 | 2   | 1   | >128| 1   | 1   | >32 | >256 | >32 | 16  | 1   | >32 | >32 | 1/19 | 0.50 | 1  | 256 |
| KPN69   | PU  | >256 | 1   | 2   | >128| 1   | 1   | >32 | >256 | >32 | 16  | 0.5 | >32 | >32 | 1/19 | 0.50 | 1  | 256 |
| KPN72   | SE  | >256 | 2   | 1   | >128| 2   | 1   | >32 | >256 | >32 | 16  | 1   | >32 | >32 | 1/19 | 0.50 | 1  | 256 |
| KPN74   | SE  | >256 | 2   | 1   | >128| 1   | 2   | >32 | >256 | >32 | 16  | 0.5 | >32 | >32 | 1/19 | 0.50 | 1  | 256 |
| KPN100  | PF  | >256 | >512| 64  | 64  | >32 | 2   | >32 | 128  | 16  | 8   | >128| 32  | 8   | 4/76 | 1   | >128| 128 |
| KPN104  | BL  | >256 | >512| 64  | 128 | >32 | 2   | >32 | 128  | 16  | 8   | >128| 64  | 8   | 4/76 | 1   | >128| 128 |
| RR (%)  | 100.0 | 30.8 | 46.2 | 100.0 | 46.2 | 0.0 | 100.0 | 100.0 | 100.0 | 84.6 | 30.8 | 100.0 | 46.2 | 100.0 | 15.4 | 0.00 | 38.5 | 100.0 |

Abbreviations: AMP, ampicillin; AMK, amikacin; ATM, aztreonam; BL, Blood; CAZ, ceftazidime; CIP, ciprofloxacin; COL, colistin; CRO, ceftiraxone; CZO, ceftazolin; ETP, ertapenem; FEP, cefepime; GEN, gentamicin; IPM, imipenem; LVX, levofloxacin; MEM, meropenem; MIC, minimum inhibitory concentrations; PF, puncture fluid; PU, Pus; RR, resistant rate; SE, secretion; SP, sputum; SPE, specimen; SXT, trimethoprim-sulfamethoxazole; TGC, tigecycline; TOB, tobramycin; TZA, piperacillin-tazobactam.
Antimicrobial susceptibility and resistance mechanism
The CR-HMKP isolates were primarily isolated from secretion/pus (N=6, 46.2%), followed by sputum (N=4, 30.8%), blood (N=2, 15.4%), and puncture fluid (N=1, 7.7%). All were resistant to imipenem, ertapenem, and meropenem but were susceptible to tigecycline and colistin (Table 2). The carbapenemase phenotype tests revealed that seven isolates were DDST-positive, and the others were MHT-positive. Consistent with these phenotypes, seven isolates harbored bla\textit{NDM-1}, while the remaining isolates harbored bla\textit{KPC-2}. Additionally, half of the bla\textit{KPC-2}-positive isolates co-harbored bla\textit{CTX-M-65} and bla\textit{SHV-11}, while all bla\textit{NDM-1}-positive isolates co-harbored bla\textit{SHV-71} (Fig. 1).

Genotyping, capsular serotyping, and virulence-associated genes
Five PTs and two STs were identified among the CR-HMKP isolates. All bla\textit{NDM-1}-positive isolates belonged to PT5 and were assigned to ST1764 (gap\textit{A}_1, inf\textit{B}_1, mdh\textit{1}, pgi\textit{1}, pho\textit{E}_9, rpo\textit{B}_4, ton\textit{B}_{283}), which is a three-locus variant of ST23 (gap\textit{A}_2, inf\textit{B}_1, mdh\textit{1}, pgi\textit{1}, pho\textit{E}_9, rpo\textit{B}_4, ton\textit{B}_{12}). The bla\textit{KPC-2}-positive isolates belonged to ST11 and were divided into four PTs. Nine of the isolates (69.2%) belonged to capsular serotype K64 (wzi\textit{-64}) and four (30.8%) to capsular serotype K47 (wzi\textit{-209}). Of the virulence genes tested, ent\textit{B}, iuc\textit{A}, kfu\textit{BC}, mrk\textit{D}, rmp\textit{A2}, ure\textit{A}, wab\textit{G}, ybt\textit{S}, and ycf\textit{M} were detected in all CR-HMKP isolates, and the detection rates for aerobactin, all\textit{S}, iro\textit{B}, and rmp\textit{A} were 53.8% (7/13), 46.2% (6/13), 53.8% (7/13), and 69.2% (9/13), respectively. The \textit{magA} gene was not detected in any of the isolates. Notably, all NDM-1-producing isolates were positive for the aerobactin gene (Fig. 1).

Serum killing assay
Five representative CR-HMKP isolates [KPN18(PT1), KPN11(PT2), KPN100(PT3), KPN34(PT4), and KPN53(PT5)] were selected based on PT, and their virulence phenotypes were established. The serum killing assay results demonstrated that KPN53 was highly resistant (Grade 5), KPN11 and KPN18 were intermediate sensitive (Grade 4), and KPN34 and KPN100 were serum-sensitive (Grade 1). Of the two reference strains, KPN54798<sup>\textit{H-control}</sup> was highly resistant (Grade 6), and KPN49<sup>\textit{L-control}</sup> was serum-sensitive (Grade 1; Fig. 2).

\textit{Galleria mellonella} lethality assay
In the \textit{G. mellonella} infection model, time- and dose-dependent deaths were observed for all tested isolates. At the dose of 1×10<sup>7</sup> CFU/larvae, 100.0% mortality was observed in all tested isolates except for the KPN49<sup>\textit{L-control}</sup>. At the dose of 1×10<sup>6</sup> CFU/larvae, only KPN53 and KPN54798<sup>\textit{H-control}</sup> resulted in 100.0% larval mortality at 24 hours post-infection, whereas KPN11, KPN18,
Fig. 2. Serum killing assays of the tested isolates. Data are presented as mean±SE, and log_{10}-transformed values were utilized to normalize the data (N=3 for each isolate). Abbreviations: H-control, hypervirulence control; L-control, low-virulence control; Lg, log_{10}-transformed values.

Table 3. lgLD_{50} values of tested isolates in G. mellonella at 72 hours post-infection

| Isolate  | lgLD_{50}^*       |
|----------|-------------------|
| KPN11    | 5.38 ± 0.06       |
| KPN18    | 5.51 ± 0.09       |
| KPN34    | 5.44 ± 0.03       |
| KPN53    | 4.95 ± 0.06†      |
| KPN100   | 5.61 ± 0.10       |
| KPN54798 | 4.81 ± 0.11       |
| KPN49    | 5.97 ± 0.14       |

*Data are presented as mean±SD; †The lgLD_{50} value of KPN53 did not differ from that of KPN54798^H−control (P=0.059 by the one-sample t-test) and is significantly lower than those of the other CR-HMKP isolates and KPN49^L−control (P<0.05 by the one-sample t-test).

Abbreviations: H-control, hypervirulence control; L-control, low-virulence control; lgLD_{50}, log_{10}(50% lethal dose).

Fig. 3. Survival curves for G. mellonella larvae inoculated with 1×10^7 (A), 1×10^6 (B), 1×10^5 (C), and 1×10^4 (D) colony-forming units of the tested isolates, and the data shown are from a single representative experiment out of three repeats. *The curves of KPN11, KPN18, KPN34, KPN100, KPN49^L−control and negative control are completely overlapping.

Abbreviations: H-control, hypervirulence control; L-control, low-virulence control.
KPN34, KPN100, and KPN49 \textsuperscript{control} killed 70.0%, 65.0%, 75.0%, 60.0%, and 40.0% of the larvae at this dose, respectively. The observed larval lethality rates due to KPN53 were higher than those of the other CR-HMKP isolates and KPN49 \textsuperscript{control} (P < 0.01 by the log-rank test) (Fig. 3). Additionally, KPN53 had a lower lgLD\textsubscript{50} than the other isolates except KPN5498 \textsuperscript{control} (P < 0.05 by the one-sample t-test) (Table 3).

**DISCUSSION**

In this study, the prevalence rate of HMKP among CRKP isolates was 12.3%, which is higher than the 7.4% rate reported in a previous study from Hangzhou, eastern China in 2015, but similar to the results of another recent study from Wenzhou, eastern China in 2017 [7, 9]. Our study and other reports indicated that the prevalence of HMKP among CRKP isolates in China is high, becoming a serious threat to public health.

The major mechanism of *K. pneumoniae* drug resistance to carbapenems is the expression of carbapenemases; the *Klebsiella pneumoniae* carbapenemase (KPC)-type carbapenemases are more prevalent than other types [27]. Similarly, most of the carbapenemases present in CR-HMKP isolates are KPC-2, and the NDM-type carbapenemase are rarely detected [11]. To the best of our knowledge, four NDM-producing HMKP isolates have been reported worldwide to date, which were distributed sporadically throughout Asia and the Middle East and belonged to different STs [12, 28-30]. We found that 53.8% of the CR-HMKP isolates harbored *bla*\textsuperscript{NDM-1}, shared the same PT, and belonged to ST1764. Hence, our findings expand the CR-HMKP molecular epidemiology database worldwide.

ST11 is the dominant KPC-producing *K. pneumoniae* clone in China [31]. In our study, most of the CR-HMKP isolates belonged to ST11 and were serotype K47 (wzi-209). Interestingly, a similar result was obtained in a recent study that described a fatal outbreak caused by KPC-2-producing HMKP ST11 isolates in a hospital in eastern China [8]. All the strains in that report were serotype K47 [8]. Thus, additional studies should be conducted to determine whether regional clonal dissemination of serotype 47 CR-HMKP belonging to ST11 has occurred.

Various virulence factors are associated with HMKP isolates. The *magA* gene was identified as the serotype K1 allele of the polymerase *wzy* gene in the *cps* gene cluster [32]. As we did not identify any serotype K1 isolates, the *magA* gene was not detected in any of the isolates. Conversely, two plasmid-carried genes, *rmfA* and *rmfA2*, which both contribute to the enhancement of capsular production, were detected in most of the CR-HMKP isolates in our study. Several studies have also demonstrated that nearly all HMKP isolates tested positive for the *rmfA/rmfA2* genes [33, 34].

Iron is essential for the survival of *K. pneumoniae*. As free iron in the host plasma is scarce, *K. pneumoniae* predominantly acquires iron via the secretion of siderophores, molecules that have a higher affinity for iron than the host transport proteins. In our study, the detection rates of the siderophore-associated genes (the *aerobactin*, *iucA*, *iroB*, *ybtS*, and *entB* genes) ranged from 53.8% to 100.0%. In contrast, the detection rates of siderophore-associated genes in cKP were <20.0%, except for the *entB* gene [5]. Of the siderophores secreted by HMKP, aerobactin is the most important virulence factor [4, 35]. Our subsequent assays also indicated that KPN53 (the representative *aerobactin*-positive strain) was highly serum-resistant, and its lgLD\textsubscript{50} value in *G. mellonella* model was significantly lower than that of the other isolates. According to a previous study, the LD\textsubscript{50} of most clinical *K. pneumoniae* isolates in the *G. mellonella* model were 10\textsuperscript{7} CFU/larva [36], higher than those observed for KPN53. Moreover, the capsular gene *wabG*, the type 1 and 3 fimbriae genes *fimH*, *mrkD*, and other virulence factors also contribute to the enhancement of the virulence potential of CR-HMKP isolates. In our data, the detection rates of the *fimH*, *mrkD*, *ureA*, *wabG*, and *ycfM* genes were >90.0%, which is consistent with a previous study [7].

Most patients infected by CR-HMKP have a history of ICU hospitalization and received invasive treatment [7, 8], which was consistent with our data. However, the outcomes of patients infected by CR-HMKP are diverse. Gu et al. [8] and Zhang et al. [9] demonstrated that all patients with CR-HMKP infections died of septic shock. Another study noted that only one patient died of septic shock and multiple organ failure [7]. In our study, 46.2% of the patients died, while the rest were discharged. Notably, seven cases occurred between July and August 2016 and were assessed as HA infections, and the seven case-related NDM-1-producing HMKP isolates had identical genotypes. Thus, ours is the first report on an outbreak of a nosocomial infection associated with NDM-1-producing HMKP ST1764. Of these cases, their inpatient beds were positioned very closely, and this proximity suggests nosocomial dissemination of NDM-1-producing-HMKP, most likely because of contact propagation during medical activities. As this was a retrospective study, the main limitation is the insufficient data for explaining how NDM-1-producing HMKP isolates was transmitted between the ICU and PS wards. Therefore, to prevent further nosocomial transmission of CR-HMKP, strict infection control measures such as hand hygiene,
contact isolation, active screening, and environmental surface disinfection should be implemented.

In summary, a high prevalence of HMKP was observed among the CRKP isolates in this study. All the CR-HMKP isolates produced carbapenemase, and NDM-1-producing HMKP ST1764 was identified among them. To the best of our knowledge, this is the first report worldwide on an outbreak of nosocomial infection caused by NDM-1-producing HMKP. These findings indicate that effective surveillance and the implementation of strict infection control strategies are needed to prevent the nosocomial dissemination of CR-HMKP.

Authors’ Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article are reported.

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