Original Article
Clinical Microbiology

Ann Lab Med 2019;39:167-175
https://doi.org/10.3343/alm.2019.39.2.167
ISSN 2234-3806 • eISSN 2234-3814

ANNALS OF LABORATORY MEDICINE

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

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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 pulsotype (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

Received: February 11, 2018
Revision received: July 28, 2018
Accepted: October 25, 2018

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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 capsular polysaccharide, HMKP exhibits a distinct hypermucoviscous phenotype when grown on Columbia blood agar plates that can be detected using the string test [2]. In addition, it can secrete various siderophores such as aerobactin, salmochelin, yersiniabactin, and enterobactin. Of these, aerobactin accounts for >90% of the siderophore activity and plays a crucial role in the growth and survival of HMKP in human ascites fluid or serum and *in vivo* mouse infection models, suggesting that aerobactin is a crucial virulence factor of HMKP [4].

Since HMKP is susceptible to commonly used antimicrobial agents, the multidrug-resistant *K. pneumoniae* population that does not exhibit the hypermucoviscosity phenotype is defined 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 bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{OXA-48}) and common extended-spectrum β-lactamase (ESBL)-encoding genes (including bla_{CTX-M}, bla_{SHM}, and bla_{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://bigsdb.pasteur.fr/klebsiella/klebsiella.html), and then compared with the reference sequences in GenBank [20]. The

https://doi.org/10.3343/alm.2019.39.2.167

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, mrkD, rmpA, rmpA2, 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 *Xba*I 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 pulsotype (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://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

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 bla_{NDM-1}) 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 (LD_{50}) 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/ T_{max} (°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 contracture	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; T_{max}, 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	TZP
KPN11	SP	>256	64	>128	64	>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	>128	>32	2	>32	>256	>32	64	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	1	>32	1/19	0.50	1	256
KPN60	SE	>256	1	2	>128	2	2	>32	>256	>32	16	0.5	>32	1	>32	1/19	0.50	1	256
KPN63	SP	>256	1	2	>128	1	1	>32	>256	>32	16	.0.5	>32	1	>32	1/19	0.50	1	256
KPN68	SE	>256	2	1	>128	1	1	>32	>256	>32	16	1	>32	1	>32	1/19	0.50	1	256
KPN69	PU	>256	1	2	>128	1	1	>32	>256	>32	16	0.5	>32	1	>32	1/19	0.50	1	256
KPN72	SE	>256	2	2	>128	2	1	>32	>256	>32	16	1	>32	2	>32	1/19	0.50	1	256
KPN74	SE	>256	1	2	>128	1	2	>32	>256	>32	16	0.5	>32	1	>32	1/19	0.50	1	256
KPN100	PF	>256	>512	64	64	>32	2	>32	128	16	8	>128	8	32	8	4/76	1	>128	128
KPN104	BL	>256	>512	64	128	>32	2	>32	128	16	8	>128	16	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, ceftriaxone; CZO, cefazolin; 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; TZP, piperacillin-tazobactam.

https://doi.org/10.3343/alm.2019.39.2.167

July 2016, and their inpatient bed positions were very close, while the other two patients were in adjacent beds during hospitalizations in the Department of Plastic Surgery (PS) in August 2016.

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_{NDM-1}, while the remaining isolates harbored bla_{KPC-2}. Additionally, half of the bla_{KPC-2}-positive isolates co-harbored $bla_{CTX-M-65}$ and bla_{SHV-11}, while all bla_{NDM-1}-positive isolates co-harbored bla_{SHV-71} (Fig. 1).

Genotyping, capsular serotyping, and virulence-associated genes

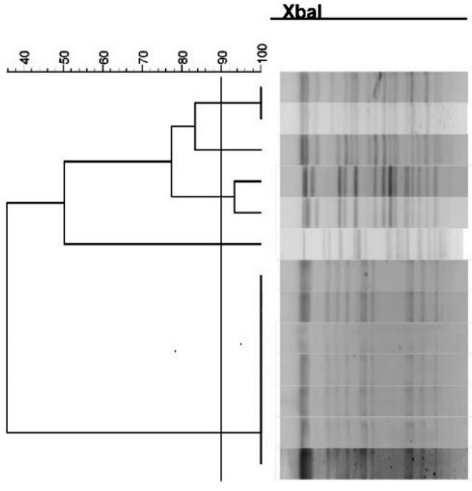
Five PTs and two STs were identified among the CR-HMKP isolates. All bla_{NDM-1}-positive isolates belonged to PT5 and were assigned to ST1764 (*gapA*_5, *infB*_3, *mdh*_1, *pgi*_1, *phoE*_9, *rpoB*_4, *tonB*_283), which is a three-locus variant of ST23 (*gapA*_2, *infB*_1, *mdh*_1, *pgi*_1, *phoE*_9, *rpoB*_4, *tonB*_12). The bla_{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*-64) and four (30.8%) to capsular serotype K47 (*wzi*-209). Of the virulence genes tested, *entB, iucA, kfuBC, mrkD, rmpA2, ureA, wabG, ybtS*, and *ycfM* were detected in all CR-HMKP isolates, and the detection rates for *aerobactin, allS, iroB*, and *rmpA* were 53.8% (7/13), 46.2% (6/13), 53.8% (7/13), and 69.2% (9/13), respectively. The *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 intermediately sensitive (Grade 4), and KPN34 and KPN100 were serum-sensitive (Grade 1). Of the two reference strains, $KPN54798^{H-control}$ was highly resistant (Grade 6), and $KPN49^{L-control}$ was serum-sensitive (Grade 1; Fig. 2).

Galleria mellonella lethality assay

In the *G. mellonella* infection model, time- and dose-dependent deaths were observed for all tested isolates. At the dose of 1×10^7 CFU/larvae, 100.0% mortality was observed in all tested isolates except for the $KPN49^{L-control}$. At the dose of 1×10^6 CFU/larvae, only KPN53 and $KPN54798^{H-control}$ resulted in 100.0% larval mortality at 24 hours post-infection, whereas KPN11, KPN18,



Isolate	Isolation data*	PT	ST	Serotype(wzi)	Resistance gene	Virulence gene†
KPN18	12-11-2013	1	11	K47(wzi-209)	KPC-2, CTX-M-65, SHV-11	allS
KPN19	12-11-2013	1	11	K47(wzi-209)	KPC-2, CTX-M-65, SHV-11	allS
KPN11	11-01-2013	2	11	K47(wzi-209)	KPC-2, CTX-M-65, SHV-11	allS
KPN100	07-06-2017	3	11	K64(wzi-64)	KPC-2, CTX-M-90	allS, rmpA
KPN104	07-09-2017	3	11	K64(wzi-64)	KPC-2, CTX-M-90	allS, rmpA
KPN34	04-07-2015	4	11	K47(wzi-209)	KPC-2, CTX-M-65	allS
KPN53	07-09-2016	5	1764	K64(wzi-64)	NDM-1, SHV-71	Aer, iroB, rmpA
KPN60	07-23-2016	5	1764	K64(wzi-64)	NDM-1, SHV-71	Aer, iroB, rmpA
KPN63	07-24-2016	5	1764	K64(wzi-64)	NDM-1, SHV-71	Aer, iroB, rmpA
KPN69	07-29-2016	5	1764	K64(wzi-64)	NDM-1, SHV-71	Aer, iroB, rmpA
KPN68	07-29-2016	5	1764	K64(wzi-64)	NDM-1, SHV-71	Aer, iroB, rmpA
KPN72	07-31-2016	5	1764	K64(wzi-64)	NDM-1, SHV-71	Aer, iroB, rmpA
KPN74	08-04-2016	5	1764	K64(wzi-64)	NDM-1, SHV-71	Aer, iroB, rmpA

Fig. 1. Pulse-field gel electrophoresis (PFGE) dendrograms, genotype, serotype, resistance and virulence genes of the CR-HMKP isolates. The isolates that exhibited PFGE dendrograms with more than 90% similarity are considered one pulsotype (PT). *The isolation date is listed as month-day-year; †Only partial results are shown. Of the other virulence genes tested, *entB, iucA, kfuBC, mrkD, rmpA2, ureA, wabG, ybtS,* and *ycfM* were detected in all isolates, and the *magA* gene was not detected in any of them.
Abbreviations: Aer, *aerobactin*; KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Delhi metallo-β-lactamase; ST, sequence type.

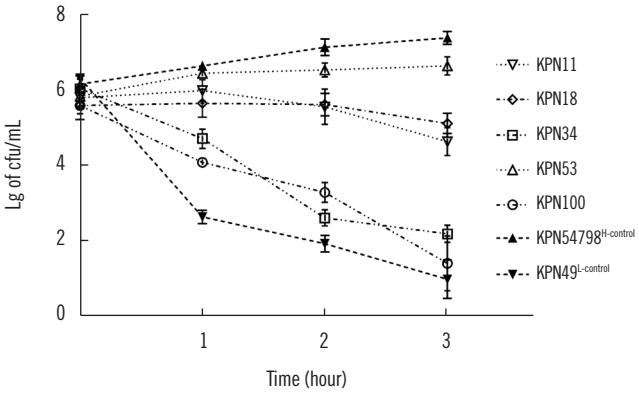


Fig. 2. Serum killing assays of the tested isolates. Data are presented as mean±SE, and log_{10}-transformed values were utilized to normalize the data (N=3 for each isolate).
Abbreviations: H-control, hypervirulence control; L-control, low-virulence control; Lg, log_{10} -transformed values.

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
KPN54798H–control	4.81±0.11
KPN49L–control	5.97±0.14

*Data are presented as mean±SD; †The $lgLD_{50}$ value of KPN53 did not differ from that of KPN54798H–control (P=0.059 by the one-sample t-test) and is significantly lower than those of the other CR-HMKP isolates and KPN49L–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).

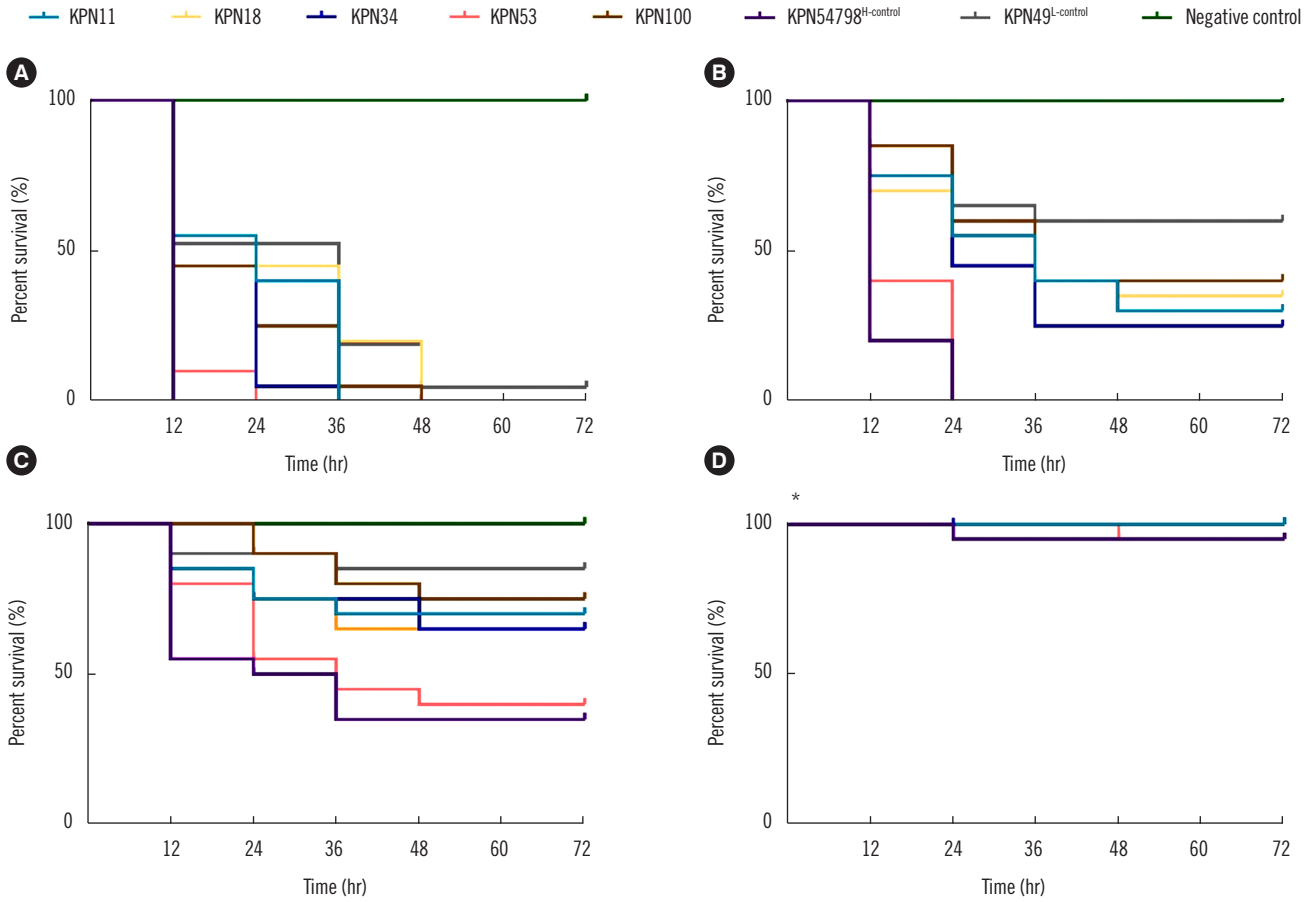


Fig. 3. Survival curves for *G. mellonella* larvae inoculated with 1×10^7 (A), 1×10^6 (B), 1×10^5 (C), and 1×10^4 (D) colony-forming units of the tested isolates, and the data shown are from a single representative experiment out of three repeats. *The curves of KPN11, KPN18, KPN34, KPN100, KPN49L-control and negative control are completely overlapping.
Abbreviations: H-control, hypervirulence control; L-control, low-virulence control.

https://doi.org/10.3343/alm.2019.39.2.167

KPN34, KPN100, and KPN49$^{L-control}$ 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$^{L-control}$ ($P<0.01$ by the log-rank test) (Fig. 3). Additionally, KPN53 had a lower $lgLD_{50}$ than the other isolates except KPN54798$^{H-control}$ ($P<0.05$ 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_{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, *rmpA* and *rmpA2*, 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 *rmpA/rmpA2* 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 $lgLD_{50}$ value in *G. mellonella* model was significantly lower than that of the other isolates. According to a previous study, the LD_{50}s of most clinical *K. pneumoniae* isolates in the *G. mellonella* model were 10^5 CFU/larva [36], higher than those observed for KPN53. Moreover, the capsular gene *wabG*, the type 1 and 3 fimbriae genes *fimH* and *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.

Acknowledgements

This work was supported by grant 81673242 from the National Natural Science Foundation of China and grant 2016QK036 from the Planned Scientific Program of Health and Family Planning Commission of Anhui Province. We thank the physicians involved in collection of the clinical data.

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Supplemental Data Table S1. Clinical and microbiological characteristics of the two reference strains

	KPN54798$^{H-control}$	KPN49$^{L-control}$
Clinical characteristics		
Patient's clinical disease	Hepatapostema	Cholangitis
Specimen	Pus	Bile
Infection type	Community-acquired	Hospital-acquired
Genotype		
MLST	ST23	ST11
Serotype	K1	K47
MIC of antimicrobial agents (μg/mL)		
AMK	1	32
ATM	2	4
CAZ	1	> 128
CIP	1	32
CRO	2	> 32
ETP	1	> 32
FEP	2	> 64
GEN	4	32
IPM	0.5	> 32
LVX	1	32
MEM	0.25	> 32
TZP	16	> 512
Resistance mechanisms		
Carbapenem resistance gene	None	bla_{NDM-1}
Virulence-associated features		
String test	+	–
Virulence gene profiles		
Aer	+	–
allS	–	–
entB	+	+
fimH	+	+
iroB	+	–
iucA	+	–
kfuBC	+	–
magA	+	–
mrkD	+	+
rmpA	+	–
rmpA2	+	–
ureA	+	–
wabG	+	–
ybtS	+	–
ycfM	+	–

Abbreviations: *Aer, aerobactin*; AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; ETP, ertapenem; FEP, cefepime; GEN, gentamicin; H-control, hypervirulence control; IPM, imipenem; L-control, low-virulence control; LVX, levofloxacin; MEM, meropenem; MIC, minimum inhibitory concentration; MLST, multilocus sequence typing; TZP, piperacillin-tazobactam.

https://doi.org/10.3343/alm.2019.39.2.167