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ORIGINAL RESEARCH

Diversity and frequency of resistance and virulence genes in *bla*$_{KPC}$ and *bla*$_{NDM}$ co-producing *Klebsiella pneumoniae* strains from China

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Background: Emergence of *bla*$_{KPC}$ and *bla*$_{NDM}$ co-producing *Klebsiella pneumoniae* strains have led to the limited therapeutic options for clinical treatment. Understanding the diversity and frequency of resistance and virulence genes of these isolates is of great significance.

Purpose: The aim of this study is to research the diversity and frequency of resistance and virulence genes in the *bla*$_{KPC}$ and *bla*$_{NDM}$ co-producing *Klebsiella pneumoniae* strains.

Methods and Results: In this study, 117 *K. pneumonia* strains were isolated from China, and among of which, 24 were found to be *bla*$_{KPC}$ and *bla*$_{NDM}$ co-producing with significant resistance against almost all the commonly used antibiotics. Additionally, 4 strains were hypermucoviscous and 8 showed high serum resistance. Overall, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *tetA* and *sul1* resistance genes found in 100% of the isolates, followed by *bla*$_{TEM}$ (95.8%), *oqxA/B* (91.7%), *qnrB* (87.5%), *aac(6')Ib-cr* (83.3%), *bla*$_{DHA}$ (79.2%), *rmtB* (66.7%), *qnrS* (54.2%), *cat*(54.2%), *floR* (50.0%), *sul2* (45.8%) *cmlA* (20.8%)and*bla*$_{CMY}$ (8.33%), respectively. What' more, seven *bla*$_{CTX-M}$ subtypes [*bla*$_{CTX-M-14}$ (n=18), *bla*$_{CTX-M-3}$(n=11), *bla*$_{CTX-M-65}$ (n=4), *bla*$_{CTX-M-15}$ (n=3), *bla*$_{CTX-M-28}$ (n=2), *bla*$_{CTX-M-55}$ (n=2), *bla*$_{CTX-M-22}$ (n=1)] and six *bla*$_{SHV}$ subtypes [*bla*$_{SHV-12}$(n=16), *bla*$_{SHV-11}$ (n=4), *bla*$_{SHV-2a}$(n=1), *bla*$_{SHV-1}$(n=1), *bla*$_{SHV-38}$ (n=1) and *bla*$_{SHV-28}$(n=1)] were detected. The frequency of virulence genes was as follows: 100% for *entB*, *ybtS* and *irp*, 95.8% for *mrkD*, 91.66% for *fimH*, 79.2% for *iutA*, 62.5% for *iroBCDE*, *aerobactin* and *kfu*, 66.7% for *allS*, 45.8% for wcaG, 37.5% for *rmpA*, 20.8% for *pagO* and 16.7% for *magA*.

Conclusion: From this study, we concluded that the *bla*$_{KPC}$ and *bla*$_{NDM}$ co-producing *Klebsiella pneumoniae* strains have a high diversity and frequency of resistance and virulence genes. This study may offer hospitals important information about the control of infections caused by *bla*$_{KPC}$ and *bla*$_{NDM}$ co-producing *Klebsiella pneumoniae.*

Keywords: *Klebsiella pneumoniae*, *bla*$_{NDM}$, *bla*$_{KPC}$, resistance genes, virulence factors

Introduction

Carbapenemase-producing bacteria can hydrolyse carbapenems and most other β-lactam antibiotics which pose significant challenges to clinical diagnosis and treatment. *Klebsiella pneumoniae* carbapenemase (KPC) and Metallo-B-Lactamases (*bla*$_{NDM}$) are the two major groups of carbapenemases that produced by the most of Carbapenemase-Resistant *Enterobacteriaceae* strains (CRE). The *bla*$_{KPC}$ and *bla*$_{NDM}$ genes are commonly found in CRE strains in recent years.[1–3] Those type of the

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carbapenem resistance genes and other resistance genes including the key Extended-Spectrum β-lactamases (ESBLs) genes (bla_{CTX-M}, bla_{SHV} and bla_{TEM}), the fluoroquinolone resistance genes (*qnrA, qnrB, qnrS, oqxA/B*), aminoglycoside resistance genes (*rmtA, rmt*B and *rmt*C), chloramphenicol resistance genes (*cat, floR, cmlA, cfr*) and tetracycline resistance genes (*tetA, tetB, tetC*) are carried by the same strain and resulting in high resistance to almost all kinds of antibiotics.[4–7] The more worrisome is hypervirulent *K. pneumoniae* strains (hvKP) emergency sharply in recent years, especially the carbapenemase-producing hvKP related infections in immunocompromised patients which is a serious threat to the patients.[8–11]

More and more researchers report that HvKP strains are characterized a number of virulence factors including *aerobactin* (encodes high-affinity iron chelators), *rmpA* (regulators of mucoid phenotype), *wcaG* (involved in the biosynthesis of the outer core lipopolysaccharide), *allS* (associated with allantoin metabolism), *kfu* (responsible for an iron uptake system), *yptS, irp* (yersiniabactin biosynthesis) and *iroBCDN* (salmochelin biosynthesis), *entB* (catecholate siderophore), *fimH* and *mrkD* (fimbrial adhesin, which mediate binding to the extracellular matrix to form the biofilm), *pagO* (involved in liver abscess formation by liver abscess-Kp.[9,12–15]

Understanding the diversity and frequency of resistance and virulence genes of these isolates is of great significance to disease prevention and control. For offer hospitals important information about the control of infections caused by bla_{KPC} and bla_{NDM} co-producing *K. pneumoniae*. In this study, we mainly present the diversity and frequency of resistance and virulence genes in the bla_{KPC} and bla_{NDM} co-producing *K. pneumoniae*.

Materials and methods

Isolates collection and screening of bla_{KPC} and bla_{NDM} genes

A total of 117 non-repetitive *K. pneumonia* strains were isolated from sputum, cerebrospinal fluid, wound, and urine samples for routine examination between Aug. 2016 and Sept.2018 at several hospitals in Sichuan, Henan, Fujian province of China. These isolates were identified by VITEK2 Compact System (bioMérieux, France) and 16sRNA sequencing. *K. pneumoniae* ATCC700603 was used as the control strain for the species identification and antimicrobial susceptibility test. The bla_{KPC} and bla_{NDM} detection were performed according to our previous work by PCR.[9,16]

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of the bla_{KPC} and bla_{NDM} co-producing *K. pneumoniae* strains were performed according to the recommendations of the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017). Antimicrobial agents (Oxoid, England) used used in this study included CXM (cefuroxime axetil), TZP (piperacillin-tazobactam), CAZ (ceftazidime), CRO (ceftriaxone), IPM (imipenem), MEM (meropenem), ATM (aztreonam), AMK (amikacin), CIP (ciprofloxacin), CHL (chloramphenicol), TMP-SMZ (trimethoprim/sulfamethoxazole). E. coli strain ATCC 25922 was used as quality control.[17]

Hypermucoviscosity, biofilm formation and serum killing assay

The hypermucoviscosity phenotype of 24 *K. pneumonia* was detected by string test.[18] The colonies were cultured on blood agar plate overnight at 37°, stretched by a bacteriology inoculation loop. The strain formed a viscous string of >5 mm was designated as hypermucoviscous. Biofilm formation assay was performed by crystal violet staining assay.[9] Biofilm formation in each well was measured by microplate reader (Bio-Rad, US) at optical density (OD) 595 nm. The susceptibility of the *K. pneumoniae* isolates to human serum was explored by an established method.[19] Briefly, *K. pneumoniae* strains were inoculated into LB Broth Medium and incubated at 37 °C with shaking until the logarithmic phase was reached (T=4 h, OD600=0.6). 25 μL of diluted culture (containing 10^6 CFU of bacteria) and 75 μL human serum were then added into a 10×75 mm Falcon polypropylene tube and incubated at 37 °C with shaking. Viable counts were checked at 0, 1, 2, and 3 h. The response to serum killing in terms of viable counts was scored on six grades as described previously method.[20]

ERIC-PCR

Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) method was used to evaluate the genetic diversity of the 24 isolates, as previously described using the primers.[21] The PCR products were loaded on a 1% agarose gel with the gelred at 90 V for 40 mins, and the banding patterns were analyzed by gel imaging and analysis system. To determine the similarity rate among the acquired outcomes, Genetic diversity were analyzed using

the unweighted pair-group method with arithmetic mean (UPGMA) and isolates with ≥80% similarity were treated as a single cluster.[22]

Detection of resistance and virulence genes

By using PCR, the carriage of carbapenemase-encoding genes (bla_{VIM}, bla_{GES}, bla_{DIM}, bla_{GIM}, bla_{SPM} and bla_{AIM}),[23] ESBL-encoding genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$ and $bla_{CTX-M-9}$),[7] AmpC β-lactamase genes (bla_{DHA}, bla_{CMY}),[24] 16 s rRNA methylase genes (*rmtA, rmtB and rmtC*),[25] sulfonamides resistance genes (*sul1, sul2* and *sul3*), chloramphenicol resistance genes (*cmlA, floR* and *catB*), multiresistance gene (*cfr*), tigecycline resistance gene (*tetA, tetB and tetC*)[26,27] and quinolone resistance genes (*qnrA, qnrB, qnrS, aac(6′)-Ib-cr, qepA and oqxAB*)[28–30] were detected as described previously. PCR assays were also used to assess the capsular serotypes (*K1, K2, K5, K20, K54* and *K57*)[31] and fourteen virulence genes (*magA, rmpA, allS, wcaG, ybtS, kfu, iroBCDE, entB, irp, iutA, aerobactin, mrkD, fimH and* pagO).[12–14,31,32] PCR amplicons were sequenced by Shanghai Sangon Bioengineering Company. Sequences were analyzed by the BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers used were shown in Table S1.

Results

Antimicrobial susceptibility, hypermucoviscosity, serotyping, biofilm, serum resistance assay and ERIC-PCR typing

A total of 24 bla_{KPC} and bla_{NDM} co-producing strains were screen from117 non-repetitive *K. pneumonia* strains. All the isolates were resistant to piperacillin-tazobactam, cefuroxime axetil, ceftazidime, ceftriaxone, imipenem, meropenem and aztreonam (Table 1). Among the 24 bla_{KPC} and bla_{NDM} co-producing strains, 16.7% (n=4) were the K1 type, while the K2, K5, K20, K57 and K54 serotype were not found (Figure 1). String test showed that 4 (KP103L, KP48L, KP97L, KP36L) bla_{KPC} and bla_{NDM} co-producing *K. pneumoniae* isolates were hypermucoviscous. Biofilm formation was observed in all the 24 strains, with values of OD595 nm ranged from 0.33 to 2.70, whereas the mean value of the negative control wells is 0.168. Serum killing assay showed that 33.3% (n=8) of the strains were high serum resistance (Grade 5 or Grade 6). Analysis of genetic linkage among isolates by ERIC-PCR showed 34–100% similarity among 24 isolates (Table 2). Genetic diversity was established among 24 bla_{KPC} and bla_{NDM} co-producing *K. pneumoniae* isolates by detecting 15 different ERIC fingerprints with the similarity cutoff of 80% (Table 2).

Diversity and frequency of resistance and virulence gene

As shown in Table 1, all isolates (100%, n=24) carried the resistance gene bla_{SHV}, bla_{CTX-M}, *tetA* and *sul1*, followed by bla_{TEM} (95.8%), *oqxA/B* (91.7%), *qnrB* (87.5%), *aac(6')Ib-cr* (83.3%), bla_{DHA}(79.2%), *rmtB* (66.7%), *qnrS* (54.2%), *cat* (54.2%), *floR* (50.0%), *sul2* (45.8%) *cmlA* (20.8%) and bla_{CMY} (8.33%), respectively. While the carbapenemase encoding genes bla_{GES}, bla_{VIM}, bla_{AIM}, bla_{GIM}, bla_{DIM} were not detected in any of those strains. Regarding the bla_{CTX-M} group (Table 2; Supplement Sequences), the most widespread subtype was $bla_{CTX-M-14}$, which was found in 75% (n=18) of the tested isolates, followed by $bla_{CTX-M-3}$ in 45.8% (n=11), $bla_{CTX-M-65}$ in 16.7% (n=4), $bla_{CTX-M-15}$ in 12.5% (n=3), $bla_{CTX-M-28}$ in 8.3% (n=2), $bla_{CTX-M-55}$ in 8.3% (n=2), $bla_{CTX-M-22}$ in 4.2% (n=1). In addition, there are 17 isolates carried two subtypes of bla_{CTX-M}. And the majority of the 8 isolates carried $bla_{CTX-M-14}$ co-existing with $bla_{CTX-M-3}$, while 2 isolates co-carried $bla_{CTX-M-14}$ and $bla_{CTX-M-65}$ (Table 2). Regarding the bla_{SHV} group, bla_{SHV-12} (66.7%; n=16) was the most prevalent bla_{SHV} in those 24 bla_{KPC} and bla_{NDM} co-producing strains, followed by bla_{SHV-11} in 16.7% (n=4), bla_{SHV-2a}, bla_{SHV-1}, bla_{SHV-38} and bla_{SHV-28} in 4.2% (n=1) (Table 2).

Diversity and frequency of virulence genes

The prevalence and distribution of virulence factors are given in Table 2. All strains carried the *ybtS, entB* and *irp* gene. 95.8% (n=23) strains harbored *mrkD* gene, 91.6% (n=22) strains harbored *fimH* gene,79.2% (n=19) strains contained *iutA* gene, 66.7% (n=16) strains carried *allS* gene, 62.5% (n=15) strains carried *iroBCDE, aerobactin* and *kfu* gene, 45.8% (n=11) strains contained *wcaG* gene, 37.5% (n=9) strains involved *rmpA* gene, 20.8% (n=5) strains involved *pagO* gene and 16.7% (n=4) carried *magA* gene.

Discussion

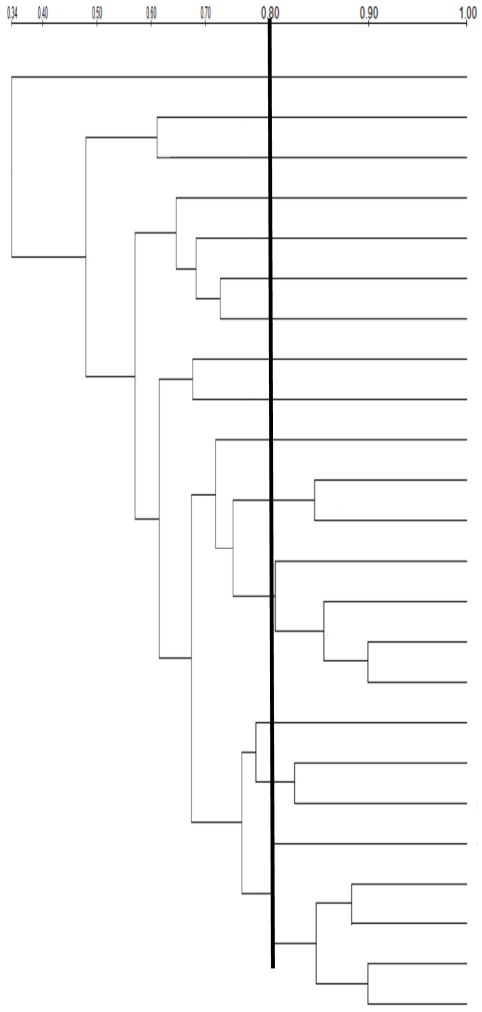
The prevalence of co-carried bla_{NDM} and bla_{KPC} in a single bacterial isolate in hospitals has led to heightened concerns because often makes the isolate an extremely drug-resistant variant.[2,3] In this study, 117 non-repetitive *K. pneumonia* strains were isolated from China, and among of which, 24

Table 1 The antibiotic resistance phenotype profile and positive rate of the resistance gene of the isolates

	Antibiotic Resistance phenotype profile	Resistance gene																	
		*bla*NDM	*bla*KPC	*bla*CTX-M	*bla*SHV	*bla*TEM	*bla*DHA	*bla*CMY	sul1	sul2	rmtB	catB	flor	cmlA	qnrB	qnrS	oqxA/B	*aac(6′)-Ib-cr*	tetA
Kp6L	TZP /CXM/CAZ/CRO/IMP/MEM/ATM/AMK/TMP-SMZ/TZP	✓	✓	✓	✓		✓		✓	✓	✓				✓		✓		✓
Kp32L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/TGC/TMP-SMZ	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓			✓	✓		✓	✓
Kp5L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/TGC	✓	✓	✓	✓	✓	✓		✓		✓				✓	✓	✓	✓	✓
Kp50L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/TMP-SMZ	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓			✓		✓		✓
Kp22L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/CHL/TMP-SMZ	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓	✓	✓	✓		✓	✓	✓
Kp49L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/TGC	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	✓		✓	✓	✓	✓	✓
Kp42L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/CIP	✓	✓	✓	✓	✓	✓		✓			✓			✓		✓	✓	✓
Kp93L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/TMP-SMZ	✓	✓	✓	✓	✓			✓	✓	✓	✓	✓				✓	✓	✓
Kp11L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/TGC/CHL	✓	✓	✓	✓	✓	✓		✓		✓	✓	✓		✓	✓	✓	✓	✓
Kp105L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/CIP/TGC/TMP-SMZ	✓	✓	✓	✓	✓			✓	✓		✓	✓		✓	✓	✓	✓	✓
Kp103L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/TGC/TMP-SMZ	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓	✓			✓	✓	✓	✓
Kp31L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP	✓	✓	✓	✓	✓	✓		✓		✓	✓			✓		✓	✓	✓
Kp48L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/TGC	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓			✓	✓	✓	✓	✓
Kp87L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/CIP	✓	✓	✓	✓	✓	✓		✓			✓			✓		✓	✓	✓
Kp104L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/CIP/TGC/CHL	✓	✓	✓	✓	✓	✓		✓			✓	✓	✓	✓	✓	✓		✓
Kp20L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/TGC/CHL	✓	✓	✓	✓	✓	✓		✓		✓	✓	✓		✓	✓	✓	✓	✓
Kp116L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/CHL/TMP-SMZ	✓	✓	✓	✓	✓			✓	✓	✓	✓	✓		✓		✓	✓	✓
Kp29L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/TGC/CHL	✓	✓	✓	✓	✓	✓		✓		✓	✓		✓	✓	✓		✓	✓
Kp12L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/TMP-SMZ	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓			✓		✓	✓	✓
Kp36L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/CIP/CHL/TMP-SMZ	✓	✓	✓	✓	✓			✓	✓		✓	✓	✓	✓		✓	✓	✓
Kp97L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/CHL/TMP-SMZ	✓	✓	✓	✓	✓			✓	✓		✓		✓			✓		✓
Kp9L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/AMK/CIP/TGC/CHL	✓	✓	✓	✓	✓	✓		✓		✓	✓	✓		✓	✓	✓	✓	✓
Kp40L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/CIP/TGC	✓	✓	✓	✓	✓	✓		✓			✓			✓	✓	✓	✓	✓
Kp13L	TZP/CXM/CAZ/CRO/IMP/MEM/ATM/CIP/TGC/CHL	✓	✓	✓	✓	✓	✓		✓			✓	✓		✓	✓	✓	✓	✓
Positive rate		100%	100%	100%	100%	95.8%	79.17%	8.33%	100%	45.8%	66.7%	54.2%	50%	20.8%	87.5%	54.2%	91.7%	83.3%	100%

Note: The green check represent the positive while the blank is the negative.

Abbreviations: TZP, piperacillin-tazobactam; CXM, cefuroxime axetil; CAZ, ceftazidime; CRO, ceftriaxone; IPM, imipenem; MEM, meropenem; ATM, aztreonam; AMK, amikacin; CIP, ciprofloxacin; CHL, chloramphenicol; TMP-SMZ, trimethoprim/sulfamethoxazole.



Isolates	bla_{CTX-M} group	bla_{SHV} group
Kp6L	$bla_{CTX-M-15}$	bla_{SHV-11}
Kp32L	$bla_{CTX-M-28}$	$bla_{SHV-2a,}$
Kp5L	$bla_{CTX-M-3}$, $bla_{CTX-M-14}$	bla_{SHV-12}
Kp50L	$bla_{CTX-M-14}$	bla_{SHV-12}
Kp22L	$bla_{CTX-M-3}$, $bla_{CTX-M-14}$	bla_{SHV-12}
Kp49L	$bla_{CTX-M-14}$	bla_{SHV-12}
Kp42L	$bla_{CTX-M-3}$, $bla_{CTX-M-14}$	bla_{SHV-11}
Kp93L	$bla_{CTX-M-14}$	bla_{SHV-12}
Kp11L	$bla_{CTX-M-3}$, $bla_{CTX-M-14}$	bla_{SHV-11}
Kp105L	$bla_{CTX-M-3}$, $bla_{CTX-M-14}$	bla_{SHV-12}
Kp103L	$bla_{CTX-M-14}$, $bla_{CTX-M-65}$	bla_{SHV-12}
Kp31L	$bla_{CTX-M-14}$, $bla_{CTX-M-65}$	bla_{SHV-11}
Kp48L	$bla_{CTX-M-3}$, $bla_{CTX-M-14}$	bla_{SHV-12}
Kp87L	$bla_{CTX-M-14}$	bla_{SHV-12}
Kp104L	$bla_{CTX-M-14}$, $bla_{CTX-M-15}$	bla_{SHV-12}
Kp20L	$bla_{CTX-M-3}$, $bla_{CTX-M-65}$	bla_{SHV-12}
Kp116L	$bla_{CTX-M-3}$	bla_{SHV-12}
Kp29L	$bla_{CTX-M-3}$, $bla_{CTX-M-14}$	bla_{SHV-12}
Kp12L	$bla_{CTX-M-14}$, $bla_{CTX-M-22}$	bla_{SHV-1}
Kp36L	$bla_{CTX-M-15}$, $bla_{CTX-M-65}$	bla_{SHV-28}
Kp97L	$bla_{CTX-M-3}$, $bla_{CTX-M-14}$	bla_{SHV-38}
Kp9L	$bla_{CTX-M-3}$, $bla_{CTX-M-55}$	bla_{SHV-12}
Kp40L	$bla_{CTX-M-14}$, $bla_{CTX-M-28}$	bla_{SHV-12}
Kp13L	$bla_{CTX-M-3}$, $bla_{CTX-M-55}$	bla_{SHV-12}

Figure 1 The dendrogram of ERIC-PCR fingerprints and diversity of the ESBLs genotypes.
Notes: The dendrogram of ERIC-PCR fingerprints was constructed using the Dice coefficient and the unweighted pair-group method with arithmetic mean (UPGMA) and the diversity of the ESBLs (bla_{CTX-M} group and bla_{SHV} group) genotypes.

were found to be bla_{KPC} and bla_{NDM} co-producing with significant resistance against almost all the commonly used antibiotics. This results showed that the positive incidence of the bla_{NDM} and bla_{KPC} co-producing *K. pneumonia* is increasing. The results were expected that all 24 isolates resist almost the all test antibiotic and biofilm formation was observed in all the 24 strains. This is a dangerous situation for antibiotic treatment because the high biofilm formation pathogenic bacteria often involved in hospital infections and always lead to the failure of antibiotic treatments.[9] Additionally, 4 strains were hypermucoviscous and 8 strains showed high serum resistance. To our knowledge, the phenotype of hypermucoviscous, biofilm formation ability and serum resistance were as the virulence evaluation criterion.[18,20] Those results indicated that there are harboring hypervirulent variant of *Klebsiella pneumonia* (hvKp) among the 24 bla_{NDM} and bla_{KPC} co-producing strains. This results suggest that urgent need to enhance clinical awareness and epidemiologic surveillance. Although the genetic diversity was established among 24 bla_{KPC} and bla_{NDM} co-producing *K. pneumoniae* isolates by detecting 15 different ERIC fingerprints with the similarity cutoff of 80%, we should pay more attention about this like strains clonal spread in the hospital.

In recent years, more and more researchers report that the co-carried bla_{NDM} and bla_{KPC} *K. pneumoniae* strains carried a large number of resistance genes, making this isolate highly resistance against almost all the commonly used antibiotics. For example, the bla_{KPC-2} and bla_{NDM-1} co-carriage strain *C. freundii* 112298 existance many resistance genes including the bla_{SHV-12}, $bla_{CTX-M-14}$, *aac (6')-Ib-cr,* bla_{OXA-1}, *catB3, arr-3, fosA3* and *sul*1.[1] The bla_{KPC-2} and bla_{NDM-5} co-carriage strain ZSH6 carried

Table 2 The string test, serotyping, Serum killing and biofilm formation assay and diversity and frequence of the virulence factors of the *bla*$_{KPC}$ and *bla*$_{NDM}$ co-producing *Klebsiella pneumoniae*

	String test	Serotype	Serum resistance	Biofilm formation (OD value)	Virulence factor													
					rmpA	ybtS	mrkD	entB	kfu	wcaG	allS	iutA	aerobactin	magA	fimH	pagO	iroBCDE	irp
Kp6L	—	ND	G1	Weak (0.33)	■	■	■	■	■	■		■			■		■	■
Kp32L	—	ND	G1	Moderate (0.70)	■	■	■	■	■			■	■		■			■
Kp5L	—	K1	G1	Strong (1.79)		■	■	■	■					■	■		■	■
Kp50L	—	ND	G2	Strong (1.95)		■	■	■			■	■	■		■			■
Kp22L	—	ND	G6	Strong (0.80)		■	■	■	■			■	■		■	■		■
Kp49L	—	ND	G6	Strong (0.94)		■	■	■	■	■		■	■		■			■
Kp42L	—	ND	G1	Strong (1.35)		■	■	■	■		■	■	■		■	■		■
Kp93L	—	ND	G3	Weak (0.33)		■	■	■			■		■		■		■	■
Kp11L	—	ND	G1	Strong (1.05)		■	■	■			■		■		■		■	■
Kp105L	—	K1	G2	Strong (1.12)	■	■	■	■	■		■	■	■	■	■		■	■
Kp103L	+	ND	G6	Strong (0.86)		■	■	■			■				■		■	■
Kp31L	—	ND	G1	Strong (1.99)		■	■	■	■		■		■		■		■	■
Kp48L	+	K1	G1	Strong (1.76)		■	■	■		■	■	■	■	■	■		■	■
Kp87L	—	ND	G6	Strong (0.99)		■	■	■		■		■	■		■		■	■
Kp104L	—	ND	G2	Strong (0.98)	■	■	■	■	■	■	■	■			■		■	■
Kp20L	—	ND	G1	Strong (1.09)	■	■	■	■	■	■	■	■			■	■	■	■
Kp116L	—	ND	G1	Strong (2.24)		■	■	■	■			■	■		■		■	■
Kp29L	—	ND	G2	Strong (2.70)	■	■	■	■		■	■		■					■
Kp12L	—	ND	G5	Strong(0.81)	■	■	■	■	■	■	■	■			■		■	■
Kp36L	+	ND	G1	Strong (2.55)		■	■	■	■		■	■			■		■	■
Kp97L	+	ND	G5	Strongp(1.04)		■		■	■	■	■	■			■	■		■
Kp9L	—	K1	G5	Strong (1.04)	■	■	■	■		■	■	■	■	■	■	■		■
Kp40L	—	ND	G6	Strong (0.83)		■	■	■	■	■	■	■			■		■	■
Kp13L	—	ND	G1	Strong (0.74)	■	■	■	■		■	■	■	■		■			■
Positive rate					37.5%	100%	95.8%	100%	62.5%	45.8%	66.7%	79.2%	62.5%	16.7%	91.66%	20.8%	62.5%	100%

Notes: "+": positive, "−": negative; The green check represent the positive while the blank is the negative. Biofilm formation expressed as crystal violet optical density value (OD at 595 nm).
Abbreviations: ND, Not Determination; OD, optical density; G, grade.

twenty resistance genes *bla*$_{KPC-2}$, *bla*$_{NDM-5}$, *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-65}$, *bla*$_{TEM-1}$, *floR, tet*(A), *tet*(B), *dfrA17, aadA5, sul1, mdf*(A), *mph*(A), *erm*(B), *aph*(3′)-*Ia, aph*(3′)-*Ib, aph*(4)-*Ia, aph*(6)-*Id, aac*(3)-*Iva, aac*(3)-*II*d.[3] In this study, we also found that the high frequency and diversity of the resistance gene were emergency in the *bla*$_{KPC-2}$ and *bla*$_{NDM-1}$ co-carriage strains. All 24 isolates carried the *bla*$_{SHV}$, *bla*$_{CTX-M}$, *tetA* and *sul1*, followed by *bla*$_{TEM}$ (95.8%), *oqxA/B*(91.7%), *qnrB*(87.5%), *aac(6')Ib-cr* (83.3%), *bla*$_{DHA}$ (79.2%), *rmtB* (66.7%), *qnrS (*54.2%), *cat* (54.2%), *floR*(50.0%), *sul2* (45.8%) and *cmlA* (20.8%). Particularly the high frequency and diversity of the ESBLs. (*bla*$_{CTX-M}$ group and *bla*$_{SHV}$ group) gene. For the *bla*$_{CTX-M}$ group, there are seven *bla*$_{CTX-M}$ subtypes including (*bla*$_{CTX-M-14}$, *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-65}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-28}$, *bla*$_{CTX-M-55}$ and *bla*$_{CTX-M-22}$) in all 24 strains. Our study showed that *bla*$_{CTX-M\ -14}$ was the most frequent. In addition, there are 17 isolates carried two subtypes of *bla*$_{CTX-M}$. And the majority of the 8 isolates carried *bla*$_{CTX-M-14}$ co-existing with *bla*$_{CTX-M-3}$, while 2 isolates co-carried *bla*$_{CTX-M-14}$ *and bla*$_{CTX-M-65}$ (Table 1). Regarding the *bla*$_{SHV}$ group, *bla*$_{SHV-12}$ (66.7%, n=16) was the most prevalent *bla*$_{SHV}$ subtype in 24 *bla*$_{KPC}$ and *bla*$_{NDM}$ co-producing strains. The threat of the high frequency and diversity of the resistance gene emergency in the *bla*$_{KPC-2}$ and *bla*$_{NDM-1}$ co-carriage strains should be strict surveillance and management, although its resist almost all the commonly used antibiotics.[2]

Besides of the high frequency and diversity of the resistance gene, the virulence genes were also high emergency in 24 *K. pneumoniae* strains. In this study, we found that the frequency of virulence genes (*ybtS, entB, irp, mrkD, fimH*) was similar to most of others researcher's reports. However, the frequency of *wcaG* (45.8%), *allS* (66.7%) and pagO (20.8%) gene was slightly higher than our previous work. This results indicated the frequency of some virulence is rising. The high frequency of virulence factors found in these *bla*$_{NDM}$ and *bla*$_{KPC}$ bacteria is a problem for treatment. Some researchers suggested that molecular typing and virulence gene analysis are powerful tools that can shed light on Klebsiella pneumonia infections.[12,15,33,34] However, in this study, we found that some isolates were high serum resistance (Grade 5 or Grade 6) but the number of the virulence factors was less to some serum resistance strains. This results showed that how to identify the hvKP is still unknown. We suspect that the comprehensive analysis the frequency of the virulence factors, phenotype (biofilm, sting test and serum killing assay) and clinical characteristics maybe a preferable method to identitfy the hvKP strains.

In conclusion, this study demonstrated that the high frequency and diversity of the resistance and virulence factors was in the *bla*$_{NDM}$ and *bla*$_{KPC}$ co-producing *K. pneumoniae* making this strain resistant to almost all antibiotics. This study may offer hospitals important information about the control of infections caused by *bla*$_{KPC}$ and *bla*$_{NDM}$ co-producing *Klebsiella pneumoniae.*

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Disclosure

The authors declare that there are no conflicts of interest in this work.

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