Diversity and frequency of resistance and virulence genes in \( \text{bla}_{\text{KPC}} \) and \( \text{bla}_{\text{NDM}} \) co-producing \textit{Klebsiella pneumoniae} strains from China

\textbf{Background:} Emergence of \( \text{bla}_{\text{KPC}} \) and \( \text{bla}_{\text{NDM}} \) co-producing \textit{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.

\textbf{Purpose:} The aim of this study is to research the diversity and frequency of resistance and virulence genes in the \( \text{bla}_{\text{KPC}} \) and \( \text{bla}_{\text{NDM}} \) co-producing \textit{Klebsiella pneumoniae} strains.

\textbf{Methods and Results:} In this study, 117 \textit{K. pneumonia} strains were isolated from China, and among which, 24 were found to be \( \text{bla}_{\text{KPC}} \) and \( \text{bla}_{\text{NDM}} \) co-producing with significant resistance against almost all the commonly used antibiotics. Additionally, 4 strains were hypermucoviscous and 8 showed high serum resistance. Overall, \( \text{bla}_{\text{SHV}}, \text{bla}_{\text{CTX-M}}, \text{mTetA} \) and \( \text{sul1} \) resistance genes found in 100% of the isolates, followed by \( \text{bla}_{\text{TEM}} \) (95.8%), \( \text{gqxA/B} \) (91.7%), \( \text{qnrB} \) (87.5%), \( \text{acc(6)Ib-cr} \) (83.3%), \( \text{bla}_{\text{OXA-26}} \) (79.2%), \( \text{rmtB} \) (66.7%), \( \text{qnrS} \) (54.2%), \( \text{cat} \) (54.2%), \( \text{floR} \) (50.0%), \( \text{sul2} \) (45.8%) \( \text{cmA} \) (20.8%) and \( \text{bla}_{\text{CMY}} \) (8.3%), respectively.

What more, seven \( \text{bla}_{\text{CTX-M}} \) subtypes \( \text{[bla}_{\text{CTX-M-14}} \) (n=18), \( \text{bla}_{\text{CTX-M-15}} \) (n=1), \( \text{bla}_{\text{CTX-M-25}} \) (n=6), \( \text{bla}_{\text{CTX-M-28}} \) (n=5), \( \text{bla}_{\text{CTX-M-31}} \) (n=4), \( \text{bla}_{\text{CTX-M-35}} \) (n=1), \( \text{bla}_{\text{CTX-M-36}} \) (n=1), \( \text{bla}_{\text{CTX-M-38}} \) (n=1) and six \( \text{bla}_{\text{SHV}} \) subtypes \( \text{[bla}_{\text{SHV-12}} \) (n=16), \( \text{bla}_{\text{SHV-11}} \) (n=4), \( \text{bla}_{\text{SHV-2a}} \) (n=1), \( \text{bla}_{\text{SHV-1}} \) (n=1), \( \text{bla}_{\text{SHV-38}} \) (n=1) and \( \text{bla}_{\text{SHV-26}} \) (n=1) were detected. The frequency of virulence genes was as follows: 100% for \text{entB}, \text{ybtS} and \text{irp}, 95.8% for \text{mrkD}, 91.66% for \text{finH}, 79.2% for \text{iutA}, 62.5% for \text{iroBCDE}, \text{aerobactin} and \text{fku}, 66.7% for \text{alli}, 45.8% for \text{wcaG}, 37.5% for \text{rmpA}, 20.8% for \text{pagO} and 16.7% for \text{magA}.

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

\textbf{Keywords:} \textit{Klebsiella pneumoniae}, \( \text{bla}_{\text{NDM}} \), \( \text{bla}_{\text{KPC}} \), resistance genes, virulence factors

\textbf{Introduction}

Carbapenemase-producing bacteria can hydrolyze carbapenems and most other \( \beta \)-lactam antibiotics which pose significant challenges to clinical diagnosis and treatment. \textit{Klebsiella pneumoniae} carbapenemase (KPC) and Metallo-\( \beta \)-Lactamases (\( \text{bla}_{\text{NDM}} \)) are the two major groups of carbapenemases that produced by the most of Carbapenem-Resistant \textit{Enterobacteriaceae} strains (CRE). The \( \text{bla}_{\text{KPC}} \) and \( \text{bla}_{\text{NDM}} \) genes are commonly found in CRE strains in recent years.\textsuperscript{1–3} Those type of the
carbapenem resistance genes and other resistance genes including the key Extended-Spectrum β-lactamas (ESBLs) genes \(\text{bla}_{\text{CTX-M}}, \text{bla}_{\text{SHV}}\) and \(\text{bla}_{\text{TEM}}\), the fluoroquinolone resistance genes \(\text{qnrA, qnrB, qnrS, qepA/B}\), aminoglycoside resistance genes \(\text{rmtA, rmtB and rmtC}\), chloramphenicol resistance genes \(\text{cat, floR, cmlA, cfr}\) and tetracycline resistance genes \(\text{tetA, tetB, tetC}\) are carried by the same strain and resulting in high resistance to almost all kinds of antibiotics.\(^4\)–\(^7\)

The more worrisome is hypervirulent \(\text{K. pneumoniae}\) 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 \textit{aerobactin} (encodes high-affinity iron chelators), \textit{rpmA} (regulators of mucoid phenotype), \textit{wcaG} (involved in the biosynthesis of the outer core lipopolysaccharide), \textit{allS} (associated with allantoin metabolism), \textit{ku} (responsible for an iron uptake system), \textit{yptS, irp} (yersiniabactin biosynthesis) and \textit{iroBCDN} (salmochelin biosynthesis), \textit{entB} (catecholate siderophore), \textit{fmH} and \textit{mrkD} (fimbrial adhesin, which mediate binding to the extracellular matrix to form the biofilm), \textit{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 \textit{bla}_{\text{KPC}} and \textit{bla}_{\text{NDM}} co-producing \textit{K. pneumoniae}. In this study, we mainly present the diversity and frequency of resistance and virulence genes in the \textit{bla}_{\text{KPC}} and \textit{bla}_{\text{NDM}} co-producing \textit{K. pneumoniae}.

**Materials and methods**

**Isolates collection and screening of \textit{bla}_{\text{KPC}} and \textit{bla}_{\text{NDM}} genes**

A total of 117 non-repetitive \textit{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. \textit{K. pneumoniae} ATCC700603 was used as the control strain for the species identification and antimicrobial susceptibility test. The \textit{bla}_{\text{KPC}} and \textit{bla}_{\text{NDM}} detection were performed according to our previous work by PCR.\(^9\)\(^,\)\(^16\)

**Antimicrobial susceptibility testing**

Antimicrobial susceptibility testing of the \textit{bla}_{\text{KPC}} and \textit{bla}_{\text{NDM}} co-producing \textit{K. pneumoniae} strains were performed according to the recommendations of the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017). Antimicrobial agents (Oxoid, England) used in this study included CXM (cefuroxime axetil), TZP (pipercillin-tazobactam), CAZ (ceftazidime), CRO (ceftiraxone), 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 \textit{K. pneumonia} was detected by string test.\(^18\) The colonies were cultured on blood agar plate overnight at 37°C, 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 \textit{K. pneumoniae} isolates to human serum was explored by an established method.\(^19\)

Briefly, \textit{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 \(\mu\)L of diluted culture (containing 10\(^6\) CFU of bacteria) and 75 \(\mu\)L human serum were then added into a 10\(\times\)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 gelled 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
Detection of resistance and virulence genes

By using PCR, the carriage of carbapenemase-encoding genes (bla\textsubscript{VIM}, bla\textsubscript{GES}, bla\textsubscript{DIM}, bla\textsubscript{GIM}, bla\textsubscript{SPM} and bla\textsubscript{AIM}),\textsuperscript{23} ESBL-encoding genes (bla\textsubscript{TEM}, bla\textsubscript{SHV}, bla\textsubscript{CTX-M}, bla\textsubscript{CTX-M-1}, bla\textsubscript{CTX-M-2} and bla\textsubscript{CTX-M-9}),\textsuperscript{7} AmpC β-lactamase genes (bla\textsubscript{DHA}, bla\textsubscript{CMY}),\textsuperscript{24} 16s rRNA methylase genes (tet\textsubscript{A}, tet\textsubscript{B} and tet\textsubscript{C}),\textsuperscript{25} sulfonamides resistance genes (sul\textsubscript{1}, sul\textsubscript{2} and sul\textsubscript{3}), chloramphenicol resistance genes (cml\textsubscript{A}, flo\textsubscript{R} and cat\textsubscript{B}), multiresistance gene (cfr), tigecycline resistance gene (tet\textsubscript{A}, tet\textsubscript{B} and tet\textsubscript{C}),\textsuperscript{26,27} and quinolone resistance genes (qnr\textsubscript{A}, qnr\textsubscript{B}, qnr\textsubscript{S}, aac(6’)-Ib-cr, qep\textsubscript{A} and oqxAB)\textsuperscript{28–30} were detected as described previously. PCR assays were also used to assess the capsular serotypes (K1, K2, K5, K20, K34 and K57),\textsuperscript{31} and fourteen virulence genes (mag\textsubscript{A}, rmp\textsubscript{A}, all\textsubscript{S}, wca\textsubscript{G}, ybt\textsubscript{S}, kfu, iro\textsubscript{BCDE}, ent\textsubscript{B}, iro, iut\textsubscript{A}, aerobactin, mrk\textsubscript{D}, fim\textsubscript{H} and pag\textsubscript{O}).\textsuperscript{12–14,31,32} PCR amplicons were sequenced by Shanghai Sangon Biotechnology 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\textsubscript{KPC} and bla\textsubscript{NDM} co-producing strains were screen from 117 non-repetitive K. pneumoniae strains. All the isolates were resistant to piperacillin-tazobactam, cefuroxime axetil, ceftazidime, ceftriaxone, imipenem, meropenem and aztreonam (Table 1). Among the 24 bla\textsubscript{KPC} and bla\textsubscript{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\textsubscript{KPC} and bla\textsubscript{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\textsubscript{KPC} and bla\textsubscript{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\textsubscript{SHV}, bla\textsubscript{CTX-M}, tet\textsubscript{A} and sul\textsubscript{I}, followed by bla\textsubscript{TEM} (95.8%), oqxA/B (91.7%), qnr\textsubscript{B} (87.5%), aac(6’)-Ib-cr (83.3%), bla\textsubscript{DHA} (79.2%), rmt\textsubscript{B} (66.7%), qnr\textsubscript{S} (54.2%), cat (54.2%), flo\textsubscript{R} (50.0%), sul2 (45.8%) cml\textsubscript{A} (20.8%) and bla\textsubscript{CMY} (8.3%), respectively. While the carbapenemase encoding genes bla\textsubscript{GES}, bla\textsubscript{VIM}, bla\textsubscript{AMM}, bla\textsubscript{GIM}, bla\textsubscript{SPM} were not detected in any of those strains. The prevalence of co-carried ESBL-encoding genes (Table 2; Supplement Sequences), the most widespread subtype was bla\textsubscript{CTX-M-14, which was found in 75% (n=18) of the tested isolates, followed by bla\textsubscript{CTX-M-3} in 45.8% (n=11), bla\textsubscript{CTX-M-65} in 16.7% (n=4), bla\textsubscript{CTX-M-15} in 12.5% (n=3), bla\textsubscript{CTX-M-28} in 8.3% (n=2), bla\textsubscript{CTX-M-55} in 8.3% (n=2), bla\textsubscript{CTX-M-22} in 2.0% (n=1). In addition, there are 17 isolates carried two subtypes of bla\textsubscript{CTX-M- and the majority of the 8 isolates carried bla\textsubscript{CTX-M-14} co-existing with bla\textsubscript{CTX-M-3, while 2 isolates co-carried bla\textsubscript{CTX-M-14} and bla\textsubscript{CTX-M-65} (Table 2). Regarding the bla\textsubscript{SHV} group, bla\textsubscript{SHV-12} (66.7%; n=16) was the most prevalent bla\textsubscript{SHV} in those 24 bla\textsubscript{KPC} and bla\textsubscript{NDM} co-producing strains, followed by bla\textsubscript{SHV-11} in 16.7% (n=4), bla\textsubscript{SHV-2a}, bla\textsubscript{SHV-1}, bla\textsubscript{SHV-38} and bla\textsubscript{SHV-2b} 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 ybt\textsubscript{S}, ent\textsubscript{B} and iro\textsubscript{A} gene. 95.8% (n=23) strains harbored mrk\textsubscript{D} gene, 91.6% (n=22) strains harbored fim\textsubscript{H} gene, 79.2% (n=19) strains contained iut\textsubscript{A} gene, 66.7% (n=16) strains carried all\textsubscript{S} gene, 62.5% (n=15) strains carried iro\textsubscript{BCDE}, aerobactin and kfu gene, 45.8% (n=11) strains contained wca\textsubscript{G} gene, 37.5% (n=9) strains involved rmp\textsubscript{A} gene, 20.8% (n=5) strains involved pag\textsubscript{O} gene and 16.7% (n=4) carried mag\textsubscript{A} gene.

Discussion

The prevalence of co-carried bla\textsubscript{NDM} and bla\textsubscript{KPC} in a single bacterial isolate in hospitals has led to heightened concerns because often makes the isolate an extremely drug-resistant variant.\textsuperscript{2,3} In this study, 117 non-repetitive K. pneumoniae 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 |  |
|---------------------------------------|-----------------|-----|
|                                       | blacem | blacev | blaczsm | blasov | blaxsm | blaxev | sul1 | sul2 | mttB | catB | flor | mlaA | qnrB | qnrS | ogxA/B | acrB (6') | tetA |
| Kp6L                                  |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp32L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp5L                                  |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp50L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp22L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp49L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp42L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp93L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp11L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp105L                                |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp103L                                |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp31L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp48L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp87L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp104L                                |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp20L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp116L                                |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp29L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp12L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp36L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp97L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp9L                                  |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp40L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |
| Kp13L                                 |        |        |          |        |        |        |      |      |      |      |      |      |      |      |      |        |        |      |

**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 represents 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.
were found to be \textit{bla}\textsubscript{KPC} and \textit{bla}\textsubscript{NDM} co-producing with significant resistance against almost all the commonly used antibiotics. This results showed that the positive incidence of the \textit{bla}\textsubscript{NDM} and \textit{bla}\textsubscript{KPC} co-producing \textit{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. 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. Those results indicated that there are harboring hypervirulent variant of \textit{Klebsiella pneumonia} (hvKp) among the 24 \textit{bla}\textsubscript{NDM} and \textit{bla}\textsubscript{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 \textit{bla}\textsubscript{KPC} and \textit{bla}\textsubscript{NDM} co-producing \textit{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 \textit{bla}\textsubscript{NDM} and \textit{bla}\textsubscript{KPC} \textit{K. pneumoniae} strains carried a large number of resistance genes, making this isolate highly resistant against almost all the commonly used antibiotics. For example, the \textit{bla}\textsubscript{KPC-2} and \textit{bla}\textsubscript{NDM-1} co-carrying strain \textit{C. freundii} 112298 existance many resistance genes including the \textit{bla}\textsubscript{SHV-12}, \textit{bla}\textsubscript{CTX-M-14}, \textit{aac (6)\textsuperscript{Ib-cr}}, \textit{bla}\textsubscript{OXA-1}, \textit{catB3}, \textit{arr-3}, \textit{fosA3} and \textit{sul}\textsuperscript{1}. The \textit{bla}\textsubscript{KPC-2} and \textit{bla}\textsubscript{NDM-5} co-carriage strain ZSH6 carried.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Isolates & \textit{bla}\textsubscript{CTX-M} group & \textit{bla}\textsubscript{SHV} group \\
\hline
Kp6L & \textit{bla}\textsubscript{CTX-M-15} & \textit{bla}\textsubscript{SHV-11} \\
Kp32L & \textit{bla}\textsubscript{CTX-M-28} & \textit{bla}\textsubscript{SHV2a} \\
Kp5L & \textit{bla}\textsubscript{CTX-M-3} , \textit{bla}\textsubscript{CTX-M-14} & \textit{bla}\textsubscript{SHV-12} \\
Kp50L & \textit{bla}\textsubscript{CTX-M-14} & \textit{bla}\textsubscript{SHV-12} \\
Kp22L & \textit{bla}\textsubscript{CTX-M-3} , \textit{bla}\textsubscript{CTX-M-14} & \textit{bla}\textsubscript{SHV-12} \\
Kp49L & \textit{bla}\textsubscript{CTX-M-14} & \textit{bla}\textsubscript{SHV-12} \\
Kp42L & \textit{bla}\textsubscript{CTX-M-3} , \textit{bla}\textsubscript{CTX-M-14} & \textit{bla}\textsubscript{SHV-11} \\
Kp93L & \textit{bla}\textsubscript{CTX-M-14} & \textit{bla}\textsubscript{SHV-12} \\
Kp11L & \textit{bla}\textsubscript{CTX-M-3} , \textit{bla}\textsubscript{CTX-M-14} & \textit{bla}\textsubscript{SHV-11} \\
Kp105L & \textit{bla}\textsubscript{CTX-M-3} , \textit{bla}\textsubscript{CTX-M-14} & \textit{bla}\textsubscript{SHV-12} \\
Kp103L & \textit{bla}\textsubscript{CTX-M-14} , \textit{bla}\textsubscript{CTX-M-65} & \textit{bla}\textsubscript{SHV-12} \\
Kp31L & \textit{bla}\textsubscript{CTX-M-14} , \textit{bla}\textsubscript{CTX-M-65} & \textit{bla}\textsubscript{SHV-11} \\
Kp48L & \textit{bla}\textsubscript{CTX-M-3} , \textit{bla}\textsubscript{CTX-M-14} & \textit{bla}\textsubscript{SHV-12} \\
Kp87L & \textit{bla}\textsubscript{CTX-M-14} & \textit{bla}\textsubscript{SHV-12} \\
Kp104L & \textit{bla}\textsubscript{CTX-M-14} , \textit{bla}\textsubscript{CTX-M-15} & \textit{bla}\textsubscript{SHV-12} \\
Kp20L & \textit{bla}\textsubscript{CTX-M-3} , \textit{bla}\textsubscript{CTX-M-65} & \textit{bla}\textsubscript{SHV-12} \\
Kp116L & \textit{bla}\textsubscript{CTX-M-3} & \textit{bla}\textsubscript{SHV-12} \\
Kp29L & \textit{bla}\textsubscript{CTX-M-3} , \textit{bla}\textsubscript{CTX-M-14} & \textit{bla}\textsubscript{SHV-12} \\
Kp12L & \textit{bla}\textsubscript{CTX-M-14} , \textit{bla}\textsubscript{CTX-M-22} & \textit{bla}\textsubscript{SHV-1} \\
Kp36L & \textit{bla}\textsubscript{CTX-M-15} , \textit{bla}\textsubscript{CTX-M-65} & \textit{bla}\textsubscript{SHV-28} \\
Kp97L & \textit{bla}\textsubscript{CTX-M-3} , \textit{bla}\textsubscript{CTX-M-14} & \textit{bla}\textsubscript{SHV-38} \\
Kp9L & \textit{bla}\textsubscript{CTX-M-3} , \textit{bla}\textsubscript{CTX-M-55} & \textit{bla}\textsubscript{SHV-12} \\
Kp40L & \textit{bla}\textsubscript{CTX-M-14} , \textit{bla}\textsubscript{CTX-M-28} & \textit{bla}\textsubscript{SHV-12} \\
Kp13L & \textit{bla}\textsubscript{CTX-M-3} , \textit{bla}\textsubscript{CTX-M-55} & \textit{bla}\textsubscript{SHV-12} \\
\hline
\end{tabular}
\caption{Isolates and their resistance profiles.}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{The dendrogram of ERIC-PCR fingerprints and diversity of the ESBLs genotypes.}
\end{figure}

\textbf{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 (\textit{bla}\textsubscript{CTX-M} group and \textit{bla}\textsubscript{SHV} group) genotypes.
Table 2 The string test, serotyping, Serum killing and biofilm formation assay and diversity and frequency of the virulence factors of the \textit{bla}_{KPC} and \textit{bla}_{NDM} co-producing \textit{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                          | Strong(1.04)    |     |     |     |     |     |     |            |      |      |      |         |     |
| Kp9L        | —        | K1               | G5                          | Strong (1.04)   |     |     |     |     |     |     |            |      |      |      |         |     |
| Kp40L       | —        | ND               | G6                          | Strong (0.83)   |     |     |     |     |     |     |            |      |      |      |         |     |
| Kp13L       | —        | ND               | G1                          | Strong (0.74)   |     |     |     |     |     |     |            |      |      |      |         |     |

**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 $\text{bla}_{\text{KPC-2}}, \text{bla}_{\text{NDM-5}}, \text{bla}_{\text{CTX-M-3}}, \text{bla}_{\text{CTX-M-65}}, \text{bla}_{\text{TEM-1}}, \text{floR}$, $\text{tet}(A)$, $\text{tet}(B)$, $\text{dfrA17}$, $\text{aadA5}$, $\text{su}2$, $\text{mdft}(A)$, $\text{mph}(A)$, $\text{erm}(B)$, $\text{aph}(3')-\text{La}$, $\text{aph}(3')-\text{Ib}$, $\text{aph} (4')-\text{La}$, $\text{aph}(6')-\text{Id}$, $\text{aac}(3')-\text{Iva}$, $\text{aac}(3')-\text{IId}$.$^{3}$ In this study, we also found that the high frequency and diversity of the resistance gene were emergency in the $\text{bla}_{\text{KPC-2}}$ and $\text{bla}_{\text{NDM-1}}$ co-carriage strains. All 24 isolates carried the $\text{bla}_{\text{SHV}}, \text{bla}_{\text{CTX-M}}, \text{tet}A$ and $\text{sul}1$, followed by $\text{bla}_{\text{TEM}}$ (95.8%), $\text{oxaA/B}$ (91.7%), $\text{qnrB}$ (87.5%), $\text{aac(6)Ib-cr}$ (83.3%), $\text{bla}_{\text{DHA}}$ (79.2%), $\text{rmtB}$ (66.7%), $\text{qnrS}$ (54.2%), $\text{cat}$ (54.2%), $\text{floR}$ (50.0%), $\text{sul}2$ (45.8%) and $\text{cmIA}$ (20.8%). Particularly the high frequency and diversity of the ESBLs, ($\text{bla}_{\text{CTX-M}}$ group and $\text{bla}_{\text{SHV}}$ group) gene. For the $\text{bla}_{\text{CTX-M}}$ group, there are seven $\text{bla}_{\text{CTX-M}}$ subtypes including ($\text{bla}_{\text{CTX-M-14}}, \text{bla}_{\text{CTX-M-3}}, \text{bla}_{\text{CTX-M-65}}, \text{bla}_{\text{CTX-M-15}}, \text{bla}_{\text{CTX-M-28}}, \text{bla}_{\text{CTX-M-55}}$ and $\text{bla}_{\text{CTX-M-23}}$) in all 24 strains. Our study showed that $\text{bla}_{\text{CTX-M-14}}$ was the most frequent. In addition, there are 17 isolates carried two subtypes of $\text{bla}_{\text{CTX-M}}$. And the majority of the 8 isolates carried $\text{bla}_{\text{CTX-M-14}}$ co-existing with $\text{bla}_{\text{CTX-M-3}}$, while 2 isolates co-carried $\text{bla}_{\text{CTX-M-14}}$ and $\text{bla}_{\text{CTX-M-65}}$ (Table 1). Regarding the $\text{bla}_{\text{SHV}}$ group, $\text{bla}_{\text{SHV-12}}$ (66.7%, n=16) was the most prevalent $\text{bla}_{\text{SHV}}$ subtype in 24 $\text{bla}_{\text{KPC}}$ and $\text{bla}_{\text{NDM}}$ co-producing strains. The threat of the high frequency and diversity of the resistance gene emergency in the $\text{bla}_{\text{KPC-2}}$ and $\text{bla}_{\text{NDM-1}}$ 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 ($\text{ygiS}$, $\text{entB}$, $\text{trp}$, $\text{mrkD}$, $\text{fimH}$) was similar to most of others researcher’s reports. However, the frequency of $\text{wcaG}$ (45.8%), $\text{allS}$ (66.7%) and $\text{pagO}$ (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 $\text{bla}_{\text{NDM}}$ and $\text{bla}_{\text{KPC}}$ 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 pneumoniae 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 identify the hvKP strains.

In conclusion, this study demonstrated that the high frequency and diversity of the resistance and virulence factors was in the $\text{bla}_{\text{NDM}}$ and $\text{bla}_{\text{KPC}}$ 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 $\text{bla}_{\text{KPC}}$ and $\text{bla}_{\text{NDM}}$ co-producing Klebsiella pneumoniae.

Acknowledgment

This research was funded by the National Natural Science Foundation of China (31500114) and by a grant from the Sichuan Province Science and Technology project (2016JY0223) and Luzhou and Southwest Medical University Natural Science Foundation [2018LZXNYD-ZK51] and Southwest Medical University Science Park funding [2019005].

Disclosure

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

References

1. Feng J, Qiu Y, Yin Z, et al. Coexistence of a novel KPC-2-encoding MDR plasmid and an NDM-1-encoding pNDM-HN380-like plasmid in a clinical isolate of Citrobacter freundii. J Antimicrob Chemother. 2015;70:2987. doi:10.1093/jac/dku445
2. Zheng B, Xu H, Yu X, et al. Identification and genomic characterization of a KPC-2, NDM-1-, and NDM-5-producing Klebsiella michiganensis isolate. J Antimicrob Chemother. 2017;73:536–538.
3. Fu L, Wang S, Zhang Z, et al. Co-carrying of KPC-2, NDM-5, CTX-M-3 and CTX-M-65 in three plasmids with serotype O89. H10 Escherichia coli strain belonging to the ST2 clone in China. Microb Pathog. 2019;128:1–6. doi:10.1016/j.micpath.2018.12.033
4. Freire Martin I, AbuOun M, Reichel R, La Ragione RM, Woodward MJ. Sequence analysis of a CTX-M-1 IncI1 plasmid found in Salmonella 4,5,12:i:-,Escherichia coli and Klebsiella pneumoniae on a UK pig farm. J Antimicrob Chemother. 2014;69:2098–2101. doi:10.1093/jac/dku098
5. Peirano G, Schreckenberger PC, Pitout JD. Characteristics of NDM-1-producing Escherichia coli isolates that belong to the successful and virulent clone ST131. Antimicrob Agents Chemother. 2011;55:2986. doi:10.1128/AAC.01763–10
6. Pan YS, Zong ZY, Yuan L, et al. Complete sequence of pEC012, a multidrug-resistant IncI1 ST71 plasmid carrying blaCTX-M-65, rmtB, fosA3, floR, and oqxAB in an Avian Escherichia coli ST171 strain. Front Microbiol. 2016;7:1117.
7. Tian GB, Wang HN, Zou KL, et al. Detection of CTX-M-15, CTX-M-22, and SHV-2 extended-spectrum beta-lactamas (ESBLs) in Escherichia coli fecal-sample isolates from pig farms in China. Foodborne Pathog Dis. 2009;6:297. doi:10.1089/fpd.2008.0164
Infection and Drug Resistance

Publish your work in this journal

Infection and Drug Resistance is an international, peer-reviewed open-access journal that focuses on the optimal treatment of infection (bacterial, fungal and viral) and the development and institution of preventive strategies to minimize the development and spread of resistance. The journal is specifically concerned with the epidemiology of antibiotic resistance and the mechanisms of resistance development and diffusion in both hospitals and the community. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use.

Submit your manuscript here: https://www.dovepress.com/infection-and-drug-resistance-journal

Liu et al

8. Chao L, Shi J, Guo J. High prevalence of hypervirulent Klebsiella pneumoniae infection in the genetic background of elderly patients in two teaching hospitals in China. Infect Drug Resist. 2018;11:1031–1041. doi:10.2147/IDR.S161075

9. Fu L, Huang M, Zhang X, et al. Frequency of virulence factors in high biofilm formation blaKPC-2 producing Klebsiella pneumoniae strains from hospitals. Microb Pathog. 2018;116:168–172. doi:10.1016/j.micpath.2018.01.030

10. Xu M, Fu Y, Fang Y, et al. High prevalence of KPC-2-producing hypervirulent Klebsiella pneumoniae causing meningitis in Eastern China. Infect Drug Resist. 2019;12:641–653. doi:10.2147/IDR.S191892

11. Struve C, Roe CC, Stegger M, et al. Mapping the evolution of Hypervirulent Klebsiella pneumoniae. mBio. 2015;6:e00630. doi:10.1128/mBio.00630-15

12. Yu WL, Ko WC, Cheng KC, Lee CC, Lai CC, Chuang YC. Comparison of prevalence of virulence factors for Klebsiella pneumoniae liver abscesses between isolates with capsular K1/K2 and non-K1/K2 serotypes. Diagn Microbiol Infect Dis. 2008;62:1. doi:10.1016/j.diagmicrobio.2008.04.007

13. Wasi R, Elkhathib WF, Ashour HM. Molecular typing and virulence analysis of multidrug resistant Klebsiella pneumoniae clinical isolates recovered from Egyptian hospitals. Sci Rep. 2016;6:38929. doi:10.1038/resp38929

14. Ye M, Tu J, Jiang J, et al. Clinical and genomic analysis of liver abscess-causing Klebsiella pneumoniae identifies new liver abscess-associated virulence genes. Front Cell Infect Microbiol. 2016;6:165. doi:10.3389/fcimb.2016.00165

15. Russo TA, Marr CM. Hypervirulent Klebsiella pneumoniae. Clin Microbiol Rev. 2019;32:e00019–19.

16. Liu Y, Zhang H, Zhang X, et al. Characterization of an NDM-19-producing Klebsiella pneumoniae strain harboring 2 resistance plasmids from China. Diagn Microbiol Infect Dis. 2019;93:355–361. doi:10.1016/j.diagmicrobio.2018.11.007

17. Fu L, Tang L, Wang S, et al. Co-location of the blaKPC-2, blaCTX-M-65, rmtB and virulence relevant factors in an IncFII plasmid from an abscess-causing Klebsiella pneumoniae isolate. J Antimicrob Chemother. 2013;68:358. doi:10.1093/jac/bdt456

18. Shon AS, Bajwa RP, Russo TA. Hypervirulent (hypermucoviscous) Klebsiella pneumoniae: a new and dangerous breed. Virulence. 2013;4:107–118. doi:10.4161/viru.22718

19. Podschan R, Sievers D, Fischer A, Ullmann U. Serotypes, hemagglutinins, siderophore synthesis, and serum resistance of Klebsiella isolates causing human urinary tract infections. J Infect Dis. 1993;168:1415–1421. doi:10.1093/infdis/168.6.1415

20. Zhang Y, Zhao C, Wang Q, et al. High prevalence of hypervirulent Klebsiella pneumoniae infection in China: geographic distribution, clinical characteristics, and antimicrobial resistance. Antimicrob Agents Chemother. 2016;60:6115–6120. doi:10.1128/AAC.01127-16

21. Versalovic J, Koeuth T, Lupski JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 1991;19:6823–6831. doi:10.1093/nar/19.24.6823

22. Duan H, Chai T, Liu J, et al. Source identification of airborne Escherichia coli of swine house surroundings using ERIC-PCR and REP-PCR. Environ Res. 2009;109:511–517. doi:10.1016/j.envres.2009.02.014

23. Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. Diagn Microbiol Infect Dis. 2011;70:119. doi:10.1016/j.diagmicrobio.2010.12.002

24. Pérez-González FJ, Hanson ND. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol. 2002;40:2153. doi:10.1128/JCM.40.6.2153-2162.2002

25. Liu Z, Ling B, Zhou L. Prevalence of 16S rRNA methylase, modifying enzyme, and extended-spectrum beta-lactamase genes among Acinetobacter baumanii isolates. J Chemother. 2015;27:207–212. doi:10.1179/1797394714Y.00000000190

26. Aminov RI, Chez-Inn JC, Garrigues N, Mehlboob A, Mackie RI. Detection of tetracycline resistance genes by PCR methods. Methods Mol Biol. 2004;268:3–13.

27. Zhang AY, Wang HN, Tian GB, et al. Phenotypic and genotypic characterisation of antimicrobial resistance in faecal bacteria from 30 Giant pandas. Int J Antimicrob Agents. 2009;33:456. doi:10.1016/j.ijantimicag.2008.10.030

28. Wu JJ, Ko WC, Tsai SH, Yan JJ. Prevalence of plasmid-mediated quinolone resistance determinants QnrA, QnrB, and QnrS among clinical isolates of Enterobacter cloacae in a Taiwanese hospital. Antimicrob Agents Chemother. 2007;51:1223–1227. doi:10.1128/AAC.01195-06

29. Andres P, Lucero C, Soler-Bistue A, et al. Differential distribution of plasmid-mediated quinolone resistance genes in clinical enterobacteria with unusual phenotypes of quinolone susceptibility from Argentina. Antimicrob Agents Chemother. 2013;57:2467–2475. doi:10.1128/AAC.01615-12

30. Kim HB, Wang M, Park CH, Kim EC, Jacoby GA, Hooper DC. oxqAB encoding a multidrug efflux pump in human clinical isolates of Enterobacteriaceae. Antimicrob Agents Chemother. 2009;53:3582–3584. doi:10.1128/AAC.01574-08

31. Turton JF, Perry C, Elgohari S, Hampton CV. PCR characterization and typing of Klebsiella pneumoniae using capsular type-specific, variable number tandem repeat and virulence gene targets. J Med Microbiol. 2010;59:541–547. doi:10.1099/jmm.0.015198-0

32. Compan F, Babosan A, Bissel S, et al. Multiplex PCR for detection of seven virulence factors and K1/K2 capsular serotypes of Klebsiella pneumoniae. J Clin Microbiol. 2014;52:4377–4380. doi:10.1128/JCM.02316-14

33. Min X, Fu Y, Kong H, et al. Bloodstream infections caused by Klebsiella pneumoniae: prevalence of bla KPC, virulence factors and their impacts on clinical outcome. BMC Infect Dis. 2018;18:358. doi:10.1186/s12879-018-3109-6

34. Wang X, Xie Y, Li G, et al. Whole-Genome-Sequencing characterization of bloodstream infection-causing hypervirulent Klebsiella pneumoniae of capsular serotype K2 and ST374. Virulence. 2019;8:510–521. doi:10.1080/21505594.2017.1421894