Molecular Characterization of Klebsiella Pneumoniae Isolated From Sputum

Yuqi Hao
Xinxiang Medical University

Yong'ang Jiang
Xinxiang Medical University

Hafiz Muhammad Ishaq
Muhammad Nawaz Shareef University of Agriculture

Wenke Liu
Xinxiang Medical University

Wei Liao
The Affiliated People's Hospital of Xinxiang Medical University

Ping Chen
Xinxiang Medical University

Fan Yang (✉️ 20.yangf77@163.com)
Xinxiang Medical University

Research Article

Keywords: Klebsiella pneumoniae, antimicrobial resistance, resistant genes, virulent genes, biofilm-forming.

Posted Date: February 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1311414/v1

License: ☑️ This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

**Background:** *K. pneumoniae* is a common opportunistic pathogen responsible in clinical practice and an important pathogen of nosocomial infection. This study aimed to analyze the trend of antimicrobial susceptibility and virulent characteristics of *K. pneumoniae* isolated from sputum in clinics and to provide data for clinical treatment of *K. pneumoniae* infection disease.

**Results:** The resistance rates of the 20 *K. pneumoniae* isolates against 13 antibiotics ranged from 15.0% to 80.0%. The detection rate of ESBLs was up to 55%, and *bla*<sub>SHV</sub> were the most prevalent ESBLs genes. Four strains (25.0%) of *K. pneumoniae* presented HMV phenotype, and 18 strains (90.0%) had the stronger biofilm-forming ability. *wzi, wab<sub>G</sub>, fim<sub>H</sub>, mrk<sub>D</sub>* were the most prevalent virulence genes. 10 strains were present capsule typing and the higher genetic diversity of colonizing *K. pneumoniae* in this region. K<sub>19</sub> exhibited a strong positive correlation with imipenem resistance, and K<sub>1</sub> showed strong correlations with *mag<sub>A</sub>*. HMV phenotype show significantly negative correlations with multidrug-resistant.

**Conclusions:** The antibiotic resistance of *K. pneumoniae* isolated from sputum in this hospital was serious, and these strains show the higher genetic diversity.

**Background**

*Klebsiella pneumoniae (K. pneumoniae)* is a common opportunistic pathogen responsible in clinical practice and an important pathogen of nosocomial infection, leading to various types of infection, such as pneumonia, blood infection, urinary tract infection, and osteomyelitis. The detection rate of Gram-negative pathogens was second only to Escherichia coli. With the widespread use and even abuse of antibiotics in clinic, resistant strains were increasing year by year. In particular, the emergence of multidrug-resistant (MDR) strains led to the failure of clinical antibacterial treatment and the delay of the course of disease. This increased the medical costs of patients and the mortality of inpatients, which has emerged as an urgent threat to public health. A variety of virulence factors are utilized in the survival and immune escape of *K. pneumoniae* infection, such as capsular polysaccharide (CPS), lipopolysaccharide (LPS), fimbriae, iron acquisition and biofilm, etc. *K. pneumoniae* carrying different virulence factors show different pathogenic characteristics and clinical characteristics. The number of studies of clinically isolated *K. pneumoniae* has been increased dramatically in recent years. However, there are limited studies about antibiotic resistance and molecular characteristics of *K. pneumoniae* isolated from sputum. Here we identify the antibiotic resistance and molecular characteristics of *K. pneumoniae* isolated from clinical sputum. This study aimed to obtain a better comprehension of the molecular epidemiological characteristics of *K. pneumoniae* in this region, which is of great clinical significance for the prevention and control of *K. pneumoniae* infection and transmission.

**Results**

*Antimicrobial susceptibility testing*
The resistance of 20 strains of *K. pneumoniae* to 13 commonly used antibiotics was shown in Table 1. Overall, the antibiotic test range of the 20 strains ranged from 15.0% to 80.0%, with multidrug-resistant strains up to 70.0%. The strains showed the highest resistance to PIP (80.0%), followed by CTX (65.0%) and CIP (65.0%), and the lowest resistance to PIT (15.0%), followed by IPN (20.0%) and CFX (25.0%). The range of other antibiotics tested was around 50.0%.

**Table 1. Antibiotic resistance profiles of *K. pneumoniae* isolated from sputum**

| Antibiotic agents | Resistance against 13 antibiotics |
|-------------------|----------------------------------|
|                   | Number of strains (n=20) | Resistant rates% |
| CAZ               | 10                       | 50.0             |
| CTX               | 13                       | 65.0             |
| CFX               | 5                        | 25.0             |
| PIP               | 16                       | 80.0             |
| AZT               | 9                        | 45.0             |
| PIT               | 3                        | 15.0             |
| CHL               | 9                        | 45.0             |
| IPN               | 4                        | 20.0             |
| DOX               | 8                        | 40.0             |
| K                 | 8                        | 40.0             |
| GAT               | 10                       | 50.0             |
| LVF               | 9                        | 45.00            |
| CIP               | 13                       | 65.0             |
| MDR               | 14                       | 70.0             |

**ESBL phenotype and resistance genes of *K. pneumoniae***

Table 2 describes the distribution of antibiotic resistance genes in clinical *K. pneumoniae*. ESBL-producing strains were prevalent in the region, with a detection rate of 55.0%, among which *bla*<sub>SHV</sub> was the most widely distributed strain, while the other two strains were less detected. Only 3 *bla*<sub>KPC</sub> positive strains of carbapenase genes were detected, but no other carbapenase genes were detected. Among plasmid-mediated quinolone resistance (*PMQR*) genes, *qnr<sub>A</sub>* was not detected, while *oqx<sub>AB</sub>* was distributed in almost all strains, *aac* and *qnr<sub>B</sub>* were scattered, with detection rates of 40.0% and 25.0%, respectively. As shown in Table S1, *bla*<sub>CTX-M</sub>+*bla*<sub>TEM</sub>+*aac*+*oqx<sub>AB</sub>* strains were widely distributed (30.0%),
all of which were multidrug-resistant. 2/3 showed co-production of \( \text{bla}_{KPC}+\text{bla}_{SHV}+\text{oqx}_{AB} \) among \( \text{bla}_{KPC} \)-producing strains.

**Table 2. Distribution of antibiotic resistance genes and ESBL phenotype in 20 strains of *K. pneumoniae***

| Resistance genes | Distribution                     |
|------------------|----------------------------------|
|                  | Number of strains (n=20) | Resistant rates (%) |
| ESBL phenotype   | 11                             | 55.0                |
| \( \text{bla}_{SHV} \) | 12                             | 60.0                |
| \( \text{bla}_{CTX-M} \) | 7                              | 35.0                |
| \( \text{bla}_{TEM} \) | 8                              | 40.0                |
| \( \text{bla}_{KPC} \) | 3                              | 15.0                |
| \( \text{bla}_{NDM} \) | 0                              | 0.0                 |
| \( \text{bla}_{OXA-48} \) | 0                              | 0.0                 |
| \( \text{bla}_{IMP} \) | 0                              | 0.0                 |
| \( qnr_A \)      | 0                              | 0.0                 |
| \( qnr_B \)      | 5                              | 25.0                |
| \( \text{oqx}_{A} \) | 18                             | 90.0                |
| \( \text{oqx}_{B} \) | 19                             | 95.0                |
| \( aac \)        | 8                              | 40.0                |
| \( \text{bla}_{KPC}+\text{bla}_{SHV}+\text{oqx}_{AB} \) | 2 | 10.0 |
| \( \text{bla}_{CTX-M}+\text{bla}_{TEM}+\text{aac}+\text{oqx}_{AB} \) | 6 | 30.0 |

**Virulence phenotypes and genes of *K. pneumoniae***

The results of the crystal violet staining showed that all 20 *K. pneumoniae* could form biofilms, and 18 strains (90.0%) had strong biofilms. String test showed that 5 strains (25.0%) presented the HMV phenotype. Table 3 characterizes the distribution of the virulence genes of *K. pneumoniae* strains isolated from sputum. The LPS-encoding \( wab_G \) gene and CPS encoding \( wzi \) were present in all strains; The genes encoding type I \( \text{fim}_H \) and type III \( \text{mrk}_D \) were 90% and 85%, respectively, and was pathogenic genes.
iucA (35.0%) and iutA (30.0%) were the genes encoding and regulating the aerobactin. The positive rates of mucoviscosity-associated genes rmpA and magA were 25.0% and 10.0%, respectively.

Table 3. Distribution of virulence genes of K. pneumoniae from sputum

| Distribution | Virulence phenotypes | Virulence genes |
|--------------|----------------------|-----------------|
|              | BF       | HMV | wzi | rmpA | magA | wabG | fimH | mrkD | iucA | iutA |
| Number of positive strains | 20 | 5    | 20  | 5  | 2   | 20  | 18  | 17  | 7    | 6    |
| positive rates (%) | 100 | 25  | 100.0 | 25.0 | 10.0 | 100.0 | 90.0 | 85.0 | 35.0 | 30.0 |

Abbreviation: BF = Biofilm, HMV = Hypermucoviscous phenotype.

Capsular serotypes and wzi phylogenetic tree

Among 20 K. pneumoniae isolates, 10 strains were identified as K1 (2 strains), K19 (2 strains), K17, K23, K24, K46.61, K14.64, and K43 (1 strain). Other 10 strains were unknown capsular serotyping, which belong wzi 168, wzi84, wzi679, wzi206, wzi187, wzi209, wzi150, wzi516, wzi275, and wzi401. Each sequence corresponds to a distinct wzi allele, as indicated. The allele number is followed by the corresponding capsular types. The phylogenetic tree showed more branches and higher genetic diversity (Fig.1).

Figure 1. Phylogenetic tree of wzi sequence of clinical isolates of K. pneumoniae

Analysis of the correlations

Pearson’s correlation analysis was used to assess the existence the potential correlation between antibiotic-resistant phenotypes and molecular characteristics of K. pneumoniae strains. Figure 2 suggests that the MDR was significantly positively correlated with blaTEM (r = 0.535, p < 0.05), blaCTX-M (r = 0.480, p < 0.05) and aac (r = 0.535, p < 0.05), and negative correlations with HMV (r = 0.630, p < 0.05) and rmpA (r = 0.630, p < 0.05). HMV exhibited positive correlations with rmpA (r = 1.000, p < 0.001), iucA (r = 0.545, p < 0.05), iutA (r = 0.630, p < 0.05) and magA (r = 0.577, p < 0.05). SBF was positively correlated with mrkD (r = 0.327, p < 0.05). Some molecular types displayed positive or negative correlations with several resistance and virulence genes. K19 and CR (r = 0.667, p < 0.001), blaKPC (r = 0.793, p < 0.001) displayed a strong positive correlation with BF (r = -1.000, P < 0.001), and a negative correlation with mrkD (r = 0.327, p < 0.05). K1 was completely correlated with magA (r = -1.000, p < 0.001). According to Figure 2, virulence genes rmpA and iutA (r = 0.630, p < 0.05) showed significantly positive correlation, and all antibiotic resistance phenotypes had huge correlation with their genes.

Figure 2. Pearson’s correlation analysis between phenotypes and genotypes of K. pneumoniae
Discussion

Reports of antibiotic resistance of *K. pneumoniae* isolated from sputum have increased year by year.\(^6\)\(^-\)\(^8\) But studies on molecular characteristics are still rare. In this study, 20 strains of *K. pneumoniae* (19 from sputum and 1 from alveolar lavage fluid) were collected from the affiliated people’s hospital of Xinxiang Medical University, Henan Province, China. The antibiotic susceptibility results showed that *K. pneumoniae* isolated from sputum showed high resistance to 15 antimicrobials. The resistance rate to PIP was the highest (80.0%), IPN and PIT were lower (20.0%, 15.0% respectively), ESBLs-producing strains accounted for up to 55.0%. These results are consistent with literature reports.\(^6\)\(^,\)\(^9\)\(^-\)\(^10\) In previous studies, resistance rates of cephalosporins and quinolone antibiotics were less than 40.0%, and carbapenems were less than 10.0%. In this study, *K. pneumoniae* strains exhibited a higher prevalence of resistance than in the above study. We analyzed that the differences might be related to the molecular characteristics and prevalent antibiotic usage habits in the region. Therefore, combined with the antimicrobial resistance gene of this study and the investigation of antibiotics in patients, further analysis of the reasons.

Many antibiotic resistance genes are involved in the occurrence of drug resistance of *K. pneumoniae*, and we studied the mechanism of antibiotic resistance commonly used in clinical practice. \(\beta\)-lactam antibiotics are the most widely used antibiotics in clinic, among which ESBLs production is the main mechanism of antibiotic resistance. In this study, ESBLs-producing strains were absolutely multiresistant, which supports the conclusion that the resistance of ESBLs-producing bacteria was enhanced, consistent with the studies report.\(^11\)\(^-\)\(^12\) \(\texttt{bla}_{\textit{SHV}}\) is more widely distributed among genes encoding ESBLs resistance, which may be the main mechanism of ESBLs epidemic distribution in this region. Carbapenems are the last line of defense for the treatment of ESBLs-producing Gram-negative pathogen infection. The main mechanism of antibiotic resistance is the production of carbapenemases that can hydrolyze penicillins, cephalosporins, monocyclic \(\beta\)-lactamides, aminoglycosides, quinolones, and other antibiotics.\(^13\) Only 3 *K. pneumoniae* of KPC-producing carbapenemases were detected in this study, which was resistant to all antibiotics except chloramphenicol and doxycycline. Details of antibiotics refer to Table S2. Fluoroquinolones are a class of powerful broad-spectrum antibiotics that have been used to treat severe or antibiotic-resistant infections since the end of the 20th century.\(^14\) However, with the widespread use and abuse of antibiotics in recent years, the resistance rate of the pathogen to fluoroquinolones has been increasing, and in this study has reached about 50%. Studies by Liam S. Redgrave et al showed that the prevalence of \(\textit{PMQR}\) genes among strains was not affected by quinolone antibiotics selection,\(^14\) which may be the key to the rapid spread of resistance. In this study, \(\textit{qnr}_A\) was not detected, \(\textit{qnr}_B\) and \(\textit{aac}\) were scattered, while the detection rate of \(\textit{oqx}_{AB}\) was high, which was consistent with some reports.\(^15\)\(^-\)\(^16\)

The emergence of MDR strains has caused great difficulties in the clinical treatment of infection caused by *K. pneumoniae*. The study of MDR mechanism is of great significance for reducing the fatality rate, improving the spread of MDR strains, and delaying the occurrence of pan-resistant strains. In this study, the rate of multidrug-resistant *K. pneumoniae* has reached 70.0%, and antibiotic resistance genes \(\texttt{bla}_{\textit{CTX}}\)-
M, \textit{blaTEM} and \textit{aac} are closely related to multidrug-resistant strains. Moreover, these three genes are plasmid-mediated antibiotic resistance genes,\textsuperscript{17} and strains co-producing three genes account for 30.0%, which may be crucial genes for spreading multiresistant strains in this region. Studies have reported that \textit{PMQR} gene is usually carried on the plasmids of other antibiotic resistance genes, especially ESBLs.\textsuperscript{15} Therefore, we speculated that the above-mentioned epidemic resistance genes in this region may be located in the same plasmid and spread, the specific mechanism needs further study.

The main virulence factors of \textit{K. pneumoniae} included capsular (CPS), lipopolysaccharide (LPS), pili, and iron carriers, which are also the main factors leading to the characteristics of hypervirulent \textit{K. pneumoniae} (hvKP).\textsuperscript{18} Many genes encoding these virulence factors have been reported, and some representative genes were selected for amplification in this study. The results showed that HMV phenotype was strongly correlated with \textit{rmp\textsubscript{A}}, \textit{map\textsubscript{A}}, \textit{iuc\textsubscript{A}} and \textit{iut\textsubscript{A}}, which was consistent with some studies: \textit{rmp\textsubscript{A}} and \textit{mag\textsubscript{A}} regulated the expression of CPS, which was closely related to the high HMV phenotype of \textit{K. pneumoniae}.\textsuperscript{18-19} \textit{iuc\textsubscript{A}} codes aerobactin, and \textit{iut\textsubscript{A}} codes aerobactin transporter,\textsuperscript{20} closely related to high virulence.\textsuperscript{19} \textit{wzi} and \textit{wab\textsubscript{G}} have been detected in 20 strains of \textit{K. pneumoniae}, \textit{wzi} encodes outer membrane protein Wzi and is involved in the attachment of the CPS to the outer membrane\textsuperscript{21} and \textit{wab\textsubscript{G}} gene encodes Wab\textsubscript{G} protein and is involved in the synthesis of LPS.\textsuperscript{22} The detection rates of \textit{fim\textsubscript{H}} and \textit{mrk\textsubscript{D}} genes encoding type I and III pili were 90% and 85%, respectively. The detection rate of the above four genes is high, which is consistent with the report.\textsuperscript{6,15,23} All \textit{K. pneumoniae} in this study can form biofilms, which may be related to the source of samples. Type I and III pili are crucial factors for \textit{K. pneumoniae} colonization, and \textit{fim\textsubscript{H}} encodes Fim\textsubscript{H} adhesion molecule at the tip of type 1 pili,\textsuperscript{23} which is closely related to urinary tract infection.\textsuperscript{24} \textit{mrk\textsubscript{D}} encodes adhesive subunit located at the tip of type 3 hair\textsuperscript{25} and mediates the binding of \textit{K. pneumoniae} with organ cells and lung tissues,\textsuperscript{26} which is related to lung infection. Studies have shown that type 3 pili have been proved to play a major role in the formation of biofilms,\textsuperscript{24} which is consistent with the results of this study.

Correlation analysis showed a strong correlation between the distribution of \textit{iut\textsubscript{A}} and \textit{rmp\textsubscript{A}}, which may be related to the fact that both are located in the same \textit{pLVPK} plasmid.\textsuperscript{27} Studies have shown that \textit{mag\textsubscript{A}} encodes CPS polymerase specific to K1 type,\textsuperscript{28} K1 serotype and \textit{mag\textsubscript{A}} also showed an absolute correlation in this study. Serotype K19 was strongly correlated with \textit{bla\textsubscript{KPC}}, which might be the main epidemic serotype of carbapenase in the local area. The antibiotic resistance of \textit{K. pneumoniae} was generally negatively correlated with the distribution of virulence characteristics, in which the multiresistant strains exhibited negative correlations with virulence phenotypes and genes, and the HMV phenotype was negatively correlated with antibiotic resistance phenotypes and genes, which was consistent with the literature reports.\textsuperscript{29} KP13 is a multiresistant strain of capsular serotype K1, carrying three types of ESBLs antibiotic-resistant genes in this study, which seriously endangers public health. Studying the prevalence mechanism of antibiotic resistance and virulence factors has crucial implications.
Conclusions

In conclusion, our study describes antibiotic resistance and virulence characteristics of 20 Strains of *K. pneumoniae* isolated from sputum from Henan, China. The results showed that local *K. pneumoniae* had severe drug resistance, and antibiotic resistance genes *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub> and *aac* were the main mechanisms. Virulence genes *magA*, *rmpA*, *iucA* and *iutA* were crucial influencing factors of HMV. In general, virulence exhibited significant negative correlations with antibiotic resistance factors, and some strains exist at the same time. Therefore, investigate their transmission mechanism to effectively slow down the emergence of such strains and reduce medical costs is necessary.

Methods

Bacterial isolates

A total of 20 *K. pneumoniae* (19 from sputum and 1 from alveolar lavage fluid) were collected from the affiliated people’s hospital of Xinxiang Medical University, Henan Province, China. The isolates were identified according to the National Clinical Examination Procedures, and the isolates were numbered KP1-KP20 in sequence.

Antibiotics and Reagents

Following antibiotics were used in this study: Ceftazidime (CAZ,30 μg), Ceftazidime-clavulanic acid (CAC,30 μg/10 μg), Cefotaxime (CTX,30 μg), Ceftazidime-clavulanic acid (CTC,30 μg/10 μg), Cefoxitin (CFX,30 μg), Aztreonam (AZT,30 μg), Piperacillin-tazobactam (PIT,100 μg/10 μg), Piperacillin (PIP,100 μg), Imipenem (IPN,10 μg), Doxycycline (DOX,30 μg), Chloramphenicol (CHL,30 μg), Ciprofloxacin (CIP,5 μg), Levofloxacin (LVF,5 μg), Gatifloxacin (GAT,5 μg), Kanamycin (KAN,30 μg). LB agar plates and Columbia blood agar plates were purchased from Guangzhou Huankai Microbiology Technology Limited Company. The reagents of PCR were purchased from Kangwei Century Co., Ltd. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The primer sequences are shown in Table 4 and Table 5.

ESBLs Phenotype confirmation tests

ESBLs phenotypes of 20 Strains of *K. pneumoniae* were detected by the combined disc test (CDT) recommended by CLSI 2020. Briefly, the bacteria with turbidity equivalent to 0.5 McFarland standards were swabbed onto MH agar plates containing 30 μg of CTX and CAZ, with and without 10 μg of clavulanic acid, were placed independently, 25 mm apart (center to center) on a lawn culture. The plates were incubated at 37 °C for up to 16-18 h. Isolates were considered ESBL positive if the inhibition zone measured around one of the combination discs was at least 5 mm larger than the corresponding cephalosporin disc.

Antimicrobial susceptibility testing
The sensitivity of *K. pneumoniae* isolates against 13 kinds of antibiotics was determined by the disc diffusion method recommended by CLSI. Drug sensitivity results were determined according to CLSI 2020 criteria. In the antimicrobial sensitivity test results, the strain resistant to more than 3 antibiotics is considered MDR bacteria.

**Amplify antimicrobial resistance genes**

Bacterial DNA template was extracted by the water boiling method. Briefly, bacteria were inoculated and cultured overnight in LB liquid medium at 37 °C. 1 ml bacterial culture was added into the 1.5 mL EP tube, centrifuged at 12,000 RPM for 2 min, and the supernatant was discarded and resuspended in 500 μL water, then centrifuged again under the same conditions, and the supernatant was discarded. Add 200 μL water, boiling at 100 °C, 20-30 min to lysate thallus. Freezing for 10-15 min and centrifugated at 12,000 rmp at 4 °C for 15 min. The supernatant (genomic DNA) was extracted into the new EP tube and stored at -20 °C. The following resistant genes of *K. pneumoniae* were amply by PCR, including encode ESBLs (*bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>), carbapenemase genes (*bla*KPC, *bla*<sub>NDM</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>IMP</sub>), quinolones resistance genes (*qnr*<sub>A</sub>, *qnr*<sub>B</sub>, *oqx*<sub>A</sub>, *oqx*<sub>B</sub>), aminoglycoside resistance gene (*aac*). The primers information of these resistance genes is shown in Table 4.

**Table 4. PCR primers sequence information of antimicrobial resistance genes**
| gene    | primer sequence                        | product size (bp) | Ta opt(°C) | references |
|---------|----------------------------------------|-------------------|------------|------------|
| **bla \textsubscript{CTX-M}** | P1: TCGGGAGGCAGACTGGGTGT  
                           P2: CCTTAGGGTGAGGCCTGGGTGA | 688              | 57.7       |            |
| **bla \textsubscript{TEM}**    | P1: TCGCCGCATACACTATTCTCAG  
                           P2: ACGCTCACC GGCTCCAGATTAT | 445              | 55.1       | 30         |
| **bla \textsubscript{SHV}**    | P1: ATGCGTTATATTCCGCTGTG  
                           P2: TGCTTTGTATTCGGGCAA | 753              | 58.4       | 30         |
| **bla \textsubscript{KPC}**    | P1: ATGTCACTGTATCGCCGTC  
                           P2: TTACTGCCCCGTGGACGCC | 882              | 57.0       |            |
| **bla \textsubscript{IMP}**    | P1: CTTGATGAAGGCGTTTATGT  
                           P2: GCCAAGCCTCTATATTGCGT | 496              | 50.9       |            |
| **bla \textsubscript{NDM}**    | P1: CCAGCTCGACCCAATG  
                           P2: AACGCCGCACAAACG | 564              | 58.8       |            |
| **bla \textsubscript{OXA-48}** | P1: ACATAAATCACAGGGCGTAG  
                           P2: TATAGTCACCATTGGCTTCG | 500              | 51.4       |            |
| **qnr \textsubscript{A}**      | P1: CAAGAGGATTTCCTCAACGAGGCTAG  
                           P2: TCGGCAAAGGTCAGGTACACG | 521              | 58.9       |            |
| **qnr \textsubscript{B}**      | P1: GCACGGAATTTCCTCGGCTGTC  
                           P2: CAACGATGCCTGGGTAGGTT | 500              | 53.4       |            |
| **oqx \textsubscript{A}**      | P1: TCCATACCAACCTCGCTCTCC  
                           P2: AGCGTGGCTTTGAACCTGGC | 529              | 59.8       |            |
| **oqx \textsubscript{B}**      | P1: TGTTGGAGAACGTGACGCTAA  
                           P2: TCGGCCTGGTTGGAACCTGC | 648              | 60.4       |            |
| **aac**                          | P1: AGTACAGCATCGTGACCAACA  
                           P2: CTGAATGCGCTGGCGT GTA | 545              | 55.8       | 18         |

PS: Primer sequences without reference were obtained by primer 5.0 design (same below)

**Determination of Mucinous phenotype**

The mucinous phenotype of *K. pneumoniae* was analyzed by “String-forming test” according to previous. Briefly, *K. pneumoniae* was transferred to Columbia blood plate for overnight culture at 37 °C. Dip the
colony was with the inoculum ring, then lift the inoculum ring. If the adhesive wire formed was larger than 0.5 cm, it was judged as positive; otherwise, it was negative. The strain with high mucilage phenotype was positive in the “String-forming test”.

**Analysis of biofilm formation ability**

Biofilm-forming ability was measured by the determination of adhesion to flat-bottomed microtiter plates (96-well). Briefly, each well of the 96-well microtitration plates was filled with 200 μl sterile broth liquid medium. Bacterial cultures during biofilm formation (18 h incubation) was added to each well(1:100 in liquid medium, 200 μl liquid medium). Only liquid medium was used as a negative control. After incubation at 37 °C for 48 h, total cell mass was measured as absorbance at 570 nm (OD₁) while the blank was OD₁₀. Each well was washed three times with PBS, dried for 1 h at 60 °C and stained for 20 min with 200 μl of 1% crystal violet. After the crystal violet solution was removed, each well was washed with PBS 4 times to remove the remaining stain. Air dried in the aseptic processing table for 30 mins, each well was added with 200 μl of 95% ethanol. After vibrating for 30 min, the absorbance was measured at 570 nm (OD₂), while the blank was OD₂₀. The biofilm formation capacity was calculated by B=(OD₂-OD₂₀)/(OD₁-OD₁₀). All the strains were classified based on the adherence capabilities into the following categories: nonbiofilm producers (B < 0.1), weak biofilm producers (B ≥ 0.1), moderate biofilm producers (0.1 < B ≤ 1.0), strong biofilm producers (B > 1.0).

**Analysis capsular serotypes and virulence genes**

Amplification of wzi genes for detection of capsular polysaccharide (antigen K) serotype is a new method for capsular serotyping, which has been used in the laboratory. In this study, wzi primers were provided for expanded PCR amplification according to document and the products were sent to Bioengineering (Shanghai) incorporated company for sequencing. Sequencing results submitted Institut Pasteur website for comparative analysis, obtained strain wzi parting and part of capsular serotyping. Virulence genes were detected by PCR, including encoding capsular polysaccharide (wzi), adhesin (fimH,markD), lipopolysaccharide (wabG), mucous phenotypic related genes (rmpA,magA), and ferritin genes (iucA,iutA). PCR primer information of virulence genes is shown in Table 5.

**Table 5. PCR primer sequence information of virulence genes**
| Gene | Primer sequence | Product size (bp) | $T_a^\text{opt}$ (°C) | References |
|------|----------------|------------------|----------------|------------|
| wzi  | P1:GTGCCGCGAGCGCTTTCTATC TTG GTATTCC  
      | P2:GAGAGCCACTGGTTCCAGAA[CorT]TT[CorG]ACCGC | 580  
      | 55 | 21 |
| magA | P1:GGTGCTCTTTACATCATTGC  
      | P2:GCAATGGCCATTGTCGTTAG | 1282  
      | 58 | 33 |
| rmpA | P1:ACTGGGCTACCTCTGCTTCA  
      | P2:CTTGCATGAGCCATCTTTCA | 516  
      | 58 | 34 |
| iucA | P1: CCCGCTTCCTACTTT  
      | P2: ATTCGCTTCGCTGTCC | 575  
      | 54.8 | |
| iutA | P1:GGCTGGACATCATGGGAACTGG  
      | P2:CGTCGGGAACGGGTAGAATCG | 300  
      | 55 | 34 |
| wabG | P1:ACCATCGGCCATTTGATAGA  
      | P2:CGGACTGGGAGGATGAAATCG | 683  
      | 49 | 34 |
| fimH | P1:TGCTGCTGGGCTGGTGATG  
      | P2:GGGAGGTTGACGGTGACATC | 688  
      | 49 | 34 |
| markD | P1:TTCTGACACAGCGGTCCC  
      | P2:GATACCGGCGCTTTTCGTTAC | 480  
      | 49 | 34 |

**Statistical analysis**

Clustal W 2.1 was used to complete the alignment of wzi gene sequence. Initial phylogenetic trees were constructed using MEGA 7 based on the neighbor-joining method (500 bootstrap replicates) and Jukes-Cantor distance. Pearson correlation analysis was conducted by SPSS software, version 19.0, and data were plotted using Hiplot for correlation Heatmap.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**
Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article (and its additional files).

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by Science and Technology Research Project of Henan Province (grant 182102310553).

Authors' contributions

YH completed the acquisition, analysis, interpretation of data and the creation of new software used in the work, and was a major contributor in writing the manuscript.

YJ completed the methodology, the data curation, the formal analysis and the visualization.

HMI completed language editing and polishing.

Wenke Liu completed the data curation, the resources and the investigation.

Wei Liao completed the conceptualization, the supervision, the validation and the writing - review & editing.

CP completed the supervision, the validation and the writing - review & editing.

FY completed the conceptualization, the funding acquisition, the validation and the writing - review & editing.

All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

References

1. Martin RM, Bachman MA. Colonization, Infection, and the Accessory Genome of Klebsiella pneumoniae. *Front Cell Infect Microbiol*. 2018;8:4.
2. Tantry BA, Rahiman S. Antibacterial resistance and trend of urinary tract pathogens to commonly used antibiotics in Kashmir Valley. *West Indian Med J.* 2012;61(7):703-707.

3. Karaiskos I, Giamarellou H. Multidrug-resistant and extensively drug-resistant Gram-negative pathogens: current and emerging therapeutic approaches. *Expert Opin Pharmacother.* 2014;15(10):1351-1370.

4. Li B, Zhao Y, Liu C, Chen Z, Zhou D. Molecular pathogenesis of Klebsiella pneumoniae. *Future Microbiol.* 2014;9(9):1071-1081.

5. Russo TA, Marr CM. Hypervirulent Klebsiella pneumoniae. *Clin Microbiol Rev.* 2019;32(3):e00001-19.

6. Wyres KL, Nguyen TNT, Lam MMC, et al. Genomic surveillance for hypervirulence and multi-drug resistance in invasive Klebsiella pneumoniae from South and Southeast Asia. *Genome Med.* 2020;12(1):11.

7. Imai K, Ishibashi N, Kodana M, et al. Clinical characteristics in blood stream infections caused by Klebsiella pneumoniae, Klebsiella variicola, and Klebsiella quasipneumoniae: a comparative study, Japan, 2014-2017. *BMC Infect Dis.* 2019;19(1):946.

8. Zhang H, Zhang G, Yang Y, et al. Antimicrobial resistance comparison of Klebsiella pneumoniae pathogens isolated from intra-abdominal and urinary tract infections in different organs, hospital departments and regions of China between 2014 and 2017. *J Microbiol Immunol Infect.* 2021;54(4):639-648.

9. Vuotto C, Longo F, Pascolini C, et al. Biofilm formation and antibiotic resistance in Klebsiella pneumoniae urinary strains. *J Appl Microbiol.* 2017;123(4):1003-1018.

10. Soltani E, Hasani A, Rezaee MA, et al. Virulence characterization of Klebsiella pneumoniae and its relation with ESBL and AmpC beta-lactamase associated resistance. *Iran J Microbiol.* 2020;12(2):98-106.

11. Gautam V, Thakur A, Sharma M, et al. Molecular characterization of extended-spectrum β-lactamases among clinical isolates of Escherichia coli & Klebsiella pneumoniae: A multi-centric study from tertiary care hospitals in India. *Indian J Med Res.* 2019;149(2):208-215.

12. Yazdansetad S, Alkhudhairy MK, Najafpour R, et al. Preliminary survey of extended-spectrum β-lactamases (ESBLs) in nosocomial uropathogen Klebsiella pneumoniae in north-central Iran. *Heliyon.* 2019;5(9):e02349.

13. Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. *Clin Microbiol Rev.* 2007;20(3):440-458.

14. Redgrave LS, Sutton SB, Webber MA, Piddock LJ. Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary success. *Trends Microbiol.* 2014;22(8):438-445.
15. Silva-Sánchez J, Cruz-Trujillo E, Barrios H, et al. Characterization of plasmid-mediated quinolone resistance (PMQR) genes in extended-spectrum β-lactamase-producing Enterobacteriaceae pediatric clinical isolates in Mexico. PLoS One. 2013;8(10):e77968.

16. Yuan J, Xu X, Guo Q, et al. Prevalence of the oqxAB gene complex in Klebsiella pneumoniae and Escherichia coli clinical isolates. J Antimicrob Chemother. 2012;67(7):1655-1659.

17. Wang G, Zhao G, Chao X, Xie L, Wang H. The Characteristic of Virulence, Biofilm and Antibiotic Resistance of Klebsiella pneumoniae. Int J Environ Res Public Health. 2020;17(17):6278.

18. Piekarska K, Wołkowicz T, Zacharczuk K, et al. Co-existence of plasmid-mediated quinolone resistance determinants and mutations in gyrA and parC among fluoroquinolone-resistant clinical Enterobacteriaceae isolated in a tertiary hospital in Warsaw, Poland. Int J Antimicrob Agents. 2015;45(3):238-243.

19. Clegg S, Murphy CN. Epidemiology and Virulence of Klebsiella pneumoniae. Microbiol Spectr. 2016;4(1):10.1128.

20. Russo TA, Olson R, Macdonald U, et al. Aerobactin mediates virulence and accounts for increased siderophore production under iron-limiting conditions by hypervirulent (hypermucoviscous) Klebsiella pneumoniae. Infect Immun. 2014;82(6):2356-2367.

21. Brisse S, Passet V, Haugaard AB, et al. wzi Gene sequencing, a rapid method for determination of capsular type for Klebsiella strains. J Clin Microbiol. 2013;51(12):4073-4078.

22. Izquierdo L, Coderch N, Piqué N, et al. The Klebsiella pneumoniae wabG gene: role in biosynthesis of the core lipopolysaccharide and virulence. J Bacteriol. 2003;185(24):7213-7221.

23. Kline KA, Dodson KW, Caparon MG, Hultgren SJ. A tale of two pili: assembly and function of pili in bacteria. Trends Microbiol. 2010;18(5):224-232.

24. Hornick DB, Thommandru J, Smits W, Clegg S. Adherence properties of an mrkD-negative mutant of Klebsiella pneumoniae. Infect Immun. 1995;63(5):2026-2032.

25. Clegg S, Murphy CN. Epidemiology and Virulence of Klebsiella pneumoniae. Microbiol Spectr. 2016;4(1):10.1128.

26. Paczosa MK, Mecsas J. Klebsiella pneumoniae: Going on the Offense with a Strong Defense. Microbiol Mol Biol Rev. 2016;80(3):629-661.

27. Tang HL, Chiang MK, Liou WJ, et al. Correlation between Klebsiella pneumoniae carrying pLVPK-derived loci and abscess formation. Eur J Clin Microbiol Infect Dis. 2010;29(6):689-698.
28. Lin TL, Yang FL, Yang AS, et al. Amino acid substitutions of MagA in Klebsiella pneumoniae affect the biosynthesis of the capsular polysaccharide. *PLoS One*. 2012;7(10):e46783.

29. Lee CR, Lee JH, Park KS, et al. Antimicrobial Resistance of Hypervirulent Klebsiella pneumoniae: Epidemiology, Hypervirulence-Associated Determinants, and Resistance Mechanisms. *Front Cell Infect Microbiol*. 2017;7:483.

30. Monstein HJ, Ostholm-Balkhed A, Nilsson MV, Nilsson M, Dornbusch K, Nilsson LE. Multiplex PCR amplification assay for the detection of blaSHV, blaTEM and blaCTX-M genes in Enterobacteriaceae. *APMIS*. 2007;115(12):1400-1408.

31. Rode TM, Langsrud S, Holck A, Møretrø T. Different patterns of biofilm formation in Staphylococcus aureus under food-related stress conditions. *Int J Food Microbiol*. 2007;116(3):372-383.

32. Institut Pasteur website. https://bigsdb.pasteur.fr/klebsiella/klebsiella.html Accessed 1 June 2021.

33. Fang CT, Chuang YP, Shun CT, Chang SC, Wang JT. A novel virulence gene in Klebsiella pneumoniae strains causing primary liver abscess and septic metastatic complications. *J Exp Med*. 2004;199(5):697-705.

34. Candan ED, Aksöz N. Klebsiella pneumoniae: characteristics of carbapenem resistance and virulence factors. *Acta Biochim Pol*. 2015;62(4):867-874.

35. Hiplot. https://hiplot.com.cn Accessed 28 August 2021.

**Figures**
Figure 1

Phylogenetic tree of wzi sequence of clinical isolates of *K. pneumoniae*
Figure 2

Pearson's correlation analysis between phenotypes and genotypes of *K. pneumoniae*

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial1.xlsx
• Supplementarymaterial2.xlsx