Infection and Drug Resistance

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ORIGINAL RESEARCH

Relationship Between Drug Resistance Characteristics and Biofilm Formation in Klebsiella Pneumoniae Strains

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Objective: To conduct epidemiological analysis of *Klebsiella pneumoniae* (*K. pneumoniae*) with *hypervirulence*, and to investigate its drug resistance phenotype, *Extended-spectrum β-lactamase* (*ESBLs*) gene, *virulence factor, capsular serotype* and biofilm formation, so as to provide theoretical basis for further understanding of the drug resistance mechanism of *K. pneumoniae* with *hypervirulence*.

Methods: *K. Pneumoniae* were isolated from clinical samples collected from inpatients. All strains were identified by *VITEK2* Compact using fully automatic microbial analyzer, the minimal inhibitory concentration (*MIC*) of antibiotics was determined by microbroth dilution test. The double disk diffusion method was used to detect the production of *ESBLs*, modified carbapenem inactivation method (*mCIM*) was used to detect the production of *carbapenemase*, and *hypermucoviscosity* phenotype was detected by wire drawing test. *PCR* was used to detect *ESBLs* gene, virulence factor and capsular serotype. Crystal violet staining was used to detect the ability of biofilm formation.

Results: The *ESBLs genes* detected in this study included strains *blaTEM* 35 (36.5%), *blaSHV* 51 (53.1%), and *blaCTX-M* 49 (51.0%). Most strains carried multiple *ESBLs genes*, but not all of them produce *ESBLs*. *K1* and *K2* accounted for 14.6% and 11.5% respectively. Most (91.7%) strains carried the *fimH* gene, and the other virulence genes were *ybtS* (53.1%), *entB* (46.9%), *rmpA* (41.7%), *aerobactin* (32.3%), *allS* (15.6%), *kfu* (15.6%). Of all the Klebsiella pneumoniae strains, 33 (34.4%) exhibited ESBLs phenotype, 16 (16.7%) were carbapenemase-producing, and 20 (20.8%) with ESBLs phenotype tested were resistant to all four drugs. The correlation between *ESBLs-producing* strains and biofilm formation was significantly increased compared to strains without *ESBLs* phenotype (P=0.035).

Conclusion: Compared to *hypervirulent Klebsiella pneumoniae* (*hvKP*), classical *Klebsiella pneumoniae* (*cKP*) has a tendency to acquire antibiotic resistance. Our study showed that genes encoding *rmpA, K1* or *K2*, and *kfu* were highly associated with *hvKP*.

Keywords: *Klebsiella pneumoniae*, antimicrobial resistance, hypermucoviscosity, virulence factors, epidemiology

Introduction

In 1920s, it was discovered that certain microorganisms inhibit the growth and reproduction of other microorganisms, a phenomenon known as *antibiotics*. Later, people extracted substances with antibiotic effect from certain microorganisms, namely *antibiotics*. There are many types of antibiotics in clinical use, and the common ones are β-lactams, aminoglycosides, quinolones, sulfonamides, nitrofurans, etc. The bactericidal effect of β-lactam antibiotics is achieved by inhibiting the activity of bacterial peptidoglycan transpeptidase, and it is usually classified into five groups, namely *penicillins, carbapenems, cephalosporins, clavams* and *monolactams*.[1] Aminoglycosides are naturally occurring antibiotics with oligosaccharide structures whose bactericidal effect is a result of their linkage to specific conserved sequences of *16SrRNA* leading to some changes in the *16SrRNA* spatial conformation, which leads to misinterpretation of the genetic code by the associated transfer *RNA* (*tRNA*). Quinolones are broad-spectrum antibiotics used to treat

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infectious diseases caused by Gram-negative bacteria. Quinolones target type II bacterial topoisomerases, which inactivate the processes of *DNA* replication, *DNA* repair, transcription, and superhelical topology, resulting in rapid bacterial cell death. Sulfonamides are synthetic broad-spectrum antibiotics that achieve antibacterial effects by inhibiting the formation of folic and nucleic acids. Nitrofuran antibiotics are a combination of nitro chemical groups and heterocycles that kill bacteria by inactivating enzymes that contribute to *DNA* and/or *RNA* and/or protein and/or cell wall biosynthesis. Over eighty years ago, antimicrobial agents began to be used for the treatment of different infectious diseases, such as urinary tract infections (*UTIs*). However, soon after the introduction of antibiotics there was an increase in the number of drug-resistant strains, leading to the worldwide spread of multidrug and pan-drug strains (*MDR* and *PDR*, respectively), with a projected 10,000,000 deaths per year by 2050, which has serious economic consequences for public health and governments.[2]

Klebsiella pneumoniae is a gram-negative bacillus that can cause serious infections such as liver abscesses, pneumonia, bacteremia and urinary tract infections (*UTIs*) in young people and other healthy people.[3] Currently, pathogenic *K. pneumoniae* can be divided into *hvKP* and *cKP*, with *cKP* and *hvkp* pathogenic forms being difficult to treat due to their antimicrobial resistance genes. At the same time, *K. pneumoniae* is highly predisposed to biofilm formation, making this challenging infection even more severe.[4] It is well known that hospital infections often involve highly biofilm-forming pathogens, such as *Escherichia coli, Pseudomonas aeruginosa*, and *K. pneumoniae*.[5] Biofilms are most notorious for their high level of resistance to antibiotics.[6] Due to the high resistance of biofilms to antibiotics, infections associated with biofilm formation isolates are very difficult to treat. And the concern about biofilm formation of *K. pneumoniae* is related to the colonization of the organism in many medical conditions, while certain virulence factors are also closely related to the ability of biofilm formation.[7] Therefore, we should continue to focus on the virulence profile of multi-drug resistant *K. pneumoniae* during biofilm formation and raise clinical awareness of this new serious threat. In this study, we examined and analyzed the virulence, drug resistance and biofilm formation ability of the isolated *K. pneumoniae* to provide a theoretical reference for further understanding of the drug resistance mechanism of *K. pneumoniae*.

Materials and Methods

Source of Strains

Ninety-six non-replicated strains of *K. pneumoniae* were isolated from sputum, urine, blood, secretions, pleural and ascitic fluid and bile specimens collected at a university teaching hospital (The Affiliated Chaohu Hospital of Anhui Medical University), from August 2018 to June 2019, A total of 96 inpatients, 64 males and 32 females, aged 23 to 93 years, with a mean of 65 years and an interquartile range of 53–76 years, were included in the observation. Blood samples were first tested for bacteria in blood cultures by the *Bact/ALERT®3D* system. Sputum, urine, blood culture positive samples and other samples were placed on blood agar plates and incubated at 37 °C for 16–18 h for bacterial isolation. All strains were identified using a *Vitek II Compact* fully automated microbiology analyzer. Ethical approval was granted by *Affiliated Chaohu Hospital of Anhui Medical University Ethics Committee.*

Instruments and Reagents

Vitek II Compaet fully automatic microbiological analyzer, turbidimeter (*bioMérieux, France*); 96-well polystyrene plate (*Costar, USA*); crystalline violet staining solution (*Beijing Solabao Technology Co., Ltd.*); *MH* agar dry powder, drug-sensitive paper sheets (*Oxoid, UK*); *PCR* reaction reagent, *DNA Marker*, agarose (*Shanghai Biotech Biological Co., Ltd.*); electrophoresis instrument (*Beijing Liuyi Instrument Factory*). Gel imaging system (*BioRad, USA*).

Antibiotic Susceptibility Testing

The antibiotic susceptibility testing was performed using the micro-broth dilution method with reference to the *2019 Clinical and Laboratory Standards Institute* (*CLSI*) guidelines for drug sensitivity analysis, the minimum inhibitory concentration (*MIC*) of the antimicrobial drug is detected. The *MIC* is the lowest concentration at which an antimicrobial drug can completely inhibit bacterial growth.[8] The specific drugs are as follows: piperacillin, ampicillin/sulbactam,

cefazolin, ceftazidime, ceftriaxone, cefotaxime, cefepime, cefoxitin, aminoglutethimide, gentamicin, tobramycin, ciprofloxacin, cotrimoxazole, furantoin (Chinese Institute for the Control of Pharmaceutical Products), imipenem, meropenem (Merck & Co.). *E. coli ATCC 25922* was used as the quality control strain for the drug sensitivity test.

β-Lactamase Identification and String Test

The double disk diffusion method was used to detect whether *ESBLs* were produced and the modified carbapenem inactivation method (*mCIM*) was used to detect whether carbapenemases were produced,[9,10] referring to the *2019 CLSI* operating standard for the specific test methods and procedures. Hypermucosity is a typical characteristic of *hvKP* strains and is commonly used to define *hvKP*. Strains with hypermucoviscous phenotype were positive, defined as *hvKP*; those that are negative were defined as classical *K. pneumoniae*.[11] *Hypermucoviscousity* (*String Test*): fresh colonies cultured overnight on blood agar plates were stretched outward by gently touching them with an inoculating ring, and if there was mucus filament formation and the length was greater than 5 mm, the strain were judged as positive for the *HM* phenotype, ie the strain was hypermucoviscous.[12] *E. coli ATCC 25922* was used as the quality control strain for the above test.

Extraction of Genomic DNA

Pick a single colony from *Mueller-Hinton agar plate* (*MH plate*) with an inoculation loop and place it in an autoclaved *EP tube* with 1mL of sterile distilled water, mix well, then place it on a foam plate and boil it in boiling water for 10 min (lysis of bacteria to release *DNA*), after boiling, mix the top and bottom upside down, immediately put it into −20 °C refrigerator and freeze it for 10 min, then put the Ep tube into a centrifuge at 12,000 rpm for 10 min and the supernatant was aspirated as the amplification template and stored at −20 °C for backup.[13]

Molecular Detection of ESBLs Genes and Virulence Genes

PCR was used to detect common *ESBLs* genes (*blaTEM, blaSHV, blaCTX-M*), capsular serotypes (*K1, K2, K5, K20* and *K57*) and virulence-related genes including mucus phenotype genes (*rmpA*), type 1 and 3 adhesins (*fimH, mrkD*), aerobactin Aerobactin, enterobactin (*entB*) and Yersinabactin (*ybtS*), allantoin regulatory factor (*allS*), iron uptake system (*kfu*), primer sequences were synthesized by *Shanghai Bioengineering Co*. The total reaction system was 50 μL: *Taq PCR Master Mix* 25 μL, bacterial *DNA* template 1 μL, upstream primers and downstream primers 2 μL each, ddH_2O 20 μL. *PCR* reaction conditions: pre-denaturation at 94 °C for 4 min; denaturation at 94 °C for 30 s, primer specific annealing temperature 30 s, extension at 72 °C for 40 s, 32 cycles; extension at 72 °C for 10 min. Amplification products were amplified in a 1.2% agarose gel containing ethidium bromide at 120 V. After electrophoresis for 40 min, the results were observed by gel imager, and the detection (predicted size band) of the target band was considered positive for the tested gene. Samples were amplified using specific primers (Extend, Sao Paulo, Brazil), as in Table 1.

Table 1 Primer Sequences Used in This Study

Target Genes	Primer Sequence (5'–3')	Amplicon Size (bp)	Tm (°C)	Reference
TEM	**F**:CATTTCCGTGTCGCCCTTATTC **R**:CGTTCATCCATAGTTGCCTGAC	800	56.2	[14]
SHV	**F**:CACTCAAGGATGTATTGTG R:TTAGCGTTGCCAGTGCTCG	885	52	[15]
CTX-M	**F**:ACAGCGATAACGTGGCGATG **R**:TCGCCCAATGCTTTACCCAG	216	58.3	[16]
K1	**F**:GGTGCTCTTTACATCATTGC **R**:GCAATGGCCATTTGCGTTAG	1283	53	[17]
K2	**F**:GACCCGATATTCATACTTGACAGAG **R**:CCTGAAGTAAAATCGTAAATAGATGGC	641	54	[17]
K5	**F**:TGGTAGTGATGCTCGCGA **R**:CCTGAACCCACCCCAATC	280	56	[17]

(*Continued*)

Table 1 (Continued).

Target Genes	Primer Sequence (5'–3')	Amplicon Size (bp)	Tm (°C)	Reference
K20	**F**:CGGTGCTACAGTGCATCATT **R**:GTTATACGATGCTCAGTCGC	741	58	[17]
K57	**F**:CTCAGGGCTAGAAGTGTCAT **R**:CACTAACCCAGAAAGTCGAG	1037	56	[17]
rmpA	**F**:ACTGGGCTACCTCTGCTTCA **R**:CTTGCATGAGCCATCTTTCA	535	54.1	[16]
fimH	**F**:TGCTGCTGGGCTGGTCGATG **R**:GGGAGGGTGACGGTGACATC	550	61	[16]
mrkD	**F**:CCACCAACTATTCCCTCGAA **R**:ATGGAACCCACATCGACATT	226	54	[16]
ybtS	**F**:GACGGAAACAGCACGGTAAA **R**:GAGCATAATAAGGCGAAAGA	242	52	[18]
allS	**F**:CCGAAACATTACGCACCTTT **R**:ATCACGAAGAGCCAGGTCAC	508	55	[12]
kfu	**F**:GGCCTTTGTCCAGAGCTACG **R**:GGGTCTGGCGCAGAGTATGC	638	59	[18]
aerobactin	**F**:GCATAGGCGGATACGAACAT **R**:CACAGGGCAATTGCTTACCT	556	54	[12]
entB	**F**:GTCAACTGGGCCTTTGAGCCGTC **R**:TATGGGCGTAAACGCCGGTGAT	400	60	[18]

Assessing Biofilm Formation

The biofilm formation ability was detected by crystalline violet staining method.[15] The strains were inoculated on *MH plates* and incubated at 37 °C for 24 h, and 0.5 *McFarland's* bacterial suspension was prepared and added to 96-well polystyrene plates with 10μL bacterial suspension and 190 μL sterile *LB broth* per well, and three replicate wells were set up with 200 μL *sterile LB broth* as a negative control. The 96-well plate was incubated at 37 °C for 24 h and then removed, washed 3 times with 200 μL sterile distilled water to remove the floating bacteria, added 200 μL of 99% methanol for 15 min, aspirated and discarded the methanol, dried and added 200 μL of 1% crystalline violet dye for 20 min, washed 3 times with sterile distilled water, dried at room temperature and then added 200 μL of 95% ethanol for 10 min to fully dissolve crystalline violet. The absorbance (*OD*) of each well was read at 570 nm using an enzyme marker and measured three times, and the *ODc* value (mean *OD* of negative control + 3×standard deviation) was used as the threshold value. If the *ODc* value is greater than this value, the biofilm is determined to be formed. Referring to the literature criteria,[19] the biofilm forming ability of strains can be classified into 4 categories, $OD \leq ODc$ as negative (-), $ODc < OD \leq 2ODc$ as weak positive (+), $2ODc < OD \leq 4ODc$ as moderate positive (++), and $OD > 4ODc$ as strong positive (+++).

Data and Statistical Analysis

The correlation test was performed using the *chi-square test* (χ^2). Therefore, correlations between antibiotics and bacterial resistance, virulence genes and two types of strains, *hvKP* strains and *ESBL-producing* strains, *ESBLs* genes and resistance phenotypes and biofilm formation were calculated by the *chi-square* (χ^2) *independence test* in *SPSS version 22.0*. *Spearman* correlation analysis was used to calculate the correlation between biofilm formation levels and cephalosporin resistance. Indeed, the chi-square (χ^2) test was suitable to determine the significance of the correlation between two nominal parameters.[20] A P value of <0.05 was considered statistically significant.

Results

Analysis of Strain Sources and Their Biofilm Characteristics

The strains in this study were mainly derived from sputum (54, 56.2%), urine (15, 15.6%), blood (11, 11.5%), secretions (9, 9.4%), thoraco-abdominal fluid (4, 4.2%) and bile (3, 3.1%). In comparison with isolates from different sources, the biofilm formation value was higher for isolates from secretions than from sputum, urine, blood, thoracic and ascitic fluid and bile, but the difference was not statistically significant ($P > 0.05$) (Figure 1).

Antibiotic Resistance Characteristics and Hypervirulent Strains

In this study, microbroth dilution method was used to determine the MIC value of the selected antibiotics against pathogenic bacteria (Table 2). As shown in Table 3, except for *ceftazidime, cefoxitin, aminotrans, imipenem*, and *meropenem, cKP* was significantly more resistant to the above antimicrobial drugs than *hvKP. MDR* strains are bacteria that are simultaneously insensitive to at least one of the three or more classes of antimicrobial drugs.[16] In this study, 53 (55.*2%*) isolates were classified as *MDR*.

Toxicity Genes and Their Distribution Characteristics in *hvKP* and *cKP*

Only one of all 96 *K. pneumoniae* strains did not carry the *mrkD* gene, most (91.7%) strains carried the *fimH* gene, and the other virulence genes were *ybtS* (53.1%), *entB* (46.9%), *rmpA* (41.7%), *aerobactin* (32.3%), *allS* (15.6%), *kfu* (15.6%). *K1* and *K2* were the most common capsular serotypes, accounting for 14.6% and 11.5%, respectively; no *K54* was found. More than half (61.5%) of the strains did not belong to the common capsular serotypes of *K1, K2, K5, K20, K54*, and *K57*. As shown in Table 4, the virulence genes (*entB, rmpA, aerobactin, kfu*) and capsular serotypes (*K1, K2, K57*) of *hvKP* isolates were significantly higher than those of *cKP* ($P < 0.05$). However, *fimH, mrkD, ybtS* genes and *K5, K20* capsular serotypes did not differ between *hvKP* and *cKP* strains. Agarose gel electrophoresis of ESBLs genes and virulence factors are shown in Figure 2.

The genes of *ESBLs* detected in this study including strain *blaTEM* 35 (36.5%), strain *blaSHV* 51 (53.1%), and strain *blaCTX-M* 49 (51.0%), most of *cKP* strains carried multiple *ESBL* genes, and all *hvKP* strains carried *ESBL* genes (Table 5).

Correlation of *hvKP* Strains with *ESBL-Producing* Strains

The correlation of *hvKP* strains with *ESBL-producing* strains are shown in Table 6. *blaTEM, blaSHV*, and *blaCTX-M* were all negatively associated with high mucus phenotype *(P*=0.001) and also highly negatively associated with *entB, rmpA* (*P*<0.001). Except for *blaTEM, blaSHV*, and *blaCTX-M* were significantly negatively associated with *allS, K1* and *K2* genes as well.

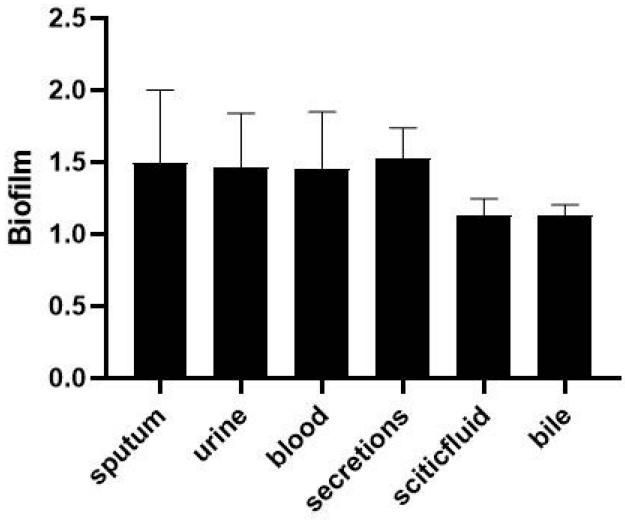

Figure 1 The value of biofilm formation of strains from different sources.

Table 2 Distribution of the Lowest Inhibitory Concentrations of 96 Strains of *Klebsiella Pneumoniae* to 16 Antibiotics

Drug	Number of Isolates with MIC (μg/mL)										
	1	2	4	8	16	32	64	128	256	512	1024
Piperacillin				1	3	10	32		9	15	26
Ampicillin/ Sulbactam		13	17	4	1		2	10	21	25	3
Cefazolin			9	28	7			10	32	9	1
Ceftazidime	1	27	30		1	16	16	4	1		
Ceftriaxone	32	8	6	20	15	13	2				
Cefotaxime					3	17	21	13	8	12	22
Cefepime	1	2	36	20		3	7	17	10		
Cefoxitin		9	20	31	1	2	1	7	23	2	
Aminotransol	1	5	38	12	2			1	20	17	
Imipenem	78	1		5	10	2					
Meropenem	80			4	11	1					
Gentamicin	12	37	8	5	7	26	1				
Tobramycin	11	32	10	9	4	5	24	1			
Ciprofloxacin	10	33	11	9	4	21	4	3			
Cotrimoxazole						1	20	32	9	29	5
Furantoin			1	6	23	3	3	7	33	18	2

Table 3 Resistance Rates of *hvKP* Vs *cKP*, n=96

Antibacterial Drugs	*hvKP* (n=32) No. (%)	*cKP* (n=64) No. (%)	*P-value*
Piperacillin	8 (16.7)	42 (65.6)	<0.001
Ampicillin / Sulbactam	13 (40.6)	48 (75.0)	0.001
Cefazolin	10 (31.3)	42 (65.6)	0.001
Ceftazidime	9 (28.1)	29 (45.3)	0.105
Ceftriaxone	9 (28.1)	41 (64.1)	0.001
Cefotaxime	9 (28.1)	38 (59.4)	0.004
Cefepime	7 (21.9)	30 (46.9)	0.018
Cefoxitin	9 (28.1)	24 (37.5)	0.362
Aminotransol	9 (28.1)	29 (45.3)	0.105
Imipenem	4 (12.5)	13 (20.3)	0.345
Meropenem	3 (9.4)	13 (20.3)	0.175
Gentamicin	6 (18.8)	33 (51.6)	0.002
Tobramycin	3 (9.4)	31 (48.4)	<0.001
Ciprofloxacin	6 (18.8)	27 (42.2)	0.023
Cotrimoxazole	3 (9.4)	31 (48.4)	<0.001
Furantoin	16 (50.0)	47 (73.4)	0.023
MDR	9 (28.1)	44 (68.8)	<0.001
ESBLs	5 (15.6)	28 (43.8)	0.006
Carbapenemase	3 (9.4)	12 (18.8)	0.233

Table 4 Distribution of Virulence Genes in cKP Strains and hvKP Strains, n=96

Toxicity Factor	*hvKP* (n=32) No. (%)	*cKP* (n=64) No. (%)	*P-value*
fimH	31 (96.9)	57 (89.1)	0.361
mrkD	32 (100.0)	63 (98.4)	1.000
entB	22 (68.8)	23 (35.9)	0.002
rmpA	28 (87.5)	12 (18.8)	<0.001
aerobactin	23 (71.9)	8 (20.7)	<0.001
ybtS	17 (53.1)	34 (53.1)	1.000
allS	6 (18.8)	9 (14.1)	0.551
kfu	9 (28.1)	6 (9.4)	0.017
K1	9 (28.1)	5 (7.8)	0.019
K2	8 (25.0)	3 (4.7)	0.009
K5	1 (3.1)	3 (4.7)	1.000
K20	0 (0.0)	2 (3.1)	0.551
K57	5 (15.6)	1 (1.6)	0.025

Biofilm Formation Characteristics of *hvKP* and *cKP* Strains

According to Table 7, PCR was used to detect common *ESBLs* genes (*blaTEM, blaSHV, blaCTX-M*) and the double disk diffusion method was used to detect whether *ESBLs* were produced and the modified carbapenem inactivation method (*mCIM*) was used to detect whether carbapenemases were produced (Figure 3), the absorbance (OD) of each pore and the formation of the biofilm were read at 570 nm using an enzyme marker, thirty-three (63%) strains of *K. pneumoniae* exhibited *ESBLs* phenotype. Sixteen of the strains were *carbapenemase-producing* and most *ESBLs* phenotype tested were resistant to all four drugs conclusively. Not all *ESBL*-positive strains produce ultra-broad-spectrum β-lactamases. The correlation between *ESBLs-producing* strains and biofilm formation was significantly increased compared to strains without *ESBLs* phenotype ($P = 0.035$). In addition, *MDR* strains were also more likely to form biofilms than sensitive strains ($P = 0.013$). *ESBLs-producing cKP* strains were significantly more correlated with biofilm formation compared to *cKP* ($P = 0.021$).

The difference in biofilm formation between *hvKP* strains and *cKP* strains was not statistically significant ($P = 0.134$). Strains carrying *blaSHV* and *blaCTX-M* were significantly associated with biofilm formation ($P = 0.007$ and $P = 0.003$, respectively). However, strains carrying the *blaTEM* were associated with biofilm formation, and the difference was not statistically significant ($P = 0.178$) (Table 7). The level of biofilm formation was significantly and negatively correlated with cephalosporin resistance ($P = 0.034$) (Table 8).

Statistical processing showed no significant correlation between sex and urinary tract infection ($P = 1.00$). Age was significantly associated with infection ($P = 0.006$). There was no significant relationship between age and sex ($P = 0.794$). Age was not significantly correlated with urinary tract infection ($P = 0.927$), neither *mrkD* ($P = 0.333$) nor *FimH* ($P = 0.602$) was significantly related to gender.

Discussion

cKP causes severe infections when infecting immunocompromised individuals,[21] and the carriage and expression of its drug resistance does not enhance the virulence of *K. pneumoniae*, although it increases the difficulty of treatment. However, *hvKP* is a hypervirulent pathogen capable of infecting both healthy and immunocompromised populations, causing purulent liver abscesses, endophthalmitis, and meningitis in a variety of serious infections.[22] *hvKP* is rarely resistant to commonly used antimicrobial agents, except for intrinsic resistance to ampicillin.[23] Our data showed that among 16 antibiotics, *cKP* strains were significantly more resistant to 11 antibiotics compared to *hvKP* ($P < 0.05$) and 68.8% of *cKP* were *MDR* strains, while only 28.1% of *hvKP* strains were *MDR* strains, suggesting a propensity for *cKP* to acquire antibiotic resistance.[24] Previous studies have shown an increased propensity for *hvKP* strains to acquire

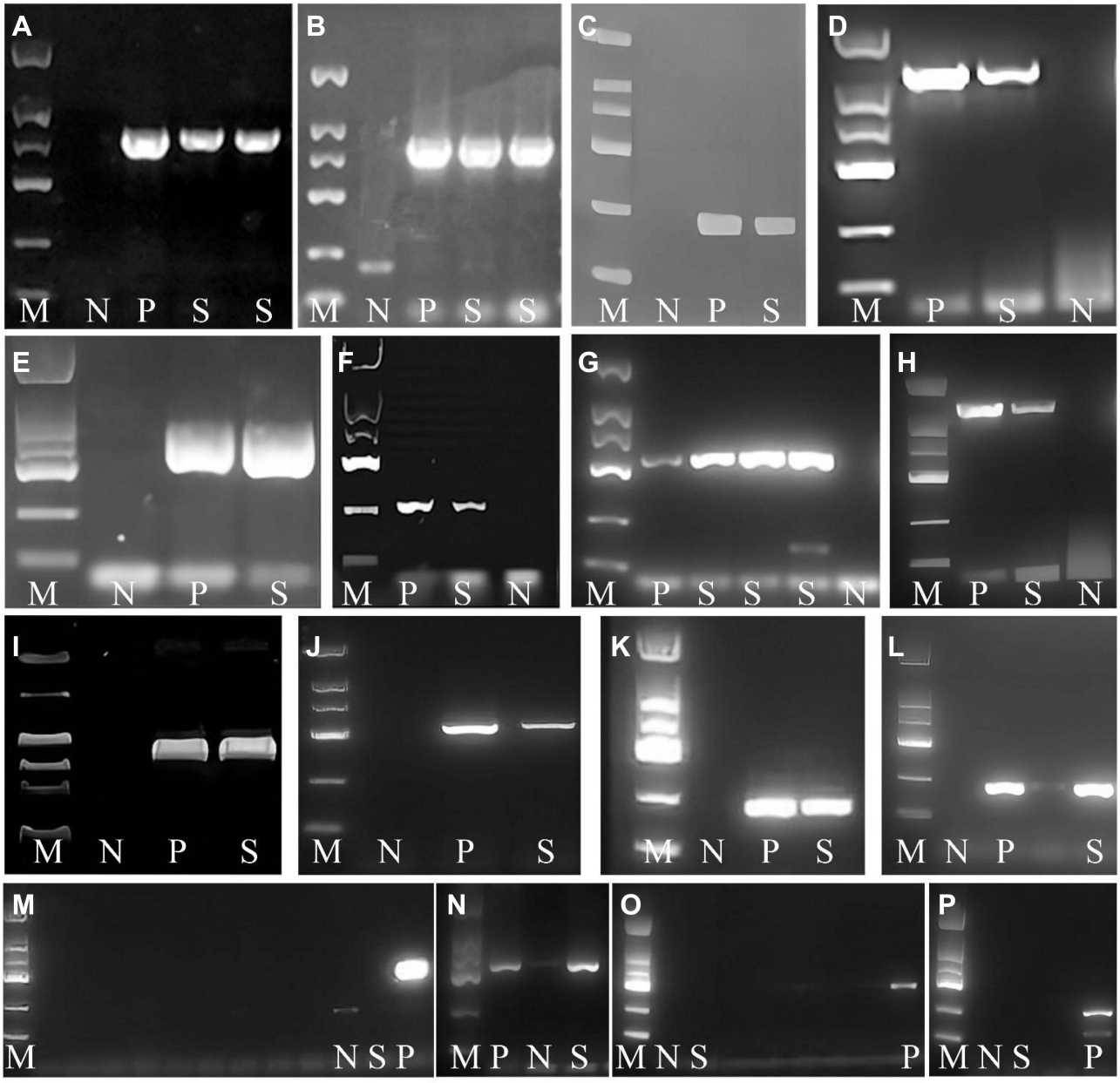

Figure 2 Agarose gel electrophoresis of ESBLs genes and virulence factors.
Notes: M, DNA marker; N, negative control; P, positive control; S, strain to be tested; (**A**) *blaTEM* (800bp); (**B**) *blaSHV* (885bp); (**C**) *blaCTX-M* (216bp); (**D**) *K1* (1283bp); (**E**) *K2* (641bp); (**F**) *K5* (280bp); (**G**) *K20* (741bp); (**H**) *K57* (1037bp); (**I**) *rmpA* (535bp); (**J**) *fimH* (550bp); (**K**) *mrkD* (226bp); (**I**) *ybtS*(242bp); (**M**) *allS* (508bp); (**N**) *kfu* (638bp); (**O**) *aerobactin* (556bp); (**P**) *entB* (400bp).

antimicrobial resistance, although *cKP* is more resistant to multiple antibiotics than *hvKP*. The present study also compared the phenotypic characteristics of *ESBL* of *hvKP* and *cKP* isolates, which is consistent with previous reports.[25,26] *hvKP* strains produced significantly less *ESBL* than *cKP* strains because *ESBL* is a class of β-lactamases that hydrolyze penicillins, cephalosporins, and monocyclic antibiotics, which explains the aforementioned higher resistance of *cKP* to most antibiotics than *hvKP*. It is also important to note, according to Table 7, thirty-three (63%) strains of *K. pneumoniae* exhibited *ESBLs* phenotype. Sixteen of the strains were *carbapenemase-producing* and most *ESBLs* phenotype tested were resistant to all four drugs conclusively. Not all *ESBL*-positive strains produce ultra-broad-spectrum β-lactamases. The reason is that a biological structural gene, consisting of several coding and non-coding regions separated from each other but continuously embedded, can be translated into a complete protein composed of

Table 5 Distribution of *ESBLs* Genes in *ESBL-Positive cKP* Strains and *hvKP* Strains, n=54

ESBL Gene	*cKP* (n=*45*) No. (%)	*hvKP* (n=*9*) No. (%)
TEM	1 (2.2)	–
SHV	2 (4.4)	–
CTX-M	1 (2.2)	–
TEM+SHV	2 (4.4)	–
TEM+CTX-M	1 (2.2)	–
SHV+CTX-M	11 (24.4)	5 (55.6)
TEM+SHV+CTX-M	27 (60.0)	4 (44.4)

Table 6 Correlation of *hvKP* Strains with *ESBL-Producing* Strains

Virulence Profiles	*TEM* (n=*35*)	*SHV* (n=*51*)	*CTX-M* (n=*49*)
hypermucoviscosity	4 ($P = 0.001$)	9 ($P = 0.001$)	9 ($P = 0.001$)
fimH	30	46	45
mrkD	35	51	49
entB	3 ($P < 0.001$)	5 ($P < 0.001$)	5 ($P < 0.001$)
rmpA	6 ($P < 0.001$)	10 ($P < 0.001$)	9 ($P < 0.001$)
aerobactin	2 ($P < 0.001$)	2 ($P < 0.001$)	2 ($P < 0.001$)
ybtS	15	27	26
allS	3	4 ($P = 0.025$)	4 ($P = 0.040$)
kfu	0 ($P = 0.001$)	0 ($P < 0.001$)	0
K1	2	2 ($P = 0.002$)	2 ($P = 0.003$)
K2	1	2 ($P = 0.014$)	2 ($P = 0.021$)
K5	0	1	0
K20	0	0	0
K57	0	0 ($P = 0.023$)	0 ($P = 0.031$)

Table 7 Statistics of the Relationship Between the Genes Carrying Different *ESBLs* and Biofilm Formation

ESBL Gene and Enzyme	Biofilm Formation (n=*72*) No. (%)	Non Biofilm Formation (n=*24*) No. (%)	*P-value*
TEM	29 (40.3)	6 (25.0)	0.178
SHV	44 (61.1)	7 (29.2)	0.007
CTX-M	43 (59.7)	6 (25.0)	0.003
ESBLs	29 (40.3)	4 (16.7)	0.035
Carbapenemase	13 (18.1)	2 (8.3)	0.417

continuous amino acids after the coding region is removed. In other words, the sequence within a structural gene that codes for an amino acid (called an exon) is discontinuous and separated by some non-coding sequence (called an intron), which is called a break gene, some *ESBL* positive strains did not produce *ESBL* enzyme because the truncated gene could not encode the protein. The nine *hvKP* strains found to be *MDR* strains in our study, all carrying two or more *ESBL* genes, may have evolved because *cKP* strains acquired virulence factors through horizontal (lateral) gene transfer by phage, plasmids or transposons,[27] or *hvKP* acquired mobile progenitors of resistance determinants.[28]

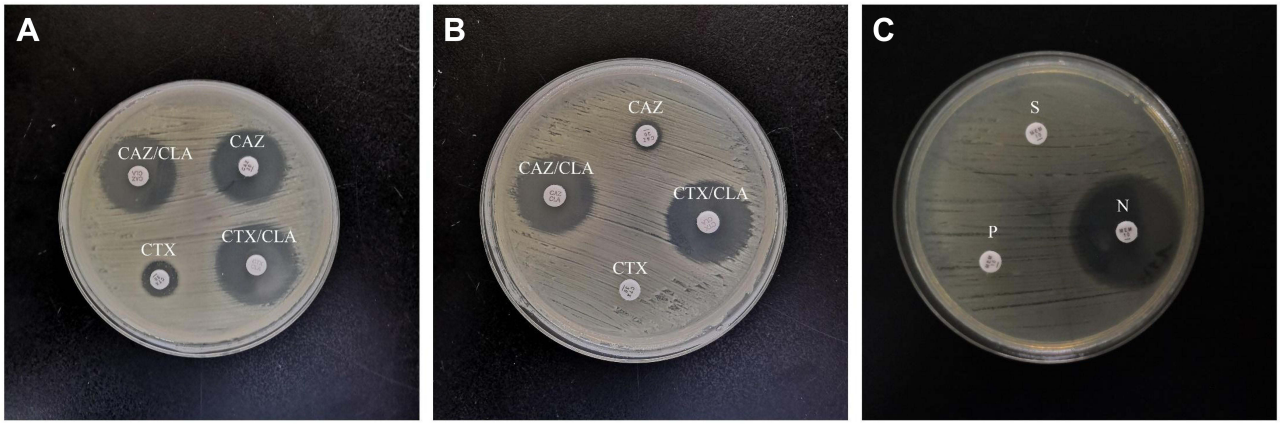

Figure 3 The results of double-disc synergy test (DDST) and modified carbapenem inactivation method (mCIM).
Notes: N, negative control; P, positive control; S, strain to be tested; (**A**) positive result of DDST; (**B**) negative result of DDST; (**C**) the result of mCIM.

In our study, the prevalence of podocyst serotypes *K1, K2, K57* and the virulence gene *rmpA* was found to be significantly higher in *hvKP* strains than in *cKP* strains, which is consistent with previous results in which genes encoding *rmpA, K1* or *K2* were highly associated with hypervirulent (highly adherent) variants of *K. pneumoniae* (*hvKP*).[29,30] Most of the hypervirulent *K. pneumoniae* podocyst serotypes are *K1, K2, K5, K20* and *K57*.[31] *K1* and *K2* capsular types are considered to be highly pathogenic to humans.[32] To date, 70%–80% of reported *hvKP* strains belong to *K1* and *K2* types of *K. Pneumoniae*.[33] The gene *rmpA* is the regulatory gene, which has been identified to be closely associated with the hyperviscosity phenotype, commonly detectable in *hvKP*.[34] Other virulence genes *entB, aerobactin*, and *kfu* were also significantly increased in *hvKP* strains. The iron carrier-related *entB* mainly mediates iron transport in Gram-negative bacteria and is widely spread in *K. pneumoniae* strains. *Kfu* is the gene encoding the iron uptake system. Our study showed that the *kfu* gene was significantly increased in strains with high mucilage. This finding is consistent with previous studies, which showed that the *kfu* gene was associated with a high-mucus phenotype.[35] Previous studies have shown that *aerobactin* (*aerobic actin*) gene and *aerobic actin* production are more common in *hvKP* strains than in *cKP* strains. *hvKP* strains produce *aerobactin* more commonly than *cKP* strains, as demonstrated by cross-feeding assays (*Yu* et al, 2007). This analysis suggests that *hvKP* strains may be more likely to acquire iron than *cKP* strains.[36,37] It has been shown that aerobic actin is the main virulence factor for increased iron carrier production in *hvKP* isolates and was used to define hvKP.[38]

K. pneumoniae contains plasmid-mediated β-lactamases with broad-spectrum hydrolytic activity,[39] carrying multiple antimicrobial drug resistance genes, including *ESBLs* and *carbapenemases*.[40] In Table 7, some strains produce carbapenase, carbapenemase includes A, B, C and D of β-lactam group classified by Ambler, class A carbapenems belong to the 2f group in Bush, including *KPC, SME, NMC-A, IMI, GES, SFC-1*, etc. As one of the most important carbapenemases at present, the emergence of *KPC*-producing carbapenemase is the main reason for increasing the resistance to carbapenems. This enzyme can hydrolyze all β-lactamases including carbapenems, penicillin, and cephalosporins of the 1st to

Table 8 Statistics on the Relationship Between Biofilm Formation Level and Drug Resistance

Biofilm Formation	Cephalosporin (n=72)		*P-value*	r_s
	Sensitive	Resistant		
Weak	10	16	0.034	−0.251
Moderate	10	17		
Strong	14	5		

4th generation, but is sensitive to monocylactam. Class B carbapenemase is a kind of metal β-lactamase, its activity needs the assistance of metal ion zinc, referred to as metal enzyme, most by plasmid coding, its genes are located in a class of integron, integron integration into the transposon or transfer plasmid, resulting in the spread of *IMP* gene between the same or different strains, so that the strains that were sensitive to antibiotics become resistant, increased resistance to hydrocarbansene antibiotics, especially imipenem was enhanced. Category C is rarely reported. Most of class D carbapenems have weak hydrolysis ability to carbapenems and ultra-broad spectrum β-lactam.[41] Factors affecting biofilms include capsular polysaccharides (capsule k antigen) lipopolysaccharide extracellular DNA and adhesion factors (including adhesion proteins and pili structures). It can be known from reading a large number of literatures that the types and abilities of the four types of antibiotics aimed at hydrolyzing are different, which have nothing to do with the formation of biofilms. In other words, the influencing factors of biofilm formation have nothing to do with carbapenase. Statistics of carbapenase and biofilm formation were calculated in the supplementary data in Table 7, and it was concluded that (P = 0.417) was not statistically significant, which was consistent with the conclusion mentioned above, that is carbapenase production had no direct relationship with biofilm formation.[42] Microorganisms that produce *ESBLs* are clinically relevant and remain an important cause of cephalosporin treatment failure.[40] There are hundreds of variants of *ESBLs*, mainly including the *blaTEM, blaSHV* and *blaCTX-M* families. In this study, *ESBL genes* were negatively correlated with *hvKP* (P = 0.001). The reason for this difference is unclear, but it can be speculated that *hvKP* strains do not have access to plasmids associated with drug resistance or that some resistance genes are lost when they become hyper-virulent.[24] The strains in our collection carry multiple virulence genes and some of them coexist with drug resistance genes. Also virulence genes were associated with *ESBL* resistance genes. *blaTEM, blaSHV* and *blaCTX-M* were highly negatively correlated with entB, rmpA and rmpA (P < 0.001), except for *blaTEM, blaSHV* and *blaCTX-M* were significantly negatively correlated with *allS, K1* and *K2. Highly mucoid Klebsiella pneumoniae strains* (*HMV*) are early associated with hypervirulence, and mutations leading to the loss of this *HMV* phenotype also lead to reduced virulence. *hvKP* strains with a high mucoid phenotype produce significantly less *ESBL* than *cKP* strains as shown by the above experimental results, and it has been shown that the virulence of *K. pneumoniae* strains is associated with the usual virulence factors (*entB, rmpA* and *rmpA*) and capsular serotypes (*K1* and *K2*) positively correlated.

Biofilms are microbial communities consisting of cellular aggregates in a matrix composed of surface polysaccharides, proteins and *DNA* that are attached by bacteria to living or non-living surfaces. The ability to produce biofilms results in increased resistance to host defense factors and antimicrobial agents, and is considered an important virulence property. The biofilm formation ability of strains with different antibiotic resistance is also different, according to the relationship between biofilm production and drug resistance of *K. pneumoniae* clinically isolated from Beijing Shijitan Hospital affiliated to Capital Medical University. Among 140 strains, 52 strains were positive for biofilm, accounting for 37.1% of all strains. The sensitive rates of biofilm-positive strains to amikacin, gentamicin, ceftazidime, cefepime and amtrannan were 44.2%, 15.4%, 28.8%, 30.8% and 11.5%, respectively. The sensitivity rates of biofilm-negative bacteria to amikacin, gentamicin, ceftazidime, cefepime and amtriaxam were 80.6%, 26.1%, 35.2%, 37.5% and 35.2%, respectively. The sensitivity rate of positive and negative biofilm bacteria to the above-mentioned drugs was statistically significant.[43] The correlation between *ESBLs-producing* strains and biofilm formation was significantly increased compared to strains without *ESBLs* phenotype (P = 0.035), and it has been suggested that this may be due to (1) the fact that biofilm is a multi-species microbial community and these species can share their genetic material in high proportions; (2) low concentrations of antibiotics can induce *ESBLs*, which is one of the reasons for reduced penetration of one of the reasons for this.[24] Strains carrying *blaSHV* and *blaCTX-M* were significantly associated with biofilm formation (P = 0.007 and P = 0.003, respectively). According to the name Proteomic and Transcriptomic Analyses Indicate Reduced Biofilm-Forming Abilities in Cefiderocol-resistant *K. pneumoniae*, it was found that drug resistance was closely related to the decreased biofilm formation ability, and the downregulation of *hdeB, stpA, yhjQ, fba, bcsZ, uvrY, bcsE, bcsC* and *ibpB* was the main factor. In addition, downregulation of ferritic transporters, including *efeO*, Tonb-dependent receptor *fecA* and ABC transporter *fbpA*, may be one of the determining factors that promote reduced biofilm formation ability and lead to cephalosporin resistance, the results are consistent with those in Table 8.[44] Several studies have shown that *ESBL-producing K. pneumoniae* form heavier biofilms than *non-ESBL-producing K. pneumoniae*, and another recent study showed that serum-resistant strains of *ESBL-producing K. pneumoniae* are more prevalent than *non-ESBL K. pneumoniae*.[24] However, strains carrying the *blaTEM* gene were

associated with biofilm formation, and the difference was not statistically significant ($P = 0.178$). This suggests that *blaSHV* and *blaCTX-M* are associated with biofilm formation. It has been proposed that (I) the production of *ESBLs* encoded by resistance plasmid increases when adhesion factors and production of extra-envelope polysaccharides are increased in *hvKP* strains. Thus strains carrying the *blaTEM* may lack adhesion factors or have other factors in combination that affect biofilm formation. In vitro studies have demonstrated the ability of *cKP* strains to produce biofilms, with *type 3 hairs, podocyanin polysaccharide* (*CP*) and *lipopolysaccharide* (*LPS), amino acid synthesis genes, l-arabinose metabolism, sugar phospho-transferase system, type 2 population-sensing regulatory system, Lys-R-type regulator oxR* and a possible cell surface protein identified as contributing factors. *hvKP* strains *NTUH- K2044* and *KpL1* were also shown to produce biofilms, and studies on *hvKP* strain *hvKP1* showed that genes encoding glutamine synthetase, the alpha subunit of succinyl coenzyme A synthase and the glycerol uptake manipulator transcriptional repressor were associated with biofilm formation.[45]

Conclusion

In conclusion, with the spread of drug-resistant *K. pneumoniae*, clinical treatment will face serious challenges. Therefore, we need to make continuous efforts to control the spread of drug-resistant *K. pneumoniae* and prevent a full-scale outbreak in the future.

Ethical Conduct of Research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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The authors report no conflicts of interest in this work.

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