Clin. Lab. 2024;70:XXX-XXX

ORIGINAL ARTICLE

Molecular Epidemiological Characteristics of Hypervirulent *Klebsiella pneumoniae* in Yakeshi City, Hulunbuir, China

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SUMMARY

Background: **This study aimed to investigate the molecular epidemiological characteristics of hypervirulent *Klebsiella pneumoniae* (hvKp) in Yakeshi City, Hulunbuir, China, analyze the resistance of hvKp to commonly used antibiotics, explore independent risk factors for hvKp infection, and provide a research basis for anti-infection treatment.**

Methods: **In total, 519 strains of *K. pneumoniae,* identified by the Inner Mongolia Forestry General Hospital from January 2020 to December 2022, were collected, and high-viscosity (HMV-Kp) and non-HMV-Kp strains were differentiated using string test. PCR and agarose gel electrophoresis were used to detect the *rmpA*, *rmpA2*, and *iutA* genes to identify hvKp strains. Sixty strains of hvKp were randomly selected for capsular serotyping by PCR and agarose gel electrophoresis. Sanger sequencing was used to sequence the housekeeping genes of 60 hvKp strains and perform ST analysis. A minimum spanning tree was drawn using capsule serotyping and ST typing. Significant differences in resistance to commonly used antibiotics between classical *K. pneumoniae* (cKp) and hvKp were analyzed by using the chi-squared test. Finally, the risk factors for hvKp infection were analyzed through binary logistic regression.**

Results: **The HMV-Kp detection rate was 39.69%, versus 37.19% for hvKp. HMV-Kp accounted for 84.97% of all hvKp isolates. The hvKp detection rate was highest in the general surgery department. In capsule serotyping, K1 was the main subtype, accounting for 63.33% of all isolates (38/60), followed by K2 (16.67%, 10/60). Through ST typing, 18 subtypes were detected, with ST23 being the most common (50.00%), followed by ST86 (8.33%), and the remaining subtypes were scattered throughout the distribution. Compared with cKp, hvKp strains exhibited higher sensitivity to commonly used antibiotics, excluding furantoin. Male gender (odds ratio (OR) = 1.977), liver abscess (OR = 15.019), and the use of macrolide antibiotics in the past 3 months (OR = 5.473) were independent risk factors for hvKp infection.**

Conclusions: **The hvKp detection rate in the local area was 37.19%, and a strong correlation was noted between hvKp and HMV-Kp strains. K1-ST23 was the dominant subtype in this study. Compared with cKp, hvKp strains were more sensitive to commonly used antibiotics. Male gender, liver abscess, suppuration or infection of other tissues and organs, and recent macrolide antibiotic use were risk factors for hvKp infection.**

(Clin. Lab. 2024;70:xx-xx. DOI: 10.7754/Clin.Lab.2024.240411)

Manuscript accepted June 19, 2024

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KEYWORDS

molecular epidemiology of hvKp, hvKp resistance, risk factors for hvKp infection

INTRODUCTION

Hypervirulent *Klebsiella pneumoniae* (hvKp) was first reported by Taiwanese scholars in 1986, and it is defined by the ability to cause multiple abscesses. Subsequently, hvKp has been reported worldwide, mainly in Asia, with China being a high-risk area [1]. Unlike classical *K. pneumoniae* (cKp), hvKp can infect healthy individuals of any age and cause liver abscess in the absence of biliary diseases, and it carries a high mortality rate [2]. The virulence phenotype of hvKp is related to the production of super sticky capsules and the virulence genes responsible for iron carriers [3]. Mucus-like phenotype regulators (*rmpA* and *rmpA2*) are forward transcriptional activators that regulate the mucus-like phenotype [4]. The wire drawing test can be used as a high-viscosity phenotype determination test [5]. The *iutA* gene encoding aerobactin is often considered one of the most critical virulence factors in hvKp, and isolates carrying the *iutA* gene are more likely to cause severe immune responses and invasive infections in the host [6]. The main capsule serotype prevalent in Asia is K1, whereas K2 is more common in Europe and North America. K1/K2 strains have significantly stronger resistance to neutrophil phagocytosis and intracellular killing, and they can cause severe invasive infections [7]. The prevalent strain of hvKp is usually associated with the sequence type ST23, and there are many reports in Asia [8]. Compared with cKp, hvKp strains, although more virulent, exhibit higher sensitivity to antibiotics other than ampicillin [2]. Multidrug-resistant cKp can acquire virulence factors, and vice versa, hvKp can also acquire antibiotic resistance genes. Although high virulence and multidrug resistance occur in different clonal lineages, convergent evolution occurs [9] and brings great challenges to clinical treatment. Some earlier studies have shown that diabetes may be an important host factor for hvKp [10]. This study aimed to systematically analyze the risk factors and molecular characteristics of hvKp. It is the first time to conduct relevant research on hvKp in the Hulun Buir region, providing a research basis for the effective prevention and treatment of hvKp infection.

MATERIALS AND METHODS

Strains and general information

In total, 519 non-repetitive strains identified as *K. pneumoniae* were collected from Inner Mongolia Forestry General Hospital from January 2020 to December 2022. The quality control strain was Escherichia coli ATCC 8739 (Wenzhou Kangtai Technology Co., Ltd.).
Clinical diagnoses, general information, underlying diseases, and antibiotic use were collected for patients with hvKp infection by consulting medical records.

Ethical approval

The approval number is NLZ-2023-008; the name of the Ethics Committee is the Medical Ethics Committee of Inner Mongolia Forestry General Hospital.

String test

In a biosafety cabinet, an inoculating loop was used to pick up a single pure colony after subculture on a blood agar plate (Bio Mérieux. Inc.). When the length of the colony picked up by the inoculating loop is more than 5mm when it is drawn into a filament, it is a positive string test [5].

Toxicity gene testing

First, the DNA of each *K. pneumoniae* strain was extracted using a bacterial DNA extraction kit (Gene-better Biotechnology Co., Ltd.), and its genome was amplified by PCR using primers (Seven Innovation Biotechnology Co., Ltd.) targeting the *iutA*, *rmpA*, and *rmpA2* virulence genes. The primer sequence is shown in Table 1. PCR reaction system: 2 x Taq PreMix (Seven Innovation Biotechnology Co., Ltd.) 12.5 μL, forward primer 1 μL, reverse primer 1 μL, sample DNA 1 μL, and $ddH2_O$ (Seven Innovation Biotechnology Co., Ltd.) are added to a total volume of 25 μL. PCR instrument (Bio-Rad Laboratories, Inc.) is used for virulence gene amplification. The basic conditions of the cycle are: predenaturation at 95℃ for 3 minutes, denaturation at 94℃ for 25 seconds, annealing at 55 - 64℃ for 25 seconds, extension at 72℃ for 10 seconds, and 30 cycles are performed.
The genes were detected by 1.5% agarose gel electrophoresis; 1 x TAE buffer (Seven Innovation Biotechnology Co., Ltd.) and agarose powder (Seven Innovation Biotechnology Co., Ltd.) are heated to prepare

Table 1. Primers` gene information.

Primer	Sequence (5′–3′)	Product size (bp)	Reference
iutA-F	AATCACCTGGGGGCTGGATGCT	683	[11]
iutA-R	CCGCACCTTCCACGCCGTAAAT		
rmpA-F	ACTGGGCTACCTCTGCTTCA	516	[12]
rmpA-R	CTTGCATGAGCCATCTTTCA		
rmpA2-F	TGTGCAATAAGGATGTTACATTAGT	590-610	[12]
rmpA2-R	TTTGATGTGCACCATTTTTCA		
Capsular type K1-F	GGTGCTCTTTACATCATTGC	1,283	[13]
Capsular type K1-R	GCAATGGCCATTTGCGTTAG		
Capsular type K2-F	GACCCGATATTCATACTTGACAGAG	641	[13]
Capsular type K2-R	CCTGAAGTAAAATCGTAAATAGATGGC		
Capsular type K5-F	TGGTAGTGATGCTCGCGA	280	[13]
Capsular type K5-R	CCTGAACCCACCCCAATC		
Capsular type K20-F	CGGTGCTACAGTGCATCATT	741	[13]
Capsular type K20-R	GTTATACGATGCTCAGTCGC		
Capsular type K54-F	CATTAGCTCAGTGGTTGGCT	881	[13]
Capsular type K54-R	GCTTGACAAACACCATAGCAG		
Capsular type K57-F	CTCAGGGCTAGAAGTGTCAT	1,037	[13]
Capsular type K57-R	CACTAACCCAGAAAGTCGAG		

80 mL of agarose gel solution. After standing at room temperature and cooling to an appropriate temperature, 4 μL of SuperRed nucleic acid dye (Seven Innovation Biotechnology Co., Ltd.) were added, mixed evenly, and cooled to prepare a 1.5% agarose gel. After the samples were loaded, they were electrophoresed for 20 minutes at 220 V using the electrophoresis apparatus (Shanghai Tanon Technology Co., Ltd.), and then the results were read with the automatic chemiluminescence image analyzer (Changzhou Weizhuiyan Engineering Medical Devices Co., Ltd.). The results refer to AL2000 DNA Marker (GeneBetter Biotechnology Co., Ltd.), defining that the *rmpA* gene/*rmpA2* gene is positive and the *iutA* gene is positive as hvKp.

Capsular typing and MLST typing of hvKp strains

For capsular serotyping, 60 hvKp strains were randomly selected for typing. DNA was extracted using the aforementioned method, and PCR amplification was performed using primers targeting the K1, K2, K5, K20, K54, and K57 type genes. The capsular K type was detected by 1.5% agarose gel electrophoresis. The primer sequences are shown in Table 1.

For MLST typing, the PCR products of seven housekeeper genes of 60 hvKp strains were expanded and recovered DNA using a DNA gel recovery kit (Jiangsu Pobo Biotechnology Co., Ltd.). The primer sequences of the seven housekeeping genes used for MLST typing of Kp strains refer to the PASTEUR database (https://bigsdb.pasteur.fr). Finally, the purified PCR products were sequenced using a sequencer (Applied Biosystems Company). The sequencing results were submitted to the PASTEUR database to compare ST types.

To draw a minimum spanning tree, the specific capsule serotyping and ST typing data of hvKp were uploaded to PHYLOViZ 2.0 to draw a minimum spanning tree.

Drug susceptibility test

Antimicrobial susceptibility testing primarily by the VITEK2-Compact II (Bio Mérieux. Inc) and supplemented by disc-diffusion on Mueller-Hinton agar (Bio Mérieux, Inc.).

The minimum inhibitory concentration (MIC) of 13 kinds of antibacterial agents was determined by the microbroth dilution method. A 0.50 McFarland turbidity bacterial suspension was prepared, 145 μl were taken and transfered to a test tube containing 3 ml of 0.45% sterile physiological saline, and the VITEK2-Compact II fully automatic bacterial identification instrument was used to conduct the drug susceptibility test. The antibacterial drugs detected by the drug susceptibility card N334 include amoxicillin/clavulanic acid, cefuroxime, ceftazidime, ceftriaxone, cefepime, cefoxitin, imipenem, ertapenem, amikacin, levofloxacin, compound sulfamethoxazole, tigecycline, and extended-spectrumβ-lactamases, using Escherichia coli ATCC 25922 as the quality control strain. The results are interpreted with reference to the Clinical and Laboratory Standards Institute (CLSI, 2023), and the tigecycline results are referred to the "Expert Consensus on the Susceptibility

Table2. Distribution of 193 hvKp strains according to patient's department.

Patient's department	n (%)
The Third Department of General Surgery	41 (21.24%)
The Second Department of General Surgery	24 (12.44%)
Interventional Department	21 (10.88 %)
Endocrinology Department	16 (8.29%)
The First Department of General Surgery	13 (6.74 %)
Intensive Care Unit	11 (5.70%)
Gastroenterology Department	9 (4.66%)
Geriatrics Department	6 (3.11%)
Urology Department	
Neurology Department	
Respiratory Department	5 (2.59%)
Gynecology Department	
Hematology Department	4 (2.07%)
General Department	
Rheumatology and Immunology Department	3 (1.55%)
Orthopedics Department	
Nephrology Department	
Otolaryngology Department	
Cardio-renal Internal Medicine Department	
Obstetrics Department	2 (1.04%)
Cardiology Department	
Thoracic and Cardiovascular Surgery Department	1 (0.52%)
Neurosurgery Department	
Pediatrics Department	

Testing Method and Reporting of Polymyxins, Tigecycline, and Ceftazidime/Avibactam".

Disk diffusion method (K-B) used the following drug-sensitive paper chips: ampicillin/sulbactam, gentamicin, ciprofloxacin, cefazolin, tobramycin, meropenem, and nitrofurantoin. The well-prepared bacterial suspension with a 0.50 McFarland turbidity was evenly spread on the M-H agar plate, and after pasting the drug susceptibility disk, it was incubated at 35℃ for 24 hours, and then the diameter of the inhibition zone was measured under a black background. The sensitivity is determined by the diameter of the inhibition zone. The results were interpreted with reference to the Clinical and Laboratory Standards Institute (CLSI, 2023).

Statistical analysis

Statistical analysis was conducted using SPSS 26.0, and general data were presented as n (%). Numerical data were analyzed using the chi-squared test to compare drug resistance between hvKp and cKp strains. The risk factors for hvKp infection were analyzed through multiple logistic regression. $p < 0.05$ was considered statistically significant.

RESULTS

String test results

According to the string test, 39.69% (206/519) of the 519 *Klebsiella pneumoniae* strains were HMV-Kp, whereas 60.31% (313/519) were non-HMV-Kp.

Toxicity gene test results

According to virulence gene testing, 37.19% (193/519) of the 519 *Klebsiella pneumoniae* strains were hvKp. The results of agarose gel electrophoresis for some virulence genes are presented in Figure 1. Out of the hvKp strains, 84.97% (164/193) were HMV-Kp.

Distribution of hvKp strains

Among the 193 strains of hvKp, the highest number was isolated from pus (36.79% (71/193)), followed by trauma infection secretions (17.62% (34/193)), urine accounts (13.47% (26/193)), whole blood (11.92% (23/193)), sputum (9.33% (18/193)), puncture fluid (8.29% (16/193)), bile (1.04% (2/193)), and ascites, amniotic fluid, and drainage fluid (0.52% (1/193)). In this study, 27 specimens were obtained from patients with liver ab-

Table 3. Comparison of drug resistance between hvKp and cKp strains.

Antimicrobial agents	Drug resistance rate of hvKp strains (%)	Drug resistance rate of cKp strains (%)	χ^2	p
Ceftriaxone	5.76	29.54	41.386	0.000
ESBL	6.22	26.90	33.223	0.000
Amoxicillin/Clavulanic acid	3.16	17.28	22.531	0.000
Ampicillin/Sulbactam	5.65	27.09	33.054	0.000
Cefuroxime	7.37	32.41	42.204	0.000
Cefepime	2.59	19.08	29.226	0.000
Gentamicin	3.96	19.00	21.691	0.000
Ciprofloxacin	3.89	18.40	20.981	0.000
Compound sulfamethoxazole	4.60	27.11	36.187	0.000
Cefazolin	8.89	36.48	33.837	0.000
Ceftazidime	2.58	18.46	27.978	0.000
Cefoxitin	3.67	13.23	12.579	0.000
Tobramycin	4.44	15.00	12.761	0.000
Levofloxacin	3.61	15.69	17.871	0.000
Nitrofurantoin	20.83	39.81	3.033	0.082
Ertapenem	0.00	3.69	—	—
Imipenem	0.00	2.46	—	—
Meropenem	0.00	2.78	—	—
Amikacin	0.00	1.81	—	—
Tigecycline	0.00	0.00	—	—

"-" represents no numerical value, and p < 0.05 is considered statistically significant.

Table 4. Results of univariate unconditional logistic regression analysis.

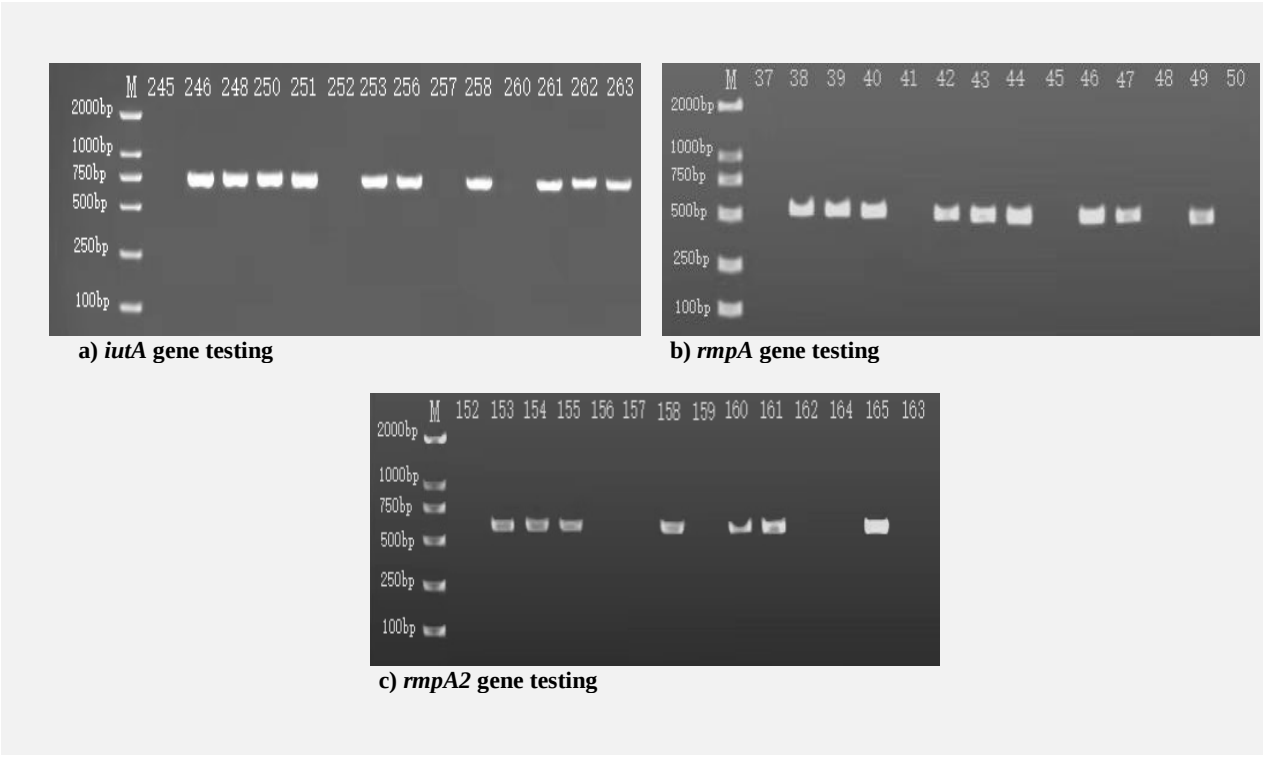
Variables	β	SE	Wals	p	OR	95% CI	
Gender (male)	0.655	0.235	7.750	0.005	1.924	1.214	3.051
Age (> 50 years old)	-0.152	0.317	0.231	0.631	0.859	0.461	1.598
Diabetes	0.618	0.274	5.082	0.024	1.855	1.084	3.175
Hypertension	-0.151	0.279	0.294	0.588	0.860	0.497	1.486
Liver abscess	2.789	0.588	22.541	0.000	16.269	5.144	51.458
Intestinal polyp	0.583	0.563	1.074	0.300	1.792	0.595	5.399
Infection and suppuration of other tissues and organs	0.793	0.317	6.268	0.012	2.211	1.188	4.113
Use of beta-lactam drugs	0.165	0.315	0.275	0.600	1.179	0.636	2.187
Use of anaerobic drugs	-0.162	0.522	0.096	0.757	0.851	0.306	2.365
Use of quinolone drugs	0.074	0.537	0.019	0.891	1.076	0.376	3.086
Use of macrolide drugs	2.102	0.912	5.305	0.021	8.179	1.368	48.899
Catheterization	-0.607	0.493	1.517	0.218	0.545	0.207	1.432
Transfer of hospital or department	0.598	0.383	2.436	0.119	1.819	0.858	3.855
Hospitalization days (> 15 days)	-0.513	0.266	3.718	0.054	0.599	0.355	1.008
Invasive operations	0.025	0.375	0.004	0.947	1.025	0.491	2.139
RDW level	0.045	0.323	0.019	0.89	1.046	0.555	1.969
Constant	-1.204	0.366	10.838	0.001	0.300	—	—

"-" represents no numerical value, and p < 0.05 is considered statistically significant.

Table 5. Results of multivariate logistic regression analysis.

Variables	*β*	SE	Wals	p	OR	95% CI	
Gender (male)	0.682	0.226	9.060	0.003	1.977	1.269	3.082
Diabetes	0.435	0.254	2.932	0.087	1.544	0.939	2.539
Liver abscess	2.709	0.568	22.72	0.000	15.019	4.930	45.757
Infection and suppuration of other tissues and organs	0.877	0.276	10.072	0.002	2.403	1.398	4.130
Use of macrolide drugs	1.700	0.844	4.053	0.044	5.473	1.046	28.637
Constant	-1.428	0.209	46.782	0.000	0.240	—	—

"-" represents no numerical value, and p < 0.05 is considered statistically significant.



a) *iutA* gene testing

b) *rmpA* gene testing

c) *rmpA2* gene testing

Figure 1. PCR electrophoresis images of three virulence genes of *Klebsiella pneumoniae.*

scess, and 85.19% (23/27) of these cases were caused by the hvKp strain.

In total, 193 hvKp strains were isolated from 24 departments, among which 21.24% (41/193) were isolated in the third treatment area for general surgery (burns) and 12.44% (24/193) were isolated in the second treatment area for general surgery. The results are presented in Table 2.

Typing of hvKp strains

Sixty hvKp strains were investigated by agarose gel electrophoresis, with representative results presented in Figure 2. Among the strains, the most common type was K1 (63.33% (38/60)), followed by K2 (16.67% (10/60)), K20 (6.67% (4/60)), K54 (3.33% (2/60)), and K57 (3.33% (2/60)), whereas 6.67% (4/60) of the strains were unclassified (NT). Among the 60 strains, 21 were isolated from patients with liver abscess, and these strains were classified as K1 (80.95% (17/21)) and K2 (19.05% (4/21).

To conduct MLST typing, the DNA amplification products of 60 hvKp strains were sequenced and uploaded to the PASTEUR public database for gene sequence alignment. In total, 18 ST types were detected in the 60

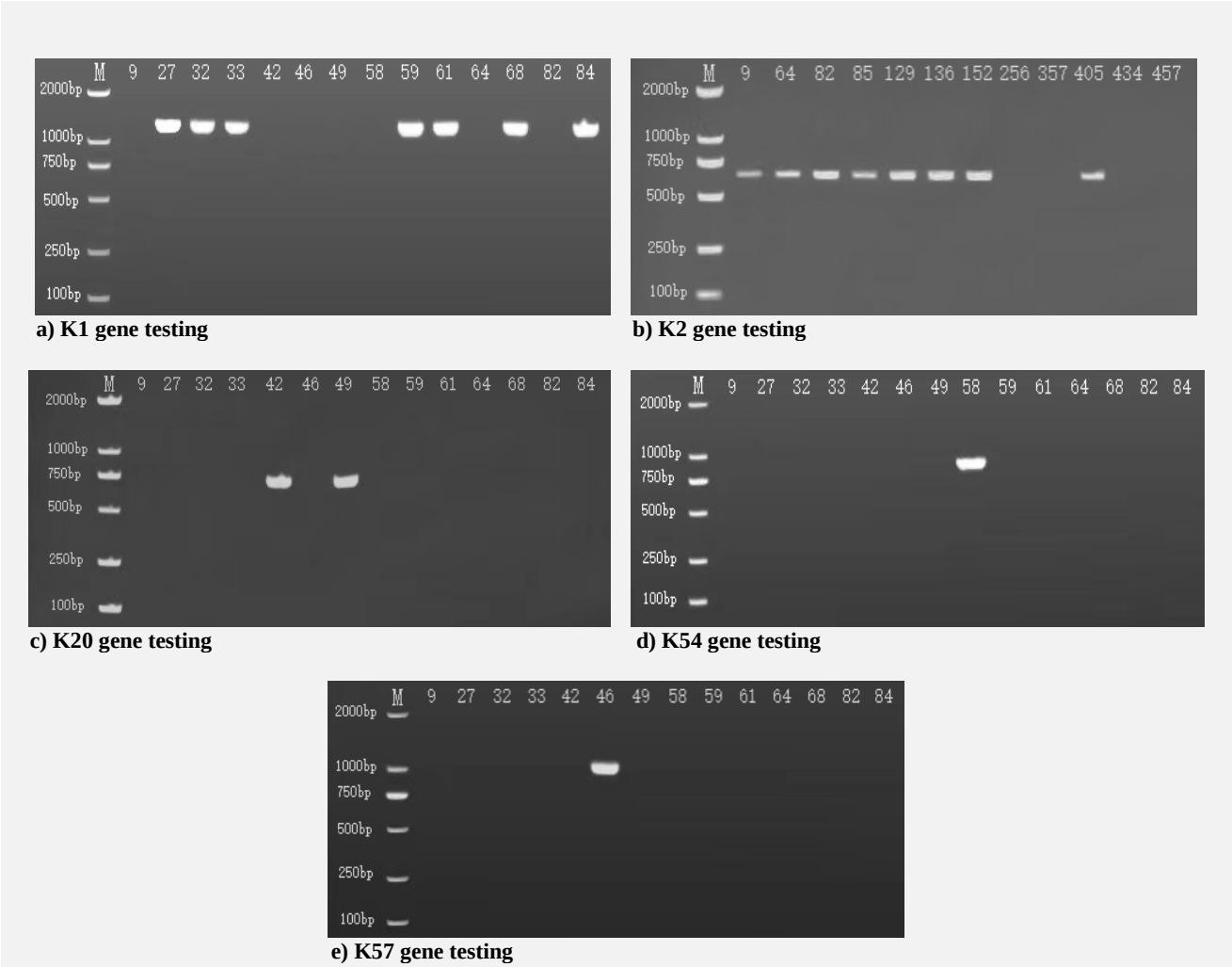


Figure 2. PCR electrophoresis images of hvKp capsule serum typing.

strains, and the dominant type was ST23 (50% (30/60)), followed by ST86 (8.33% (5/60)), ST29, ST268, ST375 (5.00% (3/60)), ST65, ST412, ST660 (3.33% (2/60)), and ST25, ST111, ST163, ST307, ST380, ST420, ST603, ST1049, ST1265, ST2159 (1.67% (1/60)). Among the 21 specimens from patients with liver abscess, ST23 accounted for 71.43% (15/21) of the strains, and the remaining subtypes were scattered.

The minimum spanning tree revealed that all ST23 strains were K1, as presented in Figure 3.

Analysis of drug resistance in hvKp strains

The chi-squared test was used to compare and analyze the resistance of hvKp and cKp strains to commonly used antibiotics. The results are presented in Table 3. The analysis could not be performed for five antibiotics because of a lack of data. Meanwhile, significant differences in susceptibility ($p < 0.05$) were observed for all antibiotics, excluding furantoin.

Analysis of risk factors for the occurrence of hvKp infection

The following variables were investigated as potential risk factors for the occurrence of hvKp infection: gender, age, hypertension, diabetes, liver abscess, intestinal polyp, suppuration or infection of other tissues and organs, use of β-lactam antibiotics in the past 3 months, use of anaerobic drugs in the past 3 months, use of quinolones in the past 3 months, use of macrolides in the past 3 months, placement of catheters in the past 3 months, transfer to another hospital or department, length of hospital stay, invasive procedures performed in the past 3 months, and red cell distribution width. The results illustrated that male (odds ratio [OR] = 1.924, 95% confidence interval [CI] = 1.214 - 3.051), diabetes (OR = 1.855, 95% CI = 1.084 - 3.175), liver abscess (OR = 16.269, 95% CI = 5.144 - 51.458), suppuration or infection of other tissues and organs (OR = 2.211, 95% CI = 1.188 - 4.113), and macrolide use in

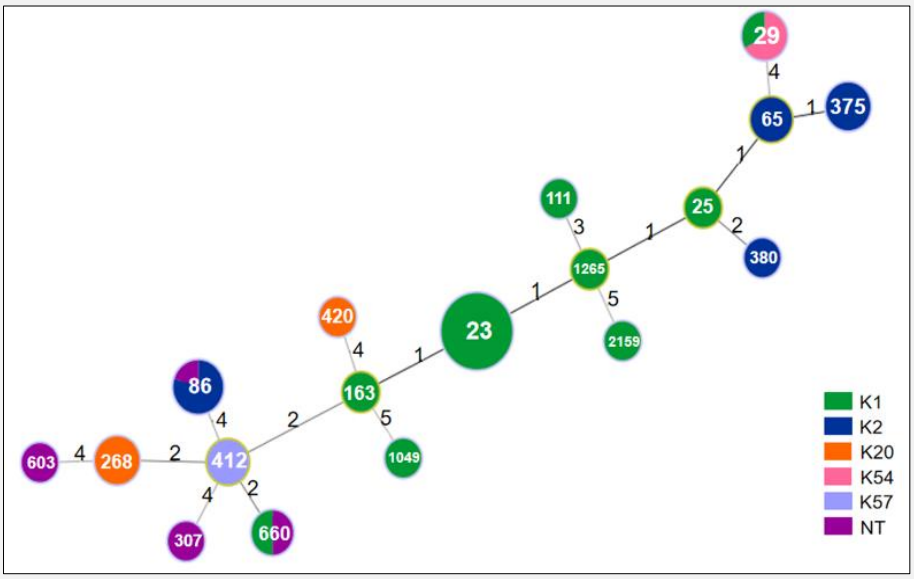


Figure 3. The relationship between the capsule serotype and ST type of hvKp strains.

The numbers inside the circle represent the ST classification. The area of the circle is directly proportional to the number of strains. The line connecting the two subtypes represents the number of differences in butler genes. The colors denote different capsule serum subtypes.

the past 3 months (OR = 8.179, 95% CI = 1.368 - 48.899) were risk factors for the occurrence of hvKp infection ($p < 0.05$), as presented in Table 4.

Multivariate logistic regression analysis was conducted using hvKp infection as the dependent variable and variables identified as significant in univariate analysis as independent variables. The results confirmed that male gender (OR = 1.977, 95% CI = 1.269 - 3.082), liver abscess (OR = 15.019, 95% CI = 4.93 - 45.757), suppuration or infection of other tissues and organs (OR = 2.403, 95% CI = 1.398 - 4.130), and macrolide use in the past 3 months (OR = 5.473, 95% CI = 1.046 - 28.637) were risk factors for hvKp infection ($p < 0.05$, Table 5).

DISCUSSION

The detection rate of hvKp varies in different regions, and a cross-regional study initiated by Peking University reported an hvKp detection rate of 37.8% [7]. The results in our hospital were similar. In this study, the general surgery department had the highest hvKp detection rate. This might be related to the invasive diagnosis and treatment measures in general surgery, which often cause bacterial migration, or the use of ventilators and built-in equipment, which can lead to biofilm formation [14]. In addition to causing purulent infections, hvKp can also spread over distances and cause severe purulent infections in various tissues and organs, explaining why pus accounted for the highest proportion of hvKp specimens in this study. A study conducted by Beijing You'an Hospital on *K. pneumoniae* infection revealed a positivity rate of 33.0% in the wire drawing test [15], in line with the present findings. The proportion of high-viscosity strains among hvKp isolates in our hospital was 84.97%, which was much higher than that in Vandhana's study [16], confirming the high correlation between the high-toxicity phenotype and high-viscosity phenotype in this study. In total, 29 hvKp strains had a non-high-viscosity phenotype in this study, and further research is needed to determine whether their virulence is higher than that of other cKp strains or lower than that of hvKp with the high-viscosity phenotype. Our hospital's dominant capsule serotype was K1, and the dominant ST type was ST23. The minimum spanning tree revealed that all ST23 type strains were K1, which is closely related to liver abscess and in line with previously reported results [17-19]. In this study, hvKp strains exhibited higher sensitivity to commonly used antibiotics than cKp, as reported previously [2]. Studies on hvKp found that males, particularly elderly men, are more susceptible to infection than females [20]. The results of this study are consistent with these findings. This might be explained by the fact that our hospital is located in the Hulunbuir area, which has a cold climate and high drinking habits among men, and studies illustrated that excessive alcohol use increases the susceptibility to hvKp infection [14]. Ding's multivariate analysis revealed that liver abscess and metastatic spread are

independent risk factors for hvKp infection [21], consistent with the current results. At present, no relevant studies have shown that the use of macrolide antibiotics is a risk factor for hvKp infection, and its mechanism needs to be further studied. This study aimed to understand the molecular epidemiological characteristics of hvKp in Hulun Buir region, but there are still some limitations as no hypervirulent and drug-resistant strains were found and 29 hvKp strains did not express the hyper adhesive phenotype. Through this study, we have identified the risk factors for hvKp infection in our hospital. Based on the results, we can develop prevention strategies and treatment pathways to control its spread.

Source of Funds:
1) Fund Project: Medical and Health Technology Plan Project of Inner Mongolia Autonomous Region Health Commission (202201607).
2) Fund Project: General Project of the Inner Mongolia Academy of Medical Sciences Public Hospital Research Joint Fund (2023GLLH0344).

Declaration of Interest:
The authors have no conflicts of interest to declare.

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