

(RESEARCH ARTICLE)



PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF *KLEBSIELLA PNEUMONIAE*: HYPERMUCOVISCOSITY, ANTIMICROBIAL RESISTANCE, AND DETECTION OF *YBT*A AND *RMP*A2 VIRULENCE-ASSOCIATED GENES

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ABSTRACT

Background: A newly emerged pathogenic subtype, Hypervirulent *Klebsiella pneumoniae* (hvKp), can cause a series of severe, systemic invasive infections. Currently, a commonly used phenotypic indicator to clinically identify hvKp is hypermucoviscosity. The conventional method to detect this feature is the string test. This study aims to detect strains with the hypermucoviscous phenotype from clinically isolated *Klebsiella pneumoniae* strains, to determine the antimicrobial susceptibility patterns of these strains, and to detect two pathogenicity-related genes, *ybtA* and *rmpA2*.

Research Methods: A descriptive cross-sectional study. After obtaining clinically isolated *Klebsiella pneumoniae* strains, they were first inoculated on MacConkey agar for subculture, purification, and reidentification using standard biochemical tests. Next, the string test was used to detect the hypermucoviscosity of the strains, and then the Kirby-Bauer disc diffusion method to determine the antimicrobial susceptibility of the strains. Then, bacterial DNA was extracted using the boiling method. After extraction, multiplex PCR technology was used to detect the two pathogenicity-related genes, *ybtA* and *rmpA2*.

Results: A total of 76 *Klebsiella pneumoniae* isolates were analyzed in this study. The *ybtA* gene was detected in 48.7% (37/76) of the strains, while the *rmpA2* gene was detected in 31.6% (24/76) of the samples. Among the test results, 12 strains (15.8%) showed a hypermucoviscous phenotypically; the remaining 64 strains (84.2%) did not have the hypermucoviscous property.

The antimicrobial resistance rates showed that ceftriaxone was the highest (93.4%), followed by gentamicin (68.4%). The resistance rates of classical cKps were all higher than those of hypervirulent hvKps: the resistance rate to gentamicin was 68.7% vs. 66.7%, to ceftriaxone was 93.7% vs. 91.7%, to meropenem was 35.9% vs. 33.3%, and to colistin was 45.3% vs. 16.7%.

Conclusion: This study confirms that both hypermucoviscous hvKp and classical cKp strains coexist among isolates. The detection rates of the two virulence-related genes, *ybtA* and *rmpA2*, are relatively high, indicating that key pathogenic traits persist in the studied strain population.

Keywords: Hypervirulent *Klebsiella pneumoniae*, Classical *Klebsiella pneumoniae*, Hypermucoviscosity, String Test, Ybta, Rmpa2, Virulence Genes, Antimicrobial Resistance, Antimicrobial Susceptibility.

1 INTRODUCTION

1.1 *Klebsiella pneumoniae*

K. pneumoniae is a Gram-negative pathogen that has a large accessory genome of plasmids. This accessory genome divides it into opportunistic pathogens that infect immunocompromised patient. These strains are common causes of healthcare-associated infections such as pneumonia, urinary tract infections (UTIs), and bloodstream infections, other strains of *K. pneumoniae* are hypervirulent (1).

1.2 Hypervirulent *Klebsiella pneumoniae*

Hypervirulent *Klebsiella pneumoniae* (hvKp) is a newly emerged strain of *Klebsiella pneumoniae* in recent years. Even otherwise healthy young people may develop multiple severe diseases after infection, including pyogenic liver abscess, meningitis, and endophthalmitis.

It was first officially reported in Taiwan, China, in the mid-1980s. In recent years, hvKp infection cases have spread worldwide, and related infection cases have been recorded in all inhabited regions of the world.

To distinguish hvKp from the more familiar classical *Klebsiella pneumoniae* (cKp), it is necessary to combine two types of features for judgment: one is the observable growth and manifestation characteristics of the bacteria itself, that is, the phenotype; the other is the inherent gene sequence characteristics of the bacteria, that is, the genotype. The combination of these two types of features can clearly separate the two strains. Hypermucoviscosity is the signature feature of hvKp. This feature needs to be confirmed by the string test on an agar plate, and it has been used as the core standard for identifying this type of strain in research over the past few decades.

The vast majority of hvKp isolates belong to the K1 and K2 capsular serotypes, and most of the recorded severe infections are related to these two serotypes.

A series of virulence-related genes, including the mucoviscosity regulatory genes *rmpA*, *rmpA2*, and the mucoviscosity-related gene *mgaA*, are all associated with the hypervirulent phenotype of this type of strain. (2)

Currently, the most recognized key genetic factors with experimental evidence supporting the hypervirulent phenotype of hvKp are all carried on plasmid sequences; these include the *iuc* biosynthetic gene responsible for synthesizing the siderophore aerobactin, as well as the regulatory genes *rmpA* and *rmpA2* that increase capsule production. (3)

1.3 Geographic Distribution of hvKp Infections:

The vast majority of currently recorded cases of hypervirulent *Klebsiella pneumoniae* (hvKp) infection occur in the Asian Pacific Rim.

Globally, the number of reported cases of this type of infection continues to rise. With the improvement of clinical awareness and the identification of validated biomarkers, we will eventually be able to accurately calculate the global incidence of hvKp infection. (3)

1.3.1 String test

The hypermucoviscous phenotype of *Klebsiella pneumoniae* isolates is confirmed by the string test. To perform this test, first inoculate the strain to be tested on an agar plate, then place the plate in a constant temperature environment of 37°C to allow the bacteria to grow overnight. After cultivation, use a microbiology-specific inoculation loop to pick the bacterial colonies grown on the plate. If a mucous strand longer than 5 millimeters can be pulled out during picking, the string test of this strain is determined to be positive. (2)

1.4 The Accessory Genome in Progression to Disease and Virulence:

K. pneumoniae virulence factors are encoded by genes in both the core and accessory genomes. These genes may determine the severity of an infection, the virulence of the infecting strain, and the ability of the strain to progress to infection, defining the pathogenic potential of the infecting strain. (1)

One of the most important pathogenic factors of *Klebsiella pneumoniae* is its polysaccharide capsule. With this capsule, the bacteria can evade attacks from the immune system after entering the human body—the body's immune cells would originally clear the bacteria through phagocytosis. In this process, the immune system first releases opsonins to mark the bacteria, allowing phagocytes to accurately identify and engulf them, and the capsule can block this marking process, helping the bacteria evade clearance by the immune system. (1)

In addition to the capsule, *Klebsiella pneumoniae* also has a type of tool for obtaining iron, which are siderophores. Siderophores are low-molecular-weight, high-affinity iron-chelating molecules that many bacteria secrete to help themselves obtain iron from the environment — iron is an essential nutrient for bacterial survival, but there is very little free iron in the human body, so bacteria rely on these molecules to capture iron. *Klebsiella pneumoniae* can secrete several types of siderophores, and mixed siderophores such as yersiniabactin (*Ybt*) and aerobactin (*Aer*) are also common in this bacterium. Different siderophores are often associated with the site of infection and the severity of the infection.

Yersiniabactin was first discovered in the genus *Yersinia*. The genes encoding the synthesis, transport, and regulatory functions of this molecule are all located on a transferable chromosomal fragment called a "high pathogenicity island". That is, this segment of genes can be transferred between different bacteria. Any bacterium that obtains it may have the ability to synthesize yersiniabactin, and its pathogenicity will increase accordingly.

If *Klebsiella pneumoniae* in the human body can secrete and utilize yersiniabactin, it often causes respiratory tract infections; applying this strain that can secrete yersiniabactin to a mouse experimental model is sufficient to cause pneumonia in mice. On a global scale, yersiniabactin is the most common among all pathogenic factors related to human *Klebsiella pneumoniae* infections (1).

1.5 Antimicrobial resistance:

There are four mechanisms by which bacteria can evade the killing effect of antibiotics and develop resistance, and strains such as hvKp may acquire resistance through all these methods.

First, hvKp strains directly acquire a plasmid carrying an antibiotic resistance determinant.

Second, they integrate an ICE (Integrative and conjugative Elements) that also carries resistance determinants into their own genetic material, either into their own chromosome or into their own virulence plasmid.

Third, genes on the chromosome mutate, such as genes encoding outer membrane proteins OMPs. After mutation, they either change the drug's target site or reduce the strain's susceptibility to the drug.

Fourth, already MDR (multiple drug resistant) or XDR (extensively drug resistant) cKp strains, in turn, acquire the virulence plasmid of hvKp (3).

1.6 Differences and similarities between hypermucoviscous and hypervirulent phenotypes:

The two phenotypes of hypermucoviscosity and hypervirulence are fundamentally different in terms of detection methods, the range of bacteria they can appear in, the associated strain serotypes, and their clinical impact on humans. hypermucoviscosity can be directly confirmed by a positive string test. This trait not only appears in *K. pneumoniae*, but also often occurs in strains of other serotypes outside K1 and K2. Even if a strain has hypermucoviscosity, this single trait alone is not enough to cause invasive infections in otherwise healthy young people. In contrast, hypervirulent strains cannot be identified by only the string test. They almost only exist in the single species of *K. pneumoniae*, and

only appear in a few specific serotypes. Their most typical feature is that they can cause invasive infections in otherwise healthy young people. (4)

1.7 Justification

Currently, infections caused by Hypervirulent *Klebsiella pneumoniae* (hvKp) are increasing worldwide, posing an increasingly serious threat to public health and safety. Unlike ordinary pathogenic bacteria, this bacterium can cause severe, even life-threatening infections, including difficult-to-treat conditions such as pyogenic liver abscess, endophthalmitis, and meningitis; even young people who usually have no underlying diseases and are in good health can be infected by it (2).

This study mainly completes two core tasks: the first is to use the string test, a common detection method, to investigate whether *Klebsiella pneumoniae* has the hypermucoviscous trait — this phenotype is one of the important references for judging hypervirulent strains; the second is to specifically screen the two genetic markers directly related to bacterial pathogenicity, *rmpA2* and *ybtA*.

If these virulence-related genes can be accurately detected in clinical tests, it can help clinical laboratories in various hospitals identify and characterize *Hypervirulent Klebsiella pneumoniae* faster, avoid confusing it with ordinary strains, and reserve sufficient time for subsequent responses.

2 MATERIALS AND METHODS

We conducted a descriptive, hospital-based cross-sectional study. Simply put, this type of study only collects samples that meet the criteria within a fixed period of time for analysis, without long-term follow-up of subsequent changes in the samples. All observations and records focus on the current situation within this research cycle.

Our research subjects are *Klebsiella pneumoniae* strains, all of which were isolated from different clinical specimens in various microbiology laboratories in Khartoum State. All strains included in the study met the pre-set collection criteria.

The entire study period started in April 2021 and lasted until August 2021, a total of five months. During this period, we included a total of 76 eligible *Klebsiella pneumoniae* strains, all included in the analysis scope of this study.

2.1 Data Collection

All patient-related data used in this study were collated and extracted from the corresponding outpatient or inpatient medical records. Which contain the patient's age, gender and the type of clinical sample collected from the patient that was used in this study.

2.2 Sub-Culture & Colonial morphology

The isolated *Klebsiella pneumoniae* were first inoculated on MacConkey agar medium for subculture, and incubated in an aerobic environment at 37°C for 24 hours (15). This step is to allow the strains isolated from clinical samples to regrow to a sufficient quantity to facilitate subsequent observation and identification. After the incubation period is reached, we can confirm their identity based on the colony characteristics of these strains grown on the medium. A typical *Klebsiella pneumoniae* colony will grow relatively large, slightly protruding from the medium surface, with a smooth and sticky surface, and appear pink overall — this color is not random, it is a characteristic of these bacteria being able to ferment lactose in the medium.

2.2.1 String test

The isolated organisms were sub-cultured on agar media, then incubated at 37°C for 24h. Hypermucoviscosity was detected, by the formation of string measuring 5mm or longer which was defined as a positive string test (2).

2.3 Antimicrobial Susceptibility Test (Kirby-Bauer Method)

A few well-isolated colonies of each *Klebsiella pneumoniae* isolate were suspended in sterile normal saline and adjusted to the turbidity of a 0.5 McFarland standard. The standardized bacterial suspension was evenly inoculated over the surface of Mueller–Hinton agar using a sterile cotton swab to produce a uniform bacterial lawn. Appropriate antibiotic-impregnated disks were then placed aseptically on the agar surface. The plates were incubated aerobically at 35°C for 16–18 hours. After incubation, the diameters of the inhibition zones were measured in millimeters and interpreted according to the applicable antimicrobial susceptibility testing guidelines (15).

Several separate, well-grown colonies of each *Klebsiella pneumoniae* strain were first picked and placed in sterile normal saline to prepare a bacterial suspension. Next, the concentration of this tube of bacterial suspension needs to be adjusted

to be uniform and accurate — the turbidity must be adjusted to completely match the 0.5 McFarland standard. Only with an appropriate concentration can the subsequent resistance test results be accurate. After adjustment, take a sterile cotton swab, dip it fully in the bacterial suspension with the adjusted concentration, and spread it over the entire surface of the Mueller–Hinton agar plate to allow the bacteria to grow a flat and uniform lawn on the plate. After coating the plate, next apply the antimicrobial discs. According to the test requirements, use sterile techniques to gently place the antimicrobial discs impregnated with the corresponding antibiotics on the agar surface that has been inoculated with bacteria, and position them appropriately. All plates with discs applied are then placed in an incubator for cultivation, and must be continuously incubated in an aerobic environment at 35°C for 16 to 18 hours to allow the bacteria to fully grow. After the incubation period is over, the plates can be taken out, and the diameter of all inhibition zones on the plates is measured one by one, recorded in millimeters. Finally, the measured values are interpreted according to the corresponding antimicrobial susceptibility test guidelines in the literature (15) to determine the susceptibility results of these bacteria to different antibiotics.

2.4 Control organisms

A quality control strain, *Klebsiella pneumoniae* ATCC 700603, was used in this experiment, which was obtained from the Department of Microbiology of the National Health Laboratory. During the experiment, we relied on this strain to confirm whether the performance of three core experimental links meets the requirements: the effect of Gram staining, whether the medium used to culture bacteria can function normally, and whether the biochemical identification test used to identify bacterial species is accurate.

2.5 DNA extraction protocol using (Boiling method)

The first step to extract Bacterial DNA using the boiling method is to pick 3-4 scattered, non-overlapping single colonies from freshly cultured bacteria, transfer them into a sterile Eppendorf tube pre-filled with 160µL of sterile distilled water, mix the colonies with water to prepare a bacterial suspension.

Place the prepared suspension in a centrifuge, centrifuge at 10,000 rpm for 5 minutes to allow all bacteria in the tube to sink to the bottom, then carefully pour off the supernatant, leaving only the bacterial pellet at the bottom of the tube.

Add another 200µL of sterile distilled water to the remaining bacterial pellet, resuspend the pellet by blowing and mixing to prepare a new bacterial suspension.

Place the resuspended tube in a BioSan Thermo-Shaker, heat at 100°C for 15 minutes to fully lyse the bacterial cells and release the DNA inside.

Then transfer the tube to a -20°C environment to freeze for 10 minutes, then take it back and heat at 100°C for 2 minutes, after heating, put it back to -20°C to freeze for 2 minutes. Repeated freezing and thawing further lyses the bacterial cells to release more DNA.

Finally, centrifuge the tube at 10,000 rpm for 10 minutes. At this time, the supernatant in the tube already contains the extracted bacterial DNA. Carefully transfer this supernatant to a clean, sterile Eppendorf tube. If long-term storage is required, store it at -20°C for later use (16).

2.6 Multiplex PCR protocol

The total volume of each PCR reaction, that is, the reaction system, is uniformly prepared to 20.0µL, with no variation. When preparing this mixed system, for each target gene to be detected, add 0.25µL of forward primer and 0.25µL of reverse primer. The two target genes correspond to the labeled *rmpA2* (10) and *ybtA* (18) respectively. After adding the primers, add 10µL of distilled water, 5µL of the previously extracted DNA template, and finally add 4µL of Green Master Mix, and mix all the components together.

The entire PCR amplification process is completed on a Prime Thermal Cycler instrument. The thermal cycle parameters of the instrument are pre-set: the first step is initial denaturation at 94°C for 5 minutes to separate the double strands of bacterial DNA, then enter the amplification stage of 10 cycles — in each cycle, first maintain at 95°C for 30 seconds to complete denaturation, then adjust the temperature to 60°C, 55°C, and 50°C in sequence, maintain at each temperature for 30 seconds to allow the primers to bind to the template, that is, complete the annealing step, and finally raise the temperature to 72°C and maintain for 30 seconds to allow the synthesis and extension of new DNA strands, which completes one cycle.

2.7 Gel electrophoresis

First, take 5.0 µL of the PCR amplification product, prepare a 50-bp DNA molecular weight ladder, and add both into the wells of a 2.0% agarose gel. Next, start the electrophoresis process. The entire process is completed in 1× Tris-borate-EDTA (TBE) buffer, set the voltage to 120 V, and continue to apply electricity for 1 hour to allow DNA fragments of different molecular weights to separate in the gel. After electrophoresis, take out the gel, stain the gel with ethidium bromide to add a chromogenic marker to the originally invisible DNA fragments, and then use a UV transilluminator imaging system to capture images under an ultraviolet (UV) light source, so that the separated nucleic acid bands can be clearly observed. To identify which specific type these amplified DNA fragments belong to, just compare their migration distance in the gel with the migration distance of the 50-bp DNA molecular weight ladder on the same gel to complete the identification.

2.8 Data management and statistical analysis

The collected data were entered, organized, and managed using Microsoft Excel 2007. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 23. Descriptive statistics were used to summarize the study variables and present the findings in appropriate tables.

2.9 Ethical consideration

Before the start of this study, we had successively obtained ethical approval from three institutions: the University of Al-Butana, the Research Department of the Khartoum State Ministry of Health, and all hospitals participating in this study, fully complying with the ethical requirements for conducting medical research. All information used in the study was extracted from patients' medical records. The entire process of data collection, storage, and subsequent analysis was strictly managed in accordance with confidentiality regulations, and the personal privacy of patients would not be leaked in any form. Moreover, these data were only used for the pre-set research purposes of this study, and would not be diverted to any other use.

3 RESULTS

3.1 Distribution of *Klebsiella pneumoniae* isolates according to the type of clinical sample:

All clinical isolates were re-identified following subculture using Gram staining and standard biochemical tests. Of the 76 isolates included in the study, all (100%) were confirmed as *Klebsiella pneumoniae*.

Sputum was the most frequently represented clinical sample, accounting for 77.6% of the isolates, followed by urine samples (10.5%), wound swabs (9.2%), and vaginal swabs (2.6%), as shown in Table 1.

Table 1: Distribution of *Klebsiella pneumoniae* isolates according to the type of clinical sample.

Type of sample	Frequency	Percent
Sputum	59	77.6
Wound	7	9.2
Urine	8	10.5
Vaginal swab	2	2.6
Total	76	100

3.2 Antimicrobial susceptibility patterns:

The antimicrobial susceptibility of each of these strains was recorded in Table 2. From the recorded data, it can be seen that among all the tested antibiotics, these strains had the highest resistance rate to ceftriaxone, with 93.4% of the strains able to tolerate this antibiotic.

Table 2: Percentage of susceptibility patterns of *Klebsiella pneumoniae* isolates to antibiotics

Antimicrobial	Reaction	Frequency	Percent	Total
Gentamicin	Sensitive	11	14.5	76 (100%)
	Resistant	52	68.4	
	Intermediate	13	17.1	
Colistin	Sensitive	45	59.2	76 (100%)
	Resistant	31	40.8	
Ceftriaxone	Sensitive	5	6.6	76 (100%)
	Resistant	71	93.4	
Meropenem	Sensitive	41	53.9	76 (100%)
	Resistant	27	35.5	
	Intermediate	8	10.5	

3.3 String test results:

We first used the string test to detect whether the isolated strains had the hypermucoviscous trait, that is, the corresponding phenotype. After the test, if the result was positive, the corresponding isolate was classified as Hypervirulent *Klebsiella pneumoniae* (HvKp); if the result was negative, the corresponding isolate was classified as classical *Klebsiella pneumonia* (cKp). Among all the strains tested in this study, 15.8% had a positive string test result, and the remaining 84.2% had a negative result, as shown in Table 3, Figure-1.

Table 3: Percentage of string test results.

String test	Frequency	Percent
Positive	12	15.8
Negative	64	84.2
Total	76	100

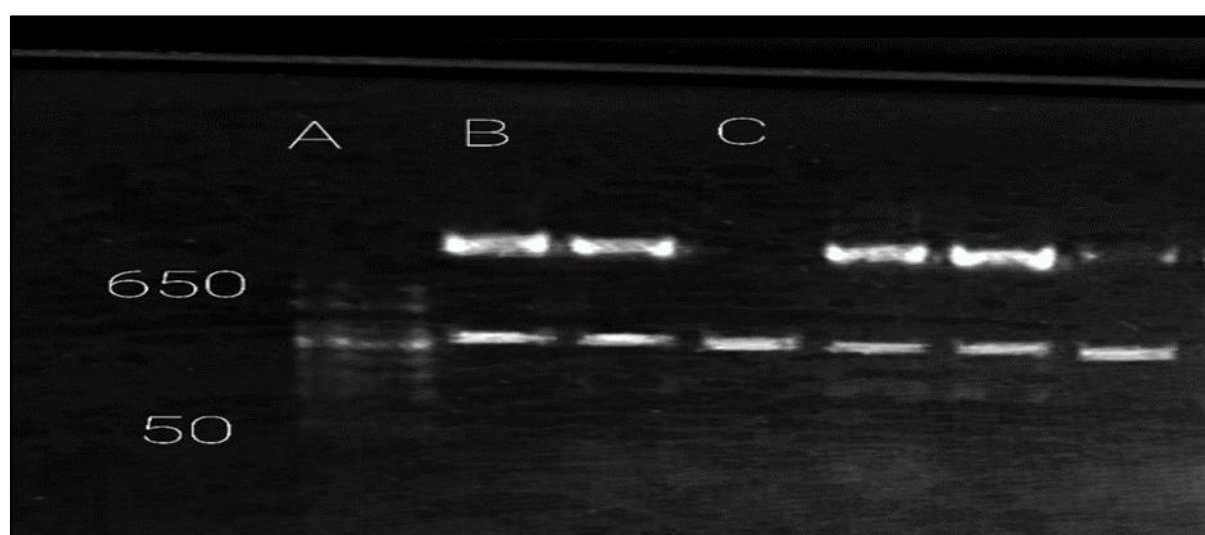
**Figure 1: String test (Positive result)**

3.4 Frequency and percentage of *ybtA* and *rmpA2* virulence genes among isolates:

Among the 76 *K. pneumoniae* isolates examined, the *ybtA* virulence gene was detected in 37 (48.7%) isolates, whereas *rmpA2* was detected in 24 (31.6%) isolates. The *ybtA* gene was therefore more frequently detected than *rmpA2* among the studied isolates, as shown in Table 4, Figure-2.

Table 4: Frequency and percentage of *ybtA* and *rmpA2* virulence genes among the studied *Klebsiella pneumoniae* isolates

Virulence gene	Status	Frequency	Percent (%)
<i>ybtA</i>	Present	37	48.7
	Absent	39	51.3
	Total	76	100.0
<i>rmpA2</i>	Present	24	31.6
	Absent	52	68.4
	Total	76	100.0

**Figure2: PCR positive amplicons in gel electrophoresis.**

- A: 100 bp ladder.
- B: Band of *ybtA* gene 960 bp.
- C: Band of *rmpA2* gene 369 bp.

3.5 Frequency of virulence genes carried by the isolates.

Overall, 44 (57.9%) isolates carried at least one of the investigated virulence genes, whereas 32 (42.1%) isolates carried neither gene. The most frequent virulence gene profile was *ybtA* alone, detected in 20 (26.3%) isolates, followed by the combined presence of *ybtA* and *rmpA2* in 17 (22.4%) isolates. The *rmpA2* gene alone was detected in 7 (9.2%) isolates and represented the least frequent virulence profile.

When considered individually, *ybtA* was present in 37 isolates (48.7%) (20 isolates carrying *ybtA* alone plus 17 carrying both genes), while *rmpA2* was present in 24 isolates (31.6%) (7 isolates carrying *rmpA2* alone plus 17 carrying both genes). Thus, *ybtA* was more commonly detected than *rmpA2* among the studied isolates.

Among the strains collected and isolated in this study, we tested each one for two pathogenicity-related genes, *ybtA* and *rmpA2*. First, let's sort out the carrying status of these genes in the strains.

A total of 44 strains, accounting for 57.9%, carried at least one pathogenic gene tested in this study. The remaining 32 strains, accounting for 42.1%, did not carry either of the two genes tested in this study.

We sorted out the gene combinations carried by the strains. The most common gene combination was carrying only *ybtA*, which was found in 20 strains, accounting for 26.3%. Next was the co-existence of both *ybtA* and *rmpA2*, which occurred in 17 strains, accounting for 22.4%. Only 7 strains carried *rmpA2* alone, accounting for 9.2%, which is the least common gene combination among all strains in this study.

If we count the occurrence of the two genes separately, *ybtA* was carried by a total of 37 strains, accounting for 48.7% — 20 of these carried only *ybtA*, and the remaining 17 carried both genes. *rmpA2* was detected in 24 strains, accounting for 31.6%, 7 carried only *rmpA2*, and the other 17 co-existed with *ybtA*. Calculated this way, among all the analyzed strains, *ybtA* appeared more frequently than *rmpA2*.

Table 5: Frequency of virulence genes carried by the isolates.

Virulence gene profile	Number of isolates	Frequency (%)
<i>ybtA</i> + <i>rmpA2</i>	17	22.4%
<i>ybtA</i>	20	26.3%
<i>rmpA2</i>	7	9.2%
No genes	32	42.1%
Total	76	100%

3.6 Association between *rmpA2* virulence gene and string test results among *K. pneumoniae* isolates:

Of the 24 isolates carrying the *rmpA2* gene, 2 (8.3%) were positive by the string test, while 22 (91.7%) were negative. Among the 52 isolates lacking *rmpA2*, 10 (19.2%) were string-test positive and 42 (80.8%) were negative.

Table 6: Association between *rmpA2* virulence gene and string test results among *K. pneumoniae* isolates

<i>rmpA2</i> gene	String test		Total n (%)
	Negative n (%)	Positive n (%)	
Present	22 (91.7)	2 (8.3)	24 (100.0)
Absent	42 (80.8)	10 (19.2)	52 (100.0)
Total	64 (84.2)	12 (15.8)	76 (100.0)

3.7 Antimicrobial susceptibility patterns according to the presence of the *ybtA* virulence gene:

A total of 76 *Klebsiella pneumoniae* strains were isolated in this study. We divided these strains into two groups — one carrying the *ybtA* virulence gene and the other not carrying it, and counted their drug susceptibility to various antibiotics, that is, their resistance status. All detailed data are listed in Table 10.

Looking at the strains carrying the *ybtA* gene, the highest resistance rate was to ceftriaxone, reaching 91.9%, followed by gentamicin, with a resistance rate of 78.4%. Of the remaining two tested antibiotics, the resistance rate to meropenem was 40.5%, and to colistin was 43.2%.

Looking at the strains not carrying the *ybtA* gene, the highest resistance rate was also to ceftriaxone, which was even higher, reaching 94.9%. The resistance rates of the subsequent antibiotics were: gentamicin 59.0%, meropenem 30.8%, and colistin 38.5%.

Combining the situations of the two groups, it can be found that regardless of whether the strains carried the *ybtA* virulence gene, their resistance rate to ceftriaxone was at a very high level.

Table 7: Antimicrobial susceptibility patterns according to the presence of the *ybtA* virulence gene

Antimicrobial	<i>ybtA</i> gene	Resistant n (%)	Sensitive n (%)	Intermediate n (%)	Total n (%)
Gentamicin	Present	29 (78.4)	4 (10.8)	4 (10.8)	37
	Absent	23 (59.0)	7 (17.9)	9 (23.1)	39
	Total	52 (68.4)	11 (14.5)	13 (17.1)	76
Colistin	Present	16 (43.2)	21 (56.8)	-	37

	Absent	15 (38.5)	24 (61.6)	-	39
	Total	31 (40.8)	45 (59.2)	-	76
Meropenem	Present	15 (40.5)	17 (45.9)	5 (13.5)	37
	Absent	12 (30.8)	24 (61.5)	3 (7.7)	39
	Total	27 (35.5)	41 (53.9)	8 (10.5)	76
Ceftriaxone	Present	34 (91.9)	3 (8.1)	-	37
	Absent	37 (94.9)	2 (5.1)	-	39
	Total	71 (93.4)	5 (6.6)	-	76

3.8 Antimicrobial susceptibility to selected antibiotics according to the presence of the *rmpA2* gene

The strains carrying the *rmpA2* gene had significantly higher resistance rates to gentamicin and meropenem than the strains not carrying this gene. Looking at the resistance to colistin, there was almost no difference between the *rmpA2*-positive and negative groups. As for ceftriaxone, regardless of whether the strains carried the *rmpA2* gene, their resistance rate to it remained high, which indicates that resistance to this antibiotic is widespread among all strains, and there will be no obvious polarization just because the strains carry the *rmpA2* gene or not.

Table 8: Antimicrobial susceptibility to selected antibiotics according to the presence of the *rmpA2* gene

Antimicrobial	<i>rmpA2</i> gene	Resistant n (%)	Sensitive n (%)	Intermediate n (%)	Total n (%)
Gentamicin	Present	20 (83.3)	3 (12.5)	1 (4.2)	24
	Absent	32 (61.5)	8 (15.4)	12 (23.1)	52
	Total	52 (68.4)	11 (14.5)	13 (17.1)	76
Colistin	Present	10 (41.7)	14 (58.3)	-	24
	Absent	21 (40.4)	31 (59.6)	-	52
	Total	31 (40.8)	45 (59.2)	-	76
Meropenem	Present	11 (45.8)	10 (41.7)	3 (12.5)	24
	Absent	16 (30.8)	31 (59.6)	5 (9.6)	52
	Total	27 (35.5)	41 (53.9)	8 (10.5)	76
Ceftriaxone	Present	23 (95.8)	1 (4.2)	-	24
	Absent	48 (92.3)	4 (7.7)	-	52
	Total	71 (93.4)	5 (6.6)	-	76

Table 9: Distribution of clinical samples among HvKp (string test positive) and cKp (string test negative) isolate.

		String test		Total
		Negative	positive	
type of sample	Sputum	50	9	59
	Wound	6	1	7
	Urine	7	1	8
	vaginal swab	1	1	2
Total		64	12	76

4 DISCUSSION

In this study, pneumonia was the most common type of infection, accounting for 77.6% of all cases. It was followed by urinary tract infections, accounting for 10.5%; wound infections, accounting for 9.2%; and vaginal infections, accounting for 2.6%. Male subjects were in the majority, reaching 67.1%, while females accounted for only 32.9%. In terms of age distribution, patients aged 40 to 49 years accounted for the highest proportion, 26.3% of the total sample size. The reason why pneumonia accounted for the highest proportion is related to the fact that *Klebsiella pneumoniae* itself is the main pathogenic bacterium for respiratory tract infections, and this type of bacteria is particularly likely to infect hospitalized patients and vulnerable groups.

The ybtA gene is related to the yersiniabactin siderophore system of *Klebsiella pneumoniae* this system helps bacteria capture iron, which is a key nutrient for bacteria to survive and cause disease. Among the 76 strains tested in this study, 37 carried this gene, accounting for 48.7%. This proportion is highly consistent with the research results of Wyres et al., who detected that about 49% of *Klebsiella pneumoniae* strains collected in South and Southeast Asia carried the ybt gene related to yersiniabactin (5). The detection rate of this gene in strains from different regions is very close, indicating that this iron acquisition system has been widely present in *Klebsiella pneumoniae* populations in multiple regions far apart. The high detection rate of ybtA in this study is worthy of attention, because bacteria carrying this gene are more likely to survive when infecting the human body, and their pathogenicity will also increase.

Another pathogenicity-related gene that can enhance bacterial virulence, rmpA2, had a detection rate of 31.6% in this study, with 24 out of 76 strains carrying this gene. This proportion is higher than the 16% reported by Wyres et al. in their South and Southeast Asia cohort (5), and also higher than the 20% reported in another study by Ahmed et al. (10). The difference between the results of this study and previous studies may be due to differences in the endemic *Klebsiella pneumoniae* strains in different regions, differences in the population composition of patients in various regions, differences in the clinical sources of collected samples, differences in the sample size used in the studies, or even differences in the molecular methods used to detect the genes. Even with these possible influencing factors, the fact that a considerable proportion of strains in the local test population carry rmpA2 still confirms the widespread existence of this key virulence factor in the local *Klebsiella pneumoniae* population.

In this study, we used the string test to detect the isolated *Klebsiella pneumoniae* strains. Among the 76 strains, 12 were found to have the hypermucoviscous trait, accounting for 15.8%. This proportion is higher than the value reported by Remya et al.'s team — in their study, only 2.4% of all tested strains had a positive string test, corresponding to 9/370, with the relevant study cited as (12). The data recorded by Shi et al. is very different. In their study of Hypervirulent *Klebsiella pneumoniae* strains, 36 out of 56, that is, 64.3% of the strains tested positive for the hypermucoviscous phenotype, with the study cited as (13). There are many possible factors for such large differences in results between different studies, including differences in the study populations of each study, the geographical environments where they were conducted, the sample sizes collected, the sources of clinical samples used, and most importantly, the different criteria used to define Hypervirulent *Klebsiella pneumoniae*. These cross-study differences also indicate that relying solely on the string test cannot accurately determine whether a strain is hypervirulent. To draw reliable conclusions, it is necessary to combine the results of the string test with the detection results of molecular virulence biomarkers and other observable phenotypic characteristics for analysis.

We also specifically analyzed the association between the hypermucoviscous phenotype and the rmpA2 gene. Among the 12 string-test positive strains in this study, all were tested for the rmpA2 gene, and only 2 strains actually carried this gene. This result indicates that the mere detection of the hypermucoviscous phenotype does not fully align with the presence of the rmpA2 gene. Not all strains with the hypermucoviscous trait carry this gene, nor do all strains carrying this gene test positive for hypermucoviscosity. There are also research conclusions that differ from this result. Shankar et al. once detected 7 string-test positive strains, each of which carried rmpA2, with the study cited as (14). In the study by Catalán-Nájera et al., 5.4%, that is, 53 out of 878 strains presented a hypermucoviscous phenotype, and only 16 of these 53 strains carried any rmpA gene variant, with the study cited as (4). The mismatch between the gene test results and the phenotypic test results is most likely because multiple unrelated genetic factors can regulate the production of bacterial capsules and the generation of hypermucoviscosity, and the expression level of the genes themselves will also affect the final test results.

In this study, *Klebsiella pneumoniae* was resistant to gentamicin (68.4%), colistin (40.8%), ceftriaxone (93.4%), and meropenem (35.5%).

Speaking of antimicrobial susceptibility, that is, whether bacteria develop resistance to antibiotics and can withstand the efficacy of the drugs, the overall resistance of the *Klebsiella pneumoniae* isolated in this study is very high.

The highest resistance rate was to ceftriaxone, accounting for 93.4%; followed by gentamicin at 68.4%, colistin at 40.8%, and meropenem at 35.5%. The high resistance rate to ceftriaxone is worthy of vigilance — third-generation cephalosporins were originally commonly used drugs to treat severe Gram-negative bacterial infections. Now, nearly 90% of strains can withstand this drug, making subsequent treatment very difficult to carry out. The resistance to gentamicin is also prominent, and even meropenem, a commonly used "last-line" antibiotic, has developed resistance. All these indicate that the strains in this study bear a heavy overall resistance burden.

Comparing the resistance of the two types of *Klebsiella pneumoniae* divided in the study — Hypervirulent *Klebsiella pneumoniae* (HvKp) and classical *Klebsiella pneumoniae* (cKp), the overall resistance level of classical strains is generally higher. Specifically for each drug, the resistance rate to ceftriaxone was 91.7% (11 out of 12 strains were resistant) in hypervirulent strains, and 93.7% (60 out of 64 strains were resistant) in classical strains; the resistance rate to meropenem was 33.3% (4 out of 12 strains were resistant) in hypervirulent strains, and 35.9% (23 out of 64 strains were resistant) in classical strains; the resistance rate to gentamicin was 66.7% (8 out of 12 strains were resistant) in hypervirulent strains, and 68.7% (44 out of 64 strains were resistant) in classical strains. The difference in resistance rates for the first several drugs is small. The most obvious difference in resistance rates between the two types of strains is for colistin: only 16.7% (2 out of 12 strains) of hypervirulent strains were resistant, while the resistance rate in classical strains reached 45.3% (29 out of 64 strains), a difference of nearly 30%.

The conclusion that classical strains have a higher overall resistance rate is basically consistent with the research results of Abd-Elmonsef et al. Abd-Elmonsef's team also found that the resistance rate of classical *Klebsiella pneumoniae* is higher than that of hypervirulent strains. In their study, the resistance rate to gentamicin was 26.09% (12 resistant strains) in hypervirulent strains, and 49.25% (33 resistant strains) in classical strains; the resistance rate to ceftriaxone was 28.26% (13 resistant strains) in hypervirulent strains, and 59.7% (40 resistant strains) in classical strains; no meropenem-resistant strains were detected in hypervirulent strains, and the resistance rate in classical strains was 5.97% (4 resistant strains) (17).

5 CONCLUSION

This study demonstrated the presence of both hypervirulent *Klebsiella pneumoniae* (HvKp) and classical *Klebsiella pneumoniae* (cKp) among clinical isolates. Isolates exhibiting the hypermucoviscous phenotype based on the string test were classified as HvKp, whereas those lacking this phenotype were classified as cKp. Classical *K. pneumoniae* isolates generally exhibited higher levels of antimicrobial resistance than HvKp isolates, particularly against gentamicin, ceftriaxone, meropenem, and colistin. The study also demonstrated the presence of important virulence-associated genes, including *ybtA* and *rmpA2*, among the investigated isolates. The coexistence of virulence determinants and antimicrobial resistance highlights the clinical and public health importance of continuous surveillance of *K. pneumoniae* infections.

This study confirms that two types of *Klebsiella pneumoniae* strains, hypervirulent *Klebsiella pneumoniae* (HvKp) and classical *Klebsiella pneumoniae* (cKp), coexist among the strains isolated from clinical cases. The criteria used in this study to divide the two types of strains rely on the hypermucoviscous phenotype detected by the string test: strains that can detect this phenotype are classified as HvKp, and strains that do not have this phenotype are classified as cKp. The overall antibiotic resistance level of classical *K. pneumoniae* strains is generally higher than that of HvKp strains, which is particularly prominent in the several drugs of gentamicin, ceftriaxone, meropenem, and colistin. The study also detected multiple key pathogenicity-related genes, including *ybtA* and *rmpA2*, among all tested strains. The co-occurrence of genes that enhance bacterial pathogenicity and resistance traits that render antibiotics ineffective in strains highlights one thing: continuous monitoring of *Klebsiella pneumoniae* infections is of great significance for both clinical disease diagnosis and treatment, and prevention and control in the entire public health field.

Recommendations

It is necessary to strengthen the daily monitoring of *K. pneumoniae*, closely track the emergence and spread trend of hypervirulent and antibiotic-resistant strains, to prevent the silent spread of these dangerous strains.

Laboratories should speed up the identification of hypervirulent *K. pneumoniae*, combine phenotypic detection methods such as the string test with molecular detection of pathogenicity-related genes, and improve the accuracy and efficiency of detection.

Future research should also detect more pathogenic genes and resistance genes, more comprehensively characterize HvKp and cKp isolates, and provide a more detailed basis for subsequent prevention, control, and treatment.

Limitations

This study has several important limitations like the short duration of the study, the sample size collected is relatively small. This deficiency may prevent our research conclusions from being generalized to a wider population.

Besides, we did not perform DNA sequencing during the entire study, so we could not conduct a more in-depth genetic analysis of the detected virulence genes.

In addition to, only tested two virulence-related genes, *ybtA* and *rmpA2*, and failed to identify other possible virulence factors in the study samples, leaving many gaps in the molecular-level analysis.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

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

Data Availability Statement

Availability of data and materials: The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request, subject to compliance with institutional data governance policies.

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