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Original Article

Clinical Insights into Klebsiella Pneumoniae: Prevalence, Antibiotic Resistance, and Virulence Gene Analysis in Hospitalized Patients

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ABSTRACT

Klebsiella pneumoniae has two pathotypes: hypervirulent (hvKP) and classical (cKP). Hypervirulent hKp produces more severe systemic infections. The study aimed to isolate and identify K. pneumoniae from clinical specimens, study the frequency of hvkp compared to cKP, evaluate the effectiveness of antibiotics against them, and examine virulence gene distribution. Samples from admitted patients were cultured on MacConkey agar, and pink colonies were identified as Klebsiella pneumoniae using colony morphology, Gram staining, urease, and Simmons citrate agar. K. pneumoniae was also confirmed, and Vitek 2 sensitivity was assessed. String tests were used for hypermucoviscosity. The molecular identification of the 16s-23s ITS gene and the virulence-associated genes entB, rmpA, and magA was evaluated using multiplex PCR. PCR amplification of the 16S–23S ITS region verified the identification of 54 isolates initially biochemically identified as K. pneumoniae; 11 (20.4%) showed as hvKP and 43 (79.6%) showed as cKP. All and cKp isolates had the entB gene; however, only one had the rmpA gene (9%). Moreover, none of the isolates had magA genes. Results of the antibiotic susceptibility test were exhibited, with complete resistance noted for ampicillin and substantial resistance for cefazolin, ceftriaxone, ceftazidime, and several other antibiotics. In contrast, hvKP isolates had reduced resistance rates for ceftriaxone, piperacillin/tazobactam, cefepime, gentamicin, ceftazidime, and trimethoprim/sulfamethoxazole. Tigecycline and colistin were the only antibiotics that worked against cKp, with 4.6% of isolates resistant. Finally, the study confirmed K. pneumoniae isolates; only a small number were classified as hvKp, all containing the entB gene, while cKp isolates showed significant antimicrobial resistance, effectively treatable only with tigecycline and colistin.

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1. Introduction

Antimicrobial resistance (AMR) is a major global health concern, causing numerous fatalities each year. Six bacterial species—Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.—are collectively referred to as ESKAPE pathogens and are recognized for their ability to evade the effects of antibiotics (Nicitra et al., 2025). An essential member of the Enterobacteriaceae family, K. pneumoniae is a rod-shaped, encapsulated, nonmotile, Gram-negative bacterium that may cause several diseases, such as bacteremia, biliary and urinary tract infections, and gastrointestinal, cutaneous, nasopharyngeal, and

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osteomyelitis (Karimi et al., 2021). Among Gram-negative bacteria, K. pneumoniae has the highest mortality rate and is the most prevalent cause of bacteremia (Kot et al., 2023). Classical (cKP) and hypervirulent (hvKP) are two distinct types of K. pneumoniae that have been identified. Nowadays, cKp strains cause most infections from K. pneumoniae, particularly in people with impaired immune systems (Choby et al., 2020). On the other hand, Taiwanese reports from the mid-1980s to the 1990s identified a novel and hypervirulent clinical variant of hvKP capable of causing severe systemic infections in otherwise healthy individuals (Guo et al., 2017). Many studies on hypervirulent K. pneumoniae (hvKP), linked to the notable death of 42% in Russia, have been released recently (Khrulnova et al., 2022). Overall, infections due to hvKp have high rates of morbidity and mortality (Chen et al., 2023).

A major hazard to public health throughout the world in recent times has been the rise of antibiotic-resistant (hvKp) strains of K. pneumoniae, which have significant resistance to several critical antimicrobial classes, including β-lactam drugs, quinolones, fluoroquinolones, and aminoglycosides. Increasing resistance occurs among polymyxin B, colistin, fosfomycin, and tigecycline (Fursova et al., 2021). Multidrug-resistant isolates generate infections that have much higher expenses for the healthcare system (Zhen et al., 2019). Thus a serious danger to global public health has emerged in the form of antibiotic resistance in (hvKp) strains (Guo et al., 2017). The ESKAPE bacteria, especially K. pneumoniae, has been recognized as a species by the World Health Organization (WHO) in response to the increasing global issue of antibiotic resistance that requires immediate attention, and it is encouraged for the discovery of new medicines (Tacconelli, 2017).

Several of the virulence factors associated with K. pneumoniae include a polysaccharide capsule that prevents the entry of serum components that kill bacteria (Zhu et al., 2021). Advances in genomic analysis enabled researchers to detect important virulence genes, which are more reliable for identification of hypervirulence. Phenotypic characters in hvKP are called hypermucoviscosity; it is a typical character of the hvKP strain, but it is not specific (Mohamed Hassan et al., 2024a). The rmpA gene, which is a positive regulator of extracapsular polysaccharide synthesis, is located on a plasmid that is 180 kilobases long (Luo et al., 2014). It has been revealed that the hypermucoviscous phenotype is related to various putative virulence factors (Mohamed Hassan et al., 2024a; Wahl et al., 2024). Two of these putative virulence factors are the regulator of mucoid phenotype A (rmpA) and the mucoviscosity-associated gene A (magA). There was an initial theory that magA mediated the hypermucoviscous phenotype. Additional research demonstrated that magA is the causative agent of K. pneumoniae capsular serotype K1(Zhang et al., 2016). Enterobactin (Ent) and other siderophores are more common in hypervirulent K. pneumoniae compared to classical K. pneumoniae (Yan et al., 2016). This study aims Isolate and identify K. pneumoniae from clinical specimens, study the frequency of hvKP compared to cKP, evaluate the effectiveness of antibiotics against them, and examine virulence gene distribution.

2. Materials and methods

2.1 Collection and identification of clinical isolates of K. pneumoniae

From November 2024 to January 2025, a total of 54 K. pneumoniae clinical isolates were collected under complete aseptic conditions, derived from a variety of clinical sources, such as urine (n=14), endotracheal aspiration (ETA) (n=12), blood (n=11), body fluid (plural fluid, seminal fluid, peritoneal fluid, drain fluid, and abdominal fluid) (n=7), wound (n=5), pus (n=3), sputum (n=1), and final vaginal swab (n=1) of patients admitted to the Shar Hospital and Smart Health Tower in Sulaimani city. Bacterial specimens were immediately cultivated for 24 hours at 37°C on the MacConkey agar medium before being incubated under aerobic conditions. Colonies with a

pink color were confirmed to be K. pneumoniae using VITEK-2 (BioMerieux, France) and standard biochemical evaluations such as colony morphology, Gram staining, urease, and Simmons citrate agar (Hashemi et al., 2018). The strains were stored on 20% glycerol at -60°C until PCR was done according to (Mohamed Hassan et al., 2024a).

2.2 String test

The string test was used to evaluate the hypermucoviscosity phenotype of the isolates and differentiate between hvkp and ckp strains. Stretching bacterial colonies allowed the inoculation loop to generate a viscous string at least 5 mm in length (figure 1). On agar plates, the string test returned negative findings when either no string was produced or the string length was less than 5 mm, as reported in (Yan et al., 2024).

2.3 Molecular identification of K. pneumoniae

The Genomic DNA Kit from Genesand Biotech was utilized to extract genomic DNA from clinical isolates of Klebsiella pneumoniae, following the manufacturer's guidelines for gram-negative bacteria. Then the concentration of all extracted DNA was determined by measuring the quantity of all extracted DNA with a Nanodrop 2000c/Thermo Scientific, USA. According to (Bunu et al., 2020). The molecular identification was validated by amplifying the 16s-23s internal transcribed spacer (ITS) region by polymerase chain reaction (PCR) utilizing the specific primers listed in Table 1. The unique amplification conditions were followed to achieve the 130 bp band. These conditions comprised a step of elongation at 72°C for 5 minutes after a 4-minute denaturation phase at 95°C and 35 cycles of 95°C, 58°C, and 72°C. A 1.5% agarose gel was used for electrophoresis to analyze the PCR findings (Turton et al., 2010). Subsequently, the PCR products (figure 2) underwent purification and sequencing using Sanger's method, with further processing performed by the QIAquick PCR Purification kit, and were sequenced on an Applied Biosystems 3500 Genetic Analyzer at Macro Gene Genome Center in South Korea.

Table 1. Primers are utilizing to discover relevant genes.

Primers	Primer Sequence (5'-3')	Product Size (bp)	References
16S-23S ITS	F: ATTTGAAGAGGTTGCAAACGAT R: TTCACTCTGAAGTTTTCTTGTGTTC	130 bp	(Liu et al., 2008)
MagA	F: GGTGCTCTTTACATCATTGC R: GCAATGGCCATTTGCGTTAG	1,283 bp	(Fang et al., 2004)
RmpA	F: CATAAGAGTATTGGTTGACAG R: CTTGCATGAGCCATCTTTCA	461 bp	(Compain et al., 2014)
EntB	F: GTCAACTGGGCCTTTGAGCCGTC R: TATGGGCGTAAACGCCGGTGAT	400 bp	(Compain et al., 2014)

2.4 Multiplex Polymerase chain reaction for detection of three virulence factors

Multiplex PCR has targeted a few virulence genes. We used a set of primers for three targets, entB, rmpA, and magA, as detailed in (Table 1.) Reactions using the final reagent concentrations and quantities were conducted in 25 µl; 1× Multiplex PCR mixture consisted of 1.0 µl of 5 pmole forward primer, 1.0 µl of 5 pmole reverse primer (Sinaclone, Iran), 10 µl of PCR Master Mix, and by addition of 2 µl of genomic DNA, the final volume became 25 µl by completing with (ddH2 O) double distilled water, then the thermal cycler was programmed for the amplification of genes, as follows: The process began with a 15-minute activation at 95°C, continued with 30-second cycles of 94°C, 60°C, and 72°C, and ended with a 10-minute extension at 72°C. For 45 minutes at 100 V, ethidium bromide was added to a 2% (wt/vol) agarose gel to separate the amplicons (Compain et al., 2014). (fig. 3).

2.5 Antibiotic susceptibility by VITEK 2 system

The VITEK-2 System employs an automated method for antibiotic susceptibility testing (AST) grounded in the minimum inhibitory concentration (MIC) technique. The following antibiotics were included: ampicillin, piperacillin/tazobactam, cefazolin, ceftazidime, ceftriaxone, cefepime, ertapenem, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, levofloxacin, nitrofurantoin, trimethoprim/sulfamethoxazole, colistin, and tigecycline. The organism suspension is adjusted to a turbidity of 0.5–0.63 McFarland (~1.5 × 10^8 CFU/mL) using a turbidimeter before being used to rehydrate the antibacterial medium within the card and is diluted in 0.45% saline to a predetermined concentration. Following the manufacturer's instructions, the cards were stacked, sealed, and placed into the VITEK-2 system, which is an instrument incubator and reader. Over the course of 18 hours, the device tracked the bacterial development in each well of the card. Each antibacterial on the card had its minimum inhibitory concentration (MIC) calculated after the incubation cycle ended. A pair of AST-417 and AST-419 cards from BioMérieux in France was utilized (Clinical & Institute, 2020; Karatuna et al., 2024).

2.6 Statistical analysis

Researchers examined the study's data using GraphPad Prism (version 9.0). Quantitative data is presented as percentages and numbers. A chi-square test of independence was used to analyze the comparability of categorical data. Differences were considered statistically significant when the p-value was less than or equal to 0.05.

3. Result

3.1 Clinical characteristics of hvKp and cKp isolates

The current study analyzed 54 K. pneumoniae isolates obtained from hospitalized patients' clinical specimens from Shar Hospital and Smart Health Tower in Sulimani City. Out of them, 11 isolates (20.4%) tested positive for the string analysis and were subsequently identified as hypervirulent K. pneumoniae (hvKP). HvKP samples were collected from various sources, including (6) endotracheal aspirate (ETA), (1) blood, (3) body fluids, and (1) urine. The remaining 43 isolates (79.6%) were identified as classical K. pneumoniae (cKP), with sources including (6) ETA, (9) blood, (4) body fluids, (5) wounds, (13) urine, (3) pus, (1) sputum, and (1) vaginal sample. Clinical specimen types were not significantly different between hvKP and cKP isolates.

3.2 Molecular identification of K. pneumoniae

To verify the molecular identity, the 16s-23s (ITS) region was amplified using specified primers (Table 1) in a PCR assay (Figure 2). Subsequently, we purified and sequenced the PCR products using Sanger's method, processed them with the QIAquick PCR Purification kit, and sequenced them on an Applied Biosystems 3500 Genetic Analyzer at Macro Gene Genome Center in South Korea.

3.3 Detecting virulence genes through multiplex PCR

The investigation of K. pneumoniae isolates focused on the presence of genes associated with capsule K antigens. Among the 11 confirmed hvKp isolates, all (100%) were found to carry the entB gene. In contrast, the RmpA gene was detected in only one isolate, representing 9% of the hvKp isolates. Additionally, among the 23 isolates that tested negative in the string test, the entB gene was also found in all (100%) of these isolates. However, the magA gene was absent in all tested isolates (Table 2 and Fig. 3).

3.4 Antibiotic-resistant profiles Characteristics of hvKp and cKp Isolates

A higher number of cKP isolates exhibited resistance to numerous antimicrobial medicines, including ampicillin (100%), cefazolin, ceftriaxone (88.3%), ceftazidime (79%), cefuroxime (76.7%), ciprofloxacin(69.7%),trimethoprim/sulfamethoxaz

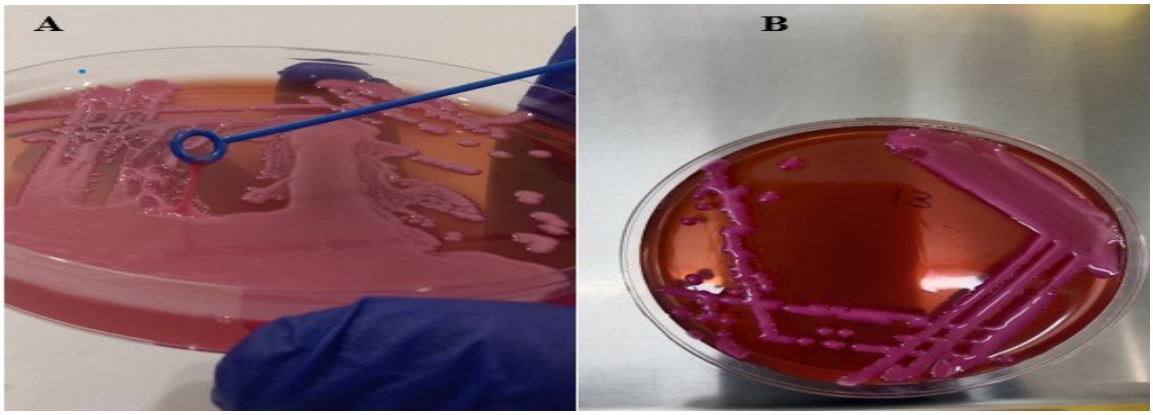

Figure 1. (A) The K. pneumoniae colonies exhibited the hypermucoviscous phenotype when stretched, forming a string ≥ 5 mm in length. (B) Colonies are round, mucoid, and convex with a full borde

ole (67.4%), piperacillin/tazobactam, cefepime, levofloxacin (62.7%), nitrofurantoin (60.4%), ertapenem (53.4%), imipenem (48.8%), meropenem (46.5%), amikacin, and gentamicin (41.8%). Antibiotics that were most effective against cKp isolates were tigecycline and colistin. Only 4.6% of the isolates were not responsive to these two drugs. The rates of resistance to the following antimicrobial drugs were noticeably reduced in (hvKp) piperacillin/tazobactam, cefepime, ceftriaxone, ceftazidime, gentamycin, and trimethoprim/sulfamethoxazole compared to cKp (Table 3).

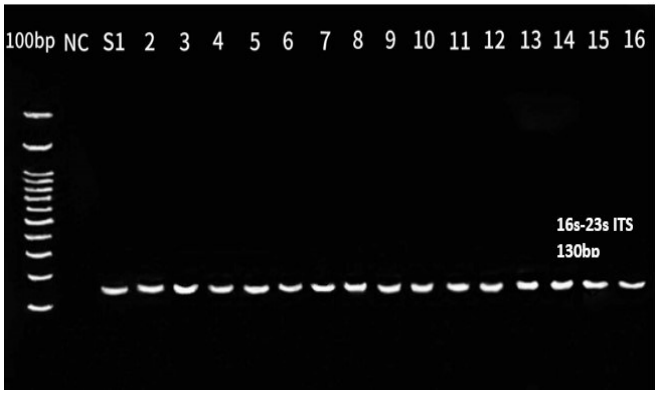

Figure 2. 16s-23s ITS gene amplification molecular identification of isolate for K. pneumoniae. Lane 1: 100 bp DNA ladder (size marker); Lane 2: Negative control (no amplification); Lanes 3 (S1): (1–16): Clinical isolates, all showing a specific band at 130 bp, confirming identification as K. pneumoniae.

Table 2. Distribution of virulence genes in classical (cKp) and hypervirulent (hvKp) Klebsiella pneumoniae strains.

Characteristic	hvKp (n = 11)	cKp (n = 23)	P-value
entB	11 (100%)	23 (100%)	n.a
rmpA	1 (9%)	0 (0.0%)	0.324
magA	0 (0.0%)	0 (0.0%)	n.a

Data are reported as the count of positive isolates, accompanied by percentages in parentheses. Statistical analysis utilized Fisher's exact test; $P < 0.05$ was deemed significant. "n.a." signifies that statistical comparison was not relevant owing to the equal presence or lack of genes in both groups.

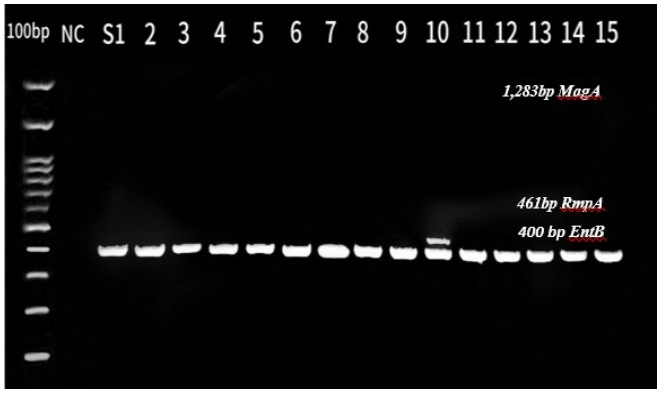

Figure 3. Multiplex PCR for *magA*, *rmpA* and *entB* for *K. pneumoniae* 100 bp DNA ladder NC: Negative Lane 1-15 is positive for entB, negative for magA, and only sample 10 is positive for rmpA.

Table 3. Antibiotic-resistant profiles of hypervirulent (hvKp) and classical (cKp) Klebsiella pneumoniae.

Antimicrobial Agent	hvKp (n = 11)	cKp (n = 43)	P-value
Ampicillin	11 (100%)	43 (100%)	n.a
Piperacillin/Tazobactam	4 (36.4%)	27 (62.7%)	0.0344[a]
Cefazolin	9 (81.8%)	38 (88.3%)	0.3371
Ceftazidime	5 (45.5%)	34 (79%)	0.0056[a]
Ceftriaxone	7 (63.6%)	38 (88.3%)	0.01[a]
Cefuroxime	7 (63.6%)	33 (76.7%)	0.1424
Cefepime	4 (36.4%)	27 (62.7%)	0.0344[a]
Ertapenem	5 (45.5%)	23 (53.4%)	0.3108
Imipenem	3 (27.3%)	21 (48.8%)	0.0665
Meropenem	3 (27.3%)	20 (46.5%)	0.0879
Amikacin	3 (27.3%)	18 (41.8%)	0.1467
Gentamicin	2 (18.2%)	18 (41.8%)	0.0423[a]
Ciprofloxacin	6 (54.5%)	30 (69.7%)	0.1319
Levofloxacin	5 (45.5%)	27 (62.7%)	0.1137
Nitrofurantoin	7 (63.6%)	25 (58.1%)	0.7452
Trimethoprim/Sulfamethoxazole	4 (36.4%)	29 (67.4%)	0.0155[a]
Colistin	0 (0%)	2 (4.6%)	1
Tigecycline	0 (0%)	2 (4.6%)	1

The difference is statistically significant when it is less than 0.05. A P value of less than 0.05 was deemed statistically significant and is denoted by the superscript a. N.A., which means not applicable statistically, while the variable remains constant. The absence of variability for comparison is due to the resistance of all isolates, rendering statistical analysis inapplicable.

4. Discussion

This study evaluates 54 K. pneumoniae isolates from patients at Shar Hospital and Smart Health Tower in Sulaimani City between November 2024 and January 2025. The identification of hypervirulent K. pneumoniae (hvKP) was based on a positive string test, resulting in 11 isolates (20.4%) being classified as hvKP. This prevalence is lower than figures reported in various studies from China (39.1%) (Li et al., 2023), Egypt (58%) (Mohamed Hassan et al., 2024a), Korea (42.4%) (Jung et al., 2013), Taiwan (38%) (Yu et al., 2006) and higher than reports from Iran (15.1%)(Rastegar et al., 2019) and Spain (13.39%) (Ballén et al., 2021). Geographical characteristics contribute to these differences in prevalence. As an example, prior research demonstrated that compared to East Asian countries, the prevalence of hvKP is lower in European nations (David et al., 2019; Maguire et al., 2025).

Previous multiplex PCRs addressed only a few virulence genes in the current study. Iron acquisition factors, including enterobactin (EntB), have been identified in K. pneumoniae strains as virulence factors, and the entB gene is very common in K. pneumoniae (Wang et al., 2025). Consistent with other prior findings, according to our analysis, the entB gene was present in the majority of hvKP and cKP isolates (Compain et al., 2014; Rastegar et al., 2019; Wang et al., 2025).

In addition to hypermucoviscosity, other virulence elements have been proposed to be involved in the pathogenesis of hvKP strains. The most often mentioned elements with direct association with hvKP virulence are RmpA and magA (Al Ismail et al., 2025). While rmpA and magA were associated with a positive string test, this was seen among the 11 isolates that tested positive we identified just Only one isolate (9%) was rmpA positive but magA negative; a previous study reports 12 (22.6%) out of 53 in Barcelona, Spain (Cubero et al., 2016), and 3 (13.6%) and 4 (18.2%) out of 22 isolates for magA and rmpA, respectively, in southeast Iran (Rastegar et al., 2019). Also, another study in Iran reported the presence of magA was 2 (3.07%) and rmpA was 10 (15.38%) out of the 22

hvKp-positive K. pneumoniae isolates (Nahavandinejad & Asadpour, 2017). These findings suggest the existence of further mucoid components besides magA and rmpA, such as differences in lipopolysaccharide composition (Cubero et al., 2016). It may be due to the presence of other regulator genes that are associated with HMV (Nahavandinejad & Asadpour, 2017). And this could be explained by the data supporting that the rmpA gene was discovered more frequently in community-acquired hvKP than in those acquired from hospitals (Abd-Elmonsef et al., 2016).

In this research, cKP isolates demonstrated a significantly higher rate of antimicrobial resistance compared to hvKP isolates against various agents, including piperacillin/tazobactam, ceftriaxone, cefepime, gentamicin, and trimethoprim/sulfamethoxazole. The result is in agreement with previous research that found a significant prevalence of ampicillin resistance among hvKP isolates (Alharbi et al., 2023; Mohamed Hassan et al., 2024b). The rationale behind this disparity is currently unclear, although previous studies have indicated that hvKP strains typically exhibit susceptibility to antimicrobial therapies. Significantly, the hvKp isolates showed reduced resistance rates to various antibiotics, especially β-lactams and aminoglycosides, in contrast to cKp isolates. Resistance to ampicillin was universally observed (100%) in both the hvKp and cKp groups, which is anticipated due to the intrinsic resistance conferred by the chromosomal bla_SHV gene in K. pneumoniae (Rastegar et al., 2019). And certain people have speculated that hypervirulent hvKP strains lose certain drug-resistant genes or that they are unable to acquire plasmids relevant to resistance (Li et al., 2014).

The authors have concluded that cKp isolates exhibited resistance to a majority of the antibiotics evaluated, including imipenem. Similarly, reports indicate that K. pneumoniae in Iran has a high rate of resistance to imipenem (Rastegar et al., 2019; Shahi et al., 2019). While our investigation did not observe a high incidence of hvKp strains resistant to imipenem and gentamicin, the increased virulence of these bacteria and their potential to acquire resistance to antibiotics are worrying. cKp isolates had a high rate of colistin and tigecycline sensitivity, similarly to a previous study (Antony et al., 2024). Resistance to last-line therapy, particularly colistin, was unusual and not significantly varied across the groups, indicating minimal exposure to or spread of plasmid-mediated colistin resistance (mcr genes) among the evaluated population. (Elbediwi et al., 2019).

5. Conclusion

This study confirmed the presence of both hvKp and cKp among clinical K. pneumoniae isolates, with hvKp representing a smaller proportion. Each isolate carried the entB gene, but only one had the rmpA gene, and none had the magA gene, indicating a limited distribution of critical virulence genes. While cKp isolates were antimicrobial-resistant. Unlike cKp, which was antibiotic-resistant, hvKp isolates were less resistant. Tigecycline and colistin were the best options. These findings emphasize the growing concern over cKp antimicrobial resistance and hvKp strains

Limitations

The sample size was relatively small (54 isolates), which may limit the generalizability of the findings. Finally, the study was limited to a single geographic region and hospital setting, which may not reflect the epidemiological patterns of K. pneumoniae in other

Recommendation

Further research to Perform more multicenter studies to look at the differences in virulence genes, clinical outcomes, and possible novel approaches to treat both hvKp and cKp strains. Antibiotic stewardship: To avoid cKp from becoming resistant, hospitals should improve their antimicrobial management policies so that broad-spectrum antibiotics, especially cephalosporins, are not used

too much. To prevent nosocomial transmission, hospitals must ensure that robust infection prevention and control policies are strictly followed, particularly in high-risk wards.

Abbreviations

hvKP, hypervirulent K. pneumoniae; cKP, classical K. pneumoniae; ITS, internal transcribed spacer; ETA, endotracheal aspirate; MIC, minimum inhibitory concentration.

Ethics

This study was conducted under approval by the medical ethics committee of the college of medicine at the University of Sulaimani, No. 341, on the date (24/10/2024). Participants provided verbal and written consent, and both participants and researchers agreed to publication.

Conflict of interest.

The authors declare that there is no conflict of interest.

CRediT authorship contribution statement.

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