Effects of silver nanoparticles on antibiotic resistance and gene expression of *Klebsiella pneumoniae* bacteria

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Abstract

Background: *Klebsiella pneumoniae* is an encapsulated gram-negative organism that can cause infections at multiple sites, including the lungs, urinary tract, bloodstream, and brain, as well as in wounds and at surgical sites. *K. pneumoniae* has emerged as a major pathogen of international concern owing to the increasing incidences of carbapenem-resistant strains. **Aims of the study:** This study aimed to identify and isolate *Klebsiella pneumoniae* bacteria from different clinical samples, investigate the effect of silver nanoparticles on the antibiotic susceptibility pattern of clinical isolates of *Klebsiella pneumonia*, and explore the effect of silver nanoparticles on the expression level of virulence genes of *Klebsiella pneumoniae.* **Materials and methods:** In this cross-sectional study, a total of (150) clinical specimens including (40) urine, (50) blood and (60) sputum specimens were collected from patients suffering from pneumonia, bacteremia, urinary tract infections and respiratory tract infections who attended Baquba Teaching Hospital and Al-Batoul Teaching Hospital/ Diyala Province. All bacterial isolates of *Klebsiella pneumonia* were characterized with morphological, microscopical, biochemical tests and Vitek II system. Antibiotic susceptibility test was conducted on eight *K. pneumoniae* isolates before and after treatment with silver nanoparticles (AgNP) to assess the antibiotic resistance or sensitivity of this bacterium and to determine the impact of silver nanoparticles on the antibiotic efficacy against *K. pneumoniae.* The real time PCR was used to detect the *Klebsiella pneumoniae* gene sequencing and gel electrophoresis was used for DNA extraction. **Results:** The results showed the higher urinary tract infection rate with *K. pneumoniae* was found in males 17 (56.6%) compared to females 13 (33.3%). Urinary tract infections with multi-drug resistant (MDR) *K. pneumoniae* were higher 20 (66.6%) than respiratory tract infections 33 (33.3%). The findings indicated that the isolates of *K. pneumoniae* exhibited sensitivity to Imipenem, Gentamicin, and Tetracycline after treatment with AgNP. Identifying *K. pneumoniae* genes rmpA, uge, wabG, wcaG and ycfM by PCR method showed improvement of these genes after AgNP treatment. **Conclusions:** It can be concluded from our study that urinary tract infections with MDR *K. pneumoniae* were higher than respiratory tract infections, and treatment of *K. pneumoniae* with silver nanoparticles was shown to be effective, and the genes rmpA, uge, wabG, wcaG and ycfM showed improvement after AgNP treatment.

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Introduction

Klebsiella pneumoniae is a widely distributed bacterium that colonizes the human skin, mouth, respiratory and gastrointestinal (GI) tracts asymptomatically. It is also one of the main causative agents of hospital infections such as urinary tract infections, pneumonia, liver abscesses, meningitis, and sepsis, being considered an opportunistic pathogen [1,2].

K. pneumoniae is among the most relevant strains when considering the increase in antibiotic resistance worldwide, being classified by the World Health Organization (WHO) as a priority pathogen for which new drugs are needed [3]. However, relying on the discovery of new drugs alone may not be enough to suppress the advance of these infections, especially when caused by multidrug-resistant isolates, so new strategies are fundamental to stop the steady increase of cases. Infections caused by multi-drug resistant bacteria (including *K. pneumoniae*) pose a great burden to healthcare systems worldwide, from the hundreds of thousands of deaths, to the reduced life expectancy and disabilities, along with the cost of the treatment. *K. pneumoniae* is among the four main causes of death by antibiotic resistance bacteria, which are responsible for 929,000 annual deaths according to a study published in 2022 [4].

K. pneumoniae strains can be classified in two major categories, namely classical (or common) and hypervirulent strains, based on traits such as the hypermucoviscosity (HMV) phenotype and increased expression of siderophores and fimbriae [5,6]. There are a few differences in the profile of the infections caused by those types of *K. pneumoniae*. Usually, most cases occur within healthcare environments and are caused by classical strains. Such strains are commonly multi-drug resistant, especially to beta-lactams, including carbapenems. The most common infections caused by the classical strains are urinary tract infections, pneumonia and bacteremia [6]. Hypervirulent strains, on the other hand, are acquired in the community and are more invasive, being able to colonize additional sites and cause further damage, when compared to the classical strains. Another important difference between classical and hypervirulent strains is the historically higher susceptibility of the hypervirulent strains to antimicrobials, which is becoming less prevalent in hospital-acquired *K. pneumoniae* in many regions, especially in lower- or middle-income countries [6,7]. Nevertheless, a very worrying development is the increase in reports of resistant hypervirulent strains leading to more severe, often fatal infections [6,8].

Whole-Genome Sequencing (WGS) has emerged as a powerful molecular diagnostic tool that is widely employed in research. WGS can predict drug resistance, trace lineage, and transmission and define outbreaks of *K. pneumoniae* [9,10]. In 2020, WGS revealed a carbapenem-resistant isolate harboring $bla_{\text{OXA-48}}$-like gene [11]. The rapid spread of antimicrobial resistance in *K. pneumoniae* is linked to the variability of virulence genes and swift dissemination within intra-hospital environments, resulting in *Klebsiella pneumoniae* carbapenemase (KPC)-producing KPC-KP being more prevalent than the other resistance types. Despite efforts to monitor this pathogen in the country, few studies have genetically characterized outbreaks using WGS [12].

Materials and methods

In this cross-sectional study, a total of (150) clinical specimens including (40) urine, (50) blood and (60) sputum specimens were collected from patients suffering from pneumonia, bacteremia, urinary tract infections and respiratory tract infections who attended Baquba Teaching Hospital and Al-Batoul Teaching Hospital/ Diyala Province. All bacterial isolates of Klebsiella pneumonia were characterized with morphological, microscopical, biochemical tests and Vitek II system. Antibiotic susceptibility test was conducted on eight *K. pneumoniae* isolates before and after treatment with silver nanoparticles (AgNP) to assess the antibiotic resistance or sensitivity of this bacterium and to determine the impact of silver nanoparticles on the antibiotic efficacy against *K. pneumoniae.*

Well-diffusion method was used to test the antibacterial effect on different nanoparticles. The wells were punched into the agar using a sterilized well cutter. The wells were loaded with AgNP solution for *K. pneumonia* bacteria, deionized water was used as a controlling factor. The dishes were incubated at 37C° for 24 hours. Results were obtained by measuring the inhibition zone. Three replicates were made for each treatment. The real time PCR was used to detect the gene sequencing and gel electrophoresis was used for DNA exraction.

All patients who had pneumonia, bacteremia, urinary tract infection, and respiratory tract infection due to *K. pneumoniae* were included in the study, while patients who showed negative results for *K. pneumoniae* and patients who had mixed infections were excluded from the present study.

AgNPs were prepared by chemical methods via using 4mM of silver nitrate, 0.4mM tri-sodium citrate dehydrates as a reducing agent, and 0.5mM sodium dodecyl sulphate-SDS as a capping agent.

The study was approved by the ethics committee of Çankiri Karatekin Universty - Graduate School of Natural and Applied Sciences, Turkey.

Statistical analysis

The SPSS program version-25 was used for statistical analysis of data in the current study. The results were expressed as (mean ± standard error) (mean ± SD).

Results

Identification of *Klebsiella pneumonia* was done by the bacteriological methods including colonial morphology on MacConkey agar, which appeared large, mucoid, regular, pink colonies, and fermented lactose sugar as shown in figure (1-A). On blood agar, the colonies of *Klebsiella pneumonia* grew with gamma type hemolysis, as shown in figure (1-B).

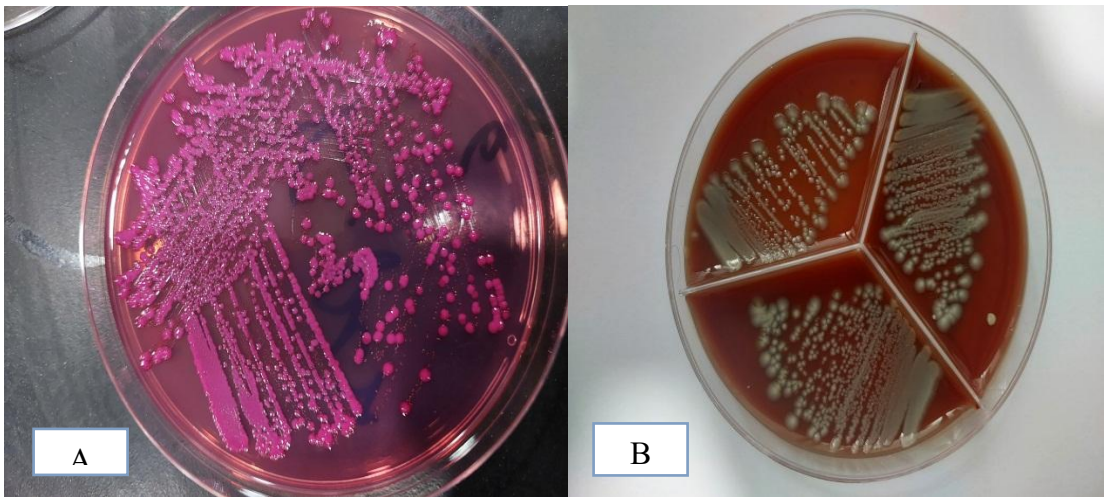

Figure (1): (A) Colonies of *K. pneumoniae* (large, mucoid, regular, pink colonies, and fermented lactose sugar grown on MacConkey agar (B): Colonies of *K. pneumoniae* (gamma hemolytic) grown on Blood agar

Microscopic examination of *K. pneumoniae* after performing the process of Gram staining, the bacterial cells appeared small, bacilli-shaped, Gram-negative (red in color), as illustrated in figure (2).

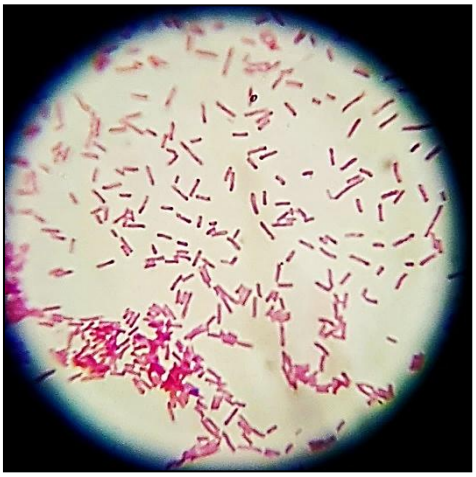

Figure (2): Gram staining of *K. pneumoniae:* the bacterial cells appeared as Gram (-ve) small bacilli

According to the biochemical properties, isolates of *K. pneumoniae* were positive for the catalase test (figure 3), negative to the oxidase test, Methyl red, and indole production, as shown in table (1) and figure (4)..

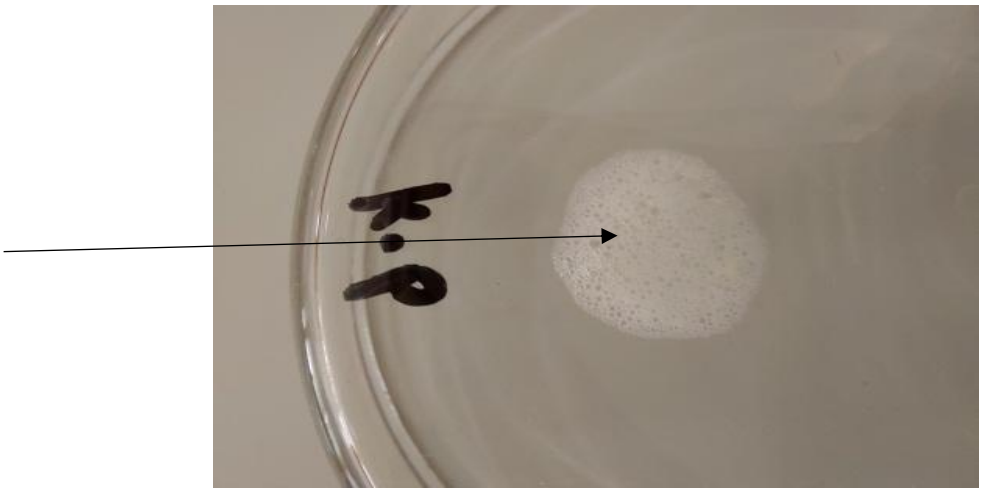

Figure (3): Biochemical identification of *K. pneumoniae:* Positive catalase test (appearance of bubbles) on nutrient agar

Table (1): Biochemical tests for characterization of *K. pneumoniae.*

Bacteria	Catalase	Oxidase	Indole	MR	VP	Citrate	KIA	Urease
K. pneumoniae	+	-	-	-	+	+	A/A	+

(+) positive result, (-) negative result, (MR) Methyl red , (VP) Voges –Proskauer test, (KIA) Kliger Iron Agar test, (A/A) Acidic Slant/ Acidic Bottom.

Figure (4) showed that the *K. pneumoniae* isolates gave clearly positive results for Citrate utilization, urease test, Voges Proskauer test, Kliger Iron Agar test, but negative for Indole test and methyl red test.

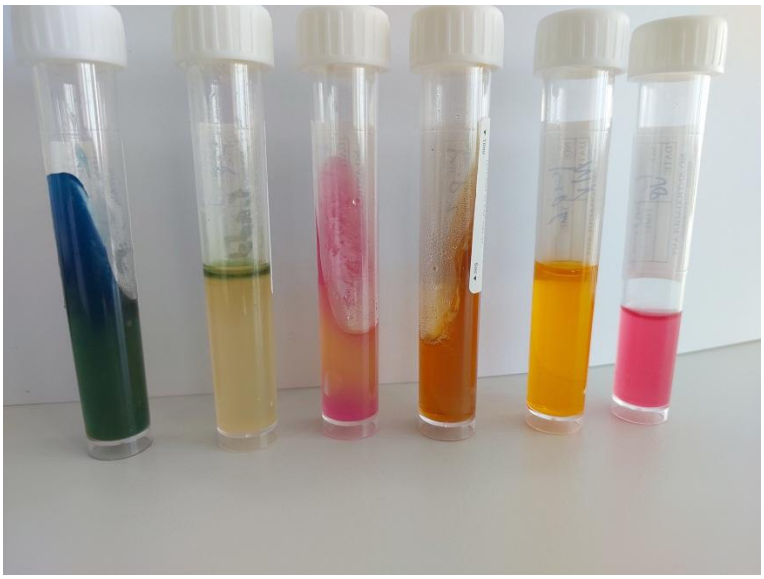

Figure (4): The results of some biochemical tests of *K. pneumoniae*; (from left to right) citrate utilization (+), indole test (-), urease test (+), Kliger Iron Agar (KIA) test, methyl red test (-) Voges Proskauer test (+)

The Vitek II system was also used for some isolates of *K. pneumoniae*, which is an automated microbiology system used for bacterial identification and to confirm the results of morphological, microscopical and biochemical identification.

Characterization of silver nanoparticles **A**: show transmission electron microscope **(TEM)** (Zeiss, Germany) used to identify the morphological feature of the silver nanoparticles, show a size range of 10 to 50 nm and

B: show Field Emission Scanning Electron Microscope (FE-SEM) analysis was used to study the particle's size, AgNPs shape, and surface morphology by scanning electron microscope (Zeiss, Germany) show a size 10 µm, at a magnification power of 50.00KX, with working distance 9.13mm with high voltage 15.0 KV.

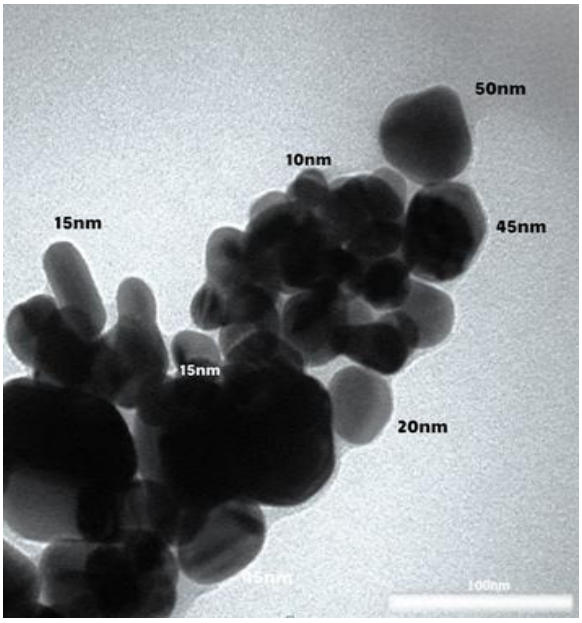

Figure (5): Transmission electron microscopy (TEM) of AgNP

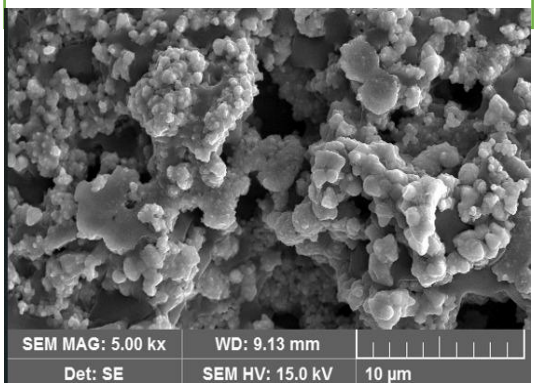

Figure (6) b: Scanning Electron Microscope (SEM) of AgNP

The results of the current study showed that out of the (150) specimens, *K. pneumonia* was isolated from only (30) clinical samples 20 (66.6%) from urine specimens and 10 (33.3%) from sputum samples, and among those (30) positive patients, 17 (56.6%) were males and 13 (43.3%) were females as shown in table (2).

Table (2): Distribution of *K. pneumonia* according to gender and infection site

Groups		Frequency N (%)
Gender	Males	17 (56.6%)
	Females	13 (43.3%)
	Total	30 (100%)
Cases	Urine	20 (66.6%)
	Sputum	10 (33.3%)
	Total	30 (100%)

Data in table (3) summerizes the results of antibiotic sensitivity test indicating that most of the *K. pneumonia* isolates were resistant to commonly prescribed antibiotics before and after treatment. The antibiotics which were resistant to *K. pneumonia* isolates before treatment and became sensitive included Imipenem, Tetracycline, Gentamicin, while Tigecycline was sensitive before and after treatment.

Table (3): Summary of antibiotic resistance before and after AgNP treatment

No.	Antibiotic	Before treatment	After treatment
1	Imipenem	**R**	**S**
2	Gentamicin	**R**	**S**
3	Tetracycline	**R**	**S**
4	Metropenem	**R**	**R**
5	Tigecycline	**S**	**S**
6	Cefoxitin	**R**	**R**
7	Ceftazidime	**R**	**R**
8	Ceftriaxone	**R**	**R**
9	Cefepime	**R**	**R**
10	Amikacin	**R**	**R**
11	Vancomycin	**R**	**R**
12	Cefazolin	**R**	**R**
13	Levofloxacin	**R**	**R**
14	Piperacillin/Tazobactam	**R**	**R**
15	Ampicillin/Sulbactam	**R**	**R**
16	Trimethoprim/Sulfamethoxazole	**R**	**R**

R: resistant S: sensitive

Table (4) showed the results of virulence genes (*Ycfm, Uge* , *WAb G, wcaG* and *rmpA*) used in this study before and after treatment with AgNP. It was shown that the No. and % of *Ycfm* gene before treatment with AgNP was 14 (46.6%) and 12 (40%) after treatment. The No. and % of *Uge* gene before treatment with AgNP was 18 (60%) and 18 (60%) after treatment. The No. and % of *WAb G* gene before treatment with AgNP was 28 (93.3%) and 28 (93.3%) after treatment. The No. and % of *wcaG* gene before treatment with AgNP was 20 (66.6%) and 20 (66.6%) after treatment. The No. and % of *rmpA* gene before treatment with AgNP was 10 (33.3%) and 8 (26.6%) after treatment. These results indicated that no change occurred in the number and percentage of *Uge, WAb G,* and *wca*G genes before and after treatment, while there was a decrease in the number of *Ycfm* and *rmpA* genes after treatment.

Table (4): The list of virulence genes used in this study before and after treatment with AgNP

	Before		**After**	
Genes	**No.**	**%**	**No.**	**%**
Ycfm	**14**	**46.6**	**12**	**40**
Uge	**18**	**60**	**18**	**60**
WAb G	**28**	**93.3**	**28**	**93.3**
wcaG	**20**	**66.6**	**20**	**66.6**
rmpA	**10**	**33.3**	**8**	**26.6**

DNA extraction and PCR product (band size 683) before and after treatment with AgNP demonstrated that all the extractions and PCR products were positive (+) before and after treatment with silver nanoparticles (AgNP) as illustrated in table (5).

Table (5): DNA extraction and PCR product before and after treatment (band size (683)

Lab. No.	DNA extraction	PCR Product 683
Before		
1	+	+
2	+	+
3	+	+
After		
4	+	+
5	+	+
6	+	+

Discussion

The results in the current study showed that among the (30) positive patients, 17 (56.6%) were males and 13 (43.3%) were females. In agreement with our results, isolated K. pneumonia was higher in males in a study by Osman., et al., 2021 [13]. However, it is inconsistent with (Jalal et al. 2023) [14], who found an increase in K. pneumonia cases in females, and a study which found that females had a higher isolation rate than males in Kirkuk city by Hmood et al., 2021 [15]. These comparisons highlight the variation in K. pneumoniae occurrence among various demographic groups and highlight how crucial it is to take these details into account when comprehending and treating the infection [16].

The results of the current study showed that out of the (150) specimens, K. pneumonia was isolated from only (30) clinical samples 20 (66.6%) from urine specimens and 10 (33.3%) from sputum samples

In one study conducted by Ulla and Ibtisam, 2024 [17], it was found that the total number of isolates from sputum samples was 9(18%) which agrees with Ahmed and Alaa, 2016 ; Temesgen et al., 2019 [18,19], both of them were 30(18%).

K. pneumoniae strains isolated from urine samples showed higher antimicrobial resistance, ESBL production, and biofilm-forming ability compared to those isolated from respiratory or blood samples. The rapid spread of clinical strains with these characteristics is of concern, and new therapeutic alternatives are essential to mitigate their harmful effects [20].

Klebsiella pneumoniae is a nosocomial pathogen that causes a broad spectrum of diseases and is increasingly resistant to antibiotics. It is becoming well known for its resistance to the majority of commonly used last-line antibiotics. It is particularly troubling in hospitals, where it causes a variety of acute infections [21].

In the current study, an antibiotic susceptibility test was conducted on eight K. pneumoniae isolates before and after treatment with silver nanoparticles (AgNP) to assess the antibiotic resistance or sensitivity of this bacterium and to determine the impact of silver nanoparticles on the antibiotic efficacy against K. pneumoniae.

The findings indicated that the isolates of K. pneumoniae exhibited sensitivity to Imipenem, Gentamicin, and Tetracycline after treatment with AgNP.

There is an urgent need to augment the efficacy of antimicrobials against pathogenic microorganisms. In recent years, there has been increased interest in the utilization of nanoparticles as therapeutic agents [22,23]. This activity is similar to that observed in other studies, being justified by AgNP's actions on Klebsiella spp, such as inhibition of metabolism and production of bacterial biofilms, in addition to structural damage [24].

Klebsiella pneumoniae, a significant pathogen, poses a serious threat to global health due to its ability to form robust biofilms and develop multidrug resistance [25]. Biofilms, communities of bacteria embedded in a protective matrix, shield K. pneumoniae from antibiotics and other stressors, making infections difficult to treat. The emergence of multidrug-resistant K. pneumoniae strains has further complicated treatment options, contributing to high mortality rates, especially in vulnerable populations [26].

Nanoparticles, with their large surface area and tunable surface chemistry, offer a promising alternative to traditional antibiotics for combating multidrug-resistant (MDR) pathogens [27]. Silver nanoparticles, in particular, have demonstrated potent antimicrobial activity and the ability to disrupt biofilm formation, a critical challenge in treating bacterial infections [28]. This has fueled increased research into nanoparticles as a potential solution to overcome antibiotic resistance [29,30].

The antimicrobial effect of silver nanoparticles is due to the damage they cause to the bacterial membrane (membrane bonding), alteration of bacterial DNA, and compromise of bacterial protein synthesis [31]. This activity is similar to that observed in other studies, being justified by AgNP's actions on Klebsiella spp, such as inhibition of metabolism and production of bacterial biofilms, in addition to structural damage [24].

Silver nanoparticles (AgNPs), characterized by minimal toxicity in ecosystems and a high surface capacity, might impede the establishment of biofilm materials that facilitate evasion and protection [32].

The rmpA is a transcriptional activator of capsular polysaccharide synthesis (CPS) gene transcription, CPS synthesis, and hyper virulence in K. pneumoniae K1/K2 [33], whereas lipopolysaccharides are encoded by the uge, wabG, ycfM genes [34]. These elements all have a role in virulence and are crucial for invasion, colonization, and pathogenicity [35].

A total of 23 isolates (12.11%) tested positive for the rmpA gene. The presence of this gene may indicate a high virulence potential in an isolate. According to a previous investigation, the rmpA gene is embedded in a 180-kb virulence plasmid. This multicopy plasmid induces K. pneumoniae to express a mucoid phenotype [36]. The rmpA-carrying plasmids of K. pneumoniae isolates have also been found to contain several other virulence-associated genes [37].

Additional virulence factors in K. pneumoniae include fimbrial and nonfimbrial adhesion genes such as mrkD, KPN, and ycfM. Among 402 K. pneumoniae isolates sourced from diverse clinical samples, the prevalence rates of specific genes were determined as follows: ycfM in 386 isolates (96.01%) [38].

YcfM, present in 96.01% of our isolates, showed a higher prevalence than the 92.8% reported by Yousefi et al., suggesting a potential increase in the gene's distribution over time or variations in sample sources. YcfM is involved in lipopolysaccharide synthesis, contributing to the bacterium's outer membrane integrity and virulence [39].

In one previous study by (Derkhashan et al., 2016) [40], the transferability of rmpA and wcaG genes were examined via the conjugation method and the data revealed transferability for the wcaG gene. The correlation between the presence of virulence genes (rmpA and wcaG) and antibiotic resistance in K. pneumoniae isolated from hospitals in Tehran, Iran was investigated [40]. One study concluded that the wcaG gene is extremely relevant for hvKP is in line with earlier studies [41].

The Uridine Diphosphate Galactose epimerase (uge) gene controls the synthesis of these factors. Sepsis, pneumoniae, and urinary tract infections are less common in K. pneumoniae which lacks this gene [42].

The absence of the uge gene makes the mutant K. pneumoniae completely a virulent in animal models of infections, and this was attributed to the presence of a truncated core oligosaccharide at the GalA residue, which reinforces the pivotal role of the uge gene in LPS biosynthesis pathways [43].

It has been demonstrated that K. pneumoniae strains lacking the uge gene are less virulent and less capable of causing UTI, pneumonia or sepsis. Mutations in the uge gene reduce the colonization ability of K. pneumoniae in experimentally induced urinary infections. Although the uge gene has previously been described as essential for K. pneumoniae virulence, future research may provide further evidence for its specific association with UTIs [44].

Al-Janaby, 2016 [45] demonstrated that K. pneumoniae strains with a mutant wab G gene are non-capsulated and less virulent [45). This demonstrates how crucial the wab G gene is for the pathogenicity of K. pneumoniae. By demonstrating that mutant strains of K. pneumoniae (without the uge gene) were not virulent in laboratory animals. The importance of the uge gene in K. pneumoniae pathogenicity of was demonstrated by Lev et al., 2018 [46].

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