

Impact of Virulence Genes on *Klebsiella pneumoniae* Isolated from Various Clinical Specimens

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ABSTRACT

Background: *Klebsiella pneumoniae* is an opportunistic pathogen responsible for a wide range of clinical infections, with increasing multidrug resistance (MDR) posing a significant challenge in healthcare settings. Virulence genes play a crucial role in antibiotic resistance and pathogenicity, necessitating molecular surveillance for effective infection control and treatment strategies. **Objective:** This study aimed to evaluate the antibiotic resistance patterns and the prevalence of selected virulence and resistance genes in *K. pneumoniae* isolates from various clinical specimens. **Methods:** A total of 22 *K. pneumoniae* isolates were collected from patients with urinary tract infections, respiratory infections (sputum), burn infections, and wound infections at Baquba Teaching Hospital and Al-Batool Maternity Hospital in Diyala Governorate, Iraq, between December 2022 and June 2023. Antibiotic susceptibility testing was performed against 12 antibiotics using standard disk diffusion and minimum inhibitory concentration (MIC) methods. The MIC values for ciprofloxacin ranged from 8 to 1024 µg/mL. Polymerase chain reaction (PCR) analysis was conducted to detect resistance-associated genes (*gyrA*, *qnrB*) and virulence-associated capsular genes (*K1*, *rmpA*). **Results:** All *K. pneumoniae* isolates exhibited multidrug resistance. PCR analysis revealed that the *gyrA* gene, associated with fluoroquinolone resistance, was present in 100% of the isolates, while *qnrB* was detected in 22.72% of the isolates. Notably, none of the isolates harbored the *K1* or *rmpA* genes, indicating the absence of hypervirulent *K. pneumoniae* (hvKP) strains in the studied samples. **Conclusion:** The findings highlight the high prevalence of multidrug-resistant *K. pneumoniae* and the significant presence of *gyrA*-mediated fluoroquinolone resistance. The absence of *K1* and *rmpA* genes suggests that these isolates may not belong to the hypervirulent *K. pneumoniae* lineage. Continuous molecular surveillance of virulence and resistance genes is essential for guiding antimicrobial stewardship and infection control measures in clinical settings.

Keywords: Antibiotics, *gyrA*, Gene, MIC.

INTRODUCTION

Klebsiella pneumoniae is within the genus *Klebsiella*; this bacterium is the most significant in terms of medicine. It is a kind of environmental organism that may be found in soil, water, and plants. Several strains of nitrogen-fixing endophytes that have been identified from plant tissues might provide the plant with nitrogen. Other strains have the potential to infect people as opportunistic diseases; they often infect immunocompromised individuals in hospitals [1, 2]. *Klebsiella* species are non-motile, encapsulated rod-shaped bacteria. These species could lysine decarboxylase production but not ornithine decarboxylase; they are positive in Voges-Proskauer; tests for urease and indole *Klebsiella* spp. are generally facultative anaerobic, with widths ranging from 0.3 to 1 mm and lengths ranging from 0.6 to 6 mm. On standard medium, all strains develop quickly. On MacConkey agar, colonies are often big, mucoid, and red, with red pigment spreading into the surrounding agar, indicating lactose fermentation and acid Generation [3]. Multi-drug resistant organisms cause severe and nosocomial infections in the community that limit therapeutic options by using available antibiotics. However, the emergence of antibiotic resistance in *K. pneumoniae* isolates worldwide has increased the risk of antibiotic treatment failure in humans [4]. Significant sites of infection include the lungs, where it causes a type of pneumonia and urinary tract infections. *Klebsiella* can also enter the bloodstream (bacteremia) and cause sepsis. Numerous pathogenicity factors are expressed by this organism, including several adhesions, capsular polysaccharides, and lipopolysaccharides for evading host defenses. For instance, the capsular polysaccharide (K antigen) inhibits phagocytosis. Attempts to eradicate the organism from infected people with antibiotics are hampered by the numerous

antibiotic-resistance genes carried on the chromosome [5]. The pathogenic bacterium causes acquired invasive syndrome and was described in 1981, being characterized by bacteremia, hepatic abscess, and metastatic infections, among which central nervous system infections and endophthalmitis (manifested as suppurative meningitis or cerebral abscess) were the most commonly found, although other abscesses in the lungs, pleura and soft tissues, prostate, necrotizing fasciitis, and osteomyelitis. Epidemiologic investigations show that *K. pneumonia* is isolated from the gastrointestinal and the liver, which matches phenotypically and genotypically, typically supporting previous hypotheses that most PLAs and systemic infections seed from the gut initially. This suggests that clinical microbiology laboratories should possibly screen high-risk patients (e.g., diabetes mellitus) for carriage of this genotype as normal flora [6]. Drug resistance has increased in gram-negative bacteria, particularly *Klebsiella pneumoniae*, in recent years as a result of incorrect antibiotic usage. Increased mortality and therapy costs are linked to drug resistance. As a result, choosing an antibiotic is a difficult task. The resistance pattern is used to determine the best therapy for illnesses caused by this bacterium, which seems to be required [7].

MATERIALS AND METHODS

Isolation and Identification of *Klebsiella pneumoniae* Isolates:

Twenty-two specimens were collected from December 2022 until June 2023 from 250 patients suffering from different clinical infections in Baquba City, ages ranging between 1 and 75. The isolates were identified by their colony characteristics on the MacConkey agar and

HiCrome agar and incubated at 37°C for 24 hours. Biochemical tests were performed, and further identification was carried out by genotypic detection using 16S rRNA.

Antimicrobial Susceptibility Test

57 *Klebsiella pneumoniae* isolates were tested for antibiotic resistance using Mueller-Hinton agar disks based on CLSI (2023). The following antigens were used: Ampicillin (10 µg), Piperacillin (100 µg), Cefoxitin (30 µg), Imipenem (10 µg), Meropenem (10 µg), Gentamycin (10 µg), Amikacin (30 µg), Streptomycin (25 µg), Levofloxacin (5 µg), Ofloxacin (5 µg), Ciprofloxacin (5 µg), and Trimethoprim – sulfamethoxazole (1.25/23.75 µg) are the antibiotics used in this study.

Molecular Study of Klebsiella Pneumonia

DNA Extraction

DNA was extracted from bacterial cells growing on brain heart infusion agar liquid, where these cells were grown on this medium for 24 hours at a temperature of 37 °C, and the extraction process was

carried out according to the ABIpure Extraction protocol. The concentration and purity of the DNA extract were determined by measuring absorbance through Quantitative Fluorimeters.

PCR Detection of Virulence-Associated Genes

The Polymerase Chain Reaction (PCR) was used to detect the presence of capsule and antibiotic genes in 22 multidrug-resistant isolates out of 57 *K. pneumoniae* isolates using proprietors assigned to each gene. The Microgen company supplied all these primers in a lyophilized form. Lyophilized primers were dissolved in nuclease-free water to give a final concentration of 100 pmol/µl as a stock solution. A working solution of these primers was prepared by adding ten µL of primer stock solution (stored in the freezer at -20 °C) to 90 µL of nuclease-free water to obtain a working primer of 10 pmol / µL. Polymerase chain reaction (PCR) is a simple and rapid method for the detection of microorganisms. PCR was performed to detect the presence of the *16S rRNA* gene and antibiotics genes (*gyrA*, *qnrB*, *K1*, *rmrA*) using primers listed in Table 1. Table (1) shows the sequence of primers for the genes used in the study.

Table 1: The Primers Sequencing of Antibiotic & Capsular Genes

Primers/Gene	Oligo Sequence (5~ → 3~)	Ann. Temp. (°C)	Product Size (bp)	Reference
Antibiotic sequence gene				
<i>gyrA</i>	F CGCGTACTATACGCCATGAACGTA	55	441	[8]
	R ACCGTTGATCACTTCGGTCAGG			
<i>qnrB</i>	F TGGCGAAAAAATTGAACAGAA	57	920	[9]
	R GAGCAACGATCGCCTGGTAG			
Capsular sequence gene				
<i>K1</i>	F GTAGGTATTGCAAGCCATGCG	57	920	[10]
	R GCCCAGGTTAATGAATCCGT			
<i>rmrA</i>	F ACTGGGCTACCTCTGCTTCA	55	535	
	R CTTGCATGAGCCATCTTTCA			
Housekeeping sequence gene				
<i>Klpn-16s</i>	F GACGATCCCTAGCTGGTCTG	60	95	[11]
	R GTGCAATATTCCCACTGCT			

The PCR reaction was carried out in 25µl of Premix (Promega, USA). 12.5 µl of Master premix was used with three ng template DNA, one µM of five primers, and the desired volume was filled with deionized distilled water. The thermal cycler programmer consisted of an initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 sec, annealing at 55-60 °C according to (table 1) for 30 sec, and extension at 72°C for 30 sec; followed by a final extension step at 72 °C for 7 min. PCR products were detected by electrophoresis on a 1.5 % agarose gel. Finally, the sizes of the PCR products were determined by comparing them with the migration of the DNA ladder [12].

Results

Collecting Samples and Recognizing *K. Pneumoniae*.

Two hundred fifty clinical samples were collected from health institutions affiliated with Diyala Governorate. The samples included Urine (121) samples, Sputum (78) samples, and Wound swabs (34) samples, and Burns smears (17) samples, and the ages of patients ranged between (1-75) years. These samples were implanted on the medium of MacConkey Agar, where the results of transplantation were (158) samples with a rate of (63.2%) showed Clear bacterial growth on the culture medium and (92) samples with a rate of (36.8%) did not give any bacterial growth. The results of the bacterial culture on the MacConkey Agar medium, according to the source of the sample, were Urine (86) out of a total of (121) samples that grew on the culture

with a percentage of (71%), Sputum out of a total of (78) samples appeared (40) with a percentage (51.3%), and Wounds (22) out of a total of (34) samples appeared at a rate of (64.7%). Burns out of a total of (17) samples appeared (10) at a rate of (58.8%).

Antibiotic Susceptibility and MIC of Ciprofloxacin:

The sensitivity of *K. pneumoniae* isolates under study, which numbered (57) was studied, as they showed high resistance towards a number of antibiotics that are used in health institutions and are widely used to treat different types of infections. By measuring the diameter of the inhibition zone surrounding the antibiotic discs, the results were compared with the standard tables contained in (CLSI-2023).

The results of the current study showed the sensitivity of *K. pneumoniae* isolates towards the group of β-lactam antibiotics, where the resistance to Ampicillin 100%, Piperacillin (100%), Cefoxitin (87.71%), Imipenem (26.31%), Meropenem (28.07%), Gentamycin (40.35%), Amikacin (50.87%), Streptomycin (80.70%), Levofloxacin (35.08%), Ofloxacin (31.57%), Ciprofloxacin (22.80%), Trimethoprim – sulfamethoxazole (64.91%). These results are consistent with the findings of the study [13]. As it was found that the percentage of resistance of *K. pneumoniae* bacteria to Beta-lactam antibiotics was (Ampicillin 100%, Cefoxitin 100%, Imipenem 50%, Meropenem 81.80%). The results of the minimum inhibitory concentration (MIC) of Ciprofloxacin showed that the MIC of this antibiotic was 8-1024 µg/ml.

Molecular detection of *Klebsiella Pneumoniae* by using the *16S rRNA* & Antibiotics Genes:

The current study's findings demonstrated that all bacterial isolates (22) carrying the (*16S rRNA*) gene with a percentage of (100%); the current study's findings concurred with those of the [22] that every *K. pneumoniae* isolate had a 100% presence rate of this gene. The *16S rRNA* gene is considered the diagnostic gene for these species depending on the primer used for the gene (*16S rRNA*). The gene has a size of (95) base pairs compared with the DNA ladder, which has bundles of known molecular size, as in Figure 1 A&B.

Molecular Diagnosis of *Klebsiella Pneumoniae* using the *gyrA* Gene:

The current study's findings demonstrated that in 22 bacterial isolates carrying the (*gyrA*) gene, the percentage of the *gyrA* gene in *K. pneumoniae* bacteria was 100%, which is consistent with the results of the current investigation regarding these two genes. The *gyrA* gene depends on the primer used for the gene (*gyrA*), as the gene has a size of (421bp.) compared with the DNA ladder that has bundles of known molecular size. As in Figure. 2.

Molecular Diagnosis of *Klebsiella Pneumoniae* using the *qnrB* Gene:

The results of the molecular detection of the *qnrB* gene with a size of (798) base pairs in (6) bacterial isolates, amounting to (22) isolates, using the (PCR) technique and a Thermocycler, showed that all (6) isolates and (22.72%) possess this gene, as shown in Figure 3.

Molecular diagnosis of *Klebsiella Pneumoniae* using the *KI* Gene:

The results of the molecular detection of the *KI* gene with a size of (920) base pairs in (1) bacterial isolates, amounting to (22) isolates, using the (PCR) technique and a Thermocycler, showed that only (1) isolates (4.54%) possess this gene, as shown in Figure 4.

Molecular Diagnosis of *Klebsiella Pneumoniae* using the *rmpA* Gene:

The results of the molecular detection of the *rmpA* gene with a size of (535) base pairs. Using the (PCR) technique and a Thermocycler showed that all the isolates don't possess this gene, as shown in Figure 5. It clarifies the context and emphasizes the negative finding regarding the presence of the *rmpA* gene in the isolates

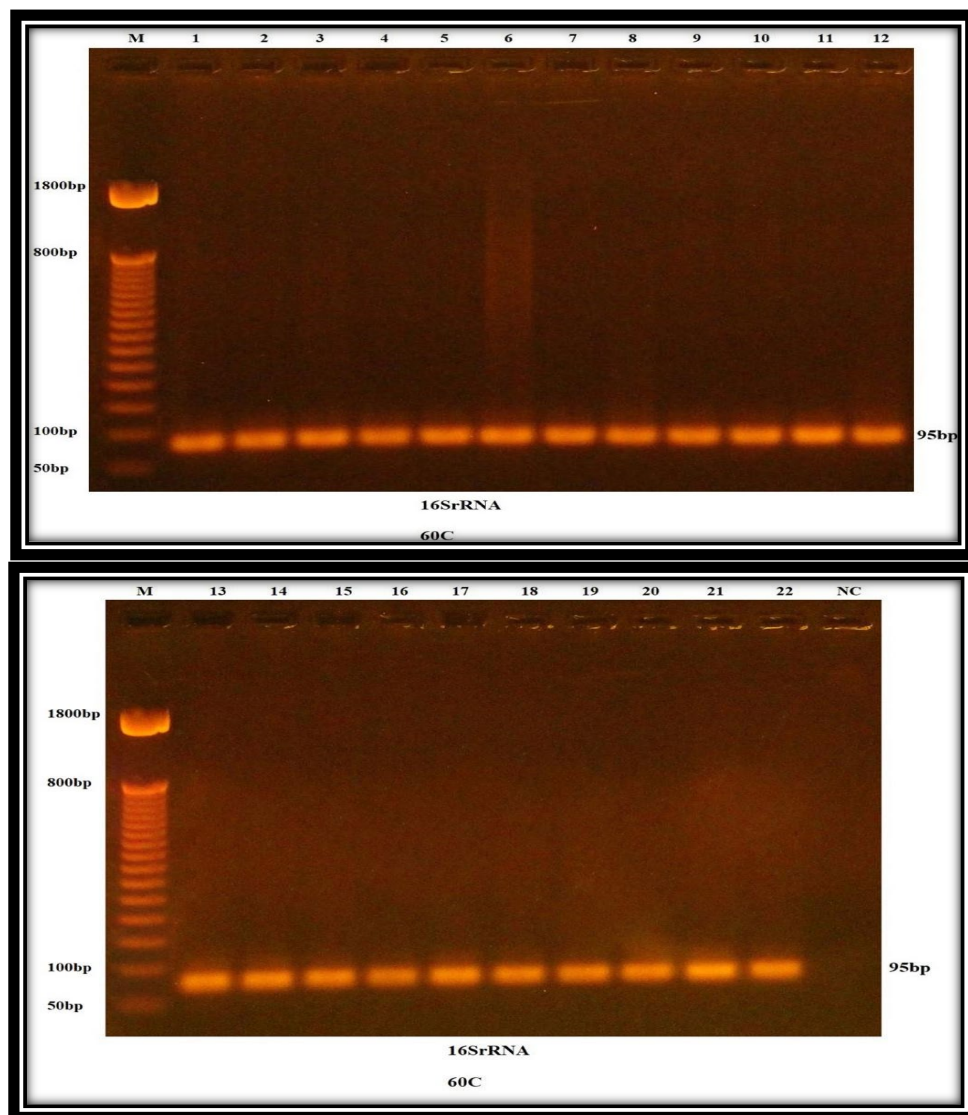


Figure 1 A&B: Electrophoresis of the 16S rRNA gene in *K. pneumoniae* bacteria by means of an agarose gel concentration of 1.5% containing 1 μ of Ethidium bromide dye and by using a DNA ladder from (95) base pairs as the path is shown in the two figures—the presence of the *16S rRNA* gene in all samples.

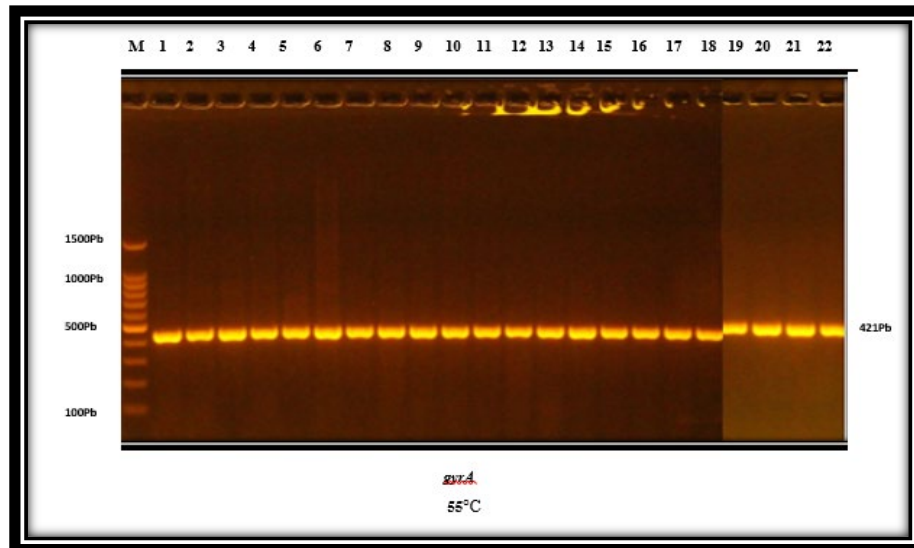


FIGURE 2: Electrophoresis of the *gyrA* gene in *K. pneumoniae* bacteria using an agarose gel concentration of 1.5% containing 1 μ of Ethidium bromide dye and a DNA ladder from (421) base pairs. The *gyrA* gene was present in all samples.

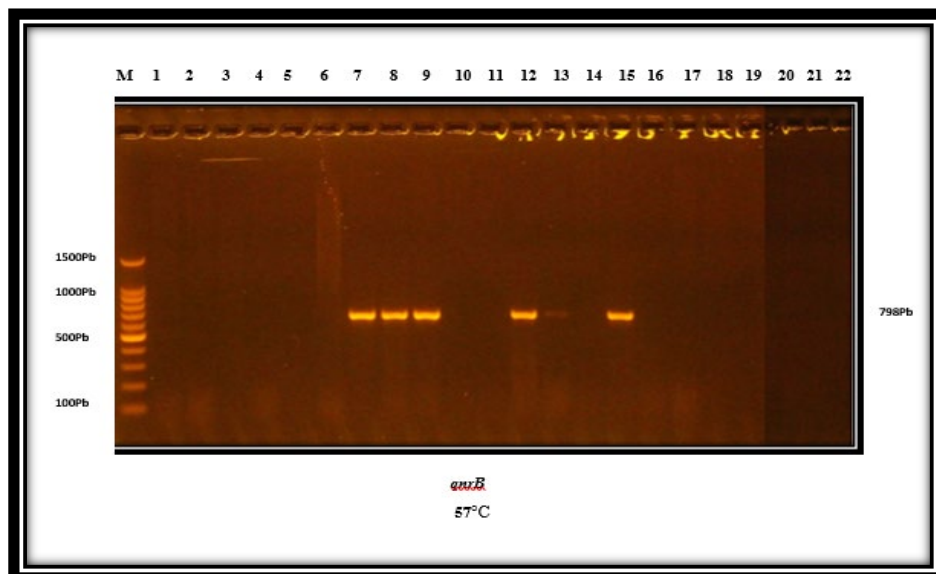


FIGURE 3: Electrophoresis of the *qnrB* gene in *K. Pneumoniae* Bacteria using an Agarose Gel Concentration of 1.5% Containing 1 μ of Ethidium Bromide Dye and a DNA Ladder from (798) Base Pairs. The *qnrB* gene was present in (6) Samples.

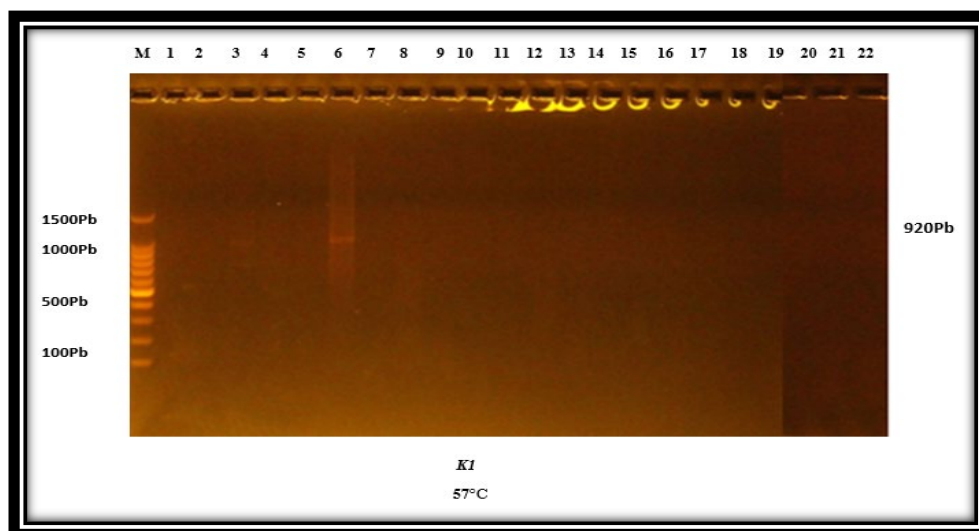


FIGURE 4: Electrophoresis of the *KI* gene in *K. Pneumoniae* Bacteria using an Agarose Gel Concentration of 1.5% containing 1 μ of Ethidium Bromide Dye and a DNA Ladder From (920) Base Pairs. The *KI* Gene was Present only in (1) sample.

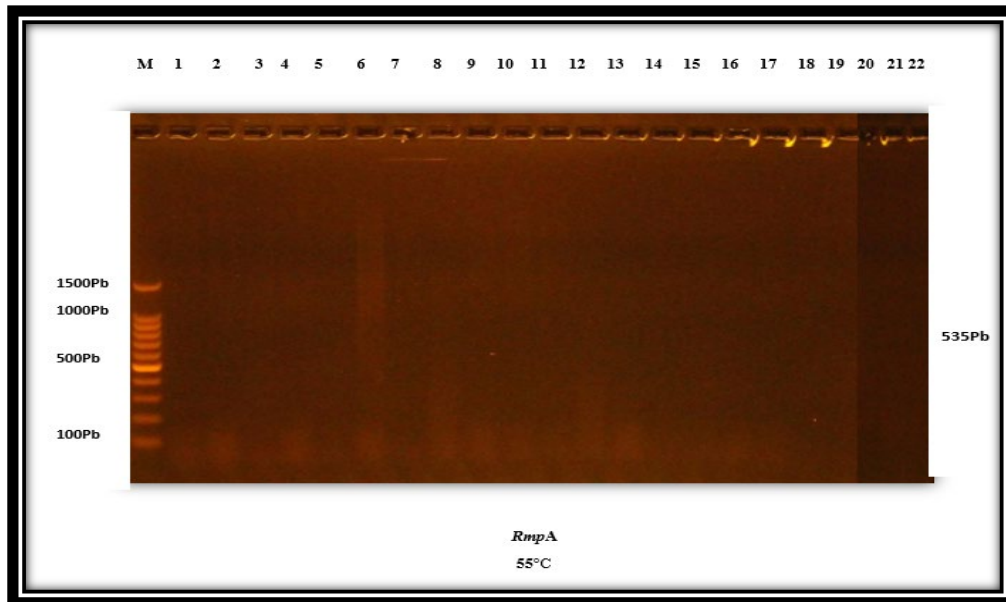


FIGURE 5: Electrophoresis of the *rmpA* Gene in *K. Pneumoniae* Bacteria using an Agarose Gel Concentration of 1.5% containing 1 μ of Ethidium Bromide Dye and a DNA Ladder from (535) Base Pairs. The *rmpA* gene wasn't present in any Samples.

Discussion

Among the results of the current study is that the percentage of infection that occurred as a bacterial infection was higher in Urine samples among all samples collected from different clinical sources, and this is consistent with what was reached by [13], where the results of his study showed that among (152) samples of Urine (115) samples gave a positive result for implantation at a rate of (75.65%). The results of [14] also showed that among (108) samples, urine samples were the most common, reaching (37), with a rate of (37%). The spread of *K. pneumoniae* and pathogenic importance comes through the events of urinary and respiratory tract infections. This may be because these bacteria are from the normal flora that lives in the intestine. The results of the current study Well, it is opportunistic, and it can adhere to surfaces of epithelial cells. The resistance of this bacteria to antibiotics converges, consistent with the study's findings [15]. The percentage of resistance of *K. pneumoniae* bacteria to Beta-lactam antibiotics was (Ampicillin 100%, Cefoxitin 100%, Imipenem 50%, Meropenem 81.80%). The results of this study converge with the findings of [16].

The resistance rate to Gentamycin was 36.23%, and the resistance rate to Amikacin was 50.87%. The findings of the presented study were inconsistent with the results of [17]. where the rate of antibiotic resistance is Piperacillin 16%, Cefoxitin 46%, Imipenem 12%, Meropenem 8%, Gentamycin 40%, Amikacin 10%, Streptomycin 78%. However, the outcome of the presented study agreed with the results given by the researchers [18, 19]. The resistance rate to levofloxacin was 38.59%, and that of ciprofloxacin was 44.73% and 27%, respectively. The outcome of the study disagreed with the researchers' results [20, 21]. They showed that the resistance rate to Levofloxacin was 13.5%, and to Ciprofloxacin and Ofloxacin was 7.7%. The relative difference in *K. pneumoniae* resistance patterns towards antibiotics is due to several factors, such as the size of the sample studied, the source of the sample, geographical differences, and the site of infection, in addition to the patient's predisposition to disease [22]. The results of the minimum inhibitory concentration (MIC) of Ciprofloxacin

showed that the MIC of this antibiotic was 8-1024 μ g/ml. This result agrees with [23], which showed that the MIC of Ciprofloxacin was 2-256. However, this result disagrees with [24], who revealed that the MIC of Ciprofloxacin was ≥ 4 . The identification of this bacteria by (*16S rRNA*) showed that all isolates possess this gene. The current study's findings concurred with those of [25] that every *K. pneumoniae* isolate had a 100% presence rate of this gene. The *16S rRNA* gene is considered the diagnostic gene for these species depending on the primer used for the gene (*16S rRNA*). The current study's findings demonstrated that all isolates carried the (*gyrA*) gene; these results were obtained by the researcher [8] Since the ratio of its isolates was 100%. However, this study's findings disagreed with the researcher [26, 27] regarding the percentages of this gene in *K. pneumoniae*, which were (80%) and (71.05%), respectively. The results of the molecular detection of the *qnrB* gene, using the (PCR) technique and a Thermocycler, showed that (6) isolates and (22.72%) possess this gene; the current study's findings were consistent with the findings of the researcher [28] where the rate to this gene was (20%). It conflicts with those of the researcher [29], who discovered that 90.9% of *K. pneumoniae* isolates carried the *qnrB* gene. The results of the molecular detection of the *k1* gene show that only one isolate possesses this gene. This result agrees with a researcher [30] Since the ratio of its isolates was 0%. However, this study's findings disagreed with the researcher [31, 32] regarding the percentages of this gene being 19.2% and 45.9%, respectively. The results of the molecular detection of the *rmpA* gene show that all isolates don't possess this gene. This result agrees with a researcher [33] Since the ratio of its isolates was 0%. However, this study's findings disagreed with the researcher [10] regarding the percentage of this gene being 48.8%.

Conclusions

B-lactam antibiotics were the most studied antibiotics that were resistant to *K. pneumoniae*. The gene under study (*16S rRNA*, *gyrA*, *qnrB*, *k1*, and *rmpA*) was detected with a percentage of 100%, 100%, and 22.72%, respectively, and *k1* and *rmpA* was 0% in *K. pneumoniae* isolated from various clinical samples.

Recommendations

Additional molecular research will be performed to identify changes in the local *K. pneumoniae* patterns, and the gene expression of antibiotics and quorum-sensing genes will be studied in the local

isolates of *K. pneumoniae* in hospitals and communities in Iraq. Moreover, we are studying phylogenetic analysis (tree of genomics) of *K. pneumoniae* isolated from different clinical cases for detected mutation.

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