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Original Article

Study of Some Virulence Genes from Uropathogenic *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* Isolated in Wasit Province, Iraq

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Abstract

Background: *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are the most common causes of nosocomial urinary tract infections (UTIs), and in recent years, these bacteria have become significantly more resistant to antibiotics. **Objective:** The objective of the study was to determine the frequency of uropathogenic *K. pneumoniae* and *P. aeruginosa* in clinical urine samples from patients with UTIs in Wasit Province. **Materials and Methods:** Initially, 100 clinical urine samples were taken for this investigation from (October 1 to December 1, 2022), and the isolates were initially identified using culture, microscopic analysis, and biochemical assays. **Results:** The findings of the biochemical results revealed that 16/100 isolates had a 16% positive response for *K. pneumonia*, and 13/100 isolates had a 13% positive response for *P. aeruginosa*. When DNA was recovered from the isolates, the nucleic acid purity was diverse from 1.8 to 2.0, and the concentration fluctuated from 50 to 360 mg/µL. By using molecular techniques, including the 16S rRNA gene, for confirmation of the diagnosis. These findings were validated by the polymerase chain reaction method used to detect the virulence genes fimH gene, where 4/16 (25%) and mrkA gene, where result 7/16 (43.75%) were positive in recovered *K. pneumoniae* isolated. And RNA polymerase subunit B (rpoB) gene where results 10/13 (76.92%) were positive in recovered *P. aeruginosa* isolated. **Conclusion:** In conclusion, the outcomes of this study presented a significant increase in biofilm-forming and multidrug-resistant *K. pneumoniae* isolates, which also harbor a wide range of virulence genes and used rpoB as high molecular identification properties to confirm the *P. aeruginosa*, the most efficient antibiotics for its treatment were Impinem and Nitrofurantoin for both *K. pneumonia* and *P. aeruginosa.*

Keywords: *fim*H *gene*, *K. pneumoniae*, *mrk*A *gene*, *P. aeruginosa*, *rpo*B, UTI

Introduction

Urinary tract infections (UTIs) are highly common infectious diseases that affect millions of people every year.[1] These infections can be triggered by various microbial agents and can target any part of the urinary system, being acquired either in healthcare settings or in the community. Among the most prevalent Gram-negative bacteria responsible for UTIs are uropathogenic *Klebsiella* pneumoniae and *Pseudomonas aeruginosa*.[2] Sticky pili or fimbriae, which are most typically associated with the Chaperone-usher (CU) pathway, are utilized as weapons by the most common uropathogenic bacteria, uropathogenic *Escherichia coli* (UPEC) and uropathogenic *Klebsiella pneumoniae* (UPKP).[3] The progress in developing antifimbrial treatment approaches has been accelerated due to the critical and indispensable role of these significant virulence components in bacterial pathogenicity and virulence.[4] On bacterial surfaces, the CU route, a common protein secretion technique, may result in a wide variety of pili. Key virulence factors

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DOI: 10.4103/MJBL.MJBL_1100_23

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Submission: 28-Jul-2023 **Accepted:** 08-Aug-2023 **Published:** 09-May-2024

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How to cite this article: Raheema RH, Hadi SH, Chabuck ZAG. Study of some virulence genes from uropathogenic *Klebsiella* pneumoniae and *Pseudomonas aeruginosa* isolated in Wasit Province, Iraq. Med J Babylon 2024;21:207-13.

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in Gram-negative bacteria, particularly within the Enterobacteriaceae family, such as UPEC, UPKP, and uropathogenic *Proteus mirabilis*, are the pili, commonly known as fimbriae or fibrillae.[5] Previous studies have shown that more than 80% of *K. pneumoniae* strains possess type 1 and type 3 CU fimbriae, also referred to as mannose-resistant Klebsiella-like hemagglutinins.[6] The widespread dissemination of high-risk *K. pneumoniae* clones has emerged as a significant public health concern.[7] This is attributed to their enhanced ability to spread and persist in healthcare settings, leading to outbreaks, as well as their rapid acquisition of plasmid-encoded carbapenemases.[8]

An initial and crucial stage in biofilm formation involves attaching to non-living surfaces, which is often facilitated by the production of fimbriae, like the type 3 fimbriae generated by the *mrk*A operon.[9] Bacterial biofilms can be formed in response to alterations in the environment or specific stressful conditions, providing protection to bacteria against potentially harmful external physical and chemical changes. *K. pneumoniae* biofilm development is linked to *mrk*A expression.[10] Most bacterial cells have 100–500 of the 2 m long, 10 nm wide Type 1 pilus on their surface.[11] The pilus exhibits mannose sensitivity due to its ability to interact with mannosylated receptors present in epithelial cells, particularly urothelial cells. This interaction is facilitated by the adhesin *fim*H, which is located at the tip of the type 1 pilus. It is worth noting that the type 1 pili of both *E. coli* and *K. pneumoniae* share high similarity in uropathogenic strains.[12,13]

Pseudomonas aeruginosa is an opportunistic pathogen that causes a variety of infections,[14] in immunocompromised individuals, such as UTIs, respiratory system infections, dermatitis, soft tissue infections, sepsis, infections, gastrointestinal infections, and a variety of systemic infections.[15,16] The rpoB gene, encoding the beta RNA polymerase subunit, was used as a bacterial identity marker as well as a locus for phylogenetic analysis; furthermore, rpoB is a highly conserved housekeeping gene and a copy present in all bacteria due to its essential role in cellular metabolism.[17] For analyzing closely related isolates, the *rpo*B gene, encoding the *β*-subunit of RNA polymerase, has become a core gene candidate for phylogenetic analysis and bacterial identification. Together with the *16S rRNA* gene, *rpo*B has significantly improved bacterial community analysis and facilitated the identification of novel bacterial species.[18] The objective of the study was to determine the prevalence of uropathogenic *K. pneumoniae* and *P. aeruginosa* in clinical urine samples collected from patients with UTIs in Wasit Province.

Materials and Methods

During the period between October 1 and December 12022, a total of 100 midstream urine samples were collected from patients at the Al-Zahra Teaching Hospital in the Wasit region. The MacConkey and Blood agar was used to cultivate the urine samples before inoculating them at 37°C for 18–24 h. It investigated colonial traits such as form, volume, color, boundaries, and texture under a microscope after being stained with gram-stain and using a biochemical test to identify *K. pneumoniae* and *P. aeruginosa* to isolate pure colonies. The isolated bacteria were identified using morphological, biochemical, and *16s RNA* testing. To assess their antibiotic susceptibility, a Kirby–Bauer technique was employed on Muller–Hinton agar. The results were interpreted using the following antibiotics and their respective concentrations: Cefepime FEP 30 µg, Ceftriaxone CTR 30 µg, Ciprofloxacin CIP 5 µg, Imipenem IPM 10 µg, Nitrofurantoin F 300 µg, and Piperacillin PRL 100 µg.[19]

DNA extraction and molecular identification

Nanodrop was used to extract DNA from isolates, and its purity and concentration were checked. The nucleic acid content of the samples ranged from 50 to 360 mg/µL, and their nucleic acid purity was measured between 1.8 and 2.0. The primers used in the study were provided by Integrated DNA Technologies, USA, in the form of lyophilized powder, each supplied in 1.5 mL Eppendorf tubes. The lyophilized primers were eliminated from the tubes by centrifuging them at 8000 rpm for 5 s before opening. Following the manufacturer's instructions, the lyophilized powder primers in 1.5 mL Eppendorf tubes were mixed with 1× TE buffer (pH 8.0) to create a stock solution with a concentration of 100 µM. This mixture was incubated for 10 min in a water bath at 65°C. After incubation, the tubes containing the primer stock solutions were stored at -20°C until they were ready to be used in the experiment.

For *K. pneumoniae* using the primer set, DNA *16S rRNA* region amplification was carried out F (5′-TTACCCGCAGAAGAAGCAC-3′) and R (5'CTACGCATTTCACCGCTAC-3′)(219 bp) *mrk*A F (5′-AAGTTAAAGCGGCAGCAG-3′) and R (5′-TGTCAGTAGACAGCACCAG-3′) (228 bp) and *fim*H F (5′-TAATCCCCCTGTTCACCACC-3′) and R (5′-GAAAAACTCGACAGCACACC-3′) (271 bp).[20]

For *P. aeruginosa* the *rpo*B primer set was used to undertake DNA *rpo*B region amplification F (5′-CAGTTCATGGACCAGAACAACCCG-3′) and rpoB R (5′-ACGCTGGTTGATGCAGGTGTTC-3′) (866 bp).

For the detection of virulence gene sequences, the monoplex polymerase chain reaction (PCR) method was employed. Each PCR reaction consisted of a 25 µL sample of the PCR master mix.[21,22] The reaction mixture included 5 µL of DNA template, 2 µL of forward primer, and 2 µL of reverse primer. ADditionally, 8 µL of DD

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Table 1: The thermal cycling programs for the two monoplex genes, *fim*H and *mrk*A

Steps	Temperatures (°C)	Time	No. of cycles
Initial denaturation	94	2 min	1
Denaturation	94	30 s	35
Annealing	52	30 s	
Extension	72	1 min	
Final extension	72	5 min	1

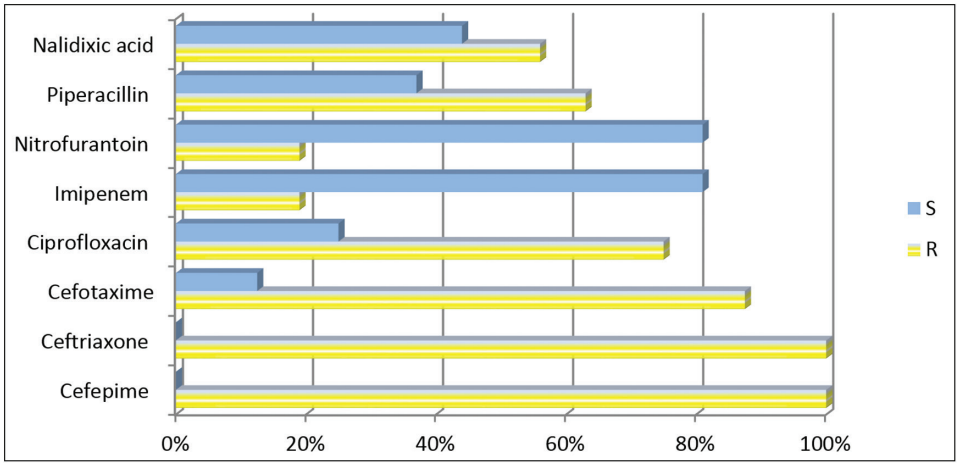

Figure 1: The percentage of antibiotics susceptibility profiles of *K. pneumonia*

water and 8 µL of PCR multiplex lyophilized powder[23,24] were added to complete the reaction. This was carried on by monoplex PCR, according to Dallenne *et al.*[25], Ugwu *et al.*[26] Each unique primer set has its own unique set of PCR amplification conditions that were carried out using a thermal cycler. As in Table 1, then electrophoresis and documentation.[27]

Statistical analysis

The Statistical Analysis System (SAS), version 9.1 (SAS Institute, Inc., Cary, NC, USA), was utilized for statistical analysis of the data. To compare percentages, the chi-square test was employed. Statistical significance was considered at a P value less than 0.05 ($P < 0.05$).

Ethical approval

The study was conducted following the ethical standards outlined in the Helsinki Declaration. Prior to obtaining any samples, the patients' verbal and written consent was obtained to ensure their voluntary and informed participation in the study. According to the document number and date on July 31, 2022, a local ethics committee evaluated and approved the study protocol, subject information, and consent form to obtain this permission.

Results

Based on the identification findings, the presence of pink, lactose fermenter, and mucoid colonies on MacConkey agar indicated the likelihood of uropathogenic *K. pneumoniae*. Results of identification of *P. aeruginosa* isolates. The TSI test results showed negative results for methyl red, Voges–Proskauer, indole, and urease tests. However, the citrate assimilation and motility tests were positive. In the TSI test, the results were alkaline/alkaline without the production of H2S and gas. However, it's worth noting that A/A (acid/acid) with gas but no H2S was detected in the TSI test.

The result of the antimicrobial susceptibility test of *K. pneumoniae* isolates revealed that Ceftriaxone displayed the highest level of resistance, with all isolates (100%) being resistant to it. Cefepime, on the other hand, showed a resistance level of 87.5%. Additionally, resistance rates of 75% and 63% were observed for Ciprofloxacin and Imipenem, respectively. Imipenem and nitrofurantoin had the highest sensitivity to *K. pneumoniae* (81% each), as illustrated in Figure 1.

Result of the antimicrobial susceptibility test of *P. aeruginosa* isolates Ceftriaxone and Cefepime had the highest degree of *P. aeruginosa* resistance, respectively, followed by cefotaxime (77%) ciprofloxacin (62%) and piperacillin (38%) in that order. According to Figure 2, nitrofurantion with (77%) and imipenem (92%) have the highest sensitivity to *K. pneumoniae*.

Result of molecular identification of *K. pneumonia isolates* and detection of the *16SrRNA* gene. It is a specialized assay that has high sensitivity and is based on the diagnosis of many bacterial genera on the *16SrRNA*

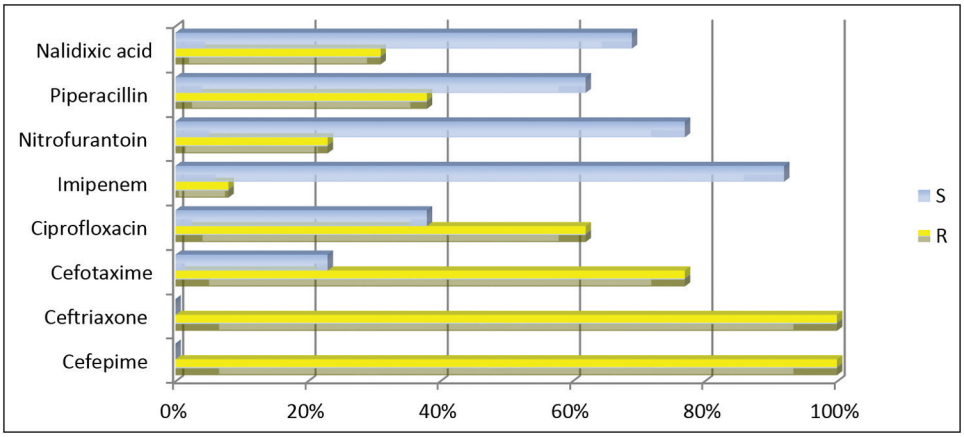

Figure 2: The percentage of antibiotics susceptibility profiles of *P. aeruginosa*

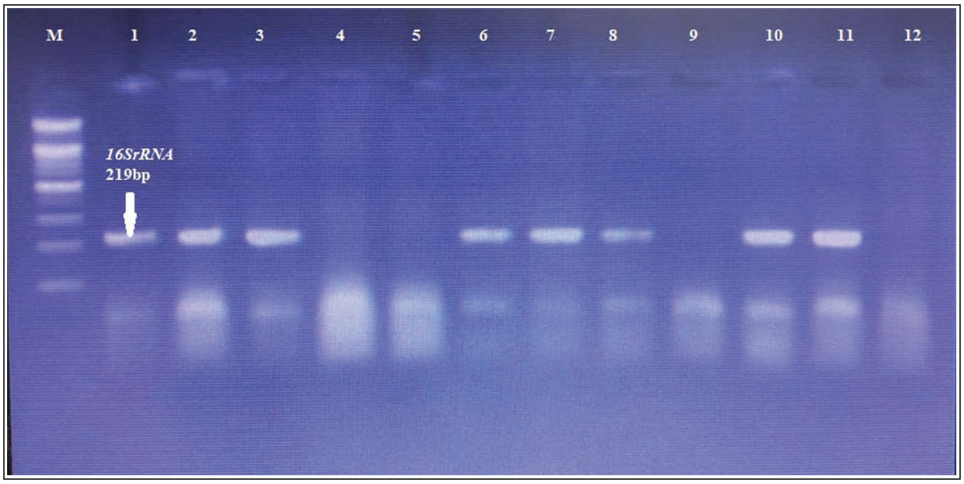

Figure 3: The *Klebsiella pneumonia 16SrRNA* gene was amplified and electrophoresed using standard PCR. Agarose 1.8%, 100 V/cm for 35 min, dye staining using ethidium bromide, and UV transilluminator viewing. Lane 2000 bp DNA ladder (M). Lanes 1–12 of *16SrRNA*, 219 bp

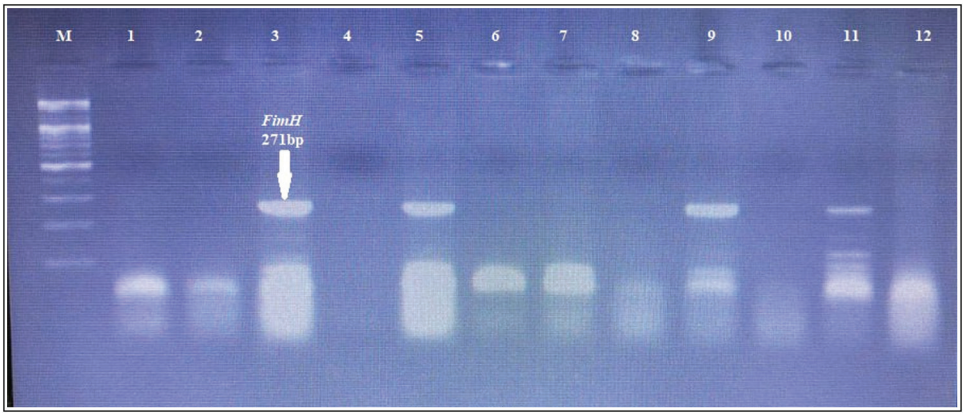

Figure 4: PCR amplicon of the *fim*H gene from *K. pneumoniae*. 271 bp is the product size. DNA marker (2000 bp ladder), lane (L), lanes 1–12

gene. It is also used as a differential gene for bacterial species. The findings of this study demonstrated that this diagnostic test successfully confirmed the results of the diagnostic test for the *16SrRNA* bacterium *K. pneumonia*, which numbered 16 (*16SrRNA*) (100%). Figure 3 displays the PCR products' agarose gel.

*K. pneumoniae's fim*H gene was discovered by molecular analysis in 4/16 isolates, according to the results of Figure 4.

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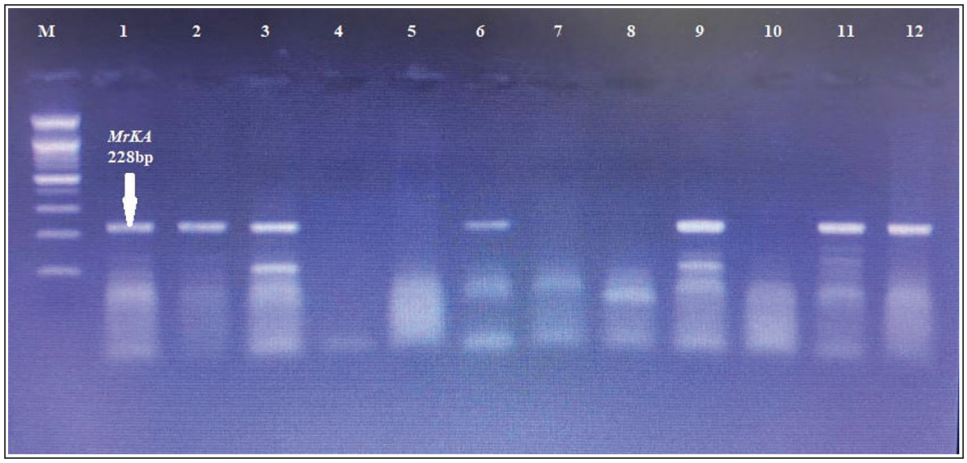

Figure 5: PCR amplicon of the *mrk*A gene PCR amplicon from *K. pneumonia*. 228 bp is the product size. DNA marker (2000-bp ladder), lane (L), lanes 1–12

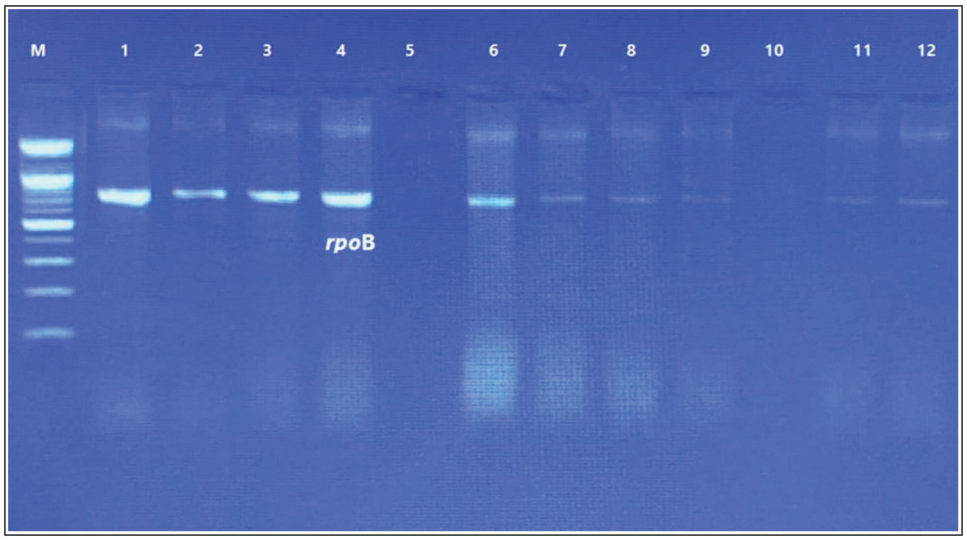

Figure 6: PCR amplicon of the *rpo*B gene PCR amplicon from *P. aeruginosa*, 866 bp is the product size. DNA marker (2000-bp ladder), lane (L), lanes 1–12

Figure 5 displays the findings of the gel electrophoresis for the PCR product using particular primers for the *mrk*A gene of *K. pneumonia*, which revealed that 7/16 (43.75%) of the isolates had the *mrk*A biofilm gene.

Result of molecular identification of *Pseudomonas aeruginosa* isolates

The findings of the gel electrophoresis for the PCR product using particular primers for the *rpoB* gene of *P. aeruginosa*, which revealed that 10/13 (76.92%) of the isolates had the RNA polymerase subunit B gene, as shown in Figure 6.

Discussion

Bacterial isolates that were obtained from the clinical specimens have been initially characterized based on the cultural morphology as well as the biochemical tests. *K. pneumoniae* and *P. aeruginosa* biochemical test findings were similar to those reported[23,29-31] and were also documented.[28] The emergence of resistance of *K. pneumoniae* and *P. aeruginosa* infections to multiple antibiotics is an emerging problem that is highly complex and complicates the management of UTIs progressively more expensive and more demanding; this limits the options for selecting appropriate agents for the treatment of infection. Outbreaks of *K. pneumoniae* and *P. aeruginosa* infections have been known widely. Contributing to the global spread of multidrug resistance. The result was in accordance with the results of other studies of resistance of *K. pneumoniae* conducted by Jassim *et al.*[28], and Guragain *et al.*[32] Similar results were found in the results of Al-Saeedi and Raheema[30] and Lob *et al.*[33] The study's findings that imipenem and nitrofurantoin had the lowest rates of resistance may be explained by the ineffective use of these antibiotics in Al-Kut hospitals.

16SrRNA gene results showed that 25% of isolates have the *fim*H *gene,* which is relatively close to Al-Kraety

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et al.[34] that found the percentage was (35%). Fimbriae are assumed to be necessary for attachment, and type-1 fimbriae are important in determining pathogenicity. *Klebsiella* needed a mannose-resistant hemagglutinin to attach to target cells, according to Jaroni's tests.[34] The current finding suggested that fimbriae 1 played a significant role in epithelial cell surface attachment (which facilitates bacterial colonization) and determining the pathogenicity of *K. pneumoniae*. By observing how bacteria cling to mucous surfaces or epithelial cells, type 1 fimbriae and bacterial pathogenicity were linked.[35] The closest results were,[36] which found the percentage (66%). The expression of the *mrk*A gene is positively linked with the buildup of biofilm biomass.[37] The study conducted by *rpo*B reported that 99.5% of the isolates examined carried the *rpo*B gene, making their findings the most closely aligned with the presence of this gene in the studied bacterial population. To confirm *P. aeruginosa*, it is thus advisable to utilize the *rpo*B gene since it provides accurate findings and has strong molecular identification qualities. ADditionally,[38] indicated that employing *rpo*B sequencing as a standard method of identifying Pseudomonas strains. Similar findings were reported by Ibrahim *et al.*[39] and Mohammed *et al.*,[40] who recommended using antibiotics only when directed to do so by medical professionals and only for the prescribed length of time. Infections caused by pathogenic bacteria are difficult to treat due to the pathogen's capacity to develop antibiotic resistance.

Conclusion

Uropathogenic *K. pneumoniae* can produce several virulence factors that enhance their ability on infection events, such as biofilm formation on surfaces, which increases their pathogenicity and makes them more difficult to treat. Multidrug resistance and pathogenicity are common in uropathogenic *K. pneumonia*, making these infections more severe and difficult to treat. The most effective antibiotics against uropathogenic *K. pneumonia* were Nitrofurantoin and Impinem.

Acknowledgements

The study received funding from the College of Medicine at the University of Wasit in Wasit, Iraq.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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