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# Molecular Detection of Some Virulence Factors and Antibiotic Resistance Genes of Klebsiella Pneumoniae Isolated from Urolithiasis Patients

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**ABSTRACT:** Urolithiasis, also known as urinary stone disease, is a prevalent and recurrent ailment that affects around 12% of people worldwide, with a higher incidence in men. Recent research has highlighted the critical role that microbial infections particularly those caused by *Klebsiella pneumoniae* play in the development of stones, specifically through processes that include urease activity and biofilm formation. In addition to performing molecular detection of important virulence and antibiotic resistance genes that may be involved in infection pathogenesis and persistence, the study sought to determine the distribution of *K. pneumoniae* among urolithiasis patients in Karbala, Iraq.

## INTRODUCTION

Urolithiasis is a term which is used to describe the development of stones in the urinary system such as the kidney, ureter, bladder, or urethra. The term "Urolithiasis" is made up of lithos (stone) and ouron (urine). About 12% of people worldwide suffer with stone formation, recurrence rate of 70–81% in men and 47–60% women (1) Men have a higher risk of kidney stones than women mainly due to differences in urine chemistry. Men's urine contains more calcium, oxalate, and uric acid and has a lower pH, all of which increase stone formation. Lifestyle also plays a role, but urine composition is the key factor (2) in female The shorter length of the female urethra, located close to the vagina, makes it more susceptible to colonization by pathogenic bacteria such as *E. coli*. This increases the likelihood of urinary tract infections and subsequent formation of infectious stones in women (3). The type of Urolithiasis classified into four primary types based on their composition: calcium stones (mostly calcium oxalate), uric acid stones struvite stones (often linked to urinary infections), and cystine stones resulting from a genetic disorder (4). Once the urinary microbiome was identified and microorganisms were found in urine, which until recently, was assumed to be sterile in healthy persons. the link between the microbiome and urological illnesses became more plausible. In patients with urolithiasis, the microbiome is affected by several parameters, amongst others, lifestyle factors and diet (6) *Klebsiella pneumoniae* facilitates struvite urolithiasis through urease activity, which elevates urinary pH and promotes magnesium ammonium phosphate crystallization. Its biofilm-forming capacity further enhances chronic infection and stone persistence (7) bacteria included *Klebsiella spp., Enterococcus spp Enterobacter spp*., *Proteus* which belong to the family

Enterobacteriaceae. They proposed that bacteria present in the stone nucleus might directly participate in the stone formation (8). *Klebsiella pneumoniae* is a Gram-negative bacterium within the Enterobacteriaceae family that can cause multiple systemic infections, such as respiratory tract, blood, liver abscesses and urinary systems (9) It is facultatively anaerobic, rod-shaped, nonsporulating, and immobile. It is named after German scientist Edwin Klebs (1834–1913). Is classified as rendering to the following. It is also known as Friedlander's bacillus (10). The pathogenicity of *K. pneumoniae* is caused by a number of virulence factors that effectively evade the host's innate immune systems and support the infection's persistence. Important elements influencing pathogenicity include bacterial capsules, adhesins, siderophores, lipopolysaccharides, and biofilm formation **(**11) *K. pneumoniae* diseases show a strong link between clinical symptoms and virulence factors. The capsular polysaccharide protects

the bacteria from phagocytosis, while type 1 and type 3 fimbriae (encoded by fimH-1 and mrkD) help in biofilm formation and infection establishment (12) one of the mechanisms of antibiotic resistance in this bacterium is the production of biofilm, and in fact, biofilm is a population of bacteria that are trapped in an extracellular matrix (13). This matrix consists of proteins, exopolysaccharides, DNA and lipopeptides. By forming a biofilm, *K. pneumoniae* isolates can stay away from the immune system and antibiotics and lead to antibiotic resistance (14). *K. pneumoniae* must secrete siderophores for reproduction and full pathogenicity. These molecules help bacteria acquire iron in vivo by chelating it from the environment, making siderophores essential virulence factors for bacterial survival and infection (15). The important genes encoding iron uptake system in *K. pneumoniae* include fhuA (ferrichrome), iucABCD and iutA (aerobactin), fepAB and entAB (enterobactin), fecAE (ferric citrate), iroNB (salmochelin), hmuR (heme), sitA and feo (ferrous, Fe2+), kfu ABC (ferric, Fe3+), fyuA and irp-1, irp-2, ybtS and yersiniaHPI (yersiniabactin) (16) *K. pneumoniae* displayed antibiotic resistance that was heterogeneous based on specimen type or gender. Different genotypes and a high association between the examined genes and antibiotic resistance profiles were found by PCR identification of the genes coding for efflux pumps and porins. Most of the K. pneumoniae that was investigated was resistant to different antibiotics; the most beneficial medicines were tigecycline and imipenem, while the least effective ones were ampicillin, piperacillin, cefuroxime, cefepime, cefazolin, and aztreonam (17).

## MATERIALS AND METHODS:

### Study design and Samples collection

130 sample were isolated from urine of patients with urolithiasis in distinct Karbala hospitals from January to Februry,2025. Samples collected by using a sterile method in a sterile, dry container with a tight lid. Where left the first urine portion and collected the intermediate sample from the second urine section. All sample was taken, cultured independently on blood agar and MacConkey agar, and then incubated for 24 hours at 37°C (18). After incubation, isolates believed to belong to the Klebsiella genus were gathered for identification based on the morphology of their colonies (dimensions, color, texture, and shape). [19]. Mucoid growth colonies on MacConkey agar and non-hemolytic mucoid colonies on sheep blood agar are gray, white [20] and we used vitik-2 system for identification and antibiotic susceptibility testing.

### Molecular detection of Virulence genes by PCR:

Total DNA was extracted using DNA extraction kit purchased from Addbio, Korea. The resistance and virulence genes, including,WabG,fimH,ycf,Kfu,entB,iut A were distinguished by the PCR technique using specific primers (Table No. 1). PCR reactions were performed in the PCR thermal cycler (Edison, NJ-USA) using PCR master mix (Microgen, South Korea). PCR reactions were performed in total volumes of 23 µl. The master mix contained 10 µL of reaction mixture with Taq DNA polymerase master mix, 1.5 µL of each of the forward and reverse primers in 10 µM/µL concentrations,3 µL of target DNA, and 7 µl of distilled water. Primer solution was prepared in a 10 pmoles/µL by addition of 10 µL of stock solution to 90 µL of deionized sterile water then mixed well and stored in ice until usage while the stock solution of primers was stored in -20°C.The PCR products were exposed by electrophoresis on a 1.5% agarose gel using 70 voltages for 50 minutes followed by consequent exposure to UV light in the presence of DNA load dye.

**Tab. No. 1:** Primers with their sequences and amplicon sizes of Klebsiella

| Genes | Primer Sequences (F/R) | Product Size (bp) | Annealing Temp. (°C) | Reference |
|---|---|---|---|---|
| **wabG** | F: ACCATCGGCCATTTGATAGA<br>R: CGGACTGGCAGATCCATATC | 683 | F: 58<br>R: 60 | (21) |
| **fimH** | F: ATGAACGCCTGGTCCTTTGCR: GCTGAACGCCTATCCCCTGC | 688 | F: 60<br>R: 66.1 | (22) |
| **ycfM** | F: ATCAGCAGTCGGGTCAGC<br>R: CTTCTCCAGCATTCAGCG | 160 | F: 62.4<br>R: 58 | (23) |
| **entB** | F: ATTTCCTCAACTTCTGGGC<br>R: AGCATCGGTGGCGTGGTCA | 371 | F: 60.7<br>R: 69.6 | (24) |

| Genes | Primer Sequences (F/R) | Product Size (bp) | Annealing Temp. (°C) | Reference |
|---|---|---|---|---|
| **kfu** | F: GAAGTGACGCTGTTTCTGGCR: TTTCGTGTGGCCAGTGACTC | 797 | F: 61<br>R: 62 | (25) |
| **mrkD** | F: GGCGTTGGCGCTCAGATAGG<br>R: AAGCTATCGCTGTACTTCCGGCA | 340 | F: 65.7<br>R: 63.8 | (26) |
| **iutA** | F: TTATTCGCCACCACGCTCTT<br>R: TTATTCGCCACCACGCTCTT | 920 | F: 63<br>R: 60.8 | (27) |

**RESULTS:**

The total 130 urolithiasis patients studied 84 were males and 46 were females. Their ages ranged from 20 years to ≥85 years. The maximum number of patients having urolithiasis was in the age group of (35 to 70) years. Among the 130 samples, 21 exhibited no growth while 109 isolates were positive cultures, of these, 82% were poly-microbial while 18% were mono microbial.overall 109 isolates were collected from 130 cultures. the 109 isolates was Gram negative and Gram positive. Gram negative bacteria k.pneumoniae. were the most prevalent as 25 (23%) isolates. e.coli 18(16%), Pseudomonas aeruginosa 15(14), Proteus mirabilis 5 (14%) while of Gram positive bacteria Staphylococcus aureus 13 (12%) Staphylococcus saprophyticus 10 (9%) Enterococcus faecalis 8 (7%) Acinetobacter baumannii 5 (5%) as mentioned in figure No. 1

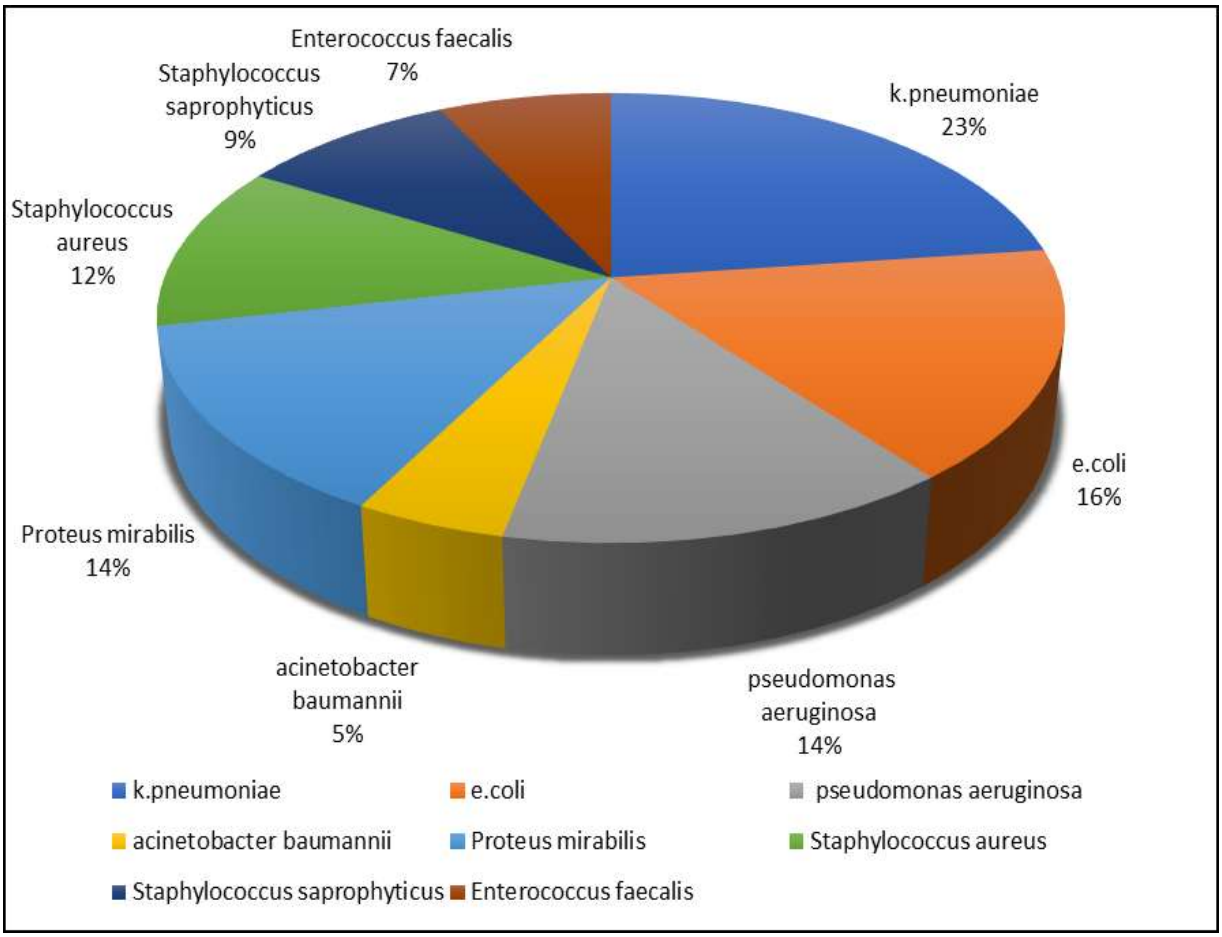


**Fig. No. 1** Distribution of bacterial isolates obtained from Urolithiasis

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**Antibiotics sensitivity**

The antimicrobial susceptibility of 25 Klebsiella pneumoniae isolates recovered from urolithiasis patients in Karbala was evaluated. Amoxicillin demonstrated sensitivity in only 30% of isolates, with resistance noted in 70%. Ampicillin produced similar results, with 32% sensitivity and 68% resistance. Third-generation cephalosporins, including cefixime, ceftriaxone, and cefotaxime, showed low sensitivity, ranging from 15% to 33%, while resistance rates were correspondingly high (67–85%). Among fluoroquinolones, ciprofloxacin exhibited the highest sensitivity at 48%, whereas norfloxacin, levofloxacin, and ofloxacin displayed moderate sensitivity between 35% and 41%. Aminoglycosides varied, as gentamicin showed only 19% sensitivity, tobramycin 36%, and amikacin 52%. Nitrofurantoin was effective in 42% of isolates. Trimethoprim achieved 48% sensitivity. Notably, carbapenems were the most effective: imipenem sensitivity reached 77%, while meropenem and ertapenem showed sensitivities of 49% and 37%, respectively.

**Genetic study:**

In this study, the PCR technique was used to detect the presence of the gene wabG and mrkD genes were detected in 100% of the Klebsiella pneumoniae isolates from urolithiasis patients in Karbala City, indicating their ability to formatin bioflim. Figure No. 2 shows the electrophoresis of the PCR products through which it is clear that the primer of the wabG gene was successful in amplifying this gene through the appearance of a PCR product of 683 bp in size and in figure No.3 show primer of mrkD gene was successful in amplifying this gene through the appearance of a PCR product of 340 bp in size. The kfu gene was found in 76% of the samples, suggesting its significant role in iron uptake and virulence Figure No.4 show primer kfu gene was successful in amplifying this gene through the appearance of a PCR product of 797bp in size. Both fimH and entB genes appeared in 64% of the isolates, reflecting their involvement in adhesion and siderophore production, respectively. Figure No.5 and No .6 shows Both fimH and entB genes were successfully amplified that has been indicated by the presence of a PCR product size of 688bp for fimH gene and 371bp for entB gene ., The ycf gene was identified in 60% of the cases the primer ycfM gene was successful in amplifying this gene through the appearance of a PCR product of 160 bp in size while the iutA gene showed the lowest detection rate at 56%, possibly indicating variability in integron-related genetic elements among the isolates in product size was 920 in Figure No.7 . These percentages highlight the prominent presence of key virulence genes in K. pneumoniae linked to urolithiasis.

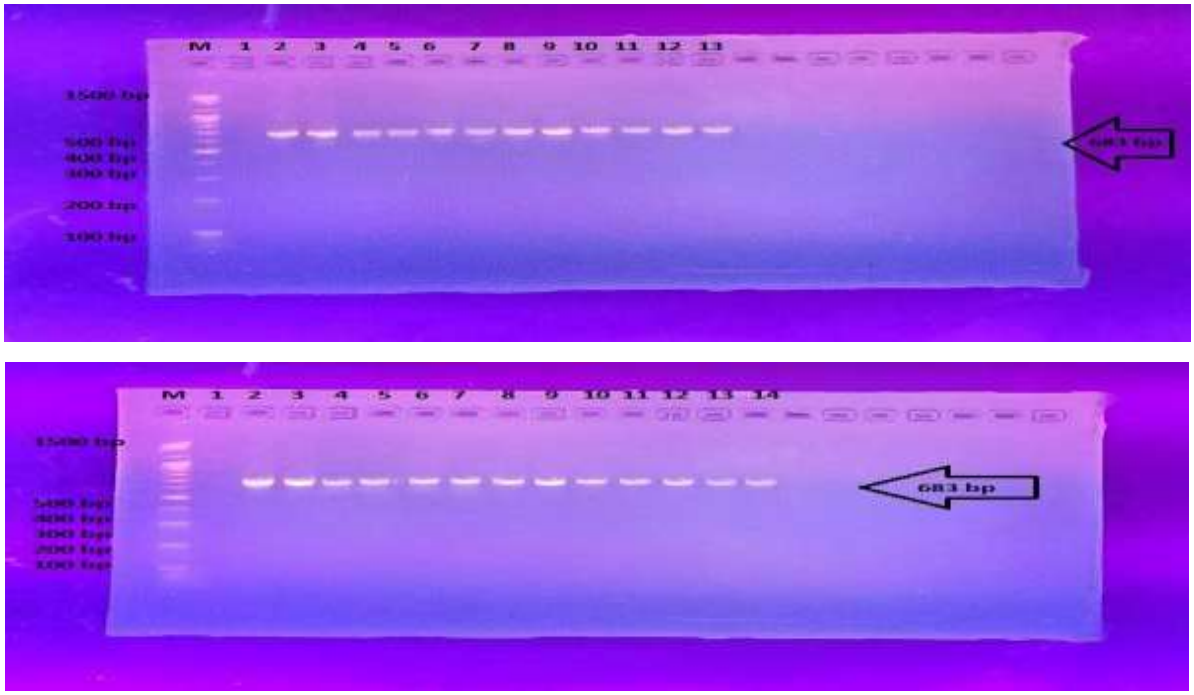


**Fig. No. 2**: Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *W*abG gene (683bp) using 1.5% Agarose gel , 70 voltages for 50 minutes

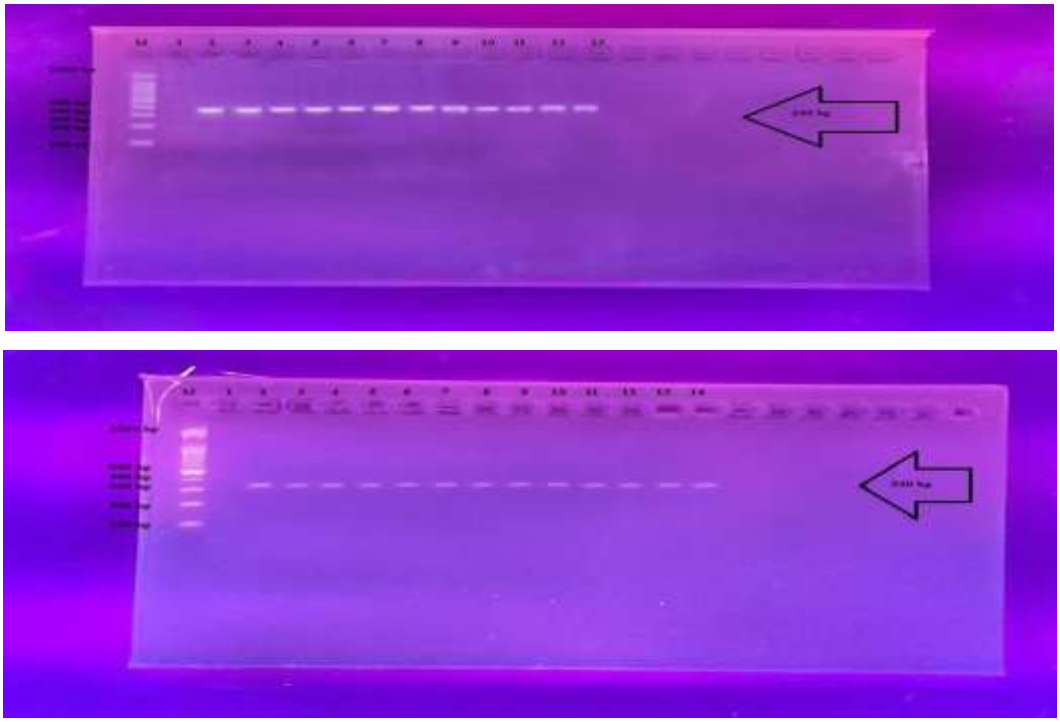

**Fig. No. 3:** Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *mrkD* gene (340bp) using 1.5% Agarose gel , 70 voltages for 65 minutes

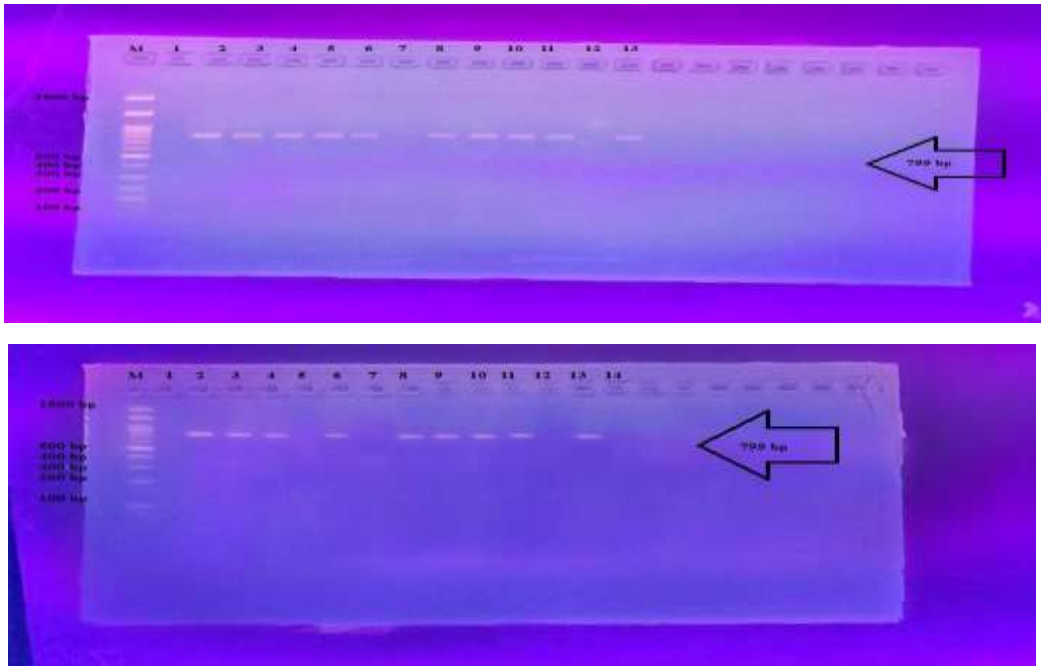

**Fig. No. 4**: Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *kfu* gene (799bp) using 1.5% Agarose gel , 70 voltages for 61 minutes

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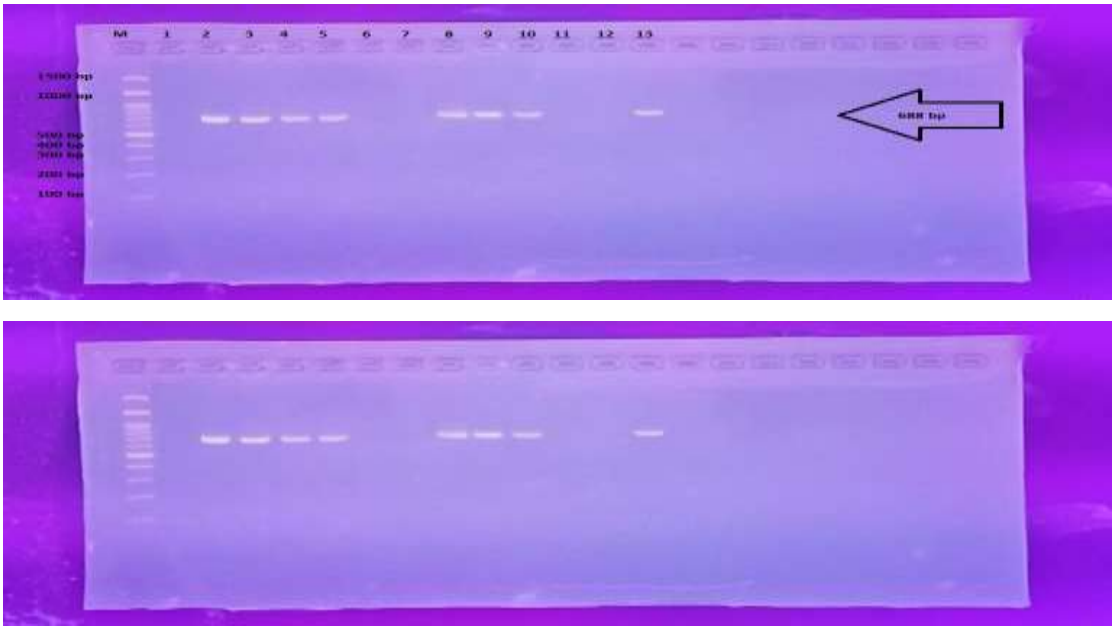


**Fig. No. 5**: Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *fimH*gene (688bp) using 1.5% Agarose gel , 70 voltages for 60 minutes

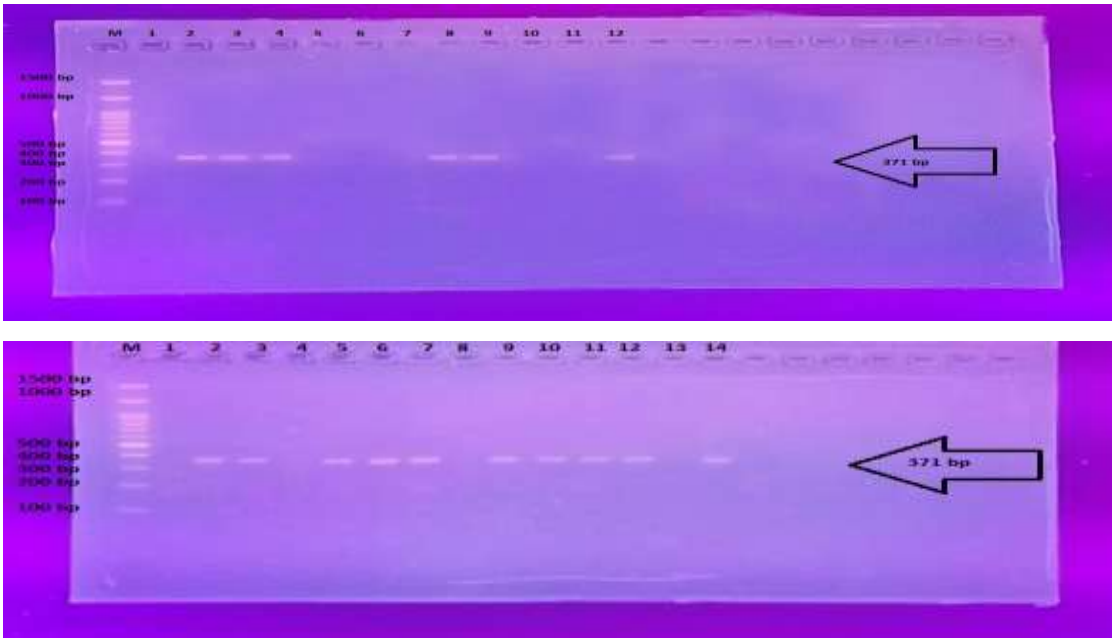


**Fig. No. 6**: Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *entB* gene (371bp) using 1.5% Agarose gel , 70 voltages for 60 minutes.

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**Fig. No. 7:** Electrophoresis of the PCR reaction product of *K. pneumoniae* using the specific primer of the *intA* gene (920bp) using 1.5% Agarose gel , 70 voltages for 60 minutes

**DISCUSSION:**

This study of 130 urolithiasis patients revealed a predominance of males (64.6%) over females, with the highest incidence observed in the 35 to 70 years age group. This demographic pattern aligns with current epidemiological data indicating a higher prevalence of kidney stones among middle-aged males, likely influenced by hormonal, metabolic, and lifestyle factors (28). Microbiological cultures were positive in 83.8% of samples with polymicrobial infections representing 82% of these positive cultures. This high rate of polymicrobial colonization reflects a complex urinary environment conducive to biofilm formation, which enhances bacterial survival, antibiotic resistance, and complicates infection management (29).

Gram-negative bacteria were predominant among the isolates, with Klebsiella pneumoniae being the most prevalent pathogen (23%), followed by Escherichia coli (16%), Pseudomonas aeruginosa (14%), and Proteus mirabilis (14%). This bacterial profile is consistent with prior studies from various regions highlighting the role of urease-producing Gram-negative bacteria in urolithiasis pathogenesis. These bacteria raise urinary pH through urease activity, facilitating the precipitation of struvite and apatite crystals that contribute to stone formation (30). Gram-positive bacteria such as Staphylococcus aureus (12%), Staphylococcus saprophyticus (9%), and Enterococcus faecalis (7%) were less frequent but remain important contributors to complicated urinary tract infections, particularly in patients with catheterization or compromised immunity (31). Acinetobacter baumannii was isolated in 5% of samples, highlighting the potential for nosocomial infections among urolithiasis patients, especially given this organism's well-documented multidrug resistance and association with hospital environments (32).

In current study,The resistance rates to beta-lactams were consistent with patterns reported in various regions, where resistance to ampicillin commonly exceeds 90% and resistance to third-generation cephalosporins surpasses 60% (33)(34). Fluoroquinolone resistance in our isolates (52–65%) corresponds with the 40–60% range reported among uropathogenic K. pneumoniae strains (35). Aminoglycoside resistance was similarly concerning, with gentamicin resistance seen in 81% of isolates, aligning with studies indicating gentamicin susceptibility in only approximately 60–70% of K. pneumoniae (36). Carbapenem resistance, though lower, remained significant: 23% resistance to imipenem and around 51–63% resistance to meropenem and ertapenem. This is higher than imipenem resistance rates of ~28% cited in recent ESBL-K. pneumoniae research (37) and matches the elevated carbapenem resistance (20–51%) observed globally among clinical K. pneumoniae isolates (38) (39).

In current study, The PCR results revealed the presence of the wabG and mrkD genes in 100% of Klebsiella pneumoniae isolates from urolithiasis patients in Karbala City, indicating a strong association of these genes with the pathogenesis of urinary

tract infections and stone formation. The wabG gene plays a crucial role in the biosynthesis of core lipopolysaccharides, which enhance bacterial adhesion and immune evasion (40). The mrkD gene encodes the type 3 fimbrial adhesin protein, facilitating bacterial adherence to epithelial surfaces and biofilm formation, key factors in urinary tract colonization and persistence (41).

The kfu gene was detected in 76% of isolates, highlighting its importance in iron acquisition, an essential process for bacterial survival under the iron-limited conditions of the urinary tract (42). Similarly, the fimH gene, present in 64% of isolates, encodes a type 1 fimbrial adhesin that mediates strong binding to host epithelial cells (43). The entB gene, also found in 64% of samples, is involved in siderophore production, allowing efficient iron scavenging which supports bacterial growth and virulence (44)

Genes ycf and intA were identified in 60% and 56% of isolates, respectively, reflecting genetic variability among clinical strains. This variability may influence bacterial virulence and antibiotic resistance profiles, particularly regarding integron-mediated gene transfer mechanisms (45). These findings emphasize the significant prevalence of key virulence genes in K. pneumoniae isolates associated with urolithiasis, underscoring their potential as targets for diagnostic and therapeutic strategies.

**CONCLUSION:**

This study shows a strong correlation between urolithiasis and K. pneumoniae infection, especially because of the high frequency of virulence genes that lead to antibiotic resistance and stone formation. Iron acquisition systems like kfu and entB are important, and the results emphasize the significance of wabG and mrkD as universal markers for biofilm-forming K. pneumoniae. The need for ongoing surveillance and the creation of alternate treatment approaches stems from the increasing antibiotic resistance among isolates, especially to widely used antibiotics. In addition, molecular profiling of virulence genes may provide new targets for diagnosis and treatment to improve the treatment of recurrent or complex cases of urolithiasis.

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