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RESEARCH ARTICLE

Bacteriological and Molecular Study of Some Urinary Tract Infections Bacteria in Human and Cows in Babylon Province

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ABSTRACT

Human and animal UTIs have increasingly become prevalent posing severe health risks to everyone infected. The purpose of this work was to identify bacterial UTIs in humans and cows, as well as to investigate antibiotic resistance of *Klebsiella pneumoniae* isolates concerning biofilm production. A total of 100 urine samples were collected: 50 from humans admitted to Al-Hashimiya and Al-Qasim General Hospital, Iraq, and 50 from cows in different areas of Babylon province in September- December 2021. Three genes were targeted: *WCAg, TRE-C,* and the housekeeping gene *rrsE*. Among 100 cases, 45 bacterial isolates were identified: *K. pneumoniae* (22 isolates, 48.88%), *Escherichia coli* (10 isolates, 22.22%), *Staphylococcus aureus* (7 isolates, 15.55%), and *Pseudomonas aeruginosa* (6 isolates, 13.33%). *Klebsiella pneumoniae* biofilm formation was determined using congo red agar and microtiter plate method, detecting that 2 exhibited strong biofilm forming ability, 8 moderate ability, 10 weak ability and 2 no biofilm forming ability. Relative expressions of *wcaG, treC, and rrsE* were validated by qPCR. AST indicated moderate susceptibility of the isolates to piperacillin-tazobactam (54.54%), cefepime (50%), cefotaxime (50%), cefoperazone-tazobactam (45.45%), ceftazidime (45.45%), and amoxicillin-clavulanic acid (40.4%). That is why in anamnestic data the sensitivity of other antibiotics such as amoxicillin, cefuroxime, cefoxitin, and ceftriaxone was below 40%. This increase has been associated with multidrug-resistant *K. pneumoniae*, through presented mutations stimulating biofilm formation – a factor difficult to treat.

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INTRODUCTION

With various important functions, the urinary tract (UT) is one of the vital organs in the animals' body; It effectively removes toxic waste from the body, regulates body fluids, and influences hormone production. UT infection (UTI) is defined by colonization and infections by one or more UT parts (Mohammed *et al.*, 2020), of which causes are mainly gastrointestinal tract-based bacteria; these infections lead to bacterial colonization and invasion of the external genitalia, bladder and urethra, leading to painfully damaging urine formation and flow. UTI also harms vasocirculation of urinary bladder, leading to kidneys damage and thus electrometabolic imbalance (Yazdi *et al.*, 2020). Bacteria adhere and persist, and multiply in the urinary tract lead to UTIs (Mohammed *et al.*, 2020).

Friedlander was first isolated it, in 1882 from lungs of died pneumonial patients (Kozlova *et al*., 2018), is ubiquitous in nature and environment, where they are found in surface water, soil, sewage, and on plant (Osińska *et al.*, 2016). Various virulence factors of *K. pneumoniae* enable them to invade the host; the *K. pneumoniae*'s virulence factors include capsular polysaccharide, lipopolysaccharide, siderophore production, serum resistance, fimbriae and production of urea and enterotoxin (Hanoon *et al*., 2023; Pu *et al*., 2024). As the predominant opportunistic uropathogens, *K. pneumonia* causes infections everywhere (i.e., from UT, liver and lungs, bones osteomyelitis (Chung *et al*., 2008;

Siu *et al.*, 2012), wound or post-surgery infection *E.coli*-related bacteraemia (Prokesch *et al.*, 2016; Virgilio *et al.*, 2016; Mahmood *et al*., 2020). *K. pneumoniae* is also considered one of the six most common nosocomial infectious pathogens worldwide (Lee *et al.*, 2017). Conventionally, *K. pneumoniae* is animated through several modes of transmission that is why the study of this important pathogen remains highly interesting and important (Yinnon *et al.*, 1996; Hamzah *et al.*, 2020).

Klebsiella pneumoniae's ability to form biofilms is well known. These biofilms, consist of clusters of bacteria embedded in an extracellular- matrix. This matrix is composed of proteins, polysaccharides, DNA, peptides, and fatty acids (Mirzaei and Ranjbar, 2022). Biofilm-producing microorganisms often exhibit- properties that are completely different from their free-floating (planktonic), counterparts of the same species, as demonstrated by various studies (Hutchins, 2018).

K. pneumoniae is predominantly a nosocomial pathogen; however, increasing attention has recently been paid to this bacterium because of the attempts to identify potential animal-borne transmission routes of the pathogen. It is still impossible for researchers to accurately identify harmful strains of *K. pneumoniae* (Davis *et al*., 2015). Genetic investigations may be useful to interrelate drug resistance and clinical *K. pneumoniae* population strains of human and bovine isolates in order to discover pathogenicity, virulence factors, and biofilm formation; expression of certain genes pathogenicity genes may be beneficial. Bacterial ear infections and UTIs that are often not treatable due to multidrug-resistant *K. pneumoniae* have gone up, and this organism is more pathogenic than other bacteria of the same species. (Mohammed and Mustafa, 2023). In this study, we evaluate the hypothesis that variations in *K. pneumoniae* virulence, nucleotide mutations, genetic characteristics associated with antibiotic resistance, and biofilm development could impact the way UTIs are treated (Sundaresan *et al*., 2024).

MATERIALS AND METHODS

Collection of urine samples: For this study, a urine specimen of 10mL was obtained from human and cows. The midstream human urine samples were collected using clean, screw- capped containers commercially available. These samples were obtained from patients attending Al-Hashimiya General Hospital, and AL-Qasim General Hospital, and were brought to the microbiological laboratory within 2 hours for bacterial analysis. For cow urine sample, massage method and repeated sterile catheterization were employed to obtain urine from cows in the Al-Qasim area. They collected transport samples from different passengers in sterile containers during the period from December 2021 to May 2022.

Antibiotic sensitivity testing: The ability of the isolates to respond to the antibacterial agents was established using the Kirby-Bauer disk diffusion technique as described by Watts, Mc Dougall and Carson (2008). The procedure included several steps:

1-Preparation of bacterial inoculum: A new culture of the bacteria was prepared by inoculating fresh selective media and incubating the cultures overnight. These cultures were standardized to 0.5 McFarland to add a similar amount of bacteria for the experiment.

2- Inoculation of agar plates: Mueller Hinton agar plates were prepared and from the standardized bacterial suspension a loopful was evenly spread over the Muellller hinton agar using a sterile cotton swab. The data generated placed the bacteria through different phases of growth, which was important for standardizing what was on every plate as a bacterial colony.

3- Placement of antibiotic disks: The antibiotic disks were equally spread on the surface of the agar by using sterile forceps and the disks did not have to touch each other and there was no gap between the two disks at least the size of a disc

4- Incubation: The plates were then incubated at 35-37°C for about 18-24 hours in other to have bacterial growth as well as bonding on the antibiotics.

5- Measurement of zones of inhibition: After the incubation period, the diameter of the inhibitory zones (the diameter of bacterial growth inhibition) was determined with a ruler or caliper, as described below.

6- Interpretation of results: antibiotics employed: The diameters of the inhibition zones were compared to standard provided by the manufacturer to give the bacteria strains tested the following classification; Sensitive, Intermediate or Resistant.

PCR for *Klebsiella pneumonia:* The obtained colonies were identified with *Klebsiella pneumoniae*, using primers that are characteristic for this microbe.

The forward primer (KP-27F3: Specific primers, KP-27F2 forward primer (5'-GGATATCTGACCAGTCGG-3') and KP-27R3 reverse primer (5'-GGGTTTTGCGTAATGATCTG-3') were use and a fragment of 176bp with GenBank date base CP028478.1 was amplified, in addition to the confirmation of previous studies. Dong *et al*. (2015).

For biofilm-related gene expression analysis, primers targeting *treC* were used: 4,5 were amplified with forward treC-F and reverse treC-R; treC-F (5'-GGGGCGTAAA TGGTAGC-3') treC-R (5'-GGACTTAAACTTCTGGG TGGT-3') that amplified a fragment of 343bp.

PCR Protocols: In this performed PCR reaction, the first stand was dried at 94°C for 5 minutes while 35 cycles were set for 94°C for 30sec, then the annealing was at 56°C for 30sec and the extension at 72°C for 1 minute. Another extension step was performed at 472°C for 5min. The cycling conditions were as follows: Semiquantitative PCR was performed on the Applied Biosystems® 7500 Fast System and for recombination PCR GoTaq® 1-Step RT-qPCR system, reverse transcription was done at 42°C for 15 minutes and the sample was denatured at 95°C for 10 minutes, PCR condition was 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C

The Biofilm Formation Assessment by using Congo Red Agar Method Congo Red Agar (CRA) was utilized to test biofilm formation. The medium was prepared with the following components:

- Sucrose: 50 g/L, Brain Heart Infusion (BHI) Broth: 37 g/L, Congo Red Indicator: 8%
- And Agar: 20 g/L. The medium was then subjected to autoclavation at 15 minutes at a temperature of 121°C and then allowed to cool to the room temperature before being aliquoted into sterile petri dishes. These plates were inoculated with bacterial samples and were grown at 37°C for 24–48 hrs. Colony morphology was used to assess biofilm production:
- Strong biofilm producers: Black, dry and hard colonies, in appearance.
- Moderate biofilm producers: It concluded that there are dark colonies without crystallinity.
- Weak or non-biofilm producers: Smooth pinkish colonies.

Biofilm assay using microtiter plate method: A standard biofilm formation assay was performed employing microtiter plate technique, which was adopted as described by Aniba *et al*. (2024). Overnight cultures of new bacterial colonies were grown in Luria-Bertani broth plus 0.5% d-glucose at 37ºC. The bacterial suspension density was standardized to 10^ 8 colony forming units /mL (approximately 0.5 McFarland standard) and 200µL of this suspension was then aliquoted into each well of a 96 well flat bottom polystyrene plate. The plates were allowed to form biofilm at 37°C for 24h and biofilm formation was measured in terms of adherence to the polymethylglycine well surface.

RESULTS

Molecular identification of *Klebsiella pneumoniae* was done by PCR Techniques/Regular PCR Protocol. The molecular identification of isolates utilized the specific capsule polysaccharide gene of *K pneumoniae*; *rcsA* regulates capsule polysaccharide synthesis. Positive controls used in the study were reference strains. First, all the isolates were biochemically characterized and identified as Klebsiella spp. PCR with the Klebsiella-specific primers detected biochemical reactions of all Klebsiella spp. isolates from humans and cows, and 22 out of 30 isolates reacted with bands characteristic of the reference strain of *K. pneumoniae* in the pattern of the gel electrophoresis PCR products. There were no bands recorded in the negative control samples. (Fig. 1). These results pointed to a high level of concordance between the biochemical and molecular method of identification of *K. pneumoniae*.

For amplification of *rcsA* gene, the primer pair used for amplification was *rcsA-F* and *rcsA-R*, and therefore these isolates were categorized under K2 serotype of *K. pneumoniae*. Finally, all the 22 samples produced PCR products of 176bp which suggest that they are all positive for *rcsA* gene, as seen in Fig. 1. The *rcsA* gene determines the mucoid phenotype through the colanic acid synthesis; low temperatures (30°C) are not necessary to trigger colanic synthesis.

There were also HvKP strains which exhibited hypermucoviscosity feature due to the mucoid colony phenotype. A major factor arising from this study is the high rate of polysaccharide production in these strains which is useful in explaining UTI infections. Biofilm production in KD-PAK-3 is proven by colony morphology where *K. pneumoniae* biofilm-producing colonies have distinct morphological features. BV and RV were first separated by their morphology and color on the base of differential media characteristics. Black colonies were labelled as biofilm producers whereas red colonies were labelled as non biofilm producers. (Kaiser *et al*., 2013) (Fig. 2). The Congo Red test outcomes represented in Fig. 2 depicted differences in the adhesion of polysaccharide in distinct groups of bacteria; a large number of XDR strains being robust producers of exopolysaccharides.

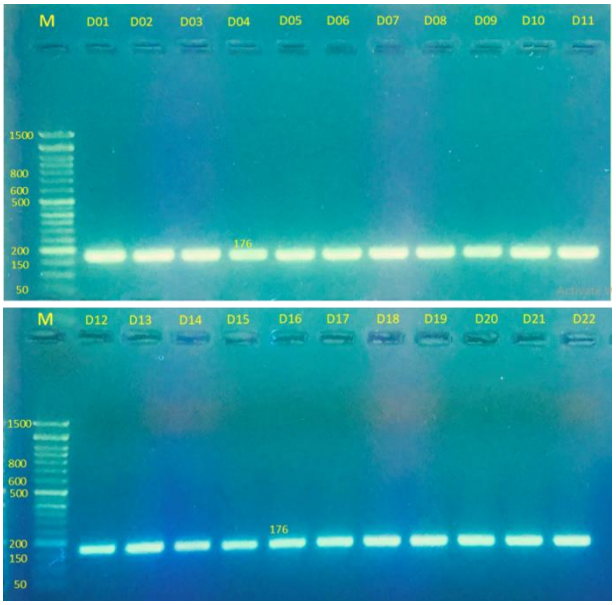

Fig. 1: Polymerase chain reaction (PCR) on areas of interest 1-22 amplified a fragment of the *Klebsiella pneumoniae* partial rcsA gene of 176bp. It was prepared as 1.5% agarose gel with 3µL RedSafe (Intron, Korea) staining solution. The gel was subjected to electrophoresis at a voltage of 90V for 45min employing a DNA Ladder as the molecular weight control.

Fig. 2: Biofilm-forming ability of *Klebsiella pneumoniae* isolates to test this ability, biofilm-forming isolates have been streaked onto Congo red agar plates and incubated for 24h at 37°C and 5% CO2.

Based on these results, the ability of bacteria to form biofilm was tested by Microtiter Plate Method (MTP) as shown in Table 2, we observed biofilm production abilities categorized as follows:
Strong producers (>0.400): 2 isolates
Moderate producers (0.2-0.4): 8 isolates
Weak producers (<0.2 to ≥0.12): 10 isolates
Little producers (<0.11): 2 isolates

Table 1: Antibiotic sensitivity test of *Klebsiella pneumonia*: Antibiotic sensitivity test profile of *Klebsiella pneumoniae* isolated from urinary tract infections (UTIs) in human and cow. Potency (mg/disc) for example in 25/10mg means concentration of antibiotics and their adjuvants

Antibiotic Agent	Symbol	Potency (mg/disc)	Number of Sensitive Isolates (n=22)	Sensitivity Rate (%)
Amoxicillin	AMX	25	4	18.18
Amoxicillin-Clavulanic Acid	AMC	25/10	9	40.40
Piperacillin-Tazobactam	TZP	75/10	12	54.54
Cefoperazone-Tazobactam	C/T	4/500	10	45.45
Cefuroxime	CXM	500	8	36.36
Cefotaxime	CTX	500	11	50.00
Cefoxitin	CFx	10	6	27.27
Ceftazidime	CAZ	30	10	45.45
Ceftriaxone	CRO	30	2	9.09
Cefepime	CEF	30	11	50.00

Table 2 contains a sequence of primers list for PCR and thermocycling conditions used in this study.

Table 2: Biofilm formation of *Klebsiella* isolates by using Microtiter plate method

Property	Non-biofilm producer (<0.12)	Biofilm producer 20 (90.9%)		
		Weak producer (<0.2 ≥0.12)	Moderate producer (0.2-0.4)	Strong producer (>0.400)
Number of strains	2 (9.09%)	10 (50%)	8 (40%)	2 (10%)

Real-time PCR analysis was conducted on the *wcaG*, *treC*, and *rrsE* genes to assess their relation to the biofilm-forming ability of *K. pneumoniae*. To compare the gene expression profiles responsive to biofilm formation of *K. pneumoniae* from the UT isolates in both human and cows, we tested the *wcaG* and *treC* genes and the housekeeping gene *rrsE* (Fig. 4). The CT values of these genes examined in the microtiter plate assay were relatively close to the values obtained in our study (Table 3 and Fig. 3) and could be classified into heavily biofilm-forming, moderately biofilm-forming and weakly biofilm-forming isolates.

Table 3. Threshold Cycle values (CT, which is directly related to the fold change of the studied genes) of the genes (calculated automatically by the Mx3005P Stratagene system).

Sample	Gene	CT	Gene	CT	Gene	CT
KA1	*wcaG*	27.33	*treC*	22.56	*rrsE*	18.28
KA1	*wcaG*	27.55	*treC*	22.24	*rrsE*	17.69
KH1	*wcaG*	No Ct	*treC*	No Ct	*rrsE*	No Ct
KH1	*wcaG*	27.13	*treC*	No Ct	*rrsE*	No Ct
KA10	*wcaG*	23.69	*treC*	20.58	*rrsE*	17.44
KA10	*wcaG*	23.35	*treC*	20.77	*rrsE*	17.25
KH7	*wcaG*	27.09	*treC*	21.9	*rrsE*	17.6
KH7	*wcaG*	26.66	*treC*	21.62	*rrsE*	17
KA15	*wcaG*	20.74	*treC*	22.14	*rrsE*	19.86
KA15	*wcaG*	21.58	*treC*	22.21	*rrsE*	19.61
KH2	*wcaG*	27.16	*treC*	22.07	*rrsE*	18.87
KH2	*wcaG*	27.18	*treC*	22.03	*rrsE*	18.82
KA4	*wcaG*	20.68	*treC*	24.96	*rrsE*	21.01
KA4	*wcaG*	20.45	*treC*	24.04	*rrsE*	21.21

Antibiotic susceptibility: The antibiotic susceptibility test indicated that the tested antibiotics have different inhibition zones around the photometric scale. The sizes of the inhibition zones were further classified according to CLSI standards as either sensitive, intermediate, or resistant as demonstrated from Table 1. This data indicates that there is need to regularly assess antibiotic resistance profiles of *K. pneumoniae* isolates.

DISCUSSION

Molecular methods are fast and sensitive methods for identifying organisms from clinical specimens. By the product involved in the formation of the K antigen capsule, *Klebsiella pneumoniae's* key gene is *rcsA* (Dong *et al*., 2015; Hamzah & Hasso, 2019; Mosa *et al*., 2022). Of all the Klebsiella strains, only *K. pneumoniae* is mostly pathogenic in nature. In addition, researchers have categorized them based on different characteristics as follows. Because *rcsA* is important for clinical application, this gene was chosen for molecular detection in this study (Dong *et al*., 2015).

The results are consistent with other studies showing that *K. pneumoniae* is a highly biofilmogenic species. Our results showed that 20 out of 22 isolates (90.9 %) were biofilm producers as it was also found by Hassan *et al*. (2011) and Cepas *et al*. (2019). Other works indicate that biofilm formation in *K. pneumoniae* may be as high as 93.6% (Seifi *et al*., 2016), and only 30% of which were characterized as high producers. Biofilm development potential varies with temperature, pH, and the surface on which it is forming (Biofilm Formation review by Cherif-Antar *et al*., 2016).

Biofilm forming ability of all the 22 Klebsiella isolates recovered were determined using microtiter plate based method as described by other workers (Makhrmash *et al*., 2022; Kot *et al*., 2023). It is worth noticing that overproduction of biofilm is well correlated with antibiotic resistance in *K. pneumoniae* (Vuotto *et al*., 2017). Among 137 isolates, 85% of biofilm producers were defined as ESBL producers (Yang & Zhang, 2008). This is in contrast to Subramanian *et al*. (2012) wherein the authors identified that samples were 73.3% resistant to cefotaxime and 83.3% against ampicillin. This should suggest that the degree of antibiotic resistance and biofilm formation in *K. pneumoniae* deserves further study.

Many researches demonstrated that genes of *K. pneumoniae* biofilm are linked with pneumonia and urinary tract infections (Jagnow & Clegg, 2003; Balestrino *et al*., 2008; Rosen *et al*., 2008). In this present study we selected two genes to check their effect on biofilm forming ability and presence of MDR genes. The *wcaG* genotype is associated with some capsular serotypes of *Cryptococcus neoformans* and *Cryptococcus gattii* (K1, K5, K58, K16). The *wcaG* gene encodes production of a polysaccharide capsule which grants a higher virulence to *K. pneumoniae* (Wu *et al*., 2008). When *treC* gene is not functional, then glucuronic acid which is required for biofilm formation in serotype K1 is not produced (Zamze *et al*., 2002; Wu *et al*., 2011). Our results pointed out that there is a strong relationship between isolated virulence genes and biofilm formation and antibiotic resistance as demonstrated by Ho *et al*. (2011). The given work also showed that variations in

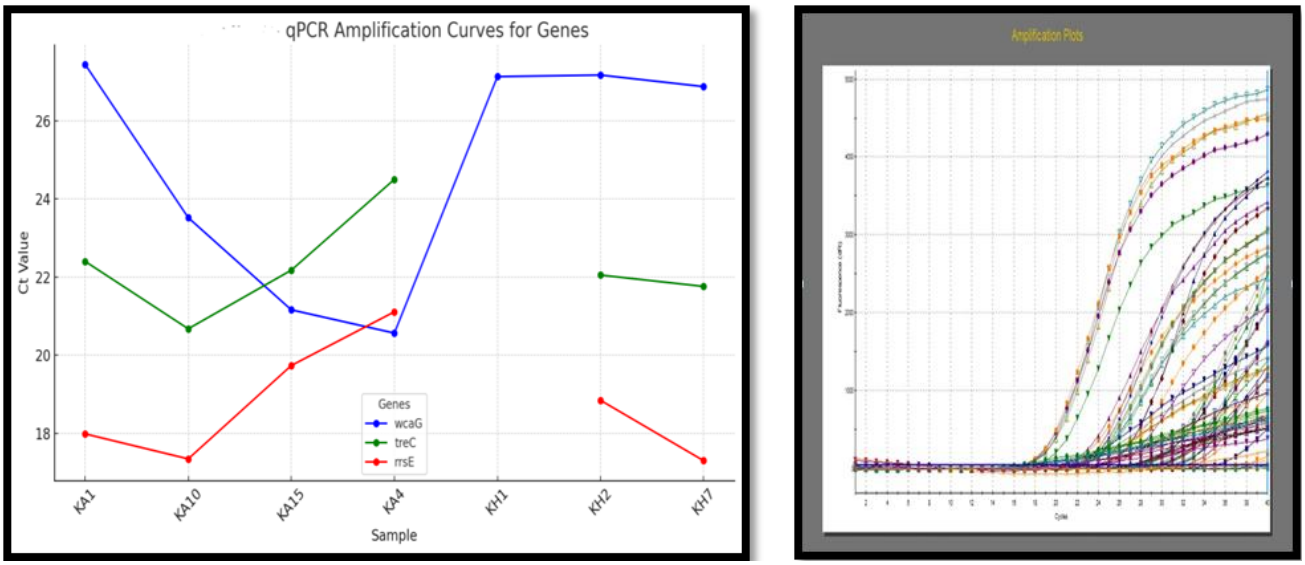

Fig. 3: Example of amplification plot of three genes, *wcaG*, *treC* and *rrsE*. and qPCR Amplification curve for genes.

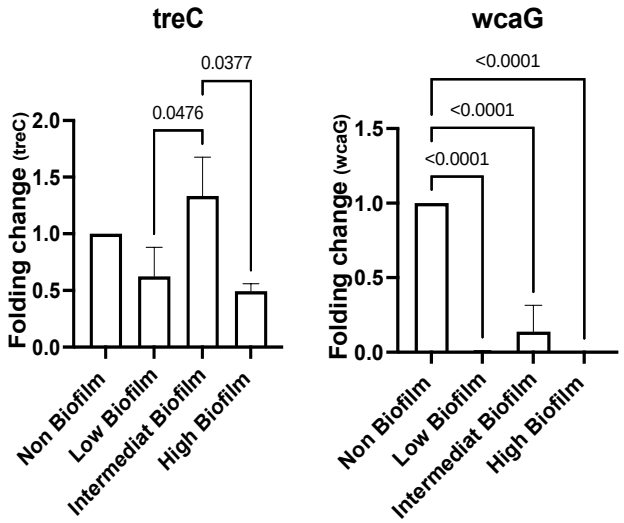

Fig. 4: Statistics of folding change of *treC* and *wcaG* genes using GRAPH PAD PRISM 9 software, calculated by $2^{-\Delta\Delta CT}$ method to identify genes that are differentially expressed relative to a fold change threshold.
The aG genes were analyzed using GraphPad Prism 9 software, calculated by $2^{-\Delta\Delta CT}$ method to identify genes that are differentially expressed relative to a fold change threshold.

wcaG might affect the structure of the polysaccharide capsule, promoting biofilm formation. Our findings were further established by noting that 80% of the *K. pneumoniae* isolates formed biofilms on the surfaces in agreement with other reported studies. Earlier, there is fairly substantial evidence to believe that biofilm-forming abilities are more pronounced among more virulent strains. (Zheng *et al*., 2018; Shadkam *et al*., 2021).

The influence of gene expression on biofilm formation shows that biofilm formation is indeed linked to the presence of antimicrobial resistance genes. Biofilm-forming capability was significantly enhanced in the isolates containing *wcaG* and *treC* resistance genes supporting earlier findings in the context of multidrug resistant Enterobacteriaceae (Corehtash *et al*., 2015; Atiyah, *et al.*, 2019). These resistance genes increase in conjunction with the observed level of biofilm formation, indicating that plasmid associated resistance genes may be involved in biofilm formation (Maeyama *et al*., 2004; Al-Bayati & Samarasinghe, 2022).

Hence, prospective prevention and treatment targets in biofilm formation of *K. pneumoniae*, besides their part in neutralizing the effects of antimicrobial therapy, should be examined. (Ashwath *et al*., 2022). It also expresses considerable genes including *treC*, *wabG* and *wcaG* to endeavor infections by attachment to epithelial and endothelial layers of the urinary and respiratory tracts (Stahlhut *et al*., 2012; El Fertas-Aissani *et al*., 2013).

Conclusions: The relatively high level of antibiotic resistance and increased virulence profile observed in *Klebsiella pneumoniae* UTI isolates in the study area from this investigation appear to be associated with the commensurate expression of biofilm formation genes including *wcaG*, *treC*, and *rrsE*. These discoveries present a rather unfavorable picture for managing UTI due to *K. pneumoniae* and, thereby, the need to develop standard antibiotic treatments in humans as well as animals. Nevertheless, more investigations are needed to understand more about the interaction between biofilm formation and antibiotic resistance in the context of *K. pneumoniae* related UTI and their outcomes.

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Authors contribution: Dhay: Research concept and design; Abdul-Kareem assembly data; Karrar data analysis, interpretation, writing the article and final approval of the article and Mehrzad critical revision.

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