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ORIGINAL ARTICLE

Evaluation of Efflux Pump activity and Biofilm Formation in Multidrug Resistant *Klebsiella pneumoniae*

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Efflux Pump, Klebsiella pneumoniae, VITEK-2

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ABSTRACT

Background: *Klebsiella pneumoniae a Gram-negative bacterium in the Enterobacteriaceae family, His non-motile, non-spore-forming, rod-shaped bacterium is characterized by its mucoid appearance on agars. Possessing a significant extracellular polysaccharide capsule that serves as a defense against host immunity K. pneumoniae is predominant nosocomial pathogen recognized for its exceptional capability to transfer resistance to multiple classes of antibiotics, which presents substantial menace to worldwide healthcare.* ***Objective:*** *This research examined the prevalence and potential interplay of biofilm formation and the OqxAB Efflux pump's role in facilitating antibiotic resistance in K. pneumoniae isolates.* ***Methodology:*** *143 clinical sample collected from the patients attending the Baghdad Medical City Hospital, during the period from September 2024 to January 2025, specimens were cultured on MacConkey's agar. Definitive identification test of the isolates was performed by VITEK-2 machine. Phenotypic detection of Biofilm generation was accomplished by the microtiter plate technique. And the activity of the efflux pump was evaluated phenotypically using the ethidium bromide cartwheel method in different concentration. To detection the OqxAB genetically, Polymerase Chain Reactin was performed.* ***Results:*** *The final identification results showed that 31 isolates were confirmed as K. pneumonia which showed high resistant (100%) to Ampicillin, piperacillin/tazobactam, Imipenem, amikacin, ceftazidime (96.77%) to cefazolin , cefoxitin ceftriaxone, Cefepime (93.55%) to Gentamicin (87.10%) to Ciprofloxacin, Levofloxacin, Trimethoprim\Sulfamethoxazol and (64.52%) to Nitrofurantion, results of Biofilm formation showed strong producers in 17(54.8%) of samples, moderate producers in 12(38.7%) of samples , weak producers in 1(3.2%) sample and Non producers in 1(3.2%) sample . results of OqxAB efflux pump activity(phenotype) revealed that the number of positive isolates at (1) mg/l concentration was 24 (77.42%), at (2)mg/l concentration was 23(74.19%), in 3(mg\l) concentration 16(50.61%). To detection the OqxAB genetically, Polymerase Chain Reactin was performed. Data showed that 29 (93.55%) of isolates harbored these genes, and only 2 isolates (6.45%) were OqxAB negative.* ***Conclusion:*** *Results revealed a high prevalence of both phenotypes, indicating that biofilm formation and OqxAB efflux pump activity are likely significant contributors to multidrug resistance in this bacterial species.These findings highlight the crucial need to consider both mechanisms when developing effective treatments for MDR K. pneumoniae infections.*

INTRODUCTION

Klebsiella pneumoniae was first identified in 1882 by Carl Friedlaender, initially termed Friedlander's bacillus, before being classified under the new genus Klebsiella[1]. *K. pneumoniae* is a member of the Gram-negative Enterobacteriaceae family. It is a rod-shaped, non-motile, non-spore-forming bacteria characterized by a unique mucoid look on agar plates. A distinguishing characteristic of K. pneumoniae is its enclosed structure The bacteria generates a prominent extracellular polysaccharide capsule that envelops the cellular structure and functions as a barrier against host natural defenses.[2]

Klebsiella pneumoniae is a leading nosocomial pathogen well known for its exemplary ability to transfer resistance to multiple classes of antibiotics, which presents a significant threat to global health care [3] Emergence of multidrug-resistant K. pneumoniae strains are threatening therapeutic maneuvers and increasing morbidity and mortality[4]. In addition to classical resistance determinants such as antibiotic-modifying enzymes and target site mutations, efflux pumps and biofilm formation have emerged as important elements of the complex interplay of mechanisms conferring resistance against antimicrobial agents in this bacterium host[5]. Efflux pumps, in particular, RND (Resistance-Nodulation-Division) pumps, aggressively expel many antibiotics from the cell, hence diminishing the intracellular concentration of medicines and resulting in resistance. The outer membrane efflux pump OqxAB of *K.pneumoniae* and its role in quinolone resistance has

been well studied with the aim of identifying the function for conferral of resistance to fluoroquinolone, tigecycline and other clinically therapeutic agents [6].

The overexpression of OqxAB can significantly reduce susceptibility to several antimicrobial drugs, resulting in a multidrug-resistant phenotype. Concurrently, biofilm development acts as a significant virulence factor and a primary contributor to antibiotic resistance in K. pneumoniae. Biofilms are organized assemblages of bacterial cells enveloped in a self-synthesized extracellular polymeric material (EPS). Forming a physical barrier that obstructs the infiltration of antibiotics and providing a conducive environment for the evolution of resistance [7].

Bacterial biofilms are more resistant to antibiotics and often require orders of magnitude more drug to be effective (vs. planktonic bacteria) [8].

The connection between efflux pumps and biofilm formation in the emergence of antibiotic resistance in multidrug resistant *K. pneumoniae* accepted as a key field in research. Biofilm production may be promoted through efflux pumps. Neurotrophic factors are positive Regulators enhanced the expression and activity of efflux pumps, further augmenting their expression on the membrane. Active agents that inhibit bacterial efflux pumps may influence bacterial resistance in vivo[9]. Comprehending the complex interplay between the OqxAB efflux pump and biofilm development in multidrug-resistant K. pneumoniae are essential for developing novel therapeutic strategies that target resistance mechanisms, ultimately improving treatment efficacy for infections caused by these resilient bacteria. This study aims to examine the role and potential synergy of the OqxAB efflux pump and biofilm formation in several multidrug-resistant K. pneumoniae strains.

METHODOLOGY

Patients and clinical specimens collection

The collected 143 clinical sample from Educational Laboratories and Surgical Hospital in Medical City, Baghdad from (September 2024 to January 2025), including Collection of 41 urine, 25 wound swabs, 7 sputum and 70 blood samples. There were both male and female of varying ages up to 60 years. The clinical sample collection and processing were conducted as standard direction.

Identification of K.pneumoniae Isolates

Each specimen was cultivated on MacConkey and blood agars ,incubated aerobically for 24 hours at 37°C [10]. The primary means of identifying bacterial isolates were their color, and morphologic traits prior to biochemical identification techniques[11]. Definitive identification test of the isolates was performed by VITEK-2 machine. The last identification results showed that 31 isolates were confirmed as *K. pneumoniae*.

Efflux pump detection (Ethidium Bromide-Agar Cartwheel Method)

This technique tests the ability of bacterial isolate to pump out ethidium bromide (EtBr), through their efflux pumps.Bacterial isolates were cultivated in broth to an optical density (OD) of 0.6 at 600 nm. The cultures were subsequently suspended in PBS at a 0.5 McFarland standard (approximately 1.5×10^8 CFU). On the day of experiment, Trypticase Soy Agar plates were developed with the integration of three separate concentrations of ethidium bromide (EtBr) i.e. 1, 2, and 3 mg/litre. The gels were placed in the dark due to the sensitivity of EtBr to light. Plates were divided into as many as 8 radial segments in a "cartwheel" configuration under different strains. Swab primary bacterial isolates from the center to the out perimeter of the marked zones. Plates were incubated for 16 hours at 37 degrees Celsius. The outcome was ascertained using field fluorescence UV light (UV transilluminator) [12]. Phenotypic recognition of efflux pump functioning Efficiency of an efflux pump in pushing the efflux of a fluoroquinolone (FQ) can readily be deter-mined through assaysof fluorometricactivity.EtBr crosses the cell wall (through the porins of Gram–negative bacteria) and accumulates, giving rise to fluorescence when observed under UV light. Bacteria use efflux pumps to sense and expel this substrate from the cell. These are pumps that are temperature sensitive, and they pump as long as the external EtBr concentration does not exceed their capacity. This permits to follow fluorescence emission as a function of controlled condition, time, and temperature.[5]

Biofilm Formation Assay (Microtiter plate's method)

The bacterial isolates were initially cultured in Brain Heart Infusion Broth (BHI) medium containing 0.5% glucose at 37°C for 18 hours. A specified volume (200 µL) of the bacterial suspension is subsequently introduced into the wells of a microtiter plate containing identical media and incubated at 37 °C for 48 hours. Following incubation, non-adherent cells and medium were meticulously eliminated by washing the wells twice with sterile PBS. The biofilm is then fixed by incubation with methanol (200 µL/well) for 15 minutes. This process helps to preserve the structure of the biofilm. The static biofilm is stained with 1% crystal violet solution (200 µL/well) for 5 min. Excess stain was removed by washing with de-ionized (distilled) water and plates were left to air-dry. The crystal violet retained by the biofilm was then dissolved in 96% ethanol. The purple was quantified at OD at 492 nm using a microplate reader for the ethanol solution. A high-absorbance value is highly correlated with a significant amount of biofilm, whereas a low-absorbance value is less-correlated with a small amount of biofilm formation [13] The experiment was performed

in triplicate per strain and the absorbance value was the mean. The average absorbance for the biofilm formation is stratified in Table (1) Values of absorbance ≥0.12 were regarded as biofilm positive.

Table (1): Classification of *K.pneumoniae* adherence by microtiter plate method

OD absorbance value	Biofilm formation
OD < 0.12	Non
OD <0.2	Weak producers
OD 0.2- 0.4	Moderate producers
OD > 0.4	Strong producers

The PCR technique steps:

DNA Extraction of K. pneumoniae isolates

Three to five isolated pure and fresh transplant colonies of MacConkey plate were suspending in Eppendorf tube with 1 mL of sterilized distilled water. Afterwards, the cells were heated (at 100 °C in a water bath for 20 minutes) to get rid of the DNA free of other organelles. The homogenates were then immediately stored after being kept on ice for ~30 min, and the remaining cellular organelles were collecte via centrifugation at 8000 revolutions per minute for 10 minutes. The supernatant was subsequently utilized as a DNA template[14].

PCR analysis cycle of Efflux Pump genes

The genetic identification of the oqxAB efflux pump was conducted utilizing the oqxAB primer sequence, listed in Table 2 through Polymerase Chain Reaction. The PCR utilized a total volume of 20 µl, comprising 10 µl of EeasyTaq PCR Super Mix (China), 2 µl of DNA, 1 µl of each primer sequence, and 6 µl of nuclease-free water.

Table 2: The oligonucleotide primer sequences used in PCR amplification

Primer Name	Primer Sequence F	Primer Sequence R	Tm.	Product size (bp)	References
OQXA	GGTGCTGTTCACGATAGATG	GAGACGAGGTTGGTATGGAC	55	144	**(**Dai & Hu,2022)
OQXB	CGGCCAGTTCTACAAACAGT	GGTAGGGAGGTCTTTCTTCG	61	136	**(**Alharbi *et al*,2023)

Polymerase chain reaction (PCR) program

The conventional PCR commenced with an initial denaturation phase at 94°C for 5 minutes one cycle, followed by 40 cycles consisting of denaturation at 94°C, annealing at 52–61°C for 20 seconds, and extension at 72°C for 20 seconds. The amplification ceases with the final extension for 10 minutes at 72 °C. A 2% agarose gel containing ethidium bromide dye was employed to detect the PCR results, and UV light was utilized to highlight the gel.

Statistical analysis

The statistical analysis was performed utilizing SPSS version 26. Categorical data was collected to clarify the relationship between the variables; when applicable, percentages were calculated, and chi-square tests were utilized. A P-value below 0.05 was considered statistically significant.

RESULTS

Isolation and identification of bacterial Isolates

The results showed that the isolates were negative for the oxidase test[15]. And positive for catalase test were included to next identification step [16]. Definitive identification test of the isolates was performed by VITEK-2 machine. The last identification results showed that 31 isolates were confirmed as *K. pneumoniae*.and showed high resistant (100%) to Ampicillin, piperacillin/tazobactam, Imipenem, amikacin, ceftazidime (96.77%) to cefazolin , cefoxitin ceftriaxone, Cefepime (93.55%) to Gentamicin (87.10%) to Ciprofloxacin, Levofloxacin, Trimethoprim\Sulfamethoxazol and (64.52%) to Nitrofurantion .

Table 3: The Bacterial Isolates Distribution According to Sample type

Sample type	No. of sample	No. of *K.pneumoniae* Isolate	Percentage (%) of *K.pneumoniae* Isolate	Chi-Square	P-Value
Blood	70	18	25.7 %	16.139	0.001
Urine	41	7	17 %		
Wound swab	25	1	4 %		
Sputum	7	5	71.4 %		
Total	143	31	21.67 %	32.081	<0.0001

Table 4: Profile of Patients Included in the Study

Patient profile	Status	No. of samples	*No .of K.Pneumoniae* isolates(n =31)	Chi-Square	P-Value
Age groups (years)	1-20 yrs	14	3	5.113	0.0037
	21-44 yrs	71	11		
	>45 yrs	58	17		
Gender	Male	79	17	3.904	0.0020
	Female	64	14		

The age of the Study subjects was between less than one year to 60 years. Table 4. The age group greater than 45 years had the highest sum total of *K. pneumoniae* isolates 17 (54.83%) although the sample size is less compared to the second age group that is 21-44 years (11 isolates (35.48%). Generation of isolates the category with the least number of isolates in the youngest age group (1-20) years, 3(9.67%)

Biofilm formation detection

Quantitative detection of Biofilm production was executed utilizing the microtiter plate method. As shown in figure (1) the deeper purple coloration indicates strong biofilm formation, while lighter or nearly clear wells indicate weaker or no biofilm. Moreover left plate exhibits that most wells are deeply stained and this indicates a high degree of biofilm production, reflecting the strong biofilm-formation ability of the isolates.

The micro titer plate method characterized by measuring biofilm density through optical density (OD) values. The results demonstrated the biofilm –forming ability of *Klebsiella pneumoniae* isolates. Biofilm formation was also qualitatively classified into four categories: “Non-producer", "Weak producers", "Moderate producers", and "Strong producers". As show Table (5) and figure (2)

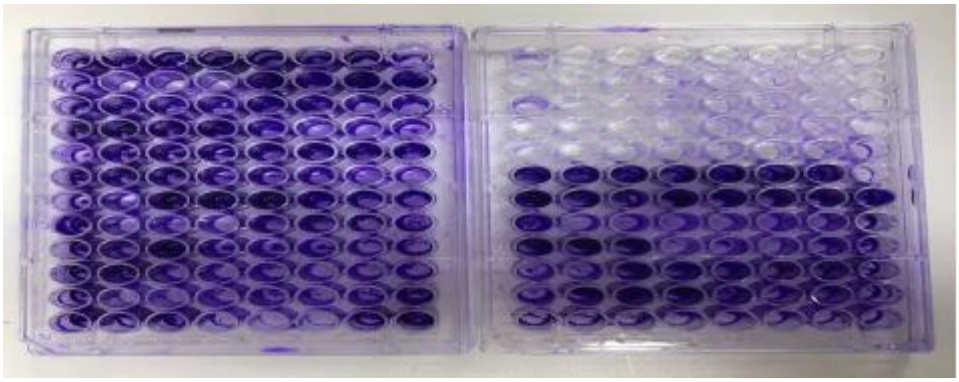


Fig. 1: Biofilm formation in micro titer plate

Table 5: Classification of *K.Pneumoniae* adherence by plate method

Category	No. of Samples	% of Total
Strong producers	17	54.8%
Moderate producers	12	38.8%
Weak producers	1	3.2%
Non producers	1	3.2%
Total Samples	31	100%

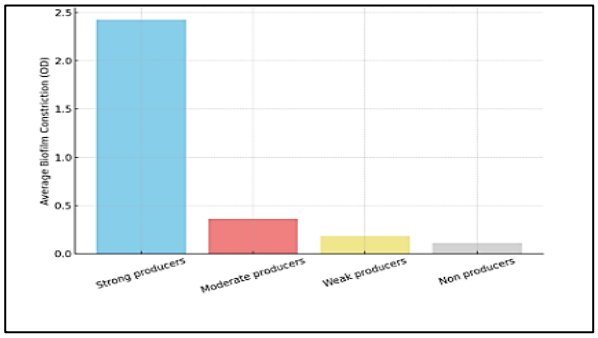


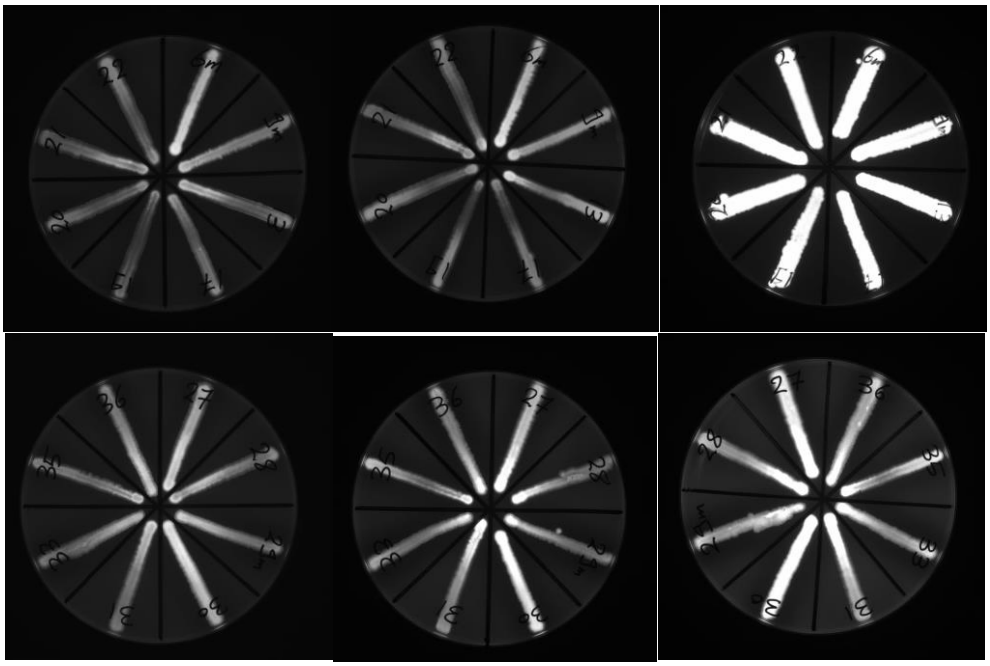
Fig. 2: Classification of *K.Penumoniae* adherence by microtiter plate method

Phenotypic detection of efflux pump activity

Strains with the highest and low fluorescence in Response reveals stains with maximal (no efflux or physiological activity) and minimal or slight (overexpressed efflux system) are depicted as show in figure (3) and resulte show at EtBr concentration (1 mg/L), were efflux positive 24 (77.42%) of the total tested isolates. With the increasing concentration to 2 mg/L, the positive rate 23 (74.19%). and increase in concentration to 3 mg/L, the number of positive is 15 isolates, (48.39%).as in table (6).

Genotypic detection of efflux pump

he oqxAB efflux pump gene was identified at a high frequency among the MDR KP analyzed in PCR test, as the outcomes of PCR analysis showed. Among the 31 isolates, as many as 29 (93.55%) isolates presented these genes, and only 2 isolates(6.45%) were oqxAB negative (Table 7).



Concentration 1mg\l 2mg\l 3mg\l

Fig. 3: Result of efflux pump activity by Ethidium Bromide-Agar Cartwheel Method in *K.pneumoniae* isolates

Table 6: Result of efflux pump activity in *K.pneumoniae* isolates

Concentration (mg\l)	Number of Positive		Number of Negative	
	Number	Percentage%	Number	Percentage%
1	24	77.42%	7	22.58%
2	23	74.19%	8	25.81%
3	16	51.6%	15	48.4%

Table 7: PCR results in *K.pneumoniae* isolates

Characteristic	Number of isolates carrying	Percentage (%)	Number of isolates not carrying	Percentage (%)	Chi-Square	P-Value
OqxA gene	29	93.55%	2	6.45%	23.516	P<0.00001
OqxB gene	29	93.55%	2	6.45%	23.516	P<0.00001

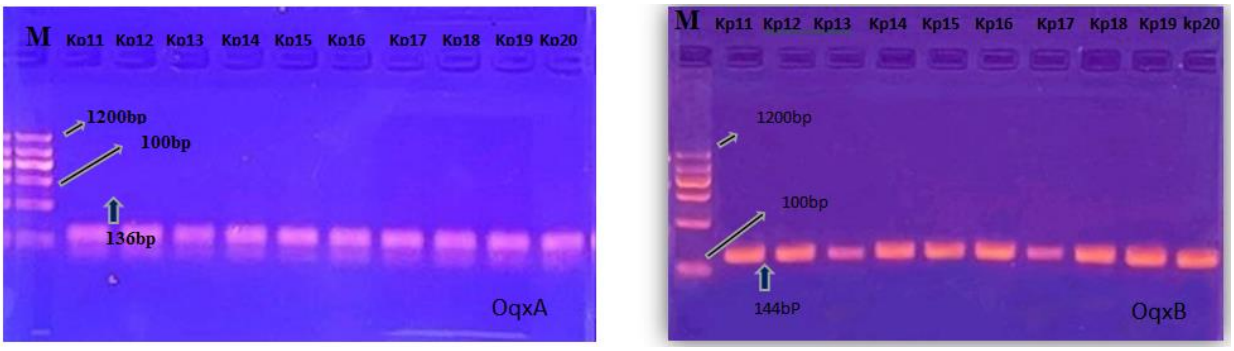


Fig. 4: PCR product for OQXAB genes was analyzed by gel electrophoresis (2% agarose gel, 50 V, 40 minutes) and visualized under UV light after ethidium bromide staining.

Table 8: Biofilm forming potential, efflux pump activity and antibiotic susceptibility profile of *Klebsiella pneumoniae*

Isolate ID	Biofilm forming	Efflux pump phenotype	Efflux pump genotype	Antibiotic resistance profile													
				AM	PTZ	KF	FOX	CAZ	CRO	FEP	IMP	AK	GM	CIP	LEV	FTN	SXT
Kp1	.+	.-	.-	R	R	S	S	R	S	R	R	R	R	S	S	S	R
Kp2	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp3	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp4	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	S
Kp5	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	S
Kp6	.+	.+	.+	R	R	R	R	R	R	R	R	R	S	R	R	R	R
Kp7	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp8	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	S
Kp9	.+	.-	.-	R	R	R	R	R	R	R	R	R	R	S	S	S	R
Kp10	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp11	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp12	.+	.-	.+	R	R	R	R	R	R	R	R	R	R	S	S	S	R
Kp13	.-	.+	.+	R	R	R	R	R	R	R	R	R	R	S	S	S	R
Kp14	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp15	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	I	R
Kp16	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp17	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	I	R
Kp18	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp19	.+	.-	.+	R	R	R	R	R	R	R	R	R	R	R	R	I	R
Kp20	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp21	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp22	.+	.-	.+	R	R	R	R	R	R	R	R	R	R	R	R	S	R
Kp23	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	I	R
Kp24	.+	.-	.+	R	R	R	R	R	R	R	R	R	R	R	R	I	R
Kp25	.+	.-	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp26	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp27	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	I	R
Kp28	.+	.+	.+	R	R	R	R	R	R	S	R	R	I	R	R	I	R
Kp29	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp30	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Kp31	.+	.+	.+	R	R	R	R	R	R	R	R	R	R	R	R	R	S

AM: Ampicillin; PTZ: piperacillin/tazobactam; KF: cefazolin; FOX: cefoxitin; CAZ: ceftazidime; CRO: ceftriaxone FEP: Cefepime; IMP: Imipenem; AK: amikacin; GM: Gentamicin; CIP: Ciprofloxacin; LEV: Levofloxacin;STX:Trimethoprim\Sulfamethoxazol; FTN:Nitrofurantion.

DISCUSSION

Klebsiella pneumoniae is a predominant nosocomial pathogen recognized for its exceptional capability to transfer resistance to multiple classes of antibiotics, which presents a significant threat to global health care.

The age of the Study subjects was between less than one year to 60 years. The age group greater than 45 years had the highest sum total of *K. pneumoniae* isolates 17 (54.83%) although the sample size is less compared to the second age group that is 21-44 years (11 isolates (35.48%). Generation of isolates The category with the least number of isolates in the youngest age group (1-20)years, 3(9.67%) . This implies that this age-specific group might be more vulnerable to opportunistic infections due to the age - related regression of immune system which. This study found

no significant female and male differences regarding bacterial infections, since these infections are generally not sex-based, excluding urinary tract infections

Biofilm production was executed utilizing the microtiter plate method, characterized by measuring biofilm density through optical density (OD) values. The results demonstrated the biofilm –forming ability of *Klebsiella pneumoniae* isolates. Biofilm Formation Categories as Strong producers which is most prevalent category with a pecent equal to 54.8%. Followed by Moderate producers as fewer than strong with a rate (38.8%), but still present in a significant number. While weak producers is Very few only (1) sample (3.2%).Finally the Non-producers is rare only 1 sample. A multitude of studies have been undertaken to investigate the capacity of *K.pneumoniae* to produce biofilm worldwide for example in a study in Iran out of 83 isolates, Sixty-two isolates, constituting 75%. Seventeen strains (20%) exhibited robust biofilm formation [١٧] .while later on, another Iran study carried out by Khoshnood *et al*.,(2023) [18] showed slightly different results from ours, where the rate of biofilm producers did not exceed (72%). In Jordan, a study bySwedan and Aldakhily [19] on *K.pneumoniae*, out of 167 isolates, 154 (92.2 %) were biofilm producers.

Our findings are quite consistent with a study in China in 2023 ,where (54%), were strong biofilm producers [20]. Another study which conducted in the same country the rate of biofilm producers were about (37%) of 140 strains involved in the study by Dan *et al*., [21]. Meanwhile our data was dissimilar to that in Brazil and Vietnam , where biofilm formation was recorded in (58.4%) and (58.2%)of the total isolates respectively[22,23]. In a study in Indonesia on ciprofloxacin resistant *K.pneumoniae* only (41.3 %) were strong biolfim producers [24]. Natively in a study performed in Baghdad the rates were collectively in a line with ours with various apabilities to form strong, moderate and weak biofilms as (63.07%), (29.23%)and, (7.69%) respectively.[25]. These findings underscore the significant propensity of MDR *Klebsiella pneumoniae* isolates to form biofilms. The high percentage of strong and moderate biofilm producers suggests that this process may be essential for the survival and persistence of these bacteria[26]

Phenotypic detection of efflux pump, the concentration dependent with EtBr used in the assay. At the least EtBr concentration (1 mg/L), were efflux positive 24 (77.42%) of the total tested isolates. With the increasing concentration to 2 mg/L, the positive rate slightly reduced to 23 (74.19%). Interestingly as a result of the increase in concentration to 3 mg/L, the number of positive results were statistically significantly reduced to 16 and negative results were shown to increase (15 isolates, 48.39%). These results are in agreement with the underlying concept of the EtBr assay (efflux the dye compared with the increased external concentrations of bacteria). Strains with highly active efflux pumps maintain a low intracellular concentration of EtBr over a wider range of EtBr added to the medium.

Previous studies have widely utilized the cartwheel method for phenotypic detection of efflux pumps in *K. pneumoniae* and other Gram-negative bacteria. In Iran, a study showed that various degrees of activity were seen in k pneumoniae isolates were nearly (28%) of isolate showed high activity and about (25%) of isolates where moderate while near (47%) where inactive[27] For instance, a study by Patil *et al*., 2021 [28] Used this method to screen MDR Enterobacteriaceae isolates from clinical samples and found a significant proportion exhibiting efflux pump activity. Similarly, other research has correlated efflux pump with resistance to various antibiotics, including fluoroquinolones and carbapenems in *K. pneumoniae* However, a study by Amereh *et al*., [29] in Iran and studies have widely utilized the genotype detection of efflux pumps gene in MDR *K. pneumoniae*

Another study, carried out in Nigeria where the finding show that total number of *K. pneumoniae* isolates where positive at the concentration of 0.5 gram per mole [30] .

Our Data aligned with another native study particularly in Baghdad where activity of efflux pump showed a rate of (75%) use varying concentrations of ethidium bromide[31].

The increased frequency of negative isolates and decreased positive isolates with increasing EBr concentration reveals the presence of some plausible interpretations. At low EBr concentrations, effIux pumps could efficiently excrete enough dye to stay Positive (wedging in as much dye as they pumps out). However, under high EBr concentration the efflux pump system would be saturated or partially inhibited, leading to high intracellular EBr concentration. This accumulated amount perhaps might change the dye-DNA interaction, resulting an “inhibitory” effect in the Cartwheel assay.

The oqxAB efflux pump gene was identified at a high frequency among the MDR KP analyzed in PCR test, as the outcomes of PCR analysis showed. Among the 31 isolates, as many as 29 (93.55%) isolates presented these genes, and only 2 isolates (6.45%) were oqxAB negative.

Numerous studies have been conducted all over the word regarding the possession of oqxA and oqxB genes.In Iran a study revealed that the prevalence of the two genes was 95% and 98 % respectively [29]. While in Turkey the real time PCR results showed that 40 fold increase in the expression of oqxA gene had been reported in 34 isolates from 46 isolates [32]. More recently, in Egypt the percentages of oqxA and oqxB genes were (94%) and (67%) respectively. [33] another

study in Egypt show the prevalence of genes were (62%) and(65%) respectively[34]

The distribution of oqxAB in these MDR isolates indicates that the MDR strains are involved in the significance of this resistance determinant in this phylogenetic group of bacteria. This realization is in line with the growing concern worldwide for acquiring efflux pump-based resistance across Gram-negative Bacteria, and *K. pneumoniae* in particular[35]. This findings suggest a potential cooperative relationship between efflux pumps and biofilm formation in MDR-*K. pneumoniae.*.

Furthermore, this finding indicates that the resistant towards multiple antibiotics might be mediated by more than one mechanism ultimately, the high detection rate suggests that the oqxAB efflux pump system is considers a significant contributor to the multidrug resistant *Klebsiella pneumoniae* isolates.

CONCLUION

The simultaneous development of biofilms and the active OqxAB efflux pump system in the K. pneumoniae isolates examined may elucidate the potential synergism of both in multidrug resistance. The high abundance of such mechanisms highlights their importance in conferring resistance to antibiotic treatment to these organisms. Further exploration of the sophisticated relationship among different resistance mechanisms is required to elucidate the novel targets or strategies for combating MDR *K.pneumoniae*

Ethical Approval Declaration The procedures followed in this study were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki). In addition, each participant provided written consent following a concise overview of the project.

Declarations: Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article. This manuscript has not been previously published and is not under consideration in another journal

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