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Assessment the relationship of Antibiotic profile and virulence factors in *Klebsiella pneumoniae*

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Abstract---Background: Nosocomial infections are frequently caused by *Klebsiella pneumoniae*. The great majority of antibiotics are no longer effective against *K. pneumoniae*. The goal of this study was to see if there was a link between antibiotic resistance and the ability to produce biofilms in *K. pneumoniae* strains isolated from UTI patients in Iraq. Methods: A total of 85 non-duplicate clinical samples of urine, blood, sputum, wound swabs, Catheters swab and endotracheal tubes swab of *K. pneumoniae* strains which were collected over the course of a year. Identification of *K. pneumoniae* isolate was done by analyzing colony morphology, microscopic examination, and by performing biochemical testing. Testing of antibiotics susceptibility by the CLSI Kirby–Bauer disk diffusion method, and biofilm-producing by crystal violet microtiter plate method as adherence quantitative assays. Virulence tests were carried out, including: string test, haemagglutination, yeast agglutination for all samples. Results: The majority of isolates were able to generate biofilms (78 isolates, 91.8 %). The most of positive string tests were from the ESBL group. Our findings revealed that major yeast agglutination is positive, while the heamagglutination test findings were varied, and it was noticed that the strong source came from the urine and catheter samples. The participants in this study were divided into four groups based on the type of biofilm they had. Imipenem (41.4%), Cefoxitin (27.6%), and Levofloxacin (27.6%) demonstrated just acceptable sensitivity in *K. pneumoniae* (21.8 %). *K. pneumoniae*, on the other hand, exhibited good sensitivity to Aztreonam (71.4%), levofloxacin, Imipenem, Cefoxitin (57.1%), Gentamicin, Trimethoprim/sulfamethoxazole, and Ceftazidime among non-biofilm producing isolates (42.9 %). There is a relationship between *K. pneumoniae*'s ability to produce biofilms and its drug resistance. Conclusions: As a result, in order to make further

progress in the prevention of hospital-acquired illnesses, new approaches to infection control will be required. All of the evidence points to the need for basic actions and novel ways to manage *K. pneumoniae* biofilm-related illnesses.

Keywords---*Klebsiella pneumoniae*, KPC, biofilm, antimicrobial resistance, MDR, ETT, VAP, CLSI, ESBL.

Introduction

Friedlander isolated *Klebsiella pneumoniae* from the lungs of pneumonia victims for the first time in 1882. Friedlander's bacillus was the original name for this encapsulated bacterium, which was termed *Klebsiella* in 1886. It was later identified as a saprophyte microbe able of colonization the human nasopharynx, gastrointestinal tract (GIT), and skin, as well as causing infections of the biliary and urinary tracts, bacteremia and osteomyelitis. *K. pneumoniae* also was thought a major cause of community-acquired infections (CAIs), such as an aggressive case of pneumonia, particularly in immune compromised, due to its high pathogenicity. The ability of *K. pneumoniae* strains to persist despite antibiotic therapy, as well as their higher adhesiveness and probably also invasiveness, may play a role in recurrent infections.

When *K. pneumoniae* established itself in the hospital setting and became a primary component of nosocomial pathogens, its epidemiology and range of illnesses changed dramatically. In fact, *K. pneumoniae*'s high colonization efficiency, combined with acquired antibiotic resistance, The most common *K. pneumoniae* strain has been able to persist and spread fast in healthcare facilities infections include the urinary tract, abdominal cavity, lungs, wounds, intravascular equipment, surgical places, soft tissues, and later bacteremia. In general, a cohort research discovered that *K. pneumoniae* was the most common pathogen, following by staphylococcal biofilms, caused the majority of infections linked to medical devices, such as intravascular catheters and urinary, and that A substantial percentage of biofilm-producing bacterial isolates that caused infection had multidrug resistant (about 90%).

The incidence of *K. pneumoniae* clinical infections in long-term acute care local hospitals was projected to be higher in 2013 than in short-stay hospital intensive care units. After *Pseudomonas aeruginosa*, *K. pneumoniae* was the 2nd most frequent Gram-negative bacterium (11 percent) in a prospective study on hospital-acquired infections. *K. pneumoniae* carbapenemase-producing *K. pneumoniae* (KPC-KP) was discovered to play a significant role in the spread of the epidemic of carbapenem-resistant Enterobacteriaceae in a countrywide cross-sectional survey. Since the decade's end in the 1980s, *Klebsiella pneumoniae* has been found to be able to develop in vitro as a biofilm. In 1992, Reid and colleagues employed scanning electron microscopy to examine some bladder epithelial cells from a spinal cord injured patient who had an asymptomatic urinary tract infection UTI caused by *K. pneumoniae*, and found unequivocal indications of an in vivo biofilm.

In vitro tests eventually found that almost 40% of *K. pneumoniae* isolates, including those from sputum, blood, urine, and wound swabs, were resistant to antibiotics, were capable of producing biofilm. In addition, In vitro biofilm detection was found in 63 percent of *K. pneumoniae* specimens from catheterized individuals with UTIs. *K. pneumoniae* strains obtained through endotracheal tubes (ETT) from patients with ventilator associated pneumonia (VAP) were recently discovered to be capable of rapidly generating an in vitro biofilm at a high rate.

Materials and Methods

Subjects

This research was place over the course of a year between Noember 2020 and October 2021. A total of 85 adult (54male and 31female patients ranging in age from 18 to 80 years old.) patient samples were collected from Al-Ramadi Teaching hospital in Al-Anbar.

Sample collection

Blood samples Bacterial isolates

A sterile syringe was used to collect 1 mL of blood, which was then combined with 20 mL of Brain-Heart Infusion broth BHIB (Liofilchem, Italy). For 7 days, the mixture was incubated at 37°C. Blood samples were streaked onto the surface of MacConkey agar and blood agar (Liofilchem, Italy) by a loopful and incubated for 24 hours at 37 degrees Celsius. Using morphological and biochemical tests, all isolates were identified. (Gram stain, capsule stain, indole production test, motility test, Methyl Red test, urease production test, Voges-Proskauer test). Susceptibility Testing for Antibiotics According to Clinical and Laboratory Standards Institute (CLSI) recommendations, the antibiotic susceptibility of *K. pneumoniae* isolates was evaluated using the Kirby-Bauer disk diffusion method. The suspension of each *K. pneumoniae* isolate was dispersed over the surface of Mueller Hinton agar MHA (Oxoid, UK) using sterilized glass rods. The antibiotic discs (Liofilchem, Italy) were then placed on the surface of the MHA plate that had been inoculated. After that, the plate was incubated at 35°C for 18 hours. The inhibition zone diameters were measured by millimeters measurement ruler and recorded. After that, these data were compared with the standard inhibition zone of CLSI, 2020.

Bacterial Isolates Urine samples

To reduce contamination, the clean catch method was utilized to collect samples. The clean catch midstream urine of each patient was collected into a 20 mL certified sterile screw-capped unified container that was supplied to them at the start. Each urine sample was autoclavable inoculated into MacConkey agar plates, blood agar plates, and cetrimide agar plates when it arrived at the laboratory. After incubation, the plates were incubated aerobically at temperature 37°C for 18-24 hours, and the samples were processed using a *K. pneumoniae* diagnosis approach previously published.

Bacterial Isolates Sputum samples

Samples of sputum were collected in sterile screw-top containers. The patient coughed deeply into the container, then instantly screwed on the cap to collect the expectorated phlegm. Samples were transferred to the laboratory within two hours and processed or chilled at 4°C as soon as practicable. After drying in the biosafety cabinet, the most purulent material in the sputum was used to produce a smear, which was then heat-fixed. We employed standard gram staining techniques. White blood cells (WBCs), squamous epithelial cells (SECs), and microbes were studied. To extract *K. pneumoniae*, sputum samples were swabbed onto MacConkey agar and blood agar plates.

Bacterial Isolates wound swab samples

One wound swab was obtained under aseptic circumstances. A dry sterile cotton swab was dipped in the wound to collect pus or other exudates. All specimens were immediately collected and analyzed before treating the wound. Macroscopic investigation: The color of the pus was initially examined macroscopically, as the color of the pus can help determine the causative agent. Each sample was cultured on blood agar plates and MacConkey agar plates. The culture plates were then incubated aerobically at 37°C overnight. After incubation, the samples were analyzed using a method for diagnosing *K. pneumoniae* that has previously been reported.

String Test

Hypervirulent *K. pneumoniae* strains had a hypermucoviscosity phenotype (as revealed by a positive string test result), while classic *K. pneumoniae* strains had a negative result. The creation of a mucoviscous string of >5 mm on a bacteriology inoculation loop used to stretch a colony grown overnight on an agar plate at 37°C was defined as a positive string test result.

Yeast agglutination and serological assay:

Yeast cell preparation. *S. cerevisiae* yeast cells were cultivated in a medium (1 liter) containing peptone (2 percent; Difco), yeast extract (1 percent), and glucose for 16 hours at 37°C with shaking (2 percent). The cells were extracted by centrifugation at 1,500 x g for 10 minutes, suspended in 1 liter of pH 7.4 phosphate-buffered saline, and sedimented once more. Glutaraldehyde was used to further treat the *S. cerevisiae* isolates. The glutaraldehyde-fixed cells (1 ml, 20 mg/ml phosphate-buffered saline) were dyed with safranine by dissolving 30 1Ld of 5 percent safranine (Fisher Scientific Co.) in 95 percent ethyl alcohol (Thomas G. Pistole, personal communication).

Hemagglutination and serological assays

Hemagglutination. Venipuncture was used to collect human group O blood, which was immediately deposited in Alsever solution and preserved at 4°C until needed. Blood cells were washed three times in Dulbecco phosphate-buffered saline (PBS), pH 7.4 (Oxoid, Basingstoke, England) before being suspended at 3 percent

(vol/vol) in this saline. By using McFarland turbidity standards, plate-grown bacteria culture suspensions in PBS yielded approximately 1.5×10^9 bacteria per milliliter. Broth-grown cultures (BGC) were harvested and washed twice in PBS before being suspended in the same turbidity as plate-grown suspensions in PBS. At room temperature, slide hemagglutinations were performed by mixing 20 microliters of blood suspension with 20 microliters of bacterial solution on a slide and gently shaking it by hand. Always included was a PBS-blood cell control. If hemagglutination did not develop within 10 minutes, strains were designated negative hemagglutinators. The fewest number of bacterial cells/ml that generated visible hemagglutination in 10 minutes was quantified as the minimum hemagglutinating dose (MHD), and the hemagglutinating activity of a culture was calculated as $10^4/\text{MHD}$.

Biofilm assay

The production of biofilms by *K. pneumoniae* isolates was measured using a semi-quantitative approach. Using brain heart infusion broth (Oxoid UK), 0.5 McFarland suspensions of *K. pneumoniae* isolates were produced, and 100 microliters of the suspensions were inoculated into wells of a 96-well polystyrene plate and incubated at 37°C for 18 hours. A sterile brain heart infusion broth was used to inoculate the negative control well. The wells were then stained for 20 minutes with 200 microliters of crystal violet at a concentration of 0.5 percent, and the excess stain was washed away three times with 200 microns sterile distilled water. The stain was then eluted with 200 microliters of 95 percent ethanol and the optical density was measured with an enzyme-linked immunoassay reader at 570 nm. Three standard deviations above the mean OD of the negative control was used to determine the positive Optical Density (OD).

Statistical Analysis

The data was gathered, edited, coded, and entered into version 23 of the Statistical Package for Social Science (SPSS). The qualitative data was given as numbers and percentages, whereas the quantitative data was provided as mean, standard deviations, and ranges. The chi-square test was used to compare qualitative data between the antibiotic sensitivity pattern and the biofilm forming capacity of *K. pneumoniae*. P-value was considered significant was set if p-value <0.01 or <0.05.

Results

The study included non-duplicate 85 of *K. pneumoniae* were isolated from 54 male (63.53%) and 31 female (36.47%) patients. Most of *K. pneumoniae* were isolated from patients aged more than 50 years old (56.47%) (Table 1). The majority of isolates were able to generate biofilms (78 isolates, 91.8%).

Table 1
Patient characteristics from which *K. pneumoniae* was isolated

Characteristics		n	%
Gender	Male	54	63.5

Characteristics		n	%
Age (years)	Female	31	36.5
	18-49	37	43.5
	50-79	48	56.5
Clinical Specimens	Sputum	23	27.1
	Pus	17	20.0
	Catheters swab	11	12.9
	Endotracheal tubes swab	3	3.5
	Wound swab	12	14.1
	Blood	4	4.7
	Urine	15	17.7
Total		85	100

Division of the patients in this study into 4 groups was done according to the biofilm type (table 2): those how are strong biofilm (18 patients 21.2%), moderate biofilm (21 patients 24.7%), weak biofilm (39 patients 45.9%), and 4th group of non-biofilm production (7 patients 8.2%).

Table 2
Biofilm grouping and virulence factors of *K.pneumonia*

Bio-Film Gps	No (%)	Mean (±SD)	Yeast Agglutination Positive: n (%)		Haem Agglutination Positive: n (%)		String Test Positive: n (%)		ESBL n (%)	
S-BF	18 (21.2)	0.851 (0.288)	18	(100)*	3	(16.7)*	7	(38.9)*	10	(55.6)*
M-BF	21 (24.7)	0.318 (0.590)	20	(95.2)*	1	(4.8)*	2	(9.5)*	9	(42.9)*
W-BF	39 (45.9)	0.169 (0.031)	30	(76.9)*	1	(2.6)*	0	(0.0)*	2	(5.1)*
A-BF	78 (91.2)	0.367 (0.308)	68	(87.2)*	5	(6.4)*	9	(11.5)*	21	(26.9)*
N-BF	7 (8.2)	0.099 (0.019)	3	(42.9)*	0	(0.0)*	0	(0.0)*	0	(0.0)*
Total	85 (100)	0.345 (0.304)	71	(83.5)**	5	(5.9)**	9	(10.6)**	21	(24.7)**

Abbreviations: Gp; group, S-BF; strong biofilm, M-BF; moderate biofilm, W-BF; weak biofilm, A-BF; all biofilm, Non-BF; non-biofilm, ESBL; extended spectrum beta lactmase.

*.percentage within group, **.percentage to all patients

Antibiotic susceptibility pattern: The majority of *K. pneumoniae* strains were resistant to a variety of antibiotics. *K. pneumoniae* demonstrated only adequate susceptibility to Imipenem (41.4%), Cefoxitin (27.6%), and Levofloxacin (21.8%) among biofilm producer isolates. *K. pneumoniae*, on the other hand, demonstrated good sensitivity to Aztreonam (71.4%), levofloxacin, Imipenem, Cefoxitin (57.1%), Gentamicin, Trimethoprim/sulfamethoxazoile and Ceftazidime (42.9%) among non-biofilm producing isolates. While the results of antimicrobial resistance are shown in Table 3.

Table 3
K. pneumoniae's antibiotic sensitivity pattern and biofilm-producing capabilities

Bio-Film Gps	Resistant %													
	LEV	AMC	IPM	CFM	FOX	CIP	CN	ATM	FEP	SXT	AMP	CAZ	Ceftazidime	
S-BF	83.3	88.9	66.7	100	88.9	94.4	100	100	100	100	100	100	93.5	
M-BF	81.0	85.7	61.9	90.5	76.2	95.2	85.7	100	95.2	95.2	100	95.2	88.5	
W-BF	74.4	82.1	48.7	89.7	61.5	94.9	69.2	84.6	87.2	89.7	100	89.7	81.0	
A-BF	78.2	83.9	58.6	90.8	72.4	93.1	80.5	90.8	90.8	92.0	97.7	92.0	92.5	
N-BF	42.9	71.4	42.9	71.4	42.9	71.4	57.1	28.6	71.4	57.1	71.4	57.1	57.1	
Total	75.3	83.5	55.3	90.6	69.4	92.9	78.8	87.1	90.6	90.6	97.6	90.6	83.5	

Abbreviations: LEV; Levofloxacin AMC; Amoxicillin/clavulanate, IPM; Imipenem, CFM; Cefixime, FOX; Cefoxitin, CIP; Ciprofloxacin, CN; Gentamicin, ATM; Aztreonam, FEP; Cefepime, SXT; Trimethoprim/sulfamethoxazole, AMP; Ampicillin, CAZ; Ceftazidime.

Based on statistical research utilizing chi-square tests, there was a strong relationship between antibiotic resistance in *K. pneumoniae* and biofilm generation capacity. (Table 4).

Table 4
 Correlation between *K. pneumoniae* biofilm production capacity and its resistance to antibiotics

	LEV	AMC	IPM	CFM	FOX	CIP	CN	ATM	FEP	SXT	AMP	CAZ
Pearson Chi Square Correlation	0.214**	0.122**	0.123	0.185*	0.254*	0.193**	0.232**	0.487**	0.229*	0.305**	0.336**	0.296*

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Discussion

The isolates of *K. pneumoniae* employed in this study were mostly from male patients. This finding matched that of Osagie *et al.* and Akter *et al.* In this investigation, the majority of *K. pneumoniae* was recovered from patients aged 50 to 79. This method was developed after a prior investigation the majority of *K. pneumoniae* isolates were found to have were from patients above the age of 70. Meanwhile, another recent study by Zheng *et al.* found that the proportion of *Klebsiella pneumoniae* was greater in patients aged 40 to 65 years old. The disparities in age distribution of patients could be connected to the immune system's ability to respond, which is typical to wane as people age. Patients under the age of 40 have more powerful immune systems, putting additional pressure on *K. pneumoniae* to overcome the host's immunity. Increased age, on the other hand, increases the risk of *K. pneumoniae* infection due to an increase in the incidence of comorbidity.

K. pneumoniae isolates were predominantly obtained from respiratory specimens in our study. (27.1 percent, as seen in table 1). Wang *et al.* came at the same conclusion in their study. The respiratory system was shown to be the most

common site of *K. pneumoniae* infection in the Republic of China. During the investigation, Nirwati *et al.* gathered 167 *K. pneumoniae* isolates from two hospitals in Klaten, Indonesia. The bulk of the samples (51.50 percent) came from respiratory specimens, with the remaining samples coming from pus, blood, and urine at 16.2, 10.8, and 7.2 percent, respectively. The majority of *K. pneumoniae* isolates were mucoid, but to varying degrees. Despite the fact that the majority of them formed as huge wet colonies, the majority of positive string tests were from the ESBL group (88.9 percent see table 2). The findings are in line with what Indian scholars Lobo and Moosabba discovered. The biofilm group, particularly the strong and moderate groups, had all positive string samples.

Our findings revealed that major yeast agglutination is positive (83.5 percent, shown in table 2), indicating that this form of fimbriae is involved in epithelial cell attachment. While the heamagglutination test findings were varied (strong biofilm 60%, moderate biofilm 20%, weak biofilm 20%, and non-biofilm 0.0%), they were classified according to the strength of the biofilm, and it was noticed that the strong source came from the urine and catheter samples. This is in line with the interpretation of the two categories by Boddicker and his research team: Bacterial adherence to a variety of epithelial cells is mediated by type 1 fimbriae (yeast agglutination), which may aid bacterial attachment to the bladder epithelium. In vitro, type 3 fimbriae (haemagglutination) have been shown to bind to the extracellular matrix of urinary and respiratory tissues, showing that they play a role in epithelial surface binding. Furthermore, type 3 fimbriae are required for *K. pneumoniae* to form biofilm on plastics and human extracellular matrix, implying that they may facilitate the formation of treatment-resistant biofilm on indwelling plastic devices such as catheters and endotracheal tubes. These devices could cause tissue damage, allowing *K. pneumoniae* to colonize exposed tissue as a biofilm.

Antibiotic resistance makes biofilm-associated infections difficult to treat. With MDR KPC-producing *K. pneumoniae* becoming more common around the world, research on their ability to form biofilms and their responsiveness to antibiotics in the biofilm state is critical. Imipenem, Cefoxitin, and Levofloxacin are found to be susceptible against the vast majority of these *K. pneumoniae* strains. The process, according to Andrei and colleagues, could be due to the existence of stationary-phase bacteria that develop antibiotic-tolerant in biofilms with nutritional restrictions, as found in free-floating bacteria. In our investigation, the betalactam antibiotic class (Amoxicillin/clavulanate, Ceftazidime, Aztreonam, Cefepime, and Cefixime) showed an increase in antibiotic resistance (R: 97.8%, 92.00%, 90.8 %, 90.8 %, and 90.8 %, respectively) due to their potential to create extended spectrum beta lactamase (ESBL).

Depending on the biofilm's strength, there was a gradual increase in antibiotic resistance (strong biofilm 93.5%, moderate biofilm 88.5 % and weak biofilm 80.0 %). Shadkam and his colleagues agreed with our conclusion. Antibiotic resistance was found to be higher in biofilm-producing *K. pneumoniae* than in non-biofilm-producing *K. pneumoniae* in this investigation. Many studies have confirmed this fact. Saha *et al.* performed a study. Despite the fact that There were higher resistance patterns in all biofilm-producing isolates than nonbiofilm producers, The processes that defend biofilms are not the same as those that cause

conventional antibiotic resistance. The multilayered defense in biofilms is thought to be formed by the protective covering of the adhesive biomaterial that prevents antibiotics from penetrating, adaptive responses to stress, and the formation of persister cells, all of which increase the difficulty of eradication, especially in combination with the bacteria's resistance to antibiotics.

There is a link between *K. pneumoniae*'s ability to produce biofilms and its resistance to antibiotics. Yang *et al.* latest [34] and Nirwati *et al.* [39] observations are in agreement with our findings in accordance with our research. A high percentage of their isolates produced biofilms. They found that medication resistance was higher in biofilm-producing *K. pneumoniae* than in non-biofilm-producing *K. pneumoniae*. Hassan *et al.*, in contrast to our findings, observed that antibiotic-susceptible isolates produce more powerful biofilms than antibiotic-resistant isolates. This mismatch shows that the ability to build biofilm perhaps a key element in non-resistant strains' survival. Because the disk diffusion method utilized in this research can't be used to investigate biofilm-mediated resistance mechanisms, the findings from this analysis should be viewed with caution. Other possible explanations for the association between biofilm production and antibiotic resistance have been proposed to circumvent this limitation. Future studies should look into these mechanisms to see if they can be clarified.

Conclusions

Infections with *Klebsiella* are frequently seen as a model for hospital-acquired illnesses. Antibiotics are being administered indiscriminately, resulting in an increase in outbreaks caused by microbes resistant to antimicrobial medicines. As a result, new approaches to infection control will be required to make significant advancements in the prevention of infections acquired in hospitals. The rising evidence of *Klebsiella pneumoniae*'s biofilm formation ability, mainly on medical equipment, as well as recently findings linking such behavior to the acquisition of antibiotic resistance, should raise even more concerns about the pathogen's hazard in hospital settings. This research uncovers fresh findings that show a link between antibiotic resistance profile and biofilm-forming ability.

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