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Biofilm development and resistance to antibiotics in *Klebsiella pneumoniae* isolated from clinical specimens in Samawwa city, Iraq

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Abstract:

Biofilm formation is a critical factor for *K. pneumoniae's* ability to cause disease, as it shields the bacteria from the immune response of serum and phagocytosis. *K. pneumoniae* commonly employs biofilm formation as a survival strategy. A global issue of bacteria developing resistance to multiple drugs causes numerous deaths each year. To address the rising prevalence of drug-resistant bacteria, various legislative actions have been implemented to restrict or eliminate the use of antibiotics. *Klebsiella pneumoniae*, a bacteria commonly associated with healthcare-related infections, exhibits high levels of antibiotic resistance and is known for its ability to form biofilms. This research aimed to isolate and identify *K. pneumoniae* strains obtained from clinical samples in Samawwa city , Iraq, and also determining their antibiotic resistance patterns and capacity for biofilm production. The isolation of *K. pneumoniae* was carried out on inpatients in Samawwa between September and December 2022. The identification process involved analyzing colony morphology on selective media (Hichrom agar and ESBL agar), microscopic examination, and biochemical testing. Antibiotic susceptibility testing and biofilm-producing capacity assessment were conducted using the Kirby-Bauer disk diffusion method and microtiter plate assays which is known as a 96-well plate, is a quantitative method used with a microplate reader to determine the production of biofilm, respectively .A total of 85(40%) *K. pneumoniae* isolates were isolated from 215 total clinical samples during the study. The majority of the samples were gathered from male and female patients spanning an age range of 3 to 90 years. These samples were primarily acquired from urine specimens.(67%). The majority of *K. pneumoniae* strains exhibited high levels of antibiotic resistance, except for meropenem, imipenem, and norfloxacin, which showed better inhibition .18%; 7% and 7.5% of resistance, respectively. In addition, 24 isolates were tested for capacity to biofilm production, 17 (63%) isolates were biofilm producers, with 10 (41%) isolates as moderate, and 6 (38%) isolates as weak biofilm producers.

Key word*:* *K. pneumoniae*, Multidrug Resistance, Biofilm production

Introduction:

The biofilm formation process comprises a series of coordinated steps, beginning with the adherence of microbial cells to the surface they are colonizing. This is followed by the formation of microcolonies, maturation of the biofilm, organization of its structure, and ultimately the detachment of planktonic cells[1]. *Klebsiella pneumoniae* is a type of bacteria that has a rod-shaped structure. It is classified as a gram-negative bacterium, meaning it does not retain a specific stain during a laboratory test. It is also capable of fermenting lactose and can survive in environments with varying oxygen levels, making it a facultative anaerobe.[2]

Antimicrobial resistance (AMR) is a growing problem in healthcare institutions worldwide. Pathogenic bacteria, like *Klebsiella pneumoniae*, are rapidly becoming resistant to multiple drugs, posing a significant threat to patients as treatment options become less effective. *K. pneumoniae*, previously associated with community infections, is now frequently found as a leading cause of hospital-acquired infections. This bacterium has a higher propensity for antibiotic resistance compared to other bacteria due to the production of enzymes such as Extended Spectrum β-Lactamase (ESBLs) and Carbapenems.[3]

K.pneumoniae is a harmful bacteria that commonly causes serious infections in healthcare settings, such as pneumonia, urinary tract infections, soft tissue infections, and septicemia. It is a major cause of both hospital-acquired and community-acquired infections. Due to the rapid increase in antibiotic resistance, it is crucial to closely monitor antibiotic usage and find effective treatment strategies to reduce the inappropriate and excessive use of antibiotics. Urinary tract infections (UTIs) are one of the most prevalent healthcare-associated infections, ranking fourth among common infectious diseases in people. UTIs pose a significant public health concern and result in substantial financial burden[4].

K. pneumoniae strains are often categorized as opportunistic, hypervirulent (hyKp), or multidrug-resistant (MDR). The classic K. pneumoniae (cKp) strains are opportunistic and commonly found in hospital-acquired infections. On the other hand, hypervirulent strains are considered community-acquired bacteria that can infect individuals of any age, even those who are healthy. The rapid dissemination of multidrug-resistant *K. pneumoniae* strains poses a significant global health risk due to their association with a large number of hospital infections, resulting in high rates of illness and death. [5].

K.pneumoniae has developed various ways to resist different types of antimicrobial drugs. These mechanisms include using efflux pumps, altering the permeability of its membrane, producing enzymes that render antibiotics ineffective, modifying the target site of antibiotics, and acquiring alternative metabolic pathways that are unaffected by antibiotics. As a result, this resistance poses a global challenge in selecting appropriate antibiotics for treating infections acquired in hospitals.[6].

K. pneumoniae exhibits various ways to resist antibiotics, including Quinolones like Ciprofloxacin and Norfloxacin. Resistance mechanisms to fluoroquinolones involve mutations in DNA gyrase and topoisomerase IV (their target proteins), the presence of PMQR determinants, efflux pumps, and alterations in cell membrane permeability. PMQR determinants carried on mobile genetic elements can facilitate their spread within the Enterobacteriaceae family. [7].

K. pneumoniae relies on biofilm formation, a crucial factor for its virulence. Biofilms are communities of bacteria, including multiple species, enclosed in an extracellular matrix made up of

polysaccharides, proteins, and DNA. This formation enhances resistance against external stresses and antimicrobial substances. Given the significance of biofilm formation in *K. pneumoniae's* spread and pathogenicity, it plays a vital role in its virulence, The formation of *K. pneumoniae* biofilms has been suggested as an important stage in the development of these bacteria, especially in KP-Catheter-associated urinary tract infections (KP-CAUTIs). [5]

The objective of this study was to use selective media for isolating *Klebsiella pneumoniae*, and then assess the antibiotic resistance and biofilm production of the obtained isolates.

Methods:

Collection of Samples:

A total of 215 clinical samples (urine, sputum, oropharyngeal swaps) were collected from patients who suffered from different cases, including UTIs, pneumonia infections from two hospitals in samawwa city .

Bacterial Isolation and Identification:

The clinical samples were placed on various types of agar (MacConkey agar, Blood agar, Hichrom *klebsiella* selective agar, and Hichrom ESBL agar) and incubated at 37°C for 24 hours. After incubation, the distinct colonies were chosen and identified through biochemical tests. [8]. Isolates were identified by standard microbiological procedure (gram staining).The Vitek 2 Compact System was utilized to confirm the identified bacteria.

Antimicrobial Susceptibility Testing:

Kirby-Bauer disc diffusion method was utilized to detect the sensitivity of isolates according to CLSI [9].

The isolates tested to Amoxicillin 25µg,Amoxicillin-clavulanate 30µg, Piperacillin-tazobactam 100/10µg, Cefotaxime30µg, Ceftriaxone 30µg,Ceftazidime 30µg,Imipenem 10µg, Meropenem 10µg, Tetracycline 30µ, Aztreonam 10µg, Ciprofloxacin 5µg, Ofloxacin 5µg,Levofloxacin 5µg, Gatifloxacin 5µg, Nitrofurazole 300µg ,Nalidixicacid 30µg, Trimethoprim sulfamethoxazole 25µg ,Norfloxacin 10µg.

MDR *K. pneumoniae* was defined as exhibiting resistance to three or more different types of antibiotics.[10].

Biofilm production:

The Microtiter plate, also known as a 96-well plate, is a quantitative method used with a microplate reader to determine the production of biofilm [11]. To prepare the bacterial culture, a single colony was cultured in 5ml of BHI broth and incubated at 37 ºC for 24 hours. The culture was then diluted by combining 50µl of bacterial broth with 950µl of tryptic soy broth (TSB) supplemented with 1% glucose in an Eppendorf tube. A U-shaped microtiter plate was utilized for the experiment. The first three wells were filled solely with TSB as a negative control, followed by transferring 200µl of bacterial suspension into three wells for each isolate. The microtiter plate was incubated at 37 ºC for 24 hours. The contents of each well were discarded and washed three times with PBS to eliminate free-floating bacteria and planktonic cells while preserving

the biofilm's integrity. The microtiter plate was then left to dry overnight in an inverted position. To assess the formed biofilm, the wells were stained with 210µl of 0.25% crystal violet, which was left for 15 minutes before being discarded. Subsequently, the wells were washed three times with distilled water. The absorbance of the stained biofilm was measured at 630nm using a micro ELISA auto reader. The biofilm formation ability of each isolate was classified in to four types,non biofilm (0) when OD ≤ ODc , weakly biofilm when ODc >OD ≤ 2ODc, moderate biofilm when 2 ODc > OD≥ 4ODc and strong biofilm when 4ODc ≥OD.[11]

Statistical Analysis:

The data underwent analysis utilizing version 9.1 of the Statistical Analysis System (SAS). One-way ANOVA and Chi-square test were utilized to assess the differences between proportions. A significance level of $P \leq 0.05$ was considered to be significant.

Results:

Characteristics of clinical samples:

The occurrence of *K. pneumoniae* in clinical samples .was 85 (40%) isolates, The urine samples yielded the highest percentage of isolates at 67% (Table 1). The isolates were divided between male individuals and female individuals ,and most of them were isolated from patients within the age range of 30-40 years old (Table 1,2,3).

Table(1). Prevalence of *K.Pneumoniae* depending on the source of specimens

Source of specimens	***NO. of samples***	***NO.of k.penumonia(%)***	***P value***
urine	153	57(67.05%)	
sputum	49	23(27.05%)	0.791
Oropharyngeal swap	13	5(5.90%)	NS
Total	215	85	

Table (2). Prevalence of *K.Pneumoniae* depending on patients' gender and age

Age Groups (yrs.)	*No. of patients (n=215)*	*K. pneumoniae (%)*	*P value*
1-20	59	23.7%	0.244
20-40	97	38.1%	NS
40-60	43	46.5%	
60-90	16	62.5%	

Table(3). Prevalence of *K.Pneumonia* among gender

gender	*No. of gender*	*NO. K. pneumoniae (%)*	*P value*
Male	108	44(41%)	0.0449 NS
Female	107	41(48%)	

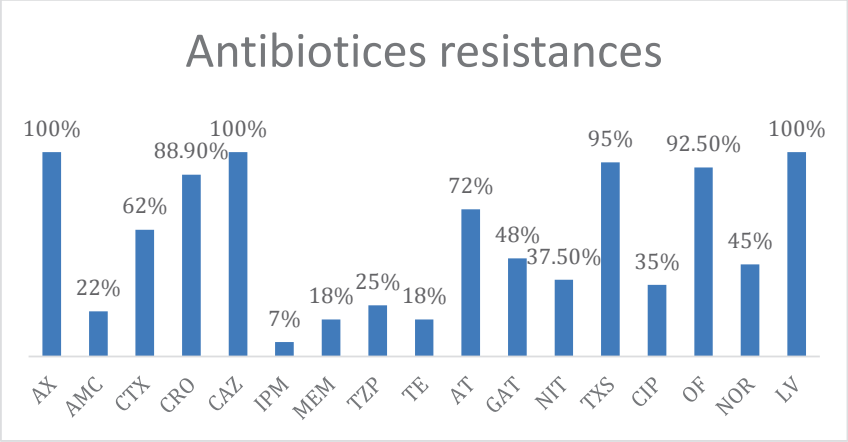
Table (4): The results of biochemical test of *K. pneumonia* isolated

Biochemical test	result
Indol Production	**-negative**
Methyl Red Production	**-negative**
Voges-Proskauer	**+positive**
Citrate Utilization	**+positive**
Urease Production	**+positive**
Oxidase Production	**-negative**
Catalase Production	**+positive**
Triple Sugar Iron	**Yellow slant and button, gas +,no H2S production.**

Antibiotic Susceptibility Pattern:

The majority of *K. pneumoniae* exhibited resistance to a broad spectrum of antibiotics, while only a small percentage of *K. pneumoniae* displayed favorable sensitivity to meropenem (70%) and Imipenem (78%).In contrast the high resistance to Amoxicillin, Ceftazidime, Trimethoprim-sulfamethoxazole and Norfloxacin with 100 %; 100%; 95% and 93% respectively.as in figure (1)

figure (1)



AX=Amoxicillin25µg,AMC=Amoxicillin-clavulanate30µg,TZP=Piperacillin-tazobactam100/10µg,CTX=Cefotaxime30µg,CRO=Ceftriaxone30µg,CAZ=Ceftazidime30µg,IMP=Imipenem10µg,MEM=Meropenem10µg,TE=Tetracycline30µ,AT=Aztreonam10µg,CIP=Ciprofloxacin5µg,OF=Ofloxacin5µg,LV=Levofloxacin5µg,GAT=Gatifloxacin5µg,NIT=Nitrofurazole300µg,NAL=Nalidixicacid30µg,TXS=Trimethoprimsulfamethoxazole25µg

Figure 1.Antibiotics resistance profile with all antibiotics used in this study.

Biofilm production:

n this study, among the 24 *K. pneumoniae* isolates tested, there were 16(66.6%) isolates as biofilm producer and 8 (34%) isolates that were not biofilm producers. Among biofilm producers, there were 6 (25%) isolates as weak, and 10 (41%) isolates identified as moderate biofilm producers.

Table 5. Biofilm production

characteristics	***NO.%***
Non Biofilm	8(34%)
Biofilm producer	16(66.6)
weak	6(25%)
moderate	10(41%)

Strong	0(0%)
Total	24

Discussion:

In this study, the majority of *K. pneumoniae* isolates were obtained from male patients, which aligns with Nirwati [12] findings indicating a higher risk of *K. pneumoniae* infection in males compared to females. However, no statistically significant differences between males and females were reported in these studies.

Furthermore, most of the *K. pneumoniae* isolates in this study were from patients ranging in age from 40 to over 60 years. This contrasts with Zheng [13] previous study, which suggested a greater number of *K. pneumoniae* isolates in patients aged between 40 and 65 years. Increased age is associated with a higher risk of *K. pneumoniae* infection due to a higher incidence of comorbid illnesses .The differences in age distribution among patients may be related to the strength of the immune system response, which is expected to decline with aging. Patients under 40 years of age typically have stronger immune systems, exerting greater pressure on K. pneumoniae to combat the host's immunity. [12].

The majority of *K. pneumoniae* showed resistance to different antibiotics, in. It was found that these isolates were resistant 100% to the following antibiotics (Amoxicillin, Ceftazidime and Nalidixic acid) and (Ceftriaxone, Trimethoprim-sulfamethoxazole , Ciprofloxacin, and Norfloxacin). As percentage (89%,95%,90% and 93%) respectively. Also, these isolates demonstrated their sensitivity towards the two antibiotics (Meropenem and Imipenem) as percentages (70% and 78%) respectively.

This finding is supported by a local study, suggesting that the higher resistance to penicillin antibiotics could be attributed to genetic mutations in penicillin binding proteins (PBPs), as reported by Jiang[14].Ceftriaxone, classified as a third-generation cephalosporin antibiotic, signifies an advancement in antibiotic resistance mechanisms. The resistance of *K. pneumoniae* to ceftazidime can be attributed to various factors, including mutations in genes ,overexpression of some genes, mutations that hinder porin function and prevent antibiotic entry, as well as the activity of efflux pumps contributing to ceftazidime resistance, [15].

Imipenem and Meropenem demonstrate high sensitivity levels of 70% and 77.7% respectively. This aligns closely with Ahmad's findings [16], which showed a resistance rate of 5.26% to imipenem in *K. pneumoniae*. The mechanisms of carbapenem resistance in *K.pneumoniae* are complex and can be attributed to factors such as carbapenemase production. When *K. pneumoniae* acquires carbapenemase, it becomes resistant to most β-lactam antibiotics, including carbapenems. Carbapenems have broad-spectrum antibacterial activity and consist of a carbapenem linked to a β-lactam ring, providing protection against various β-lactamases such as metallo-β-lactamase (MBL) and extended-spectrum β-lactamases. Carbapenem resistance primarily occurs through the acquisition of carbapenemase genes via large transferable plasmids with mobile elements or a combination of overexpression of β-lactamase with low carbapenem affinity, reduced permeability, or efflux pumps.[17]

The results of piperacillin-tazobactam, Amoxicillin-clavulanic acid and Tetracycline resistance in our study show as 25%,22% and 18% respectively , Tetracycline's limited effectiveness against *K. pneumoniae* is caused by mutations in the bacteria's outer membrane chromosomes, which reduce the penetration of tetracyclines into the cell. Additionally, *K. pneumoniae* can acquire mobile genetic elements containing specific tetracycline resistance genes like *tetB*, which are highly mobile, or possess *tetA* genes located on plasmids. Mutations within the ribosomal binding site can also occur, resulting in increased expression of intrinsic resistance mechanisms.[18].

The study found that *K.pneumonia* exhibited a high level of resistance to quinolone antibiotics, namely Ciprofloxacin, Norfloxacin, and Nalidixic acid as 90%,93% and 100%. *K.pneumoniae* employs various resistance mechanisms against Quinolones, including genetic mutations in genes responsible for DNA grease or Topoisomerase, leading to the production of enzymes that resist these antibiotics. Additionally, efflux pumps located on the outer membrane of the bacteria expel the antibiotics and hinder their permeability.[19]

The present study's findings were consistent with previous research conducted by Nirwati [12], who reported that 85.6% of the isolates were capable of producing biofilms. Similarly, Shadkam [19] found that 75% of the isolates produced biofilms while 25% did not. The biofilm-producing ability was observed in *K. pneumoniae* isolated from urine and sputum. Biofilm formation enhances resistance against external stressors and antimicrobial substances. However, the capacity to form biofilms varied among the isolates due to several factors, including the physicochemical characteristics of K. pneumoniae, physical interactions between its constituents, the type of surface where the biofilm forms, as well as temperature and pH.[5]

conclusions:

The majority of K. *pneumoniae* isolates exhibited resistance to multiple antibiotics and also demonstrated varying abilities to produce biofilms. There is a need to enhance global initiatives to control the dissemination of drug-resistant bacteria and eliminate hospital-acquired microbes, which are significantly contributing to increased mortality rates.

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