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Article

Biofilm Formation of Locales Clinical Multidrug Resistance *Klebsiella pneumoniae* Isolated from Different Sources deals with Antimicrobial Resistance

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ABSTRACT
The goal of this study was to detect antibiotic resistance and determine the ability of clinical isolates *Klebsiella pneumoniae* (*K. pneumoniae*) to form biofilms and detect the relationship between biofilm formation and antimicrobial resistance that depends on the specimen sources at the localized Baghdad Hospitals. This gram-negative rod bacterium is an opportunistic pathogen that can cause various illnesses in people and animals, such as important respiratory tract infections. For these reasons, our study included 87 isolates of *K. pneumoniae* from different clinical cases. The number and percentage of obtained isolates according to the sources distributed as specimens: 26(29.9%) urine, 25(28.7%) blood, 8(9.2%) stool, and 4(4.6%) sputum, as well as swabs:11(12.6%) burn, 9(10.3%) vagina and 4(4.6%) wound. The antibiotic susceptibility test results showed colistin, Imipenem, and meropenem were more effective against the isolates. The tissue culture plate and Congo red methods were used to evaluate biofilm formation. Finally, polymerase chain reaction was used to identify two genes linked with biofilm formation: *MrkD* and *FimH*. The isolates showed different abilities to produce biofilms based on clinical sources. The result appeared (97.7%) of isolates as biofilm producers from the following: 41(47.13%) strongly, 33 (37.93%) moderately, and 11 (12.64%) weakly. While only two isolates 2 (2.3 %) represented non-biofilm producers. 100 and 91% of the isolates, respectively, had the *MrkD* and *FimH* biofilm formation genes, according to molecular analysis. A recent study showed biofilm formation by *K. pneumoniae* strains isolated from blood specimens could form stronger biofilms. On the other hand, stool specimens formed weaker biofilm compared to them. According to this study, multidrug-resistant (MDR) *K. pneumoniae* strains' capacity to form biofilms and their antibiotic resistance profile is positively correlated. These could aid in developing therapeutic therapies for infections brought on by *K. pneumoniae* resistant to carbapenems, considered the "final line of defense" antibiotics. We can infer from this work that *K. pneumoniae* could be isolated from many sources and was MDR, as well as having the different capacity to build biofilm in various ways, especially in hospital cases of high antibiotic resistance.
Keywords: *K. pneumoniae, Biofilm formation, MDR, PDR, XDR, Congo red*

INTRODUCTION

K. pneumoniae is a Gram-negative bacterium: rod, encapsulated, nonmotile, and produces a striking mucoid colony in solid media. It is also a significant member of the Enterobacteriaceae family. It can cause several infections, including gastrointestinal, skin, nasopharyngeal, osteomyelitis, biliary, and urinary tracts, as well as bacteremia. The virulence components of K. pneumoniae include a very large polysaccharide capsule that defends against bactericidal serum factors, the types I and III pili that stick to surfaces, adhesins, and siderophore, which are crucial to the pathogenicity of the organism. [1, 2]. In coliform species, the type1- and type3-encoding genes fimH and mrkD are linked to the development of biofilms. [3]. In recent years, K. pneumoniae has drawn the attention of researchers from all over the world due to the severity of its illness, resistance to numerous medicines, and the difficulties of treating it. K. pneumoniae has also established many mechanisms for resistance to various antimicrobials. Antibiotic therapy in patients with bacterial illnesses can result in bacterial eradication and hasten the healing process. [2, 4]. Antimicrobial resistance is caused by a variety of processes that compromise the effectiveness of treatment. According to the World Health Organization (WHO), antibiotic resistance is among the top three global health issues. [5]. A biofilm is an organized bacterial colony attached to biotic or abiotic surfaces and contained in a self-produced polymeric matrix. [6]. The reduced drug diffusion through the biofilm matrix and the physiological changes in bacteria brought on by the biofilm-containing environmental circumstances are the main causes of the increasing antibiotic resistance. [7]. K. pneumoniae to build biofilms can shield the infection from the immunological responses of the host and medicines, improving its persistence on epithelial tissues and surfaces of medical devices. [8]. According to several studies, the ability of K. pneumoniae to build biofilm and antibiotic resistance were significantly correlated [9]. Treatment for infections brought on by producer biofilms K. pneumoniae strains is more challenging than for others. [10].

MATERIALS AND METHODS

In this study, 200 clinical samples from different sources as specimens of (urine, blood, stool, and sputum) also as swabs of (vagina, burns, and wounds) were collected from November 2021 to the end of April 2022 via hospitals inpatients in Baghdad city as the follows: Baghdad Teaching Hospital, Teaching Laboratories Institute, Burns hospital-Medical city, Al-Kendy Teaching Hospital. To isolate K. pneumoniae, the samples were inoculated onto CHROM agar orientation medium (Himedia, India) and incubated at 37°C for 24h. Suspected blue colonies were transferred onto blood agar) Moreover, MacConkey agar media (Himedia, India) was incubated at 37°C for 24h. for further confirmation. The presumptive isolates were confirmed as K. pneumoniae based on biochemical tests, including IMViC (indole, methyl red, Voges-Proskauer, and citrate) and their characteristic reactions on triple sugar iron agar medium (TSI; Mast UK). After recognition, identification was confirmed using the Vitek2 system (BIOME'RIEUX/France).

Antibiotic susceptibility testing: Following the Clinical and Laboratory Standards Institute's [17] recommendations, the susceptibility to antibiotics of different K. pneumoniae strains was assessed using the Kirby-Bauer disk diffusion method. Nineteen different antibiotics were used in the antimicrobial susceptibility testing. These included Amikacin (AK 30 µg), Ampicillin (AM 25 µg), Trimethoprim-Sulfamethoxazole (SXT 75 µg), Ceftriaxone (CRO 10 µg), Cefotaxime (CTX 30 µg), Ceftazidime (CAZ 30 µg), Ciprofloxacin (CIP 5 µg), Levofloxacin (LEV 5 µg), Imipenem (IPM 10 µg), Meropenem (MEM 10 µg),

Gentamicin (CN10 µg), Piperacillin (PRL100µg), Tobramycin (TOB10 µg), Aztreonam(ATM 30 µg), Cefepime(FEP 30 µg), Tigecycline(TGC15 µg) and Rifampicin(RF 5 µg), (Bioanalyses/Turkey), Colistin (CL10 µg), and Tetracycline (TE 10 µg),(HiMedia/India) The plates were incubated at 37 °C for 24 hr, after which the widths of the inhibitory zones were measured in millimeters, and interpretation was made using [17]. MDR strains are resistant to at least three different classes of antibiotics. Potentially very drug-resistant individuals were those who were resistant to at least one agent in all but two or more antimicrobial groups (XDR). Potentially pan-drug-resistant strains showed complete resistance to all antimicrobial drugs (PDR). [10].

Detection of biofilm formation:

Quantitative microtiter plate method

An overnight culture at 37 °C in trypticase soy broth was prepared for each K. pneumoniae isolate. Subsequently, 20µl of cell suspension (via MacFarland protocol) was inoculated in sterile 96 well-flat bottom polystyrene microtiter plates containing 180µl of TSB. Negative control wells that contained 200 µl of un-inoculated TSB were included in each test. Incubation was done at 37 °C for 24 h. The wells were gently washed with 200µl phosphate-buffer saline (PBS) three times at 7.2 pH. The wells were dried in an inverted position. The biofilm mass was stained with 200µl of 0.1% crystal violet. The wells were gently washed with 200µl of distilled water triple times and dried in an inverted position. Finally, the wells were dissolved in 200µl of 30% glacial acetic acid to solubilize the stain. Biofilm mass optical density (OD) was measured using a microplate reader (BIO- TEK EL800, USA) at 595 nm. The OD cut-off (ODc) was defined as “three standard deviations above the mean OD of the negative control." All the isolates were classified based on the adherence capabilities into the following categories: non-biofilm producers (OD ≤ ODc), weak biofilm producers (ODc < OD ≤ 2xODc), moderate biofilm producers (2ODc < OD ≤ 4xODc), and strong biofilm producers (4xODc < OD). [8]

Qualitative congo red agar method:

Congo red agar (CRA) medium was prepared. The CRA plates were inoculated by the pure single isolated colony and incubated aerobically for 24-48 h. at 37°C. Black colonies indicated a positive result with a dry crystalline consistency. [11]. The experiment was performed in triplicate and repeated three times.

Molecular study

DNA Extraction

Following the ABIOpure extraction process, genomic DNA was extracted from bacterial growth. The quantity of DNA that was extracted was detected using a Quantus Fluorometer.

Molecular Detection of Biofilm Formation

The PCR reaction was performed for the detection of MrkD, FimH gene of K. pneumoniae by using specific primers for the detection of biofilm formation. Which was designed according to [12] from (Macrogen/ Korea) in lyophilized form, as shown in (Table)

Using 25µl of PCR reaction, the mixture of PCR amplification consisted (2µl) DNA templet, (12.5µl) of Green Master Mix that contains (Tag DNA polymerase, $MgCl_2$, deoxy nucleosides (dNTP), reaction buffer) (Promega/USA), (1µl) of primer forward, (1µl) of primer revers each primer for each specific gene, up to the final volume (25µl) with nucleases free water (8.5µl).

All tubes were vortexed and centrifuged briefly in the microcentrifuge (My Fugene/China) for 10 seconds to bring the contents to the bottom of the tubes.

The PCR tubes were transferred to the thermal cycler (Thermo Fisher Scientific/USA) to start the amplification reaction according to the specific program for each primer, as shown in (Table 2). Amplified products were examined by 1.5% agarose gel electrophoresis containing ethidium bromide (Promega/USA) in 1X TAE buffer. A100 bp DNA ladder (Promega/USA) was used as a molecular weight marker. The amplified DNAs were electrophoresed at 100 V for 60 min. The Ethidium bromide-stained bands in gel were visualized using a Gel imaging system. (Major Science/Taiwan)

Primers		**Sequences 5`-3`**	**Size**
fimH	F	TGCTGCTGGGCTGGTCGATG	550
	R	GGGAGGGTGACGGTGACATC	
mrkD	F	AAGCTATCGCTGTACTTCCGGCA	340
	R	GGCGTTGGCGCTCAGATAGG	

Table 1. The primers used in PCR for the detection of MrkD, FimH,

Steps	**Temperatures °C**	**Minute: sec-ond**	**Cycle**
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing of MrkD	60	00:30	
Annealing of *FimH*	58		
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

Table 2. The program of PCR for *FimH, MrkD*

Statistical Analysis

The mean, SE of mean, median, minimum and maximum were calculated using [18] SPSS 28.0. Utilizing an independent T-test, the likelihood was also investigated. The probability was calculated using Pearson's chi-square test for non-parametric data. To ascertain the connection between the investigated parameters, a Pearson's correlation was performed.

RESULTS

Prevalence of K. pneumoniae in different sources

The results showed (87) isolates of a total of 200 patients samples consisting of 47 (54.22%) male and 40 (45.97%) female. Most K. pneumoniae were isolated

from patients aged between 15-59 years old 75 (86.2%), equal up to more than 60 years old 9 (10.3%), less than 15 years old 4(4.6%).

The number and percentage of isolates according to sources were as follows specimens: 26(29.9%) urine, 25(28.7%) blood, 8(9.2%) stool, and 4(4.6%) sputum, from otherwise as swabs: 11(12.6%) burn, 9(10.3%) vagina, and 4(4.6%) wound.

Diagnostic

Were revealed as *K. pneumonia,* appearing on the following agar media: CHROM (mucoid metallic blue colonies), MacConkey (lactose fermenting colonies pink to red), blood (no hemolysis), and slant Triple Sugar Iron (TSI) (yellow color without H2S). (Figures 1,2,3,4).

The biochemical characters could identify K. pneumonia simply. They were positive for Voges Proskauer, Citrate utilization, and Urease tests. However, they produce negative reactions with Indole and Methyl red tests.

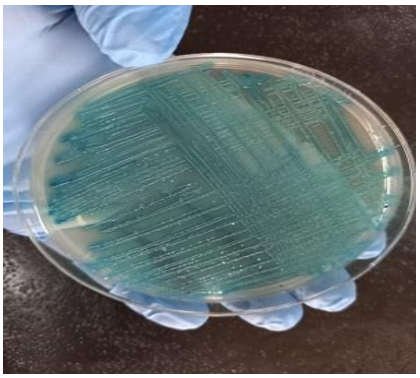

Figure (1) Growth of *K. pneumoniae* on CHROM agar media

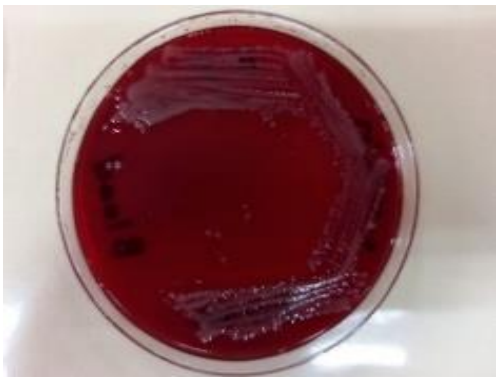

Figure 2. Growth of *K. pneumoniae* on blood agar media

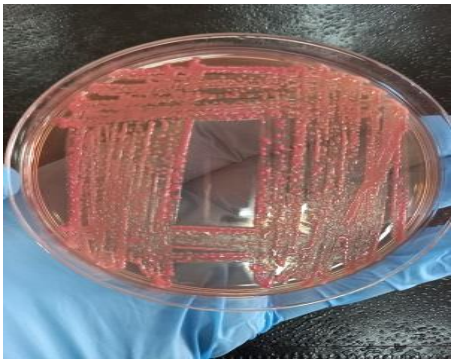

Figure 3. Growth of *K. pneumoniae* on MacConkey agar media

Figure(4) Growth of *K. pneumoniae* on TSI

Antimicrobial susceptibility results

The results explained that all K. pneumoniae clinical isolates test resistance to Rifampicin (*100*%) followed by Piperacillin (9*8.9*%), Ceftriaxone (9*8.9*%), Cefotaxime (*96.*6%), Ceftazidime (95.4%), Ampicillin (94.3%), Cefepime (92%), Trimethoprim (92%), Aztreonam (82.8%), Ciprofloxacin (71.3%), Tetracycline (69%), Tigecycline (62.1%), Tobramycin (62.1%), Levofloxacin (58.6%), Gentamicin (58.6%), Amikacin (50.6%), Colistin (44.8%), Meropenem (46%) and Imipenem (46%). A significant correlation was seen between percentages of

antimicrobial susceptibility rate of K. pneumoniae isolates against 19 antimicrobial agents (P value <0.001) and, in detail, classified from isolates as MDR- 23(26.44%), XDR- 49(56.32%), and PDR- 15(17.24%). Different antibiotic resistance was detected in various collected clinical samples. Blood and wound samples revealed the highest levels of antibiotic resistance. (Based on the source) against Cefotaxime, Piperacillin, Ampicillin, Ceftazidime, Ceftriaxone, and Rifampicin (based on the antimicrobial agents).

The biofilm formation ability of K. pneumoniae isolates

Biofilm production within the Congo red protocol was 87 (100%) isolates. Black colonies indicated a positive result with a dry crystalline consistency Figure (5). After that, biofilm production by 87 K. pneumoniae isolates was evaluated using the tissue culture plate (TCP) method (Figure 6). The results showed that 85 (97.7%) of isolates produced biofilm among them. 41 (47.13%) isolates were strongly biofilm producers, 33 (37.93%) moderately biofilm producers, and 11 (12.64%) isolates were weak. While only two isolates 2 (2.3 %) represented non-biofilm producers.

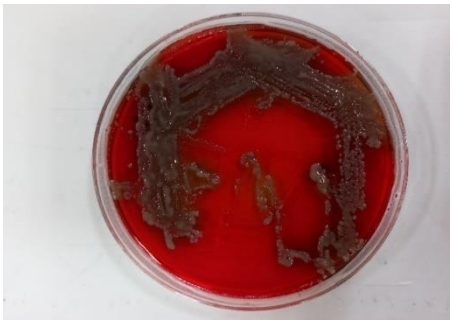

Figure 5. Biofilm formation by *K. pneumoniae* on Congo red agar medium.

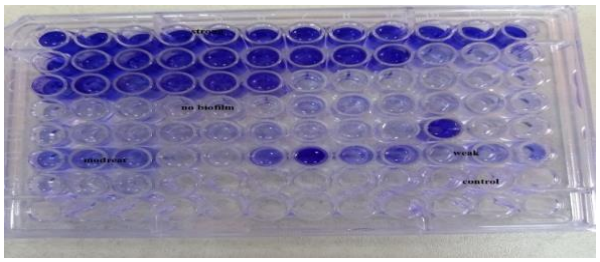

Figure 6. Biofilm formation of *K. pneumoniae*. by TCP method

According to the molecular distribution of biofilm formation genes among the isolates, MrkD and FimH were present in 100 and 91% of the isolates, respectively.

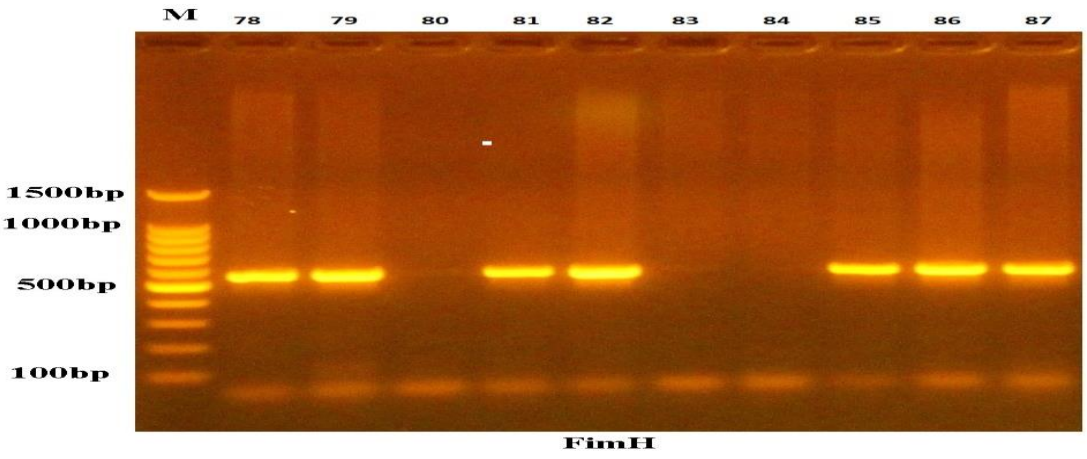

Figure 7. Agarose gel electrophoresis of PCR products *FimH* gene *K. pneumoniae*

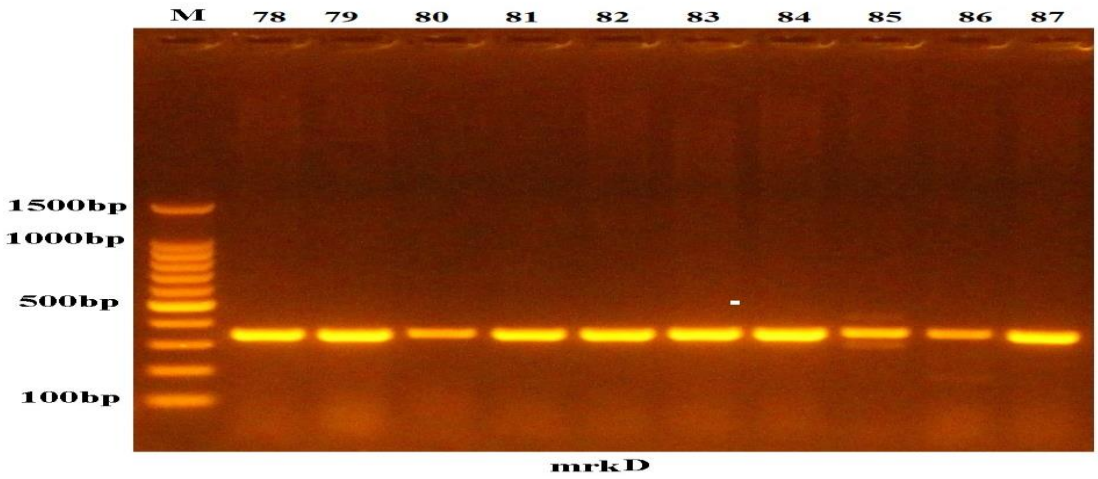

Figure 8. Agarose gel electrophoresis of PCR products *MrkD* gene in *K. pneumoniae*

Among different clinical samples, the weakly biofilm formation was seen in stool specimens, but blood specimens had the highest biofilm formation. Additionally, the specimens' sputum showed the highest moderate biofilm formation. A significant correlation was seen between the source of clinical samples, the rate of biofilm formation isolates in urine and blood specimens, and burn swabs. While no significant correlation was seen between the source of clinical samples and the rate of biofilm formation of isolates in vaginal swabs, in addition to stool and sputum specimens, the results are given in Table (3).

Source of sample	No of *K. p.*	Strong	Moderate	Weak	No. of bio-film	Probability
Urine specimen	26	16(61.5%)	6(23%)	4(15.4%)	0(0%)	P < 0.001
Blood specimen	25	16(64%)	8(32%)	1(4%)	0(0%)	P < 0.001
Burn swab	11	3(27.27%)	7(63.63%)	1(9.09%)	0(0%)	P < 0.001
vaginal swab	9	3(33.3%)	4(44.4%)	1(11.11%)	1(11.1%)	P > 0.05
Stool specimen	8	1(12.5%)	3(37.5%)	3(37.5%)	1(12.5%)	P > 0.05
Wound swab	4	0(0%)	4(100%)	0(0%)	0(0%)	—
Sputum specimen	4	2(50%)	1(25%)	1(25%)	0(0%)	P > 0.05

Table 3. Biofilm formation results of K. pneumoniae isolates.

Statistical analysis showed a meaningful correlation between biofilm formation and antibiotic resistance except for Piperacillin, Ceftriaxone, Cefotaxime, Ceftazidime, Tetracycline, Tigecycline, and Colistin. The results are given in Table (4).

Antibiotic susceptibility		Biofilm formation				Probability
		41 Strong	33 Moderate	11 Weak	2 No biofilm	
MEM	R	21 (51.2%)	17 (51.5%)	2 (18.2%)	0 (0%)	P < 0.05
	S	16 (39.0%)	13 (39.4%)	9 (81.8%)	2 (100%)	
CL	R	18 (43.9%)	17 (51.1%)	4 (36.4%)	0 (0%)	P > 0.05
	S	14 (34.1%)	10 (30.3%)	4 (36.4%)	2 (100%)	
TGC	R	25 (61%)	22 (66.7%)	6 (54.5%)	1(50%)	P > 0.05
	S	6 (14.6%)	4 (12.1%)	2 (18.2%)	0 (0%)	
AM	R	40 (97.6%)	32 (97.0%)	8 (72.7%)	2 (100%)	P < 0.05
	S	0 (0%)	1 (3%)	2 (18.2%)	0 (0%)	
TE	R	31(75.6%)	23 (69.7%)	5(45.5%)	1 (50%)	P > 0.05
	S	6 (14.6%)	5 (15.2%)	5(45.5%)	1 (50%)	
SXT	R	39 (95.1%)	30(90.9%)	9(81,8%)	2 (100%)	P < 0.05
	S	1 (2.4%)	3 (9.1%)	2 (18.2%)	0 (0%)	
AK	R	25 (61.0%)	17 (51.5%)	2 (18.2%)	0 (0%)	P < 0.001
	S	10 (24.4%)	11 (33.3%)	7 (63.6%)	2 (100%)	
ATM	R	37 (90.2%)	28 (84.8%)	6 (54.5%)	1 (50%)	P < 0.001
	S	1 (2.4%)	1 (3%)	3 (27.3%)	1 (50%)	
FEP	R	41 (100%)	29 (87.9%)	9 (81.8%)	1 (50%)	P < 0.001
	S	0 (0%)	2 (6.1%)	1 (9.1%)	1 (50%)	
IMP	R	23 (56.1%)	15 (45.5%)	2 (18.2%)	0 (0%)	P < 0.05
	S	16 (39.0%)	10 (30.3%)	8 (72.7%)	2 (100%)	
CIP	R	32 (78.0%)	26 (78.8%)	4 (36.4%)	0 (0%)	P < 0.001
	S	4 (9.8%)	6 (18.2%)	6 (54.5%)	1 (50%)	
LEV	R	29 (70.7%)	20 (60.6%)	2 (18.2%)	0 (0%)	P < 0.001
	S	4 (9.8%)	2 (6.1%)	6 (54.5%)	1 (50%)	
CTX	R	40 (97.6%)	33 (100%)	9 (81.8%)	2 (100%)	P > 0.05
	S	0 (0%)	0 (0%)	1 (9.1%)	0 (0%)	

CN	R	31 (75.6%)	18 (54.5%)	2 (18.2%)	0 (0%)	P < 0.001
	S	8 (19.5%)	13 (39.4%)	8 (72.7%)	1 (50%)	
TOB	R	29 (70.7%)	23 (69.7%)	2 (18.2%)	0 (0%)	P < 0.001
	S	10 (24.4%)	6 (18.2%)	9 (81.8%)	2 (100%)	
CRO	R	41(100%)	33 (100%)	10(90.9%)	2 (100%)	P > 0.05
	S	0 (0%)	0 (0%)	1 (9.1%)	0 (0%)	
CAZ	R	41(100%)	31 (93.9%)	9 (81.8%)	2 (100%)	P > 0.05
	S	0 (0%)	0 (0%)	1 (9.1%)	0 (0%)	
RA	R	41 (100%)	33 (100%)	11(100%)	2 (100%)	-
	S	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
PRL	R	41 (100%)	33 (100%)	10(90.9%)	2 (100%)	P > 0.05
	S	0 (0%)	0 (0%)	1 (9.1%)	0 (0%)	

Table 4. Correlation between antibiotic susceptibility test and biofilm formation of K. pneumoniae isolates

DISCUSSION

In this investigation, 87 of 200 clinical samples, or 43.5% of the total, included K. pneumoniae isolates. Given that K. pneumoniae is one of the most significant global contributors to MDR infections, this ratio should be cause for great worry. Male patients provided a greater proportion of the K. pneumoniae isolates included in this investigation than female patients., reporting that males were more likely than females to have K. pneumoniae infection. Also noted was that men were more likely than women to get a Klebsiella infection. The links between sex and the K. pneumoniae incidence were linked to unhealthy lifestyle habits like smoking and drunkenness. This result was in line with [5].

On the other hand, the isolates collected between 2021 and 2022 were also from urine and blood cultures where bacterial colonization or infections most likely occur, in agreement with [13]. Most K. pneumoniae were isolated from patients aged 15-59 (86.2%). The patients' demographics supported the theory that the bacterium primarily causes illnesses in elderly persons, with more than 60% of infections occurring in patients over 50. The most significant unavoidable risk associated with the emergence and spread of resistant K. pneumoniae, according to findings from other studies, such as [13], is senior age.

The high prevalence (56.32%) of isolates was classified as XDR, confirming that K. pneumoniae is frequently the cause of illnesses that are challenging to treat. The development of Beta-lactamase enzymes, which result in the hydrolysis of the Beta-lactam ring and the inactivation of Beta-lactam antibiotics, was the source of this resistance. [14] confirmed the same result.

The antimicrobial susceptibility profiles obtained in this study showed that the most active drugs are Meropenem, Imipenem, Colistin, and Gentamicin. The isolates resisted cephalosporin medicines from the third and even fourth generations, cefepime and piperacillin. This supports the research by [11], who

discovered Imipenem sensitivity to be 92.6%. Due to significant resistance levels, the same authors advised against cefotaxime, ceftazidime, and piperacillin.

There are different findings on the correlation between biofilm formation and the site of infection. Differences in geographical area and antibiotic use patterns can be the reason for this difference in prevalence. This study showed biofilm formation by K. pneumoniae strains isolated from blood and urine samples could form stronger biofilms, and stool specimens formed weaker biofilms. [15] gave a compatible explanation of the obtained results. Differences in the outcomes of various research can be attributed to the location, kind, and quantity of samples, as well as the features of bacterial isolates, such as antibiotic resistance trends. This commentary was clear in this study.

The recent research indicated that 97.7% of the isolates generated biofilms, which agrees with the study of [16]. Iran reported that 93.6% of K. pneumoniae isolates had the ability of biofilm formation, and 33% of them could produce biofilm strongly. [16] It has been proposed that the expression of various adhesions, their corresponding receptors, and exopolymeric components by various cell types within a biofilm community can affect how a biofilm develops. More specifically, [15] said many bacteria could use a quorum-sensing mechanism to regulate biofilm formation and other social activities.

In the present investigation, K. pneumoniae strains isolated from clinical samples carried many biofilm genes. Additionally prevalent in MDR strains were the genes linked to pathogenicity. Because K. pneumoniae attaches to the epithelial and endothelial cells of the urinary tract, Type 1 fimbriae (fimH) and Type 3 fimbrial adhesion (mrkD) are the most prevalent bacteria cell adhesive agents. The results agree with [4.]

Our findings showed that compared to non-biofilm producers, K. pneumoniae had a higher level of antimicrobial agent resistance. This finding is confirmed by [10], explaining antimicrobial resistance in biofilm production. However, biofilm production has been noted in MDR, XDR, and PDR strains, and weak and moderate biofilm producers have also been noted.

Further research is needed to elaborate novel concepts in preventing nosocomial K. pneumoniae infections because various processes are implicated. On the other side, researchers such as [14] refer to high relationships between antibiotic resistance and severe trauma in the diabetic case study, for example.

CONCLUSIONS

All K. pneumoniae isolates that produce biofilm give resistance to a variety of antibiotics, but not all antibiotic-resistant isolates can form a biofilm. This may be due to other virulence factors. Sharply increasing mortality led to the elimination of the microorganisms born in hospitals and prevented the spread of multidrug-resistant germs. Finally, K. pneumoniae isolates, even multidrug-resistant, may not have to be biofilm productive.

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