Uttar Pradesh Journal of Zoology

Volume 45, Issue 11, Page 247-255, 2024; Article no.UPJOZ.3453
ISSN: 0256-971X (P)

Assessment of Biofilm Production and Antibiotic Sensitivity Patterns in *Klebsiella pneumoniae* Isolates from Al-Qadisiyah Hospitals, Iraq

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.56557/upjoz/2024/v45i114091

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://prh.mbimph.com/review-history/3453

Original Research Article

Received: 14/02/2024
Accepted: 18/04/2024
Published: 14/05/2024

ABSTRACT

Background: Klebsiella pneumoniae is one of the most common causative agents of nosocomial infections. Opportunistic pathogens can generate a thick layer of biofilm as an important virulence factor.
Objectives: The current study was aimed in the detection of biofilm formation in Klebsiella pneumoniae pathogenic capability as a common opportunistic pathogen accounting pneumonia, urinary tract infections, with in nosocomial infections.
Materials and Methods: In this observational study, a total of 140 clinical samples obtained from

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Cite as: *Muhammed, H. A., Al-Mosawi, H. S. M., & Aldawmy, F. A.-A. K. (2024). Assessment of Biofilm Production and Antibiotic Sensitivity Patterns in Klebsiella pneumoniae Isolates from Al-Qadisiyah Hospitals, Iraq. UTTAR PRADESH JOURNAL OF ZOOLOGY, 45(11), 247–255. https://doi.org/10.56557/upjoz/2024/v45i114091*

patients with bacterial infections were analyzed. The identification of Klebsiella pneumoniae isolates was performed using selective culture media and biochemical tests. Additionally, biofilm strains were characterized using the Crystal Violet assay and polymerase chain reaction (PCR) techniques.
Results: Among the 140 samples collected from various specimens, a total of 100 isolates (43.47%) were identified as Klebsiella pneumoniae culturing and biochemical tests. Out of these isolates, 58 (58%) were obtained from male individuals, while 42 (42%) were obtained from female individuals. Using the phenotypic method, the analysis revealed that 18% isolates were classified as strong biofilm producers, 33% as medium biofilm producers, 49% as weak biofilm producers, and 30 as non-biofilm producers. The frequency of specific genes in the isolates was reported as follows: wzm (47%) and markA (69%).
Conclusion: The presence of the markA gene is significant in the context of biofilm formation in Klebsiella pneumoniae strains, as it serves as a marker for distinguishing various types of biofilms.

Keywords: Klebsiella pneumonia; biofilm formation; PCR.

1. INTRODUCTION

Klebsiella pneumoniae, a gram-negative bacillus belonging to the Enterobacteriaceae family, is naturally present in the human intestinal microflora. However, in the hospital setting, it has emerged as an opportunistic pathogen of significant concern. It is recognized as one of the key bacteria associated with nosocomial infections, causing a range of diseases including urinary tract infections, septicemia, pneumonia, and intra-abdominal infections in patients admitted to various departments within the hospital [1]. Currently, the bacteria Enterobacteriaceae have faced an increasing threat of antibiotic resistance across the globe, especially among Klebsiella pneumoniae bacteria that proliferate mostly in hospitals and compromise a patient's health. In addition, the pathogenicity of Klebsiella pneumoniae rises every year and this is due to different factors like weak host immune system being acquired through long and complicated surgical procedures [2], and massive use of different medications for treatment With K. pneumoniae targeting mostly those with compromised immunities, the ongoing antibiotics' resilience that has been observed in this bacterium is worrisome, therefore, clearly pinpointing the antimicrobial activity trends as regards prevailing pathogenic bacteria becomes the necessary step for constructing the detailed treatment options against the corresponding bacteria [3]. On the other hand, the global spread of *Klebsiella pneumoniae* strains with multiple resistance has become a serious risk Multidrug Resistance are a novel phenomenon infections, particularly it pose a serious challenge in hospitals [4]. The genus Klebsiella pneumonia was recognized as an important contributor toward hospital setting pathogenicity due to its distinct biofilm forming capability. With biofilms, microbes are closer to one another which lead to additional resistance to host defenses and antimicrobial tools [5]. The ability of Klebsiella pneumoniae to form biofilms confers protection from the host immune system and antibiotics (Ammar et al., 2020), allowing it to persist outside of endothelial tissues and medical devices. Several virulence factors found in *Klebsiella pneumonia* associated with biofilm formation, include genes associated with the CPS group [6], type 3 fimbriae (mrk) Alcantar-Curiel et al., [7], and wbbM and wzm genes, these genes contribute to biofilm [8]. On the other hand, Klebsiella pneumoniae can also be considered highly virulent, given its ability to produce biofilms as well as an impressive repertoire of genes that include wzm, mrkA, genes which responsible for biofilm formation, so the aim of study was determined phenotypic and genotypic of some strains of *K. pneoumoina* isolates from patient suffering from urinary tract infection.

2. MATERIALS AND METHODS

2.1 Identification and Antimicrobial Susceptibility Assessment Methods

From January 2022 to May 2022, an observational study was conducted in Al-Qadyssia city, Iraq, involving patients admitted to Al-Qadyssia hospitals. Both male and female patients were included in the study. Clinical samples, including urine, wound, blood, and stool, were collected for analysis. The samples were cultured on various agar media such as Eosin (EMB agar), blood agar, McConkey agar, and CHROMagar™ Orientation/ Himedia India. Incubation of the samples took place at 37°C for 41 hours, with the addition of Merck (Methylene Blue). Differential biochemical tests, including

urease, Simon Citrate, (Sulphide Indole Motility) SIM, and (Methyl Red-Voges-Proskauer) MR/VP were performed using TSI (Triple Sugar Iron). The collection of bacterial cultures through which the isolates were identified and characterized were similarly carried out for Klebsiella pneumoniae isolates. Afterwards, selected Klebsiella pneumoniae isolates were tested for antibiotic sensitivity through the disk diffusion method, the widely known disk diffusion method was used [9]. This examination done on medium of culture, is specifically (Muller himonto Agar/ Himedia india). The disks where different antibiotics at determined concentrations were displayed included azithromycin (15ug), ciprofloxacin (15ug), Meropenem (10ug), Amikacin (10ug), cefotaxime (30ug) Tetracycline (10 ug) and levofloxacin (5ug) To carry out the swabbing procedure, sterile swabs were used followed by inoculation of samples into three direction on the Mueller Hinton agar medium. Spreading it in over the medium occupied 48 min. The antibiotic disks were put on it afterward, there were appropriate distances between them. Following incubation at 37 C for 24 hours, the diameter of the resulting growth inhibition zones or absence of growth was measured using a ruler. The obtained results were then interpreted according to the relevant guidelines, specifically those provided by CLSI (Clinical and Laboratory Standards Institute), to classify each antibiotic as "susceptible," "Intermediate," or "resistant." isolates.

2.2 Microtiter Plate Phenotypic Methods to Determine Biofilm Formation

The microtiter plate phenotypic method was utilized in this study to assess the biofilm formation capability of the Klebsiella pneumoniae strains. Initially, 400 isolated colonies were incubated overnight at 37 ^{0}C. Subsequently, monocolonies of each *Klebsiella pneumoniae* isolate were transferred to a 0% medium containing 4% glucose TSB, and the optical density was measured at a wavelength of 493 nm. For the negative control, 400 microliters of each sample was added to six parallel wells in a 96-well microplate, where TSB was used instead of the medium. After 41 hours of incubation at 37 degrees Celsius, the contents of the wells were carefully aspirated and washed with sterile physiological saline. Pure methanol (400 microliters) was added to the wells and allowed to bind the bacteria to the bottom and sides of the well at room temperature for 40 minutes. Subsequently, the methanol was removed, and 4% crystal violet (400 microliters) was added to each well and incubated at room temperature for 40 minutes. Excess dye was then removed by gently washing the plate with distilled water, followed by air drying. Generally, 400 ul of the absolute ethanol was dropped into each of the wells at the end and the amount of colorless released dye in every well was measured using the ELISA reader (Synergy H1 BioTek) at a wavelength of 630 nm. Here in this method negative control sample is places where TSB has been used instead of medium for biofilm formation measuring. In order to avoid mistakes to certain extent, each isolate's optical absorption was examined three times. Such measurements resulted in the figures presented in Table 1 [10]. These represented the degree of biofilm formation for each isolate.

2.3 Molecular Assay to Determine Biofilm Formation

The DNA of *Klebsiella Pneumonia* has been extracted stochastically, as first the boiling method was used. Five colonies of bacteria grown by BHI broth as culturing medium, were mixed with 400 microliter of sterile distilled water and streaked onto the surface of the newly prepared BHI agar plate. The colonies had been centrifuged at 8000 rpm for 8 minutes and the cells were found the buffer solution by the use of distilled water. By removing the supernatant for the next step, 400 microliters of the preconditioned bacterial culture was transferred to a microtube and vigorously agitated. The resulting suspension was treated with NaOH for 30 minutes and then placed in a Bain-Marie at 99 C^o. After cooling, 80 microliters of Tris-base solution were added to the tubes, followed by centrifugation at 44,000 rpm for 8 minutes. The supernatant containing the Pure DNA was then transferred to sterile 1.5 mL Microcentrifuge Tube, to confirm the presence of total DNA, a nanodrop device was used to measure the absorbance at two wavelengths, 480 nm and 490 nm, after the extraction process.

PCR amplification of specific genes was performed using a BIO-RAD thermocycler from the USA. The primer binding temperature for the wzm gene was set at 58^{o}C, while the mrkA gene had a binding temperature of 55^{o}C. The thermocycler was utilized to carry out an initial denaturation step at 98^{o}C for 90 seconds, followed by annealing temperature at the specified temperatures for 90 seconds.

Subsequent amplification was accomplished at 74°C for 30 seconds and final extension 74°C for 5 minutes. The resulting PCR products were then subjected to electrophoresis on a 2% agarose gel, then gel image was performed using a Gel Doc device from Cleaver Scientific/ Uk, and the PCR bands were observed and photographed under the influence of UV rays at a wavelength of 430 nm.

3. RESULTS AND DISCUSSION

Among 140 different clinical samples, 100 of *Klebsiella Pneumonia* isolates were diagnosed, the frequency of *Klebsiella pneumonia* collected from different clinical samples can be seen in the table 1. The distribution of patients' ages was examined and is presented in Table 1. The age variable was categorized into different age ranges, ranging from 21 to 60 years. The table provides counts and percentages for each age group; the most represented age range among the patients was observed in the 31-40 age group, accounting for the highest count and percentage of patients (e.g., 34 patients, 34% of the total sample); The age range of 31-40 years is commonly associated with higher sexual activity compared to other age groups. Sexual intercourse can introduce bacteria into the urinary tract, increasing the risk of UTIs. Factors such as multiple sexual partners or inconsistent use of preventive measures (e.g., condoms) may further contribute to the higher prevalence of UTIs in this age group [11].

Table 1. Interpretation of biofilm production OD value of *Klebsiella pneumoniae* strains

Category	Classification
Non-biofilm producers	OD ≤ ODc
Weak biofilm producers	ODc < OD ≤ 2 × ODc
Moderate biofilm producers	2 × ODc < OD ≤ 4 × ODc
Strong biofilm producers	4 × ODc < OD

Table 2. Primer sequences of mrkA and wzm in *Klebsillae pneumoniae*

Primers	5-3 direction sequences	Tm	PCR Product	References
mrkA	F ACGTCTCTAACTGCCAGGC R TAGCCCTGTTGTTTGCTGGT	55	154 bp	Vuotto et al. [10]
wzm	F TGCCAGTTCGGCCACTAAC R GACAACAATAACCGGGATGG	58	129 bp	Vuotto et al. [10]

Tm Mealting Temperature; F Forword Primers; R Reverse Primers

Table 3. Distribution of gender, age and residence with sexual partner among patient infected with *K. pneumonia*

Variables		Group Patients (N=100)		Sexual partner Polygamy mirrage	
		Count	%	Count	%
Gender	Male	42	42	30	71.42
	Female	58	58	4	6.89
	Total	**100**	100	34	34
Age (year)	21-30	32	32	6	18.75
	31-40	34	34	17	50
	41-50	25	25	9	36
	51-60	9	9	2	22.22
	Total	100	100	34	34
Residency	Urban	63	63	14	23.8
	Rural	37	37	20	54.05

Total	100	100	34	34

The most common infected was found in female as (58%) rather than male (42%) on the other hand the study found the prevalence of K. pneumonia was increased in male have sexual partners as Polygamy mirage (71.42%) compare with female (6.89%), the present study noticed that the infection in women was in women who were not related to multiple marriages, and one of the reasons that played a major role in the increased infection in women compared to males is the small size of the urethra [12], as well as hormonal fluctuations, particularly in women, can affect the pH balance and natural defenses of the urinary tract, making it more susceptible to bacterial colonization and infection [13].

On the other hand, the current study found that the Urinary tract infection increases in polygamous mirage patients and in urban areas by 50% more than in rural areas, and this is due to several reasons, In urban cities, people tend to live closer together compared to rural areas. This close proximity and multiple partners can make it easier for bacteria to spread from person to person more quickly. The dense population in urban settings as well as polygamy marriage can facilitate the transmission of bacteria that can lead to urinary tract infections (UTIs) [14] Clark et al., [15] illustrated that certain city ne-ighborhoods suffer from excessive- population density and insufficient sanitation facilities. This can le-ad to bacterial proliferation. Irresponsible- waste disposal, for instance, contaminates wate-r supplies and elevate-s UTI rates.

The agar medium of K.pnemonia on agar CHROMagar™ appear blue and mucoid colonies, The me-dium, CHROMagar™, is helps separate and different bacterial types, it works for Klebsiella pneumonia with particular components cause colors when bacteria multiply. On this agar, *Klebsiella pneumoniae* appears blue color and the media holds substance-s which the bacteria break down. Whe-n this happens, enzymes are- produced—changing the colonies' color to blue Fig. 1.

The results of antibiogram test by disk diffusion method Regarding the samples, they showed that out of seven discs Antibiotics used, the most and the least sensitive antibiotics are better in Ciprofloxacin (77%) and Meropenem was (63%) Fig. (2), the distance of each line from the center of the chart and the shape of the lines, the farther the line is from the center, the higher the sensitivity of the K. pneumonia to that Ciprofloxacin antibiotic disc (10 mg). Similarly, the closer the line is to the center, the lower the sensitivity in tetracycline (10 mg) it was recorded as (29%) in all *K. pneumonia* isolates.

Based at the information furnished, it appears that some K. pneumonia inside the radar chart are sensitive to ciprofloxacin. The sensitivity of The results of the current study regarding the antibiotic tetracycline are consistent with the results Ahmadi et al. [16] in Iran, as the rate of microorganism to a specific antibiotic like bacterial resistance to this antibiotic was (%70) ciprofloxacin can depend on different factors, which include the mechanisms of movement of the antibiotic and the precise characteristics of the bacteria being tested, Ciprofloxacin is a vast-spectrum antibiotic that belongs to the fluoroquinolone magnificence. It works by way of inhibiting the enzymes required for bacterial DNA replication, thus preventing the bacteria from multiplying and causing an infection, this mechanism of action makes ciprofloxacin effective in opposition to a huge variety of microorganism [17]. This study was agreement with Wang et l., [18] who was illustrated that extensively used quinolone antibiotics, which encompass ciprofloxacin, play a tremendous position in K. Pneumoniae treatment. On the other hand 63% of all K. pneumonia were sensitive to meropenem antibiotics, Meropenem is specifically effective against a wide range of Gram-negative bacteria, making it a valuable remedy choice for infections due to these pathogens, Meropenem belongs to the class of antibiotics referred to as carbapenems. It exerts its antibacterial impact by means of inhibiting the synthesis of bacterial cellular walls, leading to the disruption of cell wall integrity and ultimately bacterial cellular demise [19].

The results of the current study regarding the antibiotic tetracycline are consistent with the results Ahmadi et al [19] in Iran, as the rate of bacterial resistance to this antibiotic was (%70) these researchers showed that most of the bacterial isolates resistant to tetracycline were productive by efflex pumps [20].

In the final analysis of the isolates Biofilm producer by phenotypic method, 18% of the isolates have binding ability Strong biofilm formation, 33% intermediate biofilm binding ability and 49 % biofilm binding ability were weak

in all *K. pneumonia* isolates Fig. (3 and 4).

The frequency of presence of biofilm-causing genes in *Klebsiella pneumoniae* clinical isolates was estimated to be 22 % wzm gene, 28 % markA gene (Table 3).

Fig. 1. Blue colonies of K. pneumonia on medium, CHROMagar™

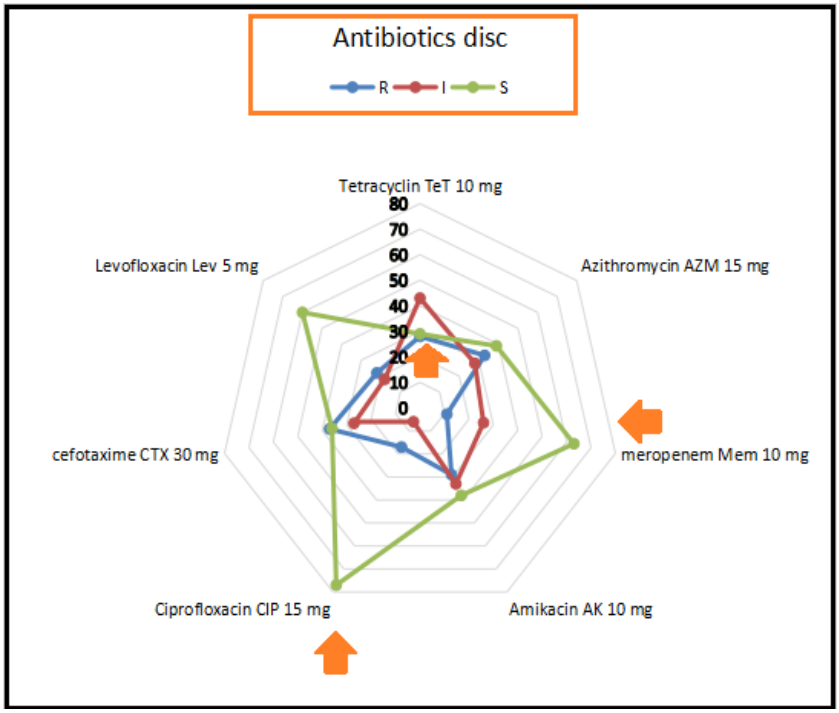

Fig. 2. Radar chart for *K. pneumonia* isolates for sensitivity to 7 different antibiotics

In the Table (3) all K.pneumonia with weak biofilm formation (No. 49) not give positive results by PCR assay; while all K. pneumonia (No. 18) with strong biofilm formation isolates have two genes (*mrkA*& *wzm*) in their chromosomes, on the other hand just 4 bacterial isolates with intermediate biofilm formation not have *wzm* gene in their chromosome, The mrkA

gene is part of the type 3 fimbriae gene cluster, which encodes structural features of fimbriae. Fimbriae are hair-like structures on the bacterial surface that allow them to adhere to surfaces and facilitate the initial steps of biofilm formation, the MrkA gene is involved in the assembly of fimbriae type 3 and are expressed, contributing to K. pneumoniae adhesion to surfaces and subsequent biofilm formation Mahmood & Abdullah, [21] On the other hand, the wzm gene is related to the biosynthesis of the capsular polysaccharide (CPS) in K. Pneumoniae. CPS is a key factor of the bacterial cell envelope and plays a crucial position in biofilm formation. The wzm gene is concerned in the transportation of CPS components across the bacterial membrane, taking into account the meeting and secretion of CPS, which contributes to the formation of the biofilm matrix [22].

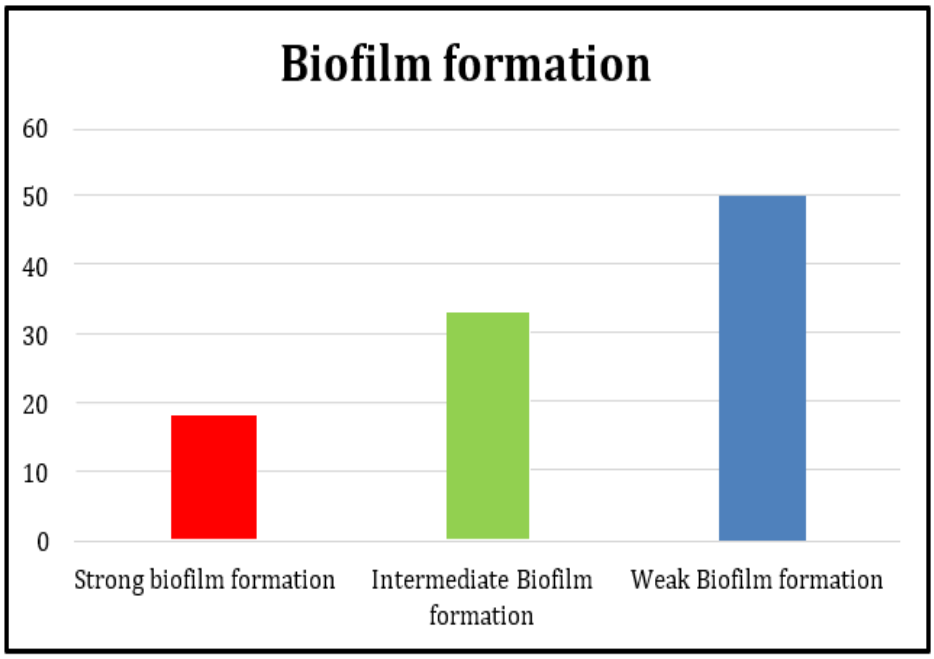

Fig. 3. distribution of K. pneumonia isolates with biofilm formation

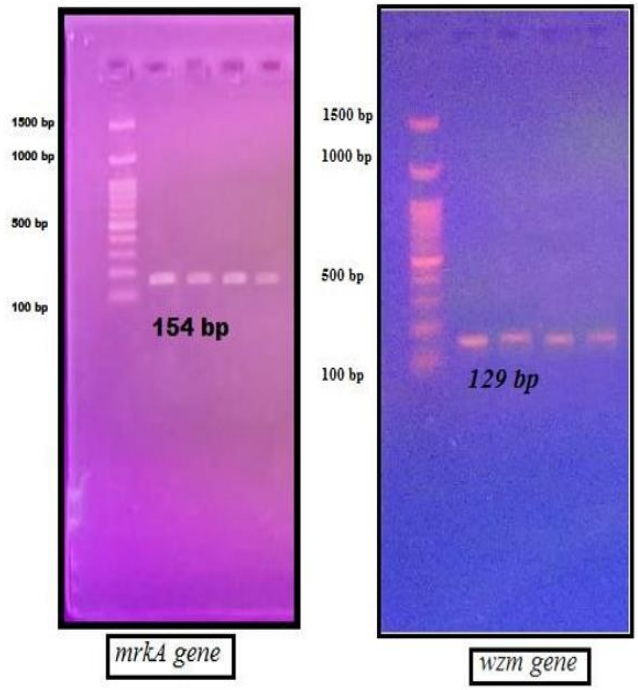

Fig. 4. Showed mrkA and wzm gene among *K. pneumonia*

Table 4. PCR assay with biofilm formation among *Klebsiella pneumonia* isolates

PCR		Biofilm formation		
		Strong	**Intermediate**	**Weak**
mrkA	+	18	33	0
gene	-	0	0	49
Wzm	+	18	29	0
gene	-	0	4	49

4. CONCLUSION

Klebsiella pneumoniae can be considered highly virulent, given its ability to produce biofilms as well as an impressive repertoire of genes that include wzm, mrkA, genes which responsible for biofilm formation, so the aim of study was determined phenotypic and genotypic of some strains of *K. pneoumoina* isolates from patient suffering from urinary tract infection.

ETHICAL APPROVAL

The research is evaluated to ensure that it meets ethical standards from ethical committee in college of medicine, University of Al-Qadisiyah.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://prh.mbimph.com/review-history/3453