Detection Of Biofilm Formation By Beta- Lactam Resistance *Klebsiella Pneumoniae* Isolated From Clinical Specimens And Aquatic Samples

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Abstract

Klebsiella pneumoniae causes a wide range of infection and it is found in different natural habitats like surface waters. It has become increasingly resistant to antibiotics, and the capacity of these bacteria to form biofilm is well known. This study aimed to determine the pattern of antibiotic resistance and biofilm-forming ability of *K. pneumoniae* isolated from clinical specimens and aquatic samples. Sixty- six (20.625%) *Klebsiella pneumoniae* clinical isolates were isolated from 320 clinical samples from Medical City Iraq, Baghdad. A total of 27 (18%) water isolates were gathered from 150 different water sources in the city of Baghdad. HiCrome UTI agar and biochemical testing were used to identify every isolate. The results show that the clinical isolates were more resistance to beta lactam antibiotics than water isolates and both of them were (100 %) resist to Amoxiclav. The resistance percentage of the clinical isolates was (6.06%) Meropenem, (13.63%) Imipenem, (19.69%) Ceftazidime, (53.03%) Cefotaxime and (86.36%) Ceftriaxone, while the resistance percentage for the water isolates was zero for the Meropenem, Imipenem, Ceftazidime, Cefotaxime and Ceftriaxone. The *infB* gene was used in molecular analysis to identify the beta- lactam antibiotic resistant isolates, and microtiter plate analysis was used to determine which isolates were most capable of forming biofilms. The results show a significant difference between biofilm formations among clinical and aquatic isolates and the aquatic isolates were more than clinical isolates in biofilm formation. The biofilm formation percentage among the most resistant for beta- lactam clinical isolates was (1 isolate (10%) strong, 2 isolates (20%) moderate, and 7 isolates (70%) weak), while the percentage for aquatic isolates was (2 isolates (20%) strong, 5 isolates (50%) moderates, and 3 isolates (30%) weak). This study showed that the aquatic isolates of *K. pneumoniae* are more than clinical beta- lactam resistant isolates in biofilm formation.

Keywords: Klebsiella pneumoniae, antibiotics resistance, beta- lactam antibiotics, biofilm, Aquatic bacteria.

Introduction

Microbial biofilms are sticky (EPS) exopolymeric substances that cause adhesion of microorganisms to abiotic surfaces, including those of medical equipment and biotic surface such as host cells causing antimicrobial resistance [1,2,3]. Biofilms play a significant role in the development of antimicrobial drug resistance because bacteria found in biofilms are more drug-resistant than other bacteria. Biofilm infections are 10–1000 times more resistant to antibiotic and antimicrobial effects than planktonic cells [4, 5, 6].

The Enterobacteriaceae family, which comprises saprophytes frequently isolated from the environment, includes *Klebsiella*. The most clinically relevant species of *Klebsiella* is *Klebsiella pneumoniae* and it causes more than 70% of human infections [7,8,9]. It is well known to be the cause of community-acquired infections, and more recently, it has also been noted to be a significant contributor to hospital-acquired pathogens. They are also quickly developing into multidrug- resistant (MDR) strains, which frequently provide a major hazard to patients due to a greater fatality rate brought on by the decreased efficacy of current therapeutic alternatives [10,11,12].

Extended spectrum -lactamase (ESBL)-producing MDR bacteria and increased *Klebsiella* carriage both are a result of extensive use of broad-spectrum antibiotics in hospitalized patients [13,14,15]. *K. pneumoniae* Carbapenem-resistant infections are challenging to treat due to the organisms' common resistance to multiple antibiotics, the patients' significant co-morbidities, and the fact that they now pose a serious risk to public health in many developed countries throughout the world [16, 17, 18].

Together with rising income and more expensive lifestyles, water pollution is becoming a significant issue on a worldwide scale [19, 20, 21]. Since untreated water intake may occur and this water may be contaminated with microbiological species like *Klebsiella*, it is necessary to frequently evaluate the levels of contamination with *Klebsiella* species in water [22, 23, 24]. *K. pneumoniae's* capacity to build biofilm shields it from both host immune responses and medicines, improving its survival on epithelial tissues and surfaces of medical devices [25, 26, 27]. At the end of the 1980s, the first *K. pneumoniae* strain to produce biofilms was reported [28, 29, 30]. It is more difficult to treat infections produced by *K. pneumoniae* strains that may form biofilms than infections caused by planktonic organisms. It is

particularly difficult to get rid of biofilms because the bacteria within them may resist phagocytosis [1, 31, 32].

With this study, we attempted to isolate *K. pneumoniae* from aquatic samples and clinical specimens, detect their ability to resist beta- lactam antibiotics, detect their ability to produce biofilm, and study the relationship between antibiotics resistance among aquatic and clinical isolates that resist beta- lactam antibiotics.

Materials and Methods

1- Bacterial isolates: From March to May 2019, 320 different clinical specimens (blood, burn, urine, sputum, wound, lung fluid and ear swa) were collected at Medical city and 150 aquatic samples were collected by sterile well screw cub were taken from different places in Baghdad city, Iraq. These samples were collected 30 cm underneath the water's surface, carried on ice, and within 4 hours of collection, treated for bacteriological examination [33]. All clinical specimens and aquatic samples were tested for biochemical tests to differentiate among *Klebsiella* species [34] and HiCrome UTI agar to distinguish it from other Enterobacteriaceae genera [35].

2- Test for antimicrobial susceptibility

Antibiotic minimum inhibitory concentrations (MICs) were measured using the agar dilution technique [36] by using 6 beta- lactam antibiotics ((Imipenem, Meropenem, Amoxiclave, Cetazidime, Ceftriaxone and Cefotaxime).

3- Molecular detection of bacterial isolates

The *infB* gene utilized for *K. pneumoniae* isolate diagnosis was used to validate the biochemical diagnosis and for future molecular investigations.

3**.1 DNA Extraction:** According to the ABIOpure Extraction/USA technique, genomic DNA was extracted from bacterial growth.

3.2 Quantitation of DNA and electrophoresis: The concentration of extracted DNA was detected by Quantus Flurometer. (199 µl) of diluted QuantyFlour Dye was combined with (1µl) of DNA. DNA concentration measurements were found after 5 min of incubation at room temperature, forward primer sequence of *infB* is 5`-CTCGCTGCTGGACTATATTCG-3 and that of reverse is 5`-CGCTTTCAGCTCAAGAACTTC-3` with (500bp) as product length with annealing temp.(50C). The existence of amplification was verified using agarose gel electrophoresis. PCR was entirely reliant on the retrieved DNA standards. Ethidium bromide (10 mg/ml), 1 X TAE buffer, and DNA ladder marker (100 bp) were the solutions utilized.

4- Detect biofilm formation: The capability of the bacteria to produce biofilm was determined using a microtiter plate assay [37].

Tryptic Soy Broth (TSB) with 1% glucose addition and sterile flat-bottomed 96-well microtiter plates with lid were used as a medium for biofilm cultivation. The bacterial broth culture used to make the inoculum for the biofilm growth was diluted 1:100 in TSB supplemented with 1% glucose before being placed into the well with 200 µl the only thing in the negative control wells is broth: Each well received 200 µl of TSB augmented with 1% glucose and each strain at least in triplicate. The lid was placed on the inoculation plate, and incubated aerobically for 24 - 30 hr. at (35–37C) in a static state, wash 3 times with sterile phosphate-buffered saline (PBS; pH 7.2), coated with 150 µl of crystal violet (1%) for (15 min) at room temperature, washed and excess stain ought to be rinsed off by tap water. 150 µl of (95%) ethanol per well was added for dye resolubilizion. Using a microtiter- plate reader (GloMax (Promega/ USA)), at 570 nm, the optical density (OD) of each crystal violet-dyed well is calculated. The strains can be categorized using the following categories

No biofilm is produced when OD≤ ODc, mild biofilm is produced when ODc < OD ≤ 2× ODc, moderate biofilm is produced when 2× ODc < OD≤ 4× ODc, and strong biofilm is produced when 4× ODc < OD.

5- Statistical Analysis:

The influence of numerous factors on the study parameters was evaluated using the Statistical Analysis System-SAS [38] software, and the Chi-square test was utilized to compare percentages (0.05 and 0.01 probability) in a significant way.

Results and Discussion

1- Bacterial isolates: Sixty-six clinical *K. pneumoniae* isolates were isolated from 320 different clinical specimens with ratio (20.625%). Twenty- seven water *K. pneumoniae* isolates were isolated from 150 aquatic samples with ratio (18%) as previously recorded by (39).The outcomes of the biochemical tests used to diagnose of clinical and aquatic isolates are shown in the table 1.

Table 1: The biochemical tests results for *K. pneumoniae*

Biochemical tests	Results
Gram stain	G-ve bacilli
Growth on MacConkey agar	Pink (lactose fermented), large, mucoid colony
Growth on EMB agar	Pink, large, mucoid colony without green

	metallic sheen
Indol	_
MR	_
VP	+
Simmone citrate	+
Motility test	Non- motile
HiCrome UTI agar	Metallic blue colony

An enzymatic reaction results in a colorless substrate in chromogenic agar changing to a recognizable color [40]. Approximately 14–20% of infections involving the respiratory system, lower biliary duct, surgical wounds, and urinary tract are caused by *K. pneumoniae* and become a relevant healthcare- association pathogen [41].

Environmental isolates and human clinical isolates have been identical in terms of virulence and biochemical mechanisms. Although environmental settings are considering potential breeding grounds for bacteria that may infect humans and animals, it is not immediately clear how important *Klebsiella* is for medical purposes [42].

2- Antibiotics resistance and MIC determination

The results of six antibiotics by agar dilution method for clinical and aquatic *K. pneumoniae* isolates as previously reported by [39]. The results show that the clinical isolates were more resistance to beta lactam antibiotics than water isolates and both of them were (100 %) resist to Amoxiclav. The resistance percentage of the clinical isolates was (6.06%) Meropenem, (13.63%) Imipenem, (19.69%) Ceftazidime, (53.03%) Cefotaxime and (86.36%) Ceftriaxone, while the resistance percentage for the water isolates was zero for the Meropenem, Imipenem, Ceftazidime, Cefotaxime and Ceftriaxone. The findings demonstrate that there are significant variations in the beta- lactam antibiotic resistance between clinical and aquatic isolates. More resistance exists in clinical isolates than in aquatic isolates.

3- Molecular Detection of the isolates

Twenty- eight *K. pneumoniae* isolates (14clinical and 14water) that have the highest resistance to the six beta- lactam antibiotics employed in this investigation were detected by PCR technique by using *infB* gene for diagnosing *K. pneumoniae*. The isolates that were chosen for detection by *infB* gene were named as (1, 2, ... , and14) for clinical isolates and (15, 16, ..., and 28) for aquatic isolates (figure 1).

Using a Quantus Flurometer, the DNA concentration (ng/µl) in the extracted DNA from clinical and water *K. pneumoniae* isolates was determined. The result for clinical isolates was (16, 22, 20, 18, 16, 19, 15, 23, 25 and 30 ng/µl) respectively, while the result for aquatic isolates was (28, 14, 17, 20, 25, 30, 22. 23, 18, 18 ng/µl) respectively.

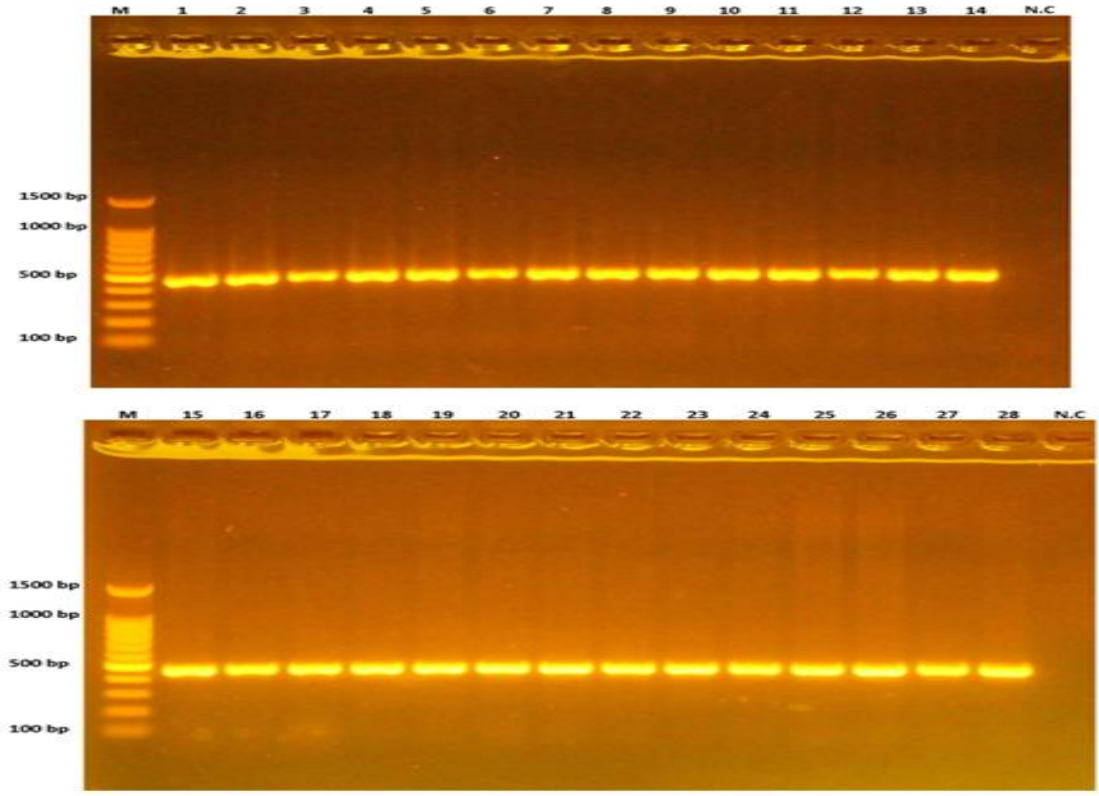

Figure1. Gel electrophoresis of amplified PCR product of infB gene (500bp) of *K. pneumoniae*. 1.5% agarose gel electrophoresis stained with ethidium bromide. TBE buffer (1X). Lane1:100bp DNA marker. The clinical *K. pneumoniae* isolates (1-14) and aquatic (15-27).

Using molecular techniques to characterize *K. pneumoniae* isolates for epidemiological reasons [43]. *infB* gene is among the seven housekeeping genes that used in Multilocus sequence typing (MLST) which is an appropriate method based on nucleotide sequences for describing the genetic relationships between bacterial isolates [44].

4- Detection of Biofilm Formation

Twenty *K. pneumoniae* isolates (ten clinical and ten aquatic) that were previously detected by *infB* gene were used for assay of biofilm formation using the microtiter plate method, by using crystal violet stain and the OD was measured by GloMax apparatus at 560 nm.

The results show that there were significant differences between biofilm formation among clinical and aquatic isolates and the aquatic isolates were more than clinical isolates in biofilm formation (Table 2 A and B). The biofilm formation percentage among clinical isolates was (1 isolate (10%) strong, 2 isolates (20%) moderate, and 7 isolates (70%) weak), while the percentage for aquatic isolates was (2 isolates (20%) strong, 5 isolates (50%) moderates, and 3 isolates (30%) weak).

Table 2 –A. The OD values of clinical *K. pneumoniae* clinical isolates. The OD was measured by GloMax apparatus at 560 nm

isolates	OD1	OD2	OD3	Average	ODC	2×ODC	4×ODC	Isolate OD	Result
1	0.454	0.478	0.449	0.460	0.086	0.172	0.344	0.374	strong
2	0.189	0.205	0.197	0.197	0.086	0.172	0.344	0.111	weak
3	0.245	0.242	0.243	0.243	0.086	0.172	0.344	0.157	weak
4	0.158	0.188	0.187	0.177	0.086	0.172	0.344	0.091	weak
5	0.288	0.322	0.289	0.299	0.086	0.172	0.344	0.213	moderate
6	0.216	0.205	0.211	0.210	0.086	0.172	0.344	0.124	weak
11	0.211	0.219	0.196	0.208	0.086	0.172	0.344	0.122	weak
12	0.318	0.295	0.302	0.3.5	0.086	0.172	0.344	0.219	moderate
13	0.165	0.187	0.166	0.172	0.086	0.172	0.344	0.086	weak
14	0.196	0.191	0.221	0.202	0.086	0.172	0.344	0.116	weak
control	0.073	0.070	0.071	0.071	0.086	0.172	0.344		
LSD	_	_	_	0.0841 *	_	_	_	0.0769 *	

* (P≤0.05).

Table 2- B. The OD values of Aquatic *K. pneumoniae* aquatic isolates The OD was measured by GloMax apparatus at 560 nm

isolates	OD1	OD2	OD3	Average	ODC	2×ODC	4×ODC	Isolate OD	Result
15	0.312	0.345	0.299	0.318	0.086	0.172	0.344	0.232	moderate
16	0.194	0.197	0.177	0.189	0.086	0.172	0.344	0.212	weak
17	0.444	0.428	0.423	0.431	0.086	0.172	0.344	0.345	strong
22	0.298	0.332	0.326	0.318	0.086	0.172	0.344	0.232	moderate
23	0.189	0.213	0.198	0.200	0.086	0.172	0.344	0.114	weak
24	0.416	0.448	0.454	0.439	0.086	0.172	0.344	0.353	strong
25	0.327	0.290	0.329	0.318	0.086	0.172	0.344	0.232	moderate
26	0.264	0.284	0.301	0.283	0.086	0.172	0.344	0.197	moderate
27	0.191	0.196	0.203	0.196	0.086	0.172	0.344	0.110	weak
28	0.280	0.275	0.263	0.272	0.086	0.172	0.344	0.186	moderate
Control	0.073	0.070	0.071	0.071	0.086	0.172	0.344		
LSD	_	_	_	0.117 *	_	_	_	0.136 *	

* (P≤0.05).

The microtiter plate, a semi-quantitative assay is a static model of biofilm formation and it is an accurate and repeatable approach for measuring biofilm development in vitro [45]. The implications of infections (chronicity, persistence, and relapse) of Gram-negative bacilli that result in increased mortality and morbidity are caused by the beta--lactamases production and biofilm formation synergistically that contributes to the widespread spread of multi-drug resistant bacteria, creating a grave health issue [46].

So far, it has been established that there are certain associations between the capacity of *K. pneumoniae* strains to form biofilms and antibiotic resistance. To assess the connection between biofilm development, resistance, and antimicrobial resistance Cusumano *et al*. [47] investigation of 139 different clinical *K. pneumoniae* isolates with distinct resistance profiles and comparison of weak vs strong biofilm-forming *K. pneumoniae* led to the discovery of predictors of strong biofilm formation. Weak biofilm formers (97.9%) tended to develop more multi-drug resistant isolates than robust biofilm formers (76%). Strong biofilm formation was (91%) less common in carbapenem-resistant *K. pneumoniae*. The suggestion that virulence may be a survival trade-off is raised by this study's statistically significant negative correlation between biofilm formation and antibiotic resistance. In a separate study, biofilm formation was seen in 41 out of 54 tested *K. pneumoniae* isolates (76%) [48].

The results that recorded by Pradhan *et al*. [49] recorded that Antibiotic resistance is further exacerbated by the fact that carbapenemase producers are greater biofilm producers than non-carbapenemase producers and that biofilm formation is extremely common with varied degrees of resistance among different antibiotics, including carbapenems. In order to choose antibiotics effectively, they advised recognizing biofilm development among carbapenemase manufacturers. In 2019, Dumaru *et al*. [50] recorded that the connection between the creation of ESBLs and the development of biofilm was statistically insignificant and 197 (62.73%) of *K. pneumoniae* isolates were bioflm positive and 84 (26.75%) were confirmed as ESBL producers. Previous research has demonstrated that a strain's capacity to form biofilms may play a significant role in infection. Biofilm formation is more common in MDR strains, and selecting highly resistant strains after treatment with sub-inhibitory antimicrobial concentrations can promote antimicrobial resistance [51,52,53].

The differences in results may be due to the kind of specimens and samples, the number of isolates, the antibiotic relation with the biofilm formation of the selected isolates, and the circumstances that affect the biofilm formation. The findings emphasize the significance of the bacterial biofilm phenotype as a possible determinant in virulence that may cause clinical recurrence of infections. The variations in biofilm development between water and clinical *K. pneumoniae* isolates may be due to the niches that the bacteria are found in, the growth conditions (pH, temperature, nutrients, light, oxygen levels, and other factors that affect bacterial growth and biofilm formation and the circumstances that effect on the behavior of the bacteria (planktonic or biofilm).

Conclusions: As a conclusion this study identifies the beta- lactam resistant *K. pneumoniae* that isolated from clinical specimens and aquatic samples and how they relate to the creation of biofilm. This research showed that the water isolates of *K. pneumoniae* are more than clinical beta- lactam resistant isolates in biofilm formation. Early identification of beta- lactam-resistant and biofilm-producing isolates would be beneficial and aid in building a patient's individualized treatment plan.

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التحري عن تكوين الاغشية الحيوية لبكتريا الكلبسيلا الرئوية المقاومة لمجموعة مضادات البيتا لاكتام والمعزولة من عينات سريرية و مائية

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مستخلص البحث

تسبب الكليبسيلا الرئوية مجموعة واسعة من الالتهابات. وهي موجودة في الطبيعة في بيئات مختلفة مثل المياه السطحية. لقد زادت قدرة هذه البكتريا على مقاومة للمضادات الحيوية و تكوين الأغشية الحيوية. هدفت هذه الدراسة إلى تحديد نمط مقاومة المضادات الحيوية والقدرة على تكوين الأغشية الحيوية لبكتيريا الكلبسيلا الرئوية المعزولة من العينات السريرية والعينات المائية. تم عزل ستة وستين (20.625%) من العزلات السريرية لبكتريا الكلبسيلا الرئوية من 320 عينة سريرية من مدينة الطب في العراق، بغداد. تم جمع 27 (18%) عزلة مائية من 150 مصادر مائية مختلفة في مدينة بغداد. تم استخدام اكار الهاي كروم يو تي اي والاختبارات البيوكيميائية للتعرف على كل عزلة. أظهرت النتائج أن العزلات السريرية كانت أكثر مقاومة للمضادات الحيوية بيتا لاكتام من العزلات المائية وكانت كلتاهما مقاومة (100%) للاموكسيكلاف. وكانت نسبة المقاومة للعزلات السريرية (6.06 (%ميروينيم، (13.63%)اميبنيم ، (19.69%)سيفتازيديم، (53.03%) سيفوتاكسيم و(86.36%) سيفترياكسون، في حين كانت نسبة المقاومة للعزلات المائية صفر للعزلات السريرية لكل من الميروبينيم، إيميبينيم، سيفتازيديم،سيفوتاكسيم، سيفترياكسون. في الاختبارات التشخيصية الجزيئية للعزلات الاكثر مقاومة للمضادات البيتا لاكتام تم استخدام الجين اي ان اف بي وتم استخدام تحليل مايكروتايتر بليت لتحديد أي العزلات كانت أكثر قدرة على تكوين الأغشية الحيوية. أظهرت النتائج وجود فرق معنوي بين تكوينات الأغشية الحيوية بين العزلات السريرية والمائية وكانت العزلات المائية أكثر من العزلات السريرية في تكوين الأغشية الحيوية. وكانت نسبة تكوين الأغشية الحيوية بين العزلات السريرية الأكثر مقاومة للبيتالاكتام (عزلة واحدة (10%) قوية، وعزلتان (20%) متوسطة، و7 عزلات (70%) ضعيفة)، بينما كانت النسبة للعزلات المائية (2 عزلات (20%) قوية، 5 عزلات (50%) متوسطة، 3 عزلات (30%) ضعيفة. أظهرت هذه الدراسة أن العزلات المائية للكلبسيلا الرئوية أكثر من العزلات السريرية المقاومة للبيتا لاكتام في تكوين الأغشية الحيوية.

الكلمات المفتاحية: الكلبسيلا الرئوية، مقاومة المضادات، مجموعة مضادات البيتا لاكتام، الاغشية الحيوية، البكتريا المعزولة من عينات مائية.