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Prevalence of biofilm and two type of betalactamase enzymes production in some klebseilla spp isolatsd in Kirkuk City

Rihab Abdulqader Ali
Prof. Dr. Salah Salman Zain Al-Abidin
epbm23009@uokirkuk.edu.iq
Salah@uokirkuk.edu.iq
College of Education for Pure Science / Department of Biology / University of Kirkuk, Iraq

Abstract

The study included the collection of 180 clinical samples from patients suffering from various septic symptoms The results showed a numerical superiority for females by 55% compared to males by 45%. Urine samples constituted the highest percentage (44.4%), followed by wound samples (25.6%) and burns (19%), while lower percentages were recorded for sputum and reproductive system samples (5.6% for each). The results of bacterial isolation and diagnosis showed that 27 isolates belonging to *Klebsiella* spp bacteria were obtained. 15% of the total samples examined, based on cultural and microscopic characteristics and biochemical tests, in addition to the VITEK-2 system, which showed high compatibility with traditional methods.The results of biofilm formation detection using Congo Red medium showed that 48.1% of the isolates were positive, while the tube agglutination method showed that 44.4% of the isolates were capable of biofilm formation, confirming the importance of this trait as a major virulence factor. As for the production of beta-lactamase enzymes, the results showed that 22.2% of the isolates were iodine positive, while 11.1% of the isolates showed the ability to produce enzymes in a catalytic manner, as well as to produce ESBLs. The results of the study confirm that *Klebsiella* spp. It possesses multiple pathogenicity and resistance, which calls for strengthening surveillance programs, rationalizing the use of antibiotics, and searching for effective therapeutic alternatives.

Keyword : Biofilm , *Klebseilla* , Pomegranate peel, antibacterial, Extended-Spectrum Beta-Lactamase.

مدى انتشار تكوّن الغشاء الحيوي وإنتاج نوعين من إنزيمات البيتا-لاكتاماز لدى بعض عزلات Klebsiella spp المعزولة في مدينة كركوك

رحاب عبدالقادر علي[1]
أ.د. صلاح سلمان زين العابدين[2]
epbm23009@uokirkuk.edu.iq[1], Salah@uokirkuk.edu.iq[2]
[1.2] كلية التربية للعلوم الصرفة / قسم علوم الحياة / جامعة كركوك، العراق

الملخص

شملت الدراسة جمع 180 عينة سريرية من مرضى يعانون من أعراض إنتانية مختلفة. أظهرت النتائج تفوقاً عددياً للإناث بنسبة 55% مقارنة بالذكور بنسبة 45%. شكّلت عينات البول النسبة الأعلى (44.4%)، تلتها عينات الجروح (25.6%) ثم الحروق (19%)، في حين سُجلت نسب أقل لعينات القشع وعينات الجهاز التناسلي (5.6% لكلٍ منهما). أظهرت نتائج العزل والتشخيص البكتيري الحصول على 27 عزلة تعود لبكتيرياKlebsiella spp ، وبنسبة 15% من مجموع العينات المفحوصة، اعتماداً على الصفات الزرعية والمجهرية والاختبارات الكيموحيوية، إضافة إلى نظام VITEK-2 الذي أظهر توافقاً عالياً مع الطرق التقليدية. أظهرت نتائج الكشف عن تكوّن الغشاء الحيوي باستخدام وسط الكونغو الأحمر أن 48.1% من العزلات كانت موجبة، في حين بيّنت طريقة أنبوب الاختبار أن 44.4% من العزلات تمتلك القدرة على تكوين الغشاء الحيوي، مما يؤكد أهمية هذه الصفة بوصفها عاملاً رئيسياً من عوامل الضراوة. أما فيما يخص إنتاج إنزيمات البيتا-لاكتاماز، فقد أظهرت النتائج أن 22.2% من العزلات كانت موجبة لاختبار اليود، بينما أبدت 11.1% من العزلات القدرة على إنتاج الإنزيمات بطريقة تحفيزية، فضلاً عن إنتاج إنزيمات البيتا-لاكتاماز واسعة الطيف .(ESBLs) تؤكد نتائج الدراسة أن بكتيريا Klebsiella spp تمتلك عوامل متعددة للإمراضية والمقاومة، مما يستدعي تعزيز برامج المراقبة، وترشيد استخدام المضادات الحيوية، والبحث عن بدائل علاجية فعّالة.

الكلمات المفتاحية: الغشاء الحيوي، Klebsiella، قشور الرمان، الفعالية المضادة للبكتيريا، البيتا-لاكتاماز واسعة الطيف.

Introduction

Klebsiella species are Gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic bacteria that cause a variety of disorders, particularly in countries with a weak healthcare system [1]. Klebseilla, its subspecies (K. pneumoniae, K. ozaenae, and K. rhinoscleromatis), and K. oxytoca are the most commonly isolated and medically significant members of this genus [2]. Despite being part of the natural flora of the human gastrointestinal system, K. pneumoniae is the most prevalent pathogenic cause of nosocomial infections among Klebsiella spp. [3]. Pneumonia, urinary tract infection (UTI), bloodstream and surgical wound infections, peritonitis, septicemia, meningitis [4], and pyogenic liver abscess [5]. K. pneumoniae causes the following main diseases in humans. Antimicrobial resistance in Enterobacteriaceae (mostly Escherichia coli, K. pneumoniae, and Salmonella spp.) has risen rapidly in recent years [4,5]. These infections have developed resistance to routinely used antimicrobial drugs, including extended-spectrum beta-lactams, aminoglycosides, fluoroquinolones, and carbapenems [4,5]. Therefore, drug-resistance concerns are responsible for reduced therapeutic alternatives, increased treatment costs, prolonged hospital admissions, and mortality [6, 7].. *Klebsiella* species' pathogenicity is due to a variety of virulence factors, including capsule, lipopolysaccharide (LPS), fimbrial and non-fimbrial adhesins, siderophores [8], and the ability to form biofilm [9]. Biofilms are

bacterial aggregation that are permanently lodged in extracellular matrices of polysaccharides, proteins, enzymes, and nucleic acid, allowing for irreversible anchoring to any surface [10]. Infections caused by K. pneumoniae are becoming increasingly troublesome due to their capacity to form biofilms, highlighting the need for novel anti-biofilm therapies [11]. A study using signature labeled mutagenesis and surfaces covered in human extracellular framework (HECM) identified a protein involved in capsule manufacturing that is required for K. pneumoniae biofilm development [12]. A recent study found that capsule genes wza and ORF14 are required for K. pneumoniae to form biofilm [13].

Extended-spectrum β-lactamase (ESBL), AmpC β-lactamases, and carbapenemase are enzymes that cause resistance to β-lactam antibiotics such penicillins, cephamycin, and carbapenem [14]. Carbapenems are utilized as the last resort treatment for life-threatening infections caused by multidrug resistant (MDR) pathogens and Enterobacteriaceae that produce ESBL [15]. However, due to the selective pressure of treating esbl and ampc infections with carbapenems in recent years, resistance has arisen, with K. pneumoniae being the most frequent carbapenem-resistant Enterobacteriaceae (cre) [16].The incidence of klebsiella pneumoniae carbapenemases (kpc) and metallo-β-lactamase generating strains has been investigated in several countries [17]. Carbapenem resistance due to metallo-beta-lactamase (MBL) synthesis has increased among clinical isolates from all over the world [18]. The rapidly increasing incidence of MBL synthesis among Enterobacteriaceae members, particularly E. coli and K. pneumoniae, has become one of the most common causes of hospital infections, with few treatment options [19]. A reliable assessment of the number of drug-resistant K. pneumoniae isolates is thus required to inform hygiene and infection control, as well as treatment choices for drug-resistant strains [20]. This study aims to isolate and diagnose Klebsiella spp. from various clinical samples in Kirkuk Governorate, studying their ability to form a biofilm and detecting the production of beta-lactamase enzymes, including AmpC and ESBLs, in addition to evaluating the pathogenic role of these factors in increasing bacterial resistance to antibiotics.

Material and Methods

Sample Collection

The study was conducted in Kirkuk City. Samples were collected between December 12, 2024, and March 15, 2025. A total of 180 samples were collected, including urine, sputum, burns, wounds, and swabs from the female reproductive tract. These samples were collected from patients admitted to and inpatients at Azadi Teaching Hospital, Kirkuk Teaching Hospital, the Children's Hospital, and

several private laboratories. Information was obtained from the patients and recorded on a questionnaire for each patient. Sample collection began after consultation with a physician, and personal protective equipment was worn. Some samples were cultured at the hospital, while others were collected using swabs containing a carrier medium (Media Amis). These were then sent to the graduate studies laboratory at Kirkuk University/College of Education for Pure Sciences, Department of Life Sciences. The samples were cultured using cotton swabs on previously prepared culture media (Blood Agar and MacConkey Agar). The plates were incubated at 37°C for 24 hours.

Diagnostic Tests

The bacteria were diagnosed based on their culture and microscopic characteristics, as well as biochemical tests (IMVIC test) and Vitik-2 Compact System.

Rapid Iodometric Method for ß-Lactamase Detection

A qualitative test was performed to detect beta-lactamase enzymes using the Rapid Iodometric Method, which is a simple and rapid method based on the degradation of penicillin G by the beta-lactamase enzyme, which leads to the disappearance of the blue color resulting from the reaction of starch with iodine. This method is one of the approved methods for detecting the activity of this enzyme, as stated in [21]. Bacterial isolates were grown on MacConkey agar medium, then incubated at a temperature of 37°C for 24 hours.

Detecting Inducible β-lactamase Enzymes

In this study, the antibiotic Cefoxitin was used to investigate the possibility of stimulating the production of beta-lactamase enzymes in the bacterial isolates under study, and to evaluate its effect on the resistance of the isolates to other third-generation beta-lactam antibiotics. The experiment was conducted according to the steps mentioned in[22].

Extended Spectrum β-Lactamases

Broad-spectrum beta-lactamases (ESBLs) were detected using the disk approximation method, as described by [23] .

Detection of biofilm production using Congo red medium

The method is based on observing the color of the colonies after incubation on Congo Red Agar medium, as stated in [24] .

Tube Adherence Test

The ability of bacterial isolates to form biofilms was assessed using a modified tube method, replacing the use of tubes by culturing samples in flat-bottomed plastic plates (Microtiter Plate), while maintaining the basic principles of the method, including initial preparation of isolates, incubation, washing to remove non-adherent cells, and staining of biofilms to facilitate visual assessment and the possibility of comparing a larger number of samples at the same time [25].

Results and discussion

Isolation and Identification

The results of the study showed that the distribution of clinical samples collected from patients suffering from various septic symptoms in Kirkuk Governorate included 180 samples, with a numerical superiority for females (55%) compared to males (45%). The majority of samples were concentrated in urine samples at a rate of 44.4%, followed by wound and burn samples, which reflects the prevalence of urinary and skin infections among the patients included in the study.Based on cultural and phenotypic tests, 27 isolates belonging to the *Klebsiell* spp bacteria were isolated and diagnosed from the total samples examined, representing 15%, which indicates the important pathogenic role of these bacteria in the causes of various infectious infections, especially in hospital settings. This is due to its high ability to colonize, and the production of a mucous capsule that contributes to increasing its virulence and its ability to resist environmental and therapeutic conditions.

At the level of biochemical diagnosis, *Klebsiella* spp showed negative results for the Oxidase, Indole, Methyl Red, Motility, and Kligler Iron Agar tests, while it was positive for the Voges–Proskauer test and the Citrate Utilization test, and positive for the Catalase test as well as the Citrate Utilization Test and Urease [26]. Diagnostic results using the VITEK device showed high accuracy in identifying the bacterial isolates under study, as the device was able to identify *Klebsiella* spp isolates. With a high degree of conformity with the results of traditional phenotypic and biochemical tests.

Biofilm detection results using the plate method

The results of the Congo Red Agar (CRA) test for Klebsiella spp. (13/27) isolates (48.1%) showed a positive ability to form a biofilm, while (6/27) isolates (22.2%) were intermediate or volatile (Intermediate), while (8/27) isolates (29.6%) did not show any ability to form a biofilm. As in Figure .1.

Figure 1. Biofilm production of *Klebsiella* spp.

These results indicate that biofilm represents one of the most important virulence factors in *Klebsiella* spp., as approximately half of the studied isolates showed a clear ability to produce it. This property is of great clinical importance, as the biofilm provides bacteria with mechanical and biochemical protection against antibiotics and host defense mechanisms, which leads to difficulty treating chronic infections and a high probability of treatment failure [27].

These results are not consistent with those reported by [28] who indicated that 57% of clinical *Klebsiella* spp. isolates were biofilm producers using the CRA method. Likewise, a study [29] showed that 61% of *Klebsiella* isolates were positive for biofilm formation, and emphasized the close relationship between this trait and increased bacterial resistance to β-lactams. As for the study [30] in Iraq, it recorded a rate of 49% of isolates producing biofilm, which is a percentage very close to the results of the current study. On the other hand, the percentage of negative isolates (29.6%) confirms that not all *Klebsiella* isolates have the ability to form a biofilm, which may be related to genetic diversity in the genes responsible for the production of extracellular polymeric substances (EPS) or different environmental conditions that stimulate this ability. As for isolates with intermediate results (22.2%), they may reflect an unstable state in the gene expression of virulence factors or may indicate a weakness in the secretion of extracellular polymers.

Results of biofilm formation using the Tube Adherence Test

The study included 27 isolates of *Klebsiella* spp. The results showed that 12/27 isolates (44.4%) were positive for biofilm formation, while 4/27 isolates (14.8%) were recorded as moderate/poor (Intermediate), and 11/27 isolates (40.7%) were negative , As show in figure .2 .These data reflect a relatively high prevalence of biofilm-forming ability among *Klebsiella* spp isolates. studied, which supports the consideration of biofilm as a major virulence factor that contributes to the establishment of bacteria on living and non-living surfaces and protecting them

from stress factors, especially antibiotics and innate immune mechanisms. The difference in the ability of *Klebsiella* spp isolates to form biofilms is due to genetic and regulatory factors, including the genes responsible for adhesion, the mrk gene, which is a gene that encodes proteins associated with Type 3 fimbriae (T3F), which are surface hairs (pili) that facilitate attachment to solid surfaces, especially implanted medical devices (such as urinary catheters), in addition to fim, a gene associated with Type 1 fimbriae. (T1F), which are filaments that help adhere to host cells and induce initial tissue colonization [31].

Clinically, biofilm-producing isolates are associated with increased antibiotic resistance due to limited permeability, intramembrane trophic gradient, and transformation of cells into tolerant states, which clearly reflects the difficulty of treating urinary tract, respiratory, and catheter-associated infections [27]Therefore, the recording of more than half of the isolates as biofilm forming partly explains the observed clinical resistance patterns of the isolates. These results are not consistent with the study of [28], as the percentage was 59% of *Klebsiella* spp. isolates producing biofilm. In Iraq, [29] mentioned percentages of about 47% for *Klebsiella* spp isolates. It has high virulence and resistance characteristics, highlighting the role of biofilm in the multi-resistance pattern, which is a percentage within the same field.

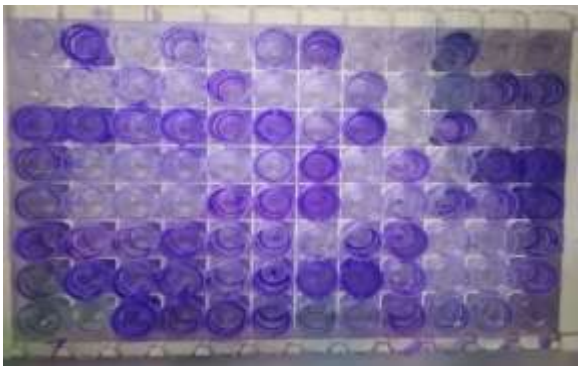

Figure.2 Results of producing cell membranes using the tube method.

The enzyme beta-lactamase is produced

The results of the iodine method on the bacteria *Klebsiella* spp showed that six isolates were positive, at a rate of 22.2%. The iodinated method confirms that these bacteria, despite their greater reliance on non-enzymatic resistance mechanisms, may possess genes capable of expressing beta-lactamase in certain circumstances, especially when exposed to selective pressures such as antibiotics. [32]. Figure .3 shows the production of the beta-lactamase enzyme by the iodinated method.

Figure .3 The production of the beta-lactamase enzyme by the iodinated method.

Induced method

The results of the study showed that only 3 out of 27 isolates of *Klebsiella* spp (11.1%) were able to produce beta-lactamase in the induced manner, This indicates that *Klebsiella* spp. They have a potential ability to express beta-lactamase genes, especially AmpC and ESBLs, when exposed to antigens, giving them an additional advantage in beta-lactam resistance [33].Figure .4 shows the production of the beta-lactamase enzyme by the induced method.

Figure .4 The production of the beta-lactamase enzyme by the induced method.

Broad spectrum method

The results of detecting the production of broad-spectrum beta-lactamase enzymes in the studied bacterial isolates showed that *Klebsiella* spp. It has a greater ability to produce this enzyme, as detection rates reached 11.1% out of 27 isolates of *Klebsiella* spp.as show in Figure .5 . This discrepancy reflects the difference in resistance mechanisms between the two species. As it is counted. *Klebsiella* spp is a bacteria known to possess genes responsible for producing beta-lactamase

enzymes, especially broad-spectrum beta-lactamases (ESBLs), which contribute to inactivating the effectiveness of beta-lactam antibiotics such as cephalosporins [33].

Figure .5 Production of the broad-spectrum beta-lactamase enzyme.

Conclusions

The bacteria Klebsiella spp. It is an important pathogen causing various infectious infections in Kirkuk Governorate, as it was recorded in a significant percentage of isolates from the examined clinical samples, as a significant percentage of the isolates showed a clear ability to form biofilm, which enhances their virulence and contributes to increasing their resistance to antibiotics and the difficulty of eliminating them. Some isolates of Klebsiella spp. Ability to produce beta-lactamase enzymes including ESBLs, which limits the effectiveness of many beta-lactam antibiotics. The interplay between biofilm formation and the production of resistance enzymes shows a complementary role in promoting bacterial survival and infection persistence, especially in hospital settings.

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