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Detection of Biofilm Formation in Classical and Hypervirulent *Klebsiella pneumoniae*

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ABSTRACT

Klebsiella pneumoniae has considered as a relevant healthcare-associated pathogen, its risk of infections is increasing in the presence of medical devices. *K. pneumoniae* is known for its ability to form biofilm on biotic and abiotic surfaces. Biofilm of *K. pneumoniae* assists in bacterial protection from host immune responses and antibiotics. Hypervirulent *Klebsiella pneumoniae* (hvKp) emerges as a new pathotype, which first appeared in Asian Pacific Rim but spread globally. Thus, this study aimed to investigate the ability of *K. pneumoniae* including hvKp and potential hvKp isolates to form biofilm. One hundred isolates of *K. pneumoniae* were collected from different hospitals in Baghdad city. These isolates were identified by phenotypic characterization on selective agar plates, biochemical tests, VITEK II, and molecular identification. Biofilm formation was tested in these isolates by two methods, congo red and Tissue Culture Plate method. In congo red method, 33% of the isolates were biofilm producer and (63%) can form biofilm by TCP method divided as: 14% strong, 15 moderate, 34% weak, and 37% non-biofilm producer. The hvKp and potential hvKp isolates showed a variable ability to form biofilm as classical *K. pneumoniae.*

KEYWORDS: *K. pneumoniae*; *Hypervirulent Klebsiella pneumoniae*; congo red; Biofilm.

الخلاصة

تعتبر بكتريا *Klebsiella pneumoniae* أحد مسببات الأمراض المرتبطة بالرعاية الصحية ، ويزداد خطر الإصابة بالعدوى من خلال وجود الأجهزة الطبية. تعرف *K. pneumoniae* بقدرتها على تكوين الغشاء الحيوي على الأسطح الحيوية وغير الحيوية. يساعد الغشاء الحيوي لهذه البكتريا بحمايتها من الاستجابات المناعية للمضيف والمضادات الحيوية. ظهر النوع hpervirulent *Klebsiella pneumoniae* (hvKp) كنمط مرضي جديد شديدة الضراوة ، ظهر لأول مرة في منطقة آسيا والمحيط الهادئ ولكنه انتشر على مستوى العالم. وبالتالي، هدفت هذه الدراسة إلى التحقق من قدرة *Klebsiella pneumoniae* بما فيها عزلات hvKp و potential hvKpعلى تكوين الغشاء حيوي. تم جمع (100) عزلة من بكتيريا *K. pneumoniae* من مستشفيات مختلفة في مدينة بغداد. تم التعرف على هذه العزلات من خلال التوصيف المظهري على الأوساط الزرعية الانتقائية والاختبارات البايوكيميائية ونظام VITEK II والتشخيص الجزيئي. تم اختبار تكوين الغشاء الحيوي في هذه العزلات بطريقتين:congo red method و tissue culture plate method ، حيث كانت(33٪) من العزلات منتجة للأغشية الحيوية باستخدام طريقة congo red و (63٪) مكونه للغشاء الحيوي بطريقة tissue culture plate مقسمة على النحو التالي: 14٪ مكونه بقوه للغشاء الحيوي ، 15% معتدلة القدرة على تكوين الغشاء الحيوي ، 34٪ لها قدره ضعيفة على تكوين الغشاء الحيوي ، و 37% غير منتجه للغشاء الحيوي . اظهرت النتائج ان قدره عزلات hvKp و potential hvKp على تكوين الغشاء حيوي متغايرة ومشابه لمااظهرته العزلات الكلاسيكية *Klebsiella pneumoniae*.

INTRODUCTION

Klebsiella pneumoniae (*K.pneumoniae*) is a member of *Enterobacteriaceae* family, Gram-negative, encapsulated, and non-motile bacterium. Primary identification of *K. pneumoniae* depends on phenotypic marker such as biochemical tests [1], which may cause misidentification sometimes. Several molecular approaches are suggested to identify of clinically important bacteria for instance, 16S rRNA gene, which widely used for accurate species identification [2]. Housekeeping genes are important tools to develop multilocus sequence typing (MLST) scheme for discriminating *K. pneumoniae*. The *rpoB* gene, which encodes the beta subunit of RNA polymerase, was showed as a powerful tool for identification of *K. pneumoniae* isolates [3]. *K. pneumoniae* possess numerous virulence factors and many genes have been discovered involved in *K. pneumoniae* pathogenesis but their role in virulence have not uncovered yet [4] [5]. K-capsular antigens, adhesion factors, O-lipopolysaccharide (LPS), and siderophores are the four primary components that have been linked to the pathogenesis of *K. pneumoniae* [6]. Hypervirulent *K. pneumoniae* (hvKp) has spread

globally since first description of this pathotype in the Asian Pacific Rim in 1980s. HvKp was emerged a new pathotype of classical K. *pneumoniae*(cKp), which was first isolated from pyogenic liver abscess in Taiwan [7] [8]. Since then, hvKp was recorded through Asia especially Japan, China, and South Korea. Furthermore, hvKp has spread globally [9]. In contrast to cKp infections, hvKp infections are spread via the community and tend to target healthy individuals. Complex surface-associated communities known as biofilms contain extracellular matrix, which can serve as a form of defense against the body's immune system as well as the pressures produced by the surrounding environment [10]. The production of biofilms by *K. pneumoniae* is widely understood, although the genetic basis for biofilm formation is not completely understood. Biofilms can be found on a variety of surfaces, there have been a variety of studies that attribute the biofilm phenotype to the capsule [11][12] and/or fimbriae [11] [13] [14]. However, other studies have demonstrated that the absence of capsule promotes the formation of biofilm [15]. Because this bacterium develops a biofilm on medical equipment, it can cause future infections that relate to health care, most commonly in the urinary and pulmonary systems [16]. In addition, biofilm is a responsible for the development of multidrug-resistant (MDR) strains [17] [18]. In a study involving more than 70 different isolates, it was found that hvKp isolates showed strong biofilm producer *in vitro* in comparison to non-tissue-invasive isolates [12]. However, many investigations have indicated that there is no difference in the production of biofilm between invasive and non-invasive isolates [19]. Thus, the aim of this study is to test the biofilm formation ability of *K. pneumoniae* including hvKp and potential hvKp isolates (previously confirmed to be hvKp by molecular method) to form biofilm.

MATERIALS AND METHODOLOGIES

Isolation of *K. pneumoniae*

One hundred isolates of *K. pneumoniae* were collected from Al-Yarmouk teaching hospital, Ibn Al balady Maternity & Children's Hospital, Baghdad teaching hospital, teaching laboratories of medical city, Al Karkh General Hospital, Al Imamain Al-Kadhimain Medical city and Al Karama Teaching Hospital, the isolates were collected under the aseptic condition and transmitted immediately to the laboratory.

Identification of *K. pneumoniae* isolates

For initial and final identification colony characteristics on culture media, biochemical Tests, catalase, oxidase, and VITEK II system were performed beside molecular identification using *ropB gene* [20].

Molecular identification of K. *pneumoniae isolates*

Genomic DNA isolation

Genomic DNA isolation was done by boiling method, the isolates were streaked on fresh agar plate. About 10 single colonies of each isolate were transferred into eppendorf tube contain 400 µl ddH_2O. Then eppendorf tubes were kept in 100ºC water bath for 10 min. After boiling, tubes immediately cooled on ice and frozen at (-20 ºC) for 20 min. Later, the tubes left to thaw at room temperature and homogenize by vortex for 10s. The samples were centrifuged at 13,362 *g* for 15 min at 4°C. The upper aqueous layer was kept and transferred into new sterile eppendorf tube, and these DNA samples were frozen until use [21].

Molecular identification using rpoB gene

PCR was used to amplify *rpoB* gene, this gene was used as identification marker for *K. pneumoniae*. The PCR reaction prepared as following: 12.5µl master mix, 1µl of each primer, 5 µl of gDNA, and final volume 25 µl adjusted by d. dH2O. Primers were used:
[Forward 5′ GTTGGCGAAATGGCGGAAAAC 3′, Reverse 5′ ACGTCCATGTAGTCAACCTGG 3′], which designed by this study. PCR conditions were used for 30 cycles as the following: 95°C for 5min as the initial denaturation of DNA; 95°C for 30sec as the denaturation step of DNA; 57°C for 30sec as the annealing step of the primers, 72°C for 45 sec as the elongation step, 72°C for 5 min as final extension step.

Antibiotic Susceptibility Test

Kirby-Bauer method was used perform the antibiotic susceptibility test for 14 different antibiotics including; (Amikacin (30µg), Aztreonam(15µg), Cefepime(10µg), Ceftazidim-e (30µg), Ceftriaxone (10 µg), Ciprofloxacin (5 µg), Colistin (10 µg), Gentamicin(10µg), Imipenem (10 µg), Ampicillin (10µg), Levofloxacin(5µg), Piperacillin/tazobactam (100/10 µg), Tetracycline

(30 µg), Trimethoprim / sulfamethoxazole (1.25/23.75 µg). The suspension of bacteria was prepared by taking a few single colonies of each isolate into 5 ml of normal saline to make turbidity equals to 0.5 McFarland standards (1.5×10^8 _CFU/ml). A sterile cotton swab was merged in the bacterial suspension and used to spread on Mueller-Hinton agar medium then it was left for 10 min to dry. The antimicrobial discs were placed on the agar with sterile forceps pushed firmly to confirm contact with the agar. Then, the plates were incubated for 18-24 hours at 37ºC. After incubation, inhibition zone around each disc was measured in millimeter (mm) using a metric ruler and the results were interpreted according to Clinical Laboratories Standards Institute (CLSI, 2022) [22].

Biofilm production

Detection of Biofilm Formation Biofilm formation test was done by:

Biofilm formation by Congo red test

To prepare Congo red agar: Brain heart infusion broth (BHI) (37 gm/L) and sucrose (50 gm/L) were dissolved in 500 ml of distilled water, and 10 gm of agar-agar and was added. After that, the volume was adjusted to 900 ml of distilled water and the medium was sterilized by autoclave. 0.8gm of congo red stain was dissolved in100 ml of distilled water to prepare the dye and sterilized by autoclave. After autoclaving, the medium and dye left to cool to 50 ºC and dye were added to the agar. The medium was poured into sterile petri plates. This medium was used to detect the biofilm formation of the isolates [23]. Congo red agar plates were inoculated with a single colony of each isolate, and then they were incubated at 37 ºC from 24 to 48 hours. A positive result was indicated by the appearance of black colonies, while pink or white colonies indicated as non-producer isolates of biofilm.

Biofilm formation by MicrotiterPlate method

Biofilm formation was detected by micro titter plate method as described by [24] as following: bacterial isolates were cultured on brain heart infusion agar, incubated at 37Cº for overnight. Then, few (4-5) colonies suspended in 5ml of normal saline in test tubes, mixed by vortex. The following step, 20µl of bacterial suspension was used to inoculate 96-well flat-bottomed microtiter plate containing 180 µl of BHI broth with 5% sucrose and 200 µl uninoculated broth was added into control wells. The plates were incubated at 37ºC for overnight. After that, the content of each well was removed, washed three times with PBS (pH 7.2). The plates were left to dry at room temperature for 15 minutes. Crystal violet (1%) was added to the wells for 15 minutes. After removing the crystal violet, wells were washed three times with PBS (pH 7.2) to remove the unbounded dye, allowed to dry at room temperature and 200 µl ethanol was added to each well. The absorbance of each well was measured at 630 nm using spectrophotometer reader. Each assay was performed in triplicate. The adherence capabilities of the bacterial was calculated as follow: Non-biofilm producers ($OD \leq ODc$), weak biofilm producers ($ODc < OD \leq 2 \times ODc$), moderate biofilm producers ($2\ ODc < OD \leq 4 \times ODc$) and strong biofilm producers ($4 \times ODc < OD$). (ODc) represents OD cut-off (three standard deviations above the mean OD of the negative control).

RESULTS AND DISCUSSION

***K. pneumoniae* isolates identification**

For preliminary identification of the isolates, they were cultured on blood agar, MacConkey agar, CHROMagar Orientation, and EMB medium. On blood agar, *K. pneumoniae* colonies appeared circular, 2-3mm in size, mucoid, greyish white, translucent-opaque and γ-Hemolysis (Non-hemolytic) as in Figure 1A. In addition, *K. pneumoniae* colonies on MacConkey agar plates were large, mucoid, and pink due to lactose fermentation Figure 1 B. To differentiate *K. pneumoniae* from *E. coli* colonies, all the isolates were cultured on EMB, and colonies appeared large, mucoid, pink to purple without green metallic Figure 1 C. In this study, CHROM agar was used as differential medium for *K. pneumoniae*, which showed metallic blue colonies as shown in Figure 1 D.

All isolates of *K. pneumoniae* were positive for catalase test, while they were negative for oxidase. In addition, IMVIC was done, and the isolates were negative for both the indole and methyl red test, while they were positive for Voges-Proskauer and citrate utilization test. In addition, the isolates were identified by VITEK II system. Finally, *rpoB* gene was showed as a powerful tool to identify *K.*

pneumoniae isolates. It was found that *rpoB* gene is more reliable in identification of *K. pneumoniae* than 16s, which could discriminate *Klebsiella* at the species and even subspecies level (He *et al*., 2016|). Thus, we investigated the presence of this gene as identification marker in our isolates. All isolates were positive and gave a PCR product 599 bp as in Figure 2.

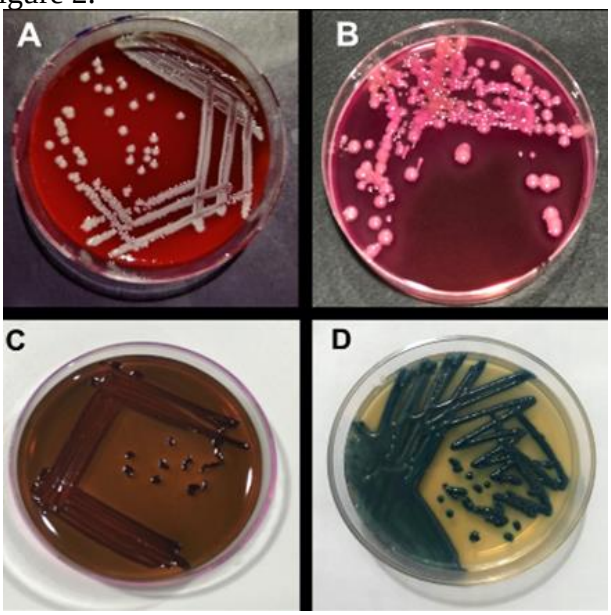

Figure 1. *K. pneumoniae* colonies on different culture medium plates, A) *K. pneumoniae* colonies on blood agar, B) *K. pneumoniae* colonies on Macckoncy agar, C) *K. pneumoniae* colonies on EMB, D) *K. pneumoniae* colonies on CHRMO agar. The isolates were streaked on agar plates from single colony and incubated for at 37 ºC for 24 hr.

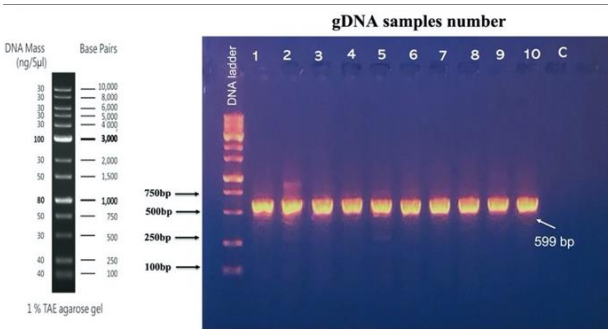

Figure 2. *Molecular identification of K. pneumoniae by PCR. RopB* gene (599bp) was used to identify K. pneumoniae isolates. The PCR products were run alongside DNA ladder on 1% agarose gel at 100 v for 45 min. The bands were visualized under UV light. The gDNA isolates were numbered from 1-10, C =negative control, 100bp DNA ladder used as DNA marker.

Antibiotic susceptibility of *K. pneumoniae* isolates

In current study, several antibiotics were tested for *K. pneumoniae* antibiotic susceptibility. The isolates showed a high resistance rate for ampicillin 100% followed by 3rd or 4th generation cephalosporins (ceftriaxone 86%, ceftazidime 82% and cefepime 69%). In addition, the isolates were 72% and 74% resistant to aztreonam and tetracycline, respectively. Trimethoprim/sulfamethoxazole resistance was 61% among the isolates. Furthermore, the isolates showed variable resistance to other antibiotics used in this study as in Figure 3. Most of these isolates (94%) were multidrug resistance (MDR) isolates. Two isolates only showed a resistance to colistin, which is used as last resort to treat infections when no other drugs available due the high side effects, which indicates the emergence of colistin resistance.

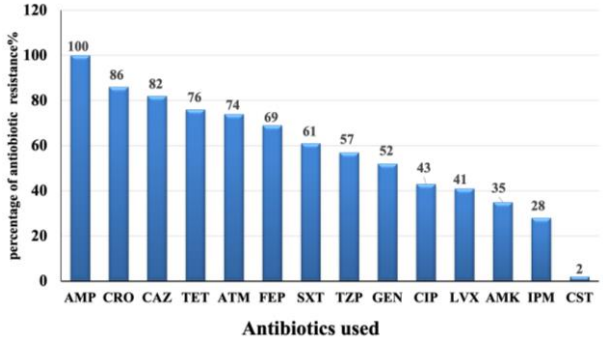

Figure 3. Percentage of antibiotic resistance of K. *pneumoniae isolates.* Ampicillin=AMP, Ceftriaxone = CRO, Ceftazidime = CAZ, Tetracycline = TET, Aztreonam = ATM, Cefepime = FEP, Trimethoprim/Sulfamethoxazole = SXT, Piperacillin /Tazobactam=TZP, Gentamicin = GEN, Ciprofloxacin = CIP, Levofloxacin = LVX, Amikacin = AMK, Imipenem = IPM, Colistin = CST.

Biofilm formation in *K. pneumoniae*

Biofilm formation in *K. pneumoniae* isolates were tested by Congo red method and Microtiter plate assay for 100 isolates. It was found out of these isolates, 2 isolates are hvKp and 19 have the potential to be hvKp depending on phenotypic and molecular identification used previously. Although, we found only two hvKp and 19 have the potential to be hvKp, this study aimed to compare the ability of these isolates to form biofilm.

Congo red test

The isolates were streaked on congo red plates and checked for black colonies on the plates. the isolates (33%) were biofilm producer and 67% non-biofilm producer as shown in Figure 4.

Microtiter Plate Assay

A quantitative adherence assay was performed to test biofilm formation of the isolates (Figure 5). The cKp, hvKp and potential hvKp isolates from this study showed variable biofilm ability as cKp isolates Table 1.

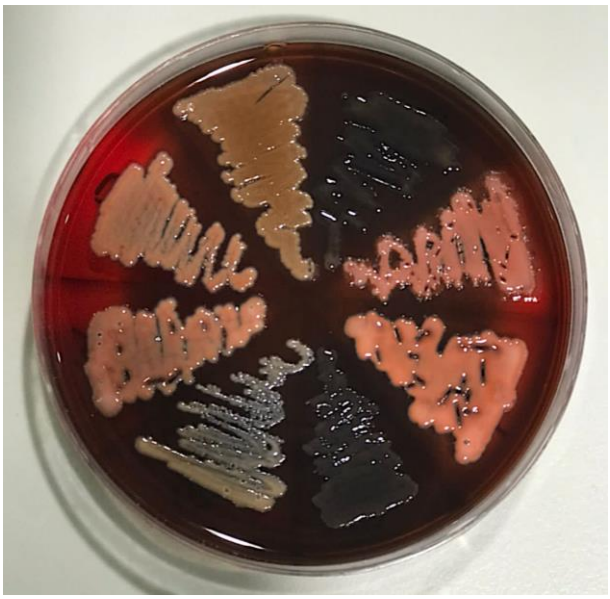

Figure 3. K. *pneumoniae* isolates on Congo red agar plates Black colonies indicated the ability to form biofilm, pink or white colonies represent non-biofilm producer isolates.

DISCUSSION

K. pneumoniae can form biofilm, which is an aggregation of cells embedded within a self-produced matrix (consists of exop-olysaccharides, proteins, DNA, and lipopeptides [25] of extracellular polymeric substance and can adhere to each other and/or to a surface. Biofilm can protect the bacteria from immune defense mechanism and effect of antibiotics. *K. pneumoniae* can form a thick layer of extracellular biofilm, that assist in attachment of bacteria living or non-living surfaces and reducing antibiotics effect [26]. Because the biofilm produced by hvKp strains enables the organisms to colonize in the gastrointestinal, respiratory, and urinary tracts, it is possible that the biofilm increases the invasiveness of the infection. Biofilm is known to assist in protection of from immune defences mechanism and antibiotics, which lead to increase hvKp resistance to antimicrobial drugs [27] [28]. Most of our isolates were resistant to antibiotics used in this study including hvKp isolates. It was showed that hvKp is sensitive to the most antibiotics but recently the emergence of antimicrobial resistance has been documented [29]. The ability to test biofilm formation for isolates in this study was performed by two methods. Studies showed that MTP is more accurate and higher sensitivity (100%) to detect the biofilm formation [30][31]. As in this study the congo red showed only 33% biofilm producer and in MTP was 63%. HvKp and potential hvKp isolates did not show any different results in ability to form biofilm as other tested isolates (cKp), this may belong to the fact that we found only two hvKp isolates and 19 potential hvKp, thus, large scale study is required. It was showed hvKp appeared a robust biofilm producer in the laboratory in comparison to non-tissue-invasive isolates [12]. However, other studies have shown that there is no difference between biofilm formation between invasive and non-invasive isolates [19], which agreed with our study.

Table 1. presents of percentage of cKp, hvKp and potential hvKp isolates to form biofilm.

***K. pneumoniae* isolates**	**Ability of the isolates to form biofilm %**				**Total number of the isolates**
	Weak	**Moderate**	**Strong**	**Non biofilm producer**	
hvKp isolates	1 (50%)	0	1 (50%)	0	2
Potential hvkp isolates	5 (26.3)	1 (5.2%)	8 (42.1%)	5 (26.3%)	19
Classical K. pneumoniae	28 (35.4 %)	14 (17.7%)	5 (6.3%)	32 (40.5%)	79
Total number of the isolates	34	15	14	37	100

CONCLUSIONS

This study concluded that hvKp and potential hvKp isolates showed a variable ability to form biofilm as classical *K. pneumoniae*. It has not known if biofilm can assist in this hypervirulence phenotype of this pathotype, thus extensive and large-scale studies are required. Finally, this study has many limitations, few hvKps has been found to get the actual comparison in biofilm formation ability. Furthermore, molecular investigation is required to study biofilm in theses isolates.

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Author Contributions: All authors contributed equally in writing original draft preparation, all authors have read and agreed to the published version of the manuscript.

Informed Consent: All patients gave their written informed consents before inclusion.

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