The effects of U.V light on *mrk*A, *mrk*D genes in local isolates of *Klebsiella pneumoniae*

Ali Hussein Alwan, Sura Mouaid Abass
Department of Biology, College of Science, Al-Mustansiriyah University, Iraq.

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ABSTRACT

Klebsiella pneumoniae is dangerous pathogens that can cause severe diseases. This study included isolation of 50 isolates of *K.pneumoniae* from different clinical sources from different hospitals in Baghdad city, the number and percentage of isolates according to the sources (urine, blood, sputum, burns, ear swabs, pus, wounds and stool) were 22(44%), 11(22%), 4(8%), 4(8%), 3(6%), 3(6%), 2(4%) and 1(2%) respectively. The ability to form biofilm was carried out using Tissue culture plate methods (-TCP-). The results showed that 80% of the isolates were producer biofilm; the genetic study was used to detect the presence of *mrk*A, *mrk*D genes that are believed to be responsible of biofilm production. The ratio was *mrk*A 87.5% and *mrk*D 50% before exposure to U.V.light to reduce to 43.7% *mrk*A and 18.7% *mrk*D in isolates after exposure to U.V.light source.

الخلاصة

بكتريا الكليبسلا هي من الممرضات الخطرة التي بامكانها ان تسبب اصابات حادة شملت الدراسة عزل 50 عزلة من البكتريا من مصادر سريرية مختلفة من مستشفيات مختلفة في مدينة بغداد (مستشفى ابن البلدي ، مستشفى الكندي التعليمي ، والمختبرات التعليمية لمدينة الطب). لغرض دراستها من محاور عدة بعد ان شخصت البكتريا اعتمادا على الطرق التقليدية بالتشخيص من حيث صفاتها الزرعية والكيموحيوية فضلا عن استعمال جعاز الفايتك compact 2 system لتاكيد التشخيص ، اظهرت نتائجنا ان عدد ونسبة عزلات بكتريا *Klebsiella pneumoniae* التي تم الحصول عليها وفقا للمصادر السريرية (الادرار ،الدم ،القشع، الحروق ، مسحات الاذن ،القيح ،الجروح والخروج). اظهرت العزلات قدرة مختلفة على انتاج الغشاء الحيوي ، حيث استخدمت في هذه الدراسة طريقة اطباق المعايرة الدقيقة 80% مكونة و20% غير قادرة على تكوين الغشاء الحيوي ، وللتحري عن نسبة وجود الجينات التي يعتقد انها مسؤلة ان انتاج الغشاء الحيوي تمت بواسطة جهاز تفاعل البلمرة التسلسلي وكانت النسبة للجين *mrkA* وللجين *mrkD*92% و 50% للعزلات الغير المشععة واصبحت وابحت النسبة للجين *mrkA*53% والجين *mrkD*23% للعزلات بعد التشعيع .

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen responsible for causing a wide spectrum of hospital community-acquired and nosocomial infection especially patients with indwelling medical devices such as urinary catheters [1]. *K. pneumonia* is member of Enterobactericeae, Gram- negative, rod shape non-motile, facultative anaerobic, lactose fermenter with a prominent capsule, present in the environment such as soil, vegetation, water and readily isolated from mammalian mucosal surfaces. [2] It was described as a saprophyte microorganism, not only colonizing the human gastrointestinal tract, skin and nasopharynx, but also able to cause urinary and biliary tract infections, osteomyelitis and bacteremia [1] . The incidence of *K. pneumoniae* infections increased in hospitals, according to the latest data from the European Centre for Disease control (ECDC) and included the six ESKAPE bacteria responsible for two-thirds of all HAIs (Human Acquired Infections) [3]. The ESKAPE pathogens are multi-drug resistant strains of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella* species, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species [4]. *K.pneumoniae* possessing two types of fimbriae or pili which are non-flagellar, and demonstrated mainly on the basis of their ability to agglutinate erythrocytes of different animal species [5]. Type 3 fimbriae have been shown to mediate the initiation of biofilm formation on biotic and abiotic surfaces, in addition to being required for mature biofilm formation [6,7]. The mannose-resistant type 3 pili (T3P or MR/K), composed of the major pilus subunit *mrkA* and the minor tip adhesin *mrkD*. T1P [8] mediate binding to mannose- containing receptors on epithelial cells of the urogenital tract and trachea, yeast cells and guinea pig erythrocytes [9]. In contrast, the MR/K pili adhesive functions are independent of D-mannose and are thought to be produced by most K. pneumoniae isolates and to promote adherence to tracheal epithelial cells, renal tubular cells, and extracellular matrix proteins, basement membranes of lung tissue and to aid in biofilm formation [8]. Type 3 fimbriae is known to be in K. pneumoniae at least two variants, a chromosomally encoded variant and a plasmid-carried variant with 60% to 96% homology, depending on the genes compared, with the *mrkD* gene being the most diverse; The plasmid-borne variant is found only in a few isolates, while the chromosomally encoded variant is found in the vast majority of *K. pneumoniae* isolates [10]. Type3 fimbriae belongs to the chaperone/usher class of fimbriae and are encoded by the *mrkA* BCDF gene cluster, in which *mrkA* encodes the major structural component and *mrkD* encodes the fimbrial adhesin, the genes *mrkB* and *mrkC* encode the chaperone and usher

proteins, respectively; The *mrkF* gene encodes a protein suggested to be involved in the stabilization or assembly of the fimbriae [11]. In this study we aimed to detect the presence of *mrkA*, *mrkD* genes and their role in biofilm formation in *Klebsiella pneumoniae*, studying the effects of U.V. Lights on these genes, determine the role of these genes in biofilm formation in *K.pneumoniae* before and after exposure to U.V. Light.

Methods

Bacterial isolates

Fifty *Klebsiella pneumoniae* isolates were isolated from some hospitals in Baghdad city included: Ibn-El Balady hospital, Al-Kendy teaching hospital and Teaching laboratories in medical city, during July 2014 to December 2014. They were isolated from different clinical sources including: urine, blood, sputum, ear swab, burn, stool, wounds and pus. Diagnosis were carried out using traditional methods such as microscopic examination cultural chrercteristics (macConkey agar), and biochemical test (catalase and oxidase) furthermore using vitek 2 compact system for confirm the diagnosis.

Tissue Culture Plate Method

The assay was performed in triplicate using 96-well flat-bottomed cell culture plates (Nunc, New York, NY, USA) as described previously [12] . Breifly 10 ml of Trypticase soy broth with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar. The culture was further diluted 1:100 with fresh medium. Flat bottom wells tissue culture plates were filled with 0.2 ml of diluted cultures individually. Similarly control organisms were also diluted and incubated. After incubation at 37 0C for 24 hours , gentle tapping of the plates was done. The wells were washed with 0.2 ml of phosphate buffer saline (pH 7.2) four times to remove free floating bacteria. Biofilms which remained adherent to the walls and the bottoms of the wells were stained with 0.1% crystal violet. Excess stain was washed with deionized water and plates were dried properly. Optical densities (O.D) with a micro ELISA auto reader at wave length 570 nm.

Susceptibility to ultraviolet

Ultraviolet was carried out according to Ahmed *et al.* [13] with some modification. In this study the isolates were chosen for other experiment depending on biofilm formation by choosing two strongest isolates from each source unless *K.p* 12, *K.p* 13 (unable to produce biofilm) and because there was only one isolates from stool, we take an extra weak (unable to produce biofilm) isolate. Sixteen isolates of *Klebsiella pneumoniae* were inoculated in brain heart infusion broth for 24 hr. at 37 0C, after incubation tubes were centrifugation at 3000 r.p.m for 10 min, to remove the media and remain the bacterial sediment, 10 ml of normal saline was added to bacterial sediment and exposure to UV light at 240 nm and 30 cm distance from the source of radiation for 30 minutes was conservation in complete darkness. The treated strains were inoculated in nutrient agar to calculate the number of remaining cells and inoculate the wild type of strain for the purpose of comparison and then by the number of developing cells in comparison.

DNA Extraction

Template DNA was prepared by using Lysis buffer according to Ibtisam [14] as follows: Isolates (16 after and before exposure to U.V. light) were inoculated on nutrient agar for 24 hr. at 37 0C, then harvested and suspended in 500 µl of Lysis buffer in Eppendrof tube, mixed gently to homogenize bacteria with Lysis buffer, the Eppendrof tube was placed in water bath at 100 ^{0}C for 10 min, after that centrifuged at 8000 r.p.m for 5 min. the supernatant was transferred to a new Eppendrof tube and the same amounts of absolute alcohol was added with mild mixed. Bubbles were appeared as sing of presence of DNA. Ependrofs tube were centrifuged at 8000 r.p.m for 10 min. neglected the supernatant and the sediment was kept, drained and turn in filter paper to eliminate alcohol. Finally 50µl of double distilled water (D.DW) was added to got extracted DNA.

Primers selection and preparation

Oligonucleotide PCR primers specific for *mrk*A, *mrk*D (Alpha DNA, USA) were used and molecular sizes of the expected amplification product and accession number were listed in table (1). The primers were diluted by adding nuclease free water to get a final concentration equal to 100 p.mole. The amplification was performed in a TECHNE (TC-3000) thermal cycler. For amplification of *mrk*A, *mrk*D genes, five micro liters of the DNA were mixed with PCR mixture that composed from 12.5µl GoTaq® Green Master Mix, 2x, 1.5µl from each primer of *mrk*A,
*mrk*D and 4.5µl of nuclease free water to get final volume 25 µl. PCR Reaction condition of *mrk*A and *mrk*D genes with modification (Table 2).

Table 1. PCR oligonucleotide primers

Primers name	primers sequence 5′----------3′	Product size (bps)	
*mrk*A F	GTTAACGGCGGCCAGGGCAGCGA	380	Ariadnna *et al* (15)
*mrk*A R	AGGTGAAACGCGCGCCATCA		
mrkD R	CCACCAACTATTCCCTCGAA	500	Bellifa *et al* (16)
mrkD F	ATGGAACCcACATCGACATT		

Table (2): PCR Reaction condition of *mrk*A and *mrk*D genes with modification.

Conditions / Primer	Initial denaturation	No. of cycle	Denaturation	Primer annealing	Elongation	Final extension
mrkA	94^{0}C / 2min	30	94 ^{0}C / 30 sec	63 ^{0}C /30 sec	72 ^{0}C /20 sec	72^{0}C /7 min
mrkD	94^{0}C / 2min	30	94^{0}C / 30 sec	69 ^{0}C /30 sec	72^{0}C / 1min	72^{0}C /7min

RESULTS AND DISCUSSION

The wildly spread of bacteria in hospitals environment in some Baghdad hospitals is the main reasons made this pathogen to multidrug resistant and cause nosocomial infections. The number and percentage of isolates according to sources were as follow: 22(44%) isolates from urine, 11(22%) blood, 4(8%) sputum, 4(8%) burn patients, 3(6%) ear swab, 3(6%) pus, 2(4%) wounds infection, and stool 1(2%). Result of TCP method depending on O.D revealed that 60% (30/50) as highest value of biofilm formation due to strong adherence, 26% (13/50) as moderate or weak biofilm former and adherence finally 14% (7/50) as non-biofilm producers. *K.pneumoniae* has a tendency to form biofilms on biotic and abiotic surfaces, including catheters and other medical devices, which is a contributing factor to their antibiotic resistance. [17] Several factors required for biofilms formation have been identified in *K.pneumoniae* clinical isolates from the gastrointestinal tract and in isolates that are associated with pneumoniae and urinary tract infection [18].

This result partially agree with Ariadnna who rev[15]eagled that 64% of isolates were high biofilm forms, while 26% were medium biofilm former and 10% were low biofilm format. On the other hand Samia *et al.*, [19] their results showed that 52% of isolates are high producer while 40% are moderate producers and 8% are non-producers. Carlos *et al*. [20], revealed that 76% of isolates were determined to be positive for biofilm formation while 24% of isolates were to be negative for biofilm formation. The 16 isolates that have been chosen as mention before were susceptibility to UV source for the study the capability of *K.pneumoniae* to mutate, and to compare the infection of wild type and mutant type of *K.pneumoniae in vivo*, and study the effects of ultraviolet on genes responsible of biofilm formation. The result of using the ultraviolet with different time shows the ability of *K.pneumoniae* to resist ultraviolet reaching to (30) cm for 30 min. at 240 nm which the ratio of Killing was (80% to 95%) and after that we study the ability of these isolates to form biofilm in a biotic surfaces. However, the results indicate that 44% of isolates lose the ability to form biofilm in TCP plate while 31% of isolates form biofilm higher than wild type (table3) the isolates that lose the ability to form biofilm that may be to the mutagenic effects of ultraviolet on the DNA (genes) especially *mrkA* and *mrkD* genes. While the isolates that produce biofilm, may be due to the effects of UVlight on *mrkH* and *mrkJ* genes that give opposite effects on biofilm formation (these genes in downstream of the cluster *mrk* genes so may in activate these genes). UV light is characterized by having a strong antimicrobial activity, particularly light with a wavelength below 260 nm leads to cell damages as it is absorbed by proteins and especially nucleotides It causes lethal mutations within the DNA leading to cell death of bacteria[21] .

Table3. The effects of U.V light on biofilm formation by *K.pneumoniae* isolates

Sources	Isolate No.	Biofilm	
		Before exposure (O.D)	After exposure (O.D)
Ear swab	K.p 45	High (0.3)	Low (0.2)
Ear swab	K.p 13	Low (0.12)	Low (0.107)
Blood	K.p 11	High (0.377)	Low (0.089)
Blood	K.p 40	High (0.3)	Low (0.117)
Burn	K.p14	High (0.344)	Low (0.112)
Burn	K.p43	High (0.309)	Low (0.113)
Wounds	K.p12	Moderate (0.2)	High (0.399)
Wounds	K.p21	High (0.247)	Low (0.068)
Sputum	K.p31	High (0.3)	High (0.57)
Sputum	K.p47	High (0.57)	High (0.417)
Pus	K.p32	High (0.34)	Low (0.126)
Pus	K.p50	High (0.3)	High (0.572)
Urine	K.p48	High (0.313)	High (0.5)
Urine	K.p34	High (0.315)	Low (0.077)
Stool	K.p20	High (0.3)	High (0.797)
Ear swb	K.p5	Low(0.12)	Low (0.11)
Number	16		

The effectiveness of UV in inhibiting the growth of pathogens can be seen in several case studies, therefor disinfection by UV radiation can be alternative antimicrobial additives [20].

In this study the presence of *mrk*A gene in wild type was detected (14/16) 87.5% while (8/16) 50% of isolates harbored gene *mrk*D, after UV light exposure the result revealed there were reduced in the percentage became for *mrk*A was 43.7% and *mrk*D 18% indicating that not all isolates harboring *mrkA* necessarily contain *mrkD* (table 5).

These results are in agreement with previous reports in which the *mrkD* gene was found more conserved among *K. oxytoca* than in *K. pneumoniae* isolates [23] , While Alcántar *et al*. [24] Partially agreement with the current results who revealed (100%) of *K. pneumoniae* strains carried the *mrkA* gene and the *mrkD* adhesin gene was present in (20%) strains. The adhesin, *mrk*D, associated with these fimbriae has been shown to mediate binding, *in vitro*, to eukaryotic tissues [25]. However, the *mrk*D

protein does not appear to be necessary for rapid and efficient biofilm formation, although this fimbria-associated polypeptide is required for binding to ECMPs. The major structural component of type 3 fimbriae (mrkA) is a hydrophobic protein[26]. Figures 1 and 2 revealed gel electrophoresis for *mrk*A and *mrk*D genes.

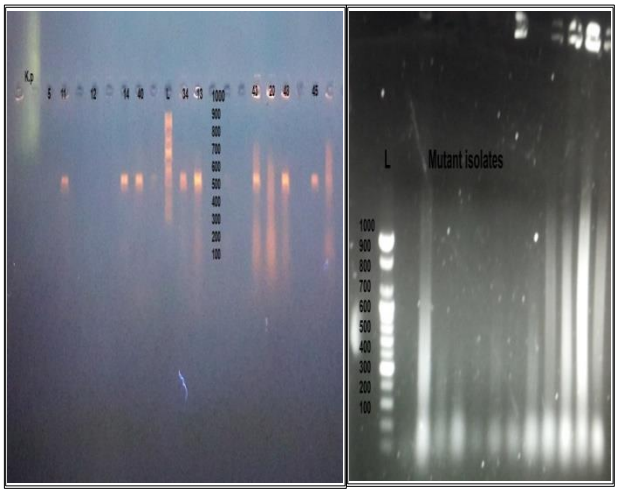

Figure1. Gel electrophoresis for *mrk*D gene in *Klebsiella pneumoniae* (left image shows the *mrk*D gene present in some isolates before exposure to U.V light while Right image shows the abscens *mrk*D gene from isolates after exposure to U.V light).

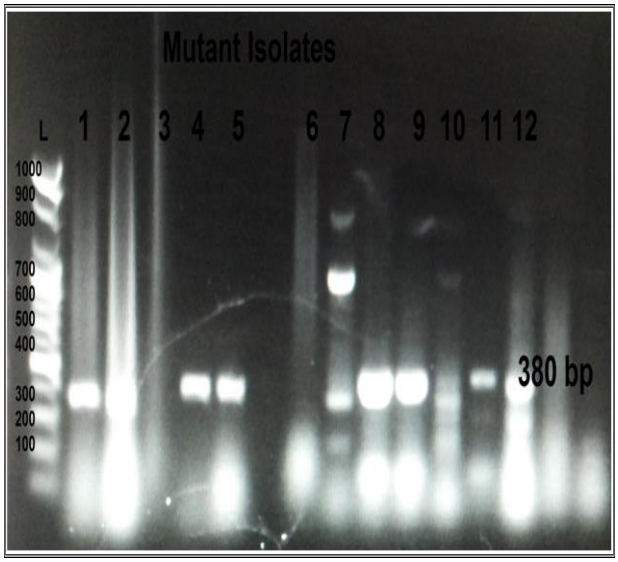

Figure2. Gel electrophoresis for *mrk*A gene in *Klebsiella pneumoniae.*

The wild type strain was found to form characteristic biofilm and development of *K. pneumoniae* biofilms occurred primarily by clonal growth, not by recruitment of planktonic cells; Type 1 fimbriae did not influence biofilm formation and the expression of type 1 fimbriae was found to be down-regulated in biofilm forming cells; In contrast, expression of type 3 fimbriae was found to strongly promote biofilm formation [27] .

Table5. Prevalence of *mrk*A and *mrk*D genes in *K.pneumoniae*

Sources	Isolate	MrkA gene		MrkD gene	
		Wild	Mutant	Wild	Mutant
Ear swab	K.p5	_	_	_	_
Ear swab	K.p13	+	_	+	_
Blood	K.p11	+	_	+	_
Blood	K.p40	+	+	_	_
Burn	K.p14	+	+	+	_
Burn	K.p43	+	_	+	_
Wounds	K.p12	_	_	_	_
Wounds	K.p21	+	+	_	_
Sputum	K.p31	+	+	+	+
Sputum	K.p47	+	_	_	_
Pus	K.p32	+	+	_	_
Pus	K.p50	+	_	_	_
Urine	K.p48	+	+	+	_
Urine	K.p34	+	_	+	+
Stool	K.p20	+	+	_	_
Ear swb	K.p45	+	_	+	+
Total	16	**87.50 %**	**43.70 %**	**50.00 %**	**18.00 %**

The formation of biofilms by *K.pneumoniae* requires the expression of type 3 fimbriae, elongate proteinaceous filaments extruded by chaperone-usher system in the bacterial outer membrane; the expression of the *mrkA* BCDF cluster that encodes this fimbrial system is strongly positively regulated by *mrk*H [7]. The *mrkD* gene is prevalent among *K. oxytoca* strains but is rare in *K. pneumoniae* strains, the *mrk*D tip adhesin is needed for tannic acid dependent hemagglutination and for adherence to extracellular matrices, However, *mrkD*-strains can still bind to host cells and form biofilms, suggesting differences in receptor specificity and the presence of other adhesins; There is a minor pilin subunit protein, known as *mrk*F, intermittently inserted into the pilus rod [29].

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