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DetectionOf genesResponsible for BiofilmsFormedby Klebsiella pneumoniaeandEsherichia coliand their effect on innate immunity

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Abstract:

Biofilms as a major virulence factor of bacteria ,therelationship between bacteria persistence in the urinary tract and genes responsible for biofilms production was studied.

The aim is to detect the presence,frequency of the,fimA, fimH, mrkA and mrkD, genes and biofilms production in two species isolated from UTI patients and their effect on some innate immunity aspects.

Sixty five isolates of E.coli and fifty of K.pneumoniae isolates were collected. All isolates were initially diagnosed as genus Esherichiacoli andKlebsiella pneumonia. Final identification for the isolates was done by Vitek 2 compact system. The ability to form biofilm was carried out by using Tissue culture plate method (TCP).Adhesion average to epithelial cells of E.coli and K.pneumoniae was studied. Detection of genes were done to by using PCR technique .

Immunological experiments were done by using bactericidal activity and opsonization factor.

UTI infection were more common in female than male. 75% (49/65) females, 25%(16/65) males ofE.coli isolates and 88% (44/50) females,12%(6/50) males of K.pneumoniaeisolates. Patients ages in children between (2 months -18years) in UTI caused by E.coli was 35%(23/65) while in k.pneumoniae was 74%(37/50),Ages between (18 years-50 years) in E.coli was 37%(24/65) while in K.pneumoniae was 18%(9/50) finally,Ages between (50 years-72years) in E.coli was 28%(18/65) while in K.pneumoniae was 8%(4/50).69% of E.coli isolates and 72% of k.pneumoniae isolates were higher biofilm producers. . Adhesion average to epithial cells of E.coli and K.pneumoniae was 42.9% and 44% respectively. Both E.coli and K.pneumoniae showed that 100% 0f the 12 isolate harbored the fimA, fimH, mrkA and mrkD genes. The bactericidal average was higher in E.coli than K.pneumoniae , P- value(0.01) for mice injected with fimbriae, Opsonization factor was higher in E.coli than K.pneumoniae the P value (0.05) .

Conclusion: A strong relationship between biofilms production and presence of genes of different species isolated from same clinical source that has effected on resistance of bacteria to innate immunity.

Key words: K.pneumoniae; E.coli; fimA; fimH; mrkA; mrkD; innate immunity.

الخلاصة:

شملت الدراسة الحالية الاغشية الحيوية والعلاقة بين قدرة البكتريا على البقاء في القناة البولية والجينات المسؤلة عن انتاج الاغشية الحيوية .هدفت الدراسة الى تحديد الجينات fimA , fimH, mrkAmrkD, وتأثيرها على انتاج الاغشية الحيوية وكذلك دراسة مدى تردد هذة الجينات في جنسين مختلفين الكليبسيلا الرئوية والاشريشيا كولاي المعزولة من نفس المصدر السريري للمصابين بألتهاب المجاري البولية وتاثير وجود هذة الجينات وترددها على المناعة المتأصلة.

خمسة وستون عزلة من بكتريا الاشريشيا القولونية وخمسون عزلة من بكتريا الكليبسيلا الرئوية جمعت من مصابين بألتهاب المجاري البولية، جميع العزلات شخصت ابتداءا وكانت تعود الى الاجناس قيد الدراسة ،التديد النهائي للتشخيص عمل بأستخدام اختبار الفايتك . Vitek 2 compact system اختبرت قابلية انتاج الاغشية الحيوية من خلال استخدام طريقة الاطباق من ناحية اخرى معدل الالتصاق على الخلايا الطلائية للاجناس السابقة تم تحديده.فيما استخدمت تقنية البي سي ار للكشف عن الجينات.اخيرا درست التاثيرات المناعية باستخدام تجربة التأثير القاتل للبكتريا bactericidal activityوتجربة عامل الطهي.opsonization factor

اظهرت النتائج ان اصابات القناة البولية في الاناث اكثر منها في الذكور 75% (65/49) في الاناث ،12%(50/6) في الذكور في الاصابات المتسببة عن بكتريا الاشيريشيا القولونية . فيما كانت النسب 88% (50/44) في الاناث ، 25%(65/16) في الذكور في الاصابات المتسببة عن بكتريا الكليبسيلا الرئوية. اعمار المصابين بين (2 شهر الى 18

سنة) كانت بنسبة 35%(65/23) للاصابات المتسببة عن الجنس الاشريشيل القولونية فيما كانت النسبة 74%(50/37) لاصابات الكليبسيلا الرئوية من ناحية اخرى كانت النسب للفئة العمرية (18 الى50 سنة)37%(65/24) للاشيريشيا القولونية و كانت 18%(50/9). الفئة العمرية الاخيرة حسب الدراسة تراوحت (50 الى 72 سنة) 28%(65/18) للاشريشيا كولاي ، 8%(50/4) لبى الكليبسيلا الرئوية . نتائج تجربة قابلية انتاج الاغشية الحيوية كشفت ان 69% من غزلات جنس الاشريشيا كانت منتجة للاغشية الحيوية ،فيما كانت النسبة اعلى فيما يخص عزلات جنس الكليبسيلا الرئوية اذ اظهرت ان 72% كانت منتجة. نتائج معدل الالتصاق لعزلات الجنسين الاشيريشيا كولاي و الكليبسيلا الرئوية كانت 42.9% و44% على التوالي . كلا الجنسين اظهرت ان 12 عزلة كانت حاوية بنسبة 100% للجينات المدروسة fimA, fimH, mrkA.mrkD

معدل نتائج اختبار الفالية القاتلة للبكتريا كانت اعلى بالنسبة الى غزلات جنس الاشيريشيا القولونية مقارنة بعزلات جنس الكليبسيلا الرئوية استنادا الى نتائج التحليل الاحصائي بمستوى معنوية (0.01) للفأران البيضاء المحقونة بالفامبيريا fimbriaeالمنقاة جزئيا من كلا الجنسين . فيما اشارت نتائج التحليل الاحصائي ان معدل عامل الطهي كان اعلى بالنسبة الى جنس الاشيريشيا كولاي مقارنة مع جنس الكليبسيلا الرئوية بمستوى معنوية (0.05).

الاستنتاج

استنادا الى هذه الدراسة المحلية نستنتج ان هنالك علاقة قوية بين قابلية انتاج الاغشية الحيوية ووجود الجينات المدروسة لاجناس مختلفة معزولة من نفس المصدر الاصابة السريرية والتي بالامكان ان تؤثر على مقاومة هذة الاجناس للمناعة المتأصلة .

Introduction:

Multiple organisms are presence in urinary tract through which urine flows from kidney viathe bladder. The urinary tractinfectionis the second most common type of infection[1]. Urinary tract infections are mostly caused by gram-negative bacteria like, E.coli, Klebsiella species, Proteus mirabilis, Pseudomonas aeruginosa, Acinetobacter, and Serratia. Ninety% of UTI cases are caused by gram-negative bacteria on the other hand only 10% of the cases are caused by gram positive bacteria[2]. E. Coli are the most common gram –negative bacteria responsible for UTI [3], 75% to 87% of UTI cases are due to Escherichia coli [4].Whereas Proteus mirabilis and Klebsiella pneumoniae infection accounts 10%, 6% in respect [5].Bacterial adherence not only contributes to colonization but also to invasion, biofilm formation, anddamage of host cell[6]. The two primary fimbrial adhesions associated with E.colistrains are type 1 and P fimbriae. Type 1 fimbriae may be mediate adherence largely by the FimH tip adhesionthatwas recognized and bound mannosylated moieties on surfaces of biotic and abiotic [7]. Within the host, FimH mediates E.colibinding to theepithelium of bladder and is required for proper formation of biofilm-likecommunities of intracellular bacterial withincells of bladder epithelial. [8].Klebsiella pneumoniae is an opportunistic pathogens responsible for a wide spectrum of hospital community-acquired and nosocomial infection especially patients suffering from indwelling devices of medicalsuch ascatheters [9].K.pneumoniae possessing two types of fimbriae or piliwhich are non-flagellar, and showed mainly on the basis of ability of agglutinating erythrocytes ofspecies of different animal [7]. Type 3 fimbriae have showedof mediating the initiation of biofilmsproducing on biotic and abiotic surfaces, in addition of being required formature biofilms formation [10,11].The mannose-resistant type 3 pili (T3P or MR/K), constituent of the major pilussubunit mrkA and the minor tip adhesin mrkD [12].Biofilms plays a main role in virulence of bacteria[1]. The possible relationship between bacteria persistence in the urinary tract andthe presence of virulence factors (VFs) lead to biofilmsproduction like adhesins, toxins, lipopolysaccharides, iron acquisition, presence of capsule and serum resistance [1,13]. Biofilms formation capable singlecellorganisms to assume a temporary multicellular lifestyle, in that organisms behavior facilitates survival in adverse environments[13]. Several advantages for

microorganisms to produce biofilms.Thatprovides enclosed of surface space which is occupied and can provide astability degree of in thegrowthenvironment [1]. The innate immune system presents the first line, the second line of defense iscomposed of antibodies, activated macrophage and T-cell [14].The urinary tractsystem reliesmainly on innate immunity for its defense and it consists of the, urinary bladder,urethra, ureters, and kidneys [15]. The urinary tract barrier defense in is the tightly joined with the epithelial lining of those components [16].

Methods

Bacterial isolates

Sixty five E.coli isolates and Fifty of K.pneumoniae isolates were isolated from some hospitals in Baghdad city ; Ibn-El Balady hospital, Al-Kandy teaching hospital and Teaching laboratories in medical city, Fatima Al-Zahraa during October 2016 to January 2016. They were isolated from UTIs patients.Diagnosis were carried out using traditional methods such as microscopic examination cultural characteristics (macConkey agar,Eosin methylene blue agar and Chromoagar), 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 [17]. Briefly 10 ml of Trypticase soy broth with1% 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.

Ability of K.pneumoniae and E.coli to adhere to epithelial cells

This test was done according to [18]:

- (0.5) ml of both K.pneumoniae and E.coli suspension were transferred to test tubes separately and these tubes each of them contain (0.5) ml of epithelial cell suspension.
- The tubes were incubated at a temperature 37°C min by using water bath in a shaking range 70 r.p.m for 60 min.
- The tubes were centrifuged at 3000 r.p.m for 10 m and the sediment was washed with phosphate buffer saline and this process was repeated 3 to 4 times to get ride of un adhered cells.
- The sediment was suspended with a drop of phosphate buffer saline and this suspension was transferred to glass slide by Pasteur pipete and left to dry in the air then the slides were stained by crystal violet to detect the adhesion average to epithelial cells.
- The slides were tested by using the oil lenses and the adhered cells were counted by using equation:

Adhesion average: the number of epithelial cells with bacteria adhered was divided by the total number of epithelial cells observed by microscope

DNA Extraction:

DNA extraction of bacteria was done using Wizard® Genomic DNA purification Kit. Isolates were inoculated on nutrient agar for 24hr at 37°C , the isolates were harvested and suspended in 1 ml of LB broth in Eppendrof tube then mixed gently. The extraction of genomic DNA according to the manufacturing of the company.

Primers selection and preparation:

The primers (Alpha DNA, USA) used in PCR amplification were specific for mrkA ,mrkDfor K.pneumoniae and fimA, fimH

for E.coli. The oligonucleotide PCR primers specific for these genes, molecular sizes of the expected amplification product and accession number were listed in (table1). Thedilution of primers weredone by adding nuclease free water according to the manufacture companies' information (Adanced Scientfic Bureau).

The amplification was performed in a TECHNE (TC-3000) thermal cycler. The amplification of all genes were done, five micro liters of the DNA were mixed with PCR mixture that composed from 12.5 µl GoTaqR Green Master Mix, 2x, 1.5 µl from each primer forward and primer reverse of all genes , then 4.5µl of nuclease free water to get final volume 25 µl.PCRReaction condition of mrkA ,mrkD ,fimA and fimH genes with modification table-2.

Table (1):- PCR oligonucleotide primers

Gene name	Primer sequence	Product size	
***fim*A**	**F 5- AGTTAGGACAGGTTCGTACCGCAT -3**	**316bp**	**Hernandes *et al*[19]**
	R 5- AAATAACGCGCCTGGAACGGAATG -3		
***fim*H**	**F 5- TGCAGAACGGATAAGCCGTGG -3**	**510bp**	**Chapman *et al* [20]**
	R 5- GCAGTCACCTGCCCTCCGGTA-3		
***mrk*A**	**F 5-GTTAACGGCGGCCAGGGCAGCGA-3**	**383bp**	**Ariadnna *et al* [21]**
	R 5-AGGTGAAACGCGCGCCATCA-3		
***mrk*D**	**F 5-CCACCAACTATTCCCTCGAA-3**	**500bp**	**Bellifa *et al* [22]**
	R 5-ATGGAACCcACATCGACATT-3		

DNA amplification was performed as follows:

initial denaturation of all genes was for 1 sec at 94°C followed by 40 cycles at 92°C for 30 s for denaturation, annealing at 58°C, 59°C of FimA and fimH respectively for 30 s and the anneling of mrkA ,mrkD was at 63 °C ,69°C respectively ,extension of all genes was at 72°C for 1 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gel. The sizes of the amplicons were determined by comparing them with a 100-bp DNA ladder.

Extraction and partial purification of fimbrial protein

This method was done according to [23].

Animal Infection Experiments

BALB/c mice were used in this experiments as follows: mice were grouped into 2 groups each group contains 9 mice , the first group used to inject Fimbriae of E.coli isolates , second group was injected by Fimbriae of K.pneumoniae isolates .

K.pneumoniae andE.coli isolates were activated in brain heart infusion broth and incubated at 370C for 24 hr, the activated isolates were diluted and compared with the turbidity of McFarland solution to obtained the concentration of cells 1.5 X 108 cell per ml, each mouse was injected with 0.2 ml of bacteria.The injection of fimbriae of E. coli and K. pneumoniae , the injection was intraperitoneal.

Immunological experiments:

Bactericidal assay

Bactericidal assay was used according to [24]:

1- 0.5 ml of overnight culture of bacteria was added to 0.1 ml serum from animals under study and 0.35 ml blood from human.

2-incubate the mixture for 3 hr. with shaking in shaker incubator.
3- 0.1 ml of mixture was placed on sterile dish, then blood agar was added, incubate in 370C for 24 hr.
4- The experiment was repeated but with non-immunized serum for control.
Percentage killing = No. of microorganism in dish contain ml of non-immune serum – No. of microorganism in dish contain ml of serum under study / No. of microorganism in dish contain ml of non-immune serum X 100%
Opsonization assay
Opsonic antibodies were detected by measuring Opsonization activity invitro according to [25]. A mixture of 0.05 ml suspension of activated bacteria, 0.05 ml serum from animals under study and 0,2 ml blood of non-immune human in sterile tube, incubated for 45 min at 37 0C in shaking incubator. After that slides stained with Giemsa stain, calculate 50 cell of polymorph nuclear cells (PMNs) to extracted percentage for Opsonization and compared to control using this formula:
Opsonization factor = No. of phagocytic PMNs / No. of phagocytic and non-phagocytic PMNs X 100%.
Statistical analysis:Statistical analyses were performed using a two-tailed Student's t-test. Values were determined to be statistically significant if the $P \leq 0.05$.

Results and discussion:

Recently wildly spread of bacteria in hospitals environment in some Baghdad hospitalswas noticedand multidrug resistant of pathogenswhich caused nosocomial infections. All isolates were collected from UTIs patients. The isolates were cultured on MacConkey agar, Eosin methylene blue and chromo agar to study the morphological characteristics of the isolates depending on bacterial growth and to confirm this diagnosis , according to results K.pneumoniae appeared large, round, pink (lactose fermenter) and mucoid colonies on MacConkey agar while E.coli showed Red or Pink; Not mucoid; Round appearance (lactose fermenter) ,K.pneumoniaegrewcolonies as brown, dark-centered, mucoid that indicating lactose fermentation and acid production on EMB agar while showed good growth of dark blue-black colonies with metallic green sheen proving vigorouslactosefermentation and production of acid that precipitates the green metallic pigmentfigure -1.

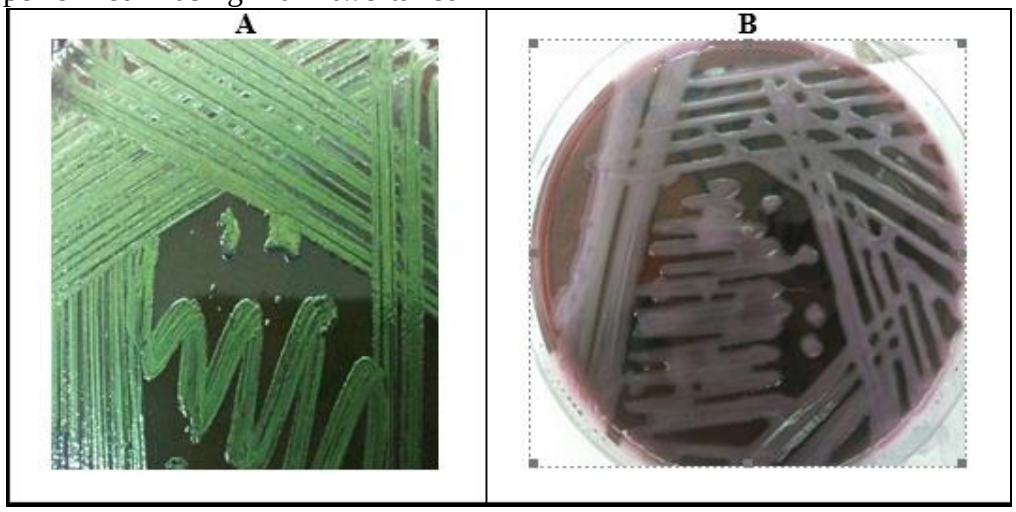

Figure (1) *E.coli* and *K.pneumoniae* on EMB agar(A=*E.coli*,B=*K.pneumoniae*)

K.pneumoniae on chromo agar showed mauve growth color on chromo agar, While *E.coli* gave metallic blue color [26]. Figure-2

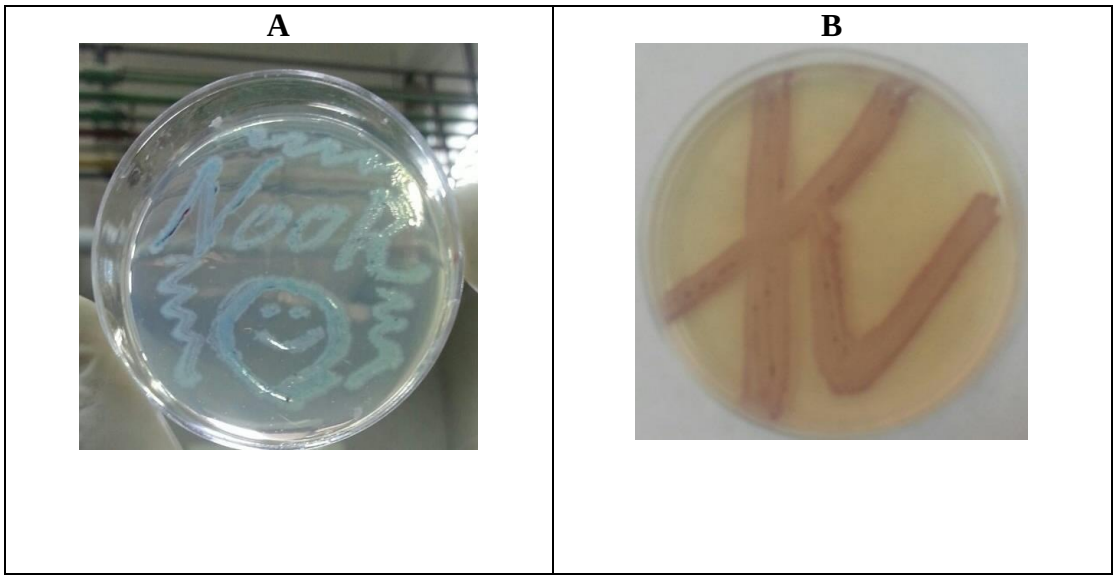

Figure-2: E.coli and K.pneumoniae on Chromo agar (A=E.coli,B=K.pneumoniae)

The finding showed a higher proportion of UTI in females more than males. 75% (49/65) isolates ofE.coliwas isolated from females while25%(16/65)wasfrom males. Alsok.pneumoniaeisolates showed 88%(44/50) fromfemales and12%(6/50)from males These results agreed with [27] .This is understandable due to the anatomy and is a consistent trend worldwide.The patients ages in children between (2 months -18years) in UTI caused by E.coli was 35%(23/65) while in k.pneumoniae was 74%(37/50),Ages between (18 years-50 years) in E.coli was 37%(24/65) while in K.pneumoniae was 18%(9/50) finally,Ages between (50 years-72years) in E.coli was 28%(18/65) while in K.pneumoniae was 8%(4/50).Iraqi people has a large infection carriage and the genito-urinary infections are very prominent, this may be belong to a less affordable personal/ hygiene community for some of the economically back word populations.Biofilms formation Results ofE.colidepending onTCP method revealed that 60% (45/65)produced highestvalue of biofilms,6%(4/65) as moderate or weak biofilm former and adherence finally 25%(16/65) as non-biofilm producers.This results partially agree with Sarojgolia et al [16] revealed that 69% of E.coli isolated from UTIs patients were higher biofilms producer.On the other hand K.pneumoniaeisolates showed that72%(36/50) as strong biofilmsproducers,12%(6/50) as moderate biofilms producers and finally16%(8/50) as non-producers table [3].This resultspartially agree with Sanhez et al. [28] revealed that 76% of isolateswere determined to be positive for biofilms formation while 24% of isolates were to be negative for biofilm formation.The present study showed that adhesion average of E.coli was 42.9% while the adhesion average of K.pneumoniae was 44% figure-3.

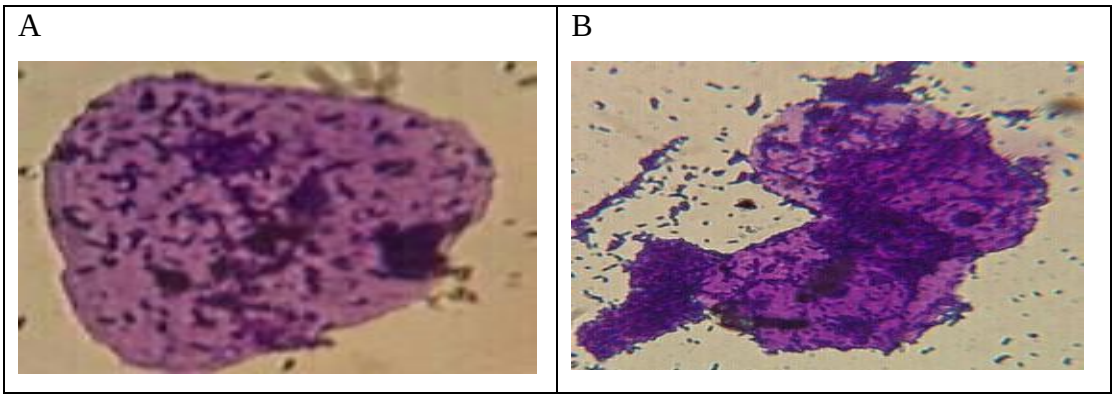

Figure-3: shows the ability of two species to adhere to epithelial cells (A=*E.coli*, B= *K.pneumoniae*

Adherence is one of virulence factors in the urinary tract human and play a critical role for thebacteriuria establishment[29].

Six isolates from E.coli were chosen for detection of fimH and fimA genes and also Six isolates of k.pneumoniae were chosen for detection of mrkA and mrkDgenes, the chosen of the 12 isolate were done according to the value of biofilms formation that showed the highest result.In this study of fimH and fimA also were 100% of the 6 E. colii.100% .All these isolates appeared the presence of type 1 fimbriae (Mannose sensitive hemagglutination -MSHA) [30].Identifying and Detection of the potential uropathogenicEscherichia coli virulence factors genes for fimA and fimHoperons has been done by using method of PCR which has impotent of being highly specific, informative and a powerful genotypic assay, used for detecting of adhesin-encoding operons also other virulence factors that can contribute to virulence in UTI. In this study, we confirmed fimH among UPEC strains in(100%) strains.Our findings showed that type 1 fimbriae is very important and relevant VF, and it can also contribute to virulence in E.coli strains. This study is agreement with [31]. Type 1-mediated adherence has been proposed to play a role in the induction of inflammation, enhancing E. coli virulence for the urinary tract.Figure[4]

Revealed gel electrophoresis for fimA and fimH genes.

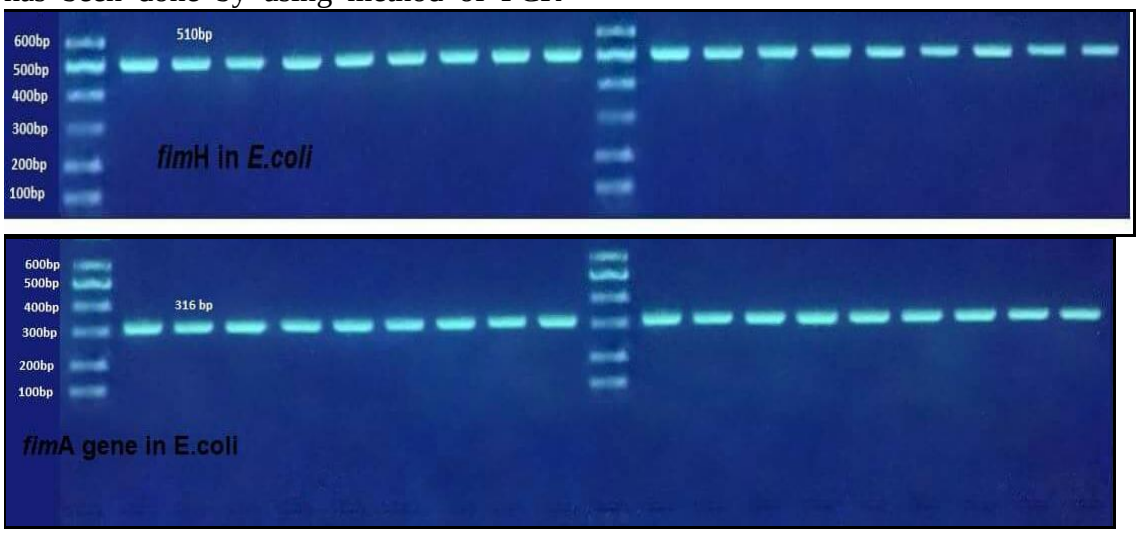

Figure-4: Gel electrophoresis for fimA and fimH gene in E.coli

The presence of mrkA and mrkD were 100% of the 6 isolates that harbored these two genes. This result agree with Alcantar et al.[32]who revealed (100%) of

k.pneumoniae strains carried the mrkA gene .Ariadnna et al.[21] showed that 84% of isolates possessed the mrkA gene Type 3 fimbriae refers to k.pneumoniae ability to adhere to extracellular matrix of cells, through the mrkA protein, type 3 fimbriae mediates bacterial attachment to the basolateral surface of various cell types as the tracheal epithelium and renal tubular cells[33]. figure [5] revealed gel electrophoresis for mrkA and mrkD genes.

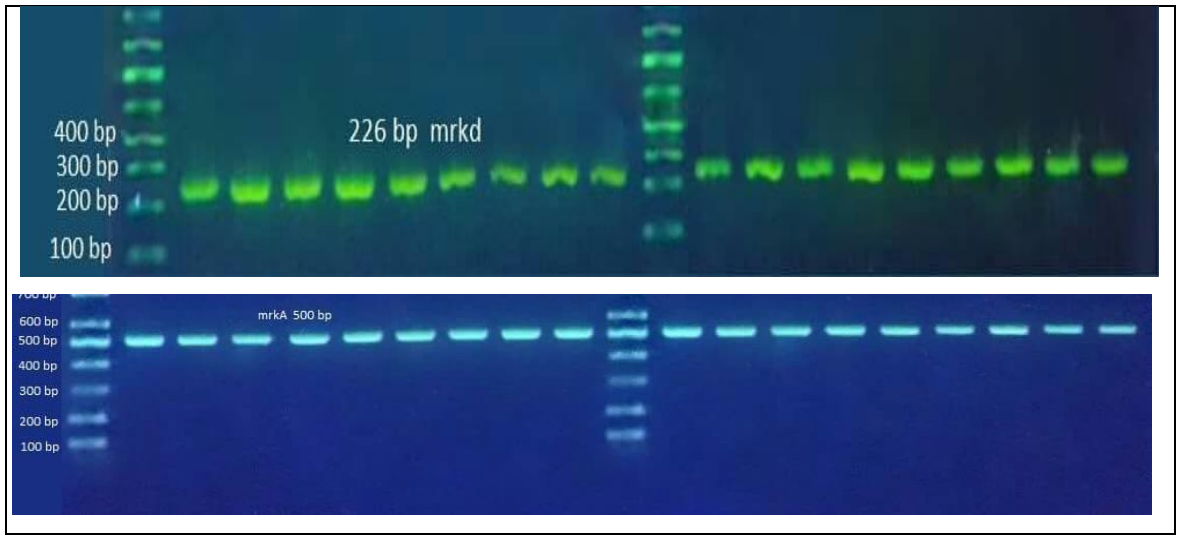

Figure-5: Gel electrophoresis for *mrk*D and mrkA gene in *Klebseilla pneumoniae*

The innate immune system responds to the presence of fimbriae ,flagella and lipopolysaccharide of both E.coli and K.pneumoniae.The adaptive immune response is specific and often delayed that usually responds after a signal from the innate system [34].

After the injection Fimbriae of E.coli and K.pneumoniaeseparately,mice were killed and blood was collected and cultured on blood agar ,after incubation in 37°C for 24 hours.

The results of culture revealed that single colonies were grown from the blood collected from mice injected with fimbriae of E.coli while they showed a heavy growth from blood collected from mice injected fimbriae of K.pneumoniae. figure -6.

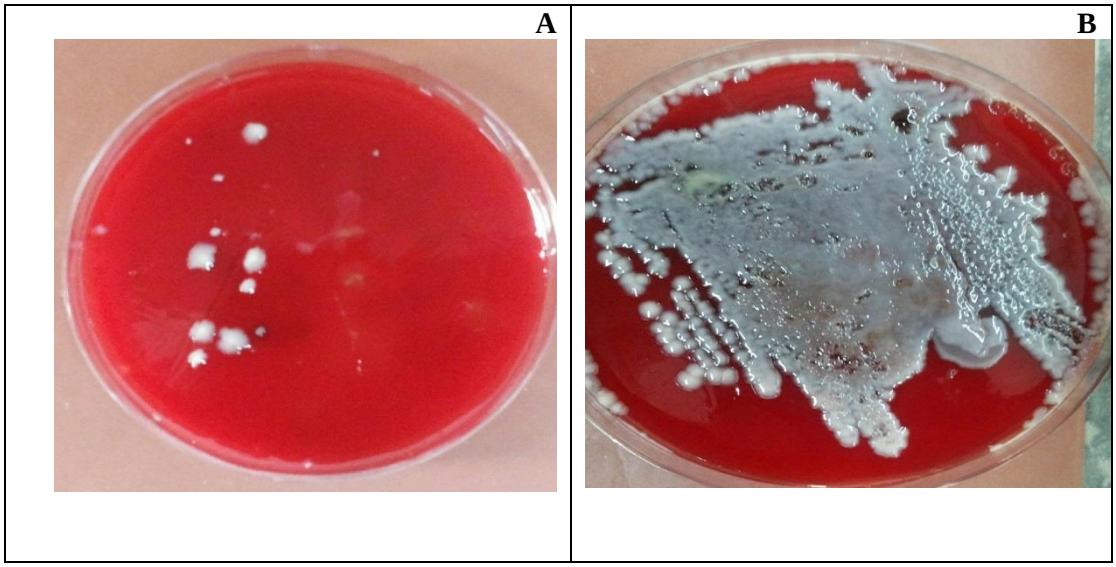

Figure-6:shows the differences between k. pneumoniae and E. coli growth on blood agar(A=E.coli ,B=K.pneumoniae)

The result of Bacteriocidalactivity revealed that use of Fimbriae of E.coli and K.pneumoniae as immunogen to study the bactericidal activity were illustrated in table-2.

Table (2) Bactericidal activity for *k.pneumoniae* and *E.coli*

Fim of *K.pneumoniae*	*mean*	*SD*	Fim of *E.coli*	*mean*	*SD*	P valve
Fim	31.6%	8.32	**Fim**	83.3%	7.63	0.001
Fim			**Fim**			
Fim			**Fim**			
Fim	29.3%	6.02	**Fim**	72.3%	6.8	0.001
Fim			**Fim**			
Fim			**Fim**			
Fim	30.6%	3.21	**Fim**	78%	5.56	0.0002
Fim			**Fim**			
Fim			**Fim**			

Fim= Fimbriae, Lps=Lipopolysaccharide

The appearance of bacterial growth of both K.pneumoniae and E.coli on blood agar showed that there was resistance to phagocytosis bybacteria. Statistical analysis revealed that the differences are significant between the two species (P value 0.01 for mice injected with Fimbriae). The results showed that The average percentage killing of mice injected with E.coli fimbriae was between (83.3%-72.3%) while in mice injected with fimbriae of K.pneumoniae was between (31.6%-26.3%) figure-7.

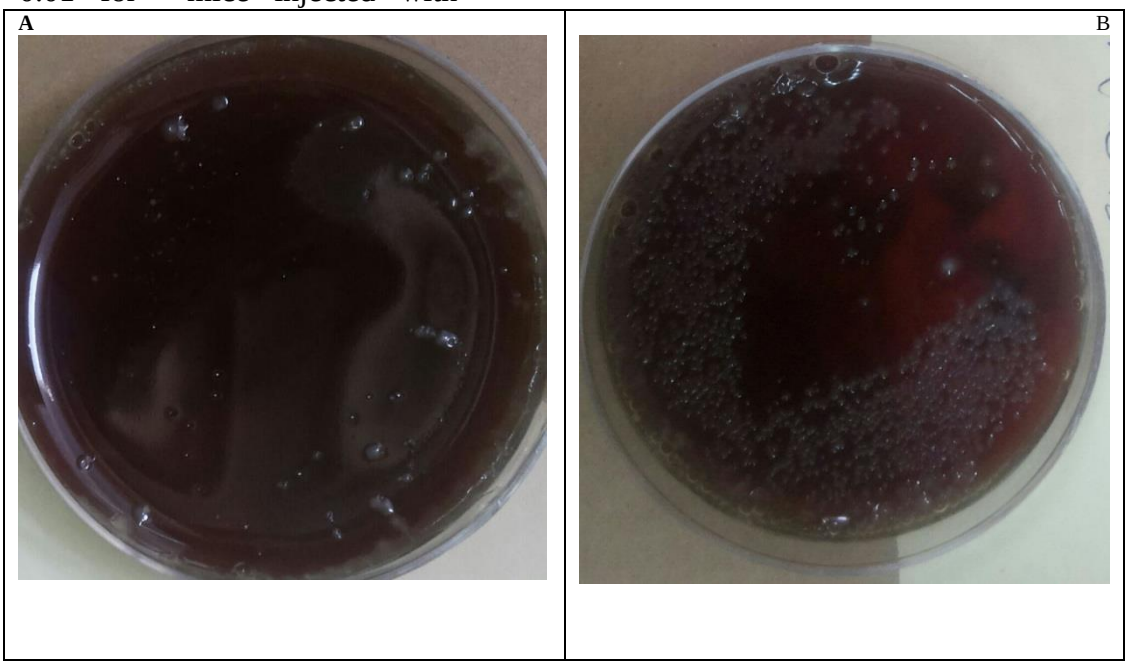

Figure -7: Bactericidal activity for mice injected with fimbriae (A=*E.coli*, B=*K.pneumoniae*)

The percentage of killing in E.coli was higher than K.pneumoniae treated with Fimbriae may be due to the differences between the two species or because that k.pneumoniae is more virulent than E.coli as revealed in the present study .The UTIis detected by TLR4 or TLR5, that can be found on, monocytes , macrophages,epithelial and immature dendritic cells [35].

The result of Opsonization experiments are shown in table (3).Our results showed that the Opsonization factor for mice injected withfimbriae of E.coli lower the p -value 0.05 was (55.5%) while for mice injected with fimbriae of k.pneumoniae was (25%) alsolower the p -value 0.05. This differences between the two species may be due to many reasons such as the differences in biofilms production ,presence of genes responsible virulence factors .

Table-3:Opsonization factor for *K.pneumoniae* and *E.coli*

Fim of *K.pneumoniae*			**Fim of *E.coli***			
Fim	25	7.07	Fim	55.5	2.12	0.02
Fim			Fim			

Fim=Fimbriae, Lps =Lipopolysaccharide

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