Detection of Urovirulence Genes (*eae,E-hly,α-hly*) of Uropathogenic *Escherichia coli* by Specific PCR

الكشف عن جينات الضراوة (*eae,E-hly,α-hly*) لبكتريا اشرشيا القولون المسببة لالتهابات المجاري البولية باستعمال تقنية سلسلة تفاعل انزيم البلمرة مع بادئات متخصصة

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Abstract

Urinary tracts infection remains a common troublesome health problem in the world. Although *E.coli* is normal intestinal flora but considered as the main important opportunistic active uropathogene because of it's pathogenesity which referred to it's different virulence factors like hemolysin, biofilm, enterotoxins, shiga like toxins, cytotoxic necrotizing factor and others. Seventy seven *E.coli* isolates which isolated from 125 midstream urine samples of patients suffering from different types of UTIs. The results showed that 44 isolates 57% were produced hemolysin. These isolates were differed in the efficiency of erythrocyte lysis because which depend on type of hemolysin and source of blood. Seventy two isolates 90.9% were formed biofilm with variety in thickness of biofilm layer. Each isolate produce hemolysin also formed biofilm. Presence of genes encoded for virulence traits of UPEC (hemolysin, biofilm) were examined by PCR with specific primers. The results showed that the percentage of *α-hly* gene encoded for *α*-hemolysin 50% while percentage of *eae* gene encoded for intimin and *E-hly* gene encoded for enterohemolysin were 40% and 20% respectively.

المستخلص

التهابات المجاري البولية هي اصابة مرضية للمجاري البولية وتعتبر من الامراض الشائعة في الكثير من دول العالم . بالرغم من ان بكتريا اشرشيا القولون هي نبيت طبيعي في الامعاء الا انها تعتبر من اكثر العوامل الانتهازية امراضية للجهاز البولي لما تمتلكه من عوامل ضراوة متنوعة مسؤولة عن امراضيتها مثل الهيمولايسين ، الغشاء الحيوي ، ذيفانات الشيكا ، السموم الداخلية وغيرها . جمعت 77 عزلة لاشرشيا القولون من 125 عينة ادرار لمرضى يعانون من انواع مختلفة من التهابات المجاري البولية . وقد اظهرت النتائج انه 44 عزلة 57% منتجة للهيمولايسين لكن هذه العزلات المنتجة للهيمولايسين اختلفت في كفائتها على تحليل كريات الدم الحمراء بالاعتماد على نوع الهيمولايسين المنتج ومصدر الدم كما كانت 72عزلة 90.9% مكونة لطبقة الغشاء الحيوي مع اختلاف في سمك الطبقة المتكونة باختلاف العزلات . كما ابرزت النتائج على انه كل عزلة منتجة للهيمولايسين هي عزلة مكونة لطبقة الغشاء الحيوي . وقد تم استخدام تقنية سلسلة تفاعل البلمرة مع استخدام بادئات متخصصة للكشف عن جينات الضراوة المشفرة للهيمولايسين و بروتينات طبقة الغشاء الحيوي وكانت نسبة وجود جين الالفا هيمولايسين 50% بينما نسبة وجود الجين المشفر لبروتين الانتمين والجين المشفر لانتاج الهيمولايسين الداخلي(20 ، 40)% على التوالي .

Introduction

Urinary tracts infections (UTIs) are condition where one or more structures in the urinary tracts become infected after bacteria overcome it's strong natural defenses, UTIs are the most common of all infections and can occur at any time in the life of the individual [1].

The most important cause of UTIs represented by Gram negative bacteria belong to *Enterobacteriacea* family specially *Escherichia coli* accounting for as much as 80% of community acquired UTIs. Several virulence factors such as hemolysin, cytotoxic necrotizing factor, aerobactin, biofilm and different types of adhesin have been responsible for *E.coli* pathogenesity causing UTIs [2].

Hemolysin is a protein can induce osmotic lysis of erythrocyte because of it's bore forming activity and cytotoxic to several types of human cell. *E.coli* can produce several types of hemolysin including extracellular protein (α-hemolysin), cell bound protein (β-hemolysin) and a hemolysin produced by nalidexic acid resistant mutant (γ-hemolysin) [3,4].

Biofilm is a microcolonies of bacteria enclosed by a complex materials of protein and polysaccharide that form on living tissue and medical device such as catheter [5]. Adhesion and biofilm formation protect the bacteria from urinary lavage, increasing antibiotic resistance and bacterial ability to multiply and invade host tissue led to successfully initiation of infection [6].

The virulence factors of *E.coli* are located on large plasmids and /or in particular region called pathogenesity island (PAIs) on the chromosome. PCR assay was developed which allow the simultaneous detection of virulence gene by using specific primers that amplify these virulence genes encoding for virulence pathogenic traits of uropathogenic bacteria. [7,8, 9].

Materials & Methods

Clinical isolates of *E.coli*

A total of *E.coli* isolates which isolated from 125 adults patients between October 2005 to May 2006 (48 isolates from females and 29 from males).The diagnosis of UTIs was established based on clinical symptoms and laboratory investigation. the laboratory criterion for acute UTIs was defined as the presence of positive urine culture with least 10^5 CFU of bacteria /ml of clean voided urine, among the hospitalized patients there were 39 cystitis case (dysuria, no fever, with or with out pain), 22 cases of acute pyelonephritis (flank pain, fever, nausea, vomiting with or without chills) and 16 cases of prostatitis. The isolation and identification of *E.coli* isolates were performed by standard bacteriological test and API-20 E assay.

Hemolysin production

E.coli isolates were testes for hemolysin production by two different methods. Hemolysin production was assayed by growing the *E.coli* isolates overnight (18 h) at 37 ºC in Luria broth (LB, Himedia, India) then inoculated on to blood agar plate (blood agar base , Himedia, India) containing defiberinated blood (sheep-rabbit-human) erythrocyte 5% v/v

separately. Production of hemolysis was read after overnight incubation at 37 °C and verified by the presence of clear hemolytic halo around the colony [10]. Micro titer plate is other method describe by [11] for detection of hemolysin production.

Biofilm formation

Method described by [12] used for detection the bacterial ability for biofilm formation, this method included inoculation 5 ml of (Trypton soya broth (Tsb, Himedia, India) with particular isolates and incubated for 48h. at 37 ºC, after that, the contents of the tubes were removed carefully and added the crystal violet stain (1%) to each tube for 15 minutes then rinsed the tubes and let tubes to dry at room temperature (20-25)ºC. The result was read by notice the formation of biofilm as a layer at the internal wall of tubes by naked eye and comprise with the negative control (tube contains Tsb medium without inoculation), thickness and color of layer consider a parameter of bacterial ability for biofilm formation.

DNA Extraction

E.coli isoloates were grown in Luria broth (LB,Himedia, India) at 37 ºC overnight then bacterial pelleted from broth and DNA extraction was done for 9 isolates which gave the best efficiency in hemolysin production and biofilm formation as well as *E.coli* MM 294 was used as standard strain by salting out method which described by [13].

Plasmid DNA Isolation

Plasmid DNA was extracted by using method described by [14].

Specific PCR

Specific primers were used to amplify sequences of the *eae*, *E-hly* and *hly* genes. Details of primer sequences, predicted size of the amplified product and specific annealing temperature are given in Table (1).

Table (1): PCR primers for virulence factor

Primers	sequence	TA (°C)	Product size (bp)	References
E-hly A1	**F/ -5 GGTGCAGCAGAAAAAGTTGTAG -3**	**60**	**1550**	**16**
E-hly A4	**R/ -5 TCTCGCCTGATAGTGTTTGGTA -3**			
hly 1	**F/-5 AACAAGGATAAGCACTGTTCTGGCT -3**	**60**	**1171**	**17**
hly 2	**R / -5 CCATATAAGCGGTCATTCCCGTCA -3**			
eae AF	**F/ -5 GACCCGGCACAAGCATAAGC -3**	**65**	**384**	**15**
eae AR	**R/ -5 CCACCTGCAGCAACAAGAGG -3**			

PCR amplification of *eae* specific sequence (384 bp product) was performed by using primers *eae* AF(′5 GACCCGGCACAAGCATAAGC 3\) and *eae* AR(′5 CCACCTGCAGCAACAAGAGG 3\) in total volume 25 µl containing 2 µl of template DNA (50 µg/ml), 2.5 µl of PCR buffer (1X), 1 µl of MgCl2 (1.5mM), 0.5 µl dNTPs(200 mM) , 5 µl of each primer (50 picamole) and 0.25 µl of *Taq* polymerase (1.25 U/reaction). (Midland/ USA). Samples were subjected to 35 PCR cycle, each one consisting of 1 min of denaturatioin at 95 ºC ; 2 min of annealing at 65 ºC for the first 10

cycle, decrementing to 60 ºC by cycle 15 and 1.5 min of elongation at 72 ºC. Incrementing to 2.5 min from cycle 25 to 35. PCR reaction mixture were electrophoresed on 1.2 % agarose [15].

PCR detection of *E-hly* specific gene sequence (1550 bp product) was performed with primers *E-hly* A1(′5 GGTGCAGCAGAAAAAGTTGTAG 3\) and *E-hly* A4 (′5 TCTCGCCTGATAGTGTTTGGTA 3\) in total volume 25 µl containing 3 µl of template DNA (50 µg/ml), 2.5 µl of PCR buffer (1X), 0.75µl of MgCl2 (1.5mM), 0.75 µl dNTPs(200 mM) , 2µl of each primer (20pica mole) and 0.25 µl of *Taq* polymerase (1.25 U/reaction). (Midland / USA). Samples were subjected to first denaturation at 94 ºC for 2 min followed by 35 cycle each one consists of denaturation at 94 ºC for 1 min, annealing at 60 ºC for 1 min and elongation at 72 ºC for min. PCR reaction mixture were electrophoresed on 2 % agarose [16].

PCR detection of *hly* specific gene sequence (1117 bp product) was performed with primers*hly1*(′5AACAAGGATAAGCACTGTTCTGGCT3\)and*hly2*(′5TCCATATAAGC GGTCATTCCCGTCA 3\) in total volume 25 µl containing 2 µl of template DNA (50 µg/ml), 2.5 µl of PCR buffer (1X), 1.15 µl of MgCl2 (1.5mM), 0.5 µl dNTPs(200 mM), 3 µl of each primer (30 picamole) and 0.2 µl of *Taq* polymerase(1U/reaction). (Midland / USA). Samples were subjected to 30 PCR cycle each one consists of denaturation at 95 ºC for 1min , annealing at 60 ºC for30 sec. (incrementing by 1 ºC after every five cycles to 65 ºC) and extension at 72 ºC for 3 min then final extension at 72 ºC for 7 min. PCR reaction mixture were electrophoresed on 2 % agarose [17].

Results and Discussion

Seventy seven bacterial isolates collected as midstream urine samples of uncomplicated UTIs patients were identified by using cultural, morphological and biochemical tests. Results showed that 77 *E.coli* isolates out of the positive cases were included 48 isolates of *E.coli* (62 %) from females, and cystitis represents the main types of UTI in females then pyelonephritis, while 29 *E.coli* isolate (38%) from males, more of them suffer from prostatitis show Figure(1)

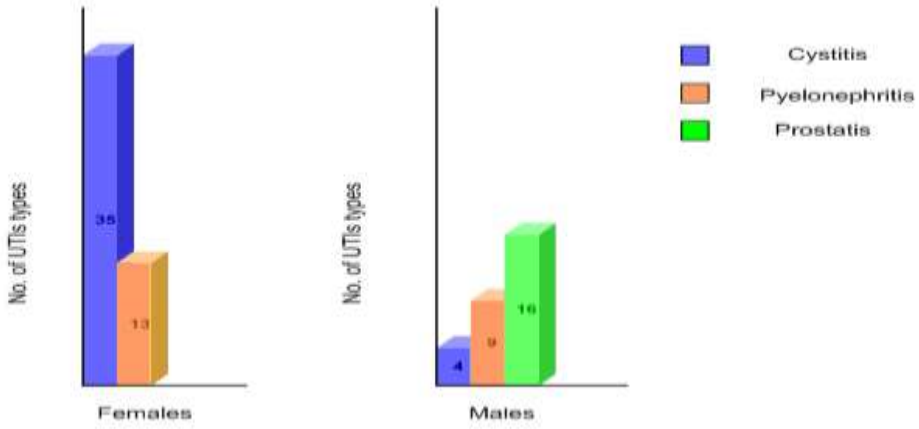


Fig (1): Distribution of UTIs types among females and males

It is known that incidence of UTIs is generally higher in females than in males world wide for several reasons like the shorter female urethra is less effective deterrent to infection than the male urethra, sexual intercourse facilitates the movement of microorganisms up the urethra particularly in females, so that the incidence of UTIs is higher among sexually active than celibate females.

Our results illustrated showed that 44 isolates 57% of *E.coli* produce hemolysin, while 33 isolates 42.9% not produce hemolysin. Detection of hemolysin production by *E.coli* isolates were done by using microtiterplate method describe by[11], more efficient than uses of blood agar plate because when the blood agar was inoculated with *E.coli*, has no hemolysis zone shown around the colony which grow as gray little convex circular colony. In micro titer plate method some strains which lysis the blood completely while others partially as shown in Figure (2).

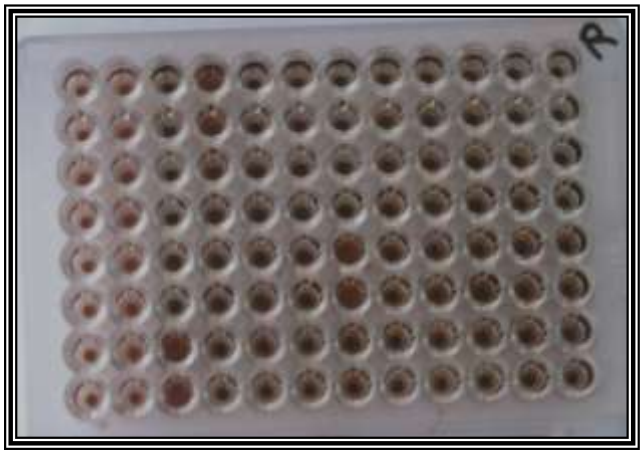


Fig (2): Hemolysis of rabbit RBCs

Several reasons explain the differences in the percentage of hemolysin production by UPEC like: source of blood, type of hemolysin was produced, source of bacteria and method to screen the production ability [18].

Different types of blood were used in this study include: sheep, rabbit and human blood (B+ve). All types were washed (defiberinated blood). The result showed that 44 isolates lysis 3 types of blood (sheep, rabbit, human), 33 isolates lysis 2 types of blood (sheep, rabbit) and 17 isolates lysis only one type human of blood.This result depends on type and amount of hemolysin, production conditions and production efficiency of strain. Hemolysin reflected high efficiency in the lysis of animals blood specifically rabbit than human blood.

Test tube method described by [12] used to detect the ability of uropathogenic *E.coli* which isolated from UTIs patients for biofilm formation. The high percent of *E.coli* isolates were able to produce biofilm. 72 isolate 90% *E.coli* produce biofilm with different degree of thickness and only 5 isolates 9.09% unable to produce biofilm. [19] reported that microorganisms differ in it's ability to produce biofilm. Thickness of biofilm differs according to the nature of producing bacteria, environmental conditions

like temperature, pH and type of UTIs. *E.coli* isolates from prostates patients give high ability to produce biofilm with large thickness more than other isolates from pyelonephritis and cystitis, as shown in Figure (3).

Fig(3): Biofilm formation by *E.coli* isolates in test tubes method

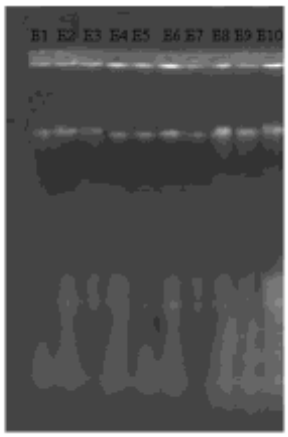
1. **Control. (Tsb only)**
2. **Biofilm produced by *E.coli* isolate from cystitis patients.**
3. **Biofilm produced by *E.coli* isolate from peylonephritis patient.**
4. **Biofilm produced by *E.coli* isolate from prostatitis patients.**

The most common bacteria caused UTI is *E.coli* with posses high ability to produce biofilm which enable them to find safe haven and subvert clearance by innate host response, because of the matrix of biofilm modify the environment of adherent cells by concentrating nutrients and protecting the cells from surfactant, biocides and phagocytic cells. Antibiotic are generally not very effective against organism embedded in biofilm [20].

Results of hemolysin production and biofilm formation by UPEC showed that 44isolates 57% of *E.coli* produce hemolysin and biofilm, 28 isolates 36.36% produce only biofilm while 5 isolates (6.49%) isolates not produce hemolysin and biofilm although they were isolated from UTIs patients and reflected resistance to antibiotic.

Nine isolates of *E.coli* with using standard strain *E.coli* MM 294 were selected for DNA extraction due to their ability for hemolysin production and biofilm formation. In this method chromosomal DNA appear as sharp bands as shown in Figure (4).

Chromosomal DNA



Plasmids

Fig(4): Agarose gel electrophoresis of DNA samples of *E.coli* isolates .
Conditions (0.8 % agarose, 2.5 hours, 60 volt)
E1: DNA of *E.coli* MM 294 .

PCR was used to detect the presence of virulence genes which play important role in pathogenesity of *E.coli* by using specific primer. 4 samples (E2, E4, E9, E10) gave positive results to produce intimin. As shown as in Figure (5, 6).

Intimin protein is responsible for the intimate adherence between bacteria and enterocyte membrane. Categories of *E.coli* that differs in their virulence factors have been contain *eae* gene encode for intimin as part of pathogencity island EPEC and EHEC.

Fig (5): Electrophoresis of 5 *E.coli* isolates for detection eae gene encoded for intimin protein.
- M: DNA marker (250-10000bp).
- E1 is negative control represented by standard strain *E.coli*MM294 (not produce intimin)
- E2 and E4 gave amplification product (positive result)

Fig (6): Electrophoresis of 5 isolates of *E.coli* (E6- E10) for detection of *eae* gene encoded for intimin protein .
- E9, E10 represented positive result of *E.coli* gave amplification product (produce intimin protein)

The gene encoded for enterohemolysin carried on plasmid, thus extraction of plasmid by using alkaline lysis method was used to explain the plasmid profile of two *E.coli* isolates E1 and E2 that gave positive result as producing of enterohemolysin after detection by PCR with using specific primer for this gene, as shown in Figure (7).

Fig (7) Electrophoresis of 10 isolates of *E.coli* for detection of *E-hly* gene encoded for enterohemolysin.

The results showed that as shown as in Figure (8) the two isolates E1 and E2 contained two large plasmids (more than 10000bp), the first isolate(E1) possessed large plasmid (10000bp) and five small plasmids less 10000 bp while the second isolate (E2) it had 3 small plasmids besides two large plasmids.

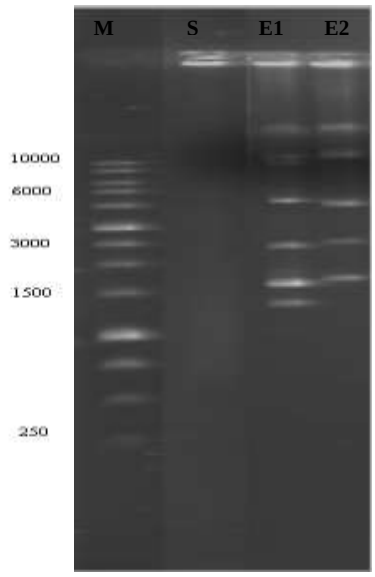


Fig (8): plasmid profile of *E.coli* isolates E1 and E2

The gene encoded for **α-**hemolysin produced by *E. coli* which isolated from UTIs patients is chromosomally determined. Five isolates E3, E5, E7,E8 and E9 gave positive result as shown in Figure (9)

Fig (9) Electrophoresis of 10 isolates of *E.coli* for detection of *hly* gene Encoded for α-hemolysin

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