Study some virulence factors of Escherichia coli isolates

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Abstract

Some Factors determining the virulence of *Escherichia coli* (*E. coli*) isolates were studied ,of 25 isolates , 17(group A) uropathogenic *E. coli* ,6 (group B) infected gastrointestinal tract , 2 (group C) infected wound , beside these group we use the standard strain *E. coli* HB101 as control group.

The twenty five isolates were tested for adherence capability to human buccal cavity epithelial cells by *in vitro* experiment .

The results showed that all isolates have different adhesion capability with mean ranging from (14.35±11.39) to (33.80 ± 22.68) bacteria / epithelial cell It was noticed that isolates EU9, ES6, EW17 displayed high adhesive capability with mean value (33.80 ± 22.68), (32.60 ± 21.19), (29.90±22.50) bacteria /epithelial cell respectively, while the isolates EU4 displayed a lowest adhesive capability with mean value of (14.35±11.39) bacteria / epithelial cell. It was also found that there was no significant difference ($P \geq 0.001$) for adhesive capability among EU9, ES6, EW17, but the adhesive capability for these isolates was significantly higher ($P \leq 0.001$) compared to standard strain which showed adhesive capability with mean value (0.55± 0.88),thus these isolates were selected for further experiments to study others virulence factors such as ability to agglutination human , rabbit , sheep erythrocytes in mannose – sensitive manner and production of haemolysin , biofilm.

The results revealed that all isolates had nearly the same hemagluttination pattern in the absence of D-mannose while the pattern is different in the presence of D- mannose, where the isolates EU9, ES6 show ability to agglutinate human and sheep erythrocytes and so considered as Mannose Resistant Hemagluttination (MRHA) while the isolate EW17 did not show this ability and considered as Mannose Sensitive Hemagluttination (MSHA). It was also found that all isolates have ability to produce haemolysin and biofilm formation but in different pattern.

Introduction

Pathogenic *E. coli* responsible for a broad spectrum of intestinal and extraintestinal diseases, including diarrhea, urinary tract infections, septicemia, and neonatal meningitis (1). The diseases caused by a particular strain of *E. coli* depend on distribution and expression of many virulence determinants such as adhesion ,biofilm formation,production of hemolysin, enterotoxin, shiga toxin ,endotoxin and capsules formation (2,3,4, 5 , 6,7) .

Adherence of bacteria to tissue surfaces is an important initial event in bacterial infections, because of allowing them to attach and colonize specifically to host cell and subsequently initiate disease (8, 9, 10).In *E. coli* there are different types of adhesion like fimbrial adhesion and non- fimbrial adhesion (Intimin) , the first one include type 1 fimbria, P- fimbria , and S-fimbria(11,12), while the second type of adhesion include α , β, γ intimin(7).The principal work for fimbriae is their capability to agglutinate erythrocytes of different animal species and the agglutinate is either inhibited by D-mannose of host cell surface and referred to as " mannose sensitive hemagglutination ,, or " mannose resistant hemagglutination ,, (13) . Epidemiologic studies in adults and children demonstrated that P- fimbriae appear to be especially important in *E. coli* pyelonephritis(11),while S-fimbriaae are a common feature of *E. coli* strains causing meningitis in newborns and are bound to epithelia via sialyl-(α -2-3)galactoside structures(14). It was found in many studies that *E. coli* expressed type-1 fimbriae and have hemolytic activity was more frequent among urinary strains than among faecal strains furthermore it was noticed besides these two categories, biofilm production play important role in the development of Urinary Tract Infection (UTI) (15,16,17,18).

The present study was designed to compare the adhesive properties of *E. coli* isolated from different sources and determine some virulence factors of pathogenic *E. coli* .

Materials and Method

A total of 25 local isolates of *E. coli* and standard strain *E. coli* HB101 were used (table1) All isolates and strain were maintained in screw-caped universal tubes containing nutrient agar(Acumedia,U.S.A.) and store at 4^{o}C.

Human buccal cavity epithelial cells were obtained from healthy adult nonsmokers using sterile swabs and placed in sterile tubes containing phosphate buffer saline (PBS) pH7,mixed well ,centrifuged at 7000 rpm for 10 minutes and resuspended in PBS again.

Bacterial suspension was obtained by culturing bacteria overnight in Brain Heart Infusion (BHI) Broth (Rashmi ,India) at 37^{o}C, the culture should give approximately 10^{8} cells/ml, and then the bacteria were washed twice in PBS for 20 min. at 5000 rpm and resuspended in PBS again.

***In-vitro* adherence test.** This was performed as described by (14,18).A mixture from 0.2 ml of bacteria suspension , 0.2 ml of epithelial cells suspension

and 0.1 ml of PBS were incubated at 37^{o}C for one hr. The unattached bacteria were removed by centrifugation in BPS for 5 min. at 7000 rpm repeated three times. The pellet was resuspended in a drop of PBS,

Table(1).Isolates and stander bacterial strain.

Bacteria	Sources and Characteristics	**Reference**
EU1 EU2 EU3 EU4 EU5 EU6 EU7 EU8	Isolated from UTI patients.	Biotechnology Department-College of Science – Baghdad University.
EU9 EU10 EU16 EU19	Isolated from UTI patients.	Biotechnology Department-College of Science – AL- Nahrain University.
EU20 EU21 EU22 EU23 EU24	Isolated from gastrointestinal disorder patients.	Biotechnology Department-College of Science – Baghdad University.
ES1 ES2 ES3 ES4 ES5 ES6	Isolated from wounds infection.	Biotechnology Department-College of Science – Baghdad University.
EW17 EW18 Standard strain *E. coli* HB101	(rec^- , F^- ,$hsdR^-$, $hsdM^-$ With pBR322plasmid Amp^r , Te^r),with non(adhesion,agglutination ability, haemolysin production, biofilm formation).	AL-Nahrain Center for Biotechnology Researches - Baghdad

dropped onto glass slide microscope, air, dried, fixed with methanol, and finally stained with methylene blue stain.

The number of attached bacteria was counted on 20 epithelial cells by light microscope. A control of epithelial cells was performed by fixing and staining the epithelial cells alone with methanol and methylene blue stain respectively.

Agglutination test. This was performed by using method as described by (18,19). Human, Rabbit and sheep blood were freshly collected, erythrocytes from each species were washed separately three times with PBS and suspended to concentration of 10% (v/v), bacterial suspension was obtained by culturing bacteria overnight in BHI broth at 37^{o}C ,15µl of bacterial suspension mixing with 15µl of 10% erythrocytes without or within 0.1ml of 0.5%(w/v)D-mannose on a glass slide. Agglutination will be examined visually and microscopically after few minutes of rocking at room temperature.

Production of haemolysin, was tested in liquid media by using microtitretion plate and according to the method as described by (7,20). Rabbit blood freshly collected , erythrocytes were washed three times with PBS and suspended to a concentration of 2% (v/v) ,the bacteria isolates were cultured overnight in BHI broth at 37^{o}C,serial twofold dilutions of the bacterial suspension in 100µl of PBS were made in microtitretion plate(96-well polystyrene microtiter U-plates , CHINA).To each well,100µl of 2% erythrocytes suspension were added and incubated at 37^{o}C for 1hr. then incubated at 4^{o}C for 5hr. A negative control were prepared from add 100µl of 2% erythrocytes suspension to 100µl of PBS in the first wells of each row of microtitretion plate. A microtitretion for each isolate were done in duplicate , the titer was read as the highest dilution showing complete hemolysis.

Biofilm formation. Ability of *E. coli* isolates for biofilm formation were detected according to the method as described by (7).The bacteria isolates were cultured 48hr. in 5ml of BHI at 37 C$^{\circ}$, add of 1% crystal violet stain solution (w/v) after discard carefully the contained of test tubes and keep it at room temperature for 15 minutes then rinsed test tubes carefully with distilled water and let tubes dry at room temperature .The Ability of bacteria isolates for biofilm formation were expressed as thickness and color of layer that lining the inner surface of test tubes compared with negative control which prepared in same way of above treatment but without inoculation with bacteria isolates.

Statistical analysis. Bacterial adherence to 20 epithelial cells was statistically analyzed by one-way analysis of variance (ANOVA). Least significant difference(LSD) was used to determine the significant difference between the mean of adherence capability. The statistical package used to determine the above statistical parameters was "Statistical Package for Social Science (SPSS)",(21).

Result and Discussion

The 25 of *E. coli* isolates were divided into three groups according to the source of isolation .The group(A) included 17 isolates which isolated from urine

of UTI patients while group(B) included 6 isolates which isolated from feces of gasrtrointestinal tract patient and group (C) included 2 isolates which isolated from infected wound ,beside these groups we use the standard strain *E. coli* HB101 as control group. The adhesive capacity of *E. coli* isolates to human buccal epithelial cells compared by the frequency of distribution of bacteria on the epithelial cells and by the mean number of bacteria adhering to 20 epithelial cells. Adhering bacteria were clearly seen by using light microscope and were easy to count .All 25 isolates adhered to more than 90% of epithelial cells with mean ranging from (14.35±11.39) to (33.80 ± 22.68) bacteria / epithelial cell as indicated in table (2) .It was noticed that isolates EU9, ES6,EW17 displayed high adhesive capability with mean value (33.80 ± 22.68), (32.60 ± 21.19), (29.90±22.50) bacteria /epithelial cell respectively, while the isolates EU4 displayed a lowest adhesive capability with mean value of (14.35±11.39) bacteria / epithelial cell. It was also found that there was no significant difference($P \geq 0.001$) for adhesive capability among EU9, ES6, EW17, but the adhesive capability for these isolates was significantly higher ($P \leq 0.001$) compared to standard strain which showed adhesive capability with mean value (0.55± 0.88), (Table 2).It was observed that there were a differences in the mean of adhesive capability of *E. coli* isolates among group, this maybe due to the variation in the sample size for these groups table(3).Any way it was noticed that higher value for adhesive capability are tend to be more frequency in group A and B and this maybe due to the isolates in these groups are more fimbriated and virulent bacteria. It was found that *E. coli* strains belonging to the most virulent bacteria adhered better to human uroepithelial cells than did avirulent strains and the fimbriated *E. coli* adhered better than did non-fimbriated(22,23).In order to investigate possible differences in fimbriae type ,we selected the isolates (EU9, ES6, EW17) as representative sample for each group and measured the hemagglutinating activities for these isolates with human rabbit and sheep erythrocytes and the mannose sensitivity of these agglutinations ,the result indicated (table 4) .

Table(2) . Adherence of *E .coli* isolates to human buccal epithelial cells

Groups	Isolate	Number of epithelial cells to which the number of adherent *E coli* was 0	1 - 5	6 – 20	21 – 50	>50	*Mean /number of adherent *E. coli* / cell ± SD
	EU1	1	4	7	5	3	24.05±23.18[a]
	EU2	1	3	9	7	0	16.70±13.39[b]
	EU3	0	2	11	7	0	18.05±13.62[b]
	EU4	0	5	8	6	1	14.35±11.39[b]
	EU5	2	2	9	7	0	16.95±11.27[b]
	EU6	0	3	9	8	0	18.90±10.94[b]
	EU7	1	0	9	9	1	29.00±17.26[a]
	EU8	2	3	6	9	0	14.90±12.30[b]
A	EU9	0	1	6	10	3	33.80±22.68[a]
	EU10	0	2	8	10	0	20.85±10.39[a]

	EU16	0	2	7	8	3	23.85±23.57[a]
	EU19	1	2	7	8	2	25.80±22.50[a]
	EU20	0	2	8	8	2	29.05±24.49[a]
	EU21	1	1	8	9	1	22.70±15.07[a]
	EU22	0	2	11	5	2	18.60±15.65[b]
	EU23	0	1	8	8	3	33.45±24.43[a]
	EU24	1	1	7	10	1	23.25±19.17[a]
	ES1	2	6	8	4	0	20.60±10.44[a]
	ES2	0	1	9	8	2	27.00±21.44[a]
B	ES3	2	3	4	8	3	28.75±27.58[a]
	ES4	1	0	9	9	1	25.40±22.09[a]
	ES5	0	2	8	10	0	20.60±10.44[a]
	ES6	0	3	5	9	3	32.60±21.19[a]
	EW17	1	1	8	9	1	29.90±22.50[a]
C	EW18	0	2	8	8	2	27.80±21.44[a]
Standard *E. coli* HB101 Strain		12	8	0	0	0	0.55±0.88[b]

LSD value = 18.50

[a,b]= Means within same column with unlike superscripts significantly different (P< 0.001)

*Each value represents Mean± standard deviation for mean (n=20)

Table (3). The mean ± SD of adhesive capability for group A,B,C.

Group	Number of bacterial isolates	Mean ± SD/ number of adherent *E. coli* / cell
A	17	22.60 ± 6.06
B	6	25.82 ± 4.70
C	2	28.85 ± 1.48
Total	25	23.87 ± 5.77

that all isolates had nearly the same hemagluttination pattern in the absence of D-mannose while the pattern is different in the presence of mannose , where the isolates EU9, ES6 show ability to agglutinate human and sheep erythrocytes and so considered as Mannose Resistant Hemagluttination (MRHA) while the isolate EW17 did not show this ability and considered as Mannose Sensitive Hemagluttination (MSHA), that mean the first and second isolates maybe have the same fimbriae type while the third isolate has different fimbriae type. It was found in many studies(18, 23,25)that most common hemagglutinating pattern of *E. coli* isolated from urine in UTI patient and from stool in patients with intestinal disorder were MRHA for human erythrocytes while hemagglutinating pattern of *E. coli* isolated from blood in septicemic patients was MSHA.

It was also found (table 4) non of the isolates agglutinated rabbit erythrocytes in the absence or presence of D-mannose and this was mean hemagglutinating pattern of *E. coli* isolates with rabbit erythrocytes was completely different from human and sheep erythrocytes, it was known that pathogenic *E. coli* exhibit different hemagglutinating pattern of erythrocytes from various species of animals and this maybe due to that these erythrocytes possess different receptors which give different patterns of bacteria-erythrocyte interaction (26,27).

Various biological properties of *E. coli* bacteria are reported as factors influencing virulence for the intestinal and extraintestinal disease(11,23) ,thus in our work the other virulence factors like production of haemolysin and biofim formation were studied by selecting the isolate EU9,ES6,EW17 which show the higher adhesive capability beside the standard strain as control treatment.

Table (4). Some virulence factors for selective *E .coli* isolates and standard strain .

Virulence factors	*E. coli* isolates			Standard strain
	EU16	ES4	EW17	*E. coli* HB101
Agglutination of indicated erythrocytes in(Phosphate Buffer Saline)				
Human Blood	+	+	+	-
Rabbit Blood	-	-	-	-
Sheep Blood	+	+	±	-
Agglutination of indicated erythrocytes in(Phosphate Buffer Saline)				
Human Blood	+	+	-	-
Rabbit Blood	-	-	-	-
Sheep Blood	+	+	-	-
Hemolysine Production	++	++	+	-
Biofilm formation	++	++	+	-

(+) = agglutination , (±) = unclear agglutination , (-) = no agglutination.
(++) = production of hemolysine with high (>16) titer, (+) = production of hemolysine with low (<16) titer, (-) = no hemolysine production .
(++) = high ability for biofilm formation, (+) = low ability for biofilm formation, (-) = no ability for biofilm formation.

The production of haemolysin was assay by microtiter technique, in order to demonstrate if there are differences in pattern of haemolysin produce from different isolates. It was found that EU9,ES6 isolates have ability to produce haemolysin with high titer while EW17 isolate produce with low titer(table 4), that mean the first and second isolates exhibit high ability in production of haemolysin compare with third isolate at the same time the standard strain did not show any ability to produce haemolysin, It was found in many studies (24, 25,28, 29,30)that haemolysin production is frequently associated with *E. coli* strains causing UTI and intestinal disorder Therefore, haemolysis is a feature of pathogenic *E. coli* , moreover hemolysin production is a virulence factor considered important for some strains of *E. coli* to overcome host defense mechanisms.

it was found (28) that haemolysin production was related to the release of iron into the bacterial environment, furthermore haemoIysin production and mannose-resistant hemagglutination (MRHA) were genetically linked in *E. coli* strains causing UTI (31).

The other virulence factor studied was biofilm formation, the result revealed (table4)that the three isolates show different ability in formation of biofilm depending on thickness and color of layer that lining the inner surface of test tubes compared with control .It found in several studies(32,33,34,35) that the phenotypic variation of biofilm in pathogenic *E. coli* depend on two factors, the environmental factor which include, temperature, pH, O_2levels, hydrodynamics, osmolarity ,while the second factor include the coordination of vast amounts of genetic information in order to regulate factors and pathways, which affect enzymatic and structural elements that are needed for biofilm formation, moreover it was noticed that there is important correlation between virulence factors especially non type-1 fimbriae and thickness of biofilm(36).

In light of the observations presented here one can conclude that there is important variation in virulence factors which was studied among pathogenic *E. coli* isolated from different sources and according to this result we recommended to do more studies on other virulence factors such as capsule formation , enterotoxins, verotoxins, endotoxins, with large sample of pathogenic *E. coli* isolated from different sources and using animals model in order to examine possible factors affecting the virulence . Beside that a genetic studies must done in order to confirm the variation in virulence factors due to the variation in the genetic contained of pathogenic *E. coli* isolated from different sources.

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دراسة بعض عوامل الضراوة لعزلات من بكتريا *Escherichia coli*

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الملخص

درست بعض العوامل المحددة لضراوة (٢٥) عزلة من بكتريا (*E. coli*) ١٧ *Escherichia coli* عزلة (المجموعة أ) سببت التهابات في المجاري البولية ، ٣ عزل (المجموعة ب) سببت التهابات مختلفة في القناة الهضمية ، عزلتان (المجموعة ج) سببت التهاب الجروح ، كما أستخدمت السلالة القياسة *E. coli* HB101 كمجموعة سيطرة .

في تجربة *in vitro* أختبرت قدرة جميع العزلات على الألتصاق بالخلايا الطلائية المبطنة لتجويف الفم لأشخاص أصحاء .

اظهرت النتائج ان لجميع العزلات قابليات مختلفة على الألتصاق و تراوح متوسط الألتصاق للعزلات البكترية من(11.39±14.35) الي (22.68±33.80) بكتريا ملتصقة / خلية . كما لوحظ أن العزلات EU9 ، EW17،ES6 قد اظهرت قدرات عالية على الألتصاق وبمتوسط(٢٢,٦٠±٣٣,٨٠)،(٢١,١٩±٣٢,٦٠)،(٢٢,٥٠±٢٩,٩٠) بكتريا ملتصقة / خلية لكل من العزلات الثلاثة وعلى التوالي،بينما أظهرت العزلة EU4 أقل مقدرة على الألتصاق حيث بلغ متوسط التصاقها (11.39±14.35) بكتريا ملتصقة / خلية .

لم يلاحظ هنالك فروقات معنوية ($P \geq 0.001$) بين قدرات العزلات الثلاثة EU9 ، EW17،ES6 على الألتصاق، بينما لوحظ هنالك فروقات عالية المعنوية ($P \leq 0.001$) في قدرات هذه العزلات على الألتصاق مقارنة بالسلالة القياسية *E. coli* HB101 والذي بلغ متوسط التصاقها (٠,٨٨±٠,٥٥) بكتريا ملتصقة / خلية.

أختيرت العزلات الثلاثة كعينات ممثلة للمجاميع العزلات المختلفة لأجراء تجارب أخرى تتضمن على دراسة بعض عناصر الضراوة مثل قابلية هذه العزلات على تلازن كريات دم الحمراء للأنسان ، الأرنب ، الأغنام وبالأعتماد على طريقة أختبار الحساسية لسكر المانوز Mannose – Sensitive Manner بالأضافة الى التعرف على قدرات هذه العزلات على أنتاج الهيمولايسين وتكوين الغشاء الحيوي.

أظهرت النتائج أن للعزلات الثلاثة نمطا تقريبا متشابها في تلازن كريات دم الحمر لكل من الأنسان و الأغنام وبغياب سكر المانوز ولكن كان نمط التلازن مختلف بوجود سكر المانوز حيث أظهرت كل من العزلتين EU9 ، ES6 قدرة على تلازن كل من كريات دم الحمراء للأنسان والأغنام لذلك أعتبرتا Mannose Resistant Hemagluttination (MRHA) بينما لم تظهر العزلة EW17 هذه القدرة وأعتبرت (Mannose Sensitive Hemagluttination (MSHA كذلك لوحظ أن لهذه العزلات قدرات متباينة في نمط انتاج الهيمولايسين وتكوين الغشاء الحيوي .