

Isolation and Identification of enterotoxigenic *Escherichia coli* (ETEC) from diarrheal patients in Basra, with detection of O169:H41 *E.coli* strains

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Abstract:

A total of 375 stool and rectal swabs specimens were collected from patients in all ages groups suffering watery diarrhoea. 167 females and 208 males were involved in this study. Specimens were cultured on MacConkey agar plates and incubated at 37°C overnight. The isolates then inoculated on Eosin methylene blue (EMB) agar medium for preliminary characterization, the colonies showing characteristic metallic sheen on EMB agar were picked up and considered as presumptive *E. coli*. All isolates were preliminary identified by conventional biochemical tests for *E.coli* bacterial identification, API 20E system and serological typing of all positive isolates by slide agglutination with *E. coli* O169:H41, 5 isolates from 13(38.46%) ETEC showed positive result for *E.coli* O169:H41. Isolates were examined by PCR to detection *ST* and *LT* gene revealed that of the 375 diarrhoeal samples, 13 (3.47) % were positive for ETEC. To the best of our knowledge, this is the first and most comprehensive report on this serogroups of *E. coli* strains isolated from diarrheic patients in Iraq.

Key word: Enterotoxigenic *E.coli* serotypes in diarrheal patients

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- **It is a part of Ph.D. thesis submitted by the first author .**

Introduction:

Enterotoxigenic *Escherichia coli* (ETEC) strains are the leading bacterial cause of diarrhea in animals and commonly to children in the developing world, it was the most common cause of traveler's diarrhea, they are non-invasive and do not leave the intestinal lumen (Rao *et al.*, 2003). Each year, ETEC causes more than 200 million cases of diarrhea and 380,000 deaths, mostly in children in developing countries (Qadri *et al.*, 2005).

Ingestion of contaminated water or food causes ETEC infection, producing watery diarrhea, nausea, abdominal cramps and low-grade fever (Nweze, 2009). The diarrhoea is watery, usually without blood, mucus, or pus and fever and vomiting are present in a minority of patients (Levine *et al.*, 2009). Administration of antibiotics to which ETEC strains are susceptible has been shown to decrease both the duration of diarrhoea and the intensity of ETEC excretion (Black *et al.*, 1982).

These pathogenic ETEC strains are characterized by the production of either large molecular weight heat labile enterotoxins (LT) or small molecular weight heat-stable peptide toxins (ST) which is serve as the main virulence

factors determinants, toxin produced by bacteria that is specific for intestinal cells and cells and causes vomiting and diarrhea associated with food poisoning called enterotoxins (Nagy and Fekete, 1999). ETEC is subdivision by serotype, which is based on major surface antigens (O antigen: part of lipopolysaccharide layerH: flagellin and K antigen: capsule (Orskov *et al.*, 1977). Determination of O serogroups associated with the cell wall lipopolysaccharides and H serogroups of the flagella, has been used to identify and characterize ETEC (Orskov *et al.*, 1976). Considerably fewer H serogroups than O serogroups are associated with ETEC (Wolf, 1997).

The first report of O169:H41 was association with a foodborne outbreak in 1991 in Japan, where this serotype continues to be isolated (Ando *et al.*, 1993; Nishikawa, 1995; Hamada *et al.*, 1999). Hamada *et al.*, (1999) reported four outbreaks that occurred from June 1997 to August 1998, the largest of these outbreaks had a 57% attack rate and resulted in approximately 2,800 cases. All four outbreaks occurred at either restaurants or catered events. Nishakawa *et al.* report that O169:H41 has become the most prevalent ETEC serotype in Japan (Nishikawa *et al.*, 1998).

In 1995, Centers for Disease Control and Prevention (CDC) detected this serotype for the first time during an outbreak on a Caribbean cruise ship. It was identified during two additional Caribbean cruise ship outbreaks before causing a domestic outbreak in Minnesota in 1997.

Materials and methods:

Samples collection: A total of 375 stool and rectal swabs specimens were collected from patients admitted to seven main hospitals in Basra city from all age groups suffering of watery diarrhoea, of 375 patients, 167 were females, and 208 were males.

Isolation of bacteria: Specimens were cultured on MacConkey agar (Microexpress, India) plates and incubated at 37°C overnight. From each plate seven to ten lactose fermenter colonies with typical appearance of *E. coli* were selected and inoculated on Eosin methylene blue (EMB) agar medium (Microexpress, India) for preliminary characterization, colonies showing metallic sheen color on EMB agar were picked up and considered as presumptive *E. coli*. All the isolates were stained by Gram's stain (Forbes *et al.*, 2007).

Biochemical test:

1. Identification of *E.coli* isolates: *E. coli* isolates were preliminary identified by

biochemical tests: Indole, Methyl-red, Voges Proskauer and citrate utilization. The isolates were further characterized for their biochemical activity by the following tests: carbohydrate fermentation, production of H₂S on TSI (Edwards and Ewing, 1972).

2. Identification by API 20E system:

Isolates were confirmed by API 20E, test strips were inoculated and incubated according to the instructions provided to the manufacturer's manual (Biomérieux, France).

Serotyping of *E.coli*: Serological typing of all positive strains was carried out by slide agglutination with *E. coli* O, H antisera (O169:H41), the antisera were obtained from State Serum Institute (Denmark).

DNA extraction: DNA was extracted from cultured *E. coli* by using High-Speed Plasmid Mini Kit (Geneaid, Taiwan). Then the DNA was used as template for PCR. The primer (Bioneer, Korea) used to amplify genes are shown in Table 1. The dried DF column was transfer to a new 1.5 ml microcentrifuge tube ,Add 20 -50 µl of elution buffer into the center DF column matrix ,Let stand for 3 minutes to allow elution buffer to be completely absorbed , Centrifuge at 15,000 xg for 2 minutes to elute the purified DNA. (DNA was stored at - 20° C).

Table (1): Program used in PCR amplification for ST and LT gene (Istituto Superiore di Sanità, 2013).

Steps	Temperature	Time	No. Of cycles
Initial denaturation	95 °C	10 min	1
Denaturation	95 °C	30 sec	35
Annealing	58 °C	60 sec	
Extension	72 °C	60 sec	
Final Extension	72°C	5min	1
Hold	5°C		

Table (2) Selected primer-set for PCR of pathogenic *E.coli*

Target genes	Primers sequences (5-3)	Amplicon size (bp)	Reference
StF StR	ATA TTA TTA ATA GCA CCC GG TCA CCT TTC CCT CAG GAT GC	179	(Kimata <i>et al.</i> ,2005)
LtF LtR	TCC TTC ATC CTT TCA ATG GCTTT GCA CAC GGA GCT CCT CAG TC	218	(Vidal <i>et al.</i> ,2004)

Table (3) distribution of patients infected with ETEC in both sex (P 0.05)

Sex	No. of patients	No. of patients positive for ETEC (%)
Females	167	5/375(1.33%)
Males	208	8/375(2.13%)
Total	375	13/375(3.47%)

Results and Discussion:

After all previous mentioned tests, the results showed that 3.47 % (13/375) of the stool specimens were positive for ETEC .There

These results are agreement with Nweze, (2010) recorded 4.2% and Bueris *et al.*, (2007) recorded 3.7%.

Because ETEC is nor identified by routine stool culture methods neither by biochemical tests, a negative stool culture might lead to the assumption that a diarrheal illness is viral in etiology. So ETEC outbreaks may go unrecognized, and opportunities for treatment and may missed (Dalton, *et al.*,1999).According to sex distribution of the overall isolates shows that ETEC are more predominant among males than women, these differences were significant ($p<0.05$). This is agreement with results of previous studies in southeastern Nigeria suggest that the incidence of ETEC is higher among males than females (Ako-Nai *et al.*,1990) it is disagreement with Basnyat and Ericsson (2011) they showed that *E.coli* causing diarrhea are at equal risk for men and women .

In the present study, 5 isolates from 13(38.46%) ETEC were *E. coli* O169:H41 strains.

Serological typing was the only convenient method for local laboratories to detect ETEC

were 5/375(1.33%) females and 8/375(2.13%) males cases investigated in Table 3

and PCR for heat-stable and heat-labile enterotoxin genes (Devasia *et al.*,2006).

These results are higher than other researchers, in the United States during 1975-1995, *E. coli* O169:H41 was identified in only one 5% of 21 ETEC outbreaks (Dalton, *et al.*,1999), the same result confirmed by Beatty *et al.*, (2004) they identified this serotype in 1(5%) of 21 confirmed ETEC outbreaks. while Konishi, *et al.*,(2011) identified *E. coli* O169:H41 in 32 strains (20.2%). In another study has been recording the highest rate than the present study but their study was for 7 years (1996-2003), *E. coli*O169:H41 was identified in 10 (63%) of 16 ETEC outbreaks (Beatty,*et al.*,2004). The reason ofthe high rate in the present study is the lack of attention to personal hygiene, with proper sanitation a much lower incidence of ETEC infection,due to continued improvements in sanitation, food handling, and hand-washing are decrease the risk of foodborne diseases that are transmitted from infected humans(Devasia *et al.*, 2006).

Through the confirmation of ETEC and *E. coli* O169:H41 isolates by PCR, all the isolates were identified as *E. coli* by

conventional biochemical methods and serology test were confirmed by amplification of enterotoxin (ST and LT) gene. The PCR results are presented and illustrated in the Figure 1.

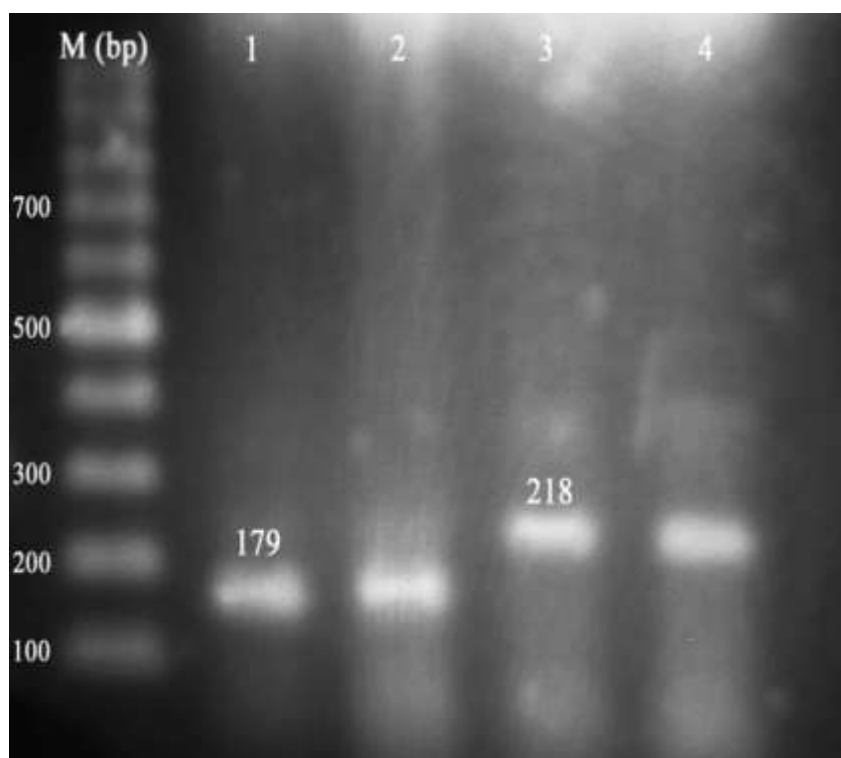


Figure 1 Agarose gel electrophoresis showing 179 bp and 218 PCR amplification products of the *ST* and *LT* gene respectively from 2 *E. coli* isolates

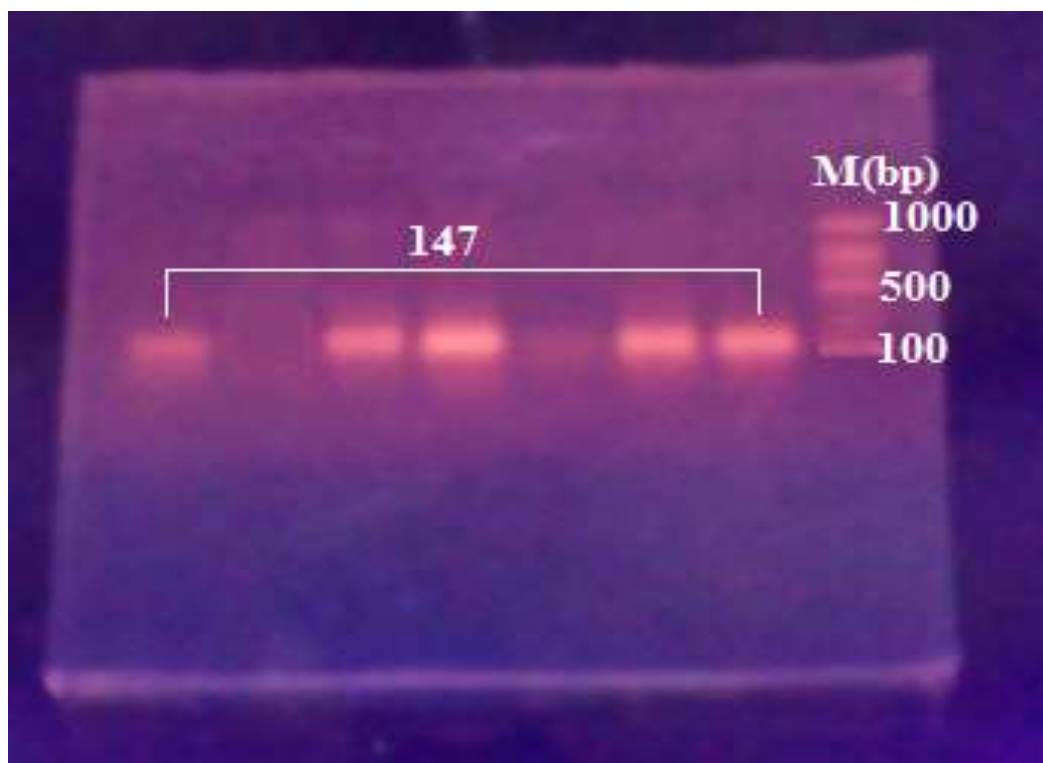


Figure (2): photograph of gel electrophoresis patterns show PCR amplified products of 5 *ST* gene from *E. coli* isolates.

The results in the present study indicate an overall agreement between serological results with a few exceptions, and molecular characterization identified of *ST* and *LT* enterotoxin genes. The prevalence and other epidemiological features of these pathogens as a causative agent of diarrhea vary from region to region. This variation is also seen between and within countries in the same

geographical area (Albert *et al.*, 2009; Kaper, *et al.*, 2004).

The high frequency of these organisms in this study reinforces the need to further studies on their epidemiology, pathogenesis and the role a different forms of diarrhea.

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عزل وتشخيص جرثومة الاشريكية القولونية المنتجة للذيفان المعوي وسلالة

O169:H41 من مرضى الإسهال في محافظة البصرة

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الخلاصة:

تم جمع (375) عينة براز و مسحات مستقيم مأخوذة من كافة الفئات العمرية لمرضى يعانون من الإسهال المائي. شملت الدراسة 167 مريض من الإناث ، و 208 من الذكور. أجري الزرع البكتيري للعينات على وسط أجار مأكوني و حضنت عند 37 درجة مئوية ليوم واحد . وقد تم اختيار من كل طبق سبعة إلى عشرة مستعمرات مخمرة لسكر اللاكتوز مع مظهر نموذجي لجرثومة الاشريكية القولونية بعد ذلك زرعت المستعمرات على وسط الايوزين مثيلين الازرق أظهرت المستعمرات لمعان معدني مميز سجلت المستعمرات افتراضيا بأنها الاشريكية القولونية. أظهرت الدراسة عزل (38.46 %) فقط من الجراثيم المنتجة للذيفان المعوي للاشريكية القولونية من بين كل العزلات التي تم تحديدها أولاً عن طريق اختبارات الكيمياء الحيوية التقليدية لتحديد البكتيريا القولونية ، ونظام API 20E والاختبارات المصلية O، H للاشريكية القولونية. سجلت 5 سلالات من 13 (38.46%) O169 : H41 من الجراثيم المنتجة للذيفان المعوي للاشريكية القولونية. ولقد تم تأكيد عزل هذه الجراثيم كذلك سلالات O169 : H41 من خلال تفاعل البوليميريز المتسلسل أظهرت النتائج أن 13 عزلات من 375 (38.46%) تعود لسلالة O169: H41 5 عزلات من 13 (38.46%) ETEC أظهر نتيجة إيجابية لاقولونية O169: H41. تم فحص العزلات بتقنية PCR لكشف جين ST و LT لجميع عينات الإسهال 375، كانت 13 (3.47%) إيجابية ل ETEC. إلى حد علمنا هذا هو التقرير الأول والأشمل في دراسة هذه السلالات O169: H41 المعزولة من المرضى المصابين بالإسهال في العراق.

البحث مستل من اطروحة الدكتوراه للباحث الاول