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Detection of stx1 and stx2 virulence genes from *Escherichia coli* O157:H7 isolated from calves by PCR assay

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Abstract

This study was conducted to detect virulence factors stx1 and stx2 on 32 isolates of *Escherichia coli* O157:H7 serotype which isolated previously from calves. The study was out in Baghdad, a province in Iraq. Three hundred and fifty fecal samples from diarrheic (n: 35) and non diarrheic calves (n: 315) were used for isolation of *Escherichia coli* O157:H7, after culturing on enrichment media and on selective media (sorbitol MacConkey agar plus cifixime potassium tellurite (SMA-CT), on Chrom agar™ *E. coli* O157). Then confirmed by using Latex agglutination test for detection of O157 and H7 antigens. The 32 *E. coli* O157:H7 isolates [4 isolated from diarrheic calves and 28from non diarrheic calves] were confirmed by PCR techniques for detection of virulence factors (stx1 and stx2) genes. The results showed that the four isolates from diarrheic calves were possessed stx1 gene (100%) and one isolate were positive for stx2 whereas only 15 isolates from 28 isolates (53.57%) were possessing (stx1) gene and 9 from 28 (32.14%) found positive for stx2 in none diarrheic calves. In conclusion, this study revealed the importance of distribution of *E. coli* O157:H7 in diarrheic and non diarrheic calves which act as a reservoir. Also, detection of stx1 and stx2 genes in most isolates suggests that these isolates were virulent and pathogenic for humans.

Keywords: *E. coli* O157:H7, stx1, stx2, PCR, calves.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) strains, of which *E.coliO157:H7* is the best-studied serotype. The main reservoirs for EHEC are ruminants, mostly cattle, which harbour the bacteria in their intestinal tracts without showing clinical symptoms (Kieckens *et al*., 2015). The Shiga toxin-producing *Escherichia coli* O157:H7 as a cause of foodborne infections and ruminants were regarded as the natural reservoir for these toxins producing in *E. coli* (STEC) especially serogroups O157 (Katani *et al*., 2015 and Bonardi *et al* 2015). The term "super-shedder" has been applied to cattle that shed concentrations of *Escherichia coli* O157: H7 10^4 CFU/g feces (Munns *et al*., 2015).

The molecular mechanisms underlying the carriage and virulence of EHEC in ruminants are poorly understood and more than 100 genes are involved in the colonization of the bovine intestine identified by biochemical and genetic analyses (Dziva *et al*., 2004).

Escherichia coli O157:H7 genes were screened in cattle, pigs, humans, beef, pork, and water samples by Ateba and Mbewe, (2011), for determining the presence of virulence genes, rfb (O157), fliC (H7), *Stx1* and *Stx2* fragments by using PCR.

The RT-PCR assay for eae (EHEC O157:H7), stx1, and stx2 proved to be a rapid test for detection of EHEC O157:H7 in complex biological matrices and could also be a potentially to use for the quantification of EHEC O157:H7 in foods or fecal samples (Sharma and Dean-Nystrom, 2003).

Enterohemorrhagic *Escherichia coli* (EHEC) serotypes O157:H7, which expresses somatic (O) antigen 157 and flagellar (H) antigen 7, causes large disease outbreaks and serious morbidity, for this reason this bacterium was considered as one of the most important waterborne and food-borne pathogens worldwide. Two main groups of Shiga toxins are harbored in STEC. Shiga toxin1 is 98% homologous to the Stx produced by *Shigella dysenteriae* type 1, while Stx2 is about 60% homologous with Stx1 and is antigenically different (Doyle *et al*., 2001; Nataro and Kapar, 1998).

Virulence markers in Shiga toxin-producing *E. coli* (STEC) and their association with diseases remain largely unknown, Chui *et al*., (2015) isolated STEC from a cattle with using surveillance program. The virulence genes tested were present in almost all *E. coli* O157:H7 isolates but highly variable in non-O157 STEC isolates.

Al- Kaabi, (2014) found that the prevalence of *E. coli* O157:H7 in cattle fecal samples in Missan province in Iraq was 9 out of 54 (16.6%) and expressed stx1 and stx2 as well as 1 (1.7%) isolate out of 59 gallbladder mucosal swabs which express rfb O157 gene only. Yousif and Al-Taii (2014) isolated *E. coli* O157:H7 from fecal samples at a percentage of (25%) in cattle in Abu- Ghraib state in Baghdad / Iraq. While Bosilevac *et al*. (2015) found that the prevalence of *Escherichia coli* O157:H7 was (10.7%) in feces of cattle in Riyadh in Saudi Arabia.

In beef cattle (steers), *Escherichia coli* O157:H7 reported at a percentage of (21.9%), diagnosis insured by primary enrichment and further confirmed by latex agglutination test and PCR (Zhang *et al*. 2015).

Osaili *et al*. (2013) characterized the 50 isolates from slaughtered cattle in Amman abattoir of *E. coli* O157:H7 for virulence factors hlyA and eaeA were present in all of the isolates. 12%, 60% and 22% of the isolates harbored stx(2), stx(1), and stx(1) and stx(2), respectively.

The aim of this study was to report the survey of *Escherichia coli* O157:H7 in calves with detection the important virulence factors stx1 and stx2 in the isolates.

Materials and Methods

Survey and bacteriological study were done in pervious study by (Yousif and Hussein, 2015) on Three hundred fifty calves aged between (one day to one year), from both sexes, found in the field at different places in Baghdad city, for six months). All methods of culturing on enrichment media, Gram stain and biochemical test for detection of *E coli.* All *E. coli* isolates were screened on sorbitol MacConkey agar plus cifixime potassium tellurite (SMA-CT), on Chrom agar™ *E .coli* O157, And serotyping by using Latex agglutination Test by using commercial kit (Wellcolex *E. coli* O157:H7, Remel) (Marky *et al*, 2013).

Detection of stx1 and stx2 gene in Isolated bacteria by PCR assay: -

PCR assay assay was performed in the laboratories of internal and preventive Veterinary Department/ College of Vet. Medicine /University of Baghdad, this assay was done on the 32 isolates of *E. coli* O157:H7 by the following methods:-

1-DNA extraction: According to manufacturing procedure. The genomic DNA of *E. coli* O157:H7 isolates was extracted by using (Presto™ Mini g DNA Bacteria Kit Geneaid. USA) .

2-Primers: The oligonucleotide primers for stx1 gene were:
F-5‘ ACA CTG GAT GAT CTC AGT GG-3‘
R-5‘ CTG AAT CCC CCT CCA TTA TG-3‘
And for stx2 gene
F-5‘ CCA TGA CAoA CGG ACA GCA-3‘
R-5‘ CCT GTC AAC TGA GCA CTT TG-3‘
The product size for stx1 614bp and for stx2 was 779bp (Gannon *et al*.,1992).
The purity and concentration of extracting DNA were recorded by using a Nanodrop spectrophotometer (NuDrops) ™ [ActGene (USA)].

3- PCR mixture components for s*tx1, stx2* genes:-

The reaction for stx1 and stx2 were included in a total volume of 25 μL in 0.5 ml eppendorf tube containing 2 μL templet DNA, 12.5 μL PCR master mix, 2 μL of each primer, 6.5 μL PCR water.

4-Thermo- cycler program

*The program of thermo-cycle for detection of stx1 and stx2 was performed as follows: - One cycle for thre minutes at 94°C to denaturate template. It was continued by 35 cycles, each cycle including denaturation 60 seconds at 94°C, annealing 30 seconds at 53°C, and extension 60 seconds at 72°C.

Final extention was done 7 minutes at 72°C (Osek 2003, Pradel *et al* 2001).

The PCR tubes containing an amplification mixture were transferred to thermal-cycler and started the program for amplification as shown in the (Table 1).

Table 1: PCR program for detection stx1 and stx2 genes

Step	Temperature (^{o}C)	Time	No. of cycles
Initial denaturation	94	3 min.	1
Denaturation	94	60 seconds	
Annealing	53	30 seconds	35
Extension	72	60 seconds	
Final extension	72	7 min.	1
Hold	4		

5- PCR Product Analysis (Agarose Gel Electrophoresis): this step used for complete PCR assay, which was used to analyses the PCR product by agarose gel electrophoresis, stained with 0.5 µg/ml ethedium bromide, the final PCR products (bands) were visualized using a UV transilluminator [Cleaver Scientific (U.K.)] and photographed by using digital camera.

Ethical Approved: This study was approved by the ethical and research committee of Veterinary Medicine of College, University of Baghdad, Ministry of High Education and Scientific Research.

Results

The confirmation process of the 32 isolates of *E. coli* O157:H7 isolates recovered from 350 fecal samples of diarrheic and non diarrheic calves. *E. coli* O157:H7 appeared in 4 isolates (11.42%) from diarrheic calves, and 28 isolates (8.88%) were isolated from non diarrheic calves. to detect the presence of specific virulence trait *stx1 and stx2* genes by PCR assay, the results showed that all four isolates from diarrheic calves were possess *stx1* gene (100%) and 1(25%) were positive for stx2 while 15(53.57%) of isolates from non-diarrheic calves were positive for *stx1* gene and 9 (32.14%) were positive for stx2 gene. The study revealed that 19(59.37%) from total isolates gave positive results with *stx1*primers equal to target product size (614bp) and 10 (31.25%) from total isolates gave positive results with stx2 primers equal to target product size (779 bp). (Table 2, Figure 1 and 2).

Table (2) number and percentage of animals and isolates carried virulence genes.

Animals		No. of *E. coli* O157:H7	No. of *Stx1*	No. of *Stx2*
Diarrheic calves(35)		4	4(100%)A	1 (25.00%)B
Non diarrheal calves(315)		28	15(53.57%)A	9 (32.14%)B
Total (350)		32	19(59.37%)A	10 (31.25%)B

Different letters denote the difference between stx1 and stx2 in *E. coli* O157:H7 **at** P>0.05

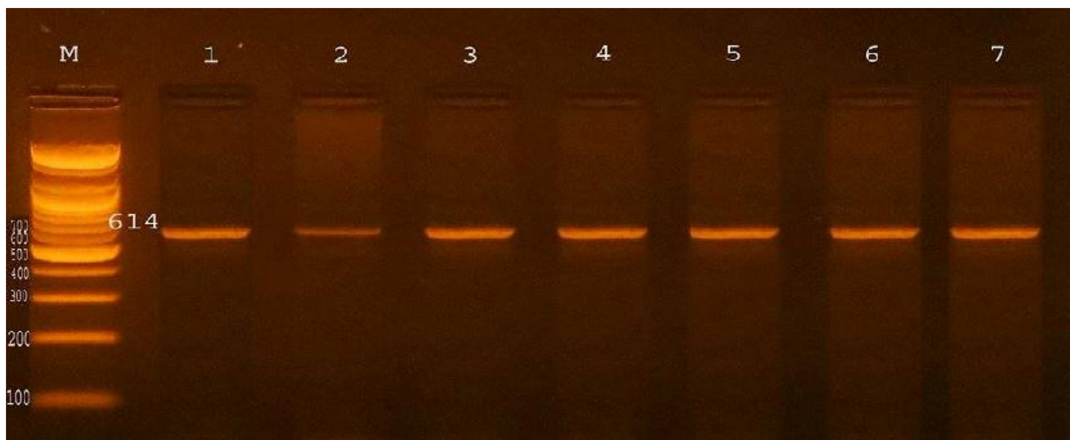

Figure 1: Agarose gel electrophoresis 2% showed amplification of 614 bp fragments of *stx1* genes of *E. coli* O157:H7 Lane M shows PCR marker.

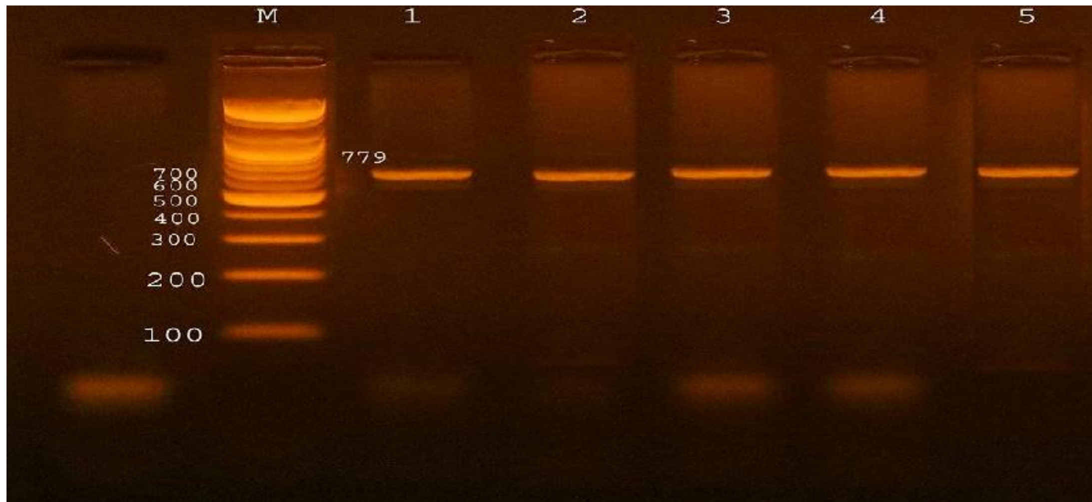

Figure 2: Agarose gel electrophoresis 2% showed amplification of 779 bp fragments of *stx2* genes of *E. coli* O157:H7 Lane M shows PCR marker.

Discussion

This is the first study which describes the detection and frequency of major virulence genes of STEC isolated from calves in Baghdad, Iraq. Study revealed 4 isolates of *E. coli* O157:H7 from 35 fecal samples at a percent (11.42%) in diarrheic calves and all these isolates possessed stx1 gene and 1(25%) were positive for stx2. Non diarrheic calves showed 28(8.88%) positive samples for *Escherichia coli* O157:H7 and 15(53.57%) possessed stx1 gene and 9 (32.14%) possessed stx2.

The percentage of *E. coli* O157 isolation from calves were compatible with Omisakin *et al*. (2003) they reported the prevalence of carriage of *E. coli* O157 in faeces of cattle was 7.5% and with study of Alam and Zurek (2006) who found the prevalence of *Escherichia coli* O157:H7 in beef cattle faeces was (9.2%).

Another study conducted by Kang *et al*. (2004) was compatible with our study as they found the prevalence of E. coli O157 in diarrheic calves at percentage 9.8% and with Kuyucuoglu *et al*. (2011) as they estimate the prevalence of *E. coli* O157:H7 in diarrheic calves at percentage (10.6). Whereas Blanco *et al*. (1993) found that the prevalence of *Eschrechia coli* O157:H7 in the faeces of dairy calves and feedlot cattle is low (0.3 to 2.2%) in the United States, the United Kingdom, Germany, and Spain. While Mechie *et al*. (1997) recorded the prevalence of *Escherichia coli* O157:H7 in calves a high percentage (56%) in England.

The prevalence of *E. coli* O157:H7 in the current study was higher than that reported by El-Shehedi *et al*. (2013) in AL-Qalyoubia Governorate in Egypt in diarrhoeic calves at level 6.97%.

In non-diarrheic calves, the results showed that the prevalence of *E. coli* O157:H7(8.88%) was higher than the percentage recorded by Kuyucuoglu *et al*. (2011), as they found 2.6% of healthy calves infected with *E. coli* O157:H7.

The occurrence of *E .coli* O157:H7 were also detected in different regions of Turkey. For instances, *E. coli* O157 was found in 14 individuals among 330 cattle slaughtered in five different abattoir in Istanbul (Yilmaz *et al*., 2002) and *E. coli* O157:H7 were isolated in 4 individuals among 312 cattle in the eastern region of Turkey (Aslantas *et al*., 2006). In another study, the rate of *E. coli* O157:H7 infection was found to be 13.6% (Cabalar *et al*., 2001), this point was very important because turkey was a neighbouring country to Iraq.

The results showed that *stx1 and stx2* genes found in a percentage 53.12%, 32.14% respectively in isolates of *E. coli* O157:H7 from diarrheic and non-diarrheic calves. This results agreed with Khanjar and Alwan, (2014) who found that the results of PCR assay of *E. coli* O157:H7 isolated from cattle in province of Missan- Iraq revealed that these bacteria were carrying *stx1* gene more than *stx2* gene. Infection in calves appeared to be associated with STEC O157 producing stx1 compared to stx2 (Moxley *et al.* 2010).

Some researcher found different percentage of Stx1, stx2 but not resemble to the results of this study, Bonardi *et al*. (2015) showed that *E. coli* O157 from

cattle harboured stx2c more than stx1, and that cattle hides could be a source of human pathogenic STEC O157 in the slaughterhouse environment. Alam and Zurek, (2006) recorded that All tested isolates of *Escherichia coli* O157:H7 in beef cattle were positive for stx2 (Shiga toxin 2) and eaeA (Intimin) genes and only 14 isolates (12.8%) were also carried stx1.

Also Karmali, (1989) and Paton and Paton, (1998) found that the Human and bovine STEC strains elaborated two potent phage-encoded cytotoxins called Shiga toxins (Stx1 and Stx2) or verotoxins (VT1 and VT2) that cattle are a major reservoir of STEC strains pathogenic for humans.

Osaili *et al*. (2013) were used the Conventional and multiplex PCR assays for serotype confirmation and virulence factor detection, respectively. Fifty *E. coli* O157:H7 isolates were identified and virulence factors eaeA and hlyA were present in all of the isolates. 60%, 12%, and 22% of the isolates harbored stx(1), stx(2), and stx(1) and stx(2), respectively. The prevalence rates of enterotoxigenic *E. coli* O157:H7 were 8.3%, 10%, and 7.8% in feces, hides and carcasses, respectively.

Al- Kaabi, (2014) found that the prevalence of *E. coli* O157:H7 in cattle fecal samples in Missan province in Iraq was 9 out of 54 (16.6%) and expressed stx1 and stx2 as wellas 1 (1.7%) isolate out of 59 gallbladder mucosal swabs which expressed rfb O157 gene only.

Epidemiological studies on EHEC in cattle are very necessary to develop control measures in order to reduce the risk of transmission from cattle to humans. Since isolation procedures are laborious and time-consuming and because of the lack of biochemical features distinguishing most EHEC strains from nonpathogenic *E. coli*, PCR approaches based on the detection of EHEC-associated genetic markers have been developed. (Bibbal *et al*., 2014).

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Author's contribution

All authors contributed equally in all details of this manuscript.

Conflict of interest: Authors declares no conflict of interest.

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