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Research Article

Molecular Detection of Enterotoxogenic Bacterial Toxins Isolated from Human Stool and Beef

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Abstract
Characterization of non-O157 enterotoxogenic Escherichia coli, particularly the important strains that cause severe illnesses has lagged considerably behind that of strain O157. In this study specification of enterotoxogenic *E coli* from other non- O157 was accomplished by PCR by using primers for *wbdI* (O antigen- encoding gene cluster). Virulence of the detected strains was determined by detection of (*stx1*, *stx2, eae and hlyA*) virulence factors genes by multiplex PCR technique in order to determine the aggressiveness of the strains. Later on the *wbdI* gene was sequenced in order to confirm the PCR results. Results showed that the number of ETEC isolates confirmed by PCR was 8; among which 6 from human diarrheal cases and 2 from beef samples. Regarding human isolates, molecular detection of virulence factors genes showed that two of human stool isolates had the 4 virulence genes and 3 had three genes. Two strains out of the three contained (*stx1, eae, hlyA*) and one contain (*stx1, stx2, eae*). One of human stool isolates contains two virulence genes (*stx1, stx2*). In the beef isolates the results showed that one of them contains (*stx1, stx2*) and the other contains (*eae, hlyA*). The present study concludes that the ETEC is found in both beef samples and human stool, this may indicate the risk of transmission from animal to the human through the contaminated meat.

INTRODUCTION

E. coli can be commensal, existing in a symbiotic state providing resistance against pathogenic organisms, and is rarely cause disease except in immunocopromised hosts or in case of breaching of gastrointestinal barrier[1], or become pathogenic and start to cause diseases in intestine or other extraintestinal sites. The most important pathogenic strains are enterotoxogenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAggEC), and diffusely adherent *E. coli* (DAEC)[2].

The variable strains of STEC are characterized by their ability to produce toxins resemble to those produced by Shigella dysenteriae type 1. There are two types of Shiga toxins, Shiga toxin 1 and Shiga toxin 2, which are encoded by the stx1 and stx2 genes respectively; these are carried on temperate bacteriophages[1]. Significant number of STEC isolates also have another pathogenicity island which is chromosomally located; locus of enterocyte effacement (LEE), first discovered in enteropathogenic E. coli (EPEC). LEE mediates attachingand-effacing lesions in the mucosal cells of the host intestinal epithelia. One of the pathogenicity island genes, called eae (for EPEC attaching and effacing), encodes for intimin, which is adhesion protein found in the outer membrane necessary to the intimate enterocytes attachment of the bacteria. The other factors adherence andcolonization, such as pili and adhesins, are also present in STEC strains negative for LEE[3].

In industrialized countries some of STEC strains are important causes of foodborne diseases[4]. These strains which related to human infections are also known as enterohemorrhagic *E. coli* (EHEC). Animals, particularly cattle, serve as reservoirs for STEC; ingestion of contaminated food or water, person- toperson contact, direct contact with animals, and exposure to the environment represent the most common modes of transmission for these strains[5].

The STEC were classified according to importance into two major categories, 157 and non-O157.

A classification model for Escherichia coli O157:H7 was performed by theUSDA, Food Safety and Inspection Service (FSIS) which considered it as a contaminant in raw ground beef, this classification model was begin in1994 in response to an outbreak associated with ingestion of undercooked ground beef. Later on, it has become evident that non-O157 (STEC) strains, particularly the serogroups O26, O45, O103, O111, O121, and O145 (known as the top six non-O157 STEC) cause similar illnesses to those of O157:H7 strain, and outbreaks due to non-O157 STEC, particularly serogroup O111 have been associated with beef or direct contact with cattle[6] Thus, FSIS stated that the top six non-O157 STEC as a contaminant in beef trim, then verify testing for these pathogens in domestic and imported beef manufacturing trimmings which was begin in June 2012[7].

MATERIALS AND METHODS

Sample Collection:

Collection of Human Stool Samples: This includes 100 stool samples which were collected from patients suffer from diarrhea or bloody diarrhea, 60 samples were collected from children between 1 day up to 14 years from different places includes, the pediatric department of AL-Sader teaching hospital-Misan-Iraq, healthcare centers in Missan province and private pediatric laboratories. 40 stool samples were collected from adults between 16 up to 70 years old. Stool samples were put in the screw tubes contain 10 ml of TSB directly after obtaining from the patients. Tubes were kept in ice box for further investigation.

Collection of Beef Samples: One hundred samples of beef were collected slaughter house. Samples were kept in plastic bags preserved in ice box[8] for processing.

Enrichment: This is a very important step to increase the number of Non-O157 STEC up to the detectable level[9].

Enrichment of the Stool Samples: Tubes containing stool samples were put in incubator at 37°C for 24 hrs to allow bacterial growth[9].

Enrichment of the Meat Samples: Three grams of beef were put in 3 ml of normal saline then minced by sterile plate. The supernatant transfer into tube containing 7 ml of TSB and incubated at 37°C for 24 hrs to allow bacterial growth [8].

Culturing of the Samples: Samples were cultured on Leven's eosin methylene blue agar media, only the bacterial colonies which showed metallic sheen were included in the study. These colonies were subcutured on CHROMagar STEC. Colonies that showed mauve color on this medium were cultured on CHROMagar O157. The bacterial colonies which showed mauve color on this media were excluded. Bacterial colonies that showed on CHROMagar O157 were considered as non- O157 according to manufacturer instructions. These colonies were kept in -20 for molecular characterization.

Extraction of Bacterial DNA: DNA extraction was performed by using (Presto™ Mini gDNA Bacteria Kit Geneaid. USA), and the procedure of extraction was done by follow the manufacturer instructions which enclosed within the kit.

Molecular Characterization of Etec Isolates: ETEC isolates were serotyped by using conventional PCR technique for detection of wbdI portion of rfb gene by using a specific primer designed by[10] Table 1. The PCR condition for amplification of the gene were, Initial denaturation includes 95°C for 15 min, 35 cycles of (denaturation: 94 °C for 60 seconds, Annealing: 53.5 for 60 seconds and Extension: 72°C for 60 seconds and the final extension step was 72 °C for 10 min[11]. Amplified gene was explored on agarose gel by gel imaging system.

Detection of the Etec Genes of Virulence Factors (stx1, stx2, eae, hlya) by Multiplex PCR: Detection of the virulence factors were carried out by amplification of the genes. Primers of the virulence factors' genes which include Stx1, Stx2, are designed by[12], eae is designed by[12] and hly is designed by[13], Table 1. Virulence factors genes were amplified by multiplex PCR according to[14]. This include preparation of the master mix by adding 2 µl of each foreword and revers of each *Stx1, Stx2, eae, hlyA* primers, and 5 µl of bacterial DNA template, the volume completed to 40 by adding 19 µl of nuclease free water (the reaction was performed in 50 µl volume of premix), then PCR tubes containing the reactant were put in the thermocycler and the following conditions was used for the amplification process; 95°C for 3min for initial denaturation step, Followed by 35 cycles of (95°C for 20 s for denaturation, 58°C for 40 for annealing and 72°C for 90s for extension). The final cycle was followed by 72°C for 5 min for final extension. The product was revealed on agarose gel by gel imaging system.

Analysis of PCR Product and Sequencing: Cast containing agarose gel was removed from the tray and put in electrophoresis apparatus which contain about 150 ml of 1X TBE buffer, then the first well was loaded with 10 µl of mixture of 7 µl (100-2000 bp) ladder and 3 µl loading dye, the second well was loaded by mixture of only loading dye and premix which was represent a negative control sample. The rest wells were loaded with 10 µl of mixture of 7 µl of the PCR product and 3 µl of the loading dye, then electrophoresis apparatus was set on 100V, 70 AM for 1 hour. The tray was transferred to the gel imaging system to show the bands and the results were imaged with canon camera. The PCR product tubes of 6 representative samples with forward primer of wbdI were sent for DNA sequencing in Korea (by Wahj Al - DNA bureau). The BLAST program (Basic Local Alignment Search Tool) accessed through the website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for analysis of the sequences data.

RESULTS AND DISCUSSIONS

Molecular Serotyping of Etec: Molecular identification of *E.coli* O111 form other non-O157 STEC was carried out by the detection of wbdI portions of the rfbo111 (O antigene- encoding) gene cluster.

The results showed that the total number of enterotoxogenic bacteria were 26 isolats. O111 was 8 out of 26, including 6 isolates out of 21 isolated from human diarrhial cases and 2 ispolats out of 5 isolated from beef, Table 2 and Fig 1.

Molecular study of ETEC virulence genes showed that all human isolates have *stx1* gene, (4 out of 6) have stx2, (5 out of 6) have eae and (4 out of 6) have hlyA genes respectively. Beef isolates have the verulence factors genes as the following; (1 out of 2) has stx1, (1 out of 2) has stx2, (1 out of 2) has eae and (1 out of 2) has hlyA genes respectively table 3 and figure 4.

Gene Sequencing: The results showed that the isolates of animal and human sources have (98%) and (95%) identity with E.coli 95NR1 (Accession number CP/021339.1 , Query cover 100% and 99%) respectively Fig (4). (strain 95NR1 is O111:H-, the source of isolation is fecal samples , country of isolation is Australia).

Molecular Detection of Etec wbdi: The accurate *E coli* serotyping is crucial for determination and surveillance of nonO157 Shiga-toxin producing strains. In the present study the molecular serotyping protocol targets the somatic antigens of O111 for its specific determination, it was implemented by using PCR for detection of wbdI gene, this was agreed with[15] who use the PCR directly to identify the O111 strain. The present study has focused on O111 STEC because of its worldwide significance as zoonotic pathogen and was an important cause of serious illnesses in human including hemorrhagic colitis and hemolytic uremic syndrome as an individual cases and outbreaks (15). The present study was found that the prevalence of O111 in human diarrheal cases were 6 isolates this was inconsistent with[16] who found the prevalence of O111 was 3/127 diarrheal cases in children, as well as[15] who found that in February 2012, a stool specimen from a 3-year-old female with HUS was submitted to the Laboratory of Gastrointestinal Pathogens at the Health Protection Agency in Colindale and identified biochemically and serotyped as *E. coli* O111:H21, Screening of household contacts revealed that

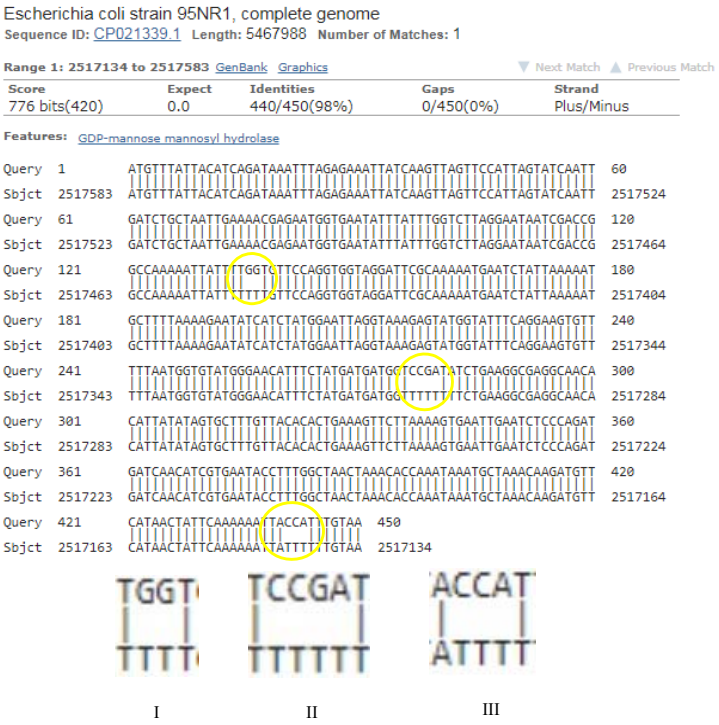

Fig. 1: Alignment of wbdI portion of rfbo111 of the strains of animal sources shows (98%) identity to 95NR1, the magnified parts (I, II, III) were the sequence differences between the local and global strain

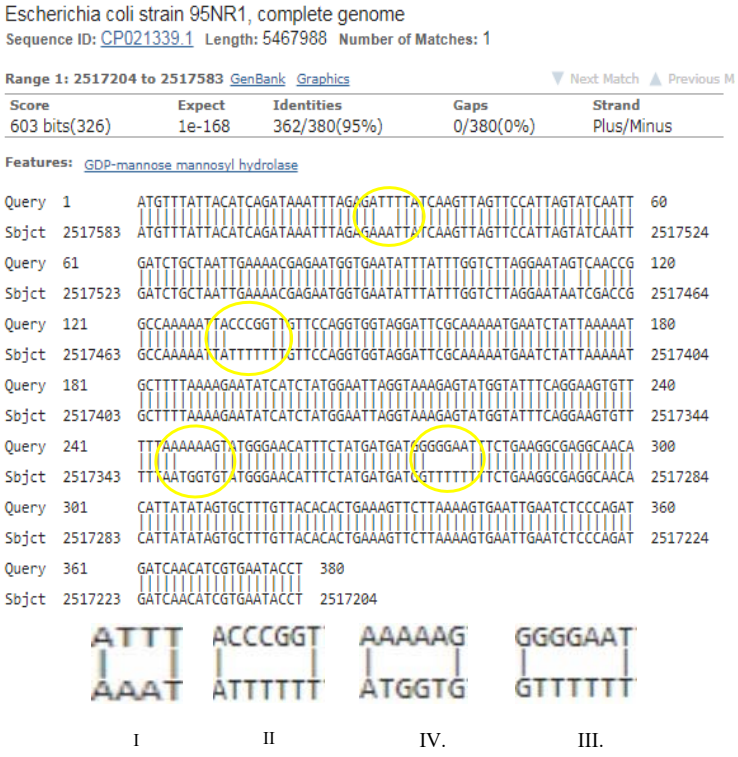

Fig. 2: Alignment of wbdI portion of rfbo111 of the strains of human sources shows (98%) identity to 95NR1, the magnified parts (I, II, III, IV) were the sequence differences between the local and global strain

the child's mother and 4-year-old male sibling each carried a strain of E. coli O111:H21 with the same pathogenic profile. Both contacts described had diarrhea, but neither were admitted to the hospital. Rasko

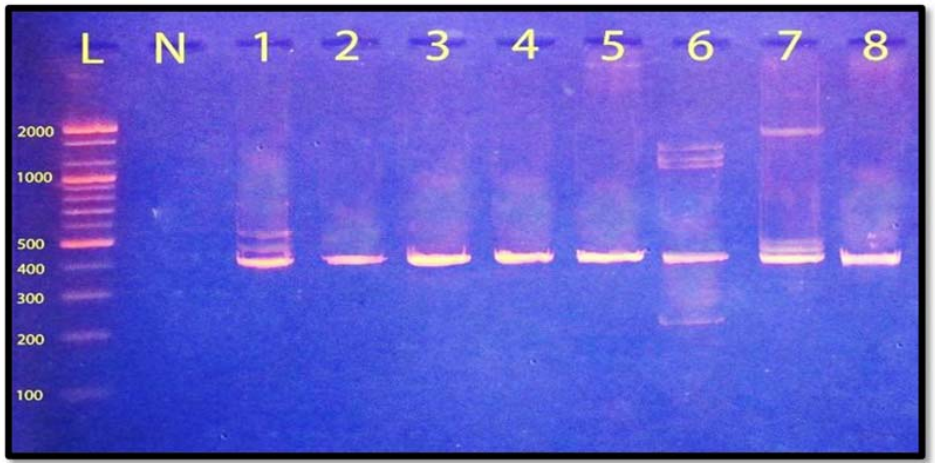

Fig. 3: The gel electrophoresis for the PCR product of wbdI portion of rfbo111 where (L) Ladder, (N) Negative control sample, Lane (1-6) represent isolates of human diarrheal samples, Lane (7-15) are isolates of bovine fecal samples and Lane (16-17) are the isolates of meat samples at (406 bp) PCR products.

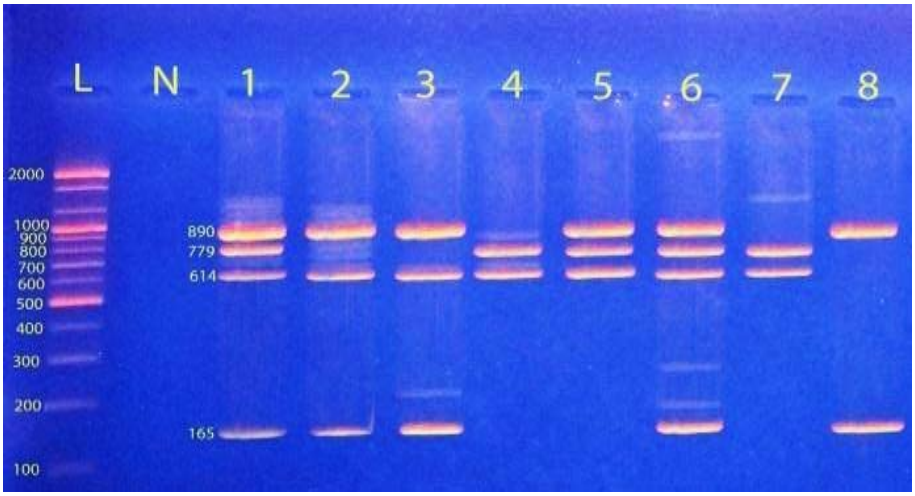

Fig. 4: The agarose gel electrophoresis for the multiplex PCR product of the stx1, stx2, eae and hlyA genes shows, (L) the ladder, (N) the negative control sample, (Lanes 1-6) represent the virulence factors genes of the human stool isolates, (Lanes 7 and 8) belong to beef isolates. (890 bp for eae), (779 bp for stx2),(614 bp for stx1) and (165 for hlyA).

reported that *E. coli* O111:H2 caused an outbreak of HUS in France in 1996[17].

The ratio of O111 from calve diarrheal cases and non-diarrheal bovine fecal samples was in consistent with the previous studies including[16] who isolated O111 from diarrheal cases in calves. Kobayashi *et al.*, 2001 isolated different types of non-O157 STEC including O111 from the calves and healthy cows in Japan. Vicente found that the prevalence of O111 in calve feces was 3.3%[18]. This variation may be from the variation of sample collection because they investigate the prevalence of pathogenic *E coli* in the feces of normal calves, and these strains were rare in normal calves because it causes diarrhea. This idea was supported by the same study when these pathogenic bacteria were investigated in the adult cattle feces; the prevalence was 50%, because large cattle can be carrier without clinical signs. Other studies pointed out that serogroup O111 has

Table 1: Primers which were used in present study with the sizes of the products

No.	Target gene	Direction	Primer sequence (5' – 3')	Fragm ent size (bases)
1	*wbdI*	Forward	AGA GAA ATTATC AAG TTA GTT CC	406
		Reverse	ATA GTTATG AAC ATC TTG TTT AGC	
2	*Stx 1*	Forward	ACACTGGATGATCTCAGTGG	614
		Reverse	CTGAATCCCCCTCCATTATG	
3	*Stx2*	Forward	CCATGACAACGGACAGCAGTT	779
		Reverse	CCTGTCAACTGAGCAGCACTTTG	
4	*eaeA*	Foreward	GTGGCGAATACTGGCGAGACT	890
		Reverse	CCCCATTCTTTTTCACCGTCG	
5	*hlyA*	Forward	ACGATGTGGTTTATTCTGGA	165
		Reverse	CTTCACGTGACCATACATAT	

Table 2: the number of etec isolates out of total number of non-o157 isolates

No.	Type of samples	No. of non- O157 STEC isolates	No. of O111 STEC isolates
1	human diarrheal samples	21	6
3	Beef samples	5	2
	Total	26	8

Table 3: The number verulence factors for each etec

Samples	NO. of isolates	Stx1	Stx2	Eae	hlyA	Total No. of virulence genes
Human stool samples	1	+	+	+	+	4
	2	+	-	+	+	3
	3	+	-	+	+	3
	4	+	+	-	-	2
	5	+	+	+	-	3
	6	+	+	+	+	4
Beef samples	7	+	+	-	-	2
	8	-	-	+	+	2

been involved in most non-O157 STEC infections. Besides, ETEC has been reported as responsible for outbreaks and also sporadic cases[18].

Molecular Detection of Etec Virulence Factors' Genes (*stx1, stx2, eae, hlya*) by Multiplex PCR: The present study was characterized the virulence factors genes profile of ETEC isolated from human and animal sources in missan province / Iraq. Isolates that carry stx genes were present among the human stool and beef samples this agreed with[19]. Both *stx1 and stx2* producing strains were found in both human stool and beef isolates, moreover; all the isolates has predominance for *stx1* gene. These results were agreed with[19,16] despite the variation in ratio of stx detection. Bonyadian reported that the detection of virulence factors from human isolates were as following 27.6% carry stx1, 6.9% of isolates carry stx2 and 13.8 carry both *stx1 and stx2*[15], while Khan reported that 36.5% of isolates carry *stx1,* 19% carry stx2, and 44.5% carry both *stx1 and stx2*[19], in the present study the virulence factors were 70.5% of total isolates carry stx1, 52.94% of total isolates carry stx2 and 64.7 % of total isolates carry both eae and hly. These variations may belong to differences in the types of samples included in each study and to differences in the number of isolates which obtained. The variations in the geographical distribution of ETEC is an important factor as the ETEC is endemic in certain areas but not in the others, as well as the difference in the type of strain which isolated. As reported by Bettelheim although the prevalence of O111 is relatively low in comparison with other non-O157 but it is the most serotype that involved in the outbreaks and sporadic cases[20]. The eae gene positive strains were isolated in this study. The highest ratio of isolates which contain eae gene where obtained from calves with diarrhea this also agreed with[21] who reported that diarrheic calves and cattle represent an important reservoirs of eae positive strains[22]. found that ETEC which isolated from cattle feces carry eae gene even in the strains that lack the stx.

The present study was agreed with[16], they found that 33.3% and 46.15 of non- O157 STEC carry eae respectively. But this study was not agreed with[15] who found that all ETEC which isolated from human stool carry no eae. Results were also in line with[23] who found that 37.6% of the ground beef isolates carried eae gene. The isolates which carry the hlyA were 55.55 in human and these results were in line with (24) 75.3%, (25) 28% , but do not agreed with[16] who found non-of the human O111 isolates carry hlyA.

All beef isolates carry hly gene, these results were in line with[23]. The present study has found that beef is one of the sources of ETEC transmission from animals to the human.

CONCLUSIONS

In conclusion the combination of virulence factor genes were observed in this study where only two isolates from calve diarrheal samples and one isolate of human stool samples have the four virulence factors, while the other

isolates have at least one deficient virulence gene this appear to be essential for ETEC to become frequent cause for diarrhea.

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