Molecular Detection of Enterotoxogenic Bacterial Toxins of Shiga Toxin Prodveing Escherichia Coli Non 0157 Serogroup 026 Isolated from Human and Calves Stool.

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Abstract
The present study includes the molecular characterization of STEC non 0157: 026 toxins. The reliable isolation as non-O157 STEC serotyping by specific latex agglutination test for the target non-O157 STEC (big six) serogroup (O26, O45, O103, O111, O121 and O145), and then confined the serotyping by targeting wzx (O-antigen) for (wzxO26, wzxO45, wzxO103, wzxO111, wzxO121, and wzxO145) by using PCR, then the positive isolate analyzed to determine the prevalence of main virulent genes (stx1, stx2, eaeA, and EHEC hlyA) among isolated non-O157 STEC. The results depending on PCR showed the prevalence of non-O157 STEC were 20 of out 127 (15.73%) in sample collected from children and 27 / 133 (20.30%) in calves sample, The serotyping showed that the O26 was 10.23% (13/127) in human sample and 8.27% (11/133) in animal sample, O103 was only detected in calves samples with 2.25%(3/133), meanwhile the O111 give positive result in both humans and calves samples, 2.36% (3/127) and 3.75% (5/133) respectively, the O145 was detected as 1.57% (2/127) in humans samples and 1.50%(2/133) of calves samples. The O45 and O121 give a negative result in both species. STEC 026 was the most frequent serotype among the other non 0157 STEC, it showed positive result in PCR analyses to the following genes (wzx026, stx1, stx2, eaeA and EHEC hlyA. Conclusion: In conclusion non-O157 STEC were important for the infection of children and calves were played important reservoir for Non-O157 STEC. And the more common strain of Non-O157 STEC was STEC O26.
Keywords: calves and human stool sample ETEC; Non 0157: 026, molecular detection.

1. Introduction

STEC infection have been described in a wide range of both domestic and wild animal species, but their natural pathogenic role has been demonstrated only in young calves, pigs and dogs. The cattle being recognized as the major reservoir for human infections [(1),(2)]. Human infection with STEC usually causes serious diseases [(3), (2)], such as bloody diarrhea, hemolytic uremic syndrome, hemorrhagic colitis, Thrombotic thrombocytopenic purpura, fever, vomiting, and possible death [(4), (5), (6)].

The STEC were classified depending on the importance of serotype O157:H7 in human disease, in to two major categories, STEC O157 and non-O157 STEC [(7)].

Center of Disease Control and Prevention (CDC) [(8)] Food Net demonstrated that approximately 70% of the infections with STEC caused by non-O157 STEC infections, belong to one of these serotypes (O) (26, 45, 103, 111, 121 and 145) and they called it as (big six) [(9), (10)].

Many virulent factors contribute to the pathogenicity of STEC and may play a role in the variability of the clinical manifestations and severity, The virulent factors of non-O157 STEC is mainly mediated by genes coding for toxin include shiga toxins (*stxl* and *stx2)* [(3)]. The Adherence factors The LEE is Pathogenicity Island that encodes for intimin, Tir, and the type III secretion system [(11)], Fimbrial adhesins Intimin is the primary adhesin in STEC, but fimbriae also contribute to adhesion, haemolytic mechanisms *(Ehly)*. At last proteases [(6)]. Over 50% of non-O157 STEC display these factors [(12)].

Humans acquire the infection by ingestion of foods and drinks contaminated with cattle products, especially undercooked meat or other foods like raw milk or homemade cheese from raw milk [(5), (6)], direct transmission from animals to humans also reported [(13)]; animal-animal contact, animal-environmental contact, and environment-animal contact have also been well documented [(14)].

2. Aims of the study

1-Isolation and serotyping of non-O157 STEC strains from children with diarrhea and calves.
2-Determine which serotype was more common by detection of serotype and virulence factors by polymerase chain reaction (PCR).

3. Material and methods

PCR Primers:

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Table (1): Primer used in this study and their designer.

No.	Primer (Target Gene)	Direction	Primer sequences	Size (bases)	Reference
	stx1	Forward Reverse	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614	Gannon *et al.* (1992)
	stx2	Forward Reverse	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779	Gannon et al. (1992)
	eaeA	Forward Reverse	GTGGCGAATACTGGCGAGACT CCCCATTCTTTTTCACCGTCG	890	Paton and Paton (1998)
	EHEC *hlyA*	Forward Reverse	ACGATGTGGTTTATTCTGGA CTTCACGTGACCATACATAT	165	Fratamico *et al.* (1995).
	wzx O26	Forward Reverse	CAA TGG GCG GAA ATT TTA GA ATA ATT TTC TCT GCC GTC GC	155	DebRoy *et al.* (2011)
	wzx O45	Forward Reverse	TGC AGT AAC CTG CAC GGG CG AGC AGG CAC AAC AGC CAC TAC T	238	DebRoy *et al.* (2011)
	wzx O103	Forward Reverse	TTG GAG CGT TAA CTG GAC CT GCT CCC GAG CAC GTA TAA AG	321	DebRoy *et al.* (2011)
	wzx O111	Forward Reverse	TGT TTC TTC GAT GTT GCG AG GCA AGG GAC ATA AGA AGC CA	438	DebRoy *et al.* (2011)
	wzx O121	Forward Reverse	TCC AAC AAT TGG TCG TGA AA AG AAG TGT GAA ATG CCC GT	628	DebRoy *et al.* (2011)
	wzx O145	Forward Reverse	TTC ATT GTT TTG CTT GCT CG GGC AAG CTT TGG AAA TGA AA	750	DebRoy *et al.* (2011)

Molecular characterization of E. coli

DNA extraction:

Genomic DNA of non-O157 STEC isolate was extracted by using (Presto™ Mini g DNA Bacteria Kit Geneaid. USA). It provides an efficient method for purifying total DNA cultured bacterial cells. This test was done according to the manufacturer company.

Measuring the purity and concentration of DNA

The purity and concentration of extracted DNA was measured using Nanodrop spectrophotometer.

Primers:

Ten primers in this study were obtained from Bioneer, Korea. These primers were used to detect somatic antigen (O26, O45, O103, O111, O121 and O14). at genus level designed by [15]. These primers were prepared according to the information of the company.*Stx1* and *Stx2* were designed by [16] while intimin (*eaeA*) gene was designed by [17], and EHEC *hlyA gene* was designed by [18].

Preparation of PCR master mix

All required reagents were thawed completely and were kept in ice, the reagent was mixed well by inversion and spin them down prior to pipetting. PCR master mix reaction was prepared by using PCR PreMix, Bioneer (Korea), these master mix were done according to the company instructions as illustrated in the tables (xx).

Detection of wzx (O-antigen-flippase) genes by PCR

In order to detect O-serogroups associated with non-O157 STEC, six O-serogroups targeting the wzx (O-antigen-flippase) genes according to [15].

Primers (4.5 µl of each forwarded and reversed) were mixed with master mix(12.5µl), and 2 µl of template DNA, and 1.5 µl of nuclease free water to complete the amplification mixture to 25µl according to Bioneer, Korea, the PCR tubes containing an amplification mixture were transferred to thermocycler and the program started as fallow, PCR amplification was conducted by initial denaturation at 95C for 15 min followed by 30 cycles of denaturation at 94C for 30 sec, primer annealing at 57C for 1.5 min followed by extension at 72C for 1.5 min, and a final extension for 10 min at 72C. The amplified DNA was electrophoresed in an agarose gel.

Detection of stx1, stx2, eaeA, and EHEC hlyA gene by PCR

For detecting of *stx1*, *stx2*, *eaeA*, and EHEC *hlyA* gene by PCR according to [16,17,18], the PCR amplification mixture was done according to Bioneer, Korea which includes master mix (12.5µl), 2 µl of template DNA, 4.5 µl of each forwarded and reversed primers and 1.5 µl of nuclease free water to complete the amplification mixture to 25µl. Then transferred to thermocycler and the program started as fallow. The Temperature conditions consisted of an initial 95°C denaturation step for 3 min followed by 35 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 90 s. The final cycle was followed by a 72°C incubation for 5 min. Amplified DNA fragments were resolved by gel electrophoresis.

PCR product analysis (Agarose Gel Electrophoresis):

It is a very important step to complete PCR assay, which is used to analyse the PCR product by agarose

gel electrophoresis as follow:

1- 2% Agarose gel was prepared in 1X TBE buffer and heated by hot magnetic stirrer until all crystals were disappeared in agarose.
2- After cooling 3µl of Ethidium bromide per 100 ml gel solution were added.
3- The gel was poured in the tray and fixing the comb at the right position and left until solidifying, then the comb was removed carefully.
4- Ten µL of PCR product were dripped into each comb gently, and 8 µL of (100 bp ladder) added in the first well which was used as molecular marker to estimate the size of the PCR products.
5- PCR product were transferred into electrophoresis machine which contained the same 1X TBE buffer that used in preparation of agarose gel.
6- An electric current was set up at 100 Volt and 70 AM for 1hour.
7- Finally; visualization with UV illumination, and imaged to determine the sensitivity.

4. Statistical analysis

Statistical analysis was conducted to determine the statistical differences between and among the groups by using ready–made statistical design: statistical package for social science (SPSS).

5. Results

Polymerase chain reaction

Extraction of genome DNA of non-O157 STEC

The DNA of isolated strains was extracted; concentration and purity of the extracted DNA were measured by Nano drop spectrophotometer and the concentration of DNA ranged from 25 – 189.9 ng/ml.

Prevalence of non-157 STEC in children and calves sample.

In our study the results showed the ratio of non-O157 STEC were 20 of out 127 (15.73%) in sample collected from children and 27 / 133 (20.30%) in calves sample as illustrated in table (1).

Table (1): Prevalence of non-157 STEC in children and calves sample.

Source	Children	Calves
Number of sample	127	133
Non-O157 STEC	20 (15.73%)	27 (20.30%)

Serotyping of non-O157 STEC by PCR with targeting wzx (O-antigen) genes (26, 45, 103, 111, 121 and 145)

The PCR assay to detect the presences of the O-antigen gene clusters in the wzx genes for (wzx_{O26}, wzx_{O45}, wzx_{O103}, wzx_{O111}, wzx_{O121}, and wzx_{O145}) showed that the isolates gave positive results to wzx_{O26} gene which was 10.23% (13/127) in children sample and 8.27% (11/133) in calves samples, the wzx_{O103} gene was only detected in calves samples with 2.25%(3/133), meanwhile the wzx_{O111} gene give positive result in both children and calves samples, 2.36% (3/127) and 3.75% (5/133) respectively, the wzx_{O145} gene was detected as 1.57% (2/127) in children samples and 1.50%(2/133) of calves samples. The *wzx* O45 and *wzx* O121 gave a negative results in both species samples Also there were 8 isolated of STEC non-157 two from children and six from calves gave negative results to the above serotypes so it is not determined serotype (ND). Table (2) and figures (1) (2) (3) (4).

Table (2): Percentage of serotyping among total collected samples.

Source	Children	Calves
Number of sample	127	133
wzx O26	13 (10.23%)	11 (8.27%)
wzx O45	0 (0%)	0 (0%)
wzx 103	0 (0%)	3 (2.25%)
wzx O111	3 (2.36%)	5 (3.75%)
wzx O121	0 (0%)	0 (0%)
wzx O145	2 (1.57%)	2 (1.50%)
N.D.	2 (1.57%)	6 (4.51%)

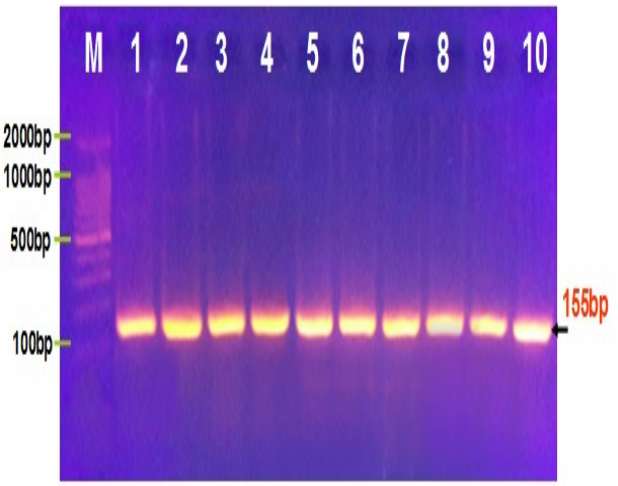

Figure (1): Agarose gel electrophoresis image showing the PCR product analysis of wzx O26 gene in STEC isolates. Where M: marker (2000-100bp), lane (1-4) positive isolates from children feces samples and lane (5-10) positive isolates from calves samples at (155bp) PCR product.

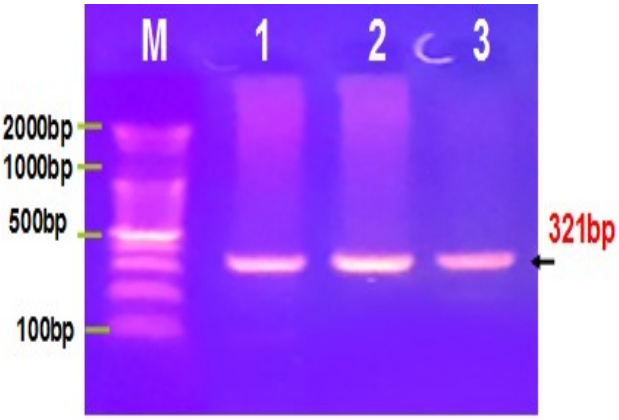

Figure (2): Agarose gel electrophoresis image showing the PCR product analysis of wzx O103 gene in STEC isolates. Where M: marker (2000-100bp), positive isolates from calves samples at (321bp) PCR product.

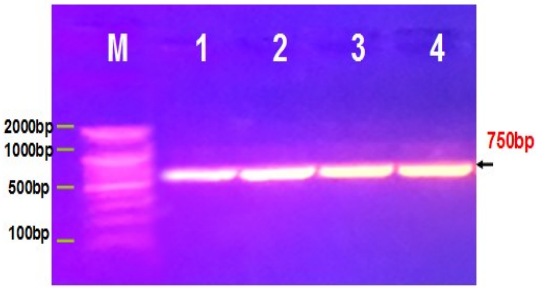

Figure (3): Agarose gel electrophoresis image showing the PCR product analysis of wzx O145 gene in STEC isolates. Where M: marker (2000-100bp), lane (1-2) positive isolates from children feces samples and lane (3-4) positive isolates from calves samples at (750bp) PCR product.

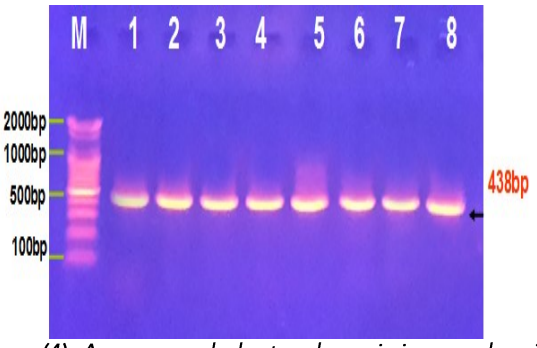

Figure (4): Agarose gel electrophoresis image showing the PCR product analysis of wzx O111 gene in STEC isolates. Where M: marker (2000-100bp), lane (1-3) positive isolates from children feces samples and lane (3-8) positive isolates from calves samples at (438bp) PCR product.

Prevalence of virulent genes (*stx1, stx2, eaeA, and EHEC hlyA)* among non-O157 STEC serogroup by PCR.

This study showed that 40% (8/20) of isolated non-O157 STEC expressed *stx1* gene in children stool sample and 48.14% (13/27) of calves samples, the *stx2* gene appeared in 30%(6/20) and 11.11%(3/27) in children and calves respectively, target *eaeA* gene gave a positive results were 40%(8/20) in children isolated non-O157 STEC and 40.47%(11/27) in calves non-O157 STEC, meanwhile the *EHEC hlyA gene* results were 55%(11/20) and 55.55%(15/27) from both children and calves isolated non-O157 STEC respectively. Table (3) and figures (5) (6) (7) (8).

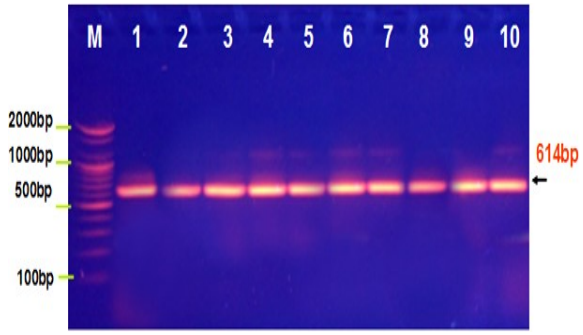

Figure (5): Agarose gel electrophoresis image showing the PCR product analysis of stx1 gene in STEC isolates. Where M: marker (2000-100bp), lane (1-6) positive isolates from children feces samples and lane (7-10) positive isolates from calves samples at (614bp) PCR product.

Table (3): Percentage of virulence factors among isolated non-O157 STEC.		
Virulence factors genes	Non-0157 STEC from children	Non-0157 STEC calves
	20	27
stx1	8 (40%)	13 (48.14%)
stx2	6 (30%)	3 (11.11%)
EaeA	8 (40%)	11 (40.47%)
EHEC hlyA	11 (55%)	15 (55.55%)

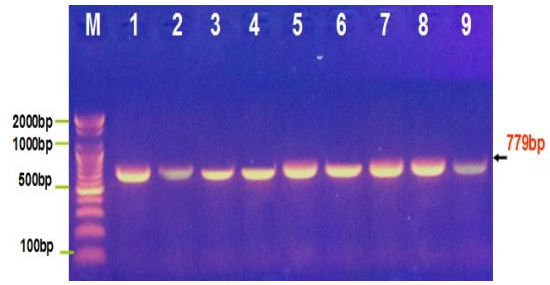

Figure (6): Agarose gel electrophoresis image showing the PCR product analysis of stx2 gene in STEC isolates. Where M: marker (2000-100bp), lane (1-6) positive isolates from children feces samples and lane (7-9) positive isolates from calves samples at (779bp) PCR product.

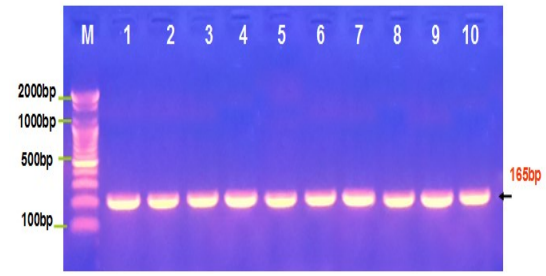

Figure (7): Agarose gel electrophoresis image showing the PCR product analysis of EHEC hlyA gene in STEC isolates. Where M: marker (2000-100bp), lane (1-5) positive isolates from children feces samples and lane (5-10) positive isolates from calves samples at (165bp) PCR product.

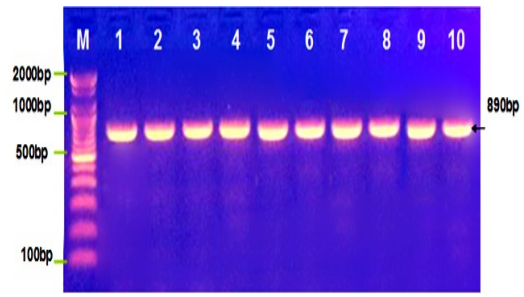

Figure (8): Agarose gel electrophoresis image showing the PCR product analysis of eaeA gene in STEC isolates. Where M: marker (2000-100bp), lane (1-8) positive isolates from children feces samples and lane (9-10) positive isolates from calves samples at (890bp) PCR product.

6. Number of virulence genes carriages by each isolated non-O157 STEC

The PCR assay demonstrates Carriage of the genes stx1, stx2, eaeA, and *EHEC hlyA* among E. coli serogroup (O) 26, 45, 103, 111, 121 and 145 isolates is shown in Tables (4) (5).

Table (4): Carriage of the genes stx1, stx2, eaeA, and EHEC hlyA by serogroup (O) 26, 45, 103, 111, 121 and 145 isolates from children.

No	Serotype	No. of isolate	*stx1*	*stx2*	*eaeA*	*EHEC hlyA*	No.of virulent genes
1	O26	1		√			One gene
		2				√	
		3				√	
		4				√	
		5				√	
		6				√	
		7	√		√		Two genes
		8			√	√	
		9		√		√	
		10	√	√	√		Three genes
		11	√	√	√		
		12	√	√	√	√	Four genes
		13	√	√	√	√	
			5(38.46%)	6(46.15%)	6(46.15%)	9(69.23%)	Percentage
2	O111	1	√				One gene
		2			√		
		3					Zero genes
			1(33.33%)	0(0%)	1(33.33%)	0(0%)	Percentage
3	O145	1	√				One gene
		2	√			√	Two genes
			2(100%)	0(0%)	0(0%)	1(50%)	Percentage
4	ND	1				√	One gene
		2			√		
			0(0%)	0(0%)	1(50%)	1(50%)	Percentage

Table (5): Carriage of the genes stx1, stx2, eaeA, and EHEC hlyA by serogroup O26, O45, O103, O111, O121 and O145 isolates from calves.

No	Serotype	No. of isolate	*stx1*	*Stx2*	*eaeA*	*EHEC hlyA*	No.of virulent genes
1	O26	1	√				One gene
		2	√				
		3	√				
		4			√		
		5				√	
		6				√	
		7	√		√		Two genes
		8			√	√	
		9			√	√	
		10	√	√	√		Three genes
		11	√	√	√	√	Four genes
			6(54.54%)	2(18.18%)	6(54.54%)	5(45.45%)	Percentage
2	O103	1				√	One gene
		2				√	
		3	√			√	Two genes
			1(33.33%)	0(0%)	0(0%)	3(100%)	
3	O111	1	√				One gene
		2				√	
		3				√	
		4				√	
		5	√	√	√		Three genes
			2(40%)	1(20%)	1(20%)	3(60%)	Percentage
	O145	1	√				One gene
		2	√			√	Two genes
			2(100%)	0(0%)	0(0%)	1(50%)	Percentage
4	ND	1			√		One gene
		2				√	
		3			√	√	Two genes
		4			√	√	
		5	√			√	
		6	√		√		
			2(33.33%)	0(0%)	4(66.66%)	4(66.66%)	Percentage

7. Discussion

Polymerase chain reaction

Prevalence of non-157 STEC in children and calves sample.

The result of this study showed a high prevalence of non-O157 STEC in calves and these finding were in agreement with many other studies. In France isolation rate was 145/415 (34.9%) [19] in USA 396/1189 (33.3%) [20]; in Argentina12/75 (16%) from Rectal Swabs [21]; in Bangladesh101/139 (72.6%) [22]; in Japan 227/605 (37.5%) [23]; and in Brazil 44/344(12.7%) [24]. Similarly [25], also reported the higher prevalence of STEC in calves.

In addition [26]. showed that the prevalence of these organisms varies from 10% to 20%, but may reach as

high as 80% to 90%.

The prevalence of non-O157 STEC in human among patients with STEC infection recorded in various region around the world; in USA 44 % [27], in Canada 20% [28], Germany 44% [29], in Italy 34% [30], Denmark 75% [31], Japan 19 % [32], Australia 69 % [33], Finland 53 % [34].

Serotyping of non-O157 STEC by PCR with targeting wzx (O-antigen) genes (O26, O45, O103, O111, O121 and O14)

This study provided a useful information about the prevalence of STEC non-157 serogroups in children due to its significant public health importance. This study showed that E. coli O26 was the most prevalent followed by O111and O145; no single sample was positive for serotypes; 103, 45 and 121 in samples from children.

This finding is in agreement with other studies done by [35] [36] [37], recorded the prevalence in human were (O26 \ 22 %, O111 \16% and O145\ 5%), In the U.S., serogroup O26, O103, and O111 accounted for 78% of non-O157 STEC infections (CDC, 8).

However, in Europe non-O157 STEC (especially O: 26, 103, 111 and 145) are considered to be the most important cause of human outbreaks [4]. It has also been observed that the number of human cases due to non-O157 STEC is on the rise worldwide, for example a 300% increase in the incidence of STEC serotypes O26, O111 and O103 has been reported from 2004 to 2007 in USA [38]; half of the STEC strains isolated from pediatric HUS patients between 1997 and 2000 in Germany and Austria were non-O157 STEC (90 of 207 strains)

Serogroup O26, according to the CDC, is the most frequent serogroup involved in human non-O157 STEC infections in the US [4]. The high prevalence of O26 serogroup-specific gene (wzxO26) also reported. in fecal prevalence estimates 23.0% [10], 80.0 % [39] and 82.5 % [40].

The sporadic cases and outbreaks caused by O145 also have been observed in different parts of the world [41]. The prevalence of O145 in human disease is low as compared to other non-O157 STEC serotypes [30]; [4]. The non-O157 STEC O111 was mostly the causes of HUS in USA [4].

In calves the prevalence of non-O157 STEC showed a high isolation rate of O26 as compared with other serogroup followed by O111,O103 and O145 respectively meanwhile the serogroup O45 and O121 were absent, Similar to this study, a Scottish study also reported a higher prevalence of O26 (4.6%) followed by O103 (2.7%) and O145 (0.7%) [35]. In addition [42], found the prevalence of O26 was 1.0%, O103 was 1.6%, and O145 was 0.8%, in 576 fecal samples.

[43], studied the occurrence of O26 and O111 in 442 in calves (257 calves with diarrhea and 185 without diarrhea) in Korea, they reported that the prevalence were 14.4% and 7.6% of 257 calves with diarrhea were tested positive for O26 and O111 respectively while 7.6% and 5.9% of 185 calves without diarrhea were tested positive for O26 and O111 respectively. An Italian study also reported a higher prevalence of E. coli O26 (3%) in fecal samples collected from cattle using IMS. However, no O103, O111 or O145 isolates were obtained [36]. Other study analyzed 809 fecal samples collected cattle aged between eight months and five years using IMS. They reported a higher prevalence of E. coli O26 (6.7%) compared to O111 (4.6%) but E. coli O103 and O145 numbers were not investigated.

The results of this study disagreed with [36], who studied the prevalence of STEC O: 26, 103, 111 and 145 in Italian cattle using IMS, and he recorded the prevalence of O26 was 0.5%. They did not isolate O103, O111 or O145 in fecal samples.

Prevalence of virulent genes (*stx1, stx2, eaeA, and EHEC hlyA)* among non-O157 STEC serogroup by PCR.

The prevalence of virulence genes of non-O157 STEC have shown that many of non-O157 STEC strains may carry the *stx* genes in both children and calves; these result are in agreement with [21], who reported that (60.0%) of STEC strains carried the stx1 gene, carried both stx1 and stx2 genes also this agreed with [35]; [39].

Stx1 gene was also higher in a study carried out by [27]. In the study of [24] among STEC strains, (50%) isolates carried stx1, (16.67%) stx2, and 8 (33.33%) carried both. The high prevalence stx1 (122/249; 49%) also documented in Scottish cattle [35]. Carriage of the stx2 gene in non-O157 STEC isolates has also been reported in German cattle (Geue *et al.*, 2002). [44] reported that stx2 gene was more prevalent than stx1 in STEC strains.

Our study indicated that highest occruing positive result to *eaeA* gene which agreed with [45], other study had done by the CDC [8] reported that over 90% of illnesses resulting in bloody diarrhea caused by non-O157 STEC contained *eaeA gene* [4]. The majority of STEC strains pathogenic to human beings were *eaeA*-positive, and *eaeA* has been identified as a risk factor for HUS development [46].

[47] reported that diarrheic calves and cattle represent an important reservoirs of *eaeA* positive[46]. The association of *eaeA*-positive clinical STEC isolates and severe diarrhea and HUS has been noted [4]. However, outbreaks and cases associated with *eaeA*-negative STEC strains were reported [48].

The highest prevalence of *EHEC hlyA* gene in our results were in both children and calves samples, the *EHEC hlyA* is considered as an important virulence marker of STEC due to its association with disease in humans [49], the role of enterohaemolysin in human disease is not clearly understood, these results agreed with many other results, Peter *et al.*, (1998) indicated that *EHEC hlyA* was detected in 35.9% of calves fecal samples tested. [50] found there is about a third of STEC strains isolated in his study from animals were *EHEC hlyA* -positive.[51] reported that STEC strains may lack the *EHEC hlyA* gene, but its presence would be a good marker for STEC.

In addition the vast majority of human clinical cases of disease caused by STEC are associated with strains that are *eaeA* and *EHEC hlyA* positive [52].

Number of virulence genes carriages by each isolated non-O157 STEC

Most of children and calves isolated strain carried one virulence factor, some had two, three and even four virulent genes in same isolated strain.

The most common virulence profile of E. coli O26 isolates was *stx1*, *eaeA*, *EHEC hlyA*. This is in agreement with another study which indicated more than 90% of O26 isolates displayed this profile [35]. This profile has a great public health significance because this is the most common profile present in O26 isolates from human diarrhoeal cases [51] [53]. Most of the non-O157 STEC isolates may have 3 virulent genes; similar study taken from isolates human diarrheal disease cases in New Zealand indicated such result for *stx1*, *eaeA*, *EHEC hlyA*. [4]

[53] showed that all O26 isolates collected between 1965 and 1994 expressed only *stx1* while most O26 isolates between 1995 and 1999 had either *stx2* or both types of stx. This new virulence was a "high pathogenic potential for humans" and more research is conducted on this STEC [5]. The incidence of VTEC O26 disease is probably because of diagnostic limitations was underestimated [54].

Non-O157 STEC that lacked major virulence genes isolated from cattle also reported in many study [55] [37].

In our study, there were eight *E. coli* isolates (2 from children and 6 from calves) fecal samples that did not belong to the top seven serogroups, but carried *stx*1 and/or *stx*2 genes. STEC isolates without *eaeA* and *EHEC hlyA* from diarrhea and HUS patients have also been recovered, but their recovery from human cases is rare [56].

[57] also demonstrated that the *eaeA* gene is present in cattle *E. coli* serogroup O: 2, 8, 10, 15, 34, 64, 77, 113, 119, 128, 156, 177 and several ONT (Oantigen nontypeable) strains. Similarly, *EHEC hlyA* is found in *E. coli* serogroups (5, 69, 76, 84, 98, and 156) of both cattle and human origins [58] [7]. The *eaeA* gene was detected in both *stx*-positive, and *stx*-negative *E. coli* serogroups [59]. Similar to this study [35] [60], reported the rare carriage of stx gene in O103 and O145 isolates.

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