Journal of Current Veterinary Research

ISSN: 2636-4026

Journal homepage: http://www.jcvr.journals.ekb.eg

Molecular Characterization of Shiga Toxin Producing *Escherichia coli* Isolated From Both Diarrheic and Apparently Health Calves

Amira Ahmed Mahmoud[1], Alaa Eldin Hussien Mostafa[2], Abdelrahman Abdel megid Abdelrahman[1] and Ahmed Elsify[3]

(1)Department of Bacteriology, Animal Health Research Institute, Alexandria Provincial Lab., Dokky, Giza, Egypt.
(2)Department of Bacteriology ,Mycology and Immunity, Faculty of Veterinary Medicine, University of Sadat City, Egypt.
(3)Department of Animal medicine and infectious diseases, Faculty of Veterinary Medicine, University of Sadat City, Egypt.
*** Corresponding Author:** amiraelabd2013@yahoo.com ***Submitted:*** *15 Aug. 2019* ***Accepted:*** *12 Sept. 2019*

ABSTRACT

Shiga toxin producing *Escherichia coli* is a contaminant of food and water that causes a diarrheal syndrome followed by more severe disease of the kidneys and symptoms of the central nervous system in humans. The isolation of Shiga toxin producing *Escherichia coli* (STEC) from diarrheic and apparently health calves is difficult due to lack of differential phenotypic characteristics from nonpathogenic *Escherichia coli.* The improvement of molecular technology allows identification of both toxin and serogroup specific genetic determinants. In this study, 300 fecal samples from diarrheic and apparently healthy calves were screened for STEC using PCR targeting Shiga toxin determinants. In addition routine culture methods for isolating O157 and non O157 STEC were also performed. The screening assays of serotyping isolates revealed 7 (4.1%) of O157H7, 156 (92.8%) of non O157 and 5 (3.1%) for untypable strains. These included STEC serotypes of O157H7 and O26 from diarrheic samples, and O78, O55 and O126 from apparently healthy calves. The high rate of STEC isolation and the diversity of STEC serogroup from calves focus the light on the importance of calves as reservoir of *E. coli* as well as motivate us to improve biosecurity measures in dairy farms.

Key words: *Escherichia coli, Shiga toxin, Serotyping, O157 H7, Non O157, diarrheic and apparently health calves.*

INTRODUCTION

Cattle are considered the main reservoir of STEC (Bertin *et al.*, 2001). *Escherichia coli* is considered as an indicator of fecal contamination of water and food. More than 700 serotypes of *Escherichia coli* have been identified and the majority of these are non-pathogenic commensally in the human and animal gastrointestinal tract (Jang *et al.*, 2017). However, a small percentage of *Escherichia coli* serotypes have the ability to cause infections ranging from wound infections to fatal meningitis in humans of all age groups Riley (2014).The pathogenic *Escherichia coli* represents a small group of highly specialized strains or serotypes which have specifically evolved to infect humans and animals (Gomes *et al.*, 2016).

The primary virulence factor of STEC is the production of one or more Shiga toxins. Shiga toxins (Stx) are of two types: Stx1, which is more or less identical to the toxins produced by *Shigella dysenteriae* Stx1 and Stx2, which has about 60% similarity with Stx1 (Krüger and Lucchesi, 2015) and (Steiner, 2016). Stx1 is more conserved compared to *Stx2* with few variants such as *Stx1c* and *Stx1d*, while several sequence variants of *Stx2*, *Stx2a, and Stx2b*, *Stx2c*, *Stx2d*, *Stx2e*, *Stx2f*, and *Stx2* genes have been reported (Baranzoni *et al.,* 2016).

Although roughly 400 serotypes of STEC produce shiga toxins, all of them are not implicated in human infections. Serotype O157:H7 is the most common serotype involved in severe cases of infections leading to HUS and HC, and majority of the large scale out breaks of STEC infections have been associated with this serotype Gould *et al.,* (2013). In addition to *Escherichia coli* O157:H7, numerous non O157 Shiga toxin producing *Escherichia coli* (STEC) serogroups caused outbreaks and severe illness, including hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) similar to STEC O157. (Gould, 2013 and Scallan *et al.,* 2011). In this study we aimed to estimate the prevalence of *E.coli* associated with calf diarrhea, with serotyping of isolated strains and molecular detection of virulence genes in isolated strains.

MATERIAL AND METHODS

Fecal Samples were collected from a total of 300 calves (200 apparently healthy and 100 were diarrheic) from rectum and immediately transported in an ice box to the laboratory, being stored at 3 ºc ± 1ºc for no longer than 2 hrs. For bacteriological examination according to Cheesbrough 1985; samples were inoculated in *modified tryptic*soya broth with novobiocin 20mg/l at 37ºC for 18-24 hrs. Then subcultured on MacConkey Sorbitol agar medium. Suspected colonies were picked up and streaked on nutrient slope and then preserved on semisolid nutrient agar for further biochemical identification and molecular identification; while for long term preservation isolates were stored at −80 ˚C in tryptic soya broth (TSB) (Becton Dickinson and Co., Sparks, MD) containing 10% glycerol.

Suspected isolates of *E. coli* were subcultured on MaCconkey agar medium and incubated for 24 hrs at 37ºC. Three to five colonies of the growth were suspended in 3 ml saline, and kept in water bath at 100ºC for 1hr, then centrifuged at 2000 rpm. The supernatant was poured and the precipitate was kept, to which 0.5 ml saline was added. *E. coli* serotyping was done in the Reference Laboratory at the Institute for Animal Health Research in Dokki, Egypt. The Mast Diagnostics Kit® (Mast Group Ltd., Merseyside,UK) was used to identify of *E. coli* using polyvalent Antisera table (1).

Seventeen strains of *E. coli* isolates were examined for the detection of the 4 genes (*eaeA*, *hly*, *stx1*, *and stx2*) which included 2 shiga toxin genes using PCR. DNA had been extracted using Wizard Genomic DNA Purification kit according to Promega® technical manual. The resulting DNA template was stored at -20°C until further use. PCR were done using DreamTaq Green PCR Master Mix (2X) (Thermo Scientific®) and Primers were supplied from Metabion® (Germany). The primers sequences shown in Table (2). The PCR mix and protocol table (3, 4, 5) were done according to according to (Dipineto et al., 2006, Bisi-Johnson et al., 2011 and Piva et al., 2003)

RESULTS

The present study was carried out for isolation of shiga toxin producing *Escherichia coli* from diarrheic and apparently health calves. The results showed in Table (6) revealed that 168 samples were positive for *Escherichia coli* with percentage of (56%) and 132 samples were negative for *E. coli* with percentage of (44%).
Serological identification of the isolates revealed that 7 (4.1%) of O157H7, 156 (92.8%) of non O157 and 5 (3.1%) were untypable strains (table 7). In this study 2 strain of O157H7,O26 and 2 untypable strains from diarrheic samples and 5 strains of O157H7, O78, O55, O126 and 3 untypable strains from apparently health calves.
In the current study, seventeen isolate strains of shiga toxin *Escherichia coli* were tested by PCR.

The test detected 8 (47.1%), of eae, 5 (29.4%) of stx1 and 6 (35.3%) for hly gene, while stx2 could not be detected in all isolates tested (Table 8).eae genes were detected at 248bp, stx1 and Stx2 gene were detected at 614 and 779bp and hly genes were detected at 1177 bp; figures (1, 2, 3, 4, 5 and 6).

Stx1 gene founded in five samples of apparently health calves which have serotypes (two of them are O157H7, one strain was untypable, another was O55and the last strain was O126).

DISCUSSION

Livestock feces may contain a variety of *Escherichiacoli* O157 strains and this study confirmed this observation. We isolated both STEC O157 and non STEC O157 from 300 calves' fecal samples. Shiga toxin producing *Escherichia coli* (STEC is recognized as an important food borne pathogen) responsible for sporadic cases to serious outbreaks worldwide. The morbidity and mortality associated with several recent outbreaks due to STEC have highlighted the risk of this organism poses to public health. Since its discovery in 1982 (Keen, 2006).

There are at least 100 serotypes of *Escherichia coli* that are capable of producing Shiga toxins. However of these serotypes *Escherichia coli* O157:H7 is the most well-known to both microbiologists and the general public Nataro and Kaper (1998). In the USA, *Escherichia coli* O157:H7 and the top six nonO157 STEC (O26, O45, O103, O111, O121, and O145) cause the majority of the food borne illnesses and outbreaks. However other emerging STEC serogroups including O113 and O91 have caused outbreaks and serious illness in the US and in other countries (Paton *et al.,* 1999, Brooks *et al.,* 2005, Johnson *et al.,* 2006, Bettelheim, 2007 and Mellmann, *et al.,* 2009).

Escherichia coli were isolated from diarrheic and apparently health calves as shown in Table (8)**.** 168 samples were positive for *Escherichia coli* with percentage of (56%) and 132 samples were negative for *Escherichia coli* with percentage of (44%).

A total of 168 isolate *Escherichia coli* strain were typed serologically. There is 7 (4.1%) of O157H7 (2 isolates isolated from diarrheic samples and 5 isolates isolated from apparently health calves), 156 (92.8%) of non O157 and 5(3.1%) for untypable strain (2) untypable isolates isolated from diarrheic sample and 3 untypable isolates isolated from apparently health). As well as O26 had been isolated from diarrheic sample, while O78, O55 and O126 had been isolated from apparently health calves.

Our results agree with reported by (Martin, 2011), Studies by (Ferreira, *et al.,* 2014 and Freitas-Filho, *et al.,* 2014); they reported that ruminants in particular healthy cattle and sheep are the main reservoirs of STEC by passing the fecal route and acting as a source of contamination of animal products thus representing a potential pathogenic threat to human health. Also Persad and LeJeune (2014); reported *Escherichia coli* O157:H7 has a high prevalence among domestic cattle and other domestic animals and is found in the feces, hides and hair of infected animals.

Serological typing showed that 7 strains of O157H7 with percentage of (4.1%), non O157H7 156 with percentage of (92.8%) while 5 (3.1%) for untypable strains. These data are in agreement with the results of other researchers by (*Brooks, et al.,* 2005; Johnson, *et al.,* 2006; Bettelheim, 2007 and (Mellmann, *et al.,* 2009) they reported *Escherichia coli* O157:H7 and the top six non O157 STEC (O26, O45, O103, O111, O121, and O145) cause the majority of the food-borne illnesses and outbreaks, however other emerging STEC serogroups including O113 and O91 have caused outbreaks and serious illness in the US and in other countries. In this work we also identified non STEC O157 in healthy and diarrheic calves included of O26, O78, O55 and O126.

Escherichia coli O157:H7 has been found in cattle of several countries including the USA, Canada, Germany, Spain, England, and Scotland (Armstrong, *et al.,* 1996). Outbreaks have also occurred in these countries as well as in Japan (Michino, *et al.,* 1999). Also our data are in agreement with the results of other researchers.

Cattle are considered to be the chief animal reservoir for *Escherichia coli* O157:H7 which is a temporary member of their normal gut microflora; *Escherichia coli* O157:H7 has been isolated from many healthy cattle and has not been shown to be a pathogen in these animals Caprioli *et al.,* (2005). Cattle seem to lack vascular receptors for shiga like toxins (PruimboomBrees *et al.,* 2000). *Escherichia coli* O157:H7 has also been isolated from other animals including deer (Diaz *et al.,* 2011), sheep (Urdahl, *et al.,* 2003), horses (Lengacher, *et al.,* 2010), goats (Mersha, *et al.,* 2010), and dogs (Kataoka, *et al.,* 2010).

Traditional methods for detecting and identifying STEC are labor intensive and time consuming. Commercially available ELISA kits and molecular methods like PCR have reduced the time and labor involved in the analysis of food products and fecal samples. The PCR technique works primarily on the detection by amplification of specific nucleotide sequences with the help of different sets of oligonucleotide primers and restriction endonucleases (RE) generally up to the size of genes. This amplified DNA/nucleic acid end product is detected by gel electrophoresis. PCR amplification of stx gene sequences present in fecal samples has been accepted as the most sensitive and specific means for STEC screening (Paton and Paton, 1998).

Several PCR-based methods are available for the detection of *Escherichia coli* O157:H7 and non O157 STEC by amplifying various target genes Chen and Real time (2012) .Target genes such as (stx, eae and fim) had been used by many researchers for detection of *Escherichia coli* as (Gannon, *et al.,* 1992; Deisingh, 2004; Barletta, *et al.,* 2009; Scallan, *et al.,* 2011 and Baliere, *et al.,* 2015).

In the current study, 17 isolate strains of *Escherichia coli* were tested by PCR (7 strain O157H7 and 5 strain non O157H7 in addition to 5 untypable strainswe found8 (47.1%) of eae, 5 (29.4%) of stx1 and 6 (35.3%) for hly gene, while stx2 could not be detected in all isolates tested. These data are in agreement with the results of other researchers which revealed the higher production of stx1 than stx2 Sidjabat-(Tambunan, 1997). Also Njage *et al.,* (2012)who reported 77.7% of the STEC were stx1 positive, 18.5 %, eae positive 3.1% and stx2 positive and 0.8 %.Also other study for PCR analysis of 49 *Escherichia coli* O157:H7 and 209 non-O157 isolates recorded by (Oporto, *et al.,* 2008) who showed a different distribution of virulence genes, all *Escherichia coli* O157:H7 were *stx 2* gene positive, *eaeA* was detected in 95.9% and the toxigenic profile *stx 2* was present in 75.5% of the isolates, among the non O15 7 STEC, prevalence of *eaeA* was significantly lower (5.3%) and *EhlyA* was present in 50.2% of the isolates but only sporadically associated with *eaeA*.

Also other study by Moura *et al.,* (2012) showed the frequency of the eae gene was higher in isolates from diarrheic calves 35/58 (60.3%) than in non-diarrheic calves 8/43(18.6%).The gene for Stx1 occurred at high frequencies in the diarrheic strains 24/58 (41.3%) as well as in non-diarrheic 19/43 (44.2%) and all strains that were Stx positive by PCR showed cytotoxicity in Vero cells.

All STEC strains are able to produce Shiga toxins (*Stx*) (their main virulence factor). A single STEC strain may carry one or more Shiga toxin-encoding genes (*stx*) in their genome. Indeed, strains carrying three or more stx subtypes have been described by (Bertin, *et al.,* 2001; Eklund, *et al.,* 2002 and Kruger*et, et al.,* 2011). STEC may be important in calves since *Stx* toxin can be involved in economic losses and important to the human health, since these animals could be carriers to humans. STEC is important for the herd since *Stx* can be responsible for economic losses and a threat to human health as animals can be carriers to humans. Cattle are considered to be the major reservoir of STEC worldwide (Aidar, *et al.,* 2007).

CONCLUSSION

It is clear that STEC are highly pathogenic where virulence is not dependent on a single gene or gene product but is a multifactorial process. The high rate of STEC isolation and the diversity of STEC serogroupsindicated that calves are important reservoirs of STEC. So that strict

biosecurity measures must be applied in dairy farms and dairy products.

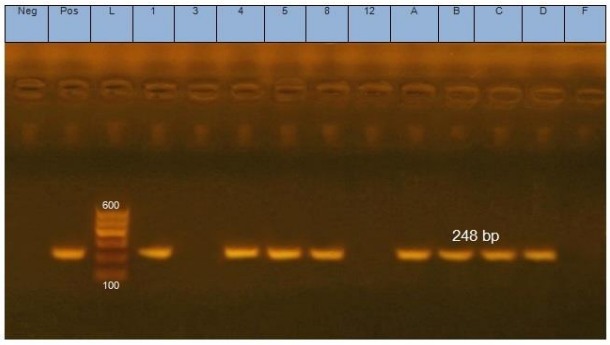

Fig. (1): Showed Electrophoretic pattern of PCR products at 248 bp for eaeA gene in 1.5%agarose gel stained with ethidium bromide: Lane (Neg): represent negative control (no amplification band was seen). Lane (L): represent the bp ladder. Lane (Pos): positive control. Lane (1): *E. coli*O26. Lane (3): *E. coli* Untypable. Lane (4): *E. coli* O26. Lane (5):Untypable *E. Coli.* Lane (8): *E. coli* O 157H7. Lane (12): *E. coli* O78. Lane (A):*E. coli* Untypable. Lane (B): *E. coli* Untypable. Lane (C): *E. coli* Untypable. Lane (D):E. coli O55. Lane (F):E. coli O126.

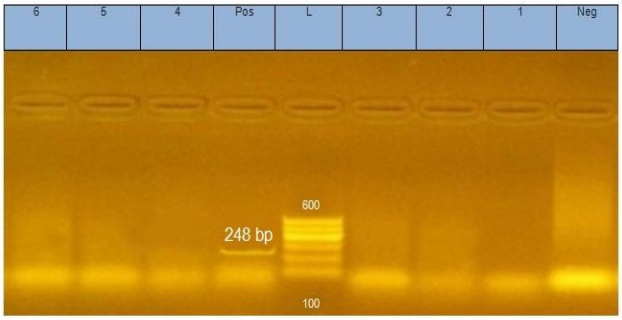

Fig. (2): Showed Electrophoretic pattern of PCR products at 248 bp for eaeA gene in 1.5%agarose gel stained with ethidium bromide: Lane (Neg): represent negative control (no amplification band was seen). Lane (L): represent the bp ladder. Lane (Pos): positive control. Lane (1): E. coli O 157H7. Lane (2): E. coli O 157H7. Lane (3): E. coli O 157H7. Lane (4): E. coli O 157H7. Lane (5): E. coli O 157H7. Lane (6): E. coli O 157H

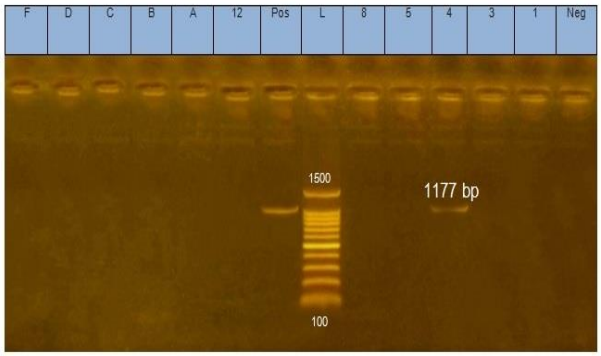

Fig. (3):Showed Electrophoretic pattern of PCR products at 1177 bp for *hly* gene in 1.5%agarose gel stained with ethidium bromide: Lane (Neg): represent negative control (no amplification band was seen). Lane (L): represent the bp ladder. Lane (Pos): positive control. Lane (1): E. coliO26. Lane (3): E. coli Untypable. Lane (4): E. coli O26. Lane (5):Untypable E. coli. Lane (F): E. coli O126. Lane (8): E. coli O 157H7. Lane (12): E. coli O78. Lane (D):E. coli O55. Lane (A):E. coli Untypable. Lane (B): E. coli Untypable. Lane (C): E. coliUntypable..

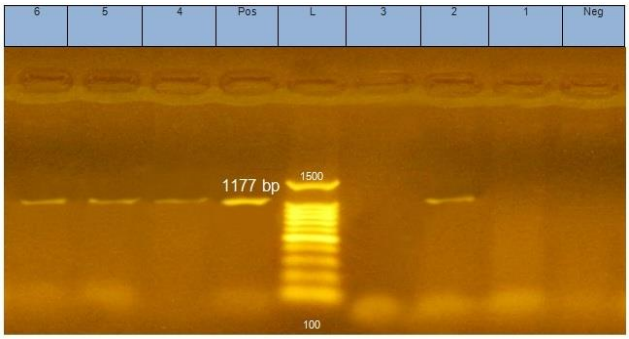

Fig. (4): Showed Electrophoretic pattern of PCR products at 1177 bp for hly gene in 1.5%agarose gel stained with ethidium bromide: Lane (Neg): represent negative control (no amplification band was seen). Lane (L): represent the bp ladder. Lane (Pos): positive control. Lane (1): E. coli O 157H7. Lane (2): E. coli O 157H7. Lane (3): E. coli O 157H7. Lane (4): E. coli O 157H7. Lane (5): E. coli O 157H7. Lane (6): E. coli O 157H.

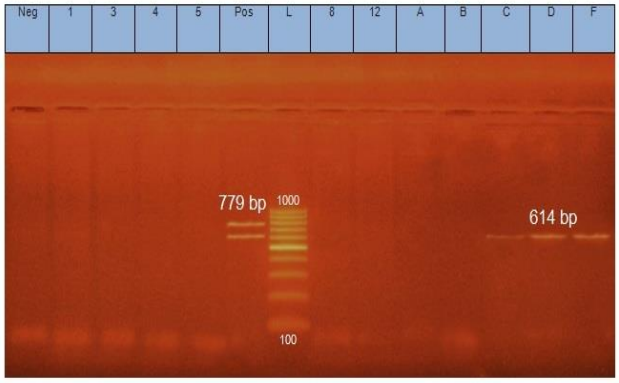

Fig. (5):Showed Electrophoretic pattern of PCR products at (614 and779) bp for stx1 and stx2 genes in 1.5%agarose gel stained with ethidium bromide: Lane (Neg): represent negative control (no amplification band was seen). Lane (L): represent the bp ladder. Lane (Pos): positive control. Lane (1): E. coliO26. Lane (3): E. coli untypable. Lane (4): E. coli O26. Lane (5):Untypable E. coli. Lane (8): E. coli O 157H7. Lane (12): E. coli O78. Lane (A):E. coli Untypable. Lane (B): E. coli Untypable. Lane (C): E. coli Untypable. Lane (D):E. coli O55. Lane (F):E. coli O126.

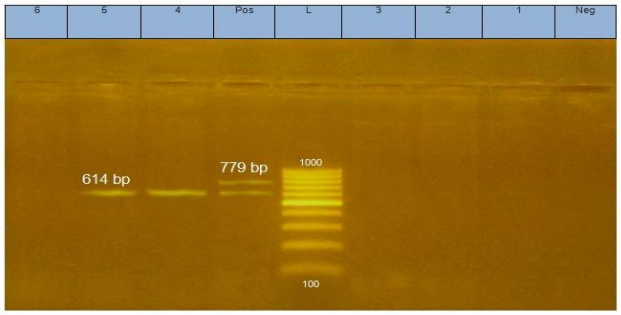

Figure (6): Showed Electrophoretic pattern of PCR products at (614 and 779)bp for stx1 and stx2 genes in 1.5%agarose gel stained with ethidium bromide: Lane (Neg): represent negative control (no amplification band was seen). Lane (L): represent the bp ladder. Lane (Pos): positive control. Lane (1): E. coli O 157H7 Lane (2): E. coli O 157H7 Lane (3): E. coli O157H7
Lane (4): E. coli O 157H7 Lane (5): E. coli O 157H7 Lane (6): E. coli O 157H7

Table (1): Showed Polyvalent and Monovalent Antisera in Mast Diagnostic kit used:

Polyvalent serum	**Monovalent serum**						
Polyvalent 1	O1	O26	O86a	O111	O119	O127a	O128
Polyvalent 2	O44	O55	O125	O126	O146	O166	
Polyvalent 3	O18	O144	O142	O151	O157	O158	
Polyvalent 4	O6	O27	O78	O148	O159	O168	
Polyvalent 5	O20	O25	O63	O153	O167		
Polyvalent 6	O8	O15	O115	O169			
Polyvalent 7	O28ac	O112ac	O124	O136	O144		
Polyvalent 8	O29	O143	O152	O164			

Table (2): Showed oligonucleotide primers encoding for 16SrRNA and *clfA* genes:

Target gene	**Primer sequence (5'-3')**	**Length of amplified product**	**Reference**
Stx1	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614 bp	(Dipineto *etal*., 2006)
Stx2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779 bp	
Hly	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1177 bp	(Piva*et, al*., 2003)
eaeA	GG TTA GGT GCT AGT CTT ATG TC CTT TCG ATT ATC TTC GCC	248 bp	(Bisi-Johnson *et al*., 2011)

Table 3: Showed preparation of uniplex PCR Master mix for *eaeA* and *hly*genes (Piva*et al*., 2003 and Bisi-Johnson *et al.*, 2011):

Component	**Volume/reaction**
2X DreamTaq Green buffer	12.5μl
PCR grade water	4.5 μl
Forward primer(20 pmol)	1 μl
Reverse primer (20 pmol)	1 μl
Template DNA	*6* μl
Total	25 μl

Table 4: Showed preparation of uniplex PCR Master mix for *stx1*and *stx2* genes (Dipineto *etal*., 2006).

Component	Volume/reaction
2X DreamTaq Green buffer	25μl
PCR grade water	13μl
Forward primer(20 pmol)	1 μleach
Reverse primer (20 pmol)	1 μleach
Template DNA	8μl
Total	50 μl

Table (5): Showed cycling conditions during PCR (Piva *et al*., 2003, Dipineto *etal*., 2006 and Bisi-Johnson *et al.*, 2011):

Target gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
*Stx1*and *stx2*	94˚C 5 min.	94˚C 30 sec	58˚C 45 sec	72˚C 45 sec.	35	72˚C 10 min.
Hly	94˚C 5 min.	94˚C 30 sec.	60˚C 50 sec.	72˚C 1 min	35	72˚C 10 min
eaeA	94˚C 5 min.	94˚C 30 sec.	51˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.

Table (6): Showed incidence and frequency distribution of *Escherichia coli* strain isolated from both diarrheic and apparently health calves:

Type of samples	No. of samples	*E. coli strains*			
		Positive samples		Negative samples	
		No	%	No	%
Diarrheic calves	100	40	40	60	60
Apparently health	200	128	64	72	36
Total	300	168	56	132	44

Table (7): showed serology results of *Escherichia coli* strains isolated from diarrheic and apparently health calves:

Strain	No. isolates	Serotyping of strains		
		O157H7 No.	Non O157H7 No.	Un typable No.
Escherichia coli	168	7 (4.1%)	156 (92.8%)	5 (3.1%)

REFERENCES

Baliere C, Rince A, Thevenot D, Gourmelon M. (2015) :Successful detection of pathogenic Shiga toxin producing Escherichia coli in shellfish, environmental waters and sediment using the ISO/TS-13136 method. LettAppl Microbiol.;60(4):315–20

Banatvala, N., Griffin, P.M., Greene, K.D., et al. (2001): The United State national prospective hemolytic uremic syndrome study: micro-biologic, serologic, clinical and epidemiological finding. J. Infect. Dis.,183, 1063-1070.

Baranzoni, G.M., Fratamico, P.M., Gangiredla, J., Patel, I., Bagi, L.K., Delannoy, S., Fach, P., Boccia, F., Anastasio, A. and Pepe, T. (2016): Characterization of Shiga toxin sub types and virulence genes in porcine Shiga

toxin producing *Escherichia coli*. Front. Microbiol., 7: 574.

Barletta F, Ochoa TJ, Ecker L, Gil AI, Lanata CF, Cleary TG.(2009):Validation of five colony pool analysis using multiplex real-time PCR for detection of diarrheagenic Escherichia coli . J Clin Microbiol.;47(6):1915 –7.

Bertin, Y., Boukhors, K., Pradel, N., Livrelli, V. and Martin, C. (2001):Stx2 subtyping of Shiga toxin producing *Escherichia coli* isolated from cattle in France: detection of a new Stx2 subtype and correlation with additional virulence factors.
J ClinMicrobiol 39, 3060–3065.

Bisi-Johnson, M.A.; Obi, C.L.; Vasaikar, S.D.; Baba, K.A. and Hattori, T. (2011): Molecular basis of virulence in clinical isolates of Escherichia coli and Salmonella species from a tertiary hospital in the Eastern Cape, South Africa. Gut Pathogens 2011, 3:9.

C. de Moura, M. Ludovico, G.F. Valadares, M.S.V. Gatti, D.S. Leite(2012) : Detection of virulence genes in Escherichia coli strains isolated from diarrheic and healthy feces of dairy calves in Brazil.Arq. Inst. Biol., São Paulo, v.79, n.2, p.273-276, abr./jun.

Cheesbrough, M. (1985): Medical laboratory manual for tropical countries. Vol. II.Microbiology. , 400-480.

Deisingh AK, Thompson M. (2004):Strategies for the detection of Escherichia coli O157:H7 in foods. J Appl Microbiol.;96(3):419 –29.

Desmarchelier PM, Bilge SS, Fegan N, Mills L, Vary JC, Jr., TarrPI(1998): A PCR specific for Escherichia coli O157 based on the rfb locus encoding O157 lipopolysaccharide. J ClinMicrobiol, 36(6):1801-1804.

Dipineto, L.; Santaniello, A.; Fontanella, M.; Lagos, K.; Fioretti, A. and Menna, L.F. (2006). Presence of Shiga toxin-producing Escherichia coli O157:H7 in living layer hens. Letters in Applied Microbiology 43 293–295.

EFSA(2009): Technical specifications for the monitoring and reporting of verotoxigenic Escherichia coli (VTEC) on animals and food (VTEC surveys on animals and food), EFSA (European Food Safety Authority); Contract no.: 1366.

Eklund, M., Leino, K. &Siitonen, A. (2002):Clinical Escherichia coli strains carrying stxgenes: stx variants and stx -positive virulenceprofiles. J ClinMicrobiol 40, 4585–4593.

Fakiha ,1D. Thirya , 1,J.-N. Duprez a , M. Saulmont b ,A. Iguchi c (2017): Identification of Shiga toxin producing (STEC) and enteropathogenic (EPEC)*Escherichia coli* in diarrheic calves and comparative genomics Of O5 bovine and human STEC Veterinary Microbiology 202 , 16 –22.

FIDOCK, D.A.; M c NICHOLAS, P.A.; LEHRBACH, P.R. (1989) :Nucleotide sequence of the F41 fimbriae subunit gene in Escherichia coli B41. Nucleic Acids Research, v.17, p.2849-2849.

Fratamico, P. M., and DebRoy, C.(2010): Detection of *Escherichia coli* O157:H7 in food using real-time multiplex PCR assays targeting the stx1 , stx2 , wzy O157 , and the fliCh7 or eae genes. Food Anal. Methods 3, 330–337.

Fratamico, P. M., Bagi, L. K., Cray, W. C. Jr., Narang, N., Yan, X., Medina, M., *et al* (2011):Detection by multiplex real time polymerase chain reaction assays and isolation of Shiga toxin-producing Escherichia coli serogroups O26, O45, O103,O111, O121, and O145 in ground beef. Food borne Pathogen. Dis. 8, 601–607.

Gannon VP, King RK, Kim JY, Thomas EJ. (1992):Rapid and sensitive method for detection of Shiga like toxin-producing *Escherichia coli* in ground beef using the

polymerase chain reaction. Appl Environ Microbiol.;58(12):3809 –15.

Gomes, T.A.T., Elias, W.P., Scaletsky, I.C.A., Guth, B.E.C., Rodrigues, J.F., Piazza, R.M.F., Ferreira, L.C. and Martinez, M.B. (2016): Diarrheagenic*Escherichia coli*. Braz. J. Microbiol., 47: 3-30.

Gould (2013): reported that Recently, it has become evident that non-O157 Shiga toxin producing *Escherichia coli*(STEC), particularly STEC serogroups O26, O45, O103, O111, O121, and O145 (referred to as the top six non-O157 STEC) cause illnesses similar to those caused by *Escherichia coli*O157:H7.

Gould, L.H., Mody, R.K., Ong, K.L., Clogher, P., Cronquist, A.B., Garman, K.N., Lathrop, S., Medus, C., Spina, N.L., Webb, T.H., White, P.L., Wymore, K., Gierke, R.E., Mahon, B.E. and Griffin, P.M. (2013): Increased recognition of non-O157 Shiga toxin producing *Escherichia coli* infections in the United States during 2000-2010: epidemiologic features and comparison with *E. coli* O157 infections. Foodborne Pathog. Dis. 10: 453-460:11827–1832.

Jang, J., Hur, H.G., Sadowsky, M.J., Byappanahalli, M.N., Yan, T. and Ishii, S. (2017): Environmental Escherichia coli Ecology and public health implications-a review. J. Appl. Microbiol., 123: 570-581.

Krü ger, A., Lucchesi, P. M. A. and Parma, A. E. (2011):Verotoxins in bovine and meat verotoxin producing Escherichia coli isolates: number of variants, and relationship to cytotoxicity.Appl Environ Microbiol 77 , 73–79.

Krüger, A. and Lucchesi, P.M. (2015):Shiga toxins and stx phages: Highly diverse entities. Microbiology,161: 451-462.

Li B, Chen JQ. Real time PCR methodology for selective detection of viable Escherichia coli O157:H7 cells by targeting Z3276 as a genetic marker. Appl Environ Microbiol.;78(15):5297 –304.

Li B, Koch WH, Cebula TA. (1997): Detection and characterization of the fimA gene of *Escherichia coli* 157:H7. Mol Cell Probes.;11(6):397 –406.

Mohammad Mehdi Aslani* and SaeidBouzariHurtado, (2009): Characterization of Virulence Genes of Non O157 Shiga Toxin Producing *Escherichia coli* Isolates from Two Provinces of Iranpn. J. Infect. Dis., 62, 16-19.

OJENIYI, B.; AHRENS, P.; MEYLING, A. (1994): Detection offimbrial and toxin genes in Escherichia coli and their prevalence in piglets with diarrhea. The application of colonyhibridization assay polymerase chain reaction and phenotype assays. Journal of Veterinary Medicine B , v.41, p.49-59.

Paton, A.W., Ratcliff, M.R., Doyle, R.M., et al. (1998): Molecular microbiological investigation of an outbreak of hemolytic uremic syndrome caused by dry fermented sausage contaminated with Shiga like toxin producing *Escherichia coli* . J. Clin. Microbiol., 34, 1622-1627.

Persad AK, LeJeuneJT(2014) :Animal reservoirs of Shiga toxin-producing *Escherichia coli*. MicrobiolSpectr 2(4):EHEC-0027.

Piva, I.C.; Pereira, A.L.; Ferraz, L.R.; Silva, R.S.N.; Vieira, A.C.; Blanco, J.E.; Blanco, M.; Blanco,J. and Giugliano, L.G. (2003): Virulence Markers of Enteroaggregative*Escherichia coli* Isolated from Children and Adults with Diarrhea in Brasília, Brazil. JOURNAL OF CLINICAL MICROBIOLOGY, May 2003, p. 1827–1832.

Restaino L., Catillo H.J., Stewart D., and Tortorello M.L. 1996):Antibody direct Epifluorescent filter technique and Immun-magnetic separation for 10-h screening and

24-h confirmation of Escherichia coli O157:H7 in beef. J. Food Prot., 59(10): 1072-1075.

Riley, L.W. (2014): Pandemic lineages of extra intesti-nal pathogenic Escherichia coli. Clin.Microbiol.Infect. 20: 380-90.

ROOSSENDAAL, B.; GAASTRA, W.; GRAAF, F.K. (1984):The nucleotide sequence of the genes encoding the K99 subunit of enterotoxigenic *Escherichiacoli* FEMS MicrobiologyLetters, v.22, and p.253-258.

Sambrook, J.; Fritscgh, E.F.; and Mentiates(1989): Molecular cloning. A laboratory manual.

Scallan E, Griffin PM, Angulo FJ, Tauxe RV, Hoekstra RM. (2011): Foodborne illness acquired in the United States unspecified agents. Emerg Infect Dis.;17(1):16–22.

Schultsz, c.; Pool, g.j.; Van ketel, r.; De wever,b:, (1994): Detection of enterotoxigenic Escherichia coli in stool samples by using nonradioactively labeled oligonucleotide DNA probes and PCR. Journal of Clinical Microbiology , v.32, p.2393-2397.

SidjabatTambunan H., and Bensink J.C., (1997): Verotoxin producing *Escherichia coli* from the faeces of sheep, calves and pigs. Aust. Vet. J. 75: 292-293.

SO, M.; Mc CARTY, B.J. (1980): Nucleotide sequence of the bacterial transposon Tn1681 encoding a heat-stable (ST) toxin and its identification in enterotoxigenic*Escherichiacoli*strains. Proceedings of the National Academy of Science USA, v.77, p.4011-4015.

Speelman, P.andDankert, J. (1994): Detection of enterotoxigenic Escherichia coli in stool samples by using nonradioactively labeled oligonucleotide DNA probes and PCR. Journal of Clinical Microbiology , v.32, p.2393-2397.

Steiner, T.S. (2016) :New insights into Shiga toxigenic *Escherichia coli* pathogenesis: When less is more. J . Infect. Dis., 213: 1214-1215.

YU, J.; KAPER, J.B. (1992) :Cloning and characterization of the Eae gene of enterohaemorragic*Escherichia coli* O157:H7. Molecular Microbiology , v.6, p.411-417.