

## Evaluation of *ehxA*, *stx1*, and *stx2* Virulence Gene Prevalence in Cattle *Escherichia Coli* Isolates by Multiplex PCR

### Short Communication

Mazaheri Nejad Fard\*<sup>1</sup>, R., Behzadian Nezhad, G.,<sup>2</sup> Zahraei Salehi, T.,<sup>3</sup> Atash Parvar, N.<sup>4</sup>

1. Clinical Science Dept., Faculty of Veterinary Medicine, University of Tehran, Tehran-Iran, P. O. Box 14115-6453

2. Bacteriology Dept., Faculty of Medical Science, Tarbiat Modares University, Tehran-Iran, P. O. Box 14115-111

3. Microbiology & Immunology Dept., Faculty of Veterinary Medicine, University of Tehran, Iran,

4. School of Veterinary Medicine, University of Lorestan, Khoramabad - Iran

Received 25 Aug 2005; accepted 20 Nov 2005

### **Summary**

Today, it is nearly 25 years past from investigation of Shiga toxigenic *Escherichia coli* (STEC) which is able to produce Shiga toxins and cause different gastroenteritis. Since incidence of gastroenteritis due to STEC is increasing, it's necessary to develop rapid, specific and accurate procedures like PCR. In this study, we used PCR method to detect and identify STEC in cultures of 55 *Escherichia coli* positive feces from cattle. The assay utilized three specific primer pairs to detect *ehxA*, *stx1*, and *stx2* (including variants of *Stx2*) genes producing 321, 348, and 584 base pairs, respectively. Finally, about 21.8% of *Escherichia coli* isolates were positive for *ehxA*, *stx1*, and/or *stx2* genes. According to other research, the prevalence of *stx2* genes was higher than *stx1* genes but not about *ehxA* genes.

**Key words:** *Escherichia coli*, EHEC, *stx*, *ehxA*, Multiplex PCR

---

\*Author for correspondence. E-mail: [raminmazaheri@gmail.com](mailto:raminmazaheri@gmail.com)

### **Introduction**

Today, it is nearly 25 years past from investigation of Shiga toxigenic *Escherichia coli* (STEC) by Konowalchuck *et al* (1977). STEC is a member of enterohemorrhagic *Escherichia coli* (EHEC) which is able to produce Shiga-like toxins (Verotoxin). In general, *Escherichia coli* includes five major groups ETEC, EPEC, EHEC, EIEC, and EAaggEC which EHEC is very important because of its ability to cause serious gastroenteritis like dysentery, hemorrhagic colitis (HC) and sever details like hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP).

Virulence factors include hemolysins and Shiga toxins (Stx) (Brooks *et al* 1998, Murray *et al* 1999). Shiga toxin first time extracted by O'Brien *et al* (1982) is similar to toxin producing by *Shigella dysantheriae* type 1 (agent of bacillary diarrhea). Members of family include Stx1 and Stx2 toxins encoded by *stx1* and *stx2* genes, respectively. Shiga toxins are the major virulence factors of EHEC which have cytotoxicity effects for human and animal eukaryotic cells and cause dangerous details like HUS and TTP (Saif *et al* 2003). Another virulence factor is enterohemolysin Hly encoded by HlyABCD gene cluster. Discovered by Beutin *et al* (1989), this hemolysin is produced widely by STEC strains named EHEC-Hly. It participates in inflammatory process of epithelial cells or amplifies effects of LPS and STX.

Epidemiology of bacterial prevalence, however, shows cattle are source of STEC strains but other animals like birds, cats, dogs, goats, pigs and sheep may infect (Beutin *et al* 1993, Caprioli *et al* 1993, Karmali *et al* 1989, Kudva *et al* 1996). Infection occurs through food contamination particularly meat products (CDC 1995, Morgan *et al* 1993, Paton *et al* 1996). In addition, transmission from person to person throughout epidemics is documented (Reida *et al* 1994), and secondary transmission which may contains hand to hand contact or indirectly via swimming contaminated water (Ackman *et al* 1997, Keene *et al* 1994).

---

Since incidence of gastroenteritis due to STEC is increasing and for its important hygienic, social, and cultural aspects, it is necessary to develop rapid, specific, and accurate procedures to detect the organism. By means of molecular methods like PCR, we would be able to diagnose early in the course of disease and with low amount of sample or low levels of contamination. Nowadays, using PCR technology gives us ability of rapid and accurate detection of STX encoding genes in different samples containing small amount of STEC. Benefits of PCR include high sensitivity, rapid diagnosing, and possibility of in situ detection and identification. In addition to, understanding of prevalence of Shiga toxin-producing bacteria increases our ability to prevent its hazards and sequels (Paton & Paton 1996). In this study, besides to routine culture of EHEC, we used multiplex PCR assay using three primer pairs to detect Shiga toxin virulence genes, *stx1* and *stx2* also enterohemolysin Hly (*ehxA*) in cattle diarrheal samples.

#### **Materials and Methods**

**Field sample.** In this study, we used 55 *Escherichia coli* isolates that had been identified from 62 cattle feces sampled in Animal Hospital of Faculty of Veterinary Medicine, University of Tehran, Iran. In order to increase probability of bacterial isolation, we decided sampling time to be near to initial time of disease symptoms. To prevent decreasing in bacterial isolation rates, we tried patients had have any antibiotic at least 48 hours before sampling. Swabs of cattle diarrhea with visible amounts of stool were placed in sterile tubes and transferred near ice to the laboratory. During short time, maintenance temperature was -20°C and for long period was -70°C. We tried to prevent freeze and thaw repeatedly (CDC 1994).

**SMTs for *Escherichia coli* detection and identification.** After received by the laboratory, stools were cultured on McConkey, and CHROMagar then incubated over night at 37°C. Lactose positive colonies were transferred to EMB and incubated over night at 37°C. Also TSI, Urea, and MR-VP were used for bacterial

identification. In this study, however, in addition to routine culture for bacterial biochemical identification, we used CHROM Agar, a new assay with simple usage, for identifying *Escherichia coli*.

**Multiplex PCR.** Newly 18-24 hour-old LB cultures of *Escherichia coli* isolates were used for PCR.

**Primers and oligonucleotide sequences.** Three sets of primer pairs were used: RH35 & RH37, specific for *Escherichia coli ehxA* gene (Pradel *et al* 2001), LP30 & LP31, specific for *Escherichia coli stx1* gene (Osek 2003) and LP43 & LP44, specific for *Escherichia coli stx2* gene (Osek 2003). Primer sequences are shown in Table1.

**DNA extraction.** DNA extraction carried out according to modified Boerlin *et al* (1998). Briefly, several colonies from newly 18-24 hour-old LB culture were resolved in 200µl sterile distilled water and vortexed. After boiled in water bath for 10 minutes, the solution was spinned off 5 minutes in 4000rpm. Then supernatant including DNA was separated.

Table1: Primers were used for multiplex PCR

Target	Primer	Sequences	Weight
ehxA	RH35 RH37	CACACGGAGCTTATAATATTCTGTCA AATGTTATCCCATTGACATCATTTGACT	321 bp
stx1	LP30 LP31	CAGTTAATGTCGTGGCGAAGG CACCAGACAATGTAACCGCTG	348 bp
stx2	LP43 LP44	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC	584 bp

**DNA amplification.** PCR was performed with 11.5µl of sterile distilled water, 2.5µl of 10x PCR buffer, 1µl of 50mM MgCl<sub>2</sub>, 1.5µl of 10mM dNTP, 1µl of each primers with 10µM density, 0.5µl of 5Unit/µl

DNA polymerase (Taq), and 2µl of extracted DNA as template in a final volume of 25µl. Condition of cycles in thermocycler (Techne TC-512, England) was as follows: Initial denaturation started 5 minutes at 95°C. It was continued by 30 cycles, each cycles including denaturation 30 seconds at 58°C, annealing 30 seconds at 95°C, and elongation 1 minute at 72°C. Final extension was done 7 minutes at 72°C (Osek 2003, Pradel *et al* 2001). Visualization of amplified products was done by UV illumination after electrophoresis on 1.2% agarose gel and ethidium bromide staining (Figure1). We used an ATCC O175:H7 strain (a kindly gift from Dr. Khashabi, Austria) as positive control and a sample without any DNA as negative control for each PCR amplification set.

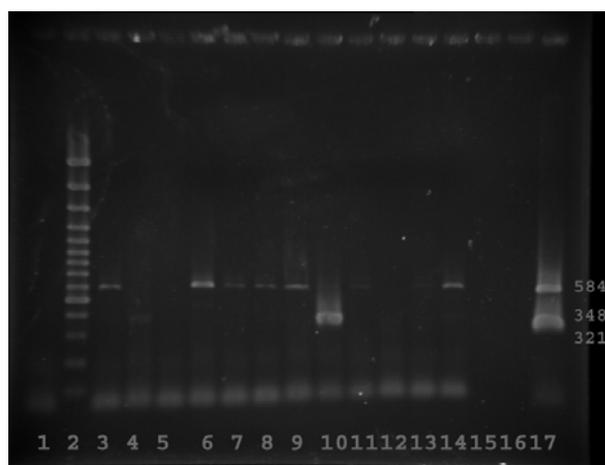


Figure1. Electrophoresis of PCR products on 1.2% agarose gel. Lane1: Negative Control, Lane 2: 100-bp Marker, Lane3; 6; 7; 8; 9 and14: *stx2*, Line10: *ehxA*<sup>+</sup> and *stx1*<sup>+</sup> and Lane17: Positive Control.

### Results and Discussion

From 62 diarrheal specimens, 55 samples (88.7%) were positive for *Escherichia coli*. During multiplex PCR on colonies from 55 *Escherichia coli* isolate cultures, 12 samples showed positive for highlighted genes. Eleven samples (20%) included

one gene (one sample included *stx1* and 10 samples included *stx2*) and one sample (1.8%) included *stx1* and *ehxA* genes, but no sample contained all three genes. In statistical study, data indicated that each *ehxA*, *stx1*, and/or *stx2* genes could be present independently. Also, there is no significance between prevalence of related genes and bloody stools ( $P>0.05$ ).

Interestingly, there is a significance between prevalence of each *ehxA* and *stx1* genes and varieties in age of cattle, as positive cases were identified in milk sucking calves ( $P>0.001$ ); whereas there is no significance between prevalence of *stx2* genes and age of cattle. Finally, however, statistical studies definite there is no significance between prevalence of *stx2* genes and gender of cattle ( $P>0.05$ ).

Stx-producing *Escherichia coli* are found in animal feces flora like cattle, sheep, pigs, cats, dogs and seagulls. It seems, however, cattle are the most important species which infect human, act as STEC source and transmit contamination through dairy and meat products or direct contact to human. High levels of Stx positive *Escherichia coli* colonization are found in breeding farms in many countries. It may contains up to 60% of flock, but value often includes 10-25% for Stx positive *Escherichia coli* strains usually isolates from healthy cattle but may be in association with initial diarrhea in young animals and to be continued with asymptomatic colonization (Nataro & Kaper, 1998). In this study, however, numbers of *stx* positive *Escherichia coli* cases were 12 from 55 *Escherichia coli* isolates (21.8%) in accord with the statistic.

In some recent studies, multiplex PCR has been developed to detect Shiga toxin genes (*stx1* and variants of *stx2*) and accessory virulence genes (*ehxA* and *eaeA*), and collaboration of these two groups of genes has been studied. Both *Stx* toxin genes and *ehxA* enterohemolysin encoded by a lambdoid bacteriophage to be inserted in bacterial chromosome. May be STEC includes subtypes of *stx1* or *stx2* or both genes together within or without *ehxA*. To study virulence genes prevalence help us to understand better completion of STEC virulence-related factors and result in to

confirm some theories, e.g. there is a relationship between producing Stx and accessory virulence factors in pathogenesis. Boerlin (1998) showed that *ehxA* gene conserved in STEC serotypes is not only important for detect STEC isolates, but also counts as a tool for determining STEC isolates with different levels of clinical relationship. Osek (2003) from national institute for veterinary research of Poland by doing multiplex PCR for 202 cattle and children *Escherichia coli* isolates found that 25 STEC strains (12.4%) were *stx* positive and 20 STEC strains included *eaeA* and *ehlyA* virulence genes. From 55 cattle *Escherichia coli* strains isolated by us, however, only one strain included *ehlyA* gene also 11 cases (about 20%) included *stx* genes. This point differs from above mentioned results due to difference in geographical prevalence of phages caring accessory virulence genes, environmental condition, or physiology of the host.

About prevalence of Shiga toxin encoding genes in animals, results show preference of *stx2* genes. Studying about Shiga toxin-producing *Escherichia coli* strains, Wieler *et al* (1996) evaluated 176 diarrheal calves, 107 strains (61%) included *stx1* genes alone, 13 strains (7%) included *stx2* genes alone, and two strains (1%) included both genes. In a study about Shiga toxin genes (*stx1* and *stx2*) of EHEC in 110 animal feces by Fagan *et al* (1999), he detected *stx1* genes alone in 7 samples (6.4%), *stx2* genes alone in 11 samples (10%), and both *stx1* and *stx2* in 9 samples (8.2%). In the study of prevalence of enterohemorrhagic *Escherichia coli* O157 in cattle feces during slaughter by Elder *et al.* (2000), it proofed that from 342 isolates, 1.4% included *stx1* genes alone, 41.2% included *stx2* genes only, and 58.4% included both *stx1* and *stx2* genes. The results are similar to others. We found two strains included *stx1* gene (3.6%) and 10 strains included *stx2* gene (18%), however, one strain (1.8%) included *stx1* and *ehxA* genes. The interesting fact is that *stx2* gene prevalence is more than *stx1*.

Noticeably, in most studies, the prevalence of *stx2* genes is greater than *stx1* genes, as Beutin *et al* (1997) reported that most of the cattle isolates were *stx2* positive,

whereas one sample was *stx1* positive. It seems that the greater prevalence of *stx2* to *stx1* may differ in different conditions. The difference in results shows that excretion of Shiga toxin-producing *Escherichia coli* is affected by several factors like food, age, stress, and seasonal changes. Although PCR is able to detect EHEC virulence factors accurately, but finding genes isn't equal to express them.

In addition to, some types of assay may have problem due to instability of phages carrying *stx* genes. Karch *et al* (1992) tested *stx* genes stability in 45 *stx* positive strains after isolation immediately. After passage on trypticase soy agar or other routine culture media, 15 of 45 strains lost one of *stx1* or *stx2* genes, and that reduce was variable related to strain. Loss of *stx* genes in serial cultures is seen after long term shedding of the organism. This phenomenon was seen in this study, in spite of prevent several cultures before doing PCR. This point leads to create doubt about bacteria.

#### **Acknowledgment**

This research was funded by grants from Tarbiat Modares and Tehran University.

#### **References**

- Ackman, D., Marks, S., Mack, P., Caldwell, M., Root, T. and Birkhead, G. (1997). Swimming associated haemorrhagic colitis due to *Escherichia coli* O157:H7 infection: evidence of prolonged contamination of a fresh water lake. *Epidemiology of Infection* 119: 1-8.
- Beutin, L., Geiger, D., Steinruck, H., Zimmermann, S. and Scheutz, F. (1993). Prevalence and some properties of verotoxin (Shiga-like toxin) producing *Escherichia coli* in seven different species of healthy domestic animals. *Journal of Clinical Microbiology* 31: 2483-2488.
- Beutin, L., Geiger, D., Zimmermann, S., Aleksic, S., Gillespie, H.A. and Whittam, T.S. (1997). Epidemiological relatedness and clonal types of natural

- populations of *Escherichia coli* strains producing Shiga toxins in separate populations of cattle and sheep. *Applied of Environmental Microbiology* 63: 2175-2180.
- Beutin, L., Montenegro, M.A., Orskov, I., Orskov, F., Prada, J., Zimmermann, S. and Stephan, R. (1989). Close association of verotoxin (Shiga-like toxin) production with enterohemolysin production in strains of *Escherichia coli*. *Journal of Clinical Microbiology* 27: 2559-2564.
- Boerlin, P., Chen, S., Colbourne, J.K., Johnson, R., De Grandis, S. and Gyles, C. (1998). Evolution of enterohemorrhagic *Escherichia coli* hemolysin plasmids and the locus for enterocyte effacement in Shiga toxin-producing *E.coli*. *Infection and Immunity* 66: 2553-2561.
- Brooks, G.F., Butel, J.S. and Morse, S.A. (1998). *Jawetz, Melnick & Adelberg's Medical Microbiology*. Pp: 218-230. Appleton & Lange,
- Caprioli, A., Nigrelli, A., Gatti, R., Zavanella, M., Blando, A.M., Minelli, F. and Donelli, G. (1993). Characterization of verocytotoxin-producing *Escherichia coli* isolated from pigs and cattle in northern Italy. *Veterinary Record* 133: 323-324.
- Centers for Disease Control and Prevention (1994). *E. coli* O157:H7: *procedure and identification from stool specimens*. CDC Wonder.
- Centers for Disease Control and Prevention (1995). *Escherichia coli* O157:H7 outbreak linked to commercially distributed dry-cured salami Washington and California, *Morbidity Mortality Weekly Report* 44: 157-160.
- Centers for Disease Control and Prevention (1995). Outbreak of acute gastroenteritis attributable to *Escherichia coli* serotype O104:H21 Helena, Montana, *Morbidity Mortality Weekly Report* 44: 501-503.
- Elder, R.O., Keen, J.E., Siragusa, G.R., Barkocy-Gallagher, G.A., Koohmaraie, M. and Laegreid, W.W. (2000). Correlation of enterohemorrhagic

- Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Applied of Biological Sciences* 97: 2999-3003.
- Fagan, P.K., Hornitzky, M.A., Bettelheim, K.A. and Djordjevic, S.P. (1999). Detection of Shiga-like toxin (*stx1* and *stx2*), intimin (*eaeA*) and enterohemorrhagic *Escherichia coli* (EHEC) hemolysin (EHEC *hlyA*) genes in animal feces by multiplex PCR. *Applied of Environmental Microbiology* 65: 868-872.
- Karch, H., Meyer, T., Rüssmann, H. and Heesemann, J. (1992). Frequent loss of Shiga-like toxin genes in clinical isolates of *Escherichia coli* upon subcultivation. *Infection and Immunity* 60: 3464-3467.
- Karmali, M.A. (1989). Infection by verotoxin-producing *Escherichia coli*. *Clinical Microbiology Review* 2: 15-38.
- Keene, W.E., McAnulty, J.M., Hoesly, F.C., Williams Jr, L.P., Hedberg, K., Oxman, G.L., Barrett, T.J., Pfaller, M.A. and Fleming, D.W. (1994). A swimming-associated outbreak of hemorrhagic colitis caused by *Escherichia coli* O157:H7 and *Shigella sonnei*. *New England Journal of Medicine* 331: 579-584.
- Keene, W.E., Sazie, E., Kok, J., Rice, D.H., Hancock, D.D., Balan, V.K., Zhao, T. and Doyle, M.P. (1997). An outbreak of *Escherichia coli* O157:H7 infections traced to jerky made from deer meat. *JAMA* 277: 1229-1231.
- Konowalchuk, J., Speirs, J.I. and Stavric, S. (1977). Vero response to a cytotoxin of *Escherichia coli*. *Infection and Immunity* 18: 775-779.
- Kudva, I.T., Hatfield, P.G. and Hovde, C.J. (1996). *Escherichia coli* O157:H7 in microbial flora of sheep. *Journal of Clinical Microbiology* 34: 431-433.
- Morgan, D., Newman, C.P., Hutchinson, D.N., Walker, A.M., Rowe, B. and Majid, F. (1993). Verotoxin producing *Escherichia coli* O157 infections associated with the consumption of yoghurt. *Epidemiology of Infection* 111: 181-187.

- Murray, P.R., Baron, E.J, Pfaller, M.A., Tenover, F.C. and Tenover, R.H. (1999). Manual of Clinical Microbiology. *American Society for Microbiology*, Pp: 450-452.
- Nataro, J.P. and Kaper, J.B. (1998). Diarrheagenic *Escherichia coli*. *Clinical Microbiology Review* 11: 142-201.
- O'Brien, A.D., LaVeck, G.D., Thompson, M.R. and Formal, S.B. (1982). Production of *Shigella dysenteriae* type 1-like cytotoxin by *Escherichia coli*. *Journal of Infectious Diseases* 146: 763-769.
- Osek, J. (2003). Development of a multiplex PCR: approach for the identification of Shiga toxin-producing *Escherichia coli* strains and their major virulence factor genes. *Journal of Applied Microbiology* 95: 1217-1225.
- Paton, A.W., Ratcliff, R., Doyle, R.M., Seymour-Murray, J., Davos, D., Lanser, J.A. and Paton, J.C. (1996). Molecular microbiological investigation of an outbreak of hemolytic uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing *Escherichia coli*. *Journal of Clinical Microbiology* 34: 1622-1627.
- Paton, J.C. and Paton, A.W. (1996). Pathogenesis and diagnosis of shiga toxin-producing *Escherichia coli* infection. *Clinical Microbiology Review* 11: 450-479.
- Pradel, N., Boukhors, K., Bertin, Y., Forestier, C., Martin, C. and Livrelli, V. (2001). Heterogeneity of Shiga toxin-producing *Escherichia coli* strains isolated from hemolytic-uremic syndrome patients, cattle, and food samples in central France. *Applied Environmental Microbiology* 67: 2460-2468.
- Reida, P., Wolff, M., Pohls, H.W., Kuhlmann, W., Lehmacher, A., Aleksic, S., Karch, H. and Bockemuhl, J. (1994). An outbreak due to enterohaemorrhagic *Escherichia coli* O157:H7 in a children day care centre characterized by person-to-person transmission and environmental contamination. *International*

*Journal of Medical Microbiology, Virology & Parasitology Infectious Diseases*  
281: 534-543.

Saif, Y.M., Barnes, H.J., Glisson, J.R., Fadly, A.M., McDougald, L.R.,  
Swayne, D.E. (2003). *Diseases of Poultry*. Pp: 631-644. Iowa State University  
Press,

Wieler, L.H., Vieler, E., Erpenstein, C., Schlapp, T., Steinruck, H., Bauerfeind, R.,  
Byomi, A. and Baljer, G. (1996). Shiga toxin-producing *Escherichia coli* strains  
from bovines: association of adhesion with carriage of *eae* and other genes.  
*Journal of Clinical Microbiology* 34: 2980-2984.