

**Full Article**

**Prevalence and molecular characterization of verotoxin-producing *Escherichia coli* O157:H7 in cattle and sheep in Shiraz-Iran**

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**ABSTRACT**

Shiga toxin producing *Escherichia coli* have been associated with HUS, HC and TTP in human. We found recto-anal mucosal sample in sheep as well in cattle is the main site for *E. coli* O157 localization. 1246 *E. coli* isolates from 872 both healthy and diarrheic animals were analyzed, by screening for the presence of Shiga toxin-producing (VT 1 and VT 2) and intimin (eae) genes used Multiplex PCR. 87(9.75%) VTEC from 52 cattle and 28(7.90%) from 28 sheep were isolated. VT2 gene was found to be more frequent than VT1 in cattle (54.02% vs 26.43%), in contrast the same genes in sheep (21.42%vs25%). There was observed significant difference in the origin of VT positive sheep in close contact with farms of cattle origin. Having cattle and sheep with each other was a possible risk factor. The animal was kept in pen was more localized than tethered. Young cattle were documented strongly significant high prevalence rate in *E. coli* O157:H7 than older, but no effect of age was observed on the occurrence of *E. coli* O157 in sheep. Both diarrheic and healthy animals were shed *E. coli* O157:H7 in their feces. Sheep and dairy cow were not illustrated any significance differences geographical region and seasonal variation with *E. coli* O157:H7 prevalence rate.

**Keywords:** *E. coli* O157, Cattle, Sheep, Iran

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**INTRODUCTION**

The bacteria that make shiga toxins are called “Shiga toxin-producing” *E. coli* (STEC) (Blanco *et al* 2004). Of the numerous VTEC serotypes identified, O157:H7 continue to be the dominant causes of illness in humans. Hemolytic-uremic syndrome (HUS), hemorrhagic colitis (HC) and thrombotic thrombocytopenic purpura (TTP) are the most

worrisome complication of *E. coli* O157:H7 infection (Garcia-Aljaro *et al* 2004, Callaway *et al* 2009). The major source for human illnesses is cattle (Callaway *et al* 2009). As for cattle, healthy sheep are also found to be a potential reservoir of *E. coli* O157:H7 (Gro *et al* 2001). But humans may serve as reservoirs for person-to-person transmission (Picozzi *et al* 2005). Ground beef is the most frequently implicated source of *E. coli* O157:H7 outbreaks (Caprioli *et al* 2005). Approximately 75% of *E. coli* O157:H7 outbreaks are linked to bovine-derived products (Yilmaz *et al* 2006).

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Accordingly, reduction of infection in humans requires preventive measures that either reduces the number of animals that carry *E. coli* O157:H7 (McGee et al 2002). To this end, a great deal of research has focused on describing the ecology and epidemiology of *E. coli* O157:H7 in cattle and sheep, with the hope of identifying interventions to reduce its prevalence in those animals (Holland et al 2000).

Albeit, *E. coli* O157:H7 can adhere and associate intimately, on the mucosal surface the terminal rectum of lambs as well (Girard et al 2005, Girard et al 2007). Therefore, if *E. coli* O157:H7 was colonized in recto-anal junction site, recto anal mucosal swab (RAMS) sampling from this site should be superior to the use of traditional fecal specimen for *E. coli* O157:H7 detection (Rice et al 2003, Sheng et al 2004).

The first reporting of *E. coli* O157:H7 diseases in Iran are not documented. In Iran, only a few studies have reported the isolation and characteristics of STEC in human (Alborzi et al 2008; Aslani and Bouzari 2003, Jomezadeh et al 2009, Salmanzadeh-Ahrabi et al 2005). The only study which investigated STEC isolation from bovine reservoir was from beef cattle (Askari Badouei et al 2009, Jamshidi et al 2008, Rahimi et al 2008, Sepehriseresh et al 2008, Tahamtan et al 2006, Zahraei Salehi et al 2006). *E. coli* O157:H7 was sporadic reported to be present up to 11.5% of cattle (Zahraei Salehi et al 2006). However, few data are available on the occurrence of *E. coli* O157:H7 in sheep (Shekarforoosh et al 2008, Tahamtan et al 2010).

Therefore, besides having a paucity of data regarding the isolation rates of STEC in animal reservoirs in our country, the occurrence of STEC in cattle, such as feed lot, dairy and calves and also sheep is unknown. No complete data were available on the fate and characteristics of *E. coli* O157:H7 isolated from healthy domestic cattle and sheep. There is no information about seasonal variations of *E. coli* O157:H7 in the area. We are not known if O157 VTEC, similar to another place of the world, is the predominant cause of HUS in Iran. No data are documented about virulence

profiles of shiga toxin. Therefore, the present study described the prevalence rate and characterizations of *E. coli* O157:H7 in cattle and sheep in Fars province of Iran.

## MATERIALS AND METHODS

*E. coli* O157:H7 strain EDL933 which possess the genes for VT1and VT2 was used as a positive control. *E. coli* O157 T-Shiraz 1387(Local collection obtained from field animal disease), which produces neither VT1 nor VT2, was used as negative control.

**Collection of samples.** RAMS samples from three groups of cattle; dairy cattle, feed lots and calves and also sheep were collected weekly in Fars province farms.

During a 2-years period from September 2006 to August 2008, 502 cattle and 370 sheep were taken. We try to get samples from farms, which kept both sheep and cattle with each other. Microbiological examination was begun immediately upon arrival, within 6 h of RAMS sampling.

**Isolation of O157 VTEC.** Each swab pre-enriched in 10 ml TSB for 4 h at 37 °C. The swab was streaked on novel protocols that use sorbitol-MacConkey agar plate supplemented with variant cefeximine (0.05 mg/L) and potassium tellurite (2.5 mg/L) (CT-SMAC). The plates were incubated at 42 °C for 18 to 20 h. On SMAC agar O157 colonies appear clear due to their inability (unlike other *E. coli* serotypes) to ferment sorbitol. Sorbitol-nonfermenting colonies (up to 8 per sample) were selected for characterization. The isolates were inoculated onto SMAC supplemented with 4-methylumbelliferyl- D-glucuronide (MUG; 0.1 g/liter; Sigma) and onto eosin methylene blue (EMB) agar (Oxoid). A typical *E. coli* metallic sheen on EMB and the isolates were both non-sorbitol fermenting colonies and β-glucuronidase negative on SMAC-MUG were tested for the somatic O157 and H fimbrial antigen before being confirmed as *E. coli* O157. O157 and H7 determination were performed with Difco antisera. Serotype O157:H7 was confirmed with positive

agglutination test result following by using an API 20E biochemical test strip (bio-Merieux, Lyon, France).

**DNA extraction.** DNA extraction was carried out by commercial DNA extraction kit (DNP Cina gene) as described by the manufacturer. Briefly, colony sweeps of the isolates identified as O157: H7 were grown overnight at 37 °C with agitation (100 rpm) in TSB, centrifuged at 3000 g in an Eppendorf centrifuge for 10 min. DNA extracts were stored at -20 °C until required.

**Nucleotide sequence.** The primer pairs specific were purchased from Roche Company, Germany. The nucleotide sequences and predicted product sizes of the primers are shown in table 1.

**PCR.** Polymerase chain reaction (PCR) was performed as described previously (Shekarforoosh *et al* 2008). Finally PCR products were run on a 1.5% agarose gel (Sigma) and visualized under UV-light gel doc (Kodak, logic gel logic 200) with ethidium bromide staining.

## RESULTS

Figure 1 was analyzed multiplex PCR of *E. coli* O157 isolates. More details were shown in table 2. The isolation rate of *E. coli* O157 was observed in 28(53.84%) of cattle were colonized with O157, while 24(46.15%) of these animals were healthy. There is no difference significant on the *E. coli* O157 isolation rate was observed between healthy and diarrheic cattle (just adult cattle, no calves). Overall 8.71% of both cattle and sheep investigated with *E. coli* O157:H7.

*E. coli* O157 strains isolates from RAMS samples of dairy cow, feed lots and calves was 7.27, 8.24 and 11.42 percent respectively, in which significance differences were observed regarding the age of the animals. A high occurrence of VT isolates was more frequently found among young cattle (calves). There was not observed any significance differences between *E. coli* O157 contamination and sex of animals.

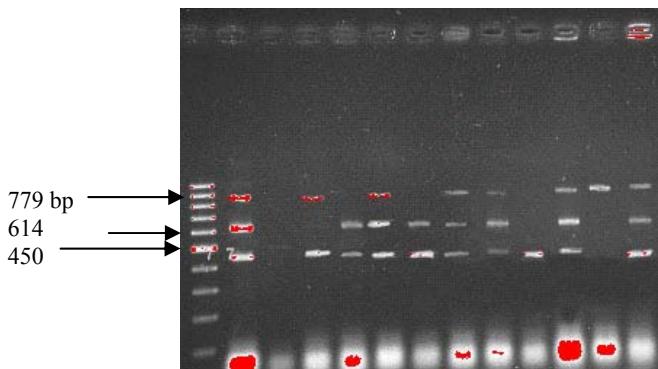
**Table 1.** Primers used in multiplex PCR for amplification of VT1, VT2, and eaeA genes.

Primers	Oligonucleotide sequence(5'-3')	Product size (bp)	References
VT2-F	CCA TGA CAA CGG ACA GCA GTT		
VT2-R	CCT GTC AAC TGA GCA CTT TG	779	(Fagan <i>et al</i> 1999)
VT1-F	ACA CTG GAT GAT CTC AGT GG		
VT1-R	CTG AAT CCC CCT CCA TTA TG	614	(Fagan <i>et al</i> 1999)
eaeA-F	AAG CGA CTG AGG TCA CT		
eaeA-R	ACG CTG CTC ACT AGA TGT	450	(Yilmaz <i>et al</i> 2006)

**Table 2.** Characteristics and distribution of vt positive animal, vt positive isolates and virulence markers encoding genes of RAMS *E. coli* O157 isolates from Iranian cattle, sheep.

Type of animal	No. of animals	No. (%) of VT+ animals	No. (%) of isolates	No. (%) of VT+ isolates	VT1 (%)	VT2 (%)	Eae(%)
Cattle	502	52(10.35)	892(71.58)	87(9.75)	23(26.43)	47(54.02)	24(27.56)
Sheep	370	24(6.48)	354(28.41)	28(7.90)	7(25.)	6(21.42)	2(7.14)
Total	872	76(8.71)	1246(100)	115(9.22)	30(26.08)	53(46.08)	26(22.6)

The VT positive cattle have been identified more readily than sheep (table 2). Both VT1 and VT2 genes were found in 31/87(35.63%) of cattle VT positive isolates. A relationship between VT profiles and cattle and sheep species was observed. VT2 is the dominant profiles in cattle. There was no existed correlation between  $\alpha$ -hemolysin production and the presence of VT or eae.



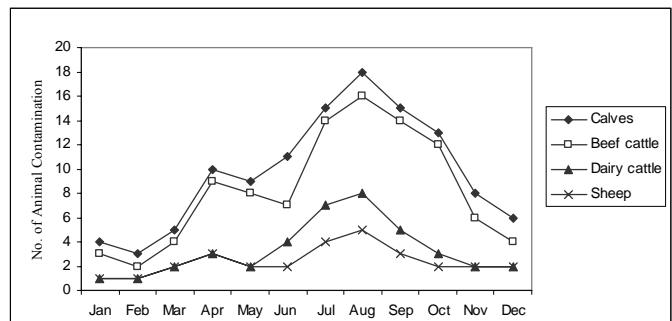
**Figure 1.** Agarose gel electrophoresis of DNA fragments amplified by multiplex PCR for virulence genes of the EHEC O157:H7 serotype. Lanes M: 1, 100-bp DNA ladder; C+: EDL 933 (O157:H7 serotype), positive control; C-: T Shiraz 1387, negative control; 1-10: *E. coli* O157 isolates. The sizes of amplified products are shown at the left of the gel. The products are (from top to bottom) Stx2, Stx1 and eae.

There is no effect of age was observed on the occurrence of VT for sheep ( $p>0.05$ ). In contrast to age of cattle with significant differences with VT isolates was observed.

The farms of mix animals, including cattle and sheep have shown monomorphic patterns of marked gene distribution. There was observed significant difference in the origin of VT positive sheep in close contact with farms of cattle origin. Effect of animals was kept in a pen or tethered was strongly observed on the distribution of VT in the cattle samples. The cattle was kept in pen was localized *E. coli* O157 than cattle tethered ( $p<0.05$ ). The late data was not shown significant differences among sheep.

No relation between geographic region and seasonal variation in the prevalence of *E. coli* O157 in sheep was observed. Although, it is plausible that the sheep was transferred from all over the state, even though the

country. Therefore, the origins of the sheep were unknown. In contrast, cattle originating from cold region farms seem to be less often infected than cattle originating from farms located in warm area (figure 2). But, no marked geographic variation in the prevalence of *E. coli* O157 in dairy cow was demonstrated. The values obtained for *E. coli* O157 organisms and gene-carrying bacteria over a 2-year period showing major variations. Monthly differences were observed for any of the measured parameters for feed lots and calves (not for sheep and dairy cow), as indicated by an analysis of variance test ( $P < 0.05$ ).



**Figure 2.** Prevalence rate of *E. coli* O157:H7 in cattle and sheep according to seasonal climate

## DISCUSSION

However, cattle sources are as far as sheep associated with human outbreaks, but some studies have indicated a higher prevalence of STEC in sheep than in cattle (Edrington *et al* 2003, Lim *et al* 2007). Culture of RAMS samples was more sensitive than culture of feces at detecting *E. coli* and *E. coli* O157:H7 in cattle (Naylor *et al* 2003) and sheep as well. The RAMS samples have minimum contamination of fecal materials, which contains high level of other bacteria (Rice *et al* 2003, Christopher *et al* 2005). *E. coli* O157 is world wide and studies performed in the Japan, China, Taiwan, European countries and USA was demonstrated that 0 to 100% of cattle have been contaminated (Blanco *et al* 2004, Best *et al* 2009). Totally 10.35% of VTEC cattle isolates recovered *E. coli* O157:H7 in the current study. VT2-producing *E. coli* was found in diarrheic calves, resulting a

pathogenic role in neonatal calf diarrhea (Blanco *et al* 2001, Yilmaz *et al* 2006). Close contact during grazing was reason the high prevalence of *E. coli* O157 in calves (Hussein & Sakuma 2005). The finding of these data revealed the higher prevalence rates of *E. coli* O157:H7 for younger than older cattle was illustrated strongly significant of animal age (Hussein & Sakuma 2005). All sheep in the present study were tethered, while all cattle particularly dairy cattle were kept in pens. Animals kept in pens will have more faecal-oral contact than tethered animals, and therefore probably maintain a higher level of Stx O157 in the intestine. The reasons for these results, in general, from young animals and adults are unknown, but may reflect differences in ruminal development, diet, resistance to infection, or other factors such as sampling and isolation methods (Urdahl *et al* 2003). Some studies explain one of a possible risk factor for *E. coli* O157 contamination was having cattle and sheep at the same farm (Urdahl *et al* 2001, Hussein & Sakuma 2005). New finding of human and bovine-associated STEC indicates differ in their ability to produce Shiga toxins (Ritchie *et al* 2003). While high prevalence of STEC in cattle in contrast of low number of human disease suggests that not all STEC are pathogenic (Acheson 2000, Samadpour *et al* 2002). Therefore, the high number of shiga toxin in studies does not represent a high human health risk (Hussein & Sakuma 2005). Both VT2 and eaeA in 20 (22.98%) and VT1, VT2 and eaeA (together) genes in 11 (12.64%) were detected in a total of 87 *E. coli* O157:H7 strains isolated from cattle. It is well known that the presence of VT2 and eaeA in a bacterium strongly associated with the human disease (Keen & Elder 2002). Unlike, the results of current study have also shown that *E. coli* O157 with VT1 was more presence in sheep isolates than VT2. However, a study in Canada and France (Pradel *et al* 2000) have shown that in spite of VT2, the presence of VT1 is associated with the presence of eaeA gene (Johnsen *et al* 2001, Omisakin *et al* 2003). The semi tropical region of Fars province and migration of animal to warm area in cold weather and vice versa, is

one of the reason why sheep is not seasonal *E. coli* O157 harboring dependent. The prevalence of *E. coli* O157:H7 in the feces of cattle has been demonstrated to be higher during the warm months (Renter *et al* 2004) and parallel with the timing of most human illness outbreaks (Farah *et al* 2003). During the winter *E. coli* O157:H7 reservoirs would likely be reduced or absent (Rice *et al* 2003). In conclusion, the findings are entirely consistent and support that the terminal rectum is the principal site of *E. coli* O157 in cattle and sheep as well. This site is associated with high-level fecal excretion, and has shown correlations between fecal prevalence and initial carcass contamination (Low *et al* 2005). Therefore abattoir has an important role in meat and environment contamination of *E. coli* O157. Decrease *E. coli* O157 shedding in feces (Hussein *et al* 2003) and stop colonized animals (Rice *et al* 2003) is the best ways to protect the food chain. The high rate of *E. coli* O157:H7 isolation presently described point the cattle particularly calves than sheep as an important reservoir of this bacteria. Also cattle isolate in Fars province with VT2 gene more frequently than sheep isolates. VT2 either or VT2 and eaeA are seems to be more important in the development of HUS than VT1. And may represent a significant risk to humans after transfer to any serotype and become a major public health problem in our country. Although the persistence and colonization of *E. coli* O157 in cattle host little characterized, but there is no more information about sheep and goats. According to this study, many characteristic in small ruminant differ those described in cattle. More investigation is needed to determine if animal and human strains belonged to the same clone or different.

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