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Full Article

Detection of verotoxin (Shiga-like toxin)-producing and *eae* harboring *Escherichia coli* in some wild captive and domestic Equidae and Canidae

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ABSTRACT

The aim of this study was to investigate the prevalence of STEC and EPEC strains and *E. coli* O157 serogroup in some Equidae and Canidae. The fecal samples of 79 animals from 6 different species were evaluated for presence of these strains. All the Isolates were tested for virulence genes using multiplex-PCR. Non-sorbitol fermenting (NSF) *Escherichia coli* isolates and positive strains for virulence factors were subjected to serogroupe specific PCR for *rfb* $_{O157}$ gene. None of the STEC, EPEC and NFS strains in this study belonged to O157 serogroupe. While 36.64% of animals carried strains positive for one or more of the virulence factors tested, and 18.9% of animals harbored STEC strains (*stx1*), *stx2* was not detected in this study. *eae* and *Ehly* positive strains were found in 3.79% and 22.7% of animals respectively. In conclusion, these species can act as a reservoir for EPEC and STEC strains. Also, since the study was conducted in some parts of Iran, a more accurate conclusion needs more distributed sampling. To our knowledge this is the first study which reports the faecal shedding of STEC and EPEC from wild captive Canidae and Equidae in Iran.

Keywords: Canidae, Equidae, STEC, EPEC, Iran

INTRODUCTION

Escherichia coli is a common normal micro flora of mammals digestive tract. Most *E. coli* strains are nonpathogenic, some strains such as enteropathogenic *E. coli* (EPEC) could cause diarrhea and other intestinal diseases (Law 2002). This pathotype, as food-borne pathogens, could result in attaching and effacing lesions on the epithelial cells *in vivo* and *in vitro* due to pathogenic mechanisms and consequently diarrhea in human (Zhang *et al* 2002). EPEC strains express outer membrane protein virulence factor entitled intimin (94-97 kDa) that is encoded by the *eae* gene (Kobayashi *et al* 2009). Some *E. coli* strains called shiga toxin-producing *E. coli* also harbor Shiga toxin (*stx*) genes (Kobayashi *et al* 2002). Shiga toxin-producing *E. coli* (STEC) strains are able to cause disease in human and

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some animal species. They are linked to hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) in humans, which requires hospitalization and intensive care with considerable mortality in children and elderly (Riley *et al* 1983, Gyles 2007). The ability of STEC strains to cause serious disease in humans is related to the production of one or more Shiga toxins (Stx1, Stx2, or their variants), which inhibit protein synthesis of host cells, and leading to cellular damage (O'Brien *et al* 1992). While ruminants are the main reservoirs of STEC, other domestic animals such as, cats, dogs and pigs could also harbor STEC and intimin encoding *E. coli* strains (Beutin *et al* 1993, Beutin *et al* 1995). Some studies have also been conducted on STEC and EPEC strains in the wild herbivorous animal (Keen *et al* 2007, Souza *et al* 1999, Beutin *et al* 1993), Equidae (Beutin *et al* 1993) and Canidae (Bentancor *et al* 2007). Cattle are considered as the primary reservoir for both O157:H7 and non-O157 STEC (Terrance *et al* 2002). A few serotypes of STEC including O157:H7, O157: NM, O26:H11, O104:H21, O111:H8, O111: NM, O48:H21 and O48: NM has been known to be closely related to food-borne associated STEC infection. The illness is often linked to the consumption of contaminated undercooked ground beef, although other means of transmission have also been reported by McDonough et al. (2002), and Gyles (2007). Different culture methods for screening fecal specimens for *E. coli* O157:H7 are available; among them, MacConkey agar containing sorbitol instead of lactose (SMAC) is commonly used for isolation of *E. coli* O157:H7. SMAC supplemented with cefixime and tellurite (CT-SMAC) is the best selective media developed for isolation of O157:H7 (Jamshidi *et al* 2008). Several sensitive and specific molecular approaches have been introduced, and serogroup-specific PCR assays targeting the genes encoding O and H antigens have also been developed (Paton and Paton 1998). The role of wild captive and domestic species of Equidae and Canidae is poorly understood in carriage of *eae* and *stx* possessing *E. coli*. Most Equidae including domestic horses and donkeys and Canidae such as domestic dogs being kept by humans may play as vehicles to infect humans by STEC and EPEC strains. Epidemiological investigation of EHEC O157, STEC and EPEC strains in animal populations has focused mainly on the bovine reservoir, so the prevalence in other animals is not well known. But some studies were mentioned the role of wild captive and zoo animal as reservoirs of STEC and EHEC strains, to our knowledge, there is no study regarding the prevalence and molecular characteristics of STEC and EPEC stains derived from the wild Equidae and Canidae in Iran. Therefore, the aim of this study was to clarify the role of the wild and domestic Equidae and Canidae as STEC and *eae* possessing *E. coli* reservoirs in some parts of Iran.

MATERIALS AND METHODS

Fecal samples from 79 wild and domestic Equidae and Canidae from 6 different species were randomly collected (Table 1). The samples were transported on Amies media (Merck,Germany) to the laboratory and then cultured on MacConkey agar (Merck, Germany). After overnight incubation at 37 ^{o}C, 3 to 4 colonies were chosen from each plate in case of observing lactose fermenting pink colonies. These isolates were characterized by evaluation of biochemical tests, including conventional lactose and glucose fermentation (using TSI medium), urease, indol, methyl red, vogesproskauer and citrate tests (Quinn *et al* 1994). Also swab samples were aseptically and separately transferred to tryptic soy broth (TSB) with cefixime (50 ng/ml) and vancomycin (40 mg/ml), After overnight incubation at 37 ^{o}C, The enriched culture was streaked on Sorbitol MacConkey agar (Merck, Germany) supplemented with cefixime (0.05 mg/ml) and potassium tellurite (2.5 mg/ml) (CT-SMAC)(Merck,Germany). The inoculated CT-SMAC plates were incubated at 37 °C for 18-24 hours. Non-sorbitol fermenting colonies from each sample were picked and sub-cultured. These isolates were characterized by evaluation of biochemical tests, including conventional lactose and glucose fermentation (using TSI medium),

Table 1. Isolation of *eae*-, *Ehly*- and *stx*-possessing *E. coli* strains from fresh fecal swabs of various species

Family	Common Name	*Nomenclature*	No. of samples tested	No. of positive samples for *stx*, *stx*, *eae* or *Ehly* genes	Genotypes of virulent strains	Location
Canidae	Domestic dog	*Canis lupus familiaris* (Terrier)	8	1	*Ehly* n:1	Mashhad Vakil abad zoo
	Domestic dog	*Canis lupus familiaris* (German Sheperd)	6	2	*stx1* n:2	Mashhad (Toos)
	Domestic dog	*Canis lupus familiaris (Sheep dog)*	10	6	(*stx1/ Ehly*) n:5 *stx1* n:1	Neyshabour (Darroud)
	Golden jackal	*Canis aureus*	6	2	*eae* n:1 *Ehly* n:1	Mashhad Vakil abad zoo
			3	-		Tehran Eram zoo
	Wolf	*Canis lupus*	4	2	(*stx1/Ehly*) n:2	Mashhad Vakil abad zoo
			3	-		Tehran Eram zoo
Equidae	Domestic horse	*Equus ferus*	4	1	*eae* n:1	Mashhad Vakil abad zoo
			8	3	*Ehly* n:3	Neyshabour (Kharv)
			13	4	(*stx1/ Ehly*) n:3 *stx1* n:1	Mashhad (Torghabeh)
	Donkey	*Equus africanus asinus*	7	2	*Ehly* n:2	Neyshabour (Boozhan)
			4	1	*eae* n:1	Karaj (Gachsar)
	Onager	*Equus hemionus*	3	1	(*stx1/ Ehly*) n:1	Tehran Eram zoo
Total			79	25	*Ehly* n:7 *eae* n:3 *stx1* n:4 *stx1/Ehly* n:11	

Table 2. PCR primers and conditions for amplification of *stx1, stx2, Ehly , eae* and rfb_{O157} genes

Name	Primer Sequence (5′to 3′)	Target Gene	PCR program	Amplicon Size (bp)	reference
O157F O157R	CGGACATCCATGTGATATGG TTGCCTATGTACAGCTAATCC	rfb_{O157}	94°C, 30 s; 52°C, 60 s; 72°C, 60 s (30 cycles)	259	Paton and Paton (1998)
Stx1-F Stx1-R	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	*stx1*	95°C 60s;65°C 120s; 72°C 60s (first 10 cycles) decrementing to 60°C (cycles 10-15)	180	
Stx2-F Stx2-R	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	*stx2*	95°C 60s;60°C 120s; 72°C 90s (cycles15-25)	255	
Eae-F Eae-R	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	*eaeA* (*intimin adherence gene)*	95 °C 60s;60°C 120s; 72°C 150s (cycles25-35)	384	
Hly-F Hly-R	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	*Ehly* (enterohemolysin gene)		534	

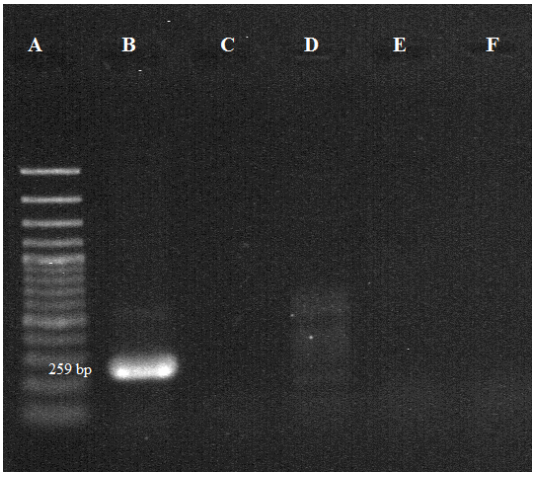

Figure 1. PCR results for detection of *rfb*$_{O157}$ gene. **A)** Marker 100bp. **B)** C+O157:H7 (control positive). **C, D and E)** NFS strains. **F)** Control negative.

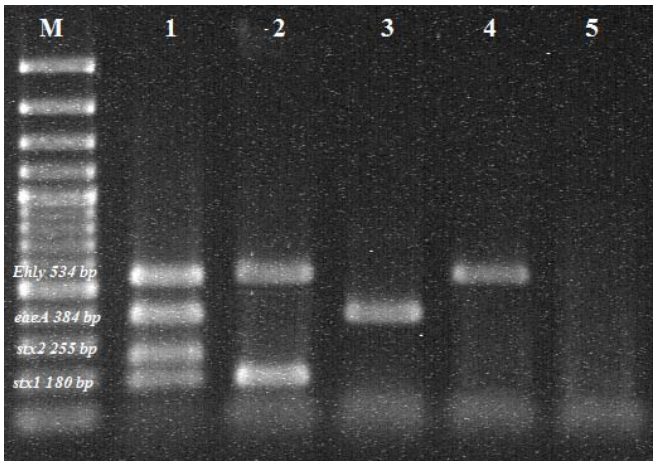

Figure 2. Multiplex PCR results for detection of *stx1*, *stx2*, *eae*, *Ehly* genes. M) marker 100bp. 1) *stx1* (180 bp), *stx2* (255 bp), *eae* (384 bp), *Ehly* (534 bp) C+O157:H7 (control positive). **2)** *stx1* /*Ehly* genotype. 3) *eae* positive strain. 4) *Ehly* positive strain. 5) Control negative.

urease, indol, methyl red, voges proskauer and citrate tests (Quinn *et al* 1994).

DNA extraction. After confirming the isolates as *E. coli*, the isolates were sub-cultured on LB Agar. After 18-20 incubation at 37 ^{o}C, DNA was extracted by boiling methoed as described previously (Zahraei *et al* 2007).

PCR for Detection Of *rfb*$_{O157}$ gene. The PCR assay for detection of O157 antigens carried out on non-sorbitol fermenting (NSF) isolates and strains positive for virulence factors. *E. coli* O157:H7 (ATCC 35218) were used as positive control and distilled water as negative control. The presence or absence of *rfb*$_{O157}$ (*O157O-antigen-encoding*) gene which encodes the O157 somatic antigen was examined. The PCR reaction was performed in a 25 µl amplification mixture consisting of 2.5µl of 10x PCR buffer, 2mM $MgCl_2$, 0.5µM primers, 1 unit *Taq* DNA polymerase, 0.2mM dNTP mix and 2 µl of template DNA. Thermal cycles were carried out according to Paton and Paton (1998), (Table 2). The PCR products were electrophoresed on 1.5% agarose gel for 1 hour at 100V and visualized by staining with ethidium bromide.

Multiplex-PCR for *stx1, stx2, eae* and *Ehly.* All of the *E. coli* isolates (obtained from CT-SMAC and MacConkey agar) were screened by multiplex PCR using four pairs of specific primers for *stx1* (*shiga toxin 1*), *stx2* (*shiga toxin 2*), *eae* (*intimin adherence gene*) and *Ehly* (*enterohemolysin*) as described by Paton and Paton 1998 (Table 2). Amplification was carried out in a total volume of 25µl containing: 3µl prepared DNA, 0.3µM of each oligonucleotide primer, 0.2mM dNTP mix, 2mM $MgCl_2$, 2.5µl of 10x PCR buffer, 1 unit *Taq* DNA polymerase (Cinnagen, Iran) and PCR grade water up to 25µl. Samples were subjected to 35 cycles of touchdown PCR according to Paton and Paton (1998) (Table 2). The PCR products were electrophoresed on 2% agarose gel for 1.5hours at 85V and visualized by staining with ethidium bromide. Positive PCR reactions were recorded by comparing the specific bands with 100bp-plus molecular size marker (Fermentas, Lithuania). Positive controls and negative controls (sterile water) were included in all PCR reactions.

RESULTS

A total number of 8 isolates which were recovered from 7 domestic horses confirmed as non-sorbitol fermenting *E. coli* (NSF) in biochemical tests. In serogroup specific PCR assay all of the NSF isolates and strains which were positive for one or more virulence genes in multiplex-PCR, were negative for *rfb*O157 gene (Figure 1). Two hundred and fifty two

fecal *E. coli* isolates obtained from 79 animal belonging to 6 different species and 8 non-sorbitol fermenting *E. coli* (NSF) strains were investigated for presence of *stx1*, *stx2*, *eae* and *Ehly* genes using multiplex PCR. After evaluating all isolates using multiplex PCR to detect STEC and EPEC strain, it was figured out that 36.64% of animals (n=25) were positive in virulence markers in multiplex PCR with strains including at least one virulence factor (Figure 2), 15 animal (18.9%) harbored shiga toxin-producing strains. The most frequent shiga toxin was type 1 (*stx1*) and was present in 18.9% of samples (n=15) and *stx2* was not detected. The predominant genotype was (*stx1* /*Ehly*) with a frequency of 13.9% (n=11). NSF strains were negative for all tested virulence factors. And individual virulence genes *stx1*, *stx2*, *eae* and *Ehly* were detected at frequencies of 60%, 0%, 12% and 72%, respectively.

DISCUSSION

Enterohemorrhagic *E. coli* (EHEC) is a major cause of food-borne disease, mostly in modern countries (Griffin *et al* 1991, Jafari *et al* 2012, Nataro and Kaper 1998) Many studies have examined the epidemiology of O157:H7 EHEC in cattle populations, but there has been only a few investigations on the relevance of other animals as reservoir for EPEC and STEC strains (Keen *et al* 2007, Souza *et al* 1999, Beutin *et al* 1993). In the present study 8 none sorbitol fermenting (NSF) *E. coli* were isolated from 7 domestic horses using pre-enrichment and CT-SMAC selective medium; but the presence of O157: H7 *E. coli* was not confirmed using serogroup-specific PCR assay for rfb_{O157} gene. While in some studies the prevalence of O157: H7 *E. coli was* 7.5% and 3.6% in domestic dogs and horses respectively (Bentancor *et al* 2007, Leotta *et al* 2007). Among 260 isolates tested (including 8 nsf isolates belonged to seven horse and 252 *E. coli* strains), 25 isolates, from 25 (36.64%) animals each, included at least one virulent gene (Table 1). The findings showed that 18.9% of samples were positive for *stx1* and *stx2* was not detected. *eae*+ strains were identified in 3.79% of animals tested. Our results support other findings which reported higher frequency of *stx1* in wild captive and domestic Equidae and Canidae (Bentacor *et al* 2007, Leotta *et al* 2006, Warshawsky *et al* 2002). The frequency of carriage of *stx* in domestic doges documented by Zahraei et al. (2011) was lower than our results, and most of the strains isolated from dogs carried *stx1* (Bentacor *et al* 2007). In Bentacor et al. (2007) study, 3.7% of dogs were carried *stx2*+ strains, while in our study *stx2* was not detected. Strains harbouring *eae* gene form a characteristic attaching and effacing lesion on intestinal epithelial cells (Gyles 2007). *eae* was detected at different frequencies in *E. coli* from wild animals (Keen *et al* 2007, Souza *et al* 1999). In the present study, 3.7% of samples contained *eae*+ strains; interestingly, all of the *eae*+ strains were negative for *stx1*, *stx2* or *Ehly* genes. Our results similarly showed that (*stx1* /*Ehly*) is the predominant virulence pattern among wild captive and domestic Equidae and Canidae (Bentacor *et al* 2007, Leotta *et al* 2006, Warshawsky *et al* 2002). Based on the literature, it is obvious that different combinations of virulence markers have been reported in different studies with dissimilar frequencies. The important criteria for these types of variations may be the geographic area, other factors have also been considered such as age, season, diet and species (Caprioli *et al* 2005). There is little information available regarding sequences and variants of genetic determinants of STEC and EPEC in wild and domestic Equidae and Canidae in Iran. According to the results of the current study and by comparing to the results of the previous studies, it seems that the prevalence of EPEC and STEC strains in mammals depend on the species and the geographical area. Also, the results may indicate the possibility that wild and domestic Equidae and Canidae have an important role for carriage of virulent strains such as STEC. On the other hand, since some species including pet dogs, horses and donkeys are kept by human, the potential infection by these animals is possible. Also this study indicated that Equidae and Canidae could act as a possible reservoir for non-O157 STEC in the studied area. To conclude more precisely about the excretion

status of STEC and EPEC strains in canines and equines of Iran, a more perfect study with more samples in different parts of Iran should be conducted.

Ethics

There was no conflict of interest to be declared.

Conflict of Interest

The authors would like to state that this study has never been published or submitted elsewhere, and conducted with regard to ethics in publication and research.

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