Prevalence, molecular characterization and serology of Shiga toxin-producing *Escherichia coli* isolated from buffaloes in West Azerbaijan, Iran

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Key Words:
Buffalo; prevalence; serotype; STEC.

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Received: 22 February 2011,
Accepted: 24 April 2011

Abstract

This present study is the first to report the presence of Shiga toxin-producing *Escherichia coli* (STEC) in buffaloes in Iran. A total of 360 fecal samples were collected from buffaloes from different regions in the west Azerbaijan province of Iran and cultured for the isolation of *E. coli* using routine biochemical tests. From the fecal samples, 340 *E. coli* were isolated and, of these, 26 STEC isolates were identified. The STEC isolates were further analyzed for the presence of specific virulence genes. Among the STEC isolates, 11 (42.3%) isolates were positive for the stx_1 gene, nine (34.6%) were positive for the stx_2 gene and six (23%) were positive for both of these genes. Six (23%) STEC isolates harbored the *hly* gene and two (7.6%) isolates were positive for the *eae* gene. Based on serotyping, only one (3.8%) isolate was of the O157 serotype, while the other 25 (96.1%) belonged to non-O157 serotypes. The results of the present study provide the first evidence that buffaloes could be a reservoir for STEC in Iran, especially those belonging to non-0157 serotypes.

Introduction

During the last 20 years, Shiga toxin-producing *Escherichia coli* (STEC) have been recognized as an important emerging group of food-borne pathogens (Bielaszewska and Karch, 2000; Conedera *et al*., 2004; Tarr *et al*., 2005) In humans, infection with STEC can cause gastroenteritis that may develop into life threatening conditions, such as hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura, especially in children, the elderly and immune-suppressed patients (Mohammad *et al*., 1986; Tarr and Neill, 1996). In animals, STEC can cause diarrhea in calves (Mohammad *et al*., 1986) and edema disease in piglets (Imberechts *et al.,* 1992).

STEC strains are classified into a considerable, still increasing, number of O:H serogroups (Pennington, 2000). Although more than 200 STEC serotypes have been reported worldwide, most outbreaks and sporadic cases of HC and HUS have been ascribed to the STEC O157 serotype strains (Eklund *et al*., 2001; Pradel *et al*., 2000). Domestic ruminants, particularly cattle, have been recognized as the natural reservoirs of STEC in the world (Conedera *et al*., 2004). In most cases transmission occurs through food and water that has been contaminated with ruminant feces (Pennington, 2000).

STEC strains are characterized by the production of one or two Shiga toxins *(Stx_1 or Stx_2)*, which are the main virulence factors, and these repress protein synthesis in the host's cells leading to cell death. The Stx_1 and Stx_2 toxins are encoded by the stx_1 and stx_2 genes from lysogenic prophages of *E. coli* (Paton and Paton, 1998). In addition to toxin production, another virulence factor expressed by STEC is enterohemolysin (*hly*), which damages eukaryotic cells by forming pores in the cell membrane (Schmidt *et al*., 1995). Furthermore, most STEC strains carry the *eae* gene, encoding a protein called intimin, which is responsible for attaching the STEC cell to host intestinal mucosa (Jerse and Kaper, 1991). Some STEC strains also encode the bifunctional catalase peroxidase (*Katp*) and serine protease (*espP*), which can cleave human coagulation factor (Brunder *et al.,* 1997).

The prevalence of STEC in cheeses produced from raw cow's milk has been reported to be as high as 4% in Iran (Mansouri-Najand and Khalili, 2007); however, the prevalence of STEC in Iranian buffaloes remains unknown. The aim of this study was to estimate the prevalence of the STEC in buffaloes in Iran and perform molecular characterization of any STEC strains isolated. STEC isolates were analyzed for the presence of virulence genes, including *stx_1*, *stx_2*, *eae* and *hly* using multiplex polymerase chain reaction (PCR).

Materials and Methods

Sampling

From April 2009 to March 2010, 360 fecal samples (50-150 g) were collected from buffaloes at random in the west Azerbaijan province of Iran. Samples were placed in a sterile plastic container and kept on ice before being transferred to the laboratory. The samples were analyzed within 6-12 h after collection.

Microbiological analyses

For each fecal sample 5-10 g was homogenized and enriched in 15 ml of nutrient broth. Then, 50 µl of the suspension was plated on MacConkey agar and incubated at 37°C for 18-24 h. Ten colonies of lactose-positive bacteria were selected and confirmed to be *E. coli* utilizing standard biochemical tests (Kudva *et al*., 1997).

Molecular characterization of E. coli isolates

E. coli isolates were screened by polymerase chain reaction (PCR) for the presence of chromosomal sequences encoding Shiga toxin 1 (*Stx_1*), Shiga toxin 2 (*Stx_2*) and the intimin protein (*eae*), and the plasmid-encoded hemolysin (*hly*) according to the procedures described by Islam *et al*. (2008). *E. coli* O157: H7 (ATCC 43895) and sterile distilled water were used as positive and negative controls, respectively.

DNA extraction

For DNA extraction, an *E. coli* colony from a pure culture was resuspended in 200 µl sterile distilled water and boiled for 10 min. The mixture was centrifuged for 10 min at 13000×*g* and placed on ice for 3 min. The supernatant was used for the PCR reaction.

Multiplex PCR

For amplification of *stx_1*, *stx_2*, *hly* and *eae* genes specific primers were used (Fitzmaurice, 2003; Paton and Paton, 1998) (Table 1). The PCR reaction was carried out in a final volume of 25 µl containing 25 µM each of dATP, dTTP, dGTP and dCTP, 0.25 µM of each primer, 2.5 µl of 10X PCR buffer (Fermentas), 2 mM $MgCl_2$, 15 U *Taq* DNA polymerase (Fermentas) and 3 µl of extracted DNA as template. Amplification of targeted fragments were carried out using an initial denaturation step of 5 min at 95˚C; followed by 35 cycles of incubations at 95˚C for 30 s, 58˚C for 60 s (for *eae* and *hly*: 59˚C for 60 s) and 72˚C for 2 min; with a final extension step of 72˚C for 5 min. Resultant PCR products were observed and analyzed on 2.0% agarose gels using ultraviolet transillumination (Figure 1 and Figure 2).

Table 1. Primer sequences used in this study and their target gene and amplicon size.

Reference	Amplicon size (bp)	Target gene	Primer Sequence (5'-3')	Primer name
9, 26	180	*Stx1*	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	*stx1F* *stx1R*
9, 26	255	*Stx2*	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	*stx2F* *stx2R*
26	384	*eae*	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	*eae F* *eae R*
26	534	*hly*	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	*hly F* *hly R*

Serotyping

Serotyping was performed by bacterial agglutination (Ørskov and Ørskov, 1984) with O antisera against O157 antigens according to the manufacturer's instructions (MAST Comp, England). Briefly, one loopful or 10 colonies of STEC isolates (from a MacConkey plate) were resuspended in 2 ml of 0.9% saline solution and incubated at 100°C for 60 min. After incubation the suspension was centrifuged at 900×*g* for 15 min and the supernatant was discarded. The bacterial pellet was resuspended in 0.5 ml saline solution and used as O-antigen solution for serotyping. *E. coli* O157: H7 (ATCC 43895) was used as the reference strain.

Antimicrobial susceptibilities

Antimicrobial susceptibilities of the isolates were determined using the disk diffusion methodology (NCCLS, 2000) on Mueller-Hinton agar according to zone size criteria described by the disk manufacturer (PadtanTeb, Iran). The antimicrobial agents used in these tests were: ampicillin (10 µg), neomycin (30 µg), streptomycin (10 µg), tetracycline (30 µg), erythromycin (15 µg), kanamycin (30 µg), amoxicillin (25 µg), tobramycin (10 µg) and cefotoxime (30 µg). *E. coli* O157: H7 (ATCC 43895) was used as a drug-sensitive control bacterium.

Results

Prevalence and molecular characterization of STEC

Characterization of the *E. coli* isolates by PCR showed that 26 (7.2%) of the strains were STEC. Of these, six isolates (23%) were positive for both *stx_1* and *stx_2* genes. Eleven isolates (42.3%) were positive for *stx_1* only and nine isolates (34.6%) were positive for *stx_2* only (Figure 1 and Table 2). The presence of the *hly* gene was confirmed in six (23%) isolates, while the *eae* gene was identified in two (7.6%) isolates (Figure 2 and Table 2).

Serotyping

Based on serotyping using the O157 antigen, only one (3.8%) isolate was the O157 serotype, while the other 25 (96.1%) isolates belonged to non-O157 serotypes.

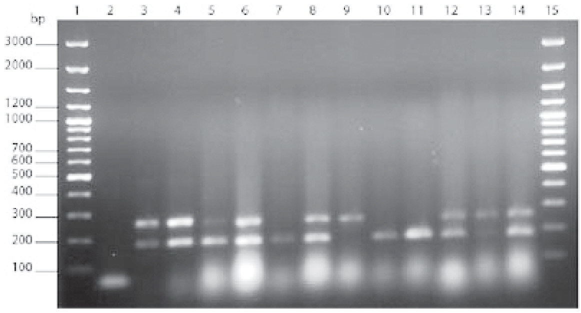

Figure 1: Gel electrophoresis of PCR products using STEC specific primers. Lanes 1 and 15: 100-bp molecular ladder (Cinnagen, Iran); lane 2: negative control; lane 3: positive control for *stx*$_1$ and *stx*$_2$ genes; lanes 4, 5, 6, 8, 12, 13 and 14: positive isolates for *stx*$_1$ and *stx*$_2$; lanes 7, 10, 11: positive isolates for *stx*$_1$; lane 9: positive isolate for *stx*$_2$.

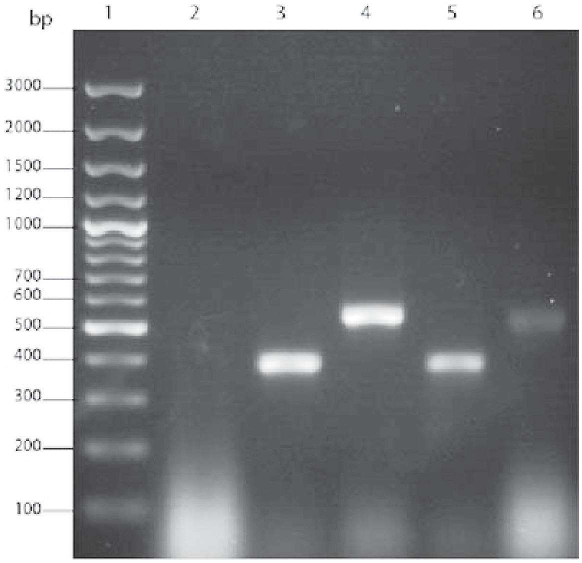

Figure 2: Gel electrophoresis of the PCR products using STEC specific primers. Lane 1: 100-bp molecular ladder (Cinnagen, Iran); lane 2: negative control; lane 3: positive control for *eae*; lane 4: positive control for *hly*; lane 5: positive isolate for *eae*; lane 6: positive isolate for *hly*.

Antimicrobial susceptibilities

The results of the antibiotic susceptibility testing are presented in Table 3. All isolates were resistant to ampicillin, erythromycin, neomycin and streptomycin. Among the 26 STEC isolates 25 (96.1%), 24 (92.3%), 18 (69.2%), 4 (15.4%) and 1 (3.8%) isolates were resistant to amoxicillin, tobramycin, kanamycin, tetracycline and cefotoxime, respectively. Multi-antibiotic resistance (resistance to seven antibiotics) was detected in 69.2% of the STEC isolates. Tetracycline and cefotoxime were the most effective antibiotics against the STEC isolates (Table 3).

Discussion

STEC is emerging as a universally important food-borne pathogen (Riley *et al*., 1983). Many studies have shown that ruminants and their food products are the main reservoir and vehicles of transmission of this pathogen (Islam *et al*., 2008; Oliveira *et al*., 2007; Pradel *et al*., 2000). To our knowledge, this is the first study to investigate the clonality of STEC in Iranian buffaloes. This present study confirms previous findings that these animals are reservoirs in Iran for this pathogen, including non-O157 serotypes. In the present study, 7.2% of examined buffaloes were positive for STEC. In a recent study on buffaloes in Bangladesh, the prevalence of STEC was 37.9% (Islam *et al*., 2008). The prevalence of STEC in water buffaloes in Brazil ranged from 0-64% depending on the farm (Oliveira *et al*., 2007). In Vietnam, the prevalence of STEC in buffaloes, goats and cattle were reported to be 27%, 38.5% and 23%, respectively (Vu-Khac and Cornick, 2008). In India, STEC was isolated from 2% and 7.6% of fecal samples collected from slaughtered cattle and diarrheic calves, respectively (Manna *et al*., 2006). The prevalence of 7.6% for STEC in west Azarbaijan is similar to other studies carried out in Iran. STEC was detected in 4% of raw milk cheeses produced in Kerman province (Mansouri-Najand and Khalili, 2007), while of 29 *E. coli* isolated in Tehran from diarrheic calves, 4 (13.7%) isolates were *stx1* positive and 16 (55.17%) carried the *stx2 gene* (Zahraei Salehi *et al.,* 2006). Moreover, 21.8% of *E. coli* isolated from cattle feces in Tehran were positive for *stx1*, and/or *stx2* genes (Mazhaheri Nejad Fard, *et al.,* 2005). The prevalence of STEC in patients with diarrhea has been reported to be 7% in Tehran (Jafari *et al*., 2008) and 10.4% in Hamedane (Iran) (Alizadeh *et al*., 2007). In Abadan (Iran), 8.7% of diarrheal cases and 2% of children without diarrhea were found to be infected

Table 2. Virulence gene typing of STEC non-O157 isolates from buffalo fecal samples.

Virulence gene(s)	Number of examined animals	Presence in STEC non-O157 isolates
stx1	360	11
stx2	360	9
stx1 and stx2	360	6
eae	360	2
hly	360	6

Table 3. Antibiotic resistance of STEC isolated from buffalo fecal samples.

Antimicrobial agent	Number of resistant isolates	Percentage of resistant isolates
Amoxicillin	25	96.1
Ampicillin	26	100
Cefotoxime	1	3.85
Erythromycin	26	100
Kanamycin	18	69.2
Neomycin	26	100
Streptomycin	26	100
Tetracycline	4	15.3
Tobramycin	24	92.3

with STEC (Alikhani *et al*., 2007). Based on the results obtained from the present study and earlier reports from Iran (Alikhani *et al*., 2007; Alizadeh *et al*., 2007; Mansouri-Najand and Khalili, 2007; Jafari *et al*., 2008), the prevalence of STEC in Iran is lower than other countries, and this may be as a result of geographical conditions, the presence of natural antibodies and differences in the natural intestinal flora present in humans and animals. The majority of STEC isolates obtained in the present study belonged to non-O157 serotypes, which is similar to results reported from Brazil where all the STEC isolated from buffaloes belonged to non-O157 serotypes (Oliveira *et al*., 2007), and results from an earlier Iranian study where all STEC isolates from children with and without diarrhea were non-O157 serotypes (Alikhani *et al*., 2007). It has been proposed that the differences in the capacity of particular STEC strains to cause severe disease in human is associated with the type and/or amount of Stx toxins produced (Paton *et al*., 1995). Production of the stx_2 toxin is an index for serious clinical consequences in infected patients, as there is a strong association between the presence of the stx_2 gene and the capacity of STEC strains to cause severe human disease (Bielaszewska *et al*., 2006; Friedrich *et al*., 2002; Jelacic *et al*., 2003). In addition, intimin is an important virulence factor associated with severe disease in humans, especially HUS (Gyles *et al*., 1998). Six and two STEC strains revealed the presence of *hly* and *eae*, respectively, (Table 2). Thus, STEC isolated in this present study were found to be carrying virulence factors clearly associated with increased human pathogenicity. However, the low frequency of the *eae* gene in the STEC isolates in the present study may be related to the isolation of certain serotypes, as it has been reported that the existence of the *eae* gene is correlated with only specific O groups of *E. coli*, such as the O157, O145, O26, O103 and O111 serotypes (Sandhu *et al*., 1996). Based on the antimicrobial susceptibility data, all of the STEC isolates were found to be resistant to ampicillin, erythromycin, streptomycin and neomycin. In another study, 66% of STEC strains that were isolated from diarrheal patients in Isfahan (Iran) were resistant to the three commonly used antibiotics (amoxicillin, tetracycline and trimethoprim-sulfamethoxazol) (Fazeli and Saheli, 2007). This means that buffaloes infected with these STEC strains may act as a reservoir for drug-resistant strains that may lead to antimicrobial treatment failures.

The results of the present study provide the first evidence that buffalo are reservoirs for STEC in Iran. STEC isolated in this study were found to be carrying various virulence factors. As buffalo farming is becoming an increasingly significant economic activity, control measures for hygienic practice, supervision and law-making have to be improved in order to prevent fecal contamination of milk and dairy products. Further studies are required to determine epidemiological aspects of STEC in buffaloes.

Acknowledgments

The authors would like to thank the Dean of Research at Urmia University for funding this project. They also would like to thank Mr Ali Kazemnia for his technical assistance.

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