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ORIGINAL ARTICLE

Survey on O157:H7 enterohemorrhagic *Escherichia coli* (EHEC) in cattle in Golestan province, Iran

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ABSTRACT

Background and Objectives: A diverse group of *Escherichia coli* are known as enterohemorrhagic *Escherichia coli* (EHEC) including O157:H7 and non-O157 EHEC. Enterohemorrhagic strains are related to severe clinical conditions in humans including hemorrhagic colitis and hemolytic uremic syndrome, and most of the recorded outbreaks occurred due to O157: H7 *E. coli*. The aim of the present study was to investigate the presence of O157:H7 *E. coli* among healthy cattle in Golestan province.

Materials and Methods: Fecal samples were collected from 180 clinically healthy cattle in Golestan province. After primary enrichment, samples were streaked on sorbitol MacConkey agar supplemented with cefixime and potassium tellurite (CT-SMAC). Non-sorbitol fermenting (NSF) *Escherichia coli* isolates were subjected to serotyping using commercial O157 antisera and *rfb* O157 gene PCR. Isolates were additionally tested for major virulence factors of EHEC including *stx1*, *stx2*, *eae* and *ehly* by multiplex-PCR.

Results: Eighteen NSF isolates were recovered from CT-SMAC confirmed as *E. coli* in biochemical tests. None of the obtained isolates belonged to O157 serogroup. Overall, two isolates harbored the tested virulence genes; one isolate possessed *stx2* and *ehly*, and the other one carried *stx2*, *eae* and *ehly*.

Conclusion: The results of this study indicated that cattle in Golestan province could be the reservoir for non-O157 EHEC.

Keywords: Cattle, Golestan province, O157:H7, PCR

INTRODUCTION

A group of pathogenic *Escherichia coli* are known as Shiga toxin-producing *E. coli* (STEC). Within this group, some strains are named enterohemorrhagic *Escherichia coli* (EHEC), because of their more pathogenic capacities and link to human infections. Importantly, EHEC strains cause 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 (1, 2). The ability of STEC strains to cause serious disease in humans is related to the production of Shiga toxins (Stx1, Stx2, or their variants), which inhibit protein synthesis in host cells, and leading to cellular damage (3).

In general, STEC strains have been categorized into five seropathotypes designated as A to E. Seropathotype A consists of O157: H7 and O157: NM strains. Seropathotype B includes O26:H11, O103:H2, O111:H8, O111: NM, O113:H21 and a number of other serotypes. Although the O157: H7

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Table 1. Primers used in this study

Gene	Primer sequence (5´-3´)	Product (bp)	Reference
rfb O157	CGGACATCCATGTGATATGG	259	7
	TTGCCTATGTACAGCTAATCC		
stx1	ATA AAT CGC CAT TCG TTG ACT AC	180	
	AGA ACG CCC ACT GAG ATC ATC		
stx2	GGC ACT GTC TCT CTG AAA CTG CTC C	255	
	TCG CCA GTT ATC TGA CAT TCT G		
eae	GAC CCG GCA CAA GCA TAA GC	384	
	CCA CCT GCA GCA ACA AGA GG		
ehly	GCA TCA TCA AGC GTA CGT TCC	534	
	AAT GAG CCA AGC TGG TTA AGC T		

was recognized to be commonly associated with severe illness and outbreaks, other serotypes can also cause sporadic cases of HUS and occasional outbreaks (2-5). Cattle are considered as the primary reservoir for both O157:H7 and non-O157 STEC and the illness in humans is often linked to the consumption of contaminated undercooked ground beef, but other means of transmission have also been described (4).

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 medium developed for isolation of O157:H7 (6). 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 (7).

The purpose of the present study was to investigate the presence of *E. coli* O157:H7 among fecal isolates from cattle in Golestan province in northeast of Iran. The major virulence determinants of the recovered isolates were additionally investigated.

MATERIALS AND METHODS

Fecal samples from 180 clinically healthy cattle of five separate farms located in Golestan province were randomly collected. Animals those had received any antimicrobial agent in the days prior to sampling were excluded from the study. Samples were collected using sterile swabs and transported to laboratory in Amies transport medium (Merck,Germany) within 24 hours. In the laboratory, each sample were aseptically transferred to E.C. broth (BBL, USA) and incubated for 4 to 6 hours at 37°C. The enriched culture was streaked on Sorbitol MacConkey agar supplemented with cefixime (0.05 mg/ml) and potassium tellurite (2.5 mg/ml) (CT-SMAC) (Biomark, India). The inoculated CT-SMAC plates were incubated at 37°C for 18-24 hours. Three 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), urease, indol, methyl red, voges proskauer, citrate and lysine decarboxylase (8). All confirmed *E. coli* isolates were subjected to slide agglutination assay using O157 antisera (Baharafshan, Iran) according to manufacturers' instruction manual. Three suspected colonies were double checked by antisera from another manufacturer (Mast, UK) according to the instruction manual.

The PCR assay for detection of O157 antigens carried out on non-sorbitol fermenting (NSF) strains. Total genomic DNA was extracted from overnight LB agar culture (Merck, Germany) by the boiling method, as described previously (9). The supernatant was used as template in the PCR mixture. *E. coli* O157:H7 (ATCC 35218) were used as positive control and distilled water as negative control. The presence or absence of *rfb*O157 gene which encodes the O157 somatic antigen was examined (Table 1). The PCR

reaction was performed in a 25 µl amplification mixture consisting of 2.5 µl of 10x PCR buffer, 2 mM $MgCl_2$, 0.5 µM primers, 1 unit *Taq* DNA polymerase, 0.2 mM dNTP mix and 2 µl of template DNA. Thermal cycles were carried out according to Paton and Paton (1998) (7). The PCR products were electrophoresed on 1.5% agarose gel and visualized by staining with ethidium bromide.

Additionally, all of the obtained NSF *E. coli* isolates were screened by multiplex-PCR using four pairs of specific primers for *stx1, stx2, eae* and *Ehly* (Table 1) (7). Amplification was carried out in a total volume of 25 µl containing: 3 µl prepared DNA, 0.3 µM of each oligonucleotide primer, 0.2 mM dNTP mix, 2mM $MgCl_2$, 2.5 µl of 10x PCR buffer, 1 unit *Taq* DNA polymerase and PCR grade water up to 25 µl. Samples were subjected to 35 cycles of touchdown PCR according to Paton and Paton (1998) (7). The PCR products were electrophoresed on 2% agarose gel for 1.5 hours 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 (as described above) were included in all PCR reactions.

RESULTS

A total number of 18 isolates, which were recovered from 16 cattle, confirmed as non-sorbitol fermenting *Escherichia coli* (NSF) in biochemical tests. None of the strains showed positive results in conventional serotyping for O157 somatic antigen. In serogroup specific PCR assay all of the NSF isolates were also negative for *rfb*O157 gene. Since all of the isolates were negative for O157 serogroup using two different methods, they were not additionally tested for H7 flagellar antigen.

All NSF isolates were subjected to multiplex-PCR for the major virulence genes. Of 18 isolates, two were positive in virulence genes multiplex PCR assay. One isolate harbored the *stx2* and *ehly* genes, and the other one possessed *stx2, eae* and *Ehly* simultaneously.

DISCUSSION

Enterohemorrhagic *E. coli* (EHEC) is a major cause of food-borne diseases, mostly in the United States, Canada, Japan and Europe (10-12). 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 these results to other serovars. For instance, O26, O91 or O113 serovars have been detected in cattle herds and have been reported to be associated with human diseases (13). Absence of routine cultural-based detection methods for non-O157 EHEC means that in many countries accurate data are not available on the prevalence of non-O157 EHEC, and there is considerable underreporting of these pathogens (14). In the present study, 18 NSF *E. coli* were isolated from 180 cattle in Golestan province using pre-enrichment and CT-SMAC selective medium, but the presence of O157: H7 *Escherichia coli* was not confirmed using conventional serotyping and serogroup-specific PCR assay for *rfb* O157gene.

Epidemiological studies in Iran have revealed that the prevalence of EHEC differs between 0.7 to 15% (15, 16). Isolation of EHEC from animal reservoirs in different regions of Iran has already been documented; however, a number of contradictory data are available on prevalence of O157 EHEC in Iran. Isolation of O157: H7 or O157: NM from different sources has been reported in some investigations conducted in Iran (6, 17 - 20). For instance, Jamshidi *et al*. (2008) detected one O157: H7 *E. coli* among 100 ground beef samples in Mashhad by PCR assay (6). In another recent study Rahimi *et al.* (2012) reported 8.2% of 85 tested beef samples to contain O157 EHEC in Khoozestan and Fars provinces. Thirteen out of 14 O157 strains isolated in the mentioned study belonged to O157: NM serotype (17). On the other hand, the results of the present study are in agreement with those studies in which the presence of O157 STEC strains in humans and animals were not documented (15, 21, 22). In the same area (Mazandaran and Golestan provinces), 3268 fecal specimens from the inhabitants were screened for O157:H7 *E. coli* but none of the isolates belonged to this serotype (15). Similarly, in other research, 297 fecal samples of diarrheic and non-diarrheic calves were tested for O26, O111 and O157 antigens, but O157:H7 was not detected (22). Considering the fact that no official outbreak due to O157:H7 has been reported in Iran, it has been suggested that diagnosis and report of O157:H7 *E. coli* should be done with caution (11). Detection of O157 antigenic marker could not solely represent the pathogenicity of the strain, and a number of phenotypic and genotypic

tests are required to establish the pathogenicity of O157: H7 (11). To the best of our knowledge the only well characterized O157:H7 *E. coli* are two strains that were isolated from one-hamped camels in Gonbad-Qabus which were analyzed using DNA-microarray method (20). It should be noted that many factors such as time of sampling, geographical area, and animal species are important criteria in epidemiology of STEC.

In the present study, the presence of major virulence factors of EHEC were investigated among 18 NSF *E. coli* isolates by a multiplex-PCR assay that has been previously validated to be an efficient method for STEC characterization (23). The results showed that only two isolates out of 18 NSF *E. coli* possessed the tested virulence genes. One isolate harbored *stx2* and *ehly* genes, and the other one possessed *stx2*, *eae* and *ehly* genes simultaneously. In fact, 11.1% of NSF isolates recovered from CT-SMAC in the current study were positive for at least one of the tested virulence genes. Nevertheless, most of the non-O157 EHEC strains ferment sorbitol and are sensitive to cefixime/potassium tellurite; therefore, the observed prevalence in the current study cannot reflect the actual prevalence of non-O157 EHEC in the area. It is of note that most HUS-associated EHEC isolates produce Stx2, and presence of both *stx2* and *eae* virulence genes increase the risk for HUS development (2). Stx2 has been found to be approximately 1000 times more toxic than Stx1 for human renal microvascular endothelial cells (24). As mentioned before, in the present study two isolates harbored the *stx2* gene. One of these isolates carried *stx2*, *eae* and *ehly* simultaneously, which represents EHEC pathotype, and the highly pathogenic virulence genes combination (2).

In summary, the most notable finding in the current study is the occurrence of non-O157 EHEC harboring highly pathogenic virulence profile in cattle in Golestan province. The results of the current research and also the previous investigations indicate that future epidemiological studies on STEC in Iran should equally consider the isolation and characterization of both O157 and non-O157 STEC using the proper and validated methodologies.

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