

Isolation and Characterization of Enterohaemorrhagic *Escherichia coli* O157:H7 and EHEC O157:NM from Raw Bovine, Camel, Water Buffalo, Caprine and Ovine Milk in Iran

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Summary

This study was conducted to determine the prevalence rate, virulence genes, and antimicrobial resistance of *Escherichia coli* O157:H7/NM isolated from dairy bovine, ovine, caprine, camel, and water buffalo milk in Iran. From August 2010 to August 2011, a total of 734 raw milk samples from bovine (192), camel (87), water buffalo (109), caprine (208), and ovine (138) were collected from 269 randomly selected herds in four provinces, Iran and they were evaluated for the presence of *Escherichia coli* O157:H7/NM using microbiological culture and PCR. Overall, 17 of 734 raw milk samples (2.3%) were found to be contaminated with *E. coli* O157. The highest prevalence of *E. coli* O157 was found in water buffalo milk samples (5.5%), followed by bovine (3.6%), ovine (1.4%) and caprine (1.0%). *E. coli* O157 was not isolated from any of camel milk samples. Of 17 *E. coli* O157 isolates, 3 were serotype O157:H7 and 14 were serotype O157:NM. All of the 3 *E. coli* O157:H7 isolates were positive for *stx1*, *stx2*, *eae A* and *ehly A* genes. The results of this study show that milk from bovine and water buffalo could be a significant source of enterohaemorrhagic *E. coli* O157:H7 and EHEC O157:NM serotypes in Iran.

Keywords: *Escherichia coli* O 157, Milk, Water buffalo, Bovine, Camel, Ovine, Caprine

İran'da İnek, Deve, Manda, Koyun ve Keçi Çiğ Sütlerinden Enterohaemorrhagic *Escherichia coli* O157:H7 ve EHEC O157:NM İzolasyonu ve Karakterizasyonu

Özet

Bu çalışma İran'da sığır, koyun, keçi, deve ve manda sütlerinden izole edilen *Escherichia coli* O157:H7/NM'nin prevalansını, virulens genlerini ve antimikrobiyal dirençlerini belirlemeyi amaçlamıştır. Ağustos 2010 - Ağustos 2011 arasında İran'da 4 farklı ildeki rastgele seçilen 269 sürüden toplam 734 çiğ süt örneği sığır (192), deve (87), manda (109), keçi (208) ve koyun (138)'den toplandı ve bu örneklerde *E. coli* O157:H7/NM varlığı bakteriyolojik kültür ve PCR ile belirlendi. Genel olarak, 734 çiğ süt örneğinin 17'sinin (%2.3) *E. coli* O157 ile kontamine olduğu bulundu. *E. coli* O157'nin en yüksek prevalans oranı manda sütlerinde (%5.5) bulundu ve bunu sığır (%3.6), koyun (%1.4), ve keçi (%1.0) örneklerindeki oranlar takip etti. Deve sütlerinden ise *E. coli* O157 izole edilmedi. On yedi *E. coli* O157 izolatu arasında, 3'ü serotip O157:H7 ve 14'ü serotip O157:NM olarak belirlendi. *E. coli* O157:H7 izolatlarının üçü de *stx1*, *stx2*, *eae A* ve *ehly A* genlerinin varlığı bakımından pozitif bulundu. Bu sonuçlar İran'da sığır, koyun, keçi, deve ve manda sütlerinin enterohemorajik *E. coli* O157:H7 ve EHEC O157:NM serotipleri bakımından önemli bir kaynak olduğunu ortaya koymaktadır.

Anahtar sözcükler: *Escherichia coli* O157, Süt, Manda, Sığır, Deve, Koyun, Keçi



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INTRODUCTION

Human diseases caused by Shiga toxin-producing *Escherichia coli* (STEC) strains have been reported throughout the world ¹. In developed countries serotype O157:H7/NM represents the major cause of human diseases; however, there have been increasing reports of non-O157 STEC strains associated with gastrointestinal infections ^{2,3}. Domestic and wild animals are the sources of *E. coli* O157, but ruminants are regarded as the main natural reservoirs ¹. Sporadic cases and outbreaks of human diseases caused by *E. coli* O157 have been linked to ground beef, raw milk, meat and dairy products, vegetables, unpasteurized fruit juices and water ⁴. Infections can also be acquired by direct contact with animals and by person-to-person spread ^{5,6}.

Escherichia coli O157:H7 is known with its high virulence and low minimal infectious dose. Multiple virulence factors contribute to the pathogenicity of *E. coli* O157:H7 and include, as a major one, the production of Shiga-like toxins (*stx1-stx2*). These toxins are associated with HC and HUS in humans, mostly children and elderly persons. Like Shiga toxins, the *eaeA* gene that encodes intimin and the *hly* gene that encodes hemolysin are the other main virulence factors ^{7,8}. In addition, the *fliCh7* gene that encodes the flagellar motility is playing a significant role in pathogenicity of the agent ⁹.

Currently, there is limited information regarding the prevalence of *E. coli* O157:H7/NM in raw milk in Iran. The present study was conducted to determine the prevalence rate, and virulence genes, of *E. coli* O157:H7/NM isolated from dairy bovine, camel, water buffalo, caprine, and ovine milk Isfahan, Chaharmahal va Bakhtiari, Yazd and Khuzestan provinces, Iran.

MATERIAL and METHODS

Sampling

Overall, 269 bovine, ovine, caprine, camel, and water

buffalo herds were randomly selected in Isfahan, Chaharmahal va Bakhtiari, Yazd and Khuzestan provinces, Iran. From August 2010 to August 2011 a total of 192 bovine bulk milk samples were collected from 84 commercial dairy herds, 138 ovine bulk milk samples were collected from 66 sheep breeding farms, 208 caprine bulk milk samples were collected from 75 goat breeding farms, 87 camel bulk milk samples were collected from 16 camel breeding farms, and 109 water buffalo bulk milk samples were collected from 28 water buffalo breeding farms. The animals whose milk samples collected for this study were clinically healthy and the milk samples showed physical (color, pH, and density) consistency. The samples were immediately transported to the laboratory in a cooler with ice packs and were processed within one hour of collection.

Microbiological Analyses

Ten mL of each sample was homogenized in 90 mL trypton soya broth (Oxoid) supplemented with novobiocin (20 mg/L, Sigma, Germany) and incubated at 37°C for 18-24 h. A total 100 µl of cultures were plated on Sorbitol MacConkey Agar (Oxoid) plates containing Cefixime-tellurite Supplement (Oxoid). After 24 h incubation at 42°C, sorbitol negative colonies were tested for the O157 antigen by latex agglutination (Oxoid) ¹⁰ and up to five agglutination positive colonies were taken for PCR analysis.

DNA Extraction

Purification of DNA was achieved using a Genomic DNA purification kit (Fermentas, GmbH, Germany) according to the manufacturer's instruction and the total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell ¹¹.

Detection of *fliCh7* Gene by PCR Analysis

All oligonucleotide primers were obtained from a commercial source (Cinna Gen, Iran). In order to determine the H7 (*fliCh7*) gene of *E. coli* O157:H7 strains, PCR analysis was used ¹². The PCR was performed with primers as

Table 1. Primer sequences and predicted lengths of PCR amplification products

Tablo 1. PCR amplikonları için primer dizilimleri ve tahmini uzunlukları

Gene	Primer	Oligonucleotide Sequence (5-3)	Fragment Size (pb)	Reference
<i>stx1</i>	SLT1-F	TGTAAGTGGAAAGGTGGAGTATACA	210	Fratamico et al. ⁹
	SLT1-R	GCTATTCTGAGTCAACGAAAAATAAC		
<i>stx2</i>	SLTII-F	GTTTTTCTCGGTATCCTATTCC	484	
	SLTII-R	GATGCATCTCTGGTCATTGTATTAC		
<i>ehlyA</i>	AE22	ATTACCATCCACACAGACGGT	397	
	AE20-2	ACAGCGTGGTTGGATCAACCT		
<i>eaeA</i>	MFS1-F	ACGATGTGGTTTATTCTGGA	166	
	MFS1-R	CTTCACGTCACCATACATAT		
<i>fliCh7</i>	FLICH7-F	GCGCTGTCGAGTTCTATCGAGC	625	
	FLICH7-R	CAACGGTGACTTTATCGCCATTCC		

described previously (Table 1)⁹ in a final volume of 50 µl containing 1×Reaction Buffer (Fermentas, GmbH, Germany), MgCl₂ (Fermentas, GmbH, Germany), each of the four deoxynucleoside triphosphates (dNTPs) (Fermentas, GmbH, Germany), Taq DNA polymerase (Fermentas, GmbH, Germany), 0.50 µM of primers and 10 µl DNA. DNA amplification reactions were carried out using a DNA thermal cycler (Master Cycler Gradient, Eppendorf, Germany) with the following program: one cycle of 2 min at 94°C, 35 cycles of denaturation at 94°C for 20 s, annealing at 54°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were stained with 1% solution of ethidium bromide and visualized under UV light after gel electrophoresis on 1.5% agarose.

Detection of Virulence Factors by Multiplex PCR Analysis

The *E. coli* O157:H7/NM isolates were screened for the presence of *stx1* (encoding for Shiga toxin 1), *stx2* (encoding for Shiga toxin 2), *eaeA* (encoding for intimin), and *ehlyA* (encoding for enterohemolysin) genes using PCR method^{9,12}. According to Fratamico et al.⁹ (Table 1), multiplex PCR protocol was used to prepare the master mix with a total concentration of 50 µl containing incomplete 1× Reaction Buffer [160 mM (NH₄)₂SO₄, 670 mM Tris-HCl (pH 8.8); 0.1% Tween-20] (Fermentas, GmbH, Germany), 3.0 mM MgCl₂ (Fermentas, GmbH, Germany), 400 µM each of the four deoxynucleoside triphosphates (dNTPs) (Fermentas, GmbH, Germany), 2.5 U Taq DNA polymerase (Fermentas,

GmbH, Germany), 0.50 µM of all primers that were used. Then, 10 µl of DNA was added to reaction mixture. Thermal cycling and gel documentation was carried out as mentioned above. *E. coli* O157:H7 ATCC 35150 and ATCC 43895 strains that carrying five virulence genes such as *fliCh7*, *stx1*, *stx2*, *eaeA* and *ehly* were used as positive controls and DNase free water was used as the negative control (Fig. 1).

Statistical Analysis

Data were transferred to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA). Using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA), a Pearson chi-square test and Fisher's exact two-tailed test analysis was performed and differences were considered significant at values of P<0.05.

RESULTS

Table 2 shows the prevalence of *E. coli* O157:H7/NM isolated from bovine, camel, water buffalo, ovine, and caprine milk in Isfahan, Chaharmahal & Bakhtyari, Yazd and Khuzestan, Iran. Overall, 17 of 734 raw milk samples (2.3%) were found to be contaminated with *E. coli* O157. The highest prevalence of *E. coli* O157 was found in water buffalo milk samples (5.5%), followed by bovine (3.6%), ovine (1.4%), and caprine (1.0%). *E. coli* O157 was not isolated from any of camel milk samples. There were not significant differences in the level of contamination with *E.*

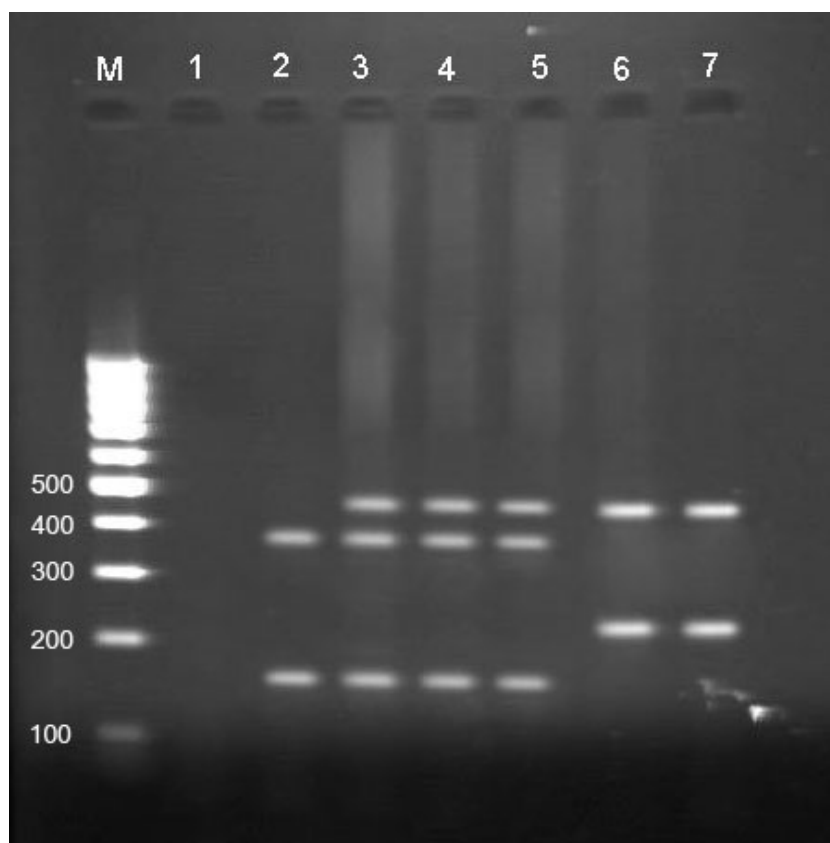


Fig1. Electropherogram of the amplification products of PCR used for the detection of *stx1* (encoding for Shiga toxin 1), *stx2* (encoding for Shiga toxin 2), *eaeA* (encoding for intimin), and *ehlyA* (encoding for enterohemolysin) genes of EHEC isolated from raw water buffalo, camel, bovine, ovine, and caprine milk in Iran. Lane M, molecular size markers (100-bp DNA ladder)

Sekil 1. İran'da manda, deve, sığıır ve keçi çiğ süt örneklerinden izole edilen EHEC'lerin *stx1* (Shiga toksin 1'i kodlar), *stx2* (Shiga toksin 2'i kodlar), *eaeA* (intimin'i kodlar), ve *ehlyA* (enterohemolizin'i kodlar) genlerinin PCR ile yapılan amplifikasyon ürünlerinin elektroforez sonrası resmi. Sıra M, moleküler büyük standardı (100-bp DNA ladder)

Table 2. Prevalence of *Escherichia coli* O157:H7/NM isolated from raw water buffalo, camel, bovine, ovine, and caprine milk in Isfahan, Chaharmahal va Bakhtyari, Yazd and Khuzestan, Iran**Table 2.** Iran'in Isfahan, Chaharmahal va Bakhtyari, Yazd ve Khuzestan illerindeki manda, deve, sığır ve keçi süt örneklerinden izole edilen *Escherichia coli* O157:H7/NM'nin prevalansı

Samples	No. of Samples Examined	<i>E. coli</i> O157	<i>E. coli</i> O157:H7	Virulence Genes				
		No. (%)	No. (%)	<i>Stx</i> ₁	<i>Stx</i> ₂	<i>Stx</i> ₁ , <i>Stx</i> ₂	<i>eaeA</i>	<i>ehlyA</i>
Water buffalo	109	6 (5.5)	1 (0.9)	0	1	1	2	2
Camel	87	0 (0.0)	0 (0.0)	0	0	0	0	0
Bovine	192	7 (3.6)	2 (1.0)	0	1	2	3	2
Ovine	138	2 (1.4)	0 (0.0)	0	0	0	1	0
Caprine	208	2 (1.0)	0 (0.0)	0	0	0	0	0
Total	734	17 (2.3)	3 (0.4)	0	2	3	6	4

coli O157 between different milk samples. Of 17 *E. coli* O157 isolates, 3 were serotype O157:H7 and 14 were serotype O157:NM. Two of the O157:H7 were isolated from bovine milk and 1 was from water buffalo milk samples. All of the 3 *E. coli* O157:H7 isolates were positive for *stx1*, *stx2*, *eaeA* and *ehlyA* genes. From among 14 *E. coli* O157:NM isolates, two, three and one strains were positive for *stx2*, *eaeA* and *ehlyA* genes, respectively.

The highest prevalence of *E. coli* O157 occurred in summer (4.4%), followed by spring (2%), fall (1.3%) and winter (0.0%). No significant differences in the prevalence rates of *E. coli* O157 were observed for ovine and caprine milk samples taken in different seasons; however, significantly higher prevalence rates of *E. coli* O157 ($P < 0.05$) were found in bovine and water buffalo samples taken in summer.

DISCUSSION

Among all the various milk tested in this study, water buffalo and cow milk samples had the highest prevalence rate of *E. coli* O157 and *E. coli* O157:H7, which is comparable with those reported by others. In a large U.S. survey focusing on the presence of *E. coli* O157:H7 in bulk tank bovine milk, only 0.02% of the samples were deemed likely to be contaminated with this organism, but the estimated contamination with other STEC serotypes was reported to be higher¹³. Solomakos et al.¹⁴ examined samples of raw bovine milk in Greece and reported that 2.2% and 0.7% of raw bovine milk samples contained *E. coli* O157 and *E. coli* O157:H7, respectively. Abdul-Raouf et al.¹⁵ reported that 6% of raw cow's milk samples examined in Egypt were contaminated with *E. coli* O157:H7. Perelle et al.¹⁶ examined samples of raw bovine milk in France and reported an overall STEC prevalence of 21% with only 4.8% of the samples containing serogroups of major public health concern including O26, O103, O111, O145 and O157. Oksuz et al.¹⁷ from Turkey, Picozzi et al.¹⁸ from Italy and Cizek et al.¹⁹ from the Czech Republic, reported that 1%, 0.7% and 2.0% raw cow samples were positive for *E. coli* O157, respectively. However, similar studies on raw

cow's milk performed in the UK (Scotland) analyzing 500 samples²⁰, in the Netherlands analyzing 1011 samples²¹, in Greece analyzing 100 samples¹⁰, and in Brazil analyzing 50 samples²² resulted in no *E. coli* O157:H7 isolation. In a study, on Verocytotoxin-producing *E. coli* O26 in raw water buffalo (*Bubalus bubalis*) milk products in Italy, of 160 analyzed samples, 1 (0.6%) tested positive for *E. coli* O26, and all of the isolates were positive for *stx1*, *stx2*, and *hlyA* genes²³. In this study, all of the 5 Shiga-toxin positive and all except one of *eaeA* and *ehlyA* positive isolates were from cattle and water buffalo milk samples. These indicate that cattle and water buffalo are important reservoirs of *E. coli* O157:H7/NM in Iran.

In the present study, 1.4% and 1.0% of ovine and caprine bulk milk samples were *E. coli* O157-positive, respectively, and no *E. coli* O157:H7 strain was isolated. Characterization of *E. coli* O157 isolates from ovine and caprine milk samples by PCR revealed that only one were positive for *eaeA*. This is in agreement with finding of Caro et al.²⁴, Rey et al.²⁵ from Spain, and Dontorou et al.¹⁰ from Greece.

In our study, no *E. coli* O157 strain was isolated from the raw camel milk samples tested. The results of this study show that raw camel milk is not an important source for *E. coli* O157 infection. Although, it has been shown that camel's milk has a bacteriostatic effect against *E. coli*²⁶, further studies are needed to confirm and explore this relationship. Also, in a study on camel fecal samples from the United Arab Emirates, no *E. coli* O157:H7 and other STEC strains was identified²⁷. The presence of other STEC strains was not investigated in that study. In another study, no STEC or anti-*stx* antibodies were detected in fecal and serum samples from 400 camels in five East African countries²⁸.

The sources of *E. coli* O157 in raw milk have been shown to be fecal and environmental contamination during milking, storage and transport, infected animals in dairy farms. High prevalence of *E. coli* O157:H7/NM have been reported in fecal samples from cattle, water buffalo, goat and sheep²⁹⁻³¹. Therefore, the contamination source of *E. coli* O157:H7/NM in raw milk in this study is likely insufficient

hygiene during milking, milk storage or transportation.

In the current study, the highest prevalence of *E. coli* O157 in bovine and water buffalo milk samples occurred in summer, however, no apparent pattern in the seasonality of *E. coli* O157 prevalence was observed in ovine and caprine milk samples. Ferens and Hovde³² reported that prevalence of *E. coli* O157 in cattle peaks between late spring and early fall. Chapman et al.³³ also reported the highest isolation rate of *E. coli* O157 from animals during the summer.

In conclusion, the presence of *E. coli* O157 has been shown in variety of raw milk samples in Iran. The results presented in this study indicate the potential risk of consumption of raw milk or unpasteurized milk and traditional dairy products in Iran. More studies on the occurrence of *E. coli* O157 are needed to establish microbiological criteria of foods in other part of the country. In addition recommendations for *E. coli* O157 in food need to be made by governmental authority.

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