Slov Vet Res
DOI 10.26873/SVR-1940-2024
UDC 579.52:637.12
Early View

Original Research Article

Prevalence of *Escherichia coli* O157:H7 and the Presence of Toxin Genes in Buffalo Feces and Milk

Key words

E. coli O157:H7;
stx toxin gene;
buffalo feces;
buffalo raw milk

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Abstract: *Escherichia coli* (*E. coli*) strains that produce Shiga toxin (*stx*) are among the most important causative agents of foodborne diseases. Cattle are the main reservoir of bacteria that can be transmitted to humans through the contaminated food chain. While Novobiocin-supplemented Tryptone Soy Broth (TSB) was used for bacterial pre-enrichment, the homogenate was first inoculated onto Cefixime Tellurite Sorbitol-MacConkey (CT-SMAC) agar and then onto 4-methylumbelliferyl-β-D-glucuronide (MUG)–Violet Red Bile Agar (VRBA) medium, respectively. Conventional and latex agglutination test methods identified colonies that did not ferment the sorbitol that grew here. Sorbitol-negative *E. coli* was detected in 32 (29.62%) of the buffalo feces samples and 23 (21.29%) of the raw milk samples. While the O157 antigen was detected in 9 (8.33%) of stool samples and 3 (2.77%) of raw milk samples, the H7 antigen was not found. With real-time Polymerase Chain Reaction, *stx* toxin was observed in 21 (18.44%) stool samples and 12 (11.11%) raw milk samples. Despite the absence of the H7 antigen in all of the samples studied, the presence of the O157 antigen and Shiga toxin-producing *E. coli* (STEC) toxin was detected at a high rate. To ensure the raw milk quality, the personnel working effectively and continuously in the milk production line should be trained on food safety/health and hygiene practices. In addition, stricter regulations and strategies should be determined to protect public health.

Received: 18 January 2024
Accepted: 30 December 2024

Introduction

STEC is a bacterium that can cause severe foodborne disease (1). While some strains cause serious infections by producing toxins, *E. coli* species that produce toxins are named as Shiga-toxin *E. coli*, enterohemorrhagic *E. coli*, verocytotoxin-producing (*vtx*) *E. coli* or Shiga toxin-producing *E. coli* (2). Cattle are the main reservoir of bacteria that can be transmitted to humans through the contaminated food chain (1,3). *E. coli* O157:H7 is transmitted to humans primarily through the consumption of contaminated foods such as raw or undercooked meat products and raw milk. Contamination of water, and other food with feces, as well as cross-contamination during food preparation (beef and other meat products, contaminated surfaces and utensils) also cause infection (1). Enteropathogenic bacteria causing foodborne infections constitute 80% of bacterial diarrheal infections. It is estimated that STEC causes 2,801,000 acute illnesses each year and causes 3890 cases of hemolytic uremic syndrome and 230 deaths (4). People infected with *E. coli* O157:H7 may experience a variety of symptoms, but these mostly include stomach cramping, diarrhea (often bloody), and vomiting. Fever is usually not present. In some cases, they have only mild diarrhea or no symptoms at all. For others, the disease is severe and even fatal (5).

Material and methods

Feces (100–125gr) and 108 raw milk samples (200–300ml) from 108 buffaloes (*Anatolian buffalo*) from different businesses in the rural part of Western Anatolia, were taken into sterile bags and brought to the laboratory by cold chain and analyzed within the same day.

For bacterial pre-enrichment, each stool (1g/10ml) and milk (25ml/225ml) sample was homogenized In Tryptone Soy Broth (6) (Oxoid, CM 0129, England) medium with 20mg/L Novobiocin (Oxoid SR 0181E, England) added and incubated at 37°C for 18–24 hours. After the incubation, the homogenate was first

inoculated in Sorbitol Mac-Conkey's Agar (Oxoid CM 0813, England) containing Cefixime-Tellurite Selective Supplement (Oxoid, SR 0172E, England), and then 4-5 colonies were selected from the sorbitol negative (colorless colonies) (7) colonies and inoculated on Violet Red Bile Agar (Oxoid, CM 0978) with MUG added to determine the ß glucuronidase activity (8) at 37°C for18–24 hours.

Typical colonies lacking β-glucuronidase activity were subjected to UV testing (Herolab UVT-20 M, Germany) at a wavelength of 366 nm. In the subsequent phase of the study, non-fluorescent colonies were first analyzed using biochemical tests, followed by the E. coli O157:H7 latex agglutination test (Wellcolex E. coli O157:H7, Remel, UK). Since the kit used in the analysis can detect O157 and H7 antigens separately, the results of the analysis were evaluated accordingly.

DNA Extraction

In the DNA extraction process of the isolated strains, extraction was carried out in accordance with the manufacturer's recommendations by using a commercial kit (GF-1 Bacterial DNA Extraction, Ver. 1.2, Vivantis, Malaysia). The amount and quality of the obtained DNA extracts were measured by spectrophotometer (NanoDrop 2000C, UV–Vis Spectrophotometer, ThermoScientific, US).

Real–time PCR

Real Time-PCR [Enterohemorrhagic *E. coli* (EHEC) Real Time PCR Kit, Rev.No. ZJ0008, Liferiver, Shanghai ZJ Bio-Tech Co., Shanghai, China] studies were performed with DNA extracts obtained from the isolates. The *stx1* gene of the primer pairs used in toxin gene detection consists of 418bp and the *stx2* gene consists of 246bp (Table 1).

Bacterial mix (*stx1/2* genes) and positive control, prepared according to the procedure specified by the manufacturer, were analyzed on the Real Time-PCR (Applied Biosystems® 7500 Real-Time PCR Systems, England) device to investigate the *stx1* and *stx2* genes.

Statistical analysis

Statistical analysis results were performed using a commercial software program (IBM SPSS Statistics, Version 23.0. IBM Corp.; Armonk, NY, USA) and the statistical significance level was accepted as 0.05.

Results

The study included 108 buffalo feces and 108 buffalo raw milk samples from different farms and a total of 216 samples were included in the study. Sorbitol-negative *E. coli* strains were identified in 29.62% of feces and 21.29% of milk samples. While the *stx* toxin gene was detected in 65.62% and 52.17% of sorbitol-negative *E. coli* strains isolated from feces and milk samples, strains without the *stx* gene were also observed (Table 2).

Table 1: Primers used for the detection of gene regions

Genes	Forward (F) / Reverse (R)		Primers	Base pair (bp)	References
stx1	STXA1-598	F-Primary	5'-AGT CGT ACG GGG ATG CAG ATA AAT-3'	418 bp	Bellin et al., 2001 (9)
	STXA1-1015	R- Primary	5'-CCG GAC ACA TAG AAG GAA ACT CAT-3'		
stx2	STXA2-679	F- Primary	5'- TTC CGG AAT GCA AAT CAG TC-3'	246 bp	Bellin et al., 2001 (9)
	STXA2-942	R- Primary	5'-CGA TAC TCC GGA AGC ACA TTG-3'		

Table 2: Presence of the "*stx*" toxin gene in feces and milk samples according to enterprises

Sample	Sorbitol Negative *Escherichia coli*		*stx*							
			Farm							
			a		b		c		d	
Sample Type	**n (+)**	**%**	**n (+)**	**%**	**n (+)**	**%**	**n (+)**	**%**	**n (+)**	**%**
Buffalo stool (n=108)	32	29,62	11	10,18	6	5,55	3	2,77	1	0,92
Buffalo milk (n=108)	23	21,29	7	6,48	3	2,77	1	0,92	1	0,92
Total	55	25,46	18	8,33	9	4,16	4	2,31	2	2,31

The presence of O157 antigen in sorbitol-negative *E. coli* strains obtained from stool and milk samples was detected at 28.12% and 13.12%, respectively, while H7 antigen was not found in either sample. STEC types other than O157:H7 were observed to be present at higher rates. However, *stx* toxin gene was detected in 18.44% of stool samples and 11.11% of milk samples (Table 3). In addition, *stx* toxin gene was detected in strains isolated from 3 milk samples and 8 stool samples in which the O157 antigen was detected. *E. coli* isolates with the *stx* toxin gene were found to have a higher proportion of *stx1/2* toxin genes. Regardless of whether the milk samples were taken from the farm, no significant difference was observed between stx gene positivity detected by the PCR method and their origin ($p > 0.05$).

Table 3: Presence of the "*stx*" in EHEC and *E. coli* O157 strains

Sample Type	Number of Samples (n)	Sorbitol Negative *E. coli* (%)	EHEC *stx* (%)				Latex Agglutination Test (%)	*Escherichia coli* O157 (%)			
			*stx*1	*stx*2	*stx*1/2	Total *stx*		*stx*1	*stx*2	*stx*1/2	Total *stx*
Buffalo stool	108	32(29,62)	2 (1,85)	6 (5,55)	13 (12,03)	21(18,44)	9(8,33)	1(0,92)	3(2,77)	4(3,7)	8(7,4)
Buffalo milk	108	23(21,29)	1 (0,92)	2 (1,85)	9(8,33)	12(11,11)	3(2,77)	0	1(0,92)	2(1,85)	3(2,77)
Total	216	55(25,46)	3(1,38)	8(3,70)	22(10,18)	32(14,81)	12(5,55)	1(0,004)	4(1,85)	6(2,77)	11(0,5)

Discussion

The main reservoir of *E. coli* O157 and non-O157 STEC is cattle, and it is frequently observed in human infections as a zoonotic agent (10). STEC has transformed from a clinically restricted strain into a global public health problem and is a disease agent that is commonly seen in food products in more than 30 countries, with symptoms ranging from asymptomatic infections to severe bloody diarrhea. In some cases, it can be life-threatening (10). Most human foodborne infections are associated with 5 serogroups of Shiga toxin-producing *E. coli* (O157, O26, O103, O145, and O111) (11). The presence of *E. coli* in food is quite important as an indicator of fecal contamination. *E. coli* O157:H7 has been the most common STEC species causing foodborne outbreaks in North America, Europe, and Japan (12), and its importance has increased in recent years.

In a study conducted in Portugal, it was reported that STEC and stx genes were found in 32.5% of cattle feces samples (13). In India, H7 antigen was detected in 7.1% of buffalo feces samples, while verotoxin was identified in 26.9% (10). In Tunisia, H7 antigen prevalence was reported at 4.2% in cattle feces samples, with stx2 and stx1 genes present in 70% and 60% of samples, respectively (14). In Cameroon, the O157 antigen and stx gene were identified in 10.9% of cattle feces samples (15), and in the United Arab Emirates, these were reported in 1.46% of cattle feces samples (16).

While the presence of STEC reported by Ballem et al. (2020) aligns with the findings of our study, the prevalence of O157 antigen appears elevated according to the results of Al-Ajmi et al. (2020) and is similar to those of Akomoneh et al. (2020). Although the H7 antigen was detected by Shinde et al. (2020) and Tayh et al. (2022), it was not identified in our study. Our findings regarding the presence of toxin genes align with the reports from Tayh et al. (2022) and Akomoneh et al. (2020). However, they appear to be higher than those indicated by Shinde et al. (2020) and Al-Ajmi et al. (2020). The ongoing detection of *E. coli* with toxin genes across various regions continues to pose a significant public health concern worldwide.

Milk plays an important role in human nutrition across many countries. Consequently, there has been substantial interest in studies aimed at improving milk quality, particularly its hygienic quality. Bacterial contamination in milk can compromise the quality of raw milk, and enzymes and toxins associated with certain bacterial species that survive pasteurization can pose significant health risks (17).

In Ethiopia, a study found that 19% of 210 raw milk samples from various dairy farms contained E. coli, while 5.2% tested positive for the H7 antigen (18). Similarly, in Turkey, 19.75% of 162 raw milk samples were contaminated with EHEC, 2.46% contained the O157 antigen, and 10.4% tested positive for the toxin gene (19). In Nigeria, 9.38% of 160 raw milk samples showed the presence of E. coli, with 1.86% containing the H7 antigen (20). Pakistan reported H7 in 12% of 100 raw milk samples, with the stx toxin gene present in all isolates (21). In Iran, the O157 antigen was found in 25% of 40 buffalo raw milk samples, with 50% of those isolates testing positive (22). In Egypt, 24% of 25 raw milk samples were positive for EHEC, and 4% for the H7 antigen (23). Furthermore, a study in northwestern Spain indicated that STEC was present in 2.34% of 214 raw milk samples (24).

The prevalence of EHEC in raw milk was found to be significantly higher in studies conducted by Ababu et al. (2020), Aslan et al. (2016), Yakubu et al. (2018), and Rios et al. (2019). In contrast, lower detection rates were reported in the studies by Ranjbar et al. (2018) and Elafify et al. (2020). The presence of the O157 antigen was similarly established at elevated levels in the research conducted by Aslan et al. (2016), Ababu et al. (2020),

Tahira et al. (2017), Ranjbar et al. (2018), and Elafify et al. (2020), compared to the findings of our study; however, Yakubu et al. (2018) reported a lower prevalence.

Regarding the H7 antigen, Ababu et al. (2020), Yakubu et al. (2018), Tahira et al. (2017), and Elafify et al. (2020) documented its presence, whereas our findings align more closely with the studies by Ranjbar et al. (2018) and Aslan et al. (2016), which reported its absence. Furthermore, our results concerning the toxin gene are consistent with those reported by Aslan et al. (2016) and Tahira et al. (2017).

The differences observed in similar studies in different regions are likely to be due to differences in season, geographical location, farm size, number and variety of animals on the farm, hygiene practices on the farm, differences in the sample and sample types studied, and differences in the applied method. The findings show that STEC types other than O157:H7 are more common and the high level of STEC in raw milk suggests that raw milk can potentially transmit infection to humans. Therefore, further studies are needed to determine the prevalence of STEC, as it can also be found in other food samples. A foodborne outbreak was reported in Great Britain in May 2024, in which the toxin genes of non-O157 STEC were determined to be caused by consuming a sandwich containing lettuce (25).

Conclusion

Cattle, which meet people's food needs, are the reservoir of STEC and *E. coli* O157/O157:H7 pathogenic microorganisms that can cause from asymptomatic infection to serious infections and even death in humans. In addition, the determination of O157 antigens in buffaloes and obtaining similar results with studies conducted with cattle samples suggest that buffaloes may also be the reservoir of the O157 isolate. In our study, the presence of *E. coli* O157:H7 in raw milk samples showed, along with similar studies, that the milking process was not carried out by less educated people and farm families in full compliance with the hygiene rules. This situation reveals that raw milk is a serious public health problem. It has shown that in order to ensure the quality of raw milk, there is a need for training on food safety/health issues and hygiene practices related to the dangers of raw milk for the personnel who work effectively and constantly on the milk production line. Additionally, stricter regulations and strategies should be determined to protect public health.

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Razširjenost bakterije Escherichia coli O157:H7 in prisotnost toksinskih genov v bizonjih iztrebkih in mleku

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Izvleček: Sevi *Escherichie coli* (*E. coli*), ki proizvajajo toksin šiga (stx), so med najpomembnejšimi povzročitelji bolezni, ki se prenašajo s hrano. Govedo je glavni rezervoar bakterij, ki se lahko prenesejo na ljudi v kontaminirani prehranski verigi. Medtem ko je bilo za predhodno obogatitev uporabljeno triptonsko sojino gojišče (TSB), dopolnjeno z novobiocinom, je bil homogenat najprej nacepljen na agar CT-SMAC (Cefixime Tellurite Sorbitol-MacConkey) in nato na gojišče VRBA-MUG (Violet Red Bile Agar – VRBA in 4-metilumbelliferil-β-D-glukuronid – MUG). S konvencionalnimi metodami in metodami lateksne aglutinacije so bile ugotovljene bakterijske kolonije, ki niso fermentirale sorbitola. Sorbitol-negativna *E. coli* je bila odkrita v 32 (29,62 %) vzorcih bivoljih iztrebkov in 23 (21,29 %) vzorcih surovega mleka. Antigen O157 je bil odkrit v 9 (8,33 %) vzorcih blata in 3 (2,77 %) vzorcih surovega mleka, antigen H7 pa v nobenem. S polimerazno verižno reakcijo v realnem času je bil toksin stx ugotovljen v 21 (18,44 %) vzorcih blata in 12 (11,11 %) vzorcih surovega mleka. Kljub odsotnosti antigena H7 v vseh proučevanih vzorcih je bila v veliki meri ugotovljena prisotnost antigena O157 in *E. coli*, ki proizvaja toksin šiga (STEC). Za zagotavljanje kakovosti surovega mleka je treba osebje, ki učinkovito in neprekinjeno dela v proizvodnji mleka, usposobiti za higieno in varnost živil. Poleg tega je treba določiti strožje predpise in strategije za varovanje javnega zdravja.

Ključne besede: *E. coli* O157:H7; gen za toksin stx; bizonji iztrebki; bizonje surovo mleko