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Original article

PREVALENCE AND MOLECULAR CHARACTERISATION OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN SHEEP FARMS OF SANANDAJ, IRAN

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Summary

Ghaderi, P., E. Ahmadi, A. M. Farrokhi, F. Moshrefi, A. Rezaei, K. Siavashi, Q. Ghavami, K. Rahmani & A. Sharifi, 2022. Prevalence and molecular characterisation of Shiga toxin-producing *Escherichia coli* in sheep farms of Sanandaj, Iran. *Bulg. J. Vet. Med.* (online first).

Shiga toxin-producing *Escherichia coli* (STEC) strains have emerged as important foodborne pathogens of global public health concern, causing life-threatening diseases. Animals and their products have been documented as important reservoirs for STECs, especially *E. coli* O157. The aim of this study was to investigate STECs from healthy and diarrhoeic sheep in Sanandaj, Iran. In the current study, a total of 81 sheep faecal samples were taken (22 from diarrhoeic sheep and 59 from healthy sheep). *E. coli* and subsequently STEC strains was detected according to standard protocol (cultural characterisation and PCR assays). Finally, the frequency of Shiga-toxin producing gene(s) (*stx1*, *stx2*), intimin (*eaeA*) and enterohaemolysin (*hlyA*) was detected among STEC isolates using duplex PCR. Totally, 42 *E. coli* were isolated from 81 faecal samples (51.85% contamination). Of these, 34 isolates (80.9%) were identified as STEC patotypes based on Sorbitol-MacConkey (SMAC) medium culturing and also the presence of *stx1* and/or *stx2*. Of these, only 3 isolates (7.1%) were identified as serotype O157:H7 based on PCR assay. In addition, the results showed that STEC bacteria were significantly more prevalent in diarrhoeic samples than in healthy samples (50% vs. 22.1%). Overall, the PCR results showed that 33 (97%), 12 (35.3%) and 8 (23.5%) isolates carried *stx1*, stx2 and *hlyA*, respectively. The *eaeA* gene was not found in any isolate. The number of isolated STEC bacteria in spring (10 isolates) and winter (14 isolates) were significantly higher than those in summer (4 isolates) and autumn (6 isolates) (P=0.039). Also, the number of STEC in diarrhoea samples was significantly higher compared to non-diarrhoea samples (P=0.032). In conclusion, the present study revealed high prevalence rate of STEC including serotype O157:H7 and non-O157:H7 in sheep faeces which highlights the importance of sheep as a reservoir of STEC pathogen in Sanandaj region. Therefore, additional control and preventive measures must be undertaken to control the contamination by this pathogen.

Key words: Sanandaj, sheep, Shiga toxin-producing *Escherichia coli*, *stx1*, *stx2*

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) or verocytotoxin-producing *E. coli* (VTEC) strains are considered important pathogens in humans and animals and a major threat to public health (Gyles, 2007). As this bacterium can be transmitted to humans through contaminated food, it is categorised as a foodborne pathogen. In addition to causing food poisoning in humans, these strains have the potential to cause serious infections ranging from bloody diarrhoea to more severe diseases, such as haemolytic-uraemic syndrome (HUS), which is a life-threatening complication especially in children below five years of age (Melton-Celsa *et al.*, 2011; Baker *et al.*, 2016). Although non-O157:H7 serotypes of STEC have the potential to cause HUS, O157:H7 is the most important serotype causing this syndrome (Smith *et al.*, 2014).

The ruminant digestive tract is known as reservoir of STEC strains as well as their predominant pathogenic serotype, O157. STEC has also been isolated from animals such as pigs, dogs, cats, and birds (Badouei *et al.*, 2015). The presence of STEC strains in the intestines of animals can lead to gastrointestinal diseases such as severe diarrhoea and dehydration. As a result, animal deaths due to infection by these pathogens have been repeatedly reported (Klein *et al.*, 2002; Melton-Celsa *et al.*, 2011).

STEC strains have the ability to produce toxins that are similar to the toxin of *Shigella dysenteriae* type I. These toxins include Shiga toxin (*Stx1* and *Stx2*), which are immunologically different. Because of the destructive effect of these toxins on Vero cell culture, they are called VT1 and VT2 and therefore *E. coli* secreting these toxins is called VTEC. Verotoxin affects host ribosomal RNA and inhibits protein synthesis (Melton-Celsa *et al.*, 2011; Singh *et al.*, 2015).

STEC strains are also able to express other important virulence factors, including intimin, an outer membrane protein encoded by *eaeA* gene, and haemolysin A protein encoded by the plasmid *hlyA* gene. Intimin is a 94-kDa outer membrane protein involved in the binding of bacteria to and damage to host intestinal epithelial cells (Franz *et al.*, 2007). Previous studies have shown that the presence of *stx1*, *stx2*, *eaeA* and *hlyA* genes in pathogenic strains is much higher than in normal flora strains. Therefore, the epidemiological study of the presence of these genes is of major importance (Franz *et al.*, 2007).

In addition, pathogenic strains and natural flora have different antibiotic resistance patterns, and pathogenic strains can be the source of antibiotic resistance genes. These strains also have the ability to transfer resistance genes to the normal intestinal flora (Zhao *et al.*, 2001). Therefore, the aim of this study was to investigate the presence of STEC among the sheep (healthy and diarrhoeic sheep) of Sanandaj city. After isolation and confirmation of STEC, the presence of toxin-producing genes (*stx1* and *stx2*) and some virulence genes (*eaeA* and *hlyA*) were examined in STEC strains.

MATERIALS AND METHODS

Sampling

Sampling for the present study was performed for one year from the spring of March 2020 to March 2021 from traditional and industrial sheep farms of Sanandaj city, west of Iran. During the study period, 22 samples were taken from diarrhoeic sheep and 59 samples – from

healthy sheep (81 samples in total). At least 5 g faecal sample was obtained directly from the rectum under sterile conditions. The samples were placed in a sterile container with a lid and immediately transferred to the laboratory.

Isolation and identification of E. coli and STEC

The bacterial isolation was performed according to standard protocol previously described (Sharaf *et al.,* 2017; Jenkins *et al.,* 2020). In details, about 1 g of each faecal sample was mixed in 9 mL of Trypticase Soya Broth (TSB) (Merck, Darmstadt, Germany) and incubated for 6–8 h at 37 °C for 6 h then transferred and plated onto MacConkey agar and incubated for 18–24 h at 37 °C. The final biochemical confirmation of *E. coli* was done using the indole, methyl red, Voges-Proskauer, and citrate tests (IMViC). Subsequently, for identification of STEC, the isolated bacteria were inoculated on CHROM agar (Merck, Darmstadt, Germany) STEC and mauve colony colour was considered as STEC pathotype. In addition, the PCR assay with a specific primer was used to detect serotypes O157 and H7. The confirmed isolates were stored in a –70 °C freezer until further testing. *E. coli* O157:H7 ATCC 43895 was used as positive control for phenotypic and genotypic assays (Dastmalchi *et al.*, 2012).

DNA extraction

To extract DNA, 18-hour bacterial culture in TSB medium was used as DNA source. One milliliter of the culture medium containing the bacteria was centrifuged at 3000 rpm for 5 min, then the bacterial DNA was extracted by commercial DNA extraction kit (SinaClon; Iran) according to the manufactory instructions. The extracted DNA molecules were stored at –70 °C for use as templates in the PCR reactions.

Polymerase chain reaction

The duplex was carried out for detection of STEC serotype and virulence genes, including O157 specific gene, H7 specific gene, *stx1*, *stx2*, *eaeA* and *hlyA*. The oligonucleotide primers are given in Table 1. For each reaction, the final volume of

Table 1. Oligonucleotides used for PCR assay

Genes	Sequence (5′–3′)	Annealing temperature (°C)	PCR product (bp)	Reference
stx1	CAGTTAATGTCGTGGCGAAGG CACCAGACAATGTAACCGCTG	53	348	Cebula *et al.* (1995)
stx2	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC	53	584	Cebula *et al.* (1995)
eaeA	GGGATCGATTACCGTCAT TTTATCAGCCTTAATCTC	53	837	Seker *et al.* (2019)
hlyA	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	53	534	Sekhar *et al.* (2017)
O157	AGCCGATGTCGATGCAATT CATGATTCCAAGCCTTGTGC	63	339	Pan *et al.* (2002)
H7	ACCATCGGTGGAAGCCAG GAAGCATACCCGGCAACAG	63	461	Pan *et al.* (2002)

mixture was 25 µL contained 5 µL of template DNA, 2.5 µL of ×10 PCR buffer, 0.5 µl of 10 mM dNTPs, 0.75 µL of 50 mM $MgCl_2$, 0.25 µL of 5 U/µL of Taq DNA polymerase, and 25 pmol of each used primer (Mohammadzadeh *et al.*, 2017).

The PCR amplification was performed under the following thermal conditions: initial denaturation at 94 °C for 4 min followed by denaturation at 94 °C for 1 min, annealing at 53 °C (duplex *stx1*+ *stx2* and duplex *eaeA* + *hlyA*) and 63 °C for (duplex O157 + H7) for 1 min, extension at 72 °C for 1 min (35 cycles), and a final extension at 72 °C for 10 min. The amplicons were stained with ethidium bromide (0.5 mg/mL) and electrophoresed in 1.5% agarose gel in 1×TBE (Tris base, boric acid and EDTA) buffer at 95 V for 55 min (Mohammadzadeh *et al.* 2017). PCR products were visualised and photographed using UVIdoc gel documentation systems (Uvitec, UK). The PCR products were compared against a 100 bp DNA marker.

Statistical analysis

Statistical analysis was performed using SPSS software. Differences between variables were analysed by Fisher's exact test small-sized samples analysis and Chi-squared test for large-sized samples. P values <0.05 were considered to be statistically significant.

RESULTS

Based on culture on selective and differential culture medium as well as biochemical tests, 42 (51.85%) out of 81 faecal samples were positive for *E. coli* bacteria. Of the 42 *E. coli* isolates, 34 isolates (80.9%) were identified as STEC pathotypes. Of these, 3 isolates (7.1%) were identified as serotype O157:H7. In addition, based on the type of sample, the results showed that 22.1% (13 out of 59) of healthy sheep samples and 50% (11 of 22) of diarrhoeic sheep samples had STEC bacteria.

Based on the number of samples in different seasons, our results showed that the number of isolated STEC bacteria in spring (10 isolates) and winter (14 isolates) were significantly higher than those in summer (4 isolates) and autumn (6 isolates) (P=0.039). Also, based on the results, diarrhoea samples had significantly more STEC bacteria (P=0.032).

Genetic profiles of *E. coli* isolates are shown in Table 2. As seen from PCR results, 33 (97%) and 12 (35.3%) of the 34 STEC isolates had the *stx1* and *stx2* genes, respectively. The *hlyA* gene was present in 8 (23.5%) STEC isolates. In other hand, the *eaeA* gene was not found in any of isolates. The predominant genotype among the tested *E. coli* was *stx1*, *stx2* accounting for 21.4% and 26.5% from total *E. coli* and STEC isolates, respectively.

Table 2. Gene profiles of STEC isolates

Genetic profile	Frequency	From all *E. coli* (%)	From STEC (%)
stx1	16	38.1	47.1
*stx*2	1	2.4	2.9
stx1, *stx2*	9	21.4	26.5
stx1, *hlyA*	6	14.3	17.6
stx1, *stx2*, *hlyA*	2	4.8	5.9

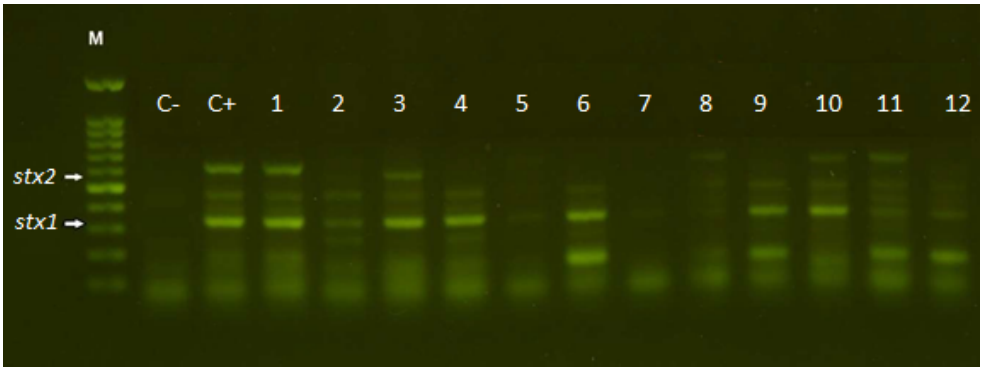

Fig. 1. Gel for representation of duplex-PCR amplification of *stx1* (348 bp) and *stx2* (584 bp). Lane M: GeneRulerTM 100 bp DNA ladder marker. Lane C+: positive control (*E. coli* O157:H7 ATCC 43895); lane C–: negative control; lanes 1–12: some isolates with different genetic profiles.

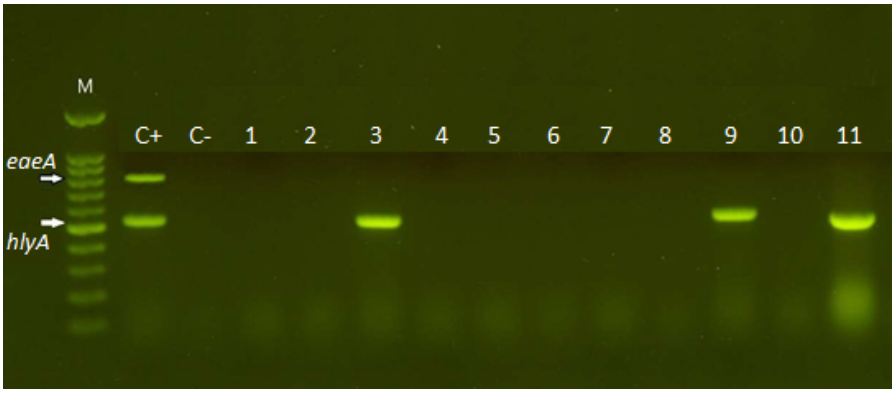

Fig. 2. Gel for representation of duplex-PCR amplification of *eaeA* (837 bp) and *hlyA* (534 bp). Lane M: GeneRulerTM 100 bp DNA ladder marker. Lane C+: positive control (*E. coli* O157:H7 ATCC 43895); lane C–: negative control; lanes 1–11: some isolates with different genetic profiles.

Electrophoresis images of the studied genes are shown on Fig. 1–3.

DISCUSSION

Although STEC can live in the intestines of many warm-blooded animals, cattle and sheep are main reservoir of this bacterium. STEC may be transmitted to humans through these animals and cause various and sometimes fatal infections (Singh *et al.*, 2015; Liu *et al.*, 2022).

Swimming in water contaminated with faeces of infected animals, drinking water from contaminated sources that are mostly used in rural areas, are considered important risk factors (Mehrabiyan *et al.*, 2013). Of course, the route of transmission to humans is not only through direct contact, but also through consumption of contaminated food – one of the most important ways of infection with this bacterium. Consumption of raw or undercooked meat, raw and unpasteurised dairy products can be considered as a source of

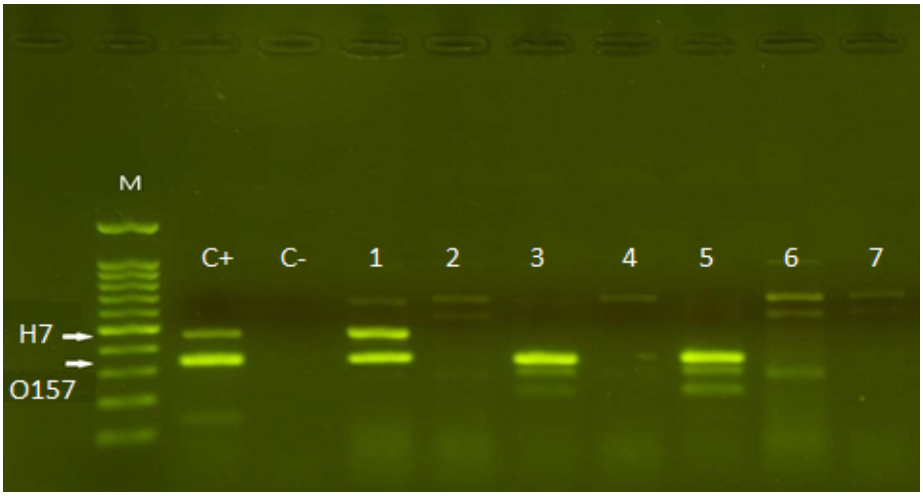

Fig. 3. Gel for representation of duplex-PCR amplification of H7 (461 bp)/O157 (339 bp) detection. Lane M: GeneRulerTM 100 bp DNA ladder marker. Lane C+: positive control (*E. coli* O157:H7 ATCC 43895); lane C−: negative control; lanes 1−7: some different strains.

contamination (McCarthy *et al.*, 2021; Condoleo *et al.*, 2022).

Regarding the country of Iran, it has been shown in previous studies that STEC was encountered with different frequency in types of meat (Zarei *et al.*, 2021) and diarrhoeic stools of Iranian children (Shams *et al.*, 2013).

Based on the results of the present study, 34 isolates (80.9%) of the 42 *E. coli* bacteria obtained from the faecal samples of the studied sheep were identified as STEC patotypes. More importantly, of these bacteria 3 isolates were related to O157:H7 serotype. These findings indicate that sheep raised in Sanandaj could be reservoirs of this bacterium. It should be noted that this number is higher than the cases studied in Iran. For example, in a study performed by Dastmalchi *et al.* (2012) in the West Azerbaijan province, Iran 20.97% of faeces from clinically healthy and diarrhoeic calves were contaminated with STEC pathotype. In another similar study performed on 384 *E. coli* isolated from cow faeces of Shahrekord area, 124 isolates (32.29%) were diagnosed as STEC (Bonyadian *et al.*, 2017). Due to the high percentage of STEC isolates compared to other studies in Iran, further studies in this field are suggested.

In the present study, the frequency of STEC in the faeces of diarrhoeic animals was significantly higher than in healthy animals (P=0.014). This is in agreement with previous studies reported by Leomil *et al.* (2003) (the frequency of carriage of STEC was higher in diarrhoeic calves than in non-diarrhoeic animals, 20% versus 8% respectively, P<0.001) in Sao Paulo and Bonyadian *et al*. (2017) and confirms the pathogenicity potential of the STEC strains.

In the present study, two genes, *stx1* and *stx2* were examined in the STEC strains, and their frequencies were 33 (97%) and 12 (35.3%), respectively. Accordingly, the frequency of *stx1* was significantly higher than that of *stx2*. This

result contradicted findings of Tahamtan *et al*. (2010) and Mohammadzadeh *et al*. (2017). These researchers stated that the prevalence of *stx2* was higher than that of *stx1*. This difference may be due to the animals being studied and the geographical location of the study (Franz *et al.*, 2007).

Another evaluated virulence factor was intimin, which is responsible for attaching/effacing (A/E) lesions on intestinal epithelial cells and completely associated with enterohaemorrhagic colitis and HUS (Al-Ajmi *et al.*, 2020). Data from previous studies have shown that *eaeA* is a clinically important virulence gene, and that the carriage of this gene was associated with the severity of human disease, especially HUS (Franz *et al.*, 2007; Al-Ajmi *et al.*, 2020). Fortunately, the *eaeA* gene was not found in any of the STEC isolates. This result was completely consistent with what Mohammadzadeh *et al*. (2017) reported about the presence of the *eaeA* gene in faecal samples. In their study of pigeon faeces, none of the STEC isolates contained the gene (Mohammadzadeh *et al.*, 2017). The absence of this gene does not mean that the isolates are not virulent or have poor pathogenicity. Nevertheless, it has been shown that the presence of major virulence genes is not essential for pathogenesis, as several sporadic cases of HUS were induced by *Stx* and *eaeA*-negative strains (Ko *et al.*, 2016).

The *hlyA* gene was another gene studied in the present research. The product of this gene is haemolysin A, which is effective on eukaryotic cells and leads to erythrocyte lysis (Islam *et al*., 2008). In the present study, the frequency of this gene in STEC isolates was 8 (23.5%). The high presence of this gene in other studies in Iran has been reported in faecal samples of various animals. This may be related to the fact that *hlyA* gene is encoded by plasmid and therefore can be easily transferred among bacterial isolates (Burgos *et al.*, 2010).

In conclusion, this study revealed high prevalence rate of STEC including serotype O157:H7 and non-O157:H7 among sheep faeces highlighting the importance of sheep as a reservoir of STEC pathogen in Sanandaj region. Besides, the present study showed significant difference (P=0.014) between healthy and diarrhoeic sheep carrying STEC in gastrointestinal tract. Therefore, more serious control and preventive measures must be implemented to control the contamination by STEC in Sanandaj area.

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