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Original Article
Özgün Araştırma

Investigation of virulence factors of shiga toxin-producing *Escherichia coli* strains isolated from sheep*

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Abstract: All over the world, *Escherichia coli* (STEC), which produces Shiga toxin, is recognized as an important zoonotic pathogen. Ruminants, particularly cattle, are their main reservoirs, but the role of small ruminants in the epidemiology of human infections has not been extensively evaluated in many countries. In this study, it was aimed to determine the Shiga toxin subtypes of *Stx1, Stx2, eae* and *ehxA* genes of Shiga toxin producing isolates within *Escherichia coli* strains in sheep and to determine the comparative effect of variables such as race and sex on them. In this study, virulence genes of STEC strains and Shiga toxin subtypes were identified in 215 *E. coli* isolates isolated from 272 rectal swab from 12 different sheep herds in and around Ankara. Of the 215 isolates, only 19 (8.84%) *Stx1*, 3 (1.4%) *Stx2*, 5 (2.33%) *eae* and 9 (4.19%) *ehxA* genes, 13 (6%, 05) *Stx1-Stx2-ehxA*, 5 (2.33%) *Stx1-eae -ehxA*, 3 (1.4%) *Stx2-eae-ehxA* together and 1 (0.47%) *Stx1- eae*, 30 (13.95%) *Stx1- ehxA*, 1 (0.47%) *Stx2- eae*, 15 (6.98%) *Stx2-ehxA* and 10 (4.65%) *eae – ehxA* were detected together. Of the 101 (46.98%) isolates, none of the *Stx1, Stx2, eae* and *ehxA* genes were detected. In stx1 subtyping in sheep, a high rate (82.35%) of *stx1c* was isolated. In *stx2* subtyping *stx2f* (%62,86), *stx2c* (%54,29), *stx2b* (%45,71), *stx2d* (%22,86), *stx2h* (%22,86) subtypes are more common in different combinations. Detected at a high rate. These data indicate the presence of STEC in sheep and their potential to cause serious infection in humans.

Keywords: Sheep, Shiga Toxin Producing *E. coli*, *Stx1, Stx2*, Subtype.

Koyunlardan İzole Edilen Shiga Toksin Üreten *Escherichia Coli* Suşlarının Virülens Faktörlerinin Saptanması

Özet: Tüm dünyada, Shiga toksin üreten *Escherichia coli* (STEC), önemli zoonotik patojen olarak kabul edilmektedir. Başta sığırlar olmak üzere geviş getirenler ana rezervuarlarıdır, ancak küçükbaş hayvanların insan enfeksiyonlarının epidemiyolojisindeki rolü pek çok ülkede kapsamlı bir şekilde değerlendirilmemiştir. Bu çalışmada koyunlarda *Escherichia coli* suşları içerisinde Shiga toksin üreten izolatların *Stx1, Stx2, eae* ve *ehxA* genlerinin shiga toksin alt tiplerini belirlenmesi ve bunlara ırk, cinsiyeti gibi değişkenlerin karşılaştırmalı etkisini belirlemek amaçlanmıştır. Bu çalışmada, Ankara ili ve çevresindeki 12 farklı koyun sürüsünden alından 272 rektal sıvaptan izole edilen 215 *E. coli* izolatlarında STEC suşlarının virulens genleri ile shiga toksin alt tipleri tanımlanmıştır. 215 adet izolattan sadece 19 (%8,84) adet *Stx1*, 3 (%1,4) adet *Stx2*, 5 (%2,33) adet *eae* ve 9 (%4,19) adet *ehxA* geni, 13 (%6,05) adet *Stx1-Stx2-ehxA*, 5 (%2,33) adet *Stx1-eae -ehxA*, 3 (%1,4) adet *Stx2-eae-ehxA* birlikte ve 1(%0,47) adet *Stx1- eae*, 30 (%13,95) adet *Stx1- ehxA*, 1(%0,47) adet *Stx2- eae*, 15 (%6,98) adet *Stx2-ehxA* ve 10 (%4,65) adet *eae – ehxA* birlikte tespit edilmiştir. 101 (%46,98) adet izolattan ise *Stx1, Stx2, eae* ve *ehxA* genlerinden herhangi biri tespit edilmemiştir. Koyunlarda *stx1* alt tiplendirmesinde ise yüksek oranda (%82,35) *stx1c* izole edilmiştir. *Stx2* alt tiplendirmesi de ise sırasıyla *stx2f* (%62,86), *stx2c* (%54,29), *stx2b* (%45,71), *stx2d* (%22,86), *stx2h* (%22,86) alt tipleri farklı kombinasyonlarda daha yüksek oranda tespit edilmiştir. Bu veriler koyunlarda STEC varlığını ve insanlarda ciddi enfeksiyona neden olma potansiyellerinin olduğunu göstermektedir.

Anahtar kelimeler: Alt Tip, Koyunlar, Shiga Toksin Üreten *E. coli* (STEC), *Stx1, Stx2*

Introduction

Shiga toxin (Stx), which was characterised as a virulence factor of *Shigella dysenteriae* by Japanese microbiologist Dr. Kiyoshi Shiga in the 19th century, was also found to be produced by *Escherichia coli (E. coli)* isolates (Amézquita-López et al., 1999). *E. coli*, which are also called Shiga toxin-producing *Escherichia coli* (STEC), cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) and emerged as a global public concern in 1980. Since then, STEC strains have been defined worldwide as a major foodborne public health concern (Jajarmi et al.,

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** Prof. Dr. Murat YILDIRIM danışmanlığında hazırlanan "Koyunlardan İzole Edilen Shiga Toksin Üreten Escherichia coli Suşlarının Virülens Faktörlerinin Araştırılması" başlıklı Doktora tezden türetilerek hazırlanmıştır.*

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2017). STEC infections, especially in children and the elderly, cause serious complications ranging from severe diarrhea outbreaks, toxin bloody diarrhea and hemorrhagic colitis to HUS, renal failure and neurological problems (Mühlen et al., 2020). STEC also causes serious gastrointestinal diseases, especially in industrialised countries (Kaper et al., 2014). Bacteria are usually spread by eating contaminated foods, notably undercooked meat, beef products, raw vegetables, sprouts and seeds (Caprioli et al., 2014).

Sheep, which have been meeting the needs of people with their meat, milk, fleece and skin for centuries, are raised in almost every region in Türkiye as well as all over the world. Although sheep breeding has an important place in Turkish economy, it has not reached the desired level yet. Considering the climate change and increasing population, it is thought that breeding sheep, which can be benefited from their meat, milk, fleece, hair and leather will increase in meeting the people's needs since sheep breeding can put inefficient pastures and fallow, stubble and areas that are not suitable for crop production and cannot be utilised by other species to good use. According to TURKSTAT data, there were 45.1 million ovine animals in Türkiye in 2019, 75% of which were sheep and 25% of which were goats. The first 5 provinces with the highest number of sheep and goats are Van, Konya, Şanlıurfa, Diyarbakır and Ankara respectively. These five provinces constitute 22% of Türkiye's ovine livestock. The production of meat in Türkiye is costly. Red meat consumption per capita in Türkiye is lower than in developed countries. When the red meat consumption in the last five years is analysed, it is seen that annual consumption per capita, which was 12.4 kgs in 2012, was 12.9 kgs in 2014 and increased to 14.1 kgs in 2017. In 2018, red meat consumption per capita was 14.84 kgs, 13.3 kgs of which was beef and 1.5 kgs was ovine meat. The countries with the highest per capita consumption of ovine meat are Australia with 8.5 kgs and Uruguay with 6.6 kgs. While the global average of ovine meat consumption is 1.7 kgs, in the United States, it was 0.4 kgs. (TEPGE, 2021).

STECs are present in the intestinal flora of many animals without any signs of symptoms and these animals spread the agent with their feces. While it is accepted that cattle are the primary reservoir of STECs (Ferhat et al., 2019), STEC vector rate of the sheep are non-negligibly high that the prevalence of fecal infection reported in various parts of the world can reach as high as 40% (Blanco et al., 2003; Chapman et al., 1997; Kudva et al., 1996; Kudva et al. 1997; Ogden et al. 2005). Even some studies prove that the prevalence of STEC in sheep feces is higher compared to cattle feces (Amézquita-López et al., 1999; Momtaz et al. 2013; Mora et al., 2011). There are few studies on the prevalence of *E. coli* O157:H7 in sheep and lamb feces in Türkiye (Göncüoğlu et al., 2010; Gökçe et al., 2010; Gülhan, 2003; Türütoğlu et al., 2007). Analysing the main virulence characteristics of STEC isolates in sheep may shed light on the epidemiology and pathogenicity of foodborne STEC infections in humans.

In epidemiological studies towards the infections such as bloody diarrhea, hemorrhagic colitis and HUS in humans report that STEC, along with the primary reservoirs, can also cause infections with the sheep reservoirs (Belanger et al., 2011; Brandal et al., 2012; Ferhat et al., 2019). Within the scope of flock health and management; mastitis, foot diseases and neonatal septicemia are diseases that should be taken into consideration, but determining the virulence factor of the pathogens that play a role in the etiology of these diseases should be the first step before implementing prevention and control practices for these diseases. In this study, the determination of the *stx1, stx2, eae and exhA* genes and Shiga toxin subtypes of Shiga toxin-producing isolates among *Escherichia coli* strains that may adversely affect flock health of the sheep and indirectly cause economic loss and the examination of the presence of STECs depending on race and sex by identifying STEC identification of isolates and important virulence genes from recto anal mucosal swabs collected from the production units in Ankara were aimed.

Materials and Methods

Samples: Rectal swabs collected from 272 sheep in 12 sheep flocks in and around Ankara were used as a samples in the study. Rectal swab samples were obtained by entering 3-5 cm into the anus with a swab and rubbing the rectal mucosa with circular movements. The swabs were brought to the laboratory in tubes with Cary-Blair transport medium in cold chain in a short time.

Isolation and identification: Swabs brought to the laboratory in Cary-Blair transport medium were inoculated on Mac Conkey Agar (Conda,1052) and incubated at 37 °C for 18-24 hours (Martins et al., 2015). After incubation, one of the large, red colonies was selected and inoculated in Nutrient Broth (Oxoid, CM0001) and incubated at 37 °C for 24 hours. Isolates grown in Nutrient Broth were pas-

saged onto Nutrient Agar (MERCK,105450) and incubated at 37 °C for 24 hours. The pure colonies were subjected to identification with both conventional and rapid diagnostic kit (BBL Crystal).

DNA isolation: In the result of the identification, one full sample of *E. coli* positive colony was taken and suspended in tubes containing 100 µl sterile distilled water. For DNA isolation, they were incubated in a 95 ºC hot plate for 10 minutes. Then centrifuged at 10.000 rpm for 2 minutes. The supernatants were transferred to sterile tubes via an automatic pipette and used for PCR test. Before the PCR step, the amounts of DNAs were measured in ng/mL with a nano-drop spectrophotometer (Dallenne et al., 2010). The concentration of DNAs used in PCR was adjusted to 100 ng/ml.

Primers: Among the primers used in the study; *stx1, stx2, eae ehxA* primers taken from the study by Paton et al. (2002); *stx1a, stx1c, stx1d* primers from the study by Scheutz et al. (2012) and *stx1e* primer from the study by Probert et al. (2014) were taken as reference. For *stx2a, stx2b, stx2c, stx2d, stx2e, stx2f, stx2g* gene regions, the study by Scheutz et al. (2012); for *stx2h*, the study by Bai et al. (2018) and for stx2k, the study by Yang, et al. (2020) were taken as reference. The accession numbers FN252457 for *stx2i*, MZ571121 for *stx2j* and AM904726 for *stx2l* were used and designed using NCBI PRIMER-BLAST. Self-mapping rations were checked using the Oligonucleotide Properties Calculator program. Primers used for the detection are shown in Table 1. Ready lyophilised primers synthesised in HPLC purity were reconstituted with Nuclease-Free Water (NEB, B1500S) to 100 pmol/µl as recommended in the synthesis report of the supplying company and stored frozen. For PCR analyses, primers were prepared and used at concentrations of 10 pmol/µl.

PCR: Multiplex PCR technique was used to investigate the presence of some virulence genes in phenotypically determined *E. coli* isolates. To detect the *stx1, stx2, eae* and *ehxA* genes; by using 1 µl, 2 µl of DNA, 4 µl of Mastermix (Solis Bio Dyne, 04-12-00S25) and 6 µl of Nuclease-Free Water (NEB, B1500S) from each primer in 20 µl volume; and to subtype *Stx1*, by using 1 µl, 2 µl DNA, 4 µl Mastermix (Solis Bio Dyne, 04-12-00S25), and 6 µl Nuclease-Free Water (NEB, B1500S) from each *stx1a, stx1c, stx1d* and *stx1e* primers, in 20 µl final volume PCR protocol was prepared. 3 different PCR protocols were used for *stx2* subtyping. First, to detect *stx2a, stx2b, stx2c, stx2d, stx2e, stx2g* genes, PCR was prepared in a final volume of 20 µl final volume, using 0.5 µl, 2 µl DNA, 4 µl Mastermix (Solis Bio Dyne, 04-12-00S25) and 6 µl Nuclease-Free Water (NEB, B1500S) from each primer. Then, to detect *stx2f, stx2h, stx2j* and *stx2k, stx2l, stx2i* genes, PCR was prepared in 20 µl volume using 0.5 µl, 2 µl DNA, 4 µl Mastermix (Solis Bio Dyne, 04-12-00S25) ad 11 µl Nuclease-Free Water from each primer with different binding temperatures.

Multiplex PCRs for determination of *stx1, stx2, eae* and *ehxA* genes and *Stx1* subtyping were started with initial denaturation at 95°C for 3 min. Denaturation at 95 °C for 20 seconds, binding at 64 °C for 30 seconds, extension at 75 °C for 45 seconds in 25 cycles and final extension at 72 °C in 5 minutes were applied. The same PCR conditions with different binding temperatures were used to determine Stx2 subtypes. The binding temperature was 60°C for *stx2a, stx2b, stx2c, stx2d, stx2e, stx2g* genes, 52°C for *stx2f, stx2h, stx2j* genes and 55°C for *stx2k, stx2l, stx2i* genes. All PCR steps were performed in iCycler (BioRad T100 PCR) thermal cycler.

Electrophoresis: To be used in the electrophoresis step, 2 grams of agarose was weighed and 100 ml of 1xTAE (ThermoFisher,15558042) was added, mixed and heated in a microwave oven until homogenised. The mixture was carefully poured into the gel dish in such a way that no bubbles were formed. The combs forming the loading dents were placed in the gel mould and allowed to cool at room temperature for 15-20 minutes. The cooled gel was removed from the mould and placed in the electrophoresis tank. The electrophoresis tank was filled with 1xTAE to cover the gels and 5 µl of PCR products were placed into each well with an automatic pipette. A 3 µl 100 bp DNA ladder (Solis Bio Dyne, 07-11-0000S / Ampliqon, A0610341) was placed in the wells on the agarose gel at certain intervals and subjected to electrophoresis at 100 Volt for 90 minutes (BioRad, USA). Agarose gels were kept in a solution prepared with Ethidium Bromide and 1xTAE (ThermoFisher,15558042) for 15 minutes and then evaluated under UV light using a Quantum ST4 gel imaging system.

Table 1. Primers used in the study

Gene	Sıra (5'-3 ')	Target gene (bp)	Referans
Stx1-F	ATAAATCGCCATTCGTTGACTAC	*Stx1 (180)*	Paton et al., 2002
Stx1-R	AGAACGCCCACTGAGATCATC		
Stx2-F	GGCACTGTCTCTCTGAAACTGCTCC	*Stx2 (255)*	
Stx2-R	TCGCCAGTTATCTGACATTCTG		
Eae-F	GACCCGGCACAAGCATAAGC	*Eae (384)*	
Eae-R	CCACCTGCAGCAACAAGAGG		
EhxA-F	GCATCATCAAGCGTACGTTCC	*EhxA (534)*	
EhxA-R	AATGAGCCAAGCTGGTTAAGCT		
Stx1a-F1	CCTTTCCAGGTACAACAGCGGTT	*Stx1a (478)*	Scheutz et al., 2012
Stx1a-R2	GGAAACTCATCAGATGCCATTCTGG		
Stx1c-F1	CCTTTCCTGGTACAACTGCGGTT	*Stx1c (252)*	
Stx1c-R1	CAAGTGTTGTACGAAATCCCCTCTGA		
Stx1d-F1	CAGTTAATGCGATTGCTAAGGAGTTTACC	*Stx1d (203)*	
Stx1d-R2	CTCTTCCTCTGGTTCTAACCCCATGATA		
Stx1e-seq-F3	TTTGTTACGGTGACAGCCGA	*Stx1e (141)*	Probert et al., 2014
Stx1e-seq-R3	CTCAGCCTTCCCCAGTTCAG		
Stx2a-F2	GCGATACTGRGBACTGTGGCC	*Stx2a*	Scheutz et al., 2012
Stx2a-R3	CCGKCAACCTTCACTGTAAATGTG	*(349)*	
Stx2a-R2	GCCACCTTCACTGTGAATGTG	*(347)*	
Stx2b-F1	AAATATGAAGAAGATATTTGTAGCGGC	*Stx2b (251)*	
Stx2b-R1	CAGCAAATCCTGAACCTGACG		
Stx2c-F1	GAAAGTCACAGTTTTTATATACAACGGGTA	*Stx2c (177)*	
Stx2c-R2	CCGGCCACYTTTACTGTGAATGTA		
Stx2d-F1	AAARTCACAGTCTTTATATACAACGGGTG	*Stx2d*	
Stx2d-R1	TTYCCGGCCACTTTTACTGTG	*(179)*	
Stx2d-O55-R	TCAACCGAGCACTTTGCAGTAG	*(235)*	
Stx2d-R2	GCCTGATGCACAGGTACTGGAC	*(280)*	
Stx2e-F1	CGGAGTATCGGGGAGAGGC	*Stx2e (411)*	
Stx2e-R2	CTTCCTGACACCTTCACAGTAAAGGT		
Stx2g-F1	CACCGGGTAGTTATATTTCTGTGGATATC	*Stx2g (573)*	
Stx2g-R1	GATGGCAATTCAGAATAACCGCT		
Stx2f-F1	TGGGCGTCATTCACTGGTTG	*Stx2f (424)*	
Stx2f-R1	TAATGGCCGCCCTGTCTCC		
Stx2h-F1	AGATCTCATTCTTTATATG	*Stx2h (146)*	Bai et al., 2018
Stx2h-R1	TCCCCATTATATTTAGAG		
Stx2j-F1	GAGAGGATGGCGTCAGAGTG	*Stx2j (97)*	This study
Stx2j-R1	GTCCCTGATGATGGCAGTT		
Stx2k-F1	GCGTTCTGTTCGCGCT	*Stx2k (387)*	Yang, et al., 2020
Stx2k-R1	ACTGGACTTGATTGTGACT		
Stx2l-F1	TGGTGTCAGAGTGGGGAGAA	*Stx2l (92)*	This study
Stx2l-R1	GCGTCCCCTGATGATGACAA		
Stx2i-F1	TTTGTAACCGTCACAGCCGA	*Stx2i (187)*	
Stx2i-R1	TTCTCCCCACTCTGACACCA		

Results

In the study, rectal swabs obtained from 272 healthy sheep from 12 different sheep flocks in Ankara were used. 215 *E. coli* were isolated and identified from rectal swabs.

Among 215 isolates, only 19 (8.84%) *Stx1*, 3 (1.4%) *Stx2*, 5 (2.33%) *eae* and 9 (4.19%) *ehxA* genes were detected. Of the isolates, 13 (6.05%) *Stx1-Stx2-ehxA*, 5 (2.33%) *Stx1-eae -ehxA*, 3 (1.4%) *Stx2-eae-ehxA* were detected together. 1 (0,47%) *Stx1- eae*, 30 (13,95%) *Stx1- ehxA*, 1 (0,47%) *Stx2- eae*, 15 (6,98%) *Stx2-ehxA* and 10 (4,65%) *eae- ehxA* were detected together. None of *Stx1, Stx2, eae* and *ehxA* genes were detected in 101 (46.98%) isolates. (Figure 1).

Among 68 isolates in which *stx1* gene was detected, only *stx1c* was detected in 56 (82.35%), *stx1a-stx1c* together in 3 (4.41%), *stx1c-stx1e* together in 2 (2.94%) and *stx1a- stx1c-stx1e* together in 1 (1.47%). In 6 isolates, none of these four gene regions were detected. (Figure 2).

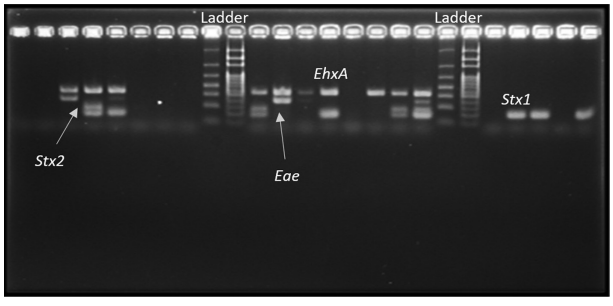

Figure 1. Gel image of *stx1, stx2, eae, ehxA* genes

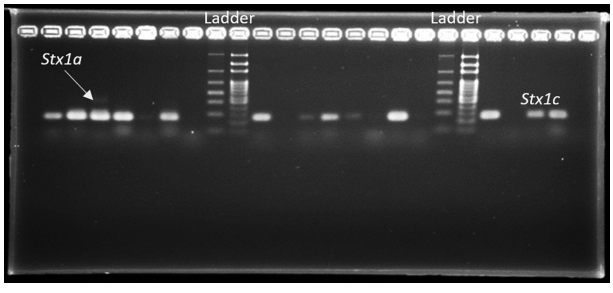

Figure 2. Gel image of *stx1* subtyping

Among 35 *stx2* isolates, 2/35 (5.71%) had *stx2a,* 16/35 (45.71%) had *stx2b*, 19/35 (54.29%) had *stx2c*, 8/35 (22.86%) had *stx2d*, 2/35(5,71%) had *stx2e*, 22/35(62,86%) had *stx2f,* 8/35(22,86%) had *stx2h*, 2/35(5,71%) had *stx2j*, 3/35(8,57%) had *stx2l*. In 8 of them, none of the investigated subtypes could be detected. (Figure 3 and Figure 4). *Stx2* subtyping results are shown in Table 5.

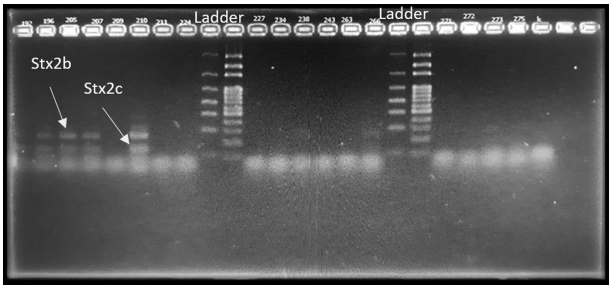

Figure 3. Gel image for *Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2g* subtypes

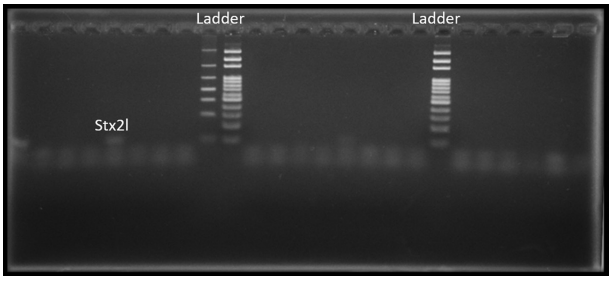

Figure 4. Gel image for *Stx2k, Stx2l, Stx2i* Subtypes

In this study, Pearson chi-square test was performed to evaluate the effect of breed and sex on STEC prevalence in sheep using Rstudio software. Two separate hypotheses were made on the isolated STECs and the effect of breed and sex were investigated. The first hypothesis was formed to determine the effect of *stx1, stx2, eae* and *ehxA* genes on sex, and it was concluded that virulence genes had no effect on sex with a confidence interval of 0.05, that is, they were independent. In our second hypothesis, it was analysed whether stx1, stx2, *eae* and *ehxA* genes have an effect on breeds (Akkaraman, Merino) and a dependency relationship was determined. Within this scope, a new hypothesis was formed to find out between which species there is a dependency relationship. As a result, it was concluded that both breeds (Akkaraman, Merino) were dependent at 0.05 confidence interval, that is, both breeds had a dependence relationship on genes and there was no difference between the breeds. The distribution by race and gender is shown in Table 2 - 3.

In the subtyping of the isolates in which stx1-stx1-ehxa genes were detected together, *stx1c-stx2b-stx2c-stx2f-stx2h-ehxA* genes were detected together more frequently. The distribution of the co-detected *Stx1-Stx2-EhxA* genes is shown in Table 4.

Table 2. Distribution of virulence genes by sex

Gender	*Stx1*	*Stx2*	*Eae*	*EhxA*	none
Female	31	17	9	34	54
Male	38	18	16	50	46

Table 3. Distribution of virulence genes according to species

Race	*Stx1*	*Stx2*	*Eae*	*EhxA*	none
Akkaraman	39	12	19	40	47
Merinos	29	23	6	44	54

Table 4. Distribution of co-detected *Stx1-Stx-ExhA* genes

Sample No	101	116	137	138	187	188	192	201	205	209	210	234	275
Stx1-Stx2-ExhA gene distribution	Stx1c	Stx1c	Stx1c	Stx1c	Stx1c	Stx1a	Stx1c	Stx1c	Stx1c	Stx1c	Stx1c	Stx1c	Stx1c
	Stx2b	Stx2b	Stx2b	Stx2c	Stx2b	Stx1c	Stx2c	Stx2c	Stx2b	EhxA	Stx2a	Stx2f	Stx2f
	Stx2f	Stx2c	Stx2c	EhxA	Stx2c	Stx2b	EhxA	EhxA	Stx2c		Stx2b	EhxA	EhxA
	EhxA	Stx2f	Stx2d		Stx2d	Stx2c			Stx2f		Stx2c		
		EhxA	Stx2f		Stx2f	Stx2d			Stx2h		Stx2f		
			EhxA		Stx2h	Stx2f			EhxA		Stx2h		
					EhxA	Stx2h					EhxA		
						EhxA							

Table 5. *Stx2* subtyping results

No	*Stx2a*	*Stx2b*	*Stx2c*	*Stx2d*	*Stx2e*	*Stx2f*	*Stx2g*	*Stx2h*	*Stx2i*	*Stx2j*	*Stx2k*	*Stx2l*
21	-	+	-	-	-	-	-	-	-	-	-	+
35	-	-	+	+	-	+	-	-	-	+	-	-
37	-	-	-	-	-	-	-	-	-	-	-	-
47	+	-	+	-	-	+	-	-	-	-	-	+
85	-	-	-	-	-	-	-	-	-	-	-	-
101	-	+	-	-	-	+	-	-	-	-	-	-
116	-	+	+	-	-	+	-	-	-	-	-	-
122	-	+	+	-	-	+	-	-	-	-	-	-
124	-	-	-	-	-	-	-	-	-	-	-	-
137	-	+	+	+	-	+	-	-	-	-	-	-
138	-	-	+	-	-	-	-	-	-	-	-	-
141	-	+	+	+	-	+	-	-	-	-	-	+
154	-	-	-	-	-	+	-	-	-	+	-	-
156	-	-	-	-	-	+	-	-	-	-	-	-
158	-	-	-	-	-	-	-	-	-	-	-	-
160	-	-	-	-	-	+	-	-	-	-	-	-
161	-	+	+	+	+	-	-	-	-	-	-	-
168	-	+	+	-	+	+	-	-	-	-	-	-
172	-	+	+	+	-	-	-	-	-	-	-	-
180	-	-	+	-	-	+	-	-	-	-	-	-
187	-	+	+	+	-	+	-	+	-	-	-	-
188	-	+	+	+	-	+	-	+	-	-	-	-
192	-	-	+	-	-	-	-	-	-	-	-	-
196	-	+	+	-	-	+	-	+	-	-	-	-
201	-	-	+	-	-	-	-	-	-	-	-	-
205	-	+	+	-	-	+	-	+	-	-	-	-

No	*Stx2a*	*Stx2b*	*Stx2c*	*Stx2d*	*Stx2e*	*Stx2f*	*Stx2g*	*Stx2h*	*Stx2i*	*Stx2j*	*Stx2k*	*Stx2l*
207	-	+	+	-	-	+	-	+	-	-	-	-
209	-	-	-	-	-	-	-	-	-	-	-	-
210	+	+	+	+	-	+	-	+	-	-	-	-
234	-	-	-	-	-	+	-	-	-	-	-	-
250	-	-	-	-	-	-	-	+	-	-	-	
251	-	-	-	-	-	-	-	-	-	-	-	-
253	-	-	-	-	-	-	-	-	-	-	-	-
266	-	+	-	-	-	+	-	-	-	-	-	-
275	-	-	-	--	-	+	-	-	-	-	-	-
Total	2	16	19	8	2	21	0	7	0	2	0	3

Discussion and Conclusion

The STEC pathotype of *E. coli*, commonly found in the gastrointestinal tract, is an important foodborne pathogen that has been causing public health problems worldwide since the 1980s. STEC is important for red meat production due to its potential to cause serious diseases that can progress from simple diarrhea to HC, HUS, end-stage renal failure and death (Cody et al., 2019). 2019 Although cattle are considered to be the main reservoir for STEC and EPEC strains, the presence of STEC and EPEC strains in sheep is not negligible (Ferhat et al., 2019; Hu et al., 2022). Determination of the main virulence factors in sheep, which are asymptomatic carriers of STEC strains, may elucidate the epidemiology and pathology of foodborne STEC infections in humans. In this study, *E. coli* was detected in 215 (79%) of 272 samples taken from 12 different sheep flocks in Ankara. The number of positive PCRs for *stx1, stx2, eae* and *ehxA* genes by multiplex-PCR was 114 (53.02%). In this study, STEC prevalence in sheep was recorded as 36.74% and EPEC prevalence was 11.62%. ETEC strains are the primary pathotype responsible for HC and HUS. Intimin plays an important role in the development of HUS (Caprioli et al., 2005). In this study, 6 isolates with *stx1* and *eae* virulence gene combinations of ETEC and 4 isolates with stx2 and *eae* combinations were detected and *stx1-eae* combination was found more frequently. This is consistent with the study conducted in Brazil (Gonzalez et al., 2016). High STEC prevalence in sheep has also been reported in different studies (Beutin et al., 1993; Martins et al., 2015). The higher prevalence of STEC in this study may be due to inadequate care and feeding conditions.

Out of 215 isolates, only 19 (8.84%) Stx1, 3 (1.4%) Stx2, 5 (2.33%) *eae* and 9 (4.19%) *ehxA* genes were detected. This result is lower than the rate reported by Martins et al. (2015) (*Stx1* 52.8%, *Stx2* 14.3%). Of the isolates, 13 (6.05%) *Stx1-Stx2-ehxA*, 5 (2.33%) *Stx1-eae -ehxA*, 3 (1.4%) *Stx2-eae-ehxA* were detected together. 1 (0,47%) *Stx1- eae,* 30 (13,95%) *Stx1- ehxA*, 1 (0,47%) *Stx2- eae*, 15 (6,98%) *Stx2-ehxA* and 10 (4,65%) *eae- ehxA* were detected together. This result was different from that of Djordjevic et al. (2001) in mother sheeps (*Stx1-Stx2-ehxA*1.53%, *Stx1-eae-ehxA*3.94%, *Stx2-eae-ehxA*0%, *Stx1-eae* 3.87%, *Stx1- ehxA* 24.23%, *Stx2-eae* 0.22%, *Stx2-ehxA* 0.11%, *eae- ehxA* 0.44%). In the study conducted by Han et al. (2022) in Jiangsu, China, among the STEC isolates obtained from sheep, *Stx1* (69.1%), *Stx2* (29.4%) and *Stx1-Stx2* (1.47%) were detected and in this study, only *Stx1* (8.84%) and *Stx2* (1.4%) are higher. However, in all positive isolates, *Stx1* was 40.27% and *Stx2* was 30.70%. In this study, the rate of detection of *Stx1* and *Stx2* genes alone was low, but the rate of co-occurrence with other genes was higher than other studies. STECs carrying both *eae* and *stx2* have been associated with severe HUS cases (Hua et al., 2020), and these two gene regions were detected together in 4 isolates in this study. All these studies suggest that *Stx1* may be the dominant gene in STECs isolated from sheep. None of the *Stx1, Stx2, eae* and *ehxA* genes were detected in 101(46.98%) isolates.

Among 68 isolates in which *stx1* gene was detected, 56 (82.35%, 56/68) isolates had only *stx1c* and 3 (4.41%, 3/68) isolates had *stx1a-stx1c* together, 2 (2.94%, 2/68) isolates had *stx1c-stx1e* together and 1 (1.47%, 1/68) isolate had *stx1a-stx1c-stx1e* together. It is known that *stx1c* subtype is frequently isolated among sheep (Blanco et al., 2003; Brett et al., 2003; Martin et al., 2011; Vettorato et al., 2003). In the study conducted by Liu et al. (2022), 64.3% *stx1c* was found; in this study, on the other hand, 82.35% was found. In the same study, *stx1a* was found as

35.7% and in our study, *stx1a* was detected at a low rate together with other genes as *stx1a-stx1c*, *stx1a-stx1c-stx1e*. In 6 isolates, none of these four gene regions were detected. In the study by Han et al. (2022), 41.2% of *stx1c* was detected, which supports that *stx1c* is the dominant subtype in sheep. Other studies support the idea that sheep are the main reservoir of *Stx1c* subtype (Brett et al., 2003; Liu et al., 2022; Zweifel et al., 2004). Although *Stx1c* is not associated with HC or HUS, it tends to trigger asymptomatic infection or mild diarrhoea (Brandal et al., 2015).

Among 35 *stx2* isolates, 2/35 (5.71%) had *stx2a*, 16/35 (45.71%) had *stx2b*, 19/35(54.29%) had *stx2c*, 8/35 (22.86%) had *stx2d,* 2/35(5,71%) had *stx2e*, 22/35(62,86%) had *stx2f,* 8/35(22,86%) had *stx2h,* 2/35(5,71%) had *stx2j*, 3/35(8,57%) had *stx2l*. In 8 isolates, none of the investigated subtypes could be detected. *Stx2* subtyping showed that *stx2f, stx2c, stx2b*, *stx2d, stx2h* subtypes were detected at higher rates in sheep, respectively. The most lethal combination for humans (Friedrich et al., 2002), *stx2a-stx2c* (2/35) and *stx2c-stx2d* (8/35) were detected. In the study conducted by Han et al. (2022) in Jiangsu, 16.17% *stx2b* (11/68), 14.7% *stx2k* (10/68) and 14.7% *stx2b* (10/68) were recorded and in this study, *stx2a* was detected at a lower rate and *stx2f* was detected at a higher rate in different combinations with other genes. *Stx2k* was not found at all. The stx2b subtype, which has been associated with sporadic HUS cases (de Boer et al., 2015), was found at a high rate in this study (16/35).

E. coli containing the *stx2e* gene region, which has been associated with oedema disease in pigs (Gyles, 2007) and is symptomless and associated with mild disease in humans (Orth et al., 2007) and rarely isolated from humans (Beutin et al., 2008), it was found in 2 isolates in this study. This showed that *E. coli* isolated from sheep may contain stx2e gene region.

The fact that *stx2a, stx2c* and *stx2d* subtypes, which are associated with severe disease in humans (Shen et al., 2022), were detected at low rates in *stx2a* and high rates in *stx2c* and *stx2d* in this study indicates that sheep have virulence potential for humans. Although *stx2h* was detected at a high rate (66.7%) only in marmots in the 2018 study (Bai et al., 2018), it was also detected in this study (22.86%), suggesting that the *stx2h* subtype may be present in other animal species. *Stx2f*, which is reported to be resistant to heat treatment and low pH, has been reported to have lower toxicity than *stx2a* (Skinner et al., 2013). This stable structure of *stx2f* increases the risk of infection with food contaminations. In this study, *stx2f* was found at the highest prevalence, suggesting that sheep are important reservoirs for *stx2f*. There were 2 (5.71%) *stx2j*, which was found to have a similar structure to *stx2f* (Gill et al., 2022). *Stx2l* is a new subtype recently associated with STEC (McCarthy et al., 2021). In a study carried out in China, 5 *Stx2l* was detected in raw sheep and beef (Yang et al., 2022), which supports the result found in this study.

Another aim of this study was to evaluate the relationship between STEC presence in sheep and breed and sex. For this purpose, *E. coli* was isolated from 107 of rectal fluid obtained from 135 female sheep. Out of 107 isolates, 53 were positive for *stx1, stx2, eae* and *ehxA* genes. 31/53(58,49%) *stx1*, 17/53(32,08%) *stx2,* 9/53(16,98%) *eae,* 34/53(64,14%) *ehxA* genes were detected. 107 *E. coli* were isolated from rectal fluid obtained from 137 male sheep. Among 107 isolates, the number of samples positive for these four gene regions was 61. *Stx1* was detected in 37/61 (60,66%), *stx2* in 20/61 (32,79%), *eae* in 16/61 (26,23%), *ehxA* in 50/61 (81,97%) and none of these four gene regions were detected in 46 isolates. In this study, STEC carriage in male animals was higher than in females, although it was not statistically significant.

To evaluate the relationship between STEC and breed, rectal swabs were taken from 143 Akkaraman sheep and 129 Merino sheep. 109 *E. coli were* isolated from rectal swabs taken from 143 Akkaraman sheep. Although 47 of 109 *E. coli* isolates did not have any of these four gene regions, 62 of them were positive for these four genes. *Stx1* genes were detected in 39/62 (62,90%), *stx2* in 12 (19,32%), *eae* in 19 (30,65%), *ehxA* in 40 (64,52%). *E. coli was* isolated from 105 of rectal swabs taken from 129 Merino sheep. 51 of 105 *E. coli* isolates were positive for these four genes. Of these, 29/51 (56.86%) were positive for *stx1*, 23/51(45.10%) for *stx2*, 6/51 (11.76%) for *eae*, and 44/51 (86.27%) for *ehxA*. Although it was not statistically significant in this study, STEC prevalence was higher in Akkaraman sheep.

In conclusion, STECs are foodborne zoonotic pathogens which cause important public health problems. This study shows that sheep represents the most important source of STECs. Identifying critical control points in food production processes and reducing the risk of cross-contamination with hygiene measures is the most effective way to reduce foodborne STEC infections in humans.

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