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RESEARCH ARTICLE

Determination of Subtypes, Serogroups, And Serotypes, Virulence, and/or Toxigenic Properties of *Escherichia coli* Isolated From Cattle, Sheep, and Goat Feces by Multiplex PCR [1]

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Abstract

In the study, rectal swabs taken from 300 ruminant animals including cattle (100), sheep (100), and goats (100) were inoculated into Mac Conkey Agar and incubated for 18 h at 37°C. *Escherichia coli* isolates were confirmed by biochemical tests and the BBL Crystal rapid diagnosis system. O26, O45, O103, O111, O121, O145, and O157 serotypes by PCR test following DNA isolation; ETEC *(elt, Stla);* EPEC *(eaeA,bfpA);* STEC *(stx1, stx2, eaeA);* EHEC *(EhlyA)*; EAEC *(CVD432)* tested for virulence and/or toxigenic genes. As a result of the isolation studies, 50 *E. coli* from cattle feces, 92 from sheep feces, and 80 from goat feces were isolated and identified. Apart from the first 5 serotypes frequently seen in studies (O157, O26, O103, O111, and O145), higher rates were found in serogroups such as O45 and O121, and subtypes such as STECs *(stx1* and *stx2),* EPEC (*eaeA* and *bfpA)* and EAEC *(CVD432)* types compared to other studies. The EAEC *(CVD432)* subtype was found to be very high in this study. It has been determined that serotypes and subtypes detected at high rates in cattle, sheep, and goat feces in our region may cause an increase in the incidence of some critical food-borne infections in humans. Within the framework of the concept of one health, taking the necessary precautions is important for public health.

Keywords: *E. coli*, Serotype, Subtype, Virulence, Toxigenic properties

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC), also known as verocytotoxigenic *E. coli* (VTEC), can have one or both of the Shiga toxin genes *(stx1* and *stx2)*. STECs are commensal in the gastrointestinal tract of ruminant livestock such as cattle, sheep, and goats and are the main source of transmission of STEC infections in humans [1,2]. During milking, slaughter, and removal of internal organs, foods of animal origin become contaminated with STECs present in feces and may pollute the environment. *E. coli* O157, O26, O103, O111, and O145 serogroups have been most commonly identified as the "top five" serogroups found to be associated with human infections. STEC infections in humans are asymptomatic or can cause the transition from non-bloody to bloody diarrhea; cause colitis, hemolytic uremic syndrome (HUS), and hemorrhagic diseases that can result in death [1,3]; cause outbreaks of diarrhea in children and the elderly [4]. O157, especially from STECs, is the main food-borne pathogen. For non-O157 STECs, cattle are considered to be the main reservoir [5]. According to the 2020 European Union One Health Zoonoses Report, the number of confirmed human cases of STEC infections was 4.446 [6].

Enterohemorrhagic *E. coli* (EHEC) is the most common foodborne pathogen. EHEC strains not only produce potent cytotoxins but have also acquired the ability to

adhere to the intestinal mucosa. There are also certain types of virulence factors. All strains produce hemolysin *(hlyA)* and at least one Shiga-like toxin *(stx1* and/or *stx2),* and most produce intimin, a 97 kDa binding and deletion protein *(eaeA)* [7]. In India, Vietnam, and the USA, EAEC has been reported to be the most common diarrheagenic *E. coli* in children with diarrhea. There are two types of EPEC: typical EPEC (Type I, *eae,* and *bfp* positive) and atypical EPEC (Type II, *eae* positive only). Typical EPEC, causes gastroenteritis in babies and is usually seen in babies under 2 years of age. Recent studies indicate an increase in the prevalence of atypical EPEC strains [8]. Enteroaggregative *E. coli* (EAggEC), even in the absence of Stx/VT, can cause diarrhea in children and adults. EAggEC is best known for causing persistent diarrhea (>14 days) in infants and developing children. In Mongolia, India, Brazil, Nigeria, Israel, Venezuela, Congo, and many other countries, EAggEC is the most common *E. coli* pathotype in infants. EAggEC is the nontoxic-secreting *E. coli* type of ETEC. It synthesizes aggregative adherence fimbriae with the *aggR* gene. EAggEC usually causes watery and often persistent diarrhea. Infection begins with the bacterium first adhering to the terminal ileum and colon of the aggregative adherence fimbriae. The damage/secretion phase occurs with the release of cytokines, mucosal toxicity, intestinal secretion, and induction of mucosal inflammation [9].

Carcasses to be contamined with intestinal contents during slaughterhouses. Therefore, bacteria present in the intestinal contents can contaminate the carcass and cause foodborne infections and toxicity. This study aimed to determine the subtypes, serotypes, virulence, and/or toxigenic properties of *E. coli*, which are at risk of causing disease in humans, especially from the feces of ruminant animals.

Material and Methods

Ethical Statement

The study has been approved by the Ethics Committee of Kırıkkale University (Approval No. E-60821397-619-177126).

Sample Collection

In this project, rectal swab samples were collected from 300 ruminants, such as healthy cattle (100), sheep (100), and goats (100). Rectal swabs from cattle were collected aseptically from slaughterhouses in Kırıkkale province, and the rectal swabs from sheep and goats were delivered to the laboratory in a transport medium with a cold chain at 4-8°C.

Isolation and Identification

The swabs were seeded on Mac Conkey Agar and incubated at 37°C for 18 h. Biochemical testing was performed on isolates that exhibited typical characteristics of *E. coli,* and the presence was confirmed using both conventional methods and the rapid diagnostic system BBL Crystal.

DNA Isolation

A 100 µL of sterile deionized water was added to each of five (n=5) *E. coli* colonies. For DNA isolation, they were boiled at 95°C for 10 min with a hot plate and centrifuged at 1000 g. for 10 min. The supernatant was transferred to sterile Eppendorf tubes, and the amount of DNA was measured with a Nanodrop spectrophotometer, and then the concentration was adjusted to 100 ng/µL [8].

PCR

For PCR: 12.5 mL of master mix, 1 µL of DNA, 5 µL of each primer, and 6.5 µL of sterile distilled water were added, and the total volume was adjusted to 25 µL. The thermocycling conditions were as follows: 1 min at 95°C; 35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and finally one cycle at 72°C for 5 min. *E. coli* serotypes and serogroups were screened for virulence and/or toxigenic genes by PCR assays: serotypes O26, O45, O103, O111, O121, O145, O157 [5] and subtypes ETEC *(elt, Stla),* EPEC *(eaeA, bfpA),* EHEC *(EhlyA),* EAEC *(CVD432)* [8] and STEC *(stx1, stx2, eaeA)* [7]. Amplicons (10 µL) generated by PCR were electrophoresed on a 2% agarose gel, in 1xTAE buffer, at 100 volts for approximately 1 h. The bands were evaluated under UV light [8].

Statistical Analysis

The KAPPA test was used to compare the analysis results of the methods used in the study for *E. coli* serogroups and serotypes. The results were evaluated according to Mc Hugh [8].

Results

Stool samples from slaughterhouses and farms in Kırıkkale were collected under sterile conditions and delivered to the laboratory as soon as possible. As a result of the isolation studies, 50 *E. coli* were isolated and identified from cattle feces, 92 from sheep feces, and 80 from goat feces.

PCR analysis of 92 *E. coli* isolates from sheep feces revealed the following serotypes and their ratios, ranking from highest to lowest: 26 of O26 (28.3%), 22 O157 (23.9%), 18 O103 (19.6%), 13 O121 (14.1%), 9 O45 (9.8%), 4 O111 (4.3%); O145 was not found. PCR analysis of 80 *E. coli* isolates from goat feces revealed the following serotypes and their ratios, ranking from highest to lowest: 20 of O45 (25%), 20 of O111 (25%), 16 O26 (20%), 12 O121 (15%), 8 O157 (10%), 4 O145 (5%); O103 was not found. PCR analysis of 50 *E. coli* isolates from bovine feces determined serotypes and their ratios as follows, ranking from highest

Table 1. *PCR analysis results of E. coli serotypes and ratios from all animal fecal samples*

Animals (Total Isolates)	**Serotypes Numbers (%)**						
	O26	**O45**	**O103**	**O111**	**O121**	**O145**	**O157**
Sheep Feces (92)	26 (28.3)	9 (9.8)	18 (19.6)	4 (4.3)	13 (14.1)	0 (0)	22 (23.9)
Goat Feces (80)	16 (20)	20 (25)	0 (0)	20 (25)	12 (15)	4 (5)	8 (10)
Cattle Feces (50)	5 (10)	11 (22)	5 (10)	7 (14)	7 (14)	2 (4)	21 (42)
Total (222)	47 (21.1)	40 (18)	23 (10.4)	31 (14)	32 (14.4)	6 (2.7)	51 (23)

Table 2. *PCR analysis results of E. coli subtypes and ratios from all animal fecal samples*

Animals (Total Isolates)	**Subtypes Numbers (%)**									
	ETEC			**EPEC**		**STEC**			**EHEC**	**EAEC**
Genes	*Elt*	*Stla*	*all*	*eaeA*	*bfpA*	*stx1*	*stx2*	*eaeA*	*EhlyA*	*CVD432*
Sheep (92)	32 (35)	9 (10)	18 (20)	5 (5)	37 (40)	32 (35)	41 (45)	32 (35)	23 (25)	**69 (75)**
Goat (80)	32 (40)	24 (30)	0 (0)	40 (50)	**52 (65)**	40 (50)	**68 (85)**	20 (25)	24 (30)	52 (65)
Cattle (50)	20 (39.1)	11 (21.7)	4 (8.7)	24 (47.8)	22 (43.5)	30 (60.9)	33 (65.2)	9 (17.4)	15 (30.4)	**46 (91.3)**
Total (222)	84 (37.8)	44 (19.8)	22 (9.9)	69 (31.1)	111 (50)	102 (45.9)	**142 (64)**	61 (27.5)	61 (27.5)	**164 (73.9)**

Table 3. *Kappa test results*

Animals and Serotypes		**K**									
		ETEC *(elt)*	**ETEC *(Stla)***	**EPEC *(eaeA)***	**EPEC *(bfpA)***	**STEC *(stx1)***	**STEC *(stx2)***	**STEC *(eaeA)***	**ETEC *(all)***	**EHEC *(EhlyA)***	**EAEC *(CVD432)***
Sheep	O45	0.146	0.002	-0.06	-1.005	0.175	0.117	0.174	0.368	-0.099	-3.294
	O157	0.434	0.62	0	0.49	1.61	0.352	0.519	-0.029	0.46	0.176
	O26	-0.048	-0.186	0.216	-0.117	0.462	-0.179	-0.514	-0.336	-0.146	-0.124
	O103	-0.193	0.497	-0.052	-0.185	-0.203	-0.08	-0.379	0.566	-0.072	-0.153
	O121	-0.19	-0.114	-0.016	0.41	0.602	-0.203	0.542	0.223	-0.174	0.06
Goat	O45	-0.019	0.068	-0.133	-0.043	-0.1	-0.037	-0.124	0.66	-0.125	0.272
	O157	-0.19	-0.171	-0.2	0.006	0.2921	-0.612	-0.16	0	-0.29	0.33
	O26	-0.136	0.21	0.2	0.22	-0.01	-0.12	-0.149	-1.3	-0.05	-0.126
	O103	0	0	0	0	0	0	0	0	0	0
	O121	0.52	0.604	0.01	0.183	0.7	0.1	0.076	0	1.09	3.568
	O145	0.146	-0.093	0.1	0.05	0.11	0.018	-0.9	0	-0.093	0.055
	O111	0	-0.125	0.288	-0.043	0.3	0	0.46	0	0.375	0.92
Bovine	O45	0.194	0.558	-0.46	0.186	0.158	0.158	0.601	0.33	-0.042	0.067
	O157	-0.131	-0.129	0.279	-0.012	-0.123	1.573	0.821	-0.19	0.029	-0.185
	O26	-0.131	-0.151	0.516	-0.174	-0.289	0.11	-0.123	-0.098	-0.098	0.02
	O103	-0.086	-0.074	0.99	-0.091	-0.082	0.061	-0.07	-0.059	-0.8	0.012
	O121	0.165	-0.201	1.041	-0.096	0.168	-3.25	0.15	-0.357	0.016	0.033
	O145	-0.126	-0.073	0.091	0.103	0.055	-0.08	0.309	0.996	0.197	-1.74
	O111	0.25	0.232	0.09	0.024	0.084	0.084	-0.07	0.581	0.194	-1.74

to lowest: 21 of O157 (42%), 11 of O45 (22%), 7 of O121 (14%), 7 of O111 (14%), 5 O26 (10%), 5 of O103 (10%), 2 O145 (4%). PCR analysis of 222 *E. coli* isolates from all animal fecal samples revealed the following serotypes and their ratios; 51 of O157 (23%), 47 of O26 (21.6%), 40 of O45 (18%), 32 of O121 (14.4%), 31 of O111 (14.4%), 23 of O103 (10.4%), 6 of O145 (2.7%). The most common serotypes of *E. coli* isolated and identified from various animal fecal samples are, respectively: In sheep: O26, O157, O103, 121, O45, O111; in goats: O45, O111, O26, O121, O157, O145; and in cattle: O157, O45, O121, O111, O26, O103, O145. PCR analysis results of *E. coli* serotypes and ratios from all animal fecal samples are shown in *Table 1*.

When evaluated as *E. coli* subtypes ratios from sheep fecal samples, ETEC *(elt)* are 35%, and ETEC *(Stla)* are 10%; EPEC *(eaeA)* are 5% and EPEC *(bfpA)* are 40%; STEC *(stx1)* are 35%, STEC *(stx2)* are 45%, STEC *(eaeA)* are 35%; ETEC *(all)* are 20%; EHEC *(EhlyA)* are 25% and EAEC *(CVD432)* are 75%.; *E. coli* subtypes ratios from goat fecal samples ETEC *(elt)* is 40%, and ETEC (Stla) is 30%; EPEC *(eaeA)* is 50% and EPEC *(bfpA)* is 65%; STEC *(stx1)* is 50%, STEC *(stx2)* is 85%, STEC *(eaeA)* is 25%; ETEC *(all)* is 0%; EHEC *(EhlyA)* is 30% and EAEC *(CVD432)* is 65%.; *E. coli* subtypes ratios from cattle fecal samples ETEC *(elt)* is 39.1%, and ETEC *(Stla)* is 21.7%, EPEC *(eaeA)* is 47.8% and EPEC *(bfpA)* is 43.5%; STEC *(stx1)* is 60.9%, STEC *(stx2)* is 65.2% STEC *(eaeA)* is17.4%; ETEC *(all)* is 8.7%; EHEC *(EhlyA)* is 30.4% and EAEC *(CVD432)* is 91.3%. PCR analysis results of *E. coli* subtypes and ratios from all animal fecal samples are shown in *Table 2*.

As a result, subspecies of *E. coli* isolated and identified from individuals most frequently seen in different animals are EAEC *(CVD432),* STEC *(stx2),* EPEC *(bfpA),* ETEC *(elt),* STEC *(stx1),* STEC *(eaeA),* EHEC *(EhlyA),* ETEC *(all),* ETEC *(Stla),* EPEC *(eaeA)* in sheep; in goats these were STEC *(stx2),* EAEC *(CVD432),* EPEC *(bfpA),* EPEC *(eaeA),* STEC *(stx1),* ETEC *(elt),* EHEC *(EhlyA),* ETEC *(Stla),* STEC *(eaeA).* In cattle, it was detected as EAEC *(CVD432),* STEC *(stx2),* STEC *(stx1),* EPEC *(eaeA),* EPEC (*bfpA*), ETEC *(elt),* EHEC *(EhlyA),* ETEC *(Stla),* STEC *(eaeA),* ETEC *(all).* The identified ones in all animals were EAEC *(CVD432),* STEC *(stx2),* EPEC *(bfpA),* STEC *(stx1),* ETEC *(elt),* EPEC *(eaeA),* EHEC *(EhlyA),* STEC *(eaeA),* ETEC *(Stla),* ETEC *(all).*

The results of the Kappa test performed were evaluated *(Table 3).* Remarkably, that there is an almost perfect correlation between the results of the sheep O517-STEC *(stx1),* goat O121-EHEC and EAEC, goat O111-EAEC, bovine O157-STEC *(stx2* and *eaeA),* bovine O121-EPEC *(eaeA),* and bovine O145-EIEC.

Discussion

Studies have reported that STECs transmitted from feces during slaughter in ruminants, especially cattle, are the main transmission source and primary reservoir of HUS infections in humans. In the studies conducted in the European Union country in the 2000s, the first 5 serotypes were O157, O26, O103, O111, and O145 [1]. In the study we conducted in Kırıkkale Region, O45 and O121 serotypes were also detected in cattle together with these 5 serotypes.

The prevalence of STEC in ready-to-eat foods detected in some countries is as follows: In Botswana, *E. coli* O157:H7 was detected at 3.8% in ground beef [10], and *E. coli* O157:H7 at 0.1% in ground beef [11]. *E. coli* O157:H7 was detected as 0.4% in ground beef in Italy. STEC was 33.1% in cattle feces in the USA, and O157 was 35.5% [12]. STECs in New Zealand were 17% in lamb and 12% in cattle [13]. In cattle feces in Italy, O157 was found at 6.3%, and O26 was found at 3.8% [14] and in addition to these literatures, STEC was detected at 3.9% in cattle and 5.1% in pig carcasses in Czechia, respectively [15].

In another study, relatively similar rates were found in cattle: *stx2* was 65.2%, *stx1* was 60.9%; less in sheep, *stx2* was 45%, *stx1* was 35%. In a study conducted in 2015, O157 was detected at a rate of 6.3%, and O26 was at 3.8% in Italy [13]. In our study, a high rate of O157 serotype was detected in cattle with a rate of 42% when compared to other countries. STEC *(stx1, stx2, eae, ehxA)* was 63.5% in cattle herds and 56.5% in sheep herds in Spain [16]. *stx2* (10%) and *hlyA* (35.9%) were the most common genes in ruminant animals in Australia [17]. 12.4% was *stx* positive in cattle and children in Poland [18]. In our study, *stx2* and *Ehly* were significantly higher than studies in Australia and Poland; it was found to be more compatible with the findings in Spain.

In a study conducted in Brazil, 52.8% *stx1* and 14.3% *stx2* were detected in sheep [19]. In another study conducted in China, 61.5% of STEC was detected in sheep and 12.9% in cattle; 69.1% of STECs in sheep were determined as *stx1*, 29.4% as *stx2*. It has been reported that STECs with *eae* gene and *stx2* gene are seen in 91.07% of HUS cases. In this study, STEC was found to be quite high; *stx1* was found to be less than this study with a rate of 32%, *stx2* was found to be higher with 41%, and *eaeA* was found to be 32% [20,21]. In another study conducted in China, 55.4% of 56 STECs isolated from cattle and sheep were found as *stx1*, which is in correlation with our study, and 3% as *stx2*, which is less than our study [22]. In another study conducted in India, 91 *E. coli* were isolated from a total of 120 infants (60) and calves (60) with diarrhea, and 30.76% *fimA* gene and 7.69% *aggR* gene were detected from EAEC genes by PCR [23]. In our study, the EAEC *(CVD432)* gene was detected at a very high rate of 73.9%. In a study conducted in France in 2023, they studied 500 fecal samples collected in a calf slaughterhouse in one year; they identified 30 STECs from

28 calves and reported that 2 of them were O103 and O26 (73%), followed by O145 and O157 [24]. In our study, O157 (42%), O26 (10%), and O45 (22%) were detected at a higher rate given in order, and O103 (10%) at a lower rate than other serotypes when compared.

Seker et al.[25], in their study on buffaloes in Afyon, Türkiye, detected *stx1* and *stx2* at 27.3%; *eaeA at* 9.1%; *ehlyA* at 72%. Kuyucuoglu et al.[26] conducted a study on cattle feces in Afyon; these rates were determined as *ehlyA* 92.8% and *eaeA* 57.1%. Yilmaz et al.[27], in their study conducted in Istanbul in 2006, found 70.3% *vt1*, *vt2,* and *eaeA*; 11.1% *eaeA* in cattle feces, carcass, and environmental samples. In Hatay, vtx was detected in 80%, *vtx1,* and *vtx2* in 3.9%, *eaeA* in 93.5%, and e*hlyA* in 96.1% in cattle rectal swabs [28]. In our study, STEC *stx2* was 65%, STEC *stx1* was 61%, EPEC *eaeA* was 48%, STEC *eaeA* was 17%, and EHEC *EhlyA* was 30%, which were found to be higher. Apart from the first 5 serotypes that are frequently seen in our study, the presence of serotypes such as O45 and O121 was also detected. STEC and *eaeA* were detected at higher rates than in other studies. In addition, the EAEC *(CVD432)* subtype was found to be very high in our study. It is seen as an important cause of diarrhea in epidemic and non-epidemic situations and causes irritable bowel syndrome. Also, STEC and EPEC genes were determined as common genes; *stx1, stx2, bfpA,* and *eaeA* had higher ratios than other genes. When considered among animals, it was found that EPECs and STECs were highest in goats and cattle. It was determined that *stx2* was highest in goat feces, and *stx1* was highest in cattle and goat feces.

E. coli serotypes and subtypes detected at high rates in cattle, sheep, and goat feces can pose a risk to human health by contaminating meat from carcasses. It can cause some important infections originating from red meat, especially in humans. Within the framework of the concept of one health, it will be beneficial to prevent *E. coli* serotypes and subtypes seen in ruminant feces such as cattle, sheep, and goats, especially in slaughterhouses, by taking necessary biosecurity measures to protect public health.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author (S. Kızıl) on reasonable request.

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Ethical Statement: The study has been approved by the ethics committee of Kırıkkale University (Approval No. E-60821397-619-177126).

Conflict of Interest: The authors declared that there is no conflict of interest.

Author Contributions: SK conceived and executed the idea, designed experiments, analyzed results, and conducted a deep revision of the manuscript. FEA, AUÖ, MY, CÖG and EMÇ collected the samples, performed experiments, and contributed to the implementation of the research. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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