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MOLECULAR DETECTION OF HEAT LABILE, HEAT STABLE AND SHIGA-TOXIN *E. COLI* GENES IN SHEEP AND GOATS

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Abstract

E. coli cause diarrhea in sheep and goats through its virulent strains, enterotoxigenic E. coli (ETEC) and shiga-toxin producing E. coli (STEC). The present study was conducted for molecular detection of E. coli and its pathogenic strains including heat labile (elt), heat stable (est) and shiga-toxin (stx1, stx2) producing genes from diarrheal fecal samples of small ruminants. About 155 fecal diarrheal fecal samples were randomly collected from sheep (n=88) and goats (n=67) and checked for the presence of E. coli by microbiological techniques. The inoculation of samples onto EMB agar revealed sheen green colonies that appeared pink rod-shaped bacilli on gram staining. DNA extraction was performed through boiling and phenol-chloroform method and PCR assay was done for the detection of uidA gene of E. coli along with elt, est, stx1 and stx2 genes of ETEC and STEC strains. PCR assay confirmed the presence of E. coli in 96% isolates by uidA gene in sheep and goats. The results revealed the presence of est (84.5%), stx1 (81.8%) and stx2 (61.1%) virulence genes but none of the isolates detected elt gene. The findings indicate that sheep and goat are a reservoir of pathogenic E. coli possessing est, stx1 and stx2 coding genes and thus, the presence of these virulence factors is a potential harm for human health.

Keywords: *Escherichia coli, Enterotoxigenic E. coli, Goats, PCR, Sheep, Shiga-toxin producing E. coli*

INTRODUCTION

Escherichia coli is an anaerobic, gram-negative bacillus, commonly reside in the gastrointestinal tract and considered as beneficial bacteria for animals as well as humans (1-2). However, there are many pathogenic strains of *E. coli* those cause disease by attaining the virulence factors like different toxins and adhesion proteins through plamids, transposons and bacteriophages that help the bacteria to colonize and cause infection in the host tissues (3-4). The common diseases caused by *E. coli* are mastitis, scours, dermatitis, botryomycosis, septicemia and diarrhea in almost all livestock species (5-7) and hemorrhagic colitis, gastroenteritis, urinary tract infection and serious inflammations like Sequelae Hemolytic Uremic Syndrome in humans (2, 8). The pathogenic strains of the bacterium include entero-toxigenic *E. coli* (ETEC) that cause diarrhea in infants and shiga-toxin producing *E. coli* (STEC) or enterohaemorrhagic *E. coli* (EHEC) are responsible for abdominal cramps diarrhea, dysentery, hemorrhagic colitis and hemolytic uremic disease both in adults and young ones (9). Hence, diarrhea is a common disease caused by ETEC and STEC in both animals and humans (10, 11).

Entero-toxigenic *E. coli* (ETEC) produce the colonization factors and enterotoxins including heat-stable enterotoxin and heat-labile enterotoxin. Heat stable (*st*) enterotoxins encode *est* (*stA* and *stB*) genes,

which are resistant to high temperature while, heat labile (*lt*) enterotoxins encode *elt* (*ltA* and *ltB*) genes, which are sensitive to high temperature. These enterotoxins bind to the receptors of columnar epithelial cells of intestine, disrupt the intestinal mucosa and cause excessive secretions leading to watery diarrhea (12). ETEC are transferred through oral to fecal route from contaminated food materials, water and environment, thus moving through to the intestinal tract of the host and colonize in the intestine tract (13). On the other hand, shiga-toxin producing *E. coli* produces shiga toxins that bind to the cellular receptors, penetrate through the epithelial cell barrier and disturb the mechanisms of protein synthesis and thus cause cell death (14). Thus, *E. coli* is a zoonotic food-borne pathogen that causes severe gastrointestinal disease in the humans including hemorrhagic colitis, bloody diarrhea and hemolytic uremic syndrome but are an important factor for acute renal failure in young animals and cause morbidity and mortality in the adults (15, 16). STEC is transferred to the humans through close contact with infected animals, waterborne transmission and contaminated food products like meat products, ground beef and unpasteurized milk products (14). Moreover, the bacterial strains are also shed in the faeces of sheep, goats, cattle, birds, bats and dogs and thus, the fecal release of the bacteria causes sickness in humans through contact with contaminated soil and water or direct handling of animal excreta through contaminated farm environments or handling of infected animals. Moreover, the meat preparation tools and areas where meat is processed, and the meat shops are likely the pathway of contamination through fecal-oral route via contaminated food, water, animals and direct contact with infected sources (14). Hence, both these small ruminants are one of the main reservoirs of different strains of *E. coli* and the diseased animals and their by-products are the foremost source of infection for humans. The study was aimed to check the presence of disease causing *E. coli* and then the molecular detection of heat labile (*elt*), heat stable (*est*) and shiga-toxin (*stx*) producing genes isolated from these bacteria from fecal samples of sheep and goats..

MATERIALS AND METHODS

COLLECTION OF SAMPLES

Fecal samples were randomly collected from sheep (n=88) and goats (n=67) at the local markets of Quetta, Pakistan. The samples were collected directly from the rectum of animal having diarrheal symptoms by using sterilized swabs. After collection, the samples were kept in an ice box and immediately transported to the laboratory at the Center of Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta. A small amount of fecal sample was added to the nutrient broth medium to make a suspension and then was kept in an incubator for 24 hours at 37ºC to promote the bacterial growth. After obtaining the growth, the pure colonies of bacteria were used for further studies.

INOCULATION OF SAMPLES ON CULTURE MEDIA AND ISOLATION OF *E. COLI*

Nutrient broth containing bacterial growth was inoculated onto nutrient agar culture media by spread plate method and incubated at 37ºC for 24 hours (10). The culture media were stored at 4ºC until further processing. Single bacterial colonies from nutrient agar media were inoculated onto EMB (Eosin Methylene Blue) agar by using streaking method and incubated at 37ºC for 24 hours. Metallic green sheen colonies of *E. coli* were obtained on EMB agar. Then, *E. coli* isolates from EMB agar were preserved in a 5 ml solution of glycerol and nutrient broth and stored at -80ºC until further molecular analysis.

GRAM STAINING AND MOLECULAR ANALYSIS

The samples were subjected to gram staining (17) and observed under the microscope to check the presence of *E. coli*.

The molecular analysis of the collected samples was carried out by DNA extraction and Polymerase chain reaction (PCR) to detect the specific genes of the bacterium (18, 19). DNA extraction was performed from 155 pure isolates of *E. coli*. DNA was extracted by both phenol-chloroform method (20) and boiling method (21). The boiling method was quick and worked well for heat stable genes (*est*, *stx1* and *stx2*), while, *elt* gene is sensitive to heat, so the phenol-chloroform method was used to prevent its degradation. For confirmation of the DNA, 10µL of the extracted DNA samples were loaded onto 1.2% agarose gels with loading dye and

subjected to electrophoresis for 10 minutes at 80 Volts (V). The analyzed DNA was visualized on gel documentation system (18).

MOLECULAR IDENTIFICATION OF *E. COLI* USING *uidA* PCR ASSAY

PCR assay was used for the identification of E. coli by amplification uidA gene using the primers (Table I). The reaction was carried out in a total volume of 25µl PCR mixture, containing 10µl WizPureTM PCR 2X Master Mix (Wizbio solutions, South Korea), 1µl forward primer, 1µl reverse primer (Macrogen Inc., Seoul, South Korea), 8µl PCR grade H_2O and 5µl DNA sample in thermocycler in the specific conditions (Table II). The size of PCR amplicons was identified by comparing with molecular weight markers of 100 bp DNA (Simply Scientific). Sterilized distilled water was used as a negative control. For the confirmation of uidA gene, 5µl of PCR product and 2µl of loading dye along with positive and negative controls were loaded into separate wells of 1.2% agarose gel and subjected to gel electrophoresis for 45 minutes at 80V. Then, the gel was visualized on gel documentation system to observe the bands.

Table I. Primer pairs used in this study to determine the *E. coli* and its virulence genes

Sr. #	Name	Primers	Sequence (5´ - 3´)	bp	References
1	*uidA*	*uidA* F	CCAAAAGCCAGACAGAGT	623	(22)
		uidA R	GCACAGCACATCAAAGAG		
2	*elt*	*elt* F	TCTCTATGTGCATACGGAGC	322	(23)
		elt R	CCATACTGATTGCCGCAAT		
3	*est*	*est* F	TCTTTCCCCTCTTTTAGTCAGTC	170	(23)
		est R	CCAGCACAGGCAGGATTAC		
4	*stx1*	*stx1* F	ACACTGGATGATCTCAGTGG	614	(24)
		stx1 R	CTGAATCCCCCTCCATTATG		
5	*stx2*	*stx2* F	CCATGACAACGGACAGCAGTT	779	(24)
		stx2 R	CCTGTCAACTGAGCACTTTG		

Table II. PCR thermocycler conditions

Primers (F and R)	Cycling				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
uidA F	95 ºC	94 ºC	52 ºC	72 ºC	72 ºC
uidA R	5 mins	1 min	30 sec	1 min	7 min
		Repeated for 35 cycles			
elt F	94 ºC	94 ºC	58 ºC	72 ºC	72 ºC
elt R	5 min	30 sec	30 sec	1 min	7 min
		Repeated for 35 cycles			
est F	94 ºC	94 ºC	58 ºC	72 ºC	72 ºC
est R	5 min	30 sec	30 sec	1 min	7 min
		Repeated for 35 cycles			
stx1 F	94 ºC	94 ºC	55 ºC	72 ºC	72 ºC
stx1 R	5 min	45 sec	45 sec	1 min	7 min
		Repeated for 30 cycles			
stx2 F	94 ºC	94 ºC	60 ºC	72 ºC	72 ºC
stx2 R	5 min	1 min	2 min	1 min	10 min
		Repeated for 35 cycles			

DETECTION OF VIRULENCE GENES

PCR was used for the detection of virulence genes including elt and est genes of enterotoxigenic E. coli (ETEC) and *stx1* and *stx2* genes of shiga-toxin producing *E. coli* (STEC) using PCR thermocycler for amplification of the genes using specific primers. The reaction mixture, with total volume of 25µl PCR mix contained 10µl WizPureTM PCR 2X Master mix (Wizbio solutions, South Korea), 0.5µl forward primer, 0.5µl reverse primer (Macrogen Inc., Seoul, South Korea), 9µl PCR grade H2O and 5µl DNA sample was carried out in thermocycler (Table II). The molecular weight marker of 100 bp DNA (Simply scientific),

positive control and negative control along with PCR product was loaded onto an agarose gel. Then, agarose gel was subjected to gel electrophoresis at 80V for 35 minutes and gel documentation was used to visualize the DNA bands.

RESULTS

MORPHOLOGICAL IDENTIFICATION OF THE BACTERIA

The results revealed the appearance of metallic sheen green color colonies depicted the presence of *E. coli* (Fig. 1a) and was further confirmed through gram staining. The microscopic examination of Gram-stained slides revealed numerous rod-shaped and uniformly pink stained microorganisms that confirmed the presence of *E. coli* (Fig. 1b).

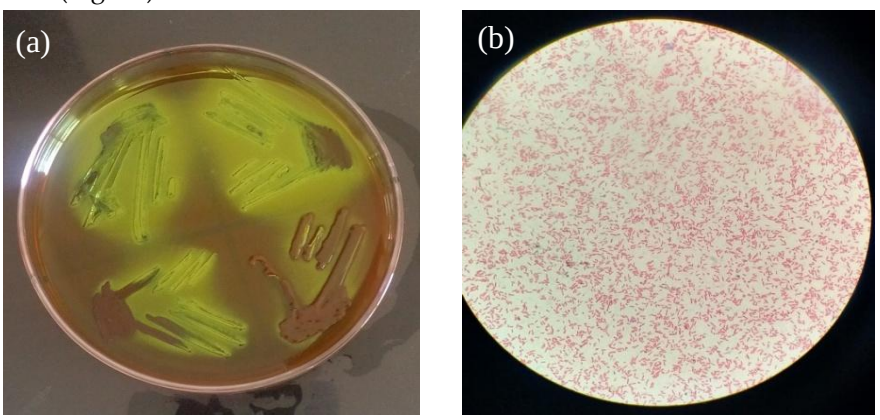

Fig. 1a. EMB agar showing metallic sheen green colonies of *E. coil;* **b.** Microscopic examination of Gram-stained slide showing numerous rod-shaped and uniformly pink stained microorganisms, confirming the presence of *E. coli*

DNA EXTRACTION AND CONFIRMATION BY GEL ELECTROPHORESIS

DNA was extracted through phenol-chloroform and boiling methods and was confirmed by running the PCR product in the agarose gel through gel electrophoresis and visualized using gel documentation system (Fig. 2a).

IDENTIFICATION OF *E. COLI* BY *uidA* PCR ASSAY

The *uidA* gene was identified by using PCR and the result revealed the amplification of *uidA* gene on 623 bp (Fig. 2b). About all the collected samples from the diseased sheep (85/88, 96.6 %) and goats (64/67, 95.5%) confirmed the presence of *E. coli* isolates by *uidA* gene. Thus, a high prevalence (95.5%) of *E. coli* was detected in both sheep and goats (Table III).

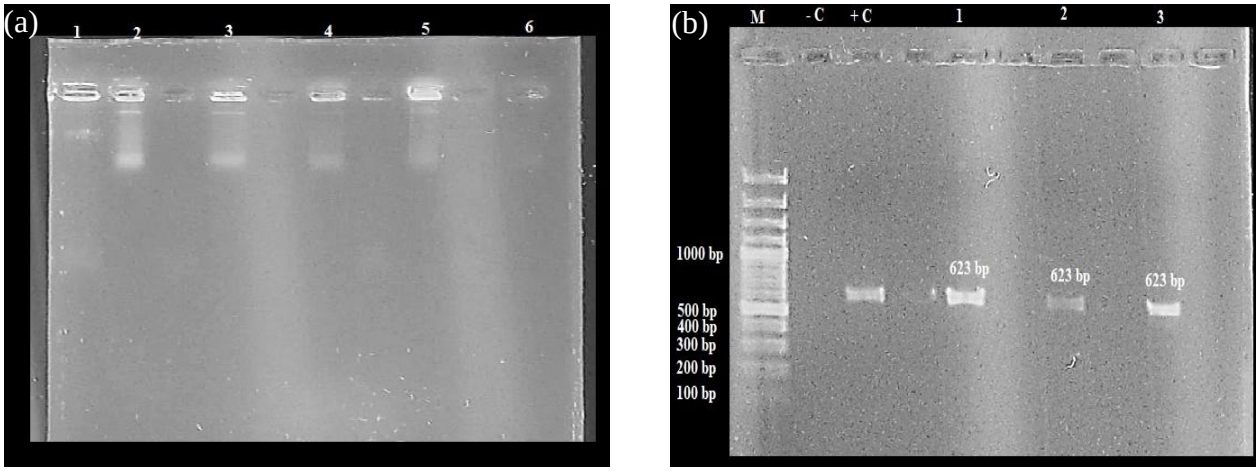

Fig. 2a. Gel Electrophoresis of extracted DNA from *E. coli* samples. The appearance of bands (Lanes 1-6) confirmed the presence of DNA. The DNA samples were loaded onto agarose gels with loading dye, subjected to electrophoresis and visualized on gel documentation system; **b.** Gel Electrophoresis of amplified PCR product of *uidA* (623 bp). Lane M: molecular weight marker (1000 bp), Lane -C: –ve control, Lane +C: +ve control, lanes 1, 2 and 3: PCR product of *uidA* (623 bp) confirmed the presence of *E. Coli*

Table III. Total prevelance of *E. coli* and its virulence genes among sheep and goat samples

Sr #	Genes	Goat	Sheep	Total
1.	*uidA*	64/67 (95.5%)	85/88 (96.6%)	149/155 (96%)
2.	*elt*	0/67 (0%)	0/85 (0%)	0/155 (0%)
3.	*est*	59/64 (92.2%)	67/85 (78.8%)	126/149 (84.5%)
4.	*stx1*	51/64 (79.6%)	71/85 (83.5%)	122/149 (81.8%)
5.	*stx2*	38/64 (59.3%)	53/85 (62.3%)	91/149 (61.1%)

DETECTION OF VIRULENCE GENES IN *E. COLI*

Four virulence genes; *est*, *elt*, *stx1* and *stx2* genes were identified in 149 *E. coli* positive fecal samples (sheep= 85, goats=64). The results revealed the amplification of est gene at 170 bp (Fig. 3a), *stx1* gene at 614 bp (Fig. 3b) and *stx2* gene at 779 bp (Fig. 3c). However, elt gene was not amplified in any of the sample. Among 64 samples from goat, 59 (92.2%) were positive for *est*, 51 (79.6%) for *stx1* and 38 (59.3%) for *stx2*. While, in 85 samples of sheep, 67 (78.8%) were positive for *est*, 71 (83.5%) for *stx1* and 53 (62.3%) for *stx2* (Table III).

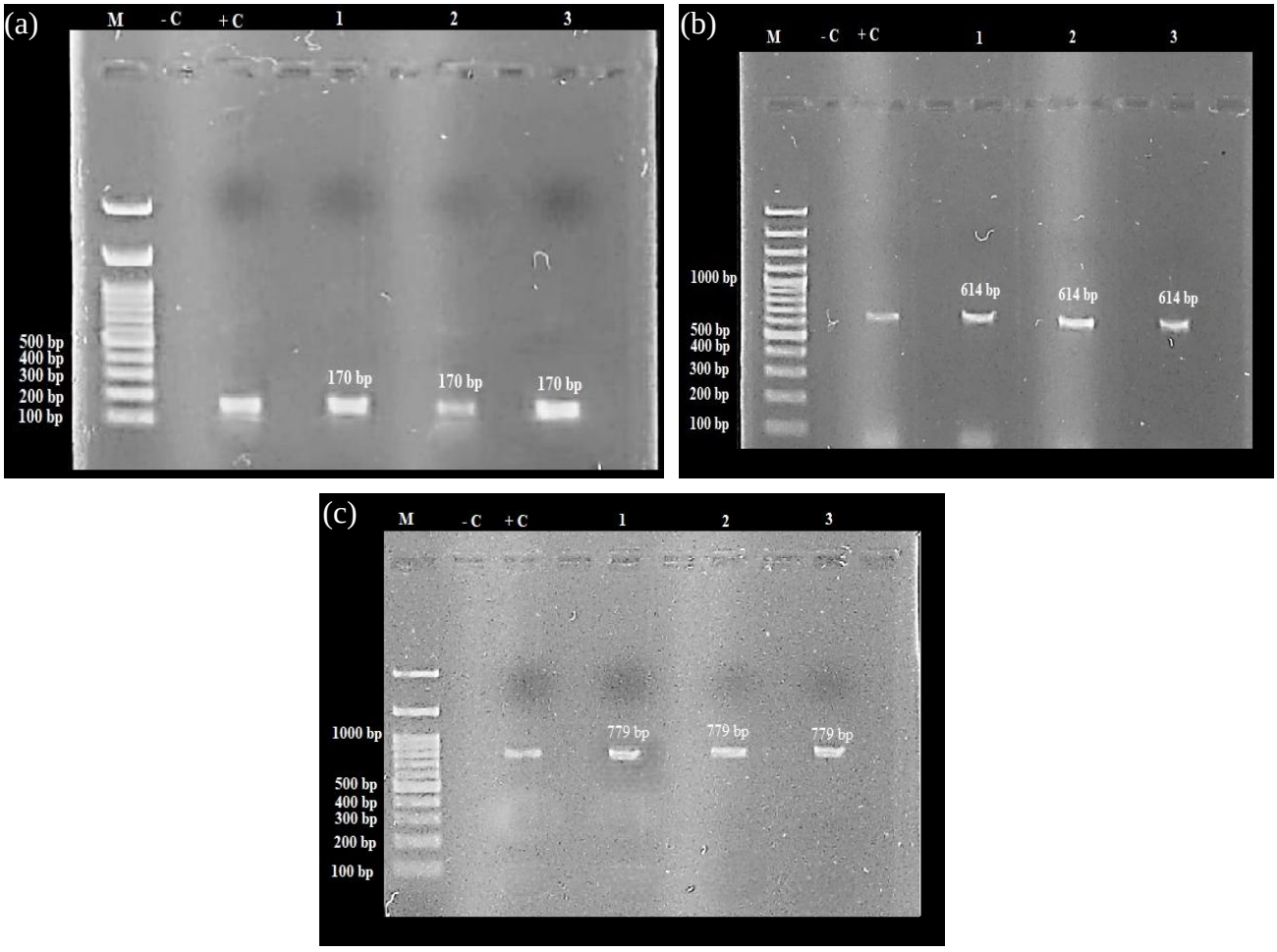

Fig. 3a. Gel Electrophoresis of amplified PCR product of *est* (170 bp). Lane M: molecular weight marker (100 bp), Lane -C: -ve control, Lane +C: +ve control, Lanes 1, 2 and 3: PCR product of *est* gene (170 bp); **b.** Gel Electrophoresis of amplified PCR product of *stx1* (614 bp). Lane M: molecular weight marker (100 bp), Lane -C: -ve control, Lane +C: +ve control, Lanes 1, 2 and 3: PCR product of *stx1* gene (614 bp); **c.** Gel Electrophoresis of amplified PCR product of *stx2* (779 bp). Lane M: molecular weight marker (100 bp), Lane -C: -ve control, Lane +C: +ve control, Lanes 1, 2 and 3: PCR product of *stx2* gene (779 bp).

DISCUSSION

Escherichia coli is an anaerobic, gram-negative bacillus acts as a beneficial bacteria that is present in the gastrointestinal tract of animals and humans (2). However, its pathogenic strains cause different diseases in small and large animals including sheep and goats. Diarrhea is a common disease caused by its pathogenic strains including enterotoxigenic and shiga-toxins producing *E. coli* in these animals (10, 25). Our results revealed that about 95.5% goats and 96.6% sheep showed the presence of E. coli isolates by *uidA*

gene PCR assay. However, in the other regions of the world like South Africa, a significantly lower incidence rate of 64.9% and 35.1% of *E. coli* was detected in sheep and goats, respectively (10) that might be due to better health and management conditions of the animals in that region.

In the current study, STEC with 81.8% *stx1* and 61.1% *stx2* virulence genes were detected in sheep and goats. Similar to our findings, the prevalence of *stx1* and *stx2* genes in sheep was reported as 67.8% and 78.6%, respectively, in Egypt (26). While, 66.85% of the diarrheic samples in the lambs at Anhui Province (China) were found to carry STEC, with 64.61% *stx1* gene and 14.61% *stx2* genes (27). In another study in South Africa, *stx1* was found in 47.2% while *stx2* was present in 100% samples in 53 *E. coli* isolates (10). In Turkey, 2.6% of neonatal lambs and goat kids with diarrhoea were found to carry *stx1* gene and 33.2% animals contained *stx2*, while 2.8% carrying both genes in 107 *E. coli* strains isolated from 43 animals farms (28). Hence, it could be depicted that these are two major serotypes of the bacterium that are mostly present in the diseased animals and are the foremost causative factor for the infection.

In a study at Brazil, the rectal swabs collected from the healthy sheep demonstrated about 115 *E. coli* isolates, wherein, 52.2% samples showed *stx1* gene, 33.3% samples were found to contain *stx2*, while 14.5% samples contained both genes (29). Similarly, 23.81% (33/138) of fecal samples carried *stx1* gene while 18.12% found to have *stx2* with 9.42% samples to carry both genes in the apparently healthy cattle and sheep at Egypt (30). In Netherlands, among the 1090 *stx*-positive samples, 31.3% were positive for *stx1* and 2.7% contained *stx2* with 66.1% of the samples carried the both genes in sheep and goats along with 5.6% (8/144) humans working at these dairy farms were found positive for STEC (31). Hence, it could be said that the apparently healthy animals could be asymptomatic reservoirs of disease causing STEC and due to direct contact with these carrier livestock species or consumption of foodstuffs or water contaminated with their feces, become a source of transfer for the infection to the healthy individuals.

In our study, ETEC with *est* and *elt* virulence genes were identified in 149 sheep and goats samples positive for *E. coil* and current findings revealed that 88.1% of the samples carried *est* gene, whereas, *elt* gene was not detected in any of the sample. In the same context, elt gene was also not detected in the *E. coli* isolates from sheep and goat feces at South Africa (10). Similarly, in India 18.46% of lambs and 8.57% of calves samples from diarrheic feces carried *est* gene, while none of the sample carried *elt* gene from ETEC isolates (32). Moreover, ETEC frequency in sheep was 61.24%, with 60.67% carrying *est* gene and only 1.12% samples carry *elt* gene (27). Furthermore, it has been reported that goats carried 6% of *est* and 4% of *elt* genes in Kenya (33). While, 11.2% of the lambs and young goats were detected to carry ETEC with 2.6% *est* and 28.2% *elt* genes in Turkey (28). These observations depict that these toxigenic genes are present in the disease causing bacteria in most of the sick animals, while the variations in the prevalence might be due to difference in the geographical regions and severity of the infection. Hence, these strains are a significant source of sickness and an emerging threat for human health and well being.

CONCLUSION

Small ruminants are an important reservoir of pathogenic bacteria and are a potential carrier to transmit the organisms to the healthy individuals. The microorganisms are a source of contamination for the animal products and the presence of virulence factors in these animal pathogens are a potential threat and a matter of serious concern for human health. *E. coli* is a pathogenic bacteria that cause diarrhea in small ruminants due to its ETEC and STEC strains that contain *est*, *elt*, *stx1* and *stx2* genes. Our study confirmed the presence of these strains and the pathogenic disease causing genes by molecular detection through PCR in the fecal samples of sick sheep and goats. The characterization of these bacterial strains was quite important from a public health and perspectives of preventive veterinary medicine.

Conflict of interest:

There is no conflict of interest in this study.

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Authors' contribution:

SI, ISS, MZM: Conceptualization; SI, DK, KA, HAA: Methodology; SI, MT, DK, HAA: Data curation; MN, TH, SA, ES: Formal analysis; ISS, MT, MZM: Investigation (Laboratory work & Biomarker assessment); SI, ES, HMA: Writing – Original Draft Preparation; ES, HMA, IA: Writing – Review & Editing; ISS, FN: Supervision.

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