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Frequency of Shiga Toxin Associated Genes in *Escherichia coli* Isolated from Salivary Abomasum Disease in Kid Goats

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ABSTRACT

Background: Salivary abomasum disease (SAD) is a devastating disease causing significant mortality in Iranian goat and sheep herds. Understanding the causative agents is essential for developing effective preventive measures. This study investigated the potential role of Shiga toxin-producing *Escherichia coli* (STEC) in SAD pathogenesis.

Methods: We isolated *E. coli* from kid goats aged 3-30 days experiencing a sudden, acute illness characterized by gait imbalance, and death within 48 hours during the kidding season. Following isolation, we employed multiplex PCR to identify the presence of Shiga toxin genes (Stx1 and Stx2) associated with virulence in STEC strains.

Results: *E. coli* was isolated from 30 (75%) out of 40 animals. Notably, 7 (23.3%) isolates harbored the *Stx2* gene, while only one isolate (3.3%) possessed the *Stx1* gene.

Conclusion: These findings suggest a potential role for STEC, particularly strains carrying the *Stx2* gene, in the development of SAD and multiple abomasal hemorrhages, in kid goats. The presence of Shiga toxin genes in a significant proportion of *E. coli* isolates highlights the importance of further research to elucidate their contribution to SAD pathogenesis and inform the development of targeted interventions.

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Introduction

High kid goat mortality rates during the neonatal period remain a significant challenge for livestock farms worldwide. Factors such as inadequate hygiene, nutrition, and husbandry practices contribute to lamb weakness and disease susceptibility, leading to production losses and economic hardship. Studies report an average global lamb mortality of approximately 15% in the first month of life, with limited improvement over the past four decades (1). Salivary abomasum disease (SAD), a complex digestive disorder primarily affecting ruminants like sheep and cattle, further exacerbates these issues. SAD disrupts the normal flow of partially digested food through the digestive tract, causing a cascade of problems that significantly impact animal health and productivity (2, 3).

SAD imposes a substantial burden on animal health and productivity. Affected animals often exhibit lethargy, absence of sucking, hind leg weakness, increasing abomasal/abdominal distension, dehydration, and frequent lying down. Untreated cases can progress to death within 1-4 days, with signs like abdominal pain, teeth grinding, and lying on one side. Bloody diarrhea might also occur in some animals. At necropsy, the abomasum is distended with gas, saliva along with mixed milk clots and gastric secretions. Additionally, multiple small abomasal hemorrhages with blood clots (‘coffee grains’) are visible on both the serosal and mucosal surfaces. Unlike watery mouth disease, minimal to no saliva production is observed throughout the disease course (8).

SAD poses a significant challenge in goat breeding, leading to substantial economic losses due to high mortality rates in kid goats less than one month old. The cause of the disease is not fully understood, and limited treatment options make it difficult to manage. Although the exact causes are still being investigated, previous research indicates that bacterial pathogens such as *Escherichia coli* and *Clostridium* species may play a role (4, 5).

Certain strains of *E. coli* bacteria harbor Shiga toxin (Stx) genes, encoding potent toxins that damage the intestinal lining and contribute to various illnesses. While research on the association between *stx*-positive *E. coli* and SAD is limited, some studies suggest a potential link between these toxin-producing bacteria and severe lamb and goat mortality (4, 5, 6). The potential involvement of Stx-producing *E. coli* in SAD is particularly concerning because these toxins could worsen the damage caused by the diseased salivary abomasum. Further investigation is crucial to understand the prevalence of Stx-producing *E. coli* in animals with SAD and definitively determine their role in disease development (7).

By elucidating the role of bacteria like *E. coli*, particularly strains harboring the *stx* gene, and implementing effective preventive and therapeutic measures, ranchers can safeguard the health and welfare of their animals, optimize production, and ensure the economic viability of their herds. This study aimed to isolate *E. coli* strains from cases of SAD in kid goats and investigate the presence of Shiga toxin-producing genes in these isolates.

Materials and Methods

Sample Collection

Forty dead kids aged 3-30 days were included in this study during the kidding season 2023. Animals with a history of sudden acute illness characterized by, gait imbalance, and death within 48 hours of sign onset were selected.

During initial clinical examination and necropsy, samples were collected exclusively from animals exhibiting characteristics, saliva, secretions, and uncoagulated milk. The abomasum mucosa displayed numerous hemorrhagic spots visible both externally and upon dissection. Additionally, blood-tinged milk resembling coffee grounds was observed within the abomasum. Samples included

swabs from the abomasum surface, abomasum contents, and tissue sections from the abomasum, liver, lung, heart, and kidney. These samples were collected aseptically using sterile swabs and transferred in 2 ml microtubes to the University of Tehran's microbiology laboratory for bacterial culture and detection.

Bacterial Isolation

For culture, 0.1 ml aliquots of abomasum contents and other samples were inoculated onto Blood Agar (HiMedia, India) and Brain Heart Infusion (BHI) broth (HiMedia, India) media under aerobic, anaerobic, and microaerophilic conditions. Additionally, 0.1 ml of abomasum samples were plated on McConkey Agar (HiMedia, India) to specifically isolate *E. coli*. All cultures were incubated at 37 °C for 18-24 hours.

Colonies observed on culture media were subjected to biochemical tests for definitive identification of *E. coli* isolates. These tests included nitrate reduction, oxidase, catalase, hydrogen sulfide (H_2S) production, indole, urease, gelatin hydrolysis, phenylalanine deaminase activity, lysine decarboxylase activity, and methyl red-Voges-Proskauer (MR-VP) test.

Molecular Analysis

According to the manufacturer's instructions, genomic DNA was extracted from biochemically confirmed *E. coli* isolates using the QIAGEN Puregene B Bacterial Genome Isolation Kit. DNA concentration was quantified using a spectrophotometer. Extracted DNA was diluted to a working concentration of 100 ng/µl and stored at -20 °C until further use.

Polymerase Chain Reaction (PCR) was employed to detect the presence of *stx* genes in the *E. coli* isolates. Primers specific for *stx1* and *stx2* genes, obtained from the study by Paton et al. (1998) (11), were used for amplification. Primer specificity was verified using BLAST on the National Center for Biotechnology Information (NCBI) website.

The PCR reaction mixture (25 µl) consisted of 5.5 µl nuclease-free water, 12.5 µl PCR buffer (Ampliqon- Denmark), 1 µl each of forward and reverse primers, and 5 µl of template DNA from each isolate. The thermal cycling program included an initial denaturation step at 94 °C for 5 minutes, followed by 40 cycles of denaturation at 94 °C for 15 seconds, annealing at 56 °C for 30 seconds, and extension at 72 °C for 45 seconds. A final extension step at 72 °C for 5 minute concluded the program.

Statistical analysis

Descriptive statistics were used to characterize the prevalence of *E. coli* and the frequency of Shiga toxin genes (*stx1* and *stx2*) identified in the *E. coli* isolates. This included calculating the proportion of samples positive for *E. coli* and the percentage of *E. coli* isolates harboring each toxin gene. A Chi-square test was employed to assess the presence of a statistically significant difference between the prevalence of *stx1* and *stx2* genes in the isolated *E. coli*. Statistical analyses were performed using SPSS software. The level of significance was set at $p<0.05$.

Results

Animal Characteristics

Forty dead kids aged 3-30 days were included in the study. Breeds consisted of Alpine goats 25% (n=10), mixed-breed (Mahabadi-Alpine) 37.5% (n=15), and Sanen 37.5% (n=15). All animals exhibited abomasal hemorrhage with varying degrees: mild 42.5% (n=17), moderate 30% (n=12), and severe 27.5% (n=11).

Bacterial Isolation

Culture of samples from the 40 animals yielded *E. coli* in 30 (75%). These isolates grew on Blood Agar and Brain Heart Infusion (BHI) broth media under aerobic, anaerobic, and microaerophilic conditions. The remaining 10 samples (25%) displayed growth of *Staphylococcus* 12.5% (n=5), *Bacillus* 7.5% (n=3), and *Pseudomonas aeruginosa* 5% (n=2) on culture media.

Gram staining and a panel of biochemical tests (nitrate reduction, oxidase, catalase, H_2S production, indole, urease, gelatin hydrolysis, phenylalanine deaminase activity, lysine decarboxylase activity, and MR-VP test) confirmed the identity of the 30 isolates as *E. coli* (Table 2).

Molecular Analysis of Stx Genes

Following confirmation of *E. coli* isolates through biochemical testing, a PCR analysis was conducted to investigate the presence of Shiga toxin genes (*stx1* and *stx2*). This analysis employed specific primers designed to target these virulence factors associated with microbial pathogenesis. The PCR results revealed that among the 30 *E. coli* isolates, 7 (23.3%) harbored the *stx2* gene. Interestingly, only one isolate (3.3%) tested positive for the *stx1* gene.

Table 1. Primers used in this study.

Gene	Primer sequence 5′ to 3′	Product size	Reference
stx1	F- ATAAATCGCCATTCGTTGACTAC R- AGAACGCCCACTGAGATCATC	180 bp	(11)
stx2	F- GGCACTGTCTGAAACTGCTCC R- TCGCCAGTTATCTGACATTCTG	255 bp	

Table 2. Staining results and differential biochemical tests of isolated *E. coli* strains.

Test	**Result**
Gram staining	Negative
Motivation	Motile
Nitrate	Positive
Oxidase	Negative
Catalase	Positive
H_2S	Negative
Indole	Positive
Gelatin	Negative
Urease	Negative
Phenylalanine deaminase	Negative
Lysin decarboxylase	Positive
MR-VP	MR+/VP-

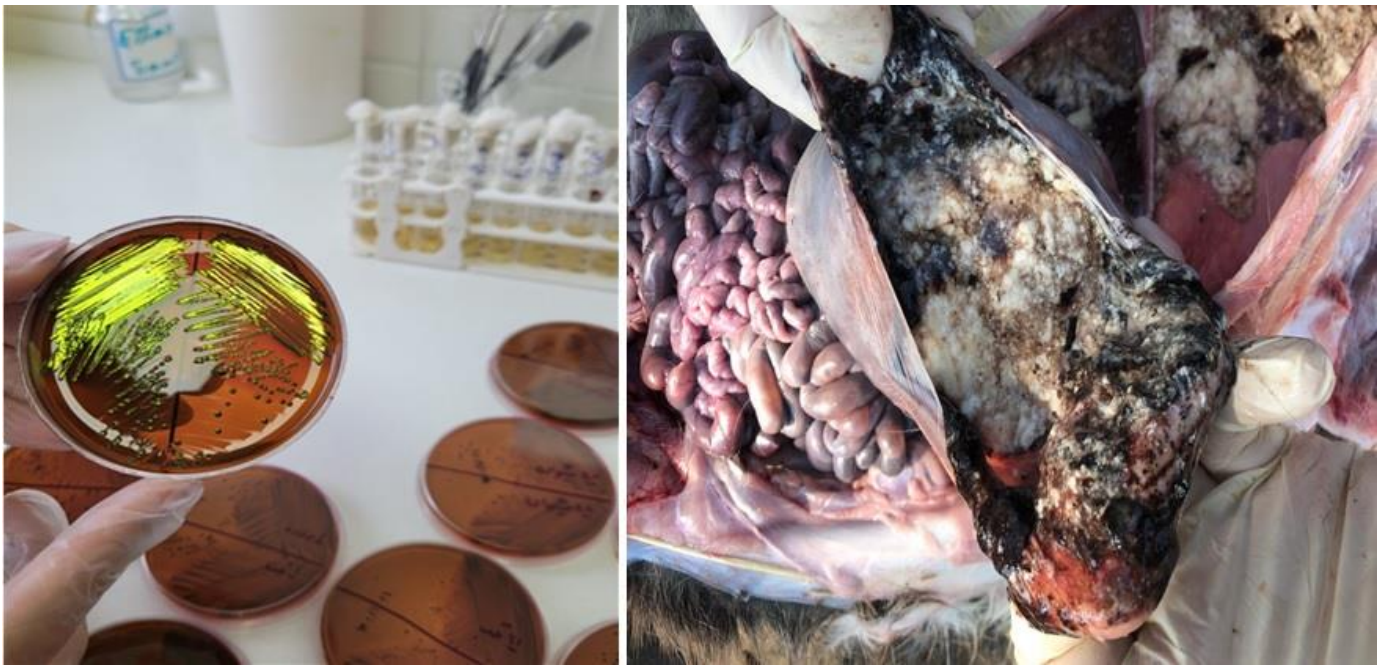

Fig 1. Image of SAD in a goat kid (right), and growth and production of green polish by *E. coli* strain isolated from sample number 5 in EMB culture medium (Left).

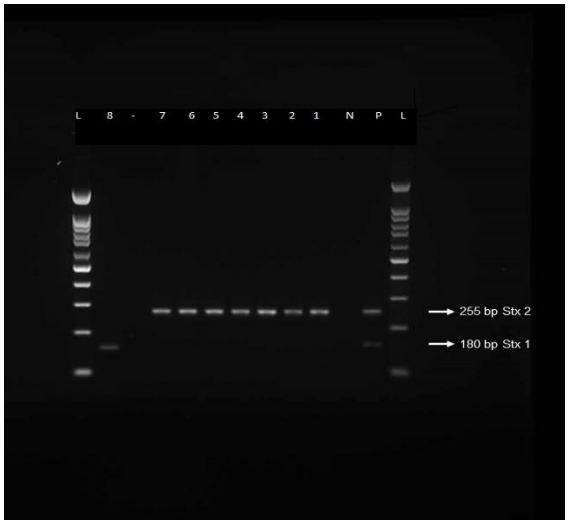

Fig 2. Electrophoresis analysis of multiplex PCR products for Stx gene detection in isolated strains. Lane L: 100 bp DNA ladder; Lane P: Positive control; Lane N: Negative control (sterile water); Lanes 1-8: Amplicons from isolated strains.

Discussion

Salivary abomasum disease is a complex digestive disorder primarily affecting young ruminants, particularly lambs and goats. It disrupts the normal flow of rumen digest, leading to a cascade of clinical signs including, lethargy, and weakness. While the etiology of SAD is not fully understood, bacterial involvement is suspected (2). This study investigated the potential role of STEC, a subset of *E. coli* known to produce Shiga toxins that damage the intestinal lining and contribute to disease (2, 12).

Our findings demonstrated that *E. coli* was isolated from 75% (30/40) of abomasum contents, liver, lung, heart, and kidney samples collected from animals with SAD. Our finding of a high *E. coli* prevalence aligns with prior studies suggesting bacterial colonization of the gastrointestinal tract in affected animals. Christodoulopoulos et al. (2013) cultured *E. coli* from six out of 37 abomasal fluid samples (16.2%), supporting this notion (3). Importantly, further analysis revealed the presence of *Stx2* genes in 23.3% (7/30) of *E. coli* isolates, while only one isolate (3.3%) harbored the *stx1* gene. Notably, isolates harboring the *stx2* gene did not possess *Stx1*. These results suggest a potential role for STEC, particularly strains carrying *stx2*, in the pathogenesis of SAD. The higher prevalence of *stx2* compared to *Stx1* aligns with observations in other ruminant diseases associated with STEC (13).

Our findings complement existing research on STEC and SAD. Christodoulopoulos et al. (2013) reported the isolation of *E. coli* from the stomachs of lambs with SAD, highlighting the potential involvement of these bacteria (3). However, their study employed traditional culturing methods, which may underestimate the prevalence of STEC compared to our more sensitive PCR approach. Additionally, Ghanbarpour et al. (14) reported a higher prevalence of *stx1* and *stx2* genes (40%) in One hundred ninety two *E. coli* isolates recovered from obtained rectal swabs from feces of healthy fat-tailed sheep in southeastern of Iran compared to our study. None of the positive isolates belonged to the O157 serogroup.

van Hoek et al. (2023) found STEC in dairy animals across Dutch farms (287 isolates) and even in some humans (8/144). Diverse STEC types were identified, including concerning serotypes. Worryingly, farm-to-human transmission was suggested by linking human isolates to animal isolates from the same farms (15). Ferreira et al. investigated Shiga toxin-producing *E. coli* in healthy sheep. A high prevalence (78.3%) of STEC was identified, with a significant proportion harboring either *stx1* (52.2%), *stx2* (33.3%), or both (14.5%). Additional virulence factors (*saa* and *cnf1*) and a high rate of antibiotic resistance (83.3%) suggest sheep could be potential reservoirs of STEC posing a public health risk. These variations could be attributed to differences in animal age, geographical location, or specific strains circulating in the studied populations.

Paton et al. (1998) validated the use of multiplex PCR for rapid and accurate identification of Shiga toxin-producing *E. coli* in stool or food samples. Their method targeted genes for *stx1*, *stx2*, and *eaeA*, providing information on both the type of Shiga toxin produced and the potential presence of intimin, a virulence factor associated with attaching to intestinal cells (11). This information is crucial for determining infection severity and guiding treatment decisions. In the present study, we employed a similar multiplex PCR approach using specific primers for *stx* genes in *E. coli* isolates. The results confirmed the findings of the initial differential biochemical investigation, further supporting the presence of STEC in animals with SAD.

While this study provides valuable insights into the potential role of STEC in SAD, limitations exist. Firstly, our investigation focused solely on the presence of Stx genes and did not assess Shiga toxin production at the protein level. Secondly, the

study design did not establish a causal link between STEC and SAD pathogenesis. Future research should employ techniques like toxin detection assays and in vivo models to solidify the connection between STEC and disease development. Additionally, investigating the mechanisms by which STEC contributes to SAD, such as through direct tissue damage or modulation of intestinal permeability, would be valuable (16, 17).

Conclusion

This study identified a high prevalence of *E. coli* in abomasum contents, and tissue sections from animals with SAD, with a significant proportion harboring *stx2* genes. These findings suggest a potential role for STEC, particularly *stx2*-positive strains, in the pathogenesis of SAD. Considering the important role of *E. coli* in the occurrence of SAD, it is necessary to implement effective prevention and treatment strategies and observe environmental hygiene to protect against the disease. Further research is necessary to elucidate the mechanisms by which STEC contributes to the disease for improved animal health and welfare.

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Ethics approval and consent to participate

Not needed.

Conflict of interest

The authors declare that they have no conflict of interest.

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