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Research Article

The Presence and Antibiotic Resistance of non-O157 STEC on Lamb Carcasses

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ABSTRACT

There is an increasing trend in the prevalence of Hemolytic Uremic Syndrome (HUS) both in Türkiye and the world. HUS might be caused by Shiga toxin (Stx)-producing *Escherichia coli* (STEC) O157 and strains of non-O157 STEC. The feces and fleece of lambs and non-hygienic conditions present in the lamb slaughtering process are the main sources of STEC contamination of lamb carcasses. In this study, the prevalence of STEC on lamb carcasses and, as an important global public health issue, the antibiotic resistance profiles of STEC strains isolated were determined. The presence of *stx* was considered as STEC indicator. STEC suspected isolates obtained by using chromogenic media were first confirmed by detecting 16S rRNA, and 16% (8/50) of the carcasses were found to be contaminated with *E. coli.* The analysis showed that none of the strains isolated were O157 serotype. Only one isolate had *hly* gene, 2 isolates carried both s*tx1* and *hly* genes together, and 3 isolates had both *stx2* and *eae* genes together. But 5 out of 8 strains isolated carried *stx1* and *stx2* genes, so they were identified as non-O157 cytotoxigenic *E. coli.* Antibiotic resistance profiles of the isolates were determined by using the Kirby Bauer Disc Diffusion method. All of the isolates were found to be resistant to at least one antibiotic investigated, and as the highest resistance rate was found, 87.5% of the isolates were resistant to both gentamycin and pefloxacine. In addition, 75% of the isolates were multidrug resistant (MDR), and the overall MAR (Multi Antimicrobial Resistant) index of isolates was 0.4. As a result, STEC contamination on lamb carcasses was considered a risk for both children and adults for HUS, and the high antibiotic resistance of the isolates observed also increased public health hesitations. Reassessment of the slaughtering process based on the HACCP (Hazard Analysis and Critical Control Points) requirements and taking necessary actions/measures to control cross contaminations are thought to be crucial steps to reduce pathogenic bacteria incidence in the food chain.

***Keywords**: Antibiotic resistance, lamb carcasses, non-O157 STEC.*

Kuzu Karkaslarında Non-O157 STEC Varlığı ve Antibiyotik Direnci

ÖZET

Son dönemde Hemolitik Uremik Sendrom'un (HUS) prevalansı dünyada ve ülkemizde artış göstermektedir. HUS Shiga toxin (Stx) üreten *Escherichia coli* (STEC) O157 ve non-O157 STEC kaynaklı meydana gelmektedir. Kuzuların dışkıları, postları ile kesim prosesindeki iyi olmayan hijyen koşulları karkaslardaki STEC kontaminasyonunun kaynakları olarak kabul edilmektedir. Araştırmada kuzu karkaslarında STEC varlığı ile halk sağlığı açısından küresel bir problem olan antibiyotik direnç profilinin belirlenmesi amaçlanmıştır. Çalışmada *stx* genlerinin varlığı STEC indikatörü olarak kullanılmıştır. Kromojenik besiyeri kullanılarak izole edilen STEC şüpheli izolatların konfirmasyonunda ilk olarak 16S rRNA geni varlığı araştırılmış, karkasların %16'sının (8/50) *E. coli* ile kontamine olduğu belirlenmiştir Yapılan analizler sonucu izolatların hiçbirinin O157 serotipi olmadığı görülmüştür. İzolatların 1'inin sadece *hyl* genini, 2'sinin *stx1* ile *hyl* genlerini, 3'ünün ise *stx2* ve *eae* genlerini birlikte taşıdığı belirlenmiştir. Diğer taraftan elde edilen 8 izolattan 5'inin *stx1* ile *stx2* geni taşıması nedeniyle non-O157 STEC *E. coli* olduğu tespit edilmiştir. Kirby Bauer yöntemi kullanılarak antibiyotik direnç profili değerlendirilmiştir. İzolatların tamamının araştırılan antibiyotiklerden en az birine dirençli olduğu, en çok da %87,5 oranda gentamisin ile pefloksasin direnci tespit edilmiştir. Bununla birlikte izolatların %75'i çoklu antibiyotik direnci göstermiş ve tüm izolatlar için de çoklu antimikrobiyal direnç (MAR) indeksi oranı 0,4 olarak belirlenmiştir. Sonuç olarak kuzu karkaslarında STEC kontaminasyonu HUS açısından başta çocuklar olmak üzere yetişkinler için de risk oluşturmasının yanı sıra yüksek orandaki antibiyotik dirençliliği de endişenin boyutunu arttırmaktadır. Kesim prosesinin HACCP gereklilikleri açısından yeniden değerlendirilmesi ile düzeltici önlemlerin/aksiyonların alınması gıda zincirine giren patojenik bakterilerin yaygınlığının azaltılmasında etkili olacaktır.

***Anahtar kelimeler**: Antibiyotik dirençliliği, kuzu karkas, non-O157 STEC.*

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Introduction

Shiga toxin producing *Escherichia coli* (STEC) may cause several foodborne diseases from bloody diarrhea, haemorogical colitis to haemolytic uremic syndrome (HUS). HUS, as one of the most frequent causes of acute kidney damages, is a multisystemic disease causing microangiopathic haemolytic anemia, thrombocytopenia and kidney failures especially in children younger than 5 years old (Tarr et al., 2005). Development of HUS requires several factors such as Stx1 and Stx2 cytotoxins encoded by *stx1* and *stx2* as well as intimine for the attachment of STEC to enterocyte and hemolysin (Etcheverría et al., 2010). Stx2-producing strains are more virulent than those of both Stx1 and Stx1 and Stx2-producing strains. Stx2 producing strains are more frequently associated with HUS than Stx1 producing strains (Paton and Paton, 2002). In addition to this, in a report produced by EFSA and ECDC (2019) stated that besides *stx1* and *stx2* genes, the presence of *eae* gene encoding intimin, which is responsible from the adhesion, should be investigated to identify STEC serogroups isolated from foodstuff.

E. coli O157 was previously identified as the primary causative agent of HUS worldwide. However, recent studies have revealed that other serotypes, such as O26, O45, O103, O111, O121, and O145, can also lead to STEC-HUS (McCarthy et al., 2021a; Alharbi et al., 2022). Foods such as raw meat, unpasteurized milk, and vegetables contaminated with these serotypes are frequently associated with HUS cases in humans (Liu et al., 2021). Over 8000 STEC infections were reported in Europe in 2018, 37% of which ended up with hospitalization of the patients (EFSA and ECDC, 2019). The EU One Health 2020 Zoonosis Report highlighted the increase in STEC infections between 2015 and 2019 (EFSA and ECDC, 2021). Although cattle are the primary source of STEC contamination, small ruminants are also stated as an important STEC reservoir and contamination source for the food industry (Mughini-Gras et al., 2018). It is also reported that small ruminants release more feces with longer period when compared to large ruminants (McCarthy et al., 2021a). The variations in contamination and cross contamination levels were reported to be due to the size of the establishment, design of the slaughterhouse, equipment, the speed of the slaughter line and the number of the animals slaughtered, geographical location, season, the hygienic conditions of holding paddocks, the animal species and age as well as the training and knowledge level of personnel (USDA, 2002). The hygienic status of the slaughtering process is affected by several contamination and cross contamination steps in the slaughter line. Applications at some processing steps such as dehiding and evisceration may result in higher carcass contamination risk than at the other processing steps. Some parts of the carcasses (brisket, shank etc.) are more susceptible to contamination and cross contamination than the other parts. Contamination of the carcasses along with the slaughtering process is considered as main port contamination in the meat supply chain for STEC (Etcheverría et al., 2010; Milios et al., 2011).

Antibiotic resistant bacteria become a serious public health problem due to the excessive usage of antibiotics in farm animals (Pan et al., 2021). Therefore, considering 'One Health' principle, the surveillance of the resistant zoonotic pathogens in animals is crucial for evaluating public health risks (Gupta et al., 2022). *E. coli* is considered an indicator of antibiotic resistance and *E. coli* strains including STEC have been used for monitoring and

Table 1. Primers and thermal cycler conditions used in this study

Gene	Primer sequence (5'-3')	Product Size	Thermal cycler conditions	References
16S rRNA	F: 5'- GACCTCGGTTTAGTTCACAGA-3' R: 5'- CACACGCTGACGCTGACCA-3'	585 bp	Initial denaturation 95˚C 5 min (1 cycle) 94˚C 1 min; 58˚C, 1 min; 72˚C 1 min; 72˚C 7min (35 cycles)	Parvin et al. (2020)
rfbE	F: 5'- CAGGTGAAGGTGGAATGGTTGTC-3' R: 5'-TTAGAATTGAGACCATCCAATAAG-3'	296 bp		
stx_1	F: 5'-TGTAACTGGAAAGGTGGAGTATACA-3' R: 5'-GCTATTCTGAGTCAACGAAAAATAAC-3'	210 bp	Initial denaturation 94˚C 2 min (1 cycle) 94˚C 20 s; 60˚C 1 min; 72˚C 1 min; 72˚C 10 min (35 cycles)	Jeshveen et al. (2012)
stx2	F: 5'-GTTTTTCTTCGGTATCCTATTCC-3' R: 5'- GATGCATCTCTGGTCATTGTATTAC-3'	484 bp		
eaeA	F: 5'- ATTACCATCCACACAGACGGT-3' R: 5'-ACAGCGTGGTTGGATCAACCT-3'	397 bp		
hyl	F: 5'-ACGATGTGGTTTATTCTGGA-3' R: 5'-CTTCACGTCACCATACATAT-3'	166 bp		

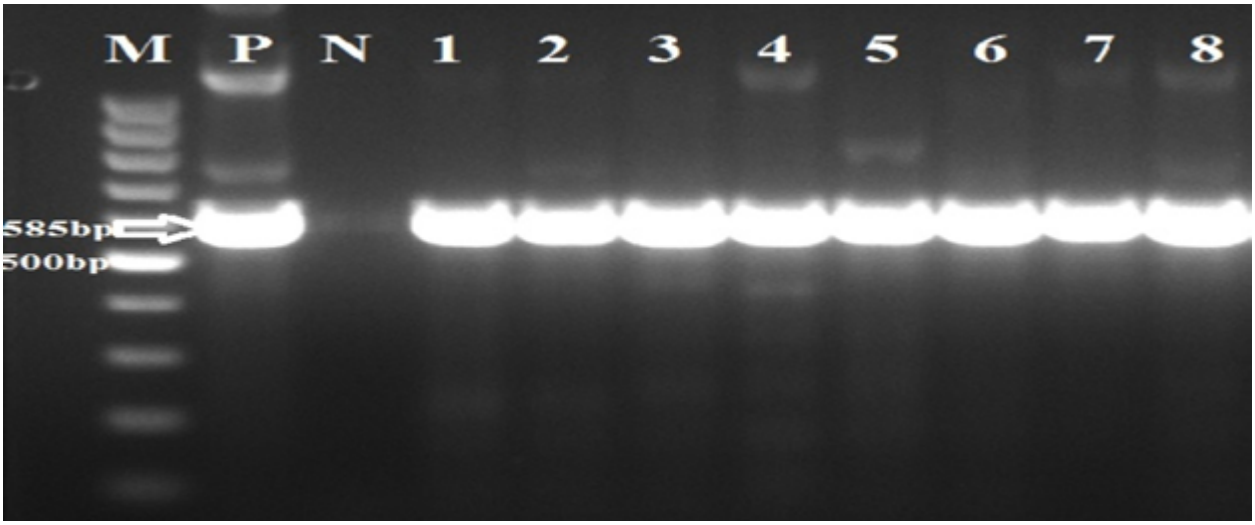

Figure 1. PCR assay for *E. coli* M: DNA ladder, P:positive control, ATCC 25922, N: negative control, 1-8 Lanes: samples isolate

surveillance of antibiotic resistance in animals, different environments, and humans (Rubab and Oh, 2021). With *E. coli* being the most widely studied bacteria, the resistance to at least two classes of antibiotic agents has been frequently documented (Karama et al, 2019; Pan et al, 2021; Rubab and Oh, 2021).

Toxins of STEC originated from animal foods might cause serious health hazards. As a natural reservoir small ruminants take an important place in disseminating antibiotic resistance to environment and foods. Determination of the prevalence of antibiotic resistant STECs and their resistance profiles may aid determining treatment alternatives for human infections. This study aimed to find out the presence of STEC on lamb carcasses and antibiotic resistance profiles of isolates.

Materials and Methods

Material

In this study, a total of 50 lamb carcasses, slaughtered at a private slaughterhouse located in Muğla, were used as material. Samples were taken according to the ISO 17604 (2015) standard by using the sponge swab technique following the slaughtering process, just before chilling. Sponge swabs were soaked in 10 ml buffered peptone water (Oxoid CM509, England) before swabbing. Sampling was carried out on a total of 400 cm^2 of area from front and rear shanks, neck and brisket areas (100 cm^2 each) of randomly selected carcasses using a sterile template (10x10 cm). Swab samples were taken to the laboratory on the same day under cold chain (+4°C) and analyzed for STEC.

Microbiological Analysis

Swab samples were homogenized in 90 ml of Modified Tryptone Soya Broth (Merck,1.090205.0500, Germany) including novobiocin then incubated at 37°C for 18-24 hrs (ISO 16654, 2001). CHROMagar STEC (CHROMagar, ST160(B), ST162(S) suppl., France) agar was used for selective isolation.

Typical colonies with violet color and smooth edges were taken and subjected to biochemical analysis. The isolates were stored at -20ºC in Tryptone Soya Broth (Oxoid, CM129, USA) containing 20% glycerol (Merck, 1.04092.100, Germany) for PCR analyses.

DNA Extraction and PCR Analysis

DNA extraction was conducted according to the instructions of the producer (Thermo Scientific Fisher, USA). Confirmation of conventionally identified isolates was carried out by using the Parvin et al. (2020) method to determine the presence of the 16S rRNA gene. Then, the presence of O157 (*rfbE*), shigatoksin 1 (*stx1*), and shigatoksin 2 (*stx2*), intimin (*eaeA*), and hemolysin (*hyl*) genes was investigated by using multiplex PCR technique developed by Jeshveen et al. (2012)(Table1).

Determination of antimicrobial susceptibility of E. coli strains

Antimicrobial susceptibility tests were conducted by Kirby Bauer disc diffusion method using *Enterobacteriaceae* specific antibiotic discs; ampicillin (10 µg), cefotaxime (5

Table 2. Virulence genes distribution among *E. coli* isolates

Isolate number	Virulence genes	Total (n=6) (%)
K5	*hyl*	1 (16.6)
K1, K8	*stx1, hyl*	2 (33.3)
K2, K6, K7	*stx2, eae*	3 (50)

µg), ceftazidime (10 µg), meropenem (10 µg), aztreonam (30 µg), pefloxacin (5 µg), gentamicin (10 µg), chloramphenicol (30 µg), tigecycline (15 µg) and trimethoprim/sulfamethoxazole (1.25-23.75 µg), referred by The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021) were used. Mueller-Hinton agar (Oxoid CM0337B, England) was used in the disk diffusion method, and the results were assessed following incubation at 37°C for 24 hours. MDR was defined as showing resistance to at least three of antimicrobials used.

Reference Strains

E. coli ATCC 25922 was used as positive control for microbiological analysis and targeting 16S rRNA genes. *E. coli* ATCC 43888 was used as positive control for *E. coli* O157:H7 *E. coli* positive strains for virulence genes were kindly supplied by Prof. Dr. Şükrü KIRKAN from Faculty of Veterinary Medicine, Aydın Adnan Menderes University, Aydın, Türkiye.

Results

A total of 8 STEC suspected isolates were isolated conventionally from 50 lamb carcasses. In order to identify *E. coli* isolates, the presence of the 16S rRNA gene was investigated. All of the isolates were found to carry 16S rRNA gene, which is specific for *E. coli* (Figure 1). None of the isolates had *rfbE* gene. 6 out of 8 isolates (75%) were reported to have at least 1 virulence genes investigated. In addition, only one isolate had *hly* gene, 2 isolates carried both *stx1* and *hly* genes together, and 3 isolates had both *stx2* and *eae* genes together (Table 2). 5 isolates had *stx1* and *stx2* genes so referred as Table 2 - Figure 1.

All of the isolates identified in this study were resistant to at least 1 antibiotic. They were also found to be susceptible to chloramphenicol, tigecycline and trimethoprim/sulfamethoxazole. When resistance profile of the isolates was evaluated, maximum resistance was observed against gentamycine and pefloxacine (87.5%) followed by ceftazidime (62.5%), ampicillin, aztreonam and meropenem (37.5%), and cefotaxime (25%). The antibiotic resistance profiles of the isolates were shown in Table 2. 25% of the isolates were classified as low drug resistant (LDR), whereas 75% as MDR. Overall Multi Antimicrobial Resistant (MAR) index of isolates was reported as 0.4. MAR index of 75% of the isolates (6/8) were higher than 0.2 (Table 1).

Discussion

Ruminants generally disseminate high amounts of STEC over a long period to the environment, which is called "super shedding." Super shedding is the most important source of STEC contamination in the food supply chain (Alharbi et al., 2022). McCarthy et al. (2021b) reported that a high percentage of lambs were STEC reservoirs, disseminating this agent by feces and taking an important role in the environmental contamination of STEC. Although cattle were referred as the primer STEC carrier, several researchers reported the carriage rate of lambs between 1.8% and 86.2% (Kalchayanand et al., 2007; Söderlund et al., 2012; Maluta et al., 2014; Oporto et al., 2019).

It is almost impossible to preclude contamination at the slaughterhouse. Several factors such as fleece, internal organs, equipment, other carcasses, aprons and hands of the personnel may affect contamination of carcasses (Hauge et al., 2011). Especially dehiding and evisceration process may disseminate microbiota including pathogens such as STEC, cause contamination and increase the microbiological load of carcasses (Hauge et al., 2011; McCarthy et al., 2021a). In this study, 10% of the lamb carcasses were found to be contaminated by STEC. Several studies conducted on lamb carcasses and meat samples showed that STEC contamination levels were ranged between 2.02% and 81.6% (Kalchayanand et al., 2007; Momtaz et al., 2013; Maluta et al., 2014; Ferhat et al., 2019). The differences between the contamination rates might be due to the isolation methods used, preslaughter and post-slaughter contamination levels of the ani-

Table 3. Antimicrobial resistance profiles of *E. coli* strains isolated from lamb carcasses

Antimicrobial resistance profile	No (%) isolates	*MAR index	Classification of isolates	
			Type of resistance	No (%) isolates
ATM, CTX, CN, CIP, CAZ, MRP	2 (25)	0.6	MDR	6 (75)
AMP, ATM, CN, CIP, CTX	1 (12.5)	0.5		
AMP, CAZ, CIP, CN	1 (12.5)	0.4		
AMP, CAZ, CN, MRP	1 (12.5)	0.4		
CIP, CN, CAZ	1 (12.5)	0.3		
CIP, CN	1 (12.5)	0.2	LDR	2 (25)
CTX, CIP	1 (12.5)	0.2		

MAR Index=Number of resistance antibiotics/total number of antibiotics tested (Krumperman, 1983)

mals, hygienic status of the premises and the slaughtering methods. In addition, although fleece cleanliness and the STEC contamination levels on the fleece were not investigated before slaughtering, the handmade fleece removal process without using an automated escalator applied on the premises might be one of the reasons for carcass contamination.

None of the 8 *E. coli* isolates did not carry *rfbE* and 5 isolates had at least one *stx* genes. Two out of five STEC isolates carried both *stx1* and *hyl*. Due to its direct association with Stx production, hemolysin is considered an important epidemiological indicator for easy and rapid identification of STEC serotypes (Alharbi et al., 2022). *eae* gene is located on a plasmid like plasmid R100 of *Shigella* spp. and pO157 of *E. coli* O157:H7 carried by a pathogenicity island called Locus of Enterocyte Effacement. These plasmids can be transferred horizontally to the other pathogens (Deng et al., 2001). On the other hand, *stx* genes are carried by mobile genetic elements. Due to mobile genetic elements, toxin genes are transferred to the other *E. coli* bacteria via transduction causing new formation of STEC clones (Quiros and Muniesa, 2017). 3 out of 5 non-O157 STEC isolates carried both *stx*2 and *eae* genes together. This genetic combination improves the virulence of pathogens causing more serious clinical symptoms in infected individuals (Werber et al., 2003; Alharbi et al., 2022). Similar to this study presented here, several previous studies conducted in the USA, Türkiye and Algeria reported that *stx2* gene was more dominant in compared to *stx1* (Kalchayanand et al., 2007; Gencay, 2014; Ferhat et al., 2019). However, studies carried out in Iran and China reported that *stx1* gene was more dominant (Babolhavaeji et al., 2021; Liu et al., 2022).

Apart from infections caused by STEC virulence factors, antibiotic resistance of bacteria/pathogens, including STEC, is another important problem for human and animal health issues worldwide. This situation increases the need for new surveillance studies on antibiotic resistance of bacteria (Rubab and Oh, 2021). Beta-lactam antibiotics have been well-known for a long time and are considered to have low toxicity and wide-range antibacterial spectra. However, they are also known as one of the most frequently seen antibiotic resistant group (Pan et al., 2021; Rubab and Oh, 2021). Unlike previous studies on the antibiotic-resistant profile of *E. coli*, the results of this study stated that beta-lactam resistance was not in the first place (Momtaz et al., 2013; Enriquez-Gómez et al., 2023). It is stated that quinolones, β-lactams and trimethoprim/sulfamethoxazole can cause bacterial SOS reactions and the massive release of Shiga toxins (Pan et al., 2021). However, the effects of various antibiotic classes on the shiga toxin production are varied. Quinolones, tetracycline, chloramphenicol and fosfomycin do not only inhibit shiga toxin release but also affect the adhesion of pathogens to intestinal membranes, which make them very usable antimicrobial tools to prevent development of HUS in the treatment of STEC infections (Mir and Kudva, 2019). It was reported that cytotoxin production of STEC could be blocked according to the dose of azithromycin, tetracycline or gentamicin exposed (Berger et al., 2019). The results of this study revealed that all of the isolates were susceptible to tetracycline, chloramphenicol and trimethoprim/sulfamethoxazole, but were greatly resistant to gentamicin and pefloxacin. Although meropenem is considered as an effective antibiotic for STEC infection treatments, 37.5% of isolates are resistant to this antibiotic. These results support the need to develop therapeutic strategies. The global increase in the rate of field isolates showing MDR is considered as an important threat for public health. The MAR index is an effective risk evaluation mark, and a MAR value of 0.2 indicates high-risk contamination, and overuse or misuse of antimicrobials in the relevant region (Krumperman, 1983). MAR index for all isolates was determined as 0.4, the MAR index of 62.5% of the isolates was found to be higher than 0.2, which was in agreement with previous studies (Elabbasy et al., 2021; Mateus et al., 2021; Rubab and Oh, 2021).

Conclusion

In this study, the presence of STEC virulence genes in isolates obtained from lamb carcasses showed that lamb carcasses are potentially pathogenic STEC reservoirs for human. The presence of pathogens on the lamb carcass surfaces indicated insufficient good hygienic practices applied along the slaughter line. Considering the antibiotic resistance problem, new strategies should be developed related to antibiotic treatments in animals by authorities, and intensive control programs should be implemented. In addition, related regulations should be updated and non-O157 STECs should be added to the pathogens list that have the potential to pose a serious threat to public health and should not be present in the foodstuff. Rapid diagnostic kits for foodborne pathogens should be used at slaughterhouses and sufficient hygiene procedures should be implemented.

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Conflict of interest

The authors declare that they have no conflict of interest in this study.

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