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Research Article

Verotoxin Production in Strains of *Escherichia coli* Isolated from Cattle and Sheep, and Their Resistance to Antibiotics

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Abstract: In this study, 130 *Escherichia coli* strains isolated from 306 cattle and sheep fecal samples were studied for their resistance to 10 antibiotics, verotoxin production, and hemolyzation.

The antibiotic resistance rates of the *E. coli* strains were as follows: tetracycline, 51.6%; streptomycin, 24.2%; ampicillin, 13.1%; amoxicillin/clavulanic acid, 5.2%; gentamycin, 4.6%; ciprofloxacin, 4.6%; trimethoprim-sulfamethoxazole, 4.3%; cefotaxime, 0.7%. None of the strains were resistant to cefepime or ceftazidime. Of all the antibiotics tested, only resistance to streptomycin was higher in the strains isolated from cattle than in the sheep strains ($P = 0.043$). The evaluation of the cattle strains, based on gender and age, indicated that the resistance to tetracycline and streptomycin was higher in the female cattle than in male cattle, whereas in cattle under 2 years of age resistance to tetracycline was significantly higher than in 3-year-old cattle. Among the cattle strains there were 4 (2.2%) of the VTEC 0157 serotype. All were sensitive to the antibiotics tested, and the isolation rate of VTEC non-0157 serotypes was 14.5%.

The overall verotoxin production rate of the *E. coli* strains was 36.9%, while it was 61.1% in sheep strains and 19.7% in cattle strains. Verotoxin production in sheep strains was significantly higher than in cattle strains ($P < 0.001$). No correlation was detected between verotoxin production in the cattle strains and antibiotic resistance; however, resistance to ampicillin and streptomycin in the sheep strains that did not produce verotoxin was higher than that observed in the sheep strains that did ($P = 0.048$ and $P = 0.009$, respectively).

The most common hemolysis type in the isolated *E. coli* strains was gamma hemolysis (46.2%). Ampicillin and amoxicillin/clavulanic acid resistance was significantly higher in the strains that hemolyzed than in the strains that did not.

In conclusion, sheep carry higher risks of verotoxin produced by *E. coli* strains than cattle do. Furthermore, due to higher resistance rates to such antibiotics as tetracycline and streptomycin, careful antibiotic selection for infections, particularly those caused by verotoxigenic *E. coli*, is of extreme importance.

Key Words: Cattle, sheep, *Escherichia coli*, verotoxin, antibiotic resistance

Sığır ve Koyunlardan İzole Edilen *Escherichia coli* Suşlarında Verotoxin Üretimi ve Antibiyotiklere Direnç Durumu

Özet: Bu çalışmada, sığır ve koyun dışkısından izole edilen toplam 306 *Escherichia coli* suşunun 10 antibiyotiğe direnç durumu ve bu suşlardan 130'unun verotoxin üretimi ve hemoliz özelliği araştırılmıştır.

E. coli suşlarında tespit edilen antibiyotik direnç oranları sırasıyla; tetrasiklin % 51,6, streptomisin % 24,2, ampisilin % 13,1, amoksisilin-klavulanik asit % 5,2, gentamisin % 4,6, siprofloksasin % 4,6 ve trimetoprim-sulfametaksazol % 4,3, sefotaxim % 0,7 olup sefepim ve seftazidim'e dirençli suş saptanmamıştır. Test edilen antibiyotikler arasında sadece streptomisin direncinin sığır suşlarında koyun suşlarından yüksek olduğu tespit edilmiştir ($P = 0,043$). Sığır suşları cinsiyete ve yaşa göre değerlendirildiğinde dişi sığırlarda tetrasiklin ve streptomisin direncinin erkek sığırlara göre yüksek olduğu, ayrıca 2 yaş ve altındaki sığırlarda 3 yaşın üzerindeki sığırlara göre tetrasiklin direncinin anlamlı oranda yüksek olduğu görülmüştür. Sığırlardan izole edilen suşlardan dört tanesinin (% 2,2) VTEC 0157 serotipine ait olduğu, bu suşların test edilen antibiyotiklere duyarlı olduğu ve VTEC non-0157 serotipinin izolasyon oranının % 14,5 olduğu görüldü.

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E. coli suşlarında verotoxin üretimi % 36,9 olup, koyun suşlarında bu oran % 61,1 ve sığır suşlarında % 19,7'dir. Koyunlardan izole edilen suşlarda verotoxin üretiminin sığırlardan izole edilen suşlardan anlamlı düzeyde yüksek olduğu görülmüştür (P < 0,001). Sığır suşlarında verotoxin üretimiyle antibiyotik direnci arasında bir ilişki tespit edilememiş olmasına rağmen koyun suşlarında ampisilin ve streptomisin direnci verotoxin salgılamayan suşlarda verotoxin salgılayan suşlara göre yüksek bulunmuştur (sırasıyla P = 0,048 ve P = 0,009).

E. coli suşlarında en sık rastlanan hemoliz tipinin gamma hemoliz (% 46,2) olduğu, ampisilin ve amoksisilin-klavulanik asit direncinin hemoliz oluşturan suşlarda hemoliz oluşturmayan suşlardan anlamlı oranda yüksek olduğu tespit edilmiştir.

Sonuç olarak, verotoxin salgılayan *E. coli* suşları açısından koyunların sığırlara göre daha fazla risk teşkil ettiği ayrıca insan enfeksiyonlarının tedavisinde de kullanılan tetrasiklin ve streptomisin gibi antibiyotiklere direnç oranlarının yüksek düzeyde bulunduğu ve bu direncin özellikle verotoksijenik türlerde de yüksek düzeyde bulunması antibiyotik seçiminde daha titiz davranılması gereğini ortaya koymuştur.

Anahtar Sözcükler: Sığır, koyun, *Escherichia coli*, verotoksin, antibiyotik direnci

Introduction

It is well known that some species of *Escherichia coli* produce verotoxin (shiga toxin) and those that do are mainly hosted by cattle and sheep, and the gastrointestinal system flora of some domestic animals. Despite more than 100 *E. coli* serotypes identified as responsible for verotoxin production in cattle, there are more than 150 such *E. coli* serotypes that cause disease in humans. Verotoxin-producing *E. coli* (VTEC) usually do not lead to disease in animals; however, they have been an important cause of food-borne illness worldwide. Currently, research is urgently needed to determine the antimicrobial resistance profiles among VTEC (1,2). The new zoonosis directive (the directives of 2003 European Council) forces EU member states to implement monitoring programs that provide comparable data on the occurrence of antimicrobial resistance in zoonotic agents, and other agents if they present a threat to public health (3).

The use of antibiotics in animal food as preservatives or for treatment purposes creates resistance, particularly in *E. coli* strains in fecal flora (4). The resultant *E. coli* strains might transfer their resistance to other bacteria through transposon, plasmid, and integrons (4,5). Antimicrobial treatment is the most important factor responsible for the formation of resistant strains, both in humans and in animals (4). Thus, this study aimed to determine the antibiotic resistance of *E. coli* strains in the fecal flora of cattle and sheep, and to investigate the transfer rate of this resistance to VTEC strains.

Materials and Methods

With swabs, 306 fecal samples were collected from the rectal mucosa of sheep and cattle (male and female, various ages) at a slaughterhouse in Kırıkkale, Turkey between March 2003 and September 2004. In all, 130 *E. coli* strains isolated from the fecal samples were evaluated for antibiotic resistance, verotoxin production, and hemolyzation. One sample isolated from each animal was studied. The specimens were obtained from different cattle and sheep strains that had undergone a cutting procedure every week; a maximum of 15 specimens were taken from every strain.

Microbiological Analysis

In the transportation of the samples for pre-enrichment, tryptic soy broth (TSB, Oxoid) plates were used. The samples were streaked on sorbitol MacConkey (SMAC, Oxoid) agar plates, aerobically incubated at 37 °C for 18-24 h, and then evaluated for sorbitol fermentation. The colonies that reproduced on SMAC agar were removed to a TSB plate and *E. coli* identification was performed according to classical methods (6). The *E. coli* strains were kept at –70 °C until verotoxin study.

Verotoxin

For the identification of verotoxin, the Ridascreen ELISA (R-Biopham AG, Darmstadt, Germany) kit was used. According the recommendations by the manufacturer, *E. coli* colonies were transferred to GN Hajna broth plates and studied after 24 h in a horizontal shaker at 37 °C. The cut-off value was determined by adding 0.1 to the absorbance value of the negative

control. Any strains with results over this value were considered verotoxin producing.

Serological method

In all the *E. coli* strains studied, the 0157 latex agglutination kit (Oxoid) was used to detect O antigen in the colonies that did not ferment sorbitol. In line with the recommendations of the manufacturer, when the samples that showed agglutination with O antigen did not agglutinate in control latex test, the result was considered positive.

To test the antibiotic sensitivity of the *E. coli* strains, the following antibiotics were used in disk diffusion tests according to NCCLS recommendations (7): ampicillin (AMP) (10 µg), amoxicillin/clavulanic acid (AMC) (20/10 µg), tetracycline (TET) (30 µg), streptomycin (STR) (10 µg), trimethoprim-sulfamethoxazole (SXT) (1.25/23.75 µg), ciprofloxacin (CIP) (5 µg), gentamycin (GN) (10 µg), cefepime (FEP) (30 µg), ceftazidime (CAZ) (30 µg), and cefotaxime (CTX) (30 µg). *E. coli* ATCC 25922 was used as the quality control strain.

Phenotype verification test

The phenotypic confirmatory test uses a disk diffusion method. CTX and CAZ disks, alone and in combination with clavulanic acid (CA), were used to detect extended spectrum beta-lactamase (ESBL), the presence of which was confirmed when there was a $\geq$ 5 mm zone size difference between the CTX/CA or CAZ/CA disks compared to the disks without CA (7).

Alpha hemolysis and enterohemolysis

To tryptic soy agar plates were added 10-mM $CaCl_2$ and 5% sheep blood (bathed in PBS, pH 7.2, 3 times). The hemolysis zone present after 3-h incubation was evaluated as γ-hemolysis, and an extra hemolysis zone after an additional night of incubation of the tryptic soy agar plates was evaluated as enterohemolysis (8).

Beta hemolysis

The hemolysis zone after 1-night incubation in 5% sheep blood agar was evaluated as β-hemolysis (9).

Gamma hemolysis

Despite the synthesis of a hemolysis zone in 5% sheep blood Columbia agar, the colonies that did not hemolyze in human blood agar were evaluated as γ-hemolytic (9).

Statistical analysis

The relationship of antibiotic resistance to other parameters was evaluated with Pearson's chi-square test in SPSS v.11.5. When one of the cells' expected count was < 5, an evaluation continuity correction was performed with the chi-square test. $P < 0.05$ was considered statistically significant.

Results

Cattle and sheep *E. coli* strains were the most resistant to TET and only STR resistance was higher in the cattle strains than in the sheep strains ($P = 0.043$) (Table 1).

When the cattle *E. coli* strains were evaluated according to age and gender, TET and STR resistance were higher in female cattle than in male cattle. Furthermore, in the cattle ≤ 2 years of age, TET resistance was significantly higher than in the 3-year-old cattle (Table 2).

Table 1. The distribution of antibiotic resistance profiles of *E. coli* strains isolated from cattle and sheep [n (%)].

Animals	AMP	AMC	TET	STR	SXT	CIP	GN
Cattle strains (n = 180)	25 (13.9)	10 (5.6)	96 (53.3)	*51 (28.3)	9 (5)	10 (5.6)	11 (6.7)
Sheep strains (n = 126)	15 (11.9)	6 (4.8)	62 (49.2)	23 (18.3)	4 (3.2)	4 (3.2)	3 (2.4)
Total (n = 306)	40 (13.1)	16 (5.2)	158 (51.6)	74 (24.2)	13 (4.3)	14 (4.6)	14 (4.6)

AMP: ampicillin; AMC: amoxicillin/clavulanic acid; TET: tetracycline; STR: streptomycin;
SXT: trimethoprim-sulfamethoxazole; CIP: ciprofloxacin; GN: gentamycin.
*significantly higher in cattle strains ($P < 0.05$)

Table 2. The effect of gender and age on antibiotic resistance [n (%)].

Antibiotic	The strains isolated from cattle (n = 180)					
	Gender			Age		
	Female (n = 63)	Male (n = 117)	P	0-2 years (n = 56)	≥ 3 years (n = 124)	P
AMP	8 (12.7)	17 (14.5)	NS	5 (8.9)	20 (16.1)	NS
AMC	4 (6.3)	6 (5.1)	NS	0	10 (8.1)	NS
TET	47 (74.6)	49 (41.9)	< 0.001	36 (64.3)	60 (48.4)	0.048
STR	26 (41.3)	25 (21.4)	0.005	15 (26.8)	36 (29)	NS
SXT	4 (6.3)	5 (4.3)	NS	2 (3.6)	7 (5.7)	NS
CIP	6 (9.5)	4 (3.4)	NS	2 (3.6)	8 (6.5)	NS
GN	5 (7.9)	6 (5.1)	NS	5 (8.9)	6 (4.8)	NS

NS: not significant (P > 0.05)

Overall verotoxin production in the *E. coli* strains was 36.9%; in sheep strains this rate was 61.1%, while in cattle strains it was 19.7%. Verotoxin production in sheep strains was significantly higher than in cattle strains (P < 0.001). Of the *E. coli* strains isolated from cattle, 4 (2.2%) were of the VTEC 0157 serotype and all of them were resistant to the antibiotics tested. Verotoxin production in the *E. coli* strains isolated from female, male, younger than 2-year-old, and over 3-year-old cattle was 7.9%, 11.8%, 2.6%, and 17.1%, respectively.

In all, 31 (35.2%) of the strains that fermented sorbitol and 17 (40.5%) of the strains that did not ferment sorbitol were VTEC. Despite higher verotoxin production in the *E. coli* strains that did not ferment sorbitol, the difference was not statistically significant. Furthermore, in the VTEC strains that did not ferment sorbitol, the resistance to TET, STR, and AMP was 35.3%, 11.8%, and 0%, respectively (data not shown). In the *E. coli* strains that fermented sorbitol, AMP resistance was higher than in the strains that did not ferment sorbitol (P = 0.029) (Table 3).

STR and SXT resistance was higher in *E. coli* strains that did not produce verotoxin than in those that did (P = 0.001 and P = 0.029), and AMP and AMC resistance in the strains that hemolyzed was higher than in those that did not (P < 0.001 and P = 0.004)

In all, 52 *E. coli* strains (40%) hemolyzed and the types of hemolysis formed were γ-hemolysin (46.2%), α-hemolysin (19.2%), β-hemolysin (13.5%), and enterohemolysin (7.7%). All the *E. coli* strains that produced enterohemolysis were verotoxin producing. The rate of Cefotaxime resistance among the *E. coli* strains was 0.7% and no strain was resistant to FEP or CAZ. With the phenotypic verification test, no GSBL producing strain was detected.

Discussion

Antibiotic use leads to resistance in pathogenic bacteria as well as the development of resistant strains in flora bacteria. The resistance that develops in flora bacteria may be transferred to other bacteria and infect humans through direct or indirect routes (meat and meat-by-products). In particular resistant strains form associations with the antibiotics used in veterinary medicine (tetracycline, streptomycin, ampicillin, trimethoprim-sulfamethoxazole, enrofloxacin, etc.). Fecal indicator bacteria (*E. coli* and enterococci) are used for determining this resistance. In a study by Sayah et al. (10), using 407 fecal *E. coli* strains isolated from cattle, antibiotic resistance rates were reported to be 23.1% for TET, 10.2% for STR, and 2.5% for AMP. In another study, conducted by Orden et al. (11) with 195 fecal *E. coli* strains isolated from dairy calves with diarrhea, TET and STR resistance was over 65%, and AMP and trimethoprim resistance was 23%-50%, while GN resistance was less than 10%. Sato et al. (12) found

Table 3. The effects of verotoxin production, sorbitol fermentation, and hemolysis on the antibiotic resistance of E. coli strains [n (%)].

Animals	AMP	AMC	TET	STR	SXT	CIP	GN
Verotoxin producing							
present (n = 48)	6 (12.5)	2 (4.2)	22 (45.8)	4 (8.3)	0	0	2 (4.2)
absent (n = 82)	21 (25.6)	11 (13.4)	50 (60.9)	*27 (32.9)	*10 (12.2)	4 (4.9)	0
Sorbitol fermentation							
present (n = 88)	*23 (26.1)	9 (10.2)	47 (53.4)	20 (22.7)	6 (6.8)	2 (2.3)	2 (2.3)
absent (n = 42)	4 (9.5)	4 (9.5)	25 (59.2)	11 (26.2)	4 (9.5)	2 (4.8)	0
Hemolysis							
present (n = 52)	*19 (36.5)	*10 (19.2)	29 (55.8)	12 (23.1)	7 (13.5)	2 (3.8)	0
absent (n = 78)	8 (10.3)	3 (3.8)	43 (55.1)	19 (24.4)	3 (3.8)	2 (2.6)	2 (2.7)

* Significant difference in antibiotic resistance ($P < 0.05$).

31.6% resistance to TET, 18.4% to STR, 16.3% to AMP, and 3.9% to GN in 570 cattle *E. coli* strains. They showed that the resistance rates of animals that had never been exposed to antibiotics were significantly lower than those reported. The resistance rates varied as TET > STR > AMP (highest to lowest) in our study as in theirs (Table 1). The higher rate of TET resistance in the sheep *E. coli* strains may have been associated with the frequent use of oxytetracycline preparations for the treatment of infection in these animals. The difference in the resistance rates of both studies may be attributed to the different strategies of antibiotic use in the areas where the studies were performed.

Antibiotic resistance determined in the cattle *E. coli* strains varied based on age and gender (Table 2). Sato et al. (12), Hinton et al. (13), Khachatryan et al. (14), and Hoyle et al. (15) all reported higher antibiotic resistance rates in young animals than in old animals. They account for this by the more frequent exposure of young animals to growth promotion and/or treatment, or to the gastrointestinal system differences of young and old animals (13,14). Higher antibiotic resistance in young animals might be explained by higher exposure to diseases (16). It was shown that healthy lactating cattle were the primary source of TET resistance and that TET resistance was higher than in non-lactating cattle (17).

VTEC, with their ability to develop infections in humans, pose a great risk. The incidence of VTEC in the gastrointestinal system flora varies among different animals. Beutin et al. (18) studied 720 healthy animals (different kinds of animals) and reported that VTEC strains were present at a particularly high rate in sheep (67%), whereas this rate was 21% in cattle. In another study by Beutin et al. (19), 26.3% of 114 fecal *E. coli* strains isolated from cattle and 44.6% of 150 fecal *E. coli* strains isolated from sheep were verotoxin producing. Gülhan (20) detected 30% VTEC isolation in sheep and 8% VTEC isolation in cattle in Turkey. Although verotoxin production was reported to be higher in the sheep *E. coli* strains, they usually did not involve the eae gene and thus had low virulence (2,15). Similarly, in our study, the prevalence of VTEC strains was significantly higher in the sheep strains (61.1%) than in the cattle strains (19.7%). Furthermore, all the strains of the VTEC 0157 serotype (2.2%) were isolated from cattle, and the prevalence rate of VTEC non-0157 serotype in cattle was 14.5%. In studies involving the *E. coli* 0157 serotype conducted in Turkey, the serotype was identified in 4.2% of 330 cattle by Yilmaz et al. (21) and in 1.3% of 312 cattle by Çabalar et al. (22). The discrepancy in the rates found in these studies may be due to variations in isolation techniques used or seasonal and geographical differences. On the other hand, Wells et al. (23) detected VTEC non-0157 strains in 14% of 322 cattle, which is compatible with the results of our study.

In recent years, TET, STR, and sulfonamides resistance has been shown to be on the rise in VTEC 0157 and non-0157 strains (24). In the USA, 56 *E. coli* O157:H7 strains were found to be sensitive to all the antibiotics tested in the years 1984-1987, while only

7.4% of 176 strains isolated in the years 1989-1991 were resistant to STR, TET, and sulfisoxazole (24). It has been concluded then that parallel to the formation of resistant *E. coli* strains in food animals the rate of isolation has increased, which is a sign of increased antibiotics use. On the other hand, the emerging antibiotic resistance of *E. coli* O157:H7 could signify an increased prevalence of these pathogens in food animals that receive antibiotics. Maidhof et al. (25) studied 48 VTEC 0118:H16 isolated from humans and cattle in 7 European countries and reported rates of STR, TET, AMP, and GN resistance of 95.8%, 75%, 66.7%, and 10.4% respectively, whereas in 119 VTEC non-0118 strains these rates were 15.1%, 10.9%, 3.4%, and 0%. The resistance rates were shown to vary depending on *E. coli* serotype and to have an increasing trend. Despite the higher resistance rates in the *E. coli* strains that did not produce verotoxin than in the VTEC strains found in the present study, their similarity in TET and STR resistance rates, particularly in the cattle strains, is suggestive of a transfer of resistance between them.

As in the VTEC 0157 serotype, some verotoxigenic serotypes that cause infection in humans (e.g., 026, 0111, and 055) have been reported to not ferment sorbitol (26). In our study, the VTEC strains that did not ferment sorbitol had resistance rates of 35.3%, 11.8%, and 0% to TET, STR, and AMP, respectively. These strains were shown to be VTEC non-0157. Accordingly, TET resistance in VTEC non-0157 was significantly high.

Data on the efficiency of γ-hemolysin forming *E. coli* strains are scarce. *E. coli* strains producing α-hemolysin were found to be associated with enterotoxigenic *E. coli* in animals, while enterohemolysin was detected in VTEC strains (9). In our study, all the enterohemolysin synthesizing *E. coli* strains were VTEC.

In conclusion, sheep constitute a greater risk for VTEC strain formation than cattle do. *E. coli* strains have high resistance rates to antibiotics, such as TET and STR. Still higher resistance in VTEC non-0157 serotypes indicates the need for greater scrutiny in antibiotic selection.

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