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Original Article

Investigation of antibiotic resistance, virulence genes, and biofilm formation of *Escherichia coli* isolated from sheep feces in Shiraz industrial slaughterhouse, South of Iran

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Abstract

Background: With the increase in human population, the consumption of livestock products such as sheep meat has also increased. Sheep are the reservoir and shedder of *Escherichia coli* that can be transmitted to humans. **Aims:** Characterization of fecal *E. coli* isolated from sheep in slaughterhouse. **Methods:** Stool specimens were collected from 30 apparently healthy sheep from different flocks in Shiraz industrial slaughterhouse. The resistance of *E. coli* isolates against 10 antibiotics was determined by disk diffusion method. The presence of three major extended spectrum beta-lactamase (ESBL) genes and five tetracycline resistance genes as well as seven virulence genes were investigated by polymerase chain reaction (PCR) technique. Using the microtiter plate method, the biofilm formation ability of *E. coli* isolates was investigated. **Results:** The highest frequency of resistance was to amoxicillin (100%) followed by tetracycline (25%). All *E. coli* isolates were susceptible to gentamicin and nitrofurantoin, and only one isolate was resistant to the tested third-generation cephalosporins. Multidrug resistance phenotype was observed in 16.7% of the isolates. bla_{TEM} (25%) was the most prevalent ESBL gene and *tetA* (62.5%) was the most prevalent tetracycline resistance gene in the isolates. *crl*, *csgA*, *fimH*, and *bcsA* genes were present in all isolates, and the prevalence of *papC* and *afa* genes was 95.8% and 83.3%, respectively. In total, 62.5% of the isolates were biofilm producers. **Conclusion:** According to the concept of One Health, the presence of virulent antibiotic-resistant biofilm producing strains of *E. coli* in sheep is a risk to public health.

Key words: Antibiotic resistance, Biofilm formation, *Escherichia coli*, Sheep, Virulence gene

Introduction

With the increase in human population, the consumption of livestock products has increased. Domestic ruminants, including healthy sheep, are natural reservoirs of *Escherichia coli* and shed it through feces (Lanumtiang *et al*., 2022). Sheep intestinal *E. coli* is under selective pressure due to the high use of antibiotics either to treat or prevent infections or to stimulate growth (Singh *et al*., 2019). Since *E. coli* often carries antibiotic resistance genes on plasmids or other mobile genetic elements, it can easily transfer resistance genes to other intestinal and even pathogenic bacteria (Cheney *et al*., 2015). The increasing prevalence of antibiotic-resistant *E. coli* in food-producing animals has not only affected the effectiveness of antibiotic treatment in these livestock, but also indirectly causes the failure of antibiotic treatment in humans. Because the antimicrobial resistance of normal flora and pathogens in the human intestine can be associated with the antimicrobial resistance of food-producing animals bacteria (Gemeda *et al*., 2023). Fecal contamination of livestock products such as sheep meat during slaughter or direct and indirect contact between livestock and humans can be the way of zoonotic transmission of antibiotic-resistant *E. coli* to humans (Shabana and Al-Enazi, 2020). In addition, slaughterhouse wastewater is contaminated with a large amount of disease-causing and/or drug-resistant bacteria that can spread if enter surface and underground water without treatment (Gemeda *et al*., 2023). Some strains of *E. coli*, depending on the genotype of virulence and resistance, have the ability to survive and grow in different environments (Nielsen *et al*., 2022). One of the reasons for their survival is the ability to form biofilms. This ability helps them to show more resistance to adverse environmental conditions and antimicrobial substances, and after entering the host, it also causes resistance to the host's defense mechanisms and persistence of the infection. Bacterial adhesion is the prerequisite for biofilm formation. Adhesion encoding genes play an important role in the process of *E. coli* colonization and contribute

to its invasiveness (Hafez, 2020).

Infections caused by biofilm-producing and multidrug-resistant (MDR) *E. coli* are less responsive to antibiotic treatment. Since *E. coli* can be transmitted from sheep to humans, especially through the food chain, genotypic and phenotypic characterization of apparently healthy sheep *E. coli* is essential both for therapeutic purposes in sheep and for public health. Although several articles have investigated the characteristics of pathogenic *E. coli* strains isolated from sheep (Tahamtan *et al*., 2011; Ghanbarpour *et al*., 2017), there is little information about characteristics of commensal *E. coli* strains isolated from healthy sheep in Iran (Aliasadi and Saei, 2015; Safavi and Shahbazi, 2017). So, we aimed to characterize fecal *E. coli* isolates from apparently healthy sheep in the slaughterhouse based on resistance to seven classes of antibiotics (third-generation cephalosporins, penicillins, tetracyclines, quinolones, nitrofuran, aminoglycosides, and sulfonamides), the presence of major extended spectrum beta-lactamase (ESBL) genes, tetracycline resistance genes, and seven virulence genes that play a role in adhesion, colonization, and biofilm formation, and their ability to form biofilm.

Materials and Methods

Specimens

During the fall of 2018, stool specimens were randomly and aseptically collected from 30 apparently healthy sheep from different flocks that were transported to Shiraz industrial slaughterhouse for slaughter. This slaughterhouse is intended for the Islamic slaughter of ruminants and is located in the southeast of Shiraz city (Fars, Iran).

Sampling of feces was done based on the principles of the Declaration of Helsinki and with the permission of livestock farmers (Shiraz University Ethical Committee registration number: MSC9731394). The specimens were placed in sterile containers with lids and transported to the laboratory for culture within a maximum of 2 h.

Isolation and identification of *E. coli*

Each stool specimen was diluted with sterile normal saline at a ratio of 1:10 and then cultured on MacConkey agar (Merck, Darmstadt, Germany) plate. After incubating the plates for 24 h at 37°C, a pink lactose positive colony was randomly selected from each plate and cultured on Eosin Methylene Blue medium (Merck, Darmstadt, Germany). After incubating the plates at 37°C for 24 h, Gram-staining and biochemical tests, including IMViC and motility tests (Merck, Darmstadt, Germany) were performed on colonies with a green metallic sheen to confirm *E. coli* isolates (Markey *et al*., 2013).

Phenotypic investigation of antibiotic resistance

In order to characterize the antibiotic resistance patterns in sheep *E. coli* isolates, antibiogram was performed by disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2018). Ten common antibiotics used in livestock and humans, including cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), amoxicillin (25 µg), tetracycline (30 µg), levofloxacin (5 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg), gentamicin (10 µg), and trimethoprim-sulfamethoxazole (25 µg) were used in this investigation. *E. coli* ATCC® 25922 was used as a positive control. Based on the diameter of the inhibition zone around each antibiotic disk (Padtan Teb, Tehran, Iran) and the information provided in the instructions (CLSI, 2018), the isolates were divided into two categories: antibiotic-resistant and antibiotic-susceptible. According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the isolates with intermediate susceptibility were considered antibiotic-susceptible (The European Committee on Antimicrobial Susceptibility Testing, 2019).

Genotypic investigation of antibiotic resistance genes

Since beta-lactams and tetracyclines are two groups of widely used antibiotics in humans and livestock, the presence of $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} genes, as the major ESBL genes, and *tetA*, *tetB*, *tetC*, *tetD*, and *tetM* genes, implying tetracycline resistance were investigated using polymerase chain reaction (PCR) technique on sheep *E. coli* isolates. For this purpose, DNA of the isolates was first extracted by boiling method (Derakhshandeh *et al*., 2014). The quality and quantity of the extracted DNA were checked by reading their absorbances (OD) at 260 nm and 280 nm by NanoDrop spectrophotometer (Spectrum Instrumentation, Grosshansdorf, Germany). The extracted DNAs with an A_{260}/A_{280} ratio of 1.7-2 were acceptable (Emett *et al*., 2020). Also, the extracted DNAs were tested by agarose gel electrophoresis (Paya Pajoohesh Pars, Tehran, Iran) to check the formation of a high molecular weight band.

For each PCR reaction, 3 µL of template DNA was added to a mixture containing 12.5 µL of *Taq* DNA Polymerase 2x Master Mix RED (Ampliqon, Odense, Denmark), 1 µL of each forward primer and reverse primer (SinaClon, Tehran, Iran), and 7.5 µL of sterile distilled water. Primer sequences, annealing temperature, and amplicon sizes are listed in Table 1.

Finally, the PCR products were electrophoresed using 1% agarose gel (Pars Tous, Mashhad, Iran) containing Safe stain (YTA, Tehran, Iran) for 1 h at 80 V. The photography of agarose gels was done on a UV transilluminator (Labnet, New Jersey, USA).

Detection of virulence genes

Detecting *afa*, *crl*, *csgA*, *fimH*, *papC*, *sfa*/*focDE*, and *bcsA* genes responsible for bacterial attachment and biofilm formation, was performed by PCR method. Primer sequences, annealing temperature, and amplicon sizes are listed in Table 1. Master mix preparation, electrophoresis, and visualization of PCR products were done similar to the methods described in the section of genotypic investigation of antibiotic resistance genes.

Table 1: Primer sequences, annealing temperature, and amplicon sizes of studied genes

Genes	Primer sequences (5´ to 3´)	Amplicon size (bp)	Annealing temperature	References
β-lactamase genes				
bla$_{CTX-M}$	F: ACCGCCGATAATTCGCAGAT R: GATATCGTTGGTGGTGCCATA	588	54°C	Tabar *et al*. (2016)
bla$_{TEM}$	F: TTCTTGAAGACGAAAGGGC R: ACGCTCAGTGGAACGAAAAC	1150	53°C	Brinas *et al*. (2002)
bla$_{SHV}$	F: CACTCAAGGATGTATTGTG R: TTAGCGTTGCCAGTGCTCG	885	50.5°C	Brinas *et al*. (2002)
Tetracycline resistance genes				
tetA	F: GGCCTCAATTTCCTGGACG R: AAGCAGGATGTAGCCTGTGC	372	65°C	Srinivasan *et al*. (2007)
tetB	F: GAGACGCAATCGAATTCGG R: TTTAGTGGCTATTCTTCCTGCC	228	66°C	Srinivasan *et al*. (2007)
tetC	F: TGCTCAACGGCCTCAACC R: AGCAAGACGTAGCCCAGCG	379	66.4°C	Srinivasan *et al*. (2007)
tetD	F: CTGGGCAGATGGTCAGATAA R: TGACCAGCACACCCTGTAGT	832	57.5°C	Srinivasan *et al*. (2007)
tetM	F: GTGGACAAAGGTACAACGAG R: CGGTAAAGTTCGTCACACAC	406	55°C	Warsa *et al*. (1996)
Virulence genes				
fimH	F: TGCAGAACGGATAAGCCGTGG R: GCAGTCACCTGCCCTCCGGTA	508	60°C	Johnson and Stell (2000)
papC	F: GACGGCTGTACTGCAGGGTGTGGCG R: ATATCCTTTCTGCAGGGATGCAATA	328	60°C	Le Bouguenec *et al*. (1992)
csgA	F: ACTCTGACTTGACTATTACC R: AGATGCAGTCTGGTCAAC	200	60°C	Maurer *et al*. (1998)
afa	F: GCTGGGCAGCAAACTGATAACTCTC R: CATCAAGCTGTTTGTTCGTCCGCCG	750	60°C	Le Bouguenec *et al*. (1992)
crl	F: TTTCGATTGTCTGGCTGTATG R: CTTCAGATTCAGCGTCGTC	250	61°C	Maurer *et al*. (1998)
sfa/focDE	F: CTCCGGAGAACTGGGTGCATCTTAC R: CGGAGGAGTAATTACAAACCTGGCA	410	61°C	Le Bouguenec *et al*. (1992)
bcsA	F: AGAGTACGTCGACTGGGTGA R: CCCACACCATACTGACGACC	140	61°C	Maurer *et al*. (1998)

Phenotypic investigation of *E. coli* biofilm formation ability

The phenotypic investigation of biofilm formation ability by sheep *E. coli* isolates was performed using the microtitre plate method according to the protocol provided by Hassan *et al*. (2011). Briefly, the isolates were inoculated in trypticase soy broth (TSB) (Merck, Darmstadt, Germany) containing 1% glucose, incubated at 37°C for 24 h, and diluted at 1:100. From each dilution, 200 µL were added to three wells of every microtitre plate (Merck, Darmstadt, Germany), and this experiment was repeated in three separate microtitre plates. An equal amount of sterile TSB was added to the negative control wells. The cultured microtitre plates were incubated at 37°C for 24 h to recognize the bacteria with the ability of biofilms formation. The wells were then washed and fixed, and the bacterial biofilm was stained. After measuring the absorbance of the contents of the wells at 570 nm by a micro-enzyme-linked immunosorbent assay (ELISA) reader (BioTek, Japan). The results were interpreted according to the instructions of Stepanović *et al*. (2007). For this purpose, ODc was determined to be three standard deviations higher than the average OD of the negative control. The average OD values of repeated wells of each sample were calculated and compared with ODc. In this way, if the average OD values of repeated wells of each sample were less than or equal to ODc, the bacterium was considered as non-biofilm producers. If this value was less than or equal to two times the ODc, the bacterium was considered as weak biofilm producers. If this value was less than or equal to four times the ODc, the bacterium was considered as a moderate biofilm producer, and if it was more than four times ODc, the bacterium was considered as a strong biofilm producer (Stepanović *et al*., 2007).

Statistical analysis

SPSS software was used to perform Chi-square or Fisher's exact tests to report and compare the results of the investigations (SPSS software version 16.0; SPSS Inc., Chicago, USA). In order to interpret the results of statistical tests, $P \leq 0.05$ was considered statistically significant.

Results

E. coli prevalence in sheep feces

Of a total of 30 sheep fecal specimens, 24 samples (80%; 95% CI 65-95%) were characterized *E. coli*.

Antibiotic resistance pattern of sheep *E. coli*

Resistance frequency of *E. coli* strains against 10 common antibiotics from seven antibiotic classes is reported in Fig. 1. In *E. coli* isolates, the highest frequency of resistance was observed against amoxicillin as a member of penicillin group, followed by tetracycline as a member of tetracyclines group, levofloxacin and

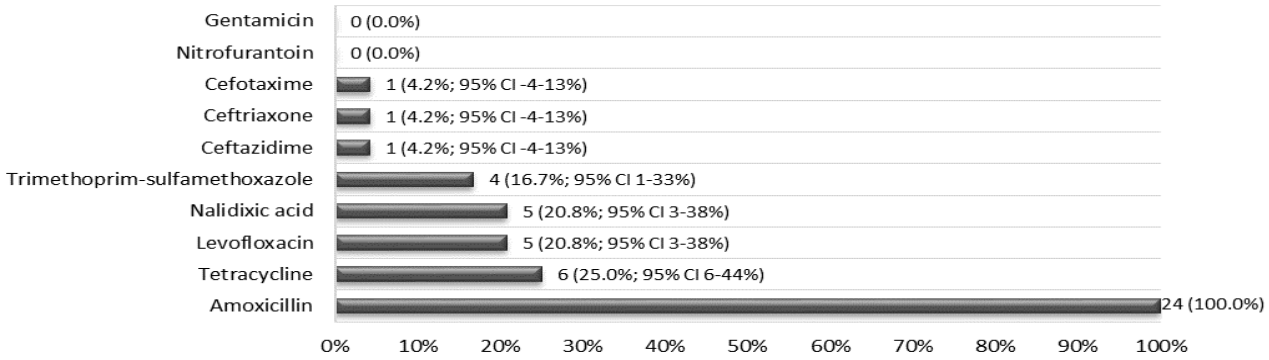

Fig. 1: Frequencies of antibiotic resistance in sheep fecal *E. coli*

nalidixic acid as members of quinolones group, and trimethoprim-sulfamethoxazole as a member of sulfonamides group. In contrast, all *E. coli* isolates were susceptible to gentamicin as a member of aminoglycosides group and nitrofurantoin as a member of nitrofuran group. The level of resistance against cefotaxime, ceftazidime and ceftriaxone as members of the third-generation cephalosporins was also low. It is noteworthy that 23 out of 24 (95.8%) isolates were susceptible to all three tested third-generation cephalosporins. One remaining isolate was simultaneously resistant to cefotaxime, ceftazidime, and ceftriaxone.

The statistical analysis of the results obtained significant associations between resistance to some antibiotics. For example, between resistance to the tested third-generation cephalosporins (P=0.042) and also between resistance to the tested quinolones (P<0.001). Additionally, significant associations were identified between resistance to the tested quinolones and tetracycline (P=0.006), as well as between resistance to the tested quinolones and trimethoprim-sulfamethoxazole (P=0.018). Furthermore, a significant association was found between resistance to tetracycline and trimethoprim-sulfamethoxazole (P=0.035).

Multidrug resistant (MDR) refers to isolates resistant to at least one member of three or more antibiotic classes. Four (16.7%; 95% CI 1-33%) sheep fecal *E. coli* isolates were found MDR. Resistance to the quinolones (P<0.001), tetracycline (P=0.001), and trimethoprim-sulfamethoxazole (P=0.008) was significantly associated with the incidence of MDR phenotype.

In the study of the resistance pattern against 10 common antibiotics, it was found that *E. coli* isolates showed resistance against the studied antibiotics with 7 different patterns (Table 2). All isolates were resistant to at least one antibiotic. The dominant resistance pattern was resistance to amoxicillin alone, which was observed in 16 (66.6%) isolates. On the other hand, the maximum number of antibiotics to which the isolates showed resistance at the same time was eight, and this pattern was observed only in 1 (4.2%) of the sheep *E. coli* isolates. Therefore, the resistance score, defining the number of antibiotics to which the isolates were resistance, for the *E. coli* isolates was between 1 and 8. The score median and the mean were respectively 1 and 1.96.

Table 2: Frequencies of different antibiotic resistance and virulence patterns and the resistance scores and the virulence scores in sheep fecal *E. coli* isolates*

Patterns	Resistance /Virulence score	Frequency
Antibiotic resistance pattern		
AMX	1	16 (66.6%)
AMX-TE	2	2 (8.3%)
AMX-SXT	2	1 (4.2%)
AMX-LEV-NA	3	1 (4.2%)
AMX-LEV-NA-TE	4	1 (4.2%)
AMX-TE-LEV-NA-SXT	5	2 (8.3%)
AMX-TE-LEV-NA-SXT-CAZ-CRO-CTX	8	1 (4.2%)
Virulence genes pattern		
fimH-papC-csgA-bcsA-crl	5	4 (16.6%)
afa-fimH-csgA-bcsA-crl	5	1 (4.2%)
afa-fimH-papC-csgA-bcsA-crl	6	19 (79.2%)

* AMX: Amoxicillin, TE: Tetracycline, SXT: Trimethoprim-sulfamethoxazole, LEV: Levofloxacin, NA: Nalidixic acid, CAZ: Ceftazidime, CRO: Ceftriaxone, and CTX: Cefotaxime

Prevalence of major ESBL and tetracycline resistance genes

Among the three tested major ESBL genes, bla_{TEM} had the highest prevalence and was found in six sheep *E. coli* isolates. In contrast, bla_{SHV} was not present in any of the isolates and $bla_{CTX\text{-}M}$ was found in only one isolate. Regarding the tetracycline resistance genes, the most prevalent gene was *tetA*, followed by *tetM*, and *tetB*. On the other hand, *tetD* was not found in any of the sheep *E. coli* isolates and *tetC* was present in only one isolate. Fig. 2 shows frequencies of major ESBL and tetracycline resistance genes in sheep fecal *E. coli* isolates (Fig. 2).

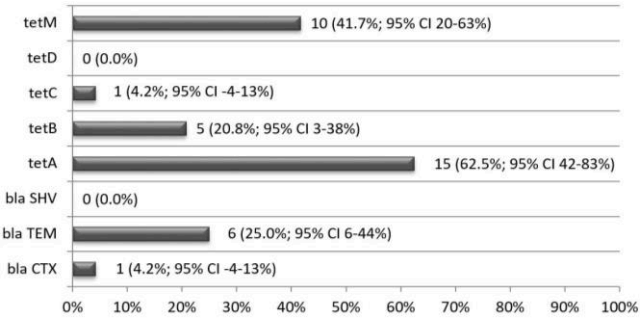

Fig. 2: Frequencies of major ESBL and tetracycline resistance genes in sheep fecal *E. coli*

In the study of the pattern of presence of ESBL genes, it was found that 18 (75%) of the *E. coli* isolates lacked the three studied ESBL genes. The most common ESBL genes pattern was the presence of bla_{TEM} gene alone in 5 (20.8%) of the isolates and then the simultaneous presence of bla_{TEM} and bla_{CTX-M} genes in 1 (4.2%) of the *E. coli* isolates.

The presence of the bla_{CTX-M} gene in *E. coli* isolates had a statistically significant association with their resistance to the three tested third-generation cephalosporin antibiotics (P=0.042). Moreover, the presence of the bla_{TEM} gene in *E. coli* isolates had statistically significant associations with their resistance to the tested quinolones antibiotics (P=0.006), trimethoprim-sulfamethoxazole (P=0.035), and MDR phenotype (P=0.035).

The highest number of tetracycline resistance genes that were simultaneously present in the isolates was three *tet* genes, which were observed in 3 (12.5%) *E. coli* isolates. In contrast, 5 (20.8%) isolates do not have any of the tested *tet* genes. The most common pattern of *tet* genes presence in *E. coli* isolates was the presence of *tetA* alone, which was observed in 6 (25%) of the isolates.

Prevalence of virulence genes

A high frequency of studied virulence genes was observed in *E. coli* isolates from sheep feces, so that except for the *sfa/focDE* gene which was not present in any of the isolates, *crl*, *csgA*, *fimH*, and *bcsA* genes were present in all (100%) of the *E. coli* isolates, and the prevalence of *papC* and *afa* genes were 95.8% and 83.3%, respectively.

The study of the presence pattern of seven virulence genes in *E. coli* isolates showed that the isolates had at least five and at most six tested virulence genes. Therefore, the virulence score of *E. coli* isolates, which is the number of their virulence genes, varied from five to six (median=6; mean=5.79). Overall, three different virulence profiles were detected in *E. coli* isolates, as reported in Table 2.

Prevalence of *E. coli* with biofilm formation ability

Among all *E. coli* isolates, 15 (62.5%; 95% CI 42-83%) isolates could form biofilm. Frequencies of isolates with weak and moderate biofilm-producing *E. coli* were 8 (33.3%) and 7 (29.2%), respectively.

Discussion

As an intestinal microflora, *E. coli* is increasingly exposed to antibiotics and is prone to antibiotic resistance, so even healthy livestock can carry antibiotic-resistant *E. coli*. This bacterium can potentially be considered a zoonotic foodborne pathogen for which ruminants such as sheep are one of the main reservoirs (Lanumtiang *et al*., 2022). In *E. coli* isolates from sheep feces examined in this study, the highest antibiotic resistance was observed against amoxicillin (100%), followed by tetracycline (25%). Since these antibiotics belong to the previous antibiotics generation, a higher level of resistance to amoxicillin and tetracycline would develop due to using a long time in livestock populations and resulting in selective pressure (Gemeda *et al*., 2023). Similar to our finding, in a study in France, the highest antibiotic resistance in *E. coli* isolates of diseased sheep and goat was observed against tetracycline and amoxicillin (>50%). The prevalence of resistance to trimethoprim-sulfamethoxazole and amoxicillin-clavulanic acid were mostly 20-40%. Also, in a study on sheep clinical *E. coli* isolates in United Kingdom, the highest resistance (35-65%) was observed against tetracyclines and ampicillin. The prevalence of resistance to trimethoprim-sulfamethoxazole, amoxicillin-clavulanic acid, and neomycin were 6-35% (Nielsen *et al*., 2022). Also, Cheney *et al*. (2015) reported the highest resistance in *E. coli* isolates from diseased farm livestock in England and Wales to tetracycline (56.4%). Frequencies of resistance to trimethoprim-sulfamethoxazole (16.8%), gentamicin (1%), cefotaxime (0%), and nalidixic acid (0%) were very similar to the present study (Cheney *et al*., 2015). In a similar study conducted in another province in Iran, 22.7% of *E. coli* isolated from sheep's rectum was resistant to trimethoprim-sulfamethoxazole, 18.2% to gentamicin, and 4.5% to cefotaxime (Safavi and Shahbazi, 2017) Studies conducted in other Asian countries also reported diverse results, for example, in a study of stool samples from healthy and diarrheal sheep and goats in Saudi Arabia, the frequency of isolation of *E. coli* from healthy sheep was 58.5%. The prevalence of resistance to nalidixic acid (23.8%), gentamicin (2.5%), cefotaxime (0%), and ceftazidime (0%) in *E. coli* isolates from healthy animals was almost similar to the results of the present study (Shabana and Al-Enazi, 2020). In comparison with the present study, a higher frequency of resistance to ceftazidime (36.2%), a relatively similar frequency of resistance to tetracycline (31%), and a same frequency of resistance to gentamicin (0%) have been reported in *E. coli* isolated from pneumonic and septicemic sheep and goats in India (Singh *et al*., 2019). Moreover, in a study in China on *E. coli* isolates of diarrheal sheep, higher frequencies of resistance were against sulfisoxazole (82.4%), tetracycline (53.2%), gentamicin (20.4%), and ceftazidime (17.8%) (Zhao *et al*., 2021). In another study in China, frequencies of resistance to trimethoprim-sulfamethoxazole and tetracycline in *E. coli* isolated from sheep feces samples were 70.83% and 29.17%, respectively (Wu *et al*., 2024). In a study in Qatar, 84.2% of sheep stool samples contained *E. coli*. Among *E. coli* isolates, 47.2% and 45.8% were resistant to nitrofurantoin and trimethoprim-sulfamethoxazole, respectively (Eltai *et al*., 2020). Moreover, *E. coli* isolates from diarrheic sheep in Egypt were resistant to cefotaxime (92%), amoxicillin (85%), gentamicin (82%), tetracycline (80%), nalidixic acid (35.7%), and nitrofurantoin (3%) (Hafez, 2020). In a study in the African country of Ethiopia, *E. coli* was

isolated from 77.8% of sheep feces samples. Of these, 5.8% was resistant to tetracycline, 4.5% to gentamicin, 4.5% to cefotaxime, 3.6% to trimethoprim-sulfamethoxazole, 1.5% to nalidixic acid, and 0.8% to nitrofurantoin (Gemeda *et al*., 2023).

MDR *E. coli* was more prevalent in diarrheal sheep in China (75.4%) (Zhao *et al*., 2021), healthy sheep stool samples in China (29.17%) (Wu *et al*., 2024), sheep stool samples in Qatar (44%) (Eltai *et al*., 2020), stool samples from healthy and diarrheal sheep and goats in Saudi Arabia (51.5%) (Shabana and Al-Enazi, 2020). In comparison, a lower frequency (16.7%) of MDR strains was observed among the sheep fecal *E. coli* isolates in our study. Although this frequency can also be an alarm for the possibility of ineffectiveness of several classes of antibiotics against *E. coli* as well as other bacteria due to the possibility of resistance genes transfer. The consequences of this issue are increased treatment failure and treatment costs, as well as zoonotic implications. On the other hand, 100% susceptibility of tested isolates to gentamicin and nitrofurantoin, as well as low resistance to tested third generation cephalosporins, can make these antibiotics potential candidates for the treatment of *E. coli* infections in sheep in the study area. This result was consistent with results reported in studies in England and Wales (Cheney *et al*., 2015), Saudi Arabia (Shabana and Al-Enazi, 2020), and Ethiopia (Gemeda *et al*., 2023). However, it is always recommended to perform antimicrobial susceptibility testing before choosing an antibiotic for treatment.

In the present study, only one *E. coli* isolate was resistant to the studied third generation cephalosporins, which was the only isolate that had two ESBL genes (bla_{TEM} and $bla_{\text{CTX-M}}$) at the same time. ESBLs are mainly plasmid-mediated enzymes that degrade the β-lactam ring of penicillins, first- to third-generation cephalosporins, and monobactams (aztreonam). But they have no inhibitory effect on cefoxitin and carbapenems (Aliasadi and Saei, 2015; Singh *et al*., 2019; Zhao *et al*., 2021). Investigating the relationship between the presence of ESBL genes and resistance to cephalosporins in the isolates showed that there is a significant association between the presence of more than one ESBL gene and the occurrence of the third generation cephalosporins resistance phenotype (P=0.042). Also, the presence of the $bla_{\text{CTX-M}}$ gene had a statistically significant association with the resistance to the tested third generation cephalosporins (P=0.042).

As in our study, bla_{TEM} had the highest prevalence (25%) among the three tested major ESBL genes, in related studies by Wu *et al*. (84.62%), Singh *et al*. (69.8%) and Hafez (64.3%) this gene was the most prevalent gene, but its frequency was higher in their studies (Singh *et al*., 2019; Hafez, 2020; Wu *et al*., 2024). Similar to our study, the bla_{SHV} gene was not detected in any of the fecal *E. coli* isolates from sheep in another study in Iran. Also, in their study, the prevalence of $bla_{\text{CTX-M}}$ and bla_{TEM} genes was reported as 27.2% and 18.2%, respectively (Aliasadi and Saei, 2015). A low prevalence (only in one isolate) of the bla_{SHV} gene was also observed in *E. coli* isolates from pneumonic and septicemic sheep and goats in India (Singh *et al*., 2019).

In past years, tetracycline has been a useful broad-spectrum antibiotic, but bacteria have become resistant to it through three mechanisms. The first mechanism is to limit the access of this antibiotic to ribosomes by pumping them out of the cell. Most of the *tet* genes, including *tetA*, *tetB*, *tetC*, and *tetD*, which were tested in the present study, are tetracycline efflux genes. A second mechanism is ribosome alternation to prevent proper binding to tetracycline. The tested *tetM* gene is one of the most common ribosome protection resistance genes. Finally, the third mechanism is the production of enzymes that inactivate tetracycline, which is not a widespread strategy (Speer *et al*., 1992; Velhner and Milanov, 2015). In the present study, *tetA* is the most common tetracycline resistance gene. This result is consistent with those observed by Hafez (2020), who reported the prevalence of 78.6% for *tetA* gene in *E. coli* of diarrheic sheep in Egypt (Hafez, 2020). In addition, in a study in China, the *tetA* gene was detected in 42.86% of *E. coli* isolates from different organs of sheep, and frequencies of *tetB* and *tetM* genes were 18.68% and 16.48%, respectively (Wu *et al*., 2024). Investigating the relationship between the presence of tetracycline resistance genes and tetracycline resistance in the isolates, showed that all six tetracycline resistant *E. coli* isolates had at least one of the studied *tet* genes. Although the presence of *tet* genes in tetracycline-resistant isolates (100%) was more common than tetracycline-susceptible isolates (72.2%), but this difference was not statistically significant. The presence of *tet* genes in tetracycline-susceptible isolates can indicate the potential of these isolates in developing the phenotype of resistance to tetracycline.

Statistically significant associations that were observed between resistance to different antibiotics as well as resistance to certain antibiotics and the presence of the bla_{TEM} gene can be attributed to the simultaneous presence of these antibiotic resistance genes on mobile genetic elements such as plasmids.

In the present study, observing the high prevalence of virulence genes involved in adhesion in *E. coli* isolated from sheep feces can indicate the potential ability of these isolates to colonize in host tissues and initiate invasion as well as biofilm formation. Frequencies of 98.9% (Wu *et al*., 2024) for *csgA*, 97.4% (Gu *et al*., 2023) and 64% (Hafez, 2020) for *crl*, 100% (Mohammed *et al*., 2020), 97.4% (Gozi *et al*., 2019), 92.24% (Gu *et al*., 2023) and 60.7% (Hafez, 2020) for *fimH*, 5.4% (Zhao *et al*., 2021) and 0% (Gozi *et al*., 2019) for *sfa/focDE*, and 3.8% (Zhao *et al*., 2021) for *papC* have been reported in related studies conducted in different countries.

Biofilm, which is a structured community of bacteria in a polymer matrix produced by them, can form on living or non-living surfaces under the influence of a series of bacterial surface structures (Van Houdt and Michiels, 2005). Genes encoding type 1 fimbriae (*fimH*), P fimbriae (*papC*), S and F1C fimbriae (*sfa/focDE*), curli

fimbriae (*csgA*, *crl*) and afimbrial adhesins (*afa*), as well as the gene involved in exopolysaccharide production (*bcsA*) can affect the ability of bacteria to produce biofilm (Naziri *et al*., 2021). The ability of biofilm formation in 62.5% of *E. coli* isolates from sheep in this study, due to the possibility of transmission of these strains among sheep and also to humans, can endanger public health. Because the bacterium can increase survival, antibiotic resistance, and pathogenicity. Similarly, 63.7% of *E. coli* isolated from different organs of sheep in China were biofilm producers (Wu *et al*., 2024). In another study conducted in China, 6.6% and 74.6% of *E. coli* isolates from sheep were moderate and weak biofilm producers, respectively (Zhao *et al*., 2021).

In general, the origin of *E. coli* isolates, the amount and type of antibiotics prescribed by veterinarians, the amount of free access of livestock farmers to antibiotics, the genetic difference of bacterial strains in different geographical areas can affect the results obtained in different studies. To overcome the limitations of the present study, a greater number of samples, a complete epidemiological study on the genotypic and phenotypic characteristics of sheep fecal *E. coli* isolates, and a study on more antibiotic resistance genes and virulence factors are recommended.

The results of the present study revealed that sheep can be the reservoir of virulent antibiotic-resistant biofilm producing *E. coli* strains. Since, according to the concept of One Health, human health depends on animals' health and their environment (CDC, 2023), the presence of these strains in sheep and their spread in the slaughterhouse environment and subsequently in sewage is a risk to public health. Therefore, continuous and strict monitoring of the health of food animals as well as the potential of pathogenicity and antibiotic resistance of their microbial population seems mandatory.

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Conflict of interest

The authors declare that they have no conflicts of interest.

References

Aliasadi, S and Saei, HD (2015). Fecal carriage of *Escherichia coli* harboring extended-spectrum beta-lactamase (ESBL) genes by sheep and broilers in Urmia region, Iran. Iran. J. Vet. Med., 9: 93-101.

Brinas, L; Zarazaga, M; Sáenz, Y; Ruiz-Larrea, F and Torres, C (2002). Beta-lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. Antimicrob. Agents Chemother., 46: 3156-3163.

CDC (2023). Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID). https://www.cdc.gov/onehealth/basics/index.html. Last Reviewed: August 17, 2023.

Cheney, TE; Smith, RP; Hutchinson, JP; Brunton, LA; Pritchard, G and Teale, CJ (2015). Cross-sectional survey of antibiotic resistance in *Escherichia coli* isolated from diseased farm livestock in England and Wales. Epidemiol. Infect., 143: 2653-2659.

CLSI (2018). Performance standards for antimicrobial susceptibility testing. CLSI Supplement M100. 28th Edn., Wayne, PA: Clinical and Laboratory Standards Institute.

Derakhshandeh, A; Firouzi, R and Naziri, Z (2014). Phylogenetic group determination of faecal *Escherichia coli* and comparative analysis among different hosts, Iran. J. Vet. Res., 15: 13-17.

Eltai, N; Al-Thani, A; Alhadidi, S; Abdfarag, A; Romaiha, H; Mahmoud, M; Alawad, O and Yassine, H (2020). Antibiotic resistance profile of commensal *Escherichia coli* isolated from healthy sheep in Qatar. J. Infect. Dev. Ctries., 14: 138-145.

Emett, J; David, R; McDaniel, J; McDaniel, S and Kingsley, K (2020). Comparison of DNA extracted from pediatric saliva, gingival crevicular fluid and site-specific biofilm samples. Methods Protoc., 3: 48.

Gemeda, BA; Wieland, B; Alemayehu, G; Knight-Jones, TJ; Wodajo, HD; Tefera, M; Kumbe, A; Olani, A; Abera, S and Amenu, K (2023). Antimicrobial resistance of *Escherichia coli* isolates from livestock and the environment in extensive smallholder livestock production systems in Ethiopia. Antibiotics. 12: 941.

Ghanbarpour, R; Askari, N; Ghorbanpour, M; Tahamtan, Y; Mashayekhi, K; Afsharipour, N and Darijani, N (2017). Genotypic analysis of virulence genes and antimicrobial profile of diarrheagenic *Escherichia coli* isolated from diseased lambs in Iran. Trop. Anim. Health Prod., 49: 591-597.

Gozi, KS; Froes, JR; Deus Ajude, LP; Da Silva, CR; Baptista, RS; Peiró, JR; Marinho, M; Mendes, LC; Nogueira, MC and Casella, T (2019). Dissemination of multidrug-resistant commensal *Escherichia coli* in feedlot lambs in Southeastern Brazil. Front. Microbiol., 10: 1394.

Gu, X; Ma, X; Wu, Q; Tao, Q; Chai, Y; Zhou, X; Han, M; Li, J; Huang, X; Wu, T and Zhang, X (2023). Isolation, identification, molecular typing, and drug resistance of *Escherichia coli* from infected cattle and sheep in Xinjiang, China. Vet. Med. Sci., 9: 1359-1368.

Hafez, AA (2020). Virulence and antimicrobial resistance genes of *E. coli* isolated from diarrheic sheep in the North-West Coast of Egypt. Sys. Rev. Pharm., 11: 609-617.

Hassan, A; Usman, J; Kaleem, F; Omair, M; Khalid, A and Iqbal, M (2011). Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz. J. Infect. Dis., 15: 305-311.

Johnson, JR and Stell, AL (2000). Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J. Infect. Dis., 181: 261-272.

Lanumtiang, Y; Jiemtaweeboon, S; Sungpradit, S; Leesombun, A and Boonmasawai, S (2022). The surveillance of antimicrobial susceptibility pattern and *bla*CTX-M gene encoding in *Escherichia coli* isolated from healthy goat farms in Sai Yok District, Kanchanaburi Province, Thailand. J. Appl. Anim. Sci., 15: 9-24.

Le Bouguenec, C; Archambaud, M and Labigne, A (1992). Rapid and specific detection of the *pap*, *afa*, and *sfa* adhesin-encoding operons in uropathogenic *Escherichia*

coli strains by polymerase chain reaction. J. Clin. Microbiol., 30: 1189-1193.

Markey, B; Leonard, F; Archambault, M; Cullinane, A and Maguire, D (2013). *Clinical veterinary microbiology*. 2nd Edn., St. Louis, MO: Mosby Ltd., PP: 239-275.

Maurer, JJ; Brown, TP; Steffens, WL and Thayer, SG (1998). The occurrence of ambient temperature-regulated adhesions, curli, and the temperature-sensitive hemagglutinin *tsh* among avian *Escherichia coli*. Avian Dis., 42: 106-118.

Mohammed, YJ; Mustafa, JY and Abdullah, AR (2020). Isolation and molecular study of some bacterial urinary tract infections of sheep in Basrah province. Iraqi J. Agric. Sci., 51: 885-893.

Naziri, Z; Kilegolan, JA; Moezzi, MS and Derakhshandeh, A (2021). Biofilm formation by uropathogenic *Escherichia coli*: a complicating factor for treatment and recurrence of urinary tract infections. J. Hosp. Infect., 117: 9-16.

Nielsen, SS; Bicout, DJ; Calistri, P; Canali, E; Drewe, JA; Garin-Bastuji, B; Gonzales Rojas, JL; Gortázar, C; Herskin, M; Michel, V and Miranda Chueca, MÁ (2022). Assessment of listing and categorisation of animal diseases within the framework of the Animal Health Law (Regulation (EU) No 2016/429): antimicrobial-resistant *Escherichia coli* in dogs and cats, horses, swine, poultry, cattle, sheep and goats. EFSA J., 20: 1-93.

Safavi, EA and Shahbazi, Y (2017). Antimicrobial resistance in *Escherichia coli* isolated from different parts of the digestive tract of sheep. Bulg. J. Vet. Med., 20: 271-275.

Shabana, II and Al-Enazi, AT (2020). Investigation of plasmid-mediated resistance in *E. coli* isolated from healthy and diarrheic sheep and goats. Saudi J. Biol. Sci., 27: 788-796.

Singh, F; Sonawane, GG; Kumar, J; Dixit, SK; Meena, RK and Tripathi, BN (2019). Antimicrobial resistance and phenotypic and molecular detection of extended-spectrum ß-lactamases among extraintestinal *Escherichia coli* isolated from pneumonic and septicemic sheep and goats in Rajasthan, India. Turkish J. Vet. Anim. Sci., 43: 754-760.

Speer, BS; Shoemaker, NB and Salyers, AA (1992). Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Clin. Microbiol. Rev., 5: 387-399.

Srinivasan, V; Gillespie, BE; Lewis, MJ; Nguyen, LT; Headrick, SI; Schukken, YH and Oliver, SP (2007). Phenotypic and genotypic antimicrobial resistance patterns of *Escherichia coli* isolated from dairy cows with mastitis. Vet. Microbiol., 124: 319-328.

Stepanović, S; Vuković, D; Hola, V; Di Bonaventura, G; Djukić, S; Cirković, I and Ruzicka, F (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS. 115: 891-899.

Tabar, MM; Mirkalantari, S and Amoli, RI (2016). Detection of *ctx-M* gene in ESBL-producing *E. coli* strains isolated from urinary tract infection in Semnan, Iran. Electron. Physician., 8: 2686-2690.

Tahamtan, Y; Pourbakhsh, S; Hayati, M; Namdar, N; Shams, Z and Namavari, M (2011). Prevalence and molecular characterization of verotoxin-producing *Escherichia coli* O157:H7 in cattle and sheep in Shiraz-Iran. Arch. Razi Inst., 66: 29-36.

The European Committee on Antimicrobial Susceptibility Testing (2019). Redefining susceptibility testing categories S, I and R. https://www.eucast.org/newsiandr/. Accessed 1 January 2022.

Van Houdt, R and Michiels, CW (2005). Role of bacterial cell surface structures in *Escherichia coli* biofilm formation. Res. Microbiol., 156: 626-633.

Velhner, M and Milanov, D (2015). Resistance to tetracycline in *Escherichia coli* and *Staphylococcus aureus*: brief overview on mechanisms of resistance and epidemiology. Arch. Vet. Med., 8: 27-36.

Warsa, UC; Nonoyama, M; Ida, T; Okamoto, R; Okubo, T; Shimauchi, C; Kuga, A and Inoue, M (1996). Detection of *tet*(K) and *tet*(M) in *Staphylococcus aureus* of Asian countries by the polymerase chain reaction. J. Antibiot. (Tokyo), 49: 1127-1132.

Wu, Z; Chi, H; Han, T; Li, G; Wang, J and Chen, W (2024). Differences of *Escherichia coli* isolated from different organs of the individual sheep: molecular typing, antibiotics resistance, and biofilm formation. Folia Microbiol., 69: 567-578.

Zhao, X; Lv, Y; Adam, FE; Xie, Q; Wang, B; Bai, X; Wang, X; Shan, H; Wang, X; Liu, H and Dang, R (2021). Comparison of antimicrobial resistance, virulence genes, phylogroups, and biofilm formation of *Escherichia coli* isolated from intensive farming and free-range sheep. Front. Microbiol., 12: 699927.