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Antibiotic resistance pattern and biofilm formation ability of *Escherichia coli* derived from beef cattle in Ilam and Kurdistan provinces

Mahmoud Kohneh Poushi[1], Aram Sharifi[2*] and Mohammad Amin Heidarzadi[3]

[1] MSc, Food Hygiene and Quality Control, Faculty of Veterinary Medicine, University of Zabol, Zabol, Iran
[2] Assistant Professor, Department of Animal Science, Faculty of Agriculture, University of Kurdistan, Sanandaj, Kurdistan, Iran
[3] PhD Student, Department of Food Hygiene, Faculty of Veterinary Medicine, University of Shahrekord, Shahrekord, Iran

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Abstract

Escherichia coli are commensal gastrointestinal microflora of human and animals, but some strains due to the presence of pathogenic factors may cause various animal and human diseases. The present study aimed to identify antimicrobial resistant pattern and biofilm formation potential of *E. coli* derived from beef cattle in Ilam and Kurdistan provinces. Samples were taken from neck, arms and thighs of 90 slaughtered cows (45 cows from Ilam and 45 cows from Kurdistan). *E. coli* isolation was done based on culturing on selective and differential culture media and biochemical tests. The antibiotic susceptibilities and biofilm formation potential were done by Kirby Bauer's disk diffusion and microtiter plate (MtP) tests, respectively. The Spearman rank correlation test performed to study the correlation between antimicrobial susceptibility and biofilm formation and *p* values less than 0.05 were considered as a significant level. Of 270 meat samples, 42 *E. coli* were isolated. In both provinces, samples taken from the thighs had significantly more *E. coli* bacteria than necks and arms. The highest resistance rate was reported to sulfamethoxazole and tetracycline (85.71%), followed by ampicillin (80.95%). Besides, all *E. coli* were sensitive to colistin. Based on MtP, 24 (57.14%), 12 (28.57%) and 6 (14.28%) isolates were categorized as strong, moderate and weak biofilm producer, respectively. The significant positive correlation was found between biofilm formation and resistance to ampicillin, amoxicillin, amikacin, ciprofloxacin, gentamicin, tetracycline and ceftriaxone. High antibiotic resistance rates and strong biofilm formation ability of *E. coli* isolates obtained from red meat suggest the need for continuous surveillance in the food chain.

Key words: *Escherichia coli*, Antibiotic resistance, Biofilm formation, Beef cattle

Introduction

Meat is consumed by many people as an important source of protein and other nutrients. Annual meat production worldwide in 2017 was about 334.2 million tons, of which 119.9, 109.1 and 66.3 million tons were related to pork, poultry and beef, respectively (Castaño-Arriba *et al.*, 2020). This shows that meat is one of the most consumed foods. In relation to meat, one of the existing problems is its contamination with various microorganisms and their transmission to the consumer body. For this reason, microbiological safety of meat products is one of the major food hygiene concerns that has been the subject of numerous epidemiological reports

[*] **Corresponding Author**: Aram Sharifi, Assistant Professor, Department of Animal Science, Faculty of Agriculture, University of Kurdistan, Sanandaj, Kurdistan, Iran
E-mail: A.sharifi@uok.ac.ir

(Bakhtiary *et al.*, 2016; Castaño-Arriba *et al.*, 2020).

E. coli is usually a commensal humans and animals organism. Pathogenic *E. coli* are common causes of intestinal and also extraintestinal infections including urinary tract infections (UTI), peritonitis, septicaemia as well as bacteraemia (Sharifi *et al.*, 2021; Torres *et al*., 2010). This pathogen is a ubiquitous, Gram-negative, motile, facultative anaerobic, non-spore forming bacteria belongs to *Enterobacteriaceae* family. The optimal *E. co*li growth temperature is 37 °C but can grow up to 49 °C temperatures. This led to its ability to survive and grow in different places and harsh environmental conditions and has made it one of the most important food contaminants (Torres *et al.*, 2010).

Based on recent studies it has seen increasing the antibiotic-resistant bacteria, which are currently considered one of the main concerns for health systems all around the world. The presence of antibiotic-resistant bacteria in foods and food related systems is a direct risk for consumers due to the potential of these microorganisms to cause hard-to-treat foodborne infections (González-Gutiérrez *et al.*, 2020).

The ability to form biofilms is one of the most important factors involved in causing disease by bacteria. A biofilm is defined as an organized collection of attached microbial communities that are embedded into a self-produced exopolymeric matrix mainly composed of proteins, polysaccharides and sometimes extracellular DNA (eDNA) (Vestby *et al.* 2020). The microbial biofilm cells are resistance to different stress condition(s) and antimicrobials agents/strategies agents such as antibiotics, preservatives, chemical sanitizers, thermal treatment etc. that are traditionally applied in food industry, thus making them robust and hard to eradicate (Vestby *et al.*, 2020).

E. coli has the ability to form a strong biofilm by using adhesion factors such as P-fimbriae, certain other mannose-resistant adhesins, type-1 fimbriae, enterotoxins, etc., which help in more effective bacterial pathogenesis and subsequently causes disease. Therefore, it is very important to study the biofilm formation ability of bacteria, especially in foods and food industries, and therefore many studies have been performed on biofilm formation potential of food-contaminating bacteria (Sharma *et al*., 2016).

Based on what was expressed above, the purpose of this study is to investigate the presence of *E. coli* in the red meat of slaughtered cows in two provinces of Kurdistan and Ilam. Also, after the isolation of bacteria, the antibiotic resistance of bacteria and the ability to form biofilm and the relationship between antibiotic resistance and biofilm formation ability in the *E. coli* isolates will be investigated.

Material and Methods

Sampling

The applied methods of the present study, which includes sampling, bacterial isolation, microbial susceptibility testing and biofilm formation ability were performed from September 2019 to February 2020 in Ilam and Kurdistan provinces, west of Iran. To investigate the level of *E. coli* contamination in red meats, a total of 270 meat samples were taken from 90 cows, of which 45 cows from Ilam province and 45 cows from Kurdistan province. The sampled parts of the neck (45, each cow one sample), arms (45, each cow one sample) and thighs (45, each cow one sample) of the cow were selected and transferred to the laboratory under completely sterile and cold conditions.

Microbiological analysis

Using sterile tweezers and a sterile scalpel, 25 g of product were taken from each sample and placed in a homogenization bag together with 225 ml of sterile 0.1% peptone water (Oxoid Ltd.). These samples were homogenized

(Masticator, IUL Instruments) for three minutes.

Isolation and counting of *E. coli* bacteria were performed by the Most Probable Number (MPN) method according to the Institute of Standards and Industrial Research of Iran. The lactose broth tubes incubated for 24 hours at 37 °C. Based on positive reaction of lactose broth the number of coliforms was reported. For confirmation of *E. coli*, from positive tubes inoculated on the Eosin-Methylene Blue (EMB) agar and incubated at 37°C for 24 h.

Presumptive *E. coli* colonies appeared as dark centered and flat, with or without a metallic sheen. These presumptive *E. coli* colonies were purified on Trypticase Soy Agar (TSA) and incubated at 37 °C for 24 h. They were identified and confirmed using Gram staining, growth and reaction on MacConkey Agar, growth in Brilliant Green Bile Broth, and *E. coli* latex agglutination test. Approved isolates were cultured for storage in Trypticase Soy Broth (TSB). Then, TSB culture medium containing bacteria was mixed with sterile glycerol in a ratio of 70 to 30 (volume-volume) and stored in a freezer at -70 °C. All bacterial were purchased from Merck, Darmstadt, Germany.

Antibiotic susceptibility test

The antibiotic susceptibilities testing for *E.coli* isolates were done by Kirby Bauer's disk diffusion method on Muller-Hinton agar (Merk, Germany) according to the Clinical and Laboratory Standards Institute (CLSI) (Goudarzi *et al.*, 2013). The applied antibiotic disks (Padtan Teb, Iran) were Nalidixic acid (30 µg), Ampicillin (10 µg), Amikacin (30 µg), Imipenem (10 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Ceftriaxone (30 µg), Tetracycline (30 µg), Amoxicillin (25 µg), Sulfamethoxazole (10 µg), Chloramphenicol (30 µg), Colistin (25 µg).

Determination of multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) isolates

According to CLSI definition, MDR isolate was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. In addition, XDR was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories for example bacterial isolates remain susceptible to only one or two categories and finally PDR was defined as non-susceptibility to all agents in all antimicrobial (Magiorakos *et al.*, 2012).

Biofilm formation potential

To investigate the biofilm formation ability the MtP method was applied. The isolates were cultured in trypticase soy broth (TSB) with 1% glucose. Overnight cultures were diluted 1:200 with TSB containing 1% glucose, and 200µL per well were inoculated in sterile 96-well polystyrene tissue culture plates and incubated in 37 °C for 24 h. Then non adhered cells were removed and wells washed three times with deionized water. The adhering bacteria in each well were fixed with adding 200µL of absolute methanol for 20 mins. The methanol was removed and the microplates left to dry overnight. Subsequently 100µL per well safranin 0.1% were added to each wells for 20 mins. Excess safranin was removed by gently washing of plates twice with distilled water. Finally, a volume of 100µL of ethanol–acetic acid (95:5 v/v) per well, was added to the plate and the optical density was measured at 490 nm using ELISA reader (ELx800). Results were scored as follows: $OD \leq 2 \times ODc$ negative for biofilm production, $2 \times ODc < OD \leq 4 \times ODc$ moderate biofilm producer and $OD > 4 \times ODc$ strong biofilm producers. (OD: Optical density and ODc: average of OD negative control + (3×SD of negative control)). *Pseudomonas aeruginosa* PA01 was used as a positive control in biofilm formation tests (Sharifi *et al.*, 2018).

Statistical Analysis

The biofilm test was repeated three times for each isolate and its mean was considered along with the standard division. To compare the susceptibility of the obtained bacteria to different antibiotics, Chi-square test was used and SPSS software version 26 was used for statistical analysis. Spearman rank correlation test performed to study the correlation between biofilm formation and antimicrobial susceptibility categories. Significant level for this study was considered $p < 0.05$.

Results

Bacterial isolates

Out of 270 meat samples from Ilam province (45 cows and 135 meat samples) and Kurdistan province (45 cows and 135 meat samples), totally, 42 samples (15.55%) were contaminated with *E. coli*. Of these 42 isolates, 22 (52.38%) and 20 (47.62%) were related to Ilam and Kurdistan provinces, respectively (Table 1). It should be noted that significant difference in terms of *E. coli* contamination was not observed between two studied provinces ($p > 0.05$). In both provinces, samples taken from the thighs had significantly more *E. coli* bacteria than necks and arms ($p = 0.033$ and 0.041 for Ilam and Kurdistan provinces, respectively).

Antibiotic resistance pattern

The highest resistance rate was related to sulfamethoxazole and tetracycline with 85.71%, followed by ampicillin with 80.95% resistance. On the other hand, all isolated bacteria were sensitive to colistin (100% sensitive). In addition, 40 (95.23%) isolates display sensitivity to imipenem. The results of antibiotic susceptibility of *E. coli* isolates are given in Table 2.

Table 1. Prevalence of *E. coli* isolated from two tested provinces

Province	Prevalence of *E. coli* from different samples			
	Necks (n= 45)	Arms (n= 45)	Thighs (n= 45)	Total
Ilam province (45 cow)	6 (13.33%)	5 (11.11%)	11 (24.44%)	22 (48.88%)
Kurdistan province (45 cow)	5 (11.11%)	6 (13.33%)	9 (20%)	20 (44.44)
Totally (90 cow)	11	11	20	42

Table 2. Antibiotic resistance patterns of the *E. coli* isolates

Antimicrobial agent	Number (Frequency)		
	R	I	S
Nalidixic acid	6 (14.28%)	0	36 (85/71%)
Ampicillin	34 (80.95%)	4 (9.52%)	4 (9.52%)
Amoxicillin	14 (33.33%)	6 (14.28%)	22 (52.38%)
Amikacin	2 (4.76%)	4 (9.52%)	36 (85/71%)
Imipenem	0	2 (4.76%)	40 (95.23%)
Ciprofloxacin	14 (33.33%)	2 (4.76%)	26 (61.90%)
Gentamicin	8 (19.04%)	6 (14.28%)	28 (66.66%)
Ceftriaxone	14 (33.33%)	2 (4.76%)	26 (61.90%)
Tetracycline	36 (85.71%)	4 (9.52%)	2 (4.76%)
Sulfamethoxazole	18 (85.71%)	2 (9.52%)	1 (4.76%)
Chloramphenicol	6 (14.28%)	2 (4.76%)	34 (80.95%)
Colistin	0	0	42 (100%)

R: resistant, I: intermediate, S: susceptible

MDR, XDR and PDR isolates

Based on definition, of tested isolates, 14 (33.33%) were in the MDR group, where 4 (9.52%) were XDR. Our results showed that 2 (4.76%) were PDR.

Biofilm formation ability and its correlation with antibiotic resistance

Using the 96-well cell culture microtiter plates with Safranin staining, the biofilm formation ability was checked. Out of 42 isolates, 24 (57.14%), 12 (28.57%) and 6 (14.28%) isolates were strong, moderate and weak biofilm producer, respectively (Figure 1).

The results revealed that isolates resistant to ampicillin, amoxicillin, amikacin, ciprofloxacin, gentamicin, tetracycline and ceftriaxone could form stronger biofilms than those susceptible or exhibiting intermediate resistance, being indicative of a positive correlation between biofilm formation potential and resistance profile (r_s = 0.215–0.241, $p < 0.05$).

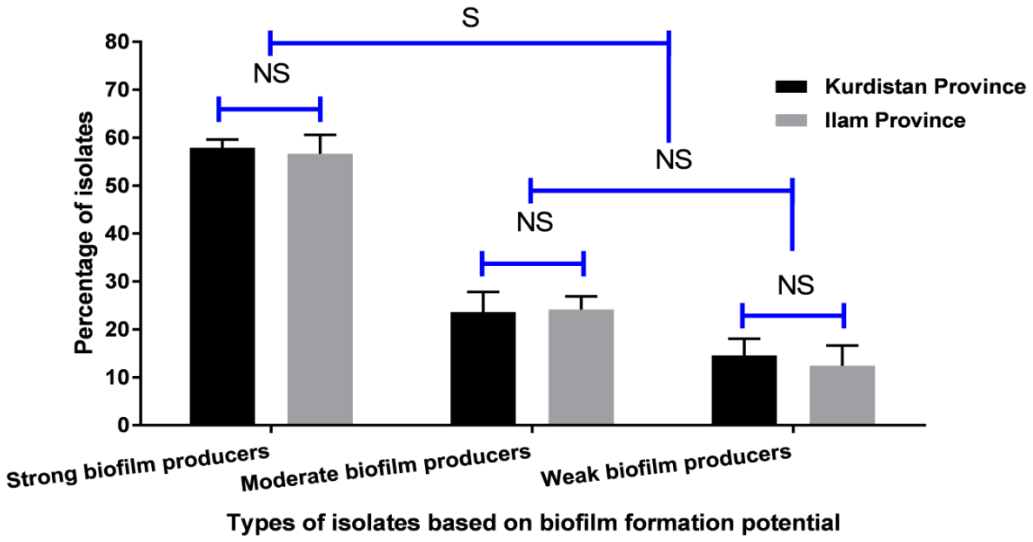

Figure 1. Biofilm formation potential of *E. coli* isolates (S: significant, NS: non-significant).

Discussion

Due to the possibility of transmission of resistant pathogens, the presence of antibiotic-resistant bacteria is one of the food industry concerns. Drug resistant *E. coli* present in the intestines of slaughtered animals can contaminate carcasses during slaughtering, and thereby transfer resistance genes to human directly or via the food chain (Aarestrup, 1999).

In the present study, out of 270 samples taken in Kurdistan and Ilam provinces, 42 isolates (15.55%) *E. coli* were isolated. This presence signifies that meat and meat products can be important sources of *E. coli* and possible risk sources for E. coli related infection.

In the present study, the antibiotic susceptibility of *E. coli* isolates obtained from red meat samples was determined. Based on results, the highest resistance rate was reported to sulfamethoxazole and tetracycline (85.71%), followed by ampicillin (80.95%). Besides, all bacteria were sensitive to colistin (100% sensitive). In a similar study which performed on the antibiotic resistance pattern of *E. coli* isolated from meat in South Africa, high resistance rate was observed for streptomycin (54.9%), ceftriaxone (54.9%), tetracycline (43.8%) and nitrofurantoin (40.1%) (Jaja *et al.*, 2020). In another study in Ghana on the antibiotic resistance of *E. coli* isolates derived from raw meat found that the highest antibiotic resistance was related to amoxicillin (70.9%), tetracycline (57.0%) and trimethoprim (55.0%). It

seems that the prevalence resistance of tetracycline and ampicillin resistance in this study could be linked to their extensive use for the treatment of bacterial infections of plants and animals (Jaja *et al.*, 2020).

On the other hand, the present study showed that all examined isolates were sensitive to colistin. Colistin (polymyxin E) is promptly bactericidal on Gram-negative bacteria (Paul *et al.* 2018). This antibiotic is one of the most often used antimicrobials in veterinary practice, particularly in underdeveloped nations such as Iran (Nikkhahi *et al.*, 2021). In previous studies on *E. coli*, either high sensitivity or complete antibiotic sensitivity has been reported regarding colistin. For example, in the study of Olowe *et al* (2019), it was reported that all isolates of *E. coli* obtained from humans, animals and food products in South-West Nigeria are sensitive to colistin.

However, studies show that *E. coli* isolated from poultry are not highly sensitive to colistin. In a meta-analysis study the prevalence of colistin-resistant *E. coli* from poultry in South Asian developing countries was 28% (Dawadi *et al.*, 2021). The studies conducted in Iran also show that colistin-resistant related genes (*mcr-1* to *mcr-9*) are present in *E. coli* animal isolates in high level rates (Ilbeigi *et al.*, 2021; Nikkhahi *et al*., 2021), so it can be expected that the presence of colistin-resistant strains in animal isolates will increase greatly in the coming years. Therefore, although complete sensitivity to this antibiotic was reported in the present study, its use in veterinary medicine should be done with caution.

Based on our finding, 40 (95.23%) isolates were sensitive to imipenem. In line with this result, in previous researches high susceptibility rates of *E. coli* isolates to imipenem have also been reported in Rwanda (Muvunyi *et al.*, 2011), India (Niranjan *et al*., 2014), Cotonou (Anago *et al.*, 2015) and Iran (Mohammadi-Mehr *et al.*, 2011). This finding may be due to the stability of imipenem and also resistant to degradation by most beta-lactamases (Olowe *et al.*, 2019), as well as limited use of this antibiotic in the veterinary medicine.

In the next part of the present study, the ability to form biofilm as an important factor in the colonization and pathogenicity of *E. coli* was investigated. Our results showed that out of 42 tested isolates, 24 (57.14%), 12 (28.57%) and 6 (14.28%) isolates were strong, moderate and weak biofilm producer, respectively. The number of strong biofilm isolates was significantly higher than other isolates, but no significant difference was observed between the two provinces in this regard. The major point of this study is that 57.14% of the isolates can form a strong biofilm, and these strains also play a role in the attachment of bacteria to food processing equipment, and if they transmitted to humans, they can cause biofilm related disease (Vestby *et al.*, 2020).

One of the problems of the food industry is the presence of biofilm-forming species in food. These pathogens are able to develop mixed biofilms on different artificial substrates common in food industry, such as stainless steel, polyethylene, wood, glass, polypropylene and rubber (Sharifi *et al.*, 2021). Based on this, the ability to form a strong biofilm by *E. coli* isolates in the present study should be noticed and monitored by food health inspectors.

The positive correlation between biofilm production and antibiotic resistance was found statistically significant ($p < 0.05$) in most of the tested antibiotics (ampicillin, amoxicillin, amikacin, ciprofloxacin, gentamicin, tetracycline and ceftriaxone) but the correlation was not found to be significant in case of nalidixic acid, imipenem, Sulfamethoxazole, chloramphenicol and colistin. There are similar reports demonstrated that various antibiotic resistance were related to biofilm formation in *E. coli* (Qian *et al.*, 2022). In a similar previous study, it was reported that, *E. coli* strains with strong biofilm formation

ability were more resistant to norfloxacin, amoxicillin-clavulanic acid, gatifloxacin, cotrimoxazole, and gentamicin, suggesting that the biofilms formed by this bacteria provide the ability to survive when exposed to these antibiotics (Mittal *et al.*, 2015). In this case it was demonstrated that microorganisms growing in a biofilm are intrinsically resistant to many antibiotics increasing the antibiotic resistance up to 1000 folds and high antimicrobial concentrations are required to inactivate organisms growing in a biofilm (Neupane *et al.*, 2016). The increase in bacterial resistance in the biofilm phase in bacteria, including *E. coli*, can be attributed to the following: decreasing the penetration of antibiotics into the biofilm and, as a result, reducing its concentration in the vicinity of the bacteria, the expression of resistance related genes, including the genes involved in the quorum sensing systems, the reduction of antibiotic uptake by bacteria due to inactive growth and reproduction, etc (Mittal *et al.*, 2015; Neupane *et al.*, 2016; Qian *et al.*, 2022).

In conclusion, based on this, the report of high antibiotic resistance and the report of high prevalence of biofilm formation in *E. coli* isolates in the present study are completely logical and scientific and therefore should be taken into consideration by public health controllers in the human and animal fields.

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Conflict of Interest

The authors declare no conflict of interest.

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الگوی مقاومت آنتی‌بیوتیکی و توانایی تشکیل بیوفیلم باکتری *اشریشیا کلی* جدا شده از گوشت گاوهای کشتار شده در کشتارگاه استان‌های ایلام و کردستان

محمود کهنه‌پوشی[۱]، آرام شریفی[۲*] و محمدامین حیدرزادی[۳]

[۱] دانش آموخته کارشناسی ارشد بهداشت و کنترل کیفی مواد غذایی، دانشکده دامپزشکی، دانشگاه زابل، زابل، ایران

[۲] استادیار، گروه علوم دامی، دانشکده کشاورزی، دانشگاه کردستان، سنندج، ایران

[۳] دانشجوی دکترای تخصصی بهداشت مواد غذایی، دانشکده دامپزشکی، دانشگاه شهرکرد، شهرکرد، ایران

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چکیده

اشریشیا کلی میکروفلورای سیستم گوارشی انسان و حیوان است، اما برخی از سویه‌ها به دلیل وجود عوامل بیماری‌زا ممکن است باعث بیماری‌های مختلف حیوانی و انسانی شوند. مطالعه‌ی حاضر با هدف شناسایی الگوی مقاومت میکروبی و پتانسیل تشکیل بیوفیلم باکتری *اشریشیا کلی* اخذ شده از گوشت گاوهای کشتارگاه استان‌های ایلام و کردستان انجام شد. از گردن، بازو و ران ۹۰ رأس گاو کشتار شده (۴۵ رأس از ایلام و ۴۵ رأس از کردستان) نمونه‌برداری شد. جداسازی *اشریشیا کلی* بر اساس کشت بر روی محیط‌های کشت انتخابی و افتراقی و آزمایش‌های بیوشیمیایی انجام شد. حساسیت آنتی‌بیوتیکی و پتانسیل تشکیل بیوفیلم به ترتیب با استفاده از آزمون انتشار دیسک کربی بائر و میکروتیتر پلیت انجام شد. برای بررسی همبستگی بین حساسیت ضد میکروبی و تشکیل بیوفیلم از آزمون همبستگی اسپیرمن استفاده شد و مقادیر p کمتراز ۰/۰۵ به عنوان سطح معنی‌داری در نظر گرفته شد. از ۲۷۰ نمونه گوشت، ۴۲ جدایه باکتری *اشریشیا کلی* جدا شد. در هر دو استان، نمونه‌های گرفته شده از ران‌ها به طور قابل توجهی بیشتر از گردن و بازوها، واجد باکتری *اشریشیا کلی* بودند. بیشترین میزان مقاومت به سولفامتوکسازول و تتراسایکلین (۸۵/۷۱ درصد) و پس از آن آمپی سیلین (۸۰/۹۵ درصد) گزارش شد. علاوه بر این، تمام جدایه‌ها به کولیستین حساس بودند. بر اساس تست میکروتیتر پلیت، ۲۴ جدایه (۵۷/۱۴ درصد) بیوفیلم قوی، ۱۲ جدایه (۲۸/۵۷ درصد) بیوفیلم متوسط و ۶ جدایه (۱۴/۲۸ درصد) بیوفیلم ضعیف بودند. بین تشکیل بیوفیلم و مقاومت به آنتی‌بیوتیک‌های آمپی‌سیلین، آموکسی‌سیلین، آمیکاسین، سیپروفلوکساسین، جنتامایسین، تتراسایکلین و سفتریاکسون همبستگی مثبت معنی‌دار مشاهده شد. درصد بالای مقاومت آنتی‌بیوتیکی و توانایی قوی تشکیل بیوفیلم جدایه‌های *اشریشیا کلی* به دست آمده از گوشت قرمز نیاز به نظارت مداوم در زنجیره غذایی را نشان می‌دهد.

کلمات کلیدی: *اشریشیا کلی*، مقاومت آنتی‌بیوتیکی، تشکیل بیوفیلم، گوشت گاو

*نویسنده مسئول : آرام شریفی، استادیار، گروه علوم دامی، دانشکده کشاورزی، دانشگاه کردستان، سنندج، ایران

E-mail: A.sharifi@uok.ac.ir