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The relationship between antibiotics resistance and biofilm formation for *Escherichia coli* isolated from sheep in Nineveh province, Iraq

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ABSTRACT

The study aimed to determine the relationship between antibiotic resistance and the ability to produce biofilm of *E.coli* isolated from sheep in Nineveh Governorate. One hundred four fecal swabs were collected from healthy sheep from 1st February to 5th March 2022. Standard microbiological methods include culture on eosin methylene blue agar (EMB) and MacConkey agar, confirmed by Gram's Stain and biochemical tests, then polymerase chain reaction (PCR) assay for the specific gene of *E.coli* (*uidA*). Antibacterial resistance for 12 types of antibiotics and biofilm production tests were done by Congo red agar. The results showed that 92 samples at a rate of 88.46% were positive for the *E.coli* isolates; the study also showed that 71 isolates at a rate of 77.17% of *E.coli* isolates could produce biofilm. The study concluded that biofilm-producing *E.coli* in both forms strong and weak appear higher resistant to antibiotics other than the non-productive ones. Therefore, searching for other methods to test bacterial sensitivity to antibiotics that consider many factors such as biofilm production and extended-spectrum beta-lactamases is necessary.

Keywords: Antibiotic resistance, biofilm production, *E.coli*, Iraq, Nineveh province, Sheep.

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INTRODUCTION

Over the past years, the increasing bacterial antibiotic resistance has been a public health attention **(Padhi, 2011)**. Recently resistance to antibiotics has been considered one of the greatest threats to food security, development, and international health, as it can cause great harm to humans. Antibiotic resistance occurs naturally and its operations' pace accelerates due to misuse. Consequently, the traditionally used antibiotics for treatment have become less effective and lead to losses, a significant increase in medical costs, and fatalities **(Tessema *et al*., 2021)**. In addition to other mechanisms of resistance possessed by some microorganisms, they are emerging, developing, and spreading widely worldwide and threatening the ability to treat diseases **(Costerton *et al*., 1999)**. Among these mechanisms that allow microorganisms to tolerate antibiotics, host defenses and other external factors is biofilm production **(De la Fuente-Núñez *et al*., 2013; Tessema *et al*., 2021).**

Biofilm is glutinous exo- polymeric substances causing adhesion of microorganisms to the surfaces of host cells or abiotic surfaces such as medical devices to cause antibacterial resistance due to their molecular contents like exo-enzymes and eDNA (toxins, *β*-lactamase, etc.) **(Beloin *et al*., 2008)**. Chemical and physical studies have revealed that the bacteria within these biofilms differ significantly from their free-swimming counterparts. The cells within the biofilm form a trilogy of complex dimensional structures consisting of micro-colonies coated with exogenous sugars **(Costerton, 1995)**.

E.coli is a facultative anaerobic species found in the mammalian digestive tract because it possesses characteristics represented by extracellular factors (appendages) which contribute to its ability to colonize surfaces and its activity of biofilm production(biological membranes), leading to the apparition of various infections and makes its elimination difficult, As well as possessing virulence factors represented by the production of Shiga toxins (*Stx1* and *Stx2*) **(Beloin *et al*., 2008)**. *E.coli* causes many types of disease in sheep, including watery mouth disease in lambs **(Collins and Carson, 2022)**, navel ill and joint illness **(Swinson, 2021)**, Scour **(Hassan *et al*., 2013)**, septicemia **(Kjelstrup *et al*., 2017)**, meningitis **(Konradt *et al*., 2017, Nataro and Kaper, 1998)**.

In the latest estimates of global bacterial resistance to antibiotics published by the World Health Organization in 2014, *E.coli* was considered one of the most common infection concerns in societies **(Beloin *et al.*, 2008; WHO, 2018; Collins and Carson, 2022)**. *E.coli* is one of the most prevalent microorganisms found in the GIT tract of cattle, sheep and goats; many cause severe diseases to animals and may be transmitted to humans through the food chain **(Ferens and Hovde, 2011; Munns *et al.*, 2015)**. Due to the majority of sheep farming in Iraq in general and in Nineveh province in particular, the study objected to setting a relationship between antibiotic resistance and the ability to produce biofilm of *E.coli* isolated from sheep in Nineveh Governorate.

MATERIALS AND METHODS

Sampling

Four different regions, including Al-Kasr, Al-Salamiya, Gogjali, and Al-Abbasiya representing sheep breeding centers in Nineveh Governorate, were selected to carry out the study. One hundred four samples of fecal swabs from healthy sheep for the period from 1st February to 5th March 2022, were collected directly from the rectum and placed in sterile containers, then transferred directly to the research unit of the Veterinary Medicine Faculty at University of Mosul for bacteriological examinations.

Bacterial isolation

Standard microbiological methods include culture in Eosin Methylene Blue Agar(EMB) and MacConkey agar (Himedia®, India). Then the isolates were confirmed using gram stain and biochemical tests (Indole-production, methyl red, Voges-Proskauer, Simmons Citrate, Triple sugar iron, Catalase, and Oxidase) **(Quinn *et al.*, 2011)**.

Molecular investigation

DNA extraction

The DNA of *E.coli* was isolated depending on the instructions of Presto™ Mini gDNA Bacteria Kit (Geneaid®, Taiwan). The concentration of DNA of *E.coli* was estimated using the (Biodrop®, UK) and stored at -20°C.

Conventional PCR amplification of uidA gene

The total volume of PCR reaction was 25 µL including 12.5 µL of 2×Go Taq Green Mix Master (Promega Corporation, USA), 1 µL of forwarding primer ($^{5'}$CCAAAAGCCAGACAGAGT$^{3'}$), 1 µL reverse primer ($^{5'}$GCACAGCACATCCCCAAAGAG^{3}) **(Moyo *et al.*, 2007)**, 5.5 µL of nuclease-free water (Promega®, USA), and 5 µL of DNA template. The mixture was placed in the PCR reaction tube (200 µL) (Biozym, Oldenhorf®, Germany). Amplification was carried out using (Biometra®, Germany) under the following conditions: initial denaturation, one cycle at 94°C for 5 minutes, followed by denaturation, 35 cycles at 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute. Then, a cycle at 72°C for 5 minutes was set for the final extension. Finally, the reactions were cooled at 4°C until the gel electrophoresis proceeded. The amplicons of the target sequence were determined by gel electrophoresis and a DNA marker (Promega®, USA) in 2% agarose gel (Peqlab, Erlangen®, Germany).

Sensitivity test

To assay antibacterial resistance of all isolates obtained under the study, Kirby Bauer disk diffusion method on Mueller Hinton agar plate according to **(Jorgensen and Turnidge, 2015)**, 12 types of antibiotics (Bioanalyse®, Turkey) were selected, which include: Amoxicillin (A.X.), Cephalothin (K.F.), Cefixime (CFM),Ceftriaxone (CRO), Erythromycin (E), Gentamycin (G.N.), Streptomycin(S), iprofloxacin (CIP), Chloramphenicol (C), Tetracycline(T.E.) , Trimethoprim (TMP) and Nitrofurantoin(F).

The diameter of bacterial inhibition zones, including the diameter of the antibiotic discs, was measured in millimeters by means of a transparent ruler and compared with the measurements of the company that supplied these discs.

Detection of biofilm

Biofilm production was detected by Congo red Agar Medium (CRA) test; according to **Mathur *et al.*, (2006)**, (CRA) was prepared by mixing two mixtures; first, one from Brain-heart infusion agar (BHIA)(Himedia®, India) 37 g/L + Sucrose 50 g/L second mixture from Congo Red Indicator (CRI) which prepared from Congo Red Dye(Himedia®, India)8 g/L + 4% NaCl, then the two mixture was sterilized (121 °C for 20 minutes), later adding (CRI) to the sterilized BHIA with sucrose in 55 °C. The isolates were classified into three classes (strong, weak, and non-producers) based on the intensity of color change on CRA, which is directly proportional to the biofilm production after isolates were cultivated and incubated for 24 hours **(Poovendran *et al.*, 2011)**.

RESULTS

Results showed that out of 104 samples, 92 samples at a rate of 88.46%, were positive for the microbiological isolation of *E.coli*, which was confirmed by biochemical assays (Indole-production +, Methyl red +, Voges-Proskauer -, Simmons Citrate -, Triple sugar iron +, Catalase + and Oxidase -). and molecular methods for *uidA* gene figure(1). The *uidA*

gene was detected in *E.coli* isolates. A 623 bp product size revealed that the 92 isolates were all positive. There was no amplification on PCR with negative control (Fig.1).

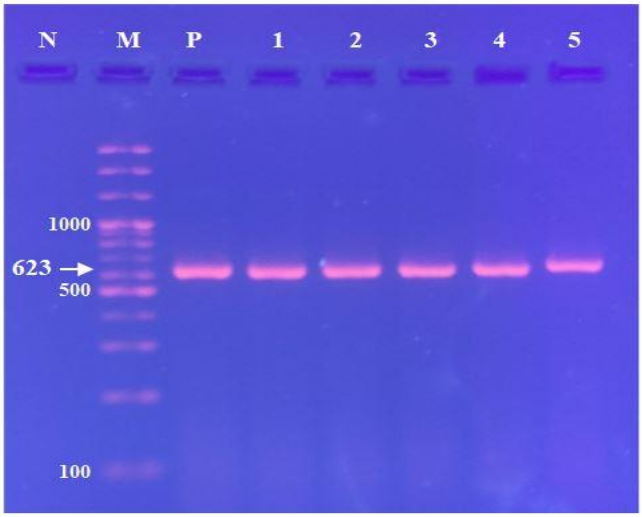

Fig. 1: PCR products of the *uidA* gene. Lane N, control negative; Lane M, DNA molecular standard; Lane P, control positive; Lanes 1-5 positive tested samples giving 623bp product size.

The results of the antibiotics susceptibility test for bacterial isolates also showed a clear difference in the antibiotic-resistant (A.R.) rates, where the highest percentage of antibiotic-resistant was Cephalothin (K.F.) at 100%, followed by Erythromycin (E), at 91.3%, and then to Amoxicillin (A.X.) by 88.04%. In comparison, it was sensitive to 100% for Nitrofurantoin (F) (Fig. 2 and Table 1). The study also showed the capability of *E.coli* isolates for biofilm production. Out of 92 isolates, 71 (77.17%) were biofilm producers with different patterns, as shown in Fig. 2.

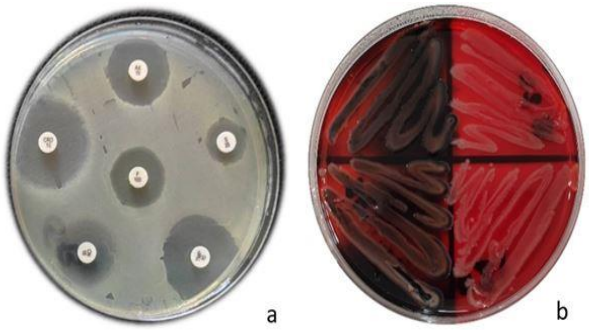

Fig. 2: Antibiotic test on Mueller–Hinton agar (a) and biofilm production test for *E.coli* on CRA(b).

Table 1: Antibiotic susceptibility and biofilm production of *E.coli* isolates.

Antibiotic susceptibility			**Biofilm production**	
No.	Antibiotics	Antibiotics resistance No. (%)	Pattern of Biofilm production	Biofilm production No. (%)
1	A.X.	81 (88.04)	Strong	42(58.33)
2	K.F.	92 (100)		
3	CFM	15 (16.3)		
4	CRO	15 (16.3)	Weak	29 (40.27)
5	E	84 (91.3)		
6	C.N.	7 (7.6)		
7	S	47 (51.08)		
8	CIP	15 (16.3)	Non	21 (22.82)
9	C	32 (34.78)		
10	T.E.	56 (60.86)		
11	TMP	35 (38.04)		
12	F	0 (0)		

DISCUSSION

Sheep are the main reservoirs of *E.coli*, which are transmitted to other animals and humans, infection with *E.coli* represents a significant challenge for people, especially those who live closely with sheep, especially since they do not have any knowledge of the pathogenesis of *E.coli* and its transmission methods **(Shabana *et al*., 2013; Ferone *et al*., 2020).**

Although several traditional procedures for *E.coli* detection are available, they pose a problem when accurately diagnosing different samples, so polymerase chain reaction (PCR) methods can overcome the problems of conventional methods. In this study, the PCR technique used a set of primers derived from the *uidA* gene sequence to detect *E.coli* strains, even those with undetectable b-d-glucuronidase activity by traditional procedure detected in this way based on the *uidA* gene **(Horakova *et al*., 2008)**.

Using CRA to detect biofilm production by *E.coli* is considered useful because these *E.coli* do not ferment other sugars that may be necessary to release certain metabolites, which combine with CRI to impart black color to the colonies indicating slime production **(Yaratha *et al*., 2017, Gajdács *et al*., 2020)**.

Despite the global trends that have occurred because of demographic, epidemiological changes and transformations, the role of non-communicable diseases has become more apparent. In contrast, infectious diseases have remained an important factor and have constituted a big global burden in previous years, including A.R. **(Costerton *et al*., 1999; Michaud, 2009, Tessema *et al*., 2021)**.

Antibiotic-resistant *E.coli* is an important concern clinically, influencing both humans and animals. Several studies have highlighted the antibiotic resistance levels of the pathogenic *E.coli* to fosfomycin, fluoroquinolone, sulfamethoxazole, trimethoprim, nitrofurantoin, and third generation. Cephalosporin (28,29,5). **(Beloin *et al*., 2008; Alizade, 2018; Gajdács *et al*., 2020)**.

In many diseases, biofilm production is an important stage, not only from a mechanistic point of view, but this may also allow extra resistance that differs in other aspects of multi-antibiotic resistance **(Giaouris *et al*., 2014; Etefia, 2021).**

Among the 92 isolates of *E.coli*, 71 isolates were biofilm-producing at a rate of 77.17%, of which 42 isolates at a rate of 58.33%, were highly biofilm producing, and 29 isolates at a rate of 40.27%, with weak biofilm production and these results, are close to what found **(Anandkumar *et al*., 2021, Beloin *et al*., 2008)**, among *E.coli* isolates, a high rate of A.R. was detected in strong and weak biofilm producers. A relation was found between production of biofilm and AR to Cephalothin(KF) Amoxicillin(AX), Cefixime(CFM), Ceftriaxone(CRO), Erythromycin(E), Gentamycin(GN), Streptomycin(S), except in the case of Ciprofloxacin(CIP), Chloramphenicol(C), Tetracycline(TE), Trimethoprim(TMP), Nitrofurantoin(F).

Ramírez-Castillo *et al*., (2018) indicated that the microorganism's product biofilm is resistant to many antibiotics. The resistance of those antibiotics increases to 1000 times, so killing bacteria inside the biofilm may require giving high concentrations to reach the effective concentration, which resists the mechanisms of decomposition of antibiotics and access to and inactivation of bacteria, and this is what the researchers **(Behzadi *et al*., 2020, Karahutová *et al*., 2021)** pointed out.

Many surface appendages of *E.coli*, like flagella, antigen43(Ag43), tortuous fibers, type I fibrils, and conjugating filaments, play a role in biofilm formation **(Sherlock *et al*., 2006, Jin and Marshall, 2020)**. Bacterial autolysis plays a critical role in the initial binding and formation of biofilm by many microorganisms **(Costerton, 1995; Nakao *et al*., 2012)**. Since biofilm production is a cooperative behavior of mutual benefit between microorganisms to enhance their survival, there is no ruling out that there will be a transfer of genes between microorganisms for the development of antibiotic resistance and biofilm production. This highlights the great and close interrelationship between the two mechanisms **(Niederdorfer *et al*., 2017; Maeusli *et al*., 2020).**

This evolutionary exchange may explain the trends of resistance to different antibiotics in the same types of bacteria producing and not producing biofilm, meaning that we find resistant and biofilm-producing isolates and other biofilm-producing isolates that are not resistant to antibiotics and vice versa or do not have both, and this is what was shown by our study of resistance to erythromycin (E) with weak biofilm production **(Nadell and Bassler, 2011).**

CONCLUSION

The study revealed that 58,33% of the isolates were strong biofilm producers, and 40.27% were weakly biofilm producers. On the other hand, these isolates recorded higher rates of antibiotic resistance other than the non-productive ones, which explains the apparent resistance of *E.coli* to deadly substances such as antibiotics; therefore, the approved methods for measuring the effect of antibiotics on microorganisms cannot give a correct picture of the efficiency of drugs against microorganisms produce or present in biofilm. It is worth noting that the nature of the composition of biofilm affects the physiology of the living organisms inside them, including the inheriting resistance to various types of antibiotics. Therefore, it is necessary to search for other methods to test bacterial sensitivity to antibiotics that take into consideration many factors such as biofilm production and extended-spectrum beta-lactamases.

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Conflict of interest

The researchers acknowledge that there is no conflict of interest regarding the research idea and tools, actual, potential and financial, directly or indirectly.

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