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Ciprofloxacin's Role in Disrupting Biofilms and Antibiotic Resistance in Uropathogenic *Escherichia coli* from Anbar Provinces

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Abstract

Escherichia coli (*E. coli*) is responsible for 70%-95% of urinary tract infections (UTIs), with biofilm formation significantly contributing to antibiotic resistance. This study investigates the relationship between biofilm formation and antibiotic resistance in uropathogenic *E. coli*, and assesses the effect of Ciprofloxacin (CIP) on biofilm development. *E. coli* strains were isolated from UTI patients and their biofilm-forming abilities were evaluated using Tube and Microtiter plate assays. The minimum inhibitory concentration (sub-MIC) of CIP was determined through resazurin assays and growth curve analysis. The impact of CIP on biofilm formation was assessed by comparing biofilm production in treated versus untreated isolates, with statistical significance determined using the t-test ($p < 0.05$) .Out of 35 samples, 30 *E. coli* isolates were identified, with 98% exhibiting biofilm production of varying intensities (strong, moderate, and weak). The sub-MIC of CIP was found to be 0.1 mg/ml, which reduced biofilm formation by 75%, lowering the mean biofilm production from 0.06 to 0.02, with statistically significant results ($p < 0.05$). The findings suggest a notable link between biofilm formation, antibiotic resistance, and *E. coli* pathogenicity. Sub-MIC CIP effectively inhibits biofilm formation without adversely affecting bacterial growth, indicating its potential as a therapeutic option for managing biofilm-associated bacterial infections.

Keywords: urinary tract infections, Uropathogenic *E. coli*, MDR, Biofilm, Ciprofloxacin, Antibiotic Resistance.

Introduction

Urinary tract infections (UTIs) are becoming increasingly challenging to treat, primarily due to higher recurrence rates and resistance to frontline treatments. Each year, 150-250 million cases of urinary tract infection (UTI) are reported worldwide, constituting 90% of all urinary tract infections, including those acquired in the community and nosocomial infections [1].

Escherichia coli (*E. coli*) is a common cause of a variety of diseases, such as prostatitis, Gram-negative bacteremia, newborn meningitis, and urinary tract infections. Both bacterial and host immunological factors influence how these illnesses progress. The virulence factors of *E. coli* are largely responsible for its pathogenicity; these factors include host colonization, tissue invasion, and promotion of the inflammatory response, immune system evasion, and biofilm formation [2].

Uropathogenic *E. coli* is the leading cause of urinary tract infections in humans, according to [3]. In addition to causing simple cystitis and asymptomatic bacteriuria, these bacteria can cause ascending infections that result in severe pyelonephritis. UPEC is associated with a limited range of serotypes and exhibits enhanced adhesion to the epithelial cells of the urinary tract, particularly those identified in instances of pyelonephritis [4]. Intracellular bacterial communities (IBCs) are microcolonies that UPEC can produce within the mucosal lining of the bladder. These IBCs resemble biofilms. This facilitates the bacterial persistence in the host [3]. The capacity to form biofilms has been connected to Infections obtained in a hospital, including catheter-associated infections of the urinary tract (CAUTI) [1].

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A biofilm is an assemblage of microorganisms adhered to surfaces that are encased in an extracellular polymeric matrix that the microorganisms themselves manufacture. Notably, bacterial cells in biofilms differ from their freely suspended planktonic counterparts in terms of emerging characteristics [5]. Previous studies have demonstrated biofilms' extracellular matrix, which promotes the chronicity and bacterial persistence of infections, defies the pressures created by urine flow, and aids *E.coli* in adhering to host cells [6] .Antibiotic resistance develops because of the structural features of the biofilm and the microorganisms that make up the biofilm. The phenomena of drug resistance linked to biofilms is intricate and might be primarily influenced by biofilms. Biofilms and related illnesses can be treated with antibiotics, disinfectants, and germicidal chemicals. Drug resistance in bacteria living in biofilms is ten to thousand times higher than that of bacteria in their planktonic stage, particularly in antibiotics [7].

Antibiotics are needed to treat bacterial infections; second-generation fluoroquinolone antibiotics like ciprofloxacin (CIP) are commonly used to treat both Gram-positive and Gram-negative bacterial infections. Low quantities of CIP have been shown to have an inhibitory effect on virulence factors, including biofilm formation. CIP is an antibiotic that is most commonly used to treat urinary tract infections [2]. The novelty of this study lies in its comprehensive analysis of the interplay between biofilm formation, antibiotic resistance, and the inhibitory effects of sub-MIC Ciprofloxacin on *E. coli* isolates from UTIs. It opens new avenues for potentially effective therapeutic strategies against biofilm-associated bacterial infections.

Methods

Sample collection

This study was included collect 35aliquots of urine from all consecutive urine specimens from patients with UTIs, differing in age and gender (28 females and 7 male) from hospitals in the Al-Anbar Provinces of Iraq. Using culture media such as Blood agar, Eosin Methyl Blue (EMB) agar, and MacConkey agar, these samples were diagnosed under a microscope. Following that, the Gram staining technique was used to differentiate between Gram-positive Cocci and Gram-negative rods. Biochemical testing was also carried out. A Vitek device was then used to confirm the diagnosis.

Antibiotic susceptibility testing

It used the Clinical and Laboratory Standards Institute's (CLSI) recommendation and employed the disk diffusion method [8]. Amoxicillin (AX), azithromycin (AZM), ciprofloxacin (CIP), meropenem (MEM), gentamicin (CN), and amoxicillin and ceftriaxone (CTR) were among the antibacterial drugs used against *E. coli*. One antibiotic from each generation of drugs was used to test *E. coli* resistance to those antibiotics. MDR isolates were those that showed resistance to at least three different antibiotic classes. XDR isolates have been defined as those that were sensitive to only one or two antibiotic classes however resistant to all except two or less antibiotic classes [9].

Biofilm formation determination

A- By (tube) method

Bacterial isolates were inoculated into a polystyrene test tube containing TSB, and the tube was then incubated for 24 h at 37°C. Following, remove of the media, bacteria were stained for 30 minutes at room temperature (18–20°C) using 3 mL of 1% crystal violet. The cells were dried at room temperature after the crystal violet coloring solution was removed by washing them in clear water until colorless water was recovered. The intersection of the liquid level and the glass tube wall's color was observed visually [10].

B - By microtiter plate method

Overnight growth was initially adjusted to a 0.5 McFarland standard using brain heart infusion broth. After that, it was diluted 100 times with 2% sucrose in the same medium. Then, 200 µl of the diluted culture for each isolate and control (broth only) were added to three wells on a 96-flat bottom microtiter plate that were assigned to each test isolate. After that, the plates were incubated for 24 hours at 37 °C. After the bacterial culture was incubated, the microtiter plate was inverted to decant the culture, and the plates were then washed three or four times with sterile saline (0.9%). After that, the washed wells received 200 µl aliquots of methanol, and the plates were allowed to sit at room temperature for 20 minutes. Following that, the methanol fixative was decanted, and the plates were left to dry naturally. To stain each well, 200 µl of 1% crystal violet was added, allowed to remain for 15 min and after this, three washes with DW were performed. Then, the plates were inverted and allowed to air dry. After that, 200 µl of 95% ethanol were added for extract the dye which attached to the cells, and a microplate reader was used to measure the optical density (OD) at 600 nm. Every clinical isolate was tested three times. After deducting the control values (non-inoculated media), the mean optical density values were determined [11].

Ciprofloxacin preparation stock solution

For this purpose, prepare 100 mg/ml as a stock solution by dissolving 500 mg of CIPn in 5 ml (D.W).

Calculate the sub inhibitory concentration (Sub MIC) and minimum inhibitory concentration (MIC).

Sub-MIC and MIC susceptibilities have been determined by use of the dilution tube technique. After the bacteria were cultivated in nutrient broth for a 24hr, the turbidity was corrected using the McFarland standard. After that, 1ml of nutrient broth was put into test tubes. After adding one milliliter of a 100 mg/ml CIP stock solution to the first tube, many half-dilutions were made to produce concentrations of (100, 50, 25, 12.5,...) mg/ml. 100 microliters of bacteria were present in each tube during a 24-hour incubation period at 37°C. After the incubation period, a 0.1% resazurin solution was added in 1 milliliter and allowed to settle for a duration of 2 hours. We contrasted the colors that emerged [12].

Impact of CIP sub-MICs on the production of biofilms

When MIC and sub-MIC concentration were determined, the sub-MIC has been employed to evaluate the effect on the production of biofilms without influencing bacterial growth. Using the same procedure as in paragraph (2-2), the antibiotic in 100 microliters at sub-MIC was added, together with adequate controls and bacteria, to carry out this procedure.

Statically significant

The student's t-test was used to statistically compare the biofilm OD values. P-values of less than 0.05 were used.

Results

Identification of E. coli

When cultivating the samples for 24 hours at 37°C on differential and selective medium, 30 isolates (85.7%) proved to be *E. coli* bacteria. The Vatic device and biochemical assays were used to confirm the diagnosis. The remaining five samples (14.3%), consisted of another (G-ve bacteria). As show in Fig.1.

Antimicrobial Susceptibility of E. coli isolates

According to the Clinical Laboratory Standard Institute (CSLI) recommendations, Antimicrobial susceptibility testing was performed on *E. coli* isolates using the Kirby Bauer Disc Diffusion technique. In this study, results showed that the isolates had a resistance rate of the following antibiotics: Meropenem 3/30 (10%), gentamicin 13/30 (43%), amoxicillin 16/30 (80%), azithromycin 12/30 (43%), CIP 19/30 (63%), and ceftriaxone 23/30 (77%). as shows in Fig. (2). 60% (n = 18) of these instances were MDR, while 40% (n = 12) were non-MDR, as shown Table 1.

Biofilm activity of E. coli isolates

According to the tube method (TM) results, 29 of *E. coli* isolates were found to generate biofilm, whereas the remaining isolate did not. Biofilm generation was identified by the apparent violet film on the tube bottoms and walls. Fig. (3).

The microtiter plate (MTP) test results, out of the 30 test isolates, 28 (93.33%) showed evidence of biofilm formation capability. The *E. coli* isolates were found to have different levels of biofilm forming capacity; they were categorized as strong (23.3%), moderate (23.3%), and weak (46.6%). Fig. (4).

Determination of MIC and sub-MIC for CIP

The ability of the bacteria to reduce the dye using resazurin was tested, indicating the presence of growing bacteria in the tube. The results showed that the lowest growth-inhibitory dose, at which no bacteria changed the dye's color to pink, was 0.5 mg/ml. The initial concentration used as a stock solution was 100 mg/ml. As shown in Fig. (5), the sub-inhibitory concentration was 0.1 mg/ml.

Determination the activity of CIP on biofilm inhibition

According to the current study findings, using sub-MIC CIP resulted in 73% biofilm inhibition rates. These treatments caused the biofilm strength to change from strong to weak and non-biofilm in contrast to the untreated, with significant changes seen at the P value of 0.05 for the isolates treatments. As seen in Fig. (6), the mean (±SD) of the biofilm formation decreased from 0.8 (±0.02) to 0.06 (±0.007).

Discussion

The findings of the investigation are consistent with earlier research showing a range of bacteria were linked to UTIs, with *E. coli* being the most common, accounting for almost 80% of infections obtained in the community and 50% of infections obtained in hospitals [13].In contrast to other isolates, 30 *E. coli* isolates were collected from individuals with urinary tract infections, showing a high bacterial proportion. Uropathogenic *E. coli* strains are *E. coli* strains that have acquired disease-causing potential and cause sickness outside of the gastrointestinal system [14].These isolates showed variable resistance to the antibiotics employed in this investigation, with penicillin, cephalosporin, and fluoroquinolones showing the highest resistance. Similarly, to several recent studies, it appears that uropathogenic *E. coli* in Iran exhibits resistance to fluoroquinolones and cephalosporins at a rate of roughly between 66% and 60%, respectively [15].

Furthermore, multidrug-resistant bacteria were present in 60% of these isolates, which is in agreement with previously reported results. High-level resistance to piperacillin, tetracycline, amoxicillin/clavulanic acid (92%, 91%, and 88%) and imidazoline was found in uropathogenic *E. coli* isolates from southern Iraq. Very sensitive to amikacin according to Allami et al. [16]. Antimicrobial resistance, however, differs depending on the period and location. According to Flament-Simon et al., in 2016 urinary tract infections and other extra intestinal infections were caused by 37.2% of *E. coli* in Spain and France [17]. In our study, 60% of the isolates appeared to be MDR, and this percentage is consistent with previous studies, where the resistance rate appeared to be 75% of total *E. coli* isolates [16], and 52.9 % MDR *E. coli* prevalence in a study made by [18], MDR *E. coli* causing risk factorsthat increase the pathogenicity of these isolates, such as diabetes mellitus, chronic renal disease, and posterior urethral valve [19].

In our recent investigation, *E. coli* produced substantial biofilms, with a production rate of 98%. Furthermore, the majority of the resistant isolates produced strong to moderate biofilms. The results show that a considerable percentage of *E. coli* clinical isolates have the ability to form biofilms; specifically, 38.67% of the isolates show strong to moderate biofilm generation, which is in line with earlier research. Furthermore, isolates obtained from urine showed a higher frequency of strong to moderate biofilm development [20]. A biofilm is a community of bacteria that sticks to a surface, either living or dead, to create a matrix made of extracellular polymeric substances such proteins, polysaccharides, and extracellular DNA. Studies reveal their involvement in a range of microbial diseases, with bacteria and fungi capable of producing biofilms accounting for around 80% of infections [21].

More of MDR isolate were produce biofilm, in our finding revealing a positive correlation between biofilm forming capabilities and antibiotic resistance phenotypes. Comparably, MDR *E. coli* isolates showed a higher ability for biofilm production than non-MDR *E. coli* isolates. These findings were consistent with another study that showed biofilm-forming *E. coli* isolates from clinical isolates in Uganda had higher resistance than non-biofilm formers, with 64% of the formed being MDR compared to 36% of non-biofilm forming *E. coli* isolates [22].

CIP was used in this study to decrease biofilm development in *E. coli* without impacting bacterial growth, with the goal of reducing antibiotic resistance in bacteria. The antibiotic CIP was selected because to its ability to break biofilms, exhibiting antimicrobial actions such as biofilm disintegration, bacterial outer membrane alteration, and inhibition of virulence factor expression [23]. Through modifying penicillin-binding protein binding, CIP prevents the production of cell walls (PBPs). A porin allows the drug to selectively penetrate the outer membrane of *E. coli* and deliver basic of amino acids [24]. Sub-MIC CIP (0.1 mg/ml) was utilized in the current study, and its impact on biofilm was measured using a microtiter plate. Sub-MICs of CIP were applied, and the result was a 75% suppression of biofilm development with statistically significant differences at $p < 0.05$, without impacting bacterial growth. This suggests that CIP sub-MICs disrupted the structures of biofilms. These findings are consistent with previous investigations that found that sub-MICs from CIP lowered *E. coli* pathogenicity. These findings support some studies but contradict others. Sub-minimum inhibitory doses (MIC) of amikacin and CIP were reported to suppress bacterial virulence factors by interfering with bacterial cell activity in a prior investigation [25].

Conclusion

This study highlights the significant role of biofilm formation in antibiotic resistance among uropathogenic *E. coli* strains, which are responsible for the majority of urinary tract infections. By isolating *E. coli* from UTI patients and assessing biofilm formation and antibiotic resistance, the study demonstrates that Ciprofloxacin (CIP) at a sub-MIC of 0.1 mg/ml effectively reduces biofilm formation by 75% without inhibiting bacterial growth. This reduction in biofilm could help mitigate the challenges associated with antibiotic resistance in UTIs. Consequently, CIP, when administered in low doses, shows promise as a viable treatment option for infections involving biofilm-producing bacteria.

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Conflicts of interest

All Authors declare that there is no conflict of interest.

Author`s contributions

All authors shared equally in conceptualization, study design, sample collection, and Ultrasonography, Data analyses, Manuscript drafting, and Manuscript finalization.

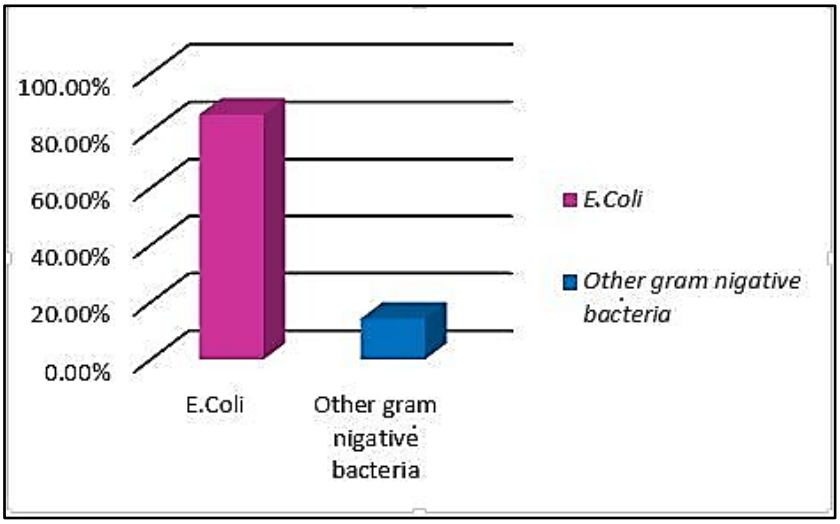

Fig. 1. Distribution of bacterial isolates

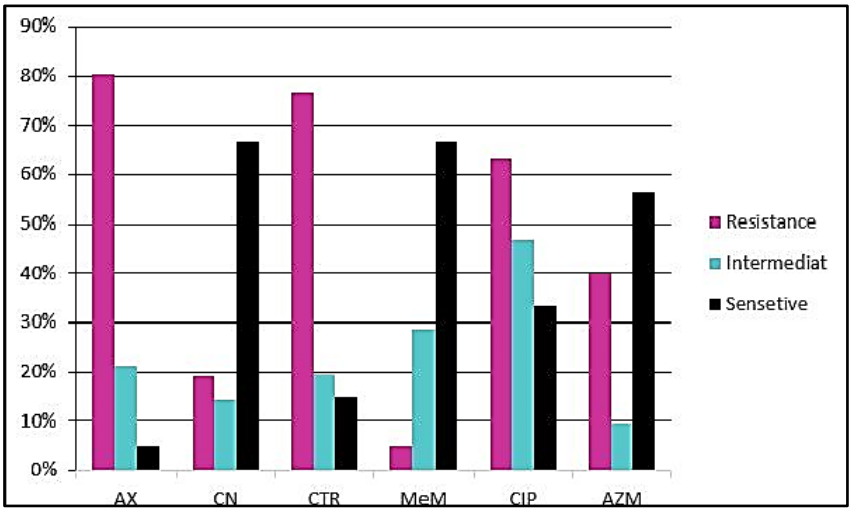

Fig.2. Antibiotic susceptibility patterns of *E. coli* isolates: AX (amoxicillin), CN (gentamicin), CTR (ceftriaxone), MeM (meropenem), CIP (ciprofloxacin), AZM (azithromycin).

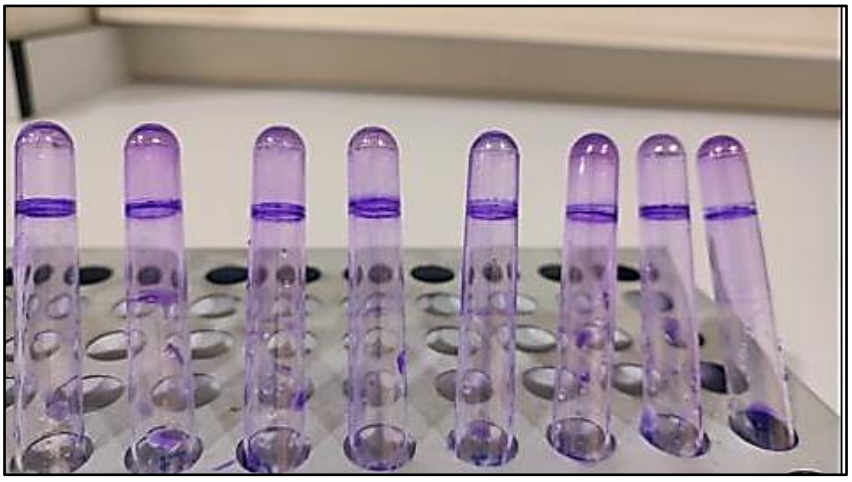

Fig. 3. Biofilms formation by *E. coli* as determined by test tube method

TABLE 1. MDR *E. coli* resistant to different numbers of antibiotics used in this study

Sample id	MDR	Resistance against
1	-	CIP
2	-	AX
3	MDR	AX,CTR,AZM
4	MDR	CIP,AX,CTR,AZM
5	MDR	CIP,AX,AZM
6	MDR	CIP,AX,CTR,AZM
7	MDR	CIP,AX,CTR,AZM
8	MDR	CIP,AX,CN,CTR,MEM
9	-	-
10	MDR	CIP,AX,CTR,AZM
11	MDR	AX,CN,CTR,AZM
12	MDR	AX,CN,CTR,
13	-	CTR,AZM
14	-	AX,CTR,
15	MDR	CIP,AX, CTR
16	-	AX,CN
17	-	AX
18	-	CIP,CTR
19	-	AX
20	-	CIP,AX
21	-	CIP,CTR
22	MDR	CN,CTR
23	MDR	CIP,CN,CTR
24	MDR	CIP,AX,CN,CTR
25	MDR	CIP,AX,CN,CTR
26	MDR	CIP,AX,CN,CTR
27	MDR	CIP,AX,CN,CTR
28	MDR	CIP,AX,CN,CTR
29	MDR	CIP,AX,CN,CTR,MEM
30	-	AX , MEM

*The (-) indicates non-multi- Drug resistant.

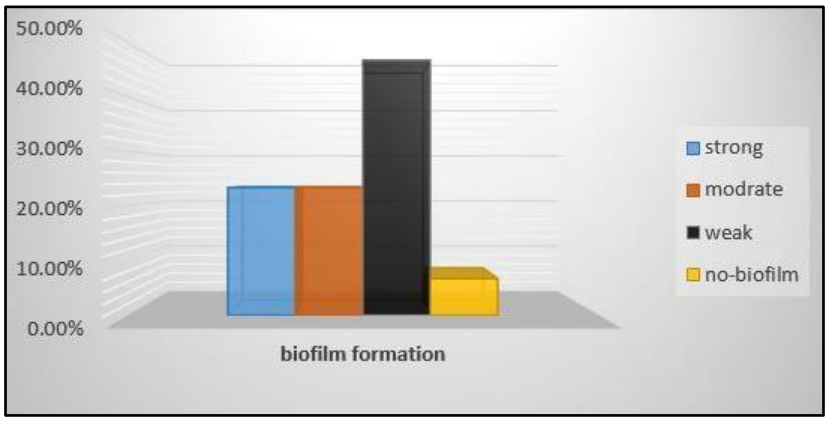

Fig. 4. Biofilm formation percentage in *E. coli* isolates

TABLE 2. Correlation between Multi-drug resistance and Biofilm formation

Biofilm formation		Antibiotic susceptibility	
Type	**%**	**MDR%**	**Sensitive %**
Strong	23.3	71.43	28.57
Moderate	23.3	57.14	42.86
Weak	46.7	68.4	53.3

This result indicates a moderate positive correlation of (rs 0.313, 0.21,0.1)at p <0.05 between percentage of biofilm formation (Strong , Moderate, Weak) and MDR.

Fig.5. Sub-MIC estimation of CIP by resazurin method,the blue color at the ninth dilution is MIC while the further dilution is Sub-MIC. Arrow denote to the MIC.

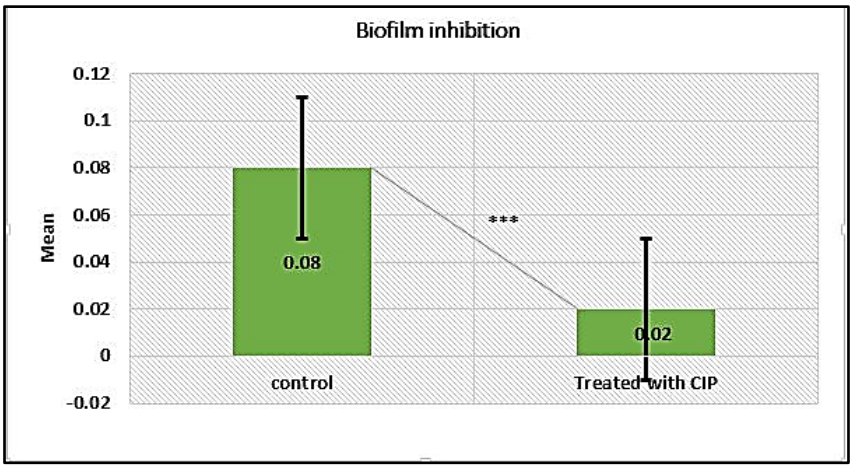

Fig. 6. Inhibition rate of biofilm by sub-MIC of CIP

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دور عقار السيبروفلوكساسين في تعطيل الأغشية الحيوية ومقاومة المضادات الحيوية في الإشريكية القولونية المسببة لعدوى المسالك البولية في محافظات الأنبار

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قسم تقنيات المختبرات الطبية، كلية التقنيات الصحية والطبية، جامعة المعارف، الانبار ٣١٠٠١ ، العراق.

الملخص

تُعد بكتيريا الإشريكية القولونية (Escherichia coli) مسؤولة عن ٧٠%-٩٥% من حالات التهابات المسالك البولية (UTIs)، حيث يُسهم تكوين الأغشية الحيوية بشكل كبير في مقاومة المضادات الحيوية. تستقصي هذه الدراسة العلاقة بين تكوين الأغشية الحيوية ومقاومة المضادات الحيوية في الإشريكية القولونية الممرضة في المسالك البولية، وتقيّم تأثير عقار سيبروفلوكساسين (CIP) على تطور تكوين الأغشية الحيوية. تم عزل سلالات الإشريكية القولونية من مرضى التهاب المسالك البولية وتم تقييم قدرتها على تكوين الأغشية الحيوية باستخدام اختبار الأنابيب واختبار لوحة الميكروتيتر. تم تحديد التركيز المثبط الأدنى (sub-MIC) لعقار سيبروفلوكساسين من خلال اختبار الريسازورين وتحليل منحنى النمو. تم تقييم تأثير سيبروفلوكساسين على تكوين الأغشية الحيوية من خلال مقارنة إنتاج الأغشية الحيوية في العينات المعالجة وغير المعالجة، وتم تحديد الأهمية الإحصائية باستخدام اختبار .t ($p < 0.05$) من بين ٣٥ عينة، تم تحديد ٣٠ عينة إشريكية قولونية، حيث أظهرت ٩٨% منها تكوين الأغشية الحيوية بدرجات متفاوتة (قوية، متوسطة، وضعيفة). وتم تحديد أن التركيز المثبط الأدنى لعقار سيبروفلوكساسين هو ٠.١ ملغم/مل، مما قلل من تكوين الأغشية الحيوية بنسبة ٧٥%، حيث خفض متوسط إنتاج الأغشية الحيوية من ٠.٠٦ إلى ٠.٠٢، مع نتائج ذات دلالة إحصائية .($p < 0.05$) تشير النتائج إلى وجود علاقة ملحوظة بين تكوين الأغشية الحيوية، مقاومة المضادات الحيوية، والإمراضية للإشريكية القولونية. يُعد التركيز المثبط الأدنى لعقار سيبروفلوكساسين فعالًا في تثبيط تكوين الأغشية الحيوية دون التأثير سلبًا على نمو البكتيريا، مما يشير إلى إمكانيته كخيار علاجي لإدارة الالتهابات البكتيرية المرتبطة بتكوين الأغشية الحيوية.

الكلمات المفتاحية: عدوى المسالك البولية، الإشريكية القولونية الممرضة للمسالك البولية، مقاومة الأدوية المتعددة، الأغشية الحيوية، سيبروفلوكساسين، مقاومة المضادات الحيوية.