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A Potential Role of Extracellular DNA in Biofilm and Ciprofloxacin Resistance

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Abstract
Objectives: This study aims to broaden our knowledge of the role of eDNA in bacterial biofilms and antibiotic-resistance gene transfer among isolates.
Methods: *Staphylococcus aureus, E. coli,* and *Pseudomonas aeruginosa* were isolated from different non-repeated 170 specimens. The bacterial isolates were identified using morphological and molecular methods. Different concentrations of genomic DNA were tested for their potential role in biofilms formed by study isolates employing microtiter plate assay. Ciprofloxacin resistance was identified by detecting a mutation in *gyrA* and *parC*.
Results: The biofilm intensity significantly decreased ($P < 0.05$) concerning *S. aureus* isolates and insignificantly ($P > 0.05$) concerning *E. coli* isolates. Yet, one *E. coli* isolate's biofilm was significantly decreased ($P < 0.05$) linearly with increasing eDNA. Of considerable interest, the addition of eDNA led to a significant increase ($P < 0.05$) in the biofilm of the two-tested *P. aeruginosa* isolates. Moreover, eDNA participated in transferring Ciprofloxacin resistance to the sensitive isolate when it presents in its biofilm.
Conclusion: eDNA has a dual effect on bacterial biofilms either supportive or suppressive following bacterial species *per se*. Also, it seems to play an important role in antibiotic resistance within the biofilm.
Keywords: eDNA, Biofilm, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*

Introduction

Staphylococcus aureus inhabited approximately 30% of healthy people, mostly in the anterior nares. Nevertheless, it is also a leading cause of hospital-associated and community-associated bacterial infections in humans, associated with numerous mild skin and soft tissue infections and life-threatening pneumonia, aimeretcab, osteomyelitis, endocarditis, sepsis, and toxic shock syndrome. The increasing prevalence of methicillin-resistant *S. aureus* (MRSA) and its ability to resist multiple drugs has posed a serious challenge to infection control.[1,2]

Escherichia coli is one of the earliest colonizers of the gastrointestinal tract; although eventually, it is a minor component of the colonic gut microbiome in humans, where it represents less than 0.1% of the total bacterial cells. Nevertheless, due to the overall high cell density in the colon, this small percentage translates into around 108 cells/ml.[3] Indeed, *E. coli* is the causative agent of various intestinal and extra-intestinal diseases, including being suspected to be the cause of sudden infant death syndrome.[4,5]

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen and the leading cause of diverse nosocomial infections and it is commonly difficult to eradicate with conventional antibiotic therapy, particularly when established as biofilms.[6] Although *P. aeruginosa* rarely infects healthy people, those individuals whose skin, mucous membranes, or immune system are affected, are more susceptible to becoming infected by this organism; for example, burn victims, patients with cystic fibrosis, or cancer patients treated with chemotherapy.[7]

Biofilms are surface-associated bacterial communities embedded in an extracellular matrix that is considered to be a major problem in the context of chronic infections because biofilm-dwelling cells have increased antibiotic resistance compared to their planktonic counterparts.[8] The critical roles of the matrix for microbial interactions and virulence, as well as for antimicrobial tolerance, are being increasingly recognized. The matrix production enhances bacterial cell adhesion and cohesion (resulting in densely packed cell aggregates), providing mechanical stability.[9]

Extracellular deoxyribonucleic acid (eDNA) is widely recognized as an integral component of biofilms' extracellular polymeric matrix (ECM). Many studies mentioned that eDNA plays a pivotal role in bacterial biofilm formation. The involvement of eDNA in biofilms includes providing nutrition and energy for sessile cells promoting horizontal gene transfer (HGT) in naturally competent cells or maintaining the biofilm integrity.[10] While others have proved that eDNA could destabilize the biofilm formation process and that effect would depend on the bacterial species or its serotypes.[11]

Upon the aforementioned facts, this study aimed at 1) investigating the effect of increasing concentration of eDNA on biofilm formation and 2) inspecting the transferring possibility of the antibiotic-resistant gene from eDNA to bacterial cell within the biofilm.

Materials and Methods

Ethical Statement

This work is approved by the College of Science Research Ethics Committee (ref. CSEC/1220/0081). All participants agreed to provide the investigator with the specimens. Informed consent according to the Declaration of Helsinki was obtained from all participants.

Specimen Collection

A total of 170 different non-repeated specimens were collected from patients referring to hospitals in Baghdad, Iraq. These specimens comprised anterior nares swabs ($n = 20$) were taken from healthcare workers as well as the patients, sputum ($n = 30$), mid-stream urine ($n = 95$), burn swabs ($n = 13$), and

blood (n = 12). The specimens were cultured on different selective culture media; Mannitol salt agar, MacConkey agar, Eosin Methylene Blue (EMB) Agar, and Cetrimide agar and subsequently subjected to conventional biochemical tests including Catalase, Oxidase, Coagulase, Acetoin production, IMViC, Motility, and Haemolysin Production Test) to identify *Staphylococcus aureus, E. coli,* and *Pseudomonas aeruginosa, Salmonella enterica* serovar Typhi and *Klebsiella pneumoniae* isolates.[12] All the bacterial isolates were then tested for Ciprofloxacin resistance by measuring the minimal inhibitory concentration for Ciprofloxacin using the Agar diffusion method following the method described by Jennifer.[13]

Polymerase Chain Reaction

Bacterial genomic DNA was extracted using Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan) and all amplifications were carried out using AccuPower® PCR PreMix, and Gradient master cycler (Eppendorf, Germany).

All *S. aureus*-suspected isolates were screened for the presence of the *S. aureus* species-specific *16S rDNA* gene using specific primers, SA1: (AATCTTTGTCGGTACACGATATTCTTCACG) and SA2: (CGTAATGAGATTTCAGTAGATAATACAACA) were used to amplify 108 bp segment of *S. aureus* species-specific 16S rDNA gene. The reaction protocol was as followed: Initial denaturation at 92°C for 3 min followed by 30 cycles of 92°C 1 min, 56°C 1 min, and 72°C 1 min; following that 3 min at 72°C for final extension.[14] *S. aureus* isolates were also screened for methicillin resistance by detecting *mecA* gene using specific primers MecA1: (GTAGAAATGACTGAACGTCCGATAA) and MecA2: (CCAATTCCACATTGTTTCGGT); The reaction condition included initial denaturation at 94°C for 10 min followed by 10 cycles of 94°C 45 sec, 55°C 45 sec and 72°C 75 sec; followed by 25 cycles of 94°C 45 sec, 50°C 45 sec and 72°C 75 sec.[15]

Escherichia coli-suspected isolates were also screened for the presence of the uspA gene by the same technique employing specific primers uspA-F (CCGATACGCTGCCAATCAGT) and uspA-R (ACGCAGACCGTAGGCCAGAT), the conditions were: Initial denaturation at 95°C for 5 min followed by 30 cycles of 94°C 30 sec, 56°C 30 sec and 72°C 30 sec; following that 5 min at 72°C for final extension.[16]

Two Ciprofloxacin-resistant *S. aureus* isolates were selected to detect any possible mutation in *gyrA* and *parC* coding for DNA gyrase subunit A and DNA topoisomerase IV, respectively using the specific primers. GyrA-F: AAATCTGCCCGTGTCGTTGGT and GyrA-R GCCATACCTACGGCGATACC for *gyrA;* ParC-F: GTATGCGATGTCTGAACT and ParC-R TTCGGTGTAACGCATTGC for *parC.* The amplification program involved initial denaturation at 95°C for 2 min followed by 35 cycles of 95°C 30 sec, 55.4°C 60 sec, and 72°C 60 sec.[17]

The sequences of the PCR products were obtained using the Sanger method and then were aligned with gene sequences from National Center for Biotechnological Information (NCBI) (https://www.ncbi.nlm.nih.gov/) to investigate for mutations.

All Ciprofloxacin resistant isolates were screened for the presence of *acrA* gene coding for acrAB efflux pump. The primers that were used are AcrA-F: (ATGAACAAAAACAGAGG) and AcrA-R: (TTTCAACGGCAGTTTTCG) in a PCR reaction program of initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C 1 min, 52°C 1 min and 72°C 1 min followed by 5 min at 72°C for final extension.[18]

Biofilm Formation Assay

Quantification of biofilm formation by *E. coli, S. aureus,* and *P. aeruginosa* on abiotic surfaces was assessed as previously described.[19] In brief; wells of sterile 96-well U-shaped-bottomed polystyrene microplates were filled with 200 µl of an overnight TSB (bacteria concentration was adjusted to in equivalence to McFarland standard no. 0.5) before the plates were covered and incubated aerobically at 37°C for 24 h. Each bacterium was tested in triplicate. Control wells were performed by adding bacteria-free TSB. The wells were aspirated and washed three times with 200 µl sterile phosphate-buffered saline (PBS); the remaining attached bacteria were fixed with 200 µl methanol for 15 min. After drying in air, the wells were stained with 200 µl 0.1% crystal violet solution for 15 min at room temperature. The excess stain was rinsed off by placing the plate under running tap water. Thereafter, the plates were dried. Subsequently, the adherent cells were resolubilized with 200 µl of 33% glacial acetic acid for 15 minutes. Finally, the optical density (OD) of each well was obtained at 600 nm using a microplate reader (Biotek, UK). Cut off value (ODc) was calculated as the mean of OD of control wells plus 3 standard deviations. The isolates were then interpreted as Non–producer (OD ≤ ODc), weak producer (ODc < OD ≤ 2*ODc), moderate producer (2*ODc < OD ≤ 4*ODc), or strong producer (4*ODc < OD).

To investigate the impact of eDNA concentration on biofilms of *E. coli, S. aureus,* and *P. aeruginosa,* the same protocol described previously was followed; nonetheless, different concentrations (400 ng/µl, 200 ng/µl, 100 ng/µl, and 50 ng/µl as a final concentration) of purified eDNA were added to each well separately. Moreover, 100 µl of TE buffer was added to the control wells instead of purified eDNA. Thereafter, plates were incubated, stained, and quantified as it is mentioned earlier.

Determining the Role of eDNA in Gene Transfer

An aliquot of 100 µl of the bacterial growth (compatible with McFarland standard no. 0.5) of Ciprofloxacin sensitive isolates of *E. coli* (E4), *S. aureus* (S4) and *P. aeruginosa* (P1) was added to wells of sterile 6-well U shaped-bottomed polystyrene microplates; thereafter, three ml of sterile tryptic soy broth were added to each well. A volume of one ml of eDNA (400 ng/µl) extracted from Ciprofloxacin-resistant isolate (*S. aureus* isolate S17) was added to each well. All plates were covered and incubated at 37°C for 24 h. then washed thrice with sterile PBS. Biofilms were removed from each well by scraping, suspended in a sterile broth medium, and incubated at 37°C for 18 h. The minimal inhibitory concentration to Ciprofloxacin was determined and further investigation was carried out using PCR technique for *gyrA*, *parC,* and *acrA* genes as it is mentioned previously, followed by sequencing of amplified products.

Statistical Analysis

Biofilm data were analyzed using two-way ANOVA followed by $LSD_{0.05}$. The differences were considered significant when $P < 0.05$.

Results and Discussion

identification results revealed that 25, 24, and 2 isolates were identified as *S. aureus, E. coli,* and *P. aeruginosa*, respectively. Furthermore, all *S. aureus* isolates were found to be methicillin-resistant due to harboring the *mecA* gene.

The polymerase chain reaction was also employed to detect the presence of acrAB efflux pump using primers that are specific for *acrA* gene encoding for this pump in all Ciprofloxacin-resistant isolates (two *S. aureus* & 13 *E. coli* isolates). The result revealed the presence of a single gene with 495 bp in all of these isolates. The present results are in line with those obtained by Pakzad et al.[18] in that all the resistant isolates harbored the *acrA* gene. On the other hand, these results differ considerably from those reported by the same authors as they reported that not all Ciprofloxacin-sensitive isolates contained this gene.

Detection of gyrA and parC Mutations

Ciprofloxacin-resistant *S. aureus* isolates (S17 and S18) were carefully chosen to be investigated for mutations in *gyrA* and *parC* genes. Two specific sets of primers were used to amplify *gyrA* and *parC* genes in separate PCR reaction tubes; after electrophoresis of the products and illumination under UV light, specific bands were obtained at 344 and 230 bp for *gyrA* and *parC*, respectively. Such results were expected as these genes are considered to be part of the structural genes of the bacterial cell.

The sequences of the PCR product of the isolate S17 were obtained and compared to sequences of *gyrA* and *parC* genes from NCBI; as illustrated in Table 1 and Table 2, about 40 and 51 mutations in the forward and reverse strands, respectively, were detected in *gyrA* of the tested isolate; since *gyrA* encodes for DNA gyrase, these mutations while leading to amino acid substitutions, alter the target protein for fluoroquinolone structure and subsequently the fluoroquinolone binding affinity of the enzyme, leading to drug resistance.[20] On the other hand; after comparing the obtained sequence of *parC* from the tested isolate with sequences from NCBI, the result revealed complete similarity, and no mutations were recorded.

In plain words, resistance to Ciprofloxacin in the tested isolates is due to a mutation in gyrase rather than topoisomerase IV.

Table 1. **List of mutations in *gyrA* forward strand**

No.	Mutation	Type	No.	Mutation	Type
1	G→C	Transversion	21	A→C	Transversion
2	T→C	Transition	22	C→T	Transition
3	T→G	Transversion	23	A→C	Transversion
4	T→A	Transversion	24	C→T	Transition
5	G→-	Deletion	25	T→A	Transversion
6	A→C	Transversion	26	C→T	Transition
7	G→A	Transition	27	G→A	Transition
8	A→T	Transversion	28	T→C	Transition
9	A→C	Transversion	29	A→G	Transition
10	T→A	Transversion	30	A→T	Transversion
11	A→C	Transversion	31	A→C	Transversion
12	G→A	Transition	32	C→A	Transversion
13	C→T	Transition	33	A→C	Transversion
14	A→C	Transversion	34	T→G	Transversion
15	A→T	Transversion	35	C→A	Transversion
16	A→C	Transversion	36	G→T	Transversion
17	G→T	Transversion	37	A→T	Transversion
18	C→A	Transversion	38	C→T	Transition
19	G→A	Transition	39	C→T	Transition
20	A→T	Transversion	40	G→C	Transversion

Table 2. **List of mutations in *gyrA* reverse strand**

No	Mutation	Type	No	Mutation	Type
1	A→–	Deletion	27	T→G	Transversion
2	T→–	Deletion	28	A→G	Transition
3	C→A	Transversion	29	G→A	Transition
4	G→T	Transversion	30	A→G	Transition
5	A→C	Transversion	31	C→T	Transition
6	G→T	Transversion	32	C→T	Transition
7	T→G	Transversion	33	G→A	Transition
8	T→A	Transversion	34	A→T	Transversion
9	A→G	Transition	35	T→G	Transversion
10	T→C	Transition	36	C→T	Transition
11	A→G	Transition	37	A→T	Transversion
12	C→T	Transition	38	A→T	Transversion
13	G→A	Transition	39	G→T	Transversion
14	A→T	Transversion	40	T→G	Transversion
15	G→A	Transition	41	T→G	Transversion
16	T→C	Transition	42	T→A	Transversion
17	G→A	Transition	43	A→T	Transversion
18	T→C	Transition	44	G→A	Transition
19	A→C	Transversion	45	A→T	Transversion
20	C→T	Transition	46	A→G	Transition
21	G→T	Transversion	47	G→A	Transition
22	T→G	Transversion	48	T→C	Transition
23	T→G	Transversion	49	T→G	Transversion
24	T→A	Transversion	50	T→A	Transversion
25	T→G	Transversion	51	G→A	Transition
26	G→A	Transition			

Biofilm Formation Assay

The Microtiter plate assay is the most widely used and was considered a standard test for the detection of biofilm formation. This method has been reported to be the most sensitive, accurate, and reproducible screening method for the determination of biofilm production by clinical isolates of *S. aureus*, *E. coli*, and *P. aeruginosa* and has the advantage of being a quantitative tool for comparing the adherence of different strains.[21]

The result revealed that only 8% of *S. aureus* isolates were strong biofilm producers; while 60% and 32% of the isolates were moderate and weak producers, respectively. On the other hand, none of the tested *E. coli* isolates were strong biofilm producers; whereas 68% and 32% of the isolates were moderate and weak producers, respectively.

Similar trends have been reported by Mohammed et al.[22] in that 14% of their local *S. aureus* isolates were strong biofilm-producers, 43% were low biofilm intensity and 43% were biofilm-negative. Mathur et al.[21] similarly conclude from their data that about 14.47% and 39.4% of *S. aureus* isolates exhibited high and moderate biofilm formation, respectively; while 46% were weak isolates. These data are not consistent with those reported by Saeed et al.[23] who stated that about 12.5% of isolated local strains of *E. coli* were strong biofilm producers while it agreed partially with their findings in that 87.5% of *E. coli* were moderate biofilm producers. It also disagrees greatly with Fattahi et al.[24] who found 38% of *E. coli* isolates were strong biofilm producers while 22%, 32%, and 8% of the isolates were moderate, weak, and non-biofilm producers respectively. The results are generally consistent with the findings of Ghafil[25] in that the ability of *S. aureus* to form biofilm was higher than that of *E. coli*.

S. aureus biofilms, once established, are recalcitrant to antimicrobial treatment and the host response, and therefore are the etiological agent of many recurrent infections that have a demonstrated biofilm component.[26] Chronic infections are associated with the biofilm mode of growth where *S. aureus* can attach and persist on host tissues, such as bone and heart valves, to cause osteomyelitis and endocarditis respectively, or on implanted materials, such as prosthetic joints,[27] catheters,[28] and pacemakers.[29] Chronic *S. aureus* infections that are associated with biofilm frequently lead to significant increases in both morbidity and mortality, mainly when the infection is associated with indwelling medical devices.[30] Implanted materials become coated with host proteins upon insertion, and the matrix-binding proteins on the surface of *S. aureus* facilitate attachment to these proteins and the development of a biofilm. In cases of infected medical devices, removal of the device is often necessary to treat the infection.[31]

Complications in *E. coli*-related infection have been mainly attributed to biofilm formation. *E. coli* biofilm formation is an intricate process that involves several steps such as initial adhesion, early development, maturation, and dispersion. These steps are governed by many genes that serve specific functions in the formation of the biofilm. *E. coli* biofilm has frequently been resistant to numerous antibiotics, mostly accredited to putative multidrug resistance pumps. The development of the extracellular matrix and the observed increased resistance to common antibiotics create a challenge to control the infections caused by *E. coli* biofilms.[32]

Impact of eDNA Concentration on Biofilm Intensity

To investigate the impact of eDNA on biofilm, Different concentrations of genomic DNA were added to the wells of a microtiter plate containing selected bacterial isolates of the species *E. coli*, *S. aureus, P. aeruginosa, Salmonella enterica* serovar Typhi and *Klebsiella pneumonia.* The result presented in Table 3 revealed that the addition of increasing concentrations

Table 3. **Impact of eDNA on biofilm**

Isolate code	OD_{600}				*P*-value	$LSD_{0.05}$
	Control (No eDNA)	50 ng/µl of eDNA	100 ng/µl of eDNA	200 ng/µl of eDNA		
S1	0.151	0.114	0.108	0.112	0.004000	0.018
S2	0.181	0.152	0.149	0.113	0.000024	0.012
S3	0.193	0.182	0.154	0.167	0.016000	0.020
S4	0.178	0.133	0.143	0.134	0.035000	0.027
S5	0.151	0.150	0.133	0.127	0.160000	–
E1	0.135	0.124	0.122	0.112	0.246066	–
E2	0.131	0.133	0.118	0.109	0.204402	–
E3	0.166	0.178	0.204	0.159	0.221004	–
E4	0.142	0.173	0.164	0.151	0.501460	–
E5	0.148	0.131	0.119	0.099	0.000206	0.011
P1	0.143	0.156	0.220	1.036	0.000001	0.012
P2	0.112	0.111	0.124	0.138	0.000010	0.004
K1	0.097	0.130	0.235	0.751	0.000004	–
K2	0.113	0.171	0.185	0.217	0.000024	–
Se1	0.112	0.134	0.115	0.109	0.006000	0.019
Se2	0.154	0.162	0.142	0.126	0.033009	–
Se3	0.164	0.162	0.159	0.148	0.449729	–
Se4	0.150	0.142	0.127	0.116	0.084083	–
Se5	0.301	0.291	0.289	0.266	0.399151	–

of eDNA resulted in a significant decrease ($P < 0.05$) in biofilm intensity for the majority of the tested *S. aureus* isolates.

S1-S5: S. aureus isolates 1-5; E1-E5: E. coli isolates 1-5; P1 and P2: P. aeruginosa isolate 1 and 2; K1 and K2: *Klebsiella pneumoniae* isolates; Se1-Se5: S. Typhi isolates

Moreover, the biofilm intensity significantly decreased ($P < 0.05$) linearly with increasing concentrations of eDNA; on the other hand, although eDNA addition had led to thinner biofilm in the tested *E. coli* and *S.* Typhi isolates, the increasing concentration did not have a significant effect ($P > 0.05$) on the biofilm intensity; nevertheless, the biofilm of one strain of *E. coli* and two strains of *S.* Typhi was significantly decreased ($P < 0.05$) linearly with increasing eDNA. Surprisingly, the addition of eDNA led to a significant increase ($P < 0.05$) in the biofilm of the tested isolates of *P. aeruginosa* and *K. pneumonia*.

The findings of this study agreed with the findings of Berne et al.[33] who had informed that the biofilm formation of *Caulobacter crescentus* is significantly inhibited by the presence of eDNA.

Those results suggested that the bacteria would probably have a better chance for attachment to abiotic surfaces in the presence of DNase I, hence their ability to form more compact biofilm would increase; additionally, the biofilm formation in *Salmonella* had significantly been inhibited upon the addition of exogenous eDNA. Another study conducted by Özdemir et al.[34] revealed that eDNA could either enhance or decrease the biofilm formation by *Salmonella* and such effect of eDNA would be reliant on *Salmonella* serotype.

Other studies that were conducted on the biofilm of *Listeria monocytogenes* and *Neisseria meningitides* come in contrast to our findings in which the biofilm formation had not been significantly affected by the addition of purified eDNA. However, crude extracts of eDNA in combination with probably some specific proteins or cell wall fragments promote the process of biofilm formation.[35,36]

Due to the interaction of eDNA with one or more of the biofilm components needs further investigation. inhibiting role of eDNA in the biofilm development of either *S. aureus*, *E. coli* or *S.* Typhi from our findings was Another study carried out by Wang et al.[11] demonstrated the inhibitory effect of eDNA on *Salmonella enterica* biofilm who stated that *Salmonella* strains formed a thicker layer of biofilm in the presence of DNase I. of *C. crescentus*, which prevented the cells from settling into and encouraged the dispersal of cells.

Determination of Minimal Inhibitory Concentration (MIC) Using Agar Diffusion Method

The susceptibility of the bacterial isolates (*S. aureus, E. coli,* and *P. aeruginosa*) towards Ciprofloxacin was tested by determining the MIC using the agar diffusion method. From the findings of the present study, various levels of susceptibilities to Ciprofloxacin among isolates were observed. The results are summarized in Figure 1.

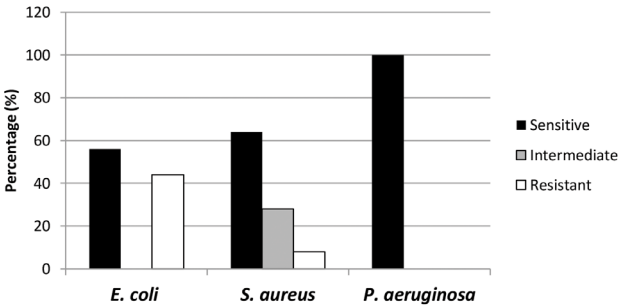

Fig. 1 **Susceptibility of bacterial isolates to Ciprofloxacin.**

From Figure 1, it can be noted that only 8% of *S. aureus* isolates were resistant to Ciprofloxacin; whereas 28% developed intermediate resistance and 64% were sensitive to this antibiotic. On the other hand, about 44% of *E. coli* isolates were resistant to Ciprofloxacin while no intermediate resistance was observed among the tested isolates; nevertheless, 56% were sensitive. Regarding *P. aeruginosa*, the two tested isolates were Ciprofloxacin-sensitive.

These findings confirm those of earlier studies, such as Mohamed et al.[37] who found that the resistance of locally isolated *E. coli* strains from Iraqi patients to Ciprofloxacin was about 40.7%. Whereas they differ slightly from those reported by Al-Jebouri and Mdish[38] who found that only 25% of *E. coli* and 40% of *S. aureus* isolates were resistant to Ciprofloxacin. Furthermore, our findings are in good agreement with Al-Marjani et al.[39] who stated that about 16% of *S. aureus* isolates were resistant to Ciprofloxacin.

The increasing resistance of bacteria to Ciprofloxacin could probably be augmented by using it to treat many infections including prostatitis, UTI, endocarditis, gastroenteritis, infections of bones and joints, lower respiratory tract infection, and enteric fever, among others, even though the risk of tendon rupture could increase upon using it. Notably, another factor contributing to the problem is the availability of Ciprofloxacin as an oral suspension that is currently flooding the market; even though, it is not licensed by the FDA to treat children with Ciprofloxacin due to the high risk of permanent injury to the musculoskeletal system except for inhalation anthrax and cystic fibrosis.[40]

Determining the Role of eDNA in Gene Transfer

This experiment was designed to assess the possible role of eDNA in the transfer of antibiotic-resistance genes. Since the addition of eDNA has increased the biofilm intensity of *P. aeruginosa* isolate P1 only, our study was focused on that isolate. The MIC of Ciprofloxacin was measured before and after the growth of the sensitive isolate in the presence of the DNA of Ciprofloxacin-resistant isolate (*S. aureus* isolate S17), the result revealed that the MIC value increased significantly ($P < 0.05$) from 1 to 4 µg/ml turning the bacterial isolate from sensitive to resistant to Ciprofloxacin. The acquired resistance was also tested after three successive generations and it was shown that the MIC value remained at 4 µg/ml. The same experiment was repeated using Ciprofloxacin-sensitive *E. coli* E4 and *S. aureus* S4 isolates as a recipient for gene transfer. Nonetheless, when measuring the MIC values before and after the gene transfer, it remained at 1 µg/ml; hence, the isolates

remained sensitive to Ciprofloxacin; consequently, no gene transfer occurred. The sequence analysis of the *gyrA* gene for the isolate before and after the addition of eDNA revealed slight variation, which furthermore confirms that the gene transfer process might have occurred and eDNA was responsible for that process. Correspondingly, no PCR product was obtained when trying to amplify the *acrA* gene after the gene transfer which implies that the *acrA* gene has not been transferred during the process.

The pool of eDNA found in bacterial biofilms provides a rich substrate for naturally occurring genetic transformation, which is the only alternative to mobile genetic elements and bacteriophage-induced gene transfer. This observation led to investigations into the role of DNA donor cells in biofilms and the conclusion that biofilm cells actively donate DNA to their prokaryotic neighbors.[41] Extracellular DNA active in the natural transformation was shown to be released by both Gram-positive and Gram-negative members of soil bacteria, thereby facilitating naturally occurring genetic transformation. Natural habitats suitable for horizontal gene transfer are not limited to the soil. The majority of bacterial populations on earth are accompanied by eDNA, and it is known that such eDNA is suitable for horizontal gene transfer.[42] Furthermore, it is well established that gene transfer occurs with enhanced efficiency in biofilms.[43-46] Such horizontal gene transfer is facilitated by a biofilm lifestyle, which is characterized by cohabitation in close vicinity. This sharing of genetic material may function as the prokaryotic equivalent to sexual selection,[47] leading to beneficial adaptations such as antibiotic resistance[48] or pathogenicity.[49]

Conclusion

Extracellular DNA has a major role in the gene transfer process to biofilms. Given that, the addition of increasing concentrations of eDNA resulted in a significant decrease ($P < 0.05$) in biofilm intensity for the majority of the tested *S. aureus* and *E. coli* isolates. Whereas, it has led to a significant increase ($P < 0.05$) in the biofilm of the two tested *P. aeruginosa* isolates.

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Conflict of Interest

"The authors declare no conflict of interest".

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