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Research Article

Sub-inhibitory doses of Ofloxacin reduce adhesion and biofilm formation of *Pseudomonas aeruginosa* to biotic and abiotic surfaces

Lubna Ali Abd Al-mutalib, Ayaid Khadem Zgair*

Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

ABSTRACT

Biofilm formation of *Pseudomonas aeruginosa* (*P. aeruginosa*) and its adherence increases bacterial virulence to cause the infection. The role of treating *P. aeruginosa* with sub-inhibitory concentrations of ofloxacin *in vitro* in reducing bacterial adherence to biotic and abiotic surfaces was evaluated. Ten isolates of *P. aeruginosa* were isolated from urine samples. Biofilm formation on polystyrene microtiter plates and Minimum inhibitory concentrations (MICs) of ofloxacin against all isolates were evaluated. The effect of sub-MICs of ofloxacin (0.5×MIC, 0.25×MIC, 0.125×MIC, and 0.06×MIX) on biofilm formation (to polystyrene) and adhesion to prepared human epithelial cells (HECs) *in vitro* was evaluated. The MICs of ofloxacin were lower than 64 µg/ml and all isolates produced biofilm. There was no relationship between the susceptibility of bacterial isolates to ofloxacin and biofilm formation (r:-0.11; P>0.05). It was found that all sub-MIC concentrations of ofloxacin reduced significantly the biofilm formation on polystyrene and adhesion to HECs in a concentration-dependent manner. Electron microscope images showed that the sub-MIC concentrations of ofloxacin prevent biofilm development to maturation. It can be concluded the negative effect of sub-MIC concentrations of ofloxacin on biofilm production and adhesion to biotic and abiotic surfaces *in vitro*.

Keywords:
Adhesion, Biofilm, Ofloxacin, Sub-inhibitory concentrations

1. INTRODUCTION

The majority of morbidity and mortality related to different infections are caused by *Pseudomonas aeruginosa*. Persistent *P. aeruginosa* infection leads to the development of a distinctive phenotype that is surrounded by an excessive amount of the polysaccharide known as alginate. This allows the bacteria to survive within the infected area[1-3]. One of the virulence factors of *P. aeruginosa* is the adherence to different surfaces[4]. The adhesion of these bacteria to medical devices such as urinary catheter leads to health complications for patients because the formation of a biofilm increases the resistance of these bacteria to antibiotics[5].

Antibiotics are the most important strategy for treating bacterial infections, and increasing antibiotic resistance represents the biggest challenge in treating bacterial infectious diseases[6]. The use of antibiotics not only for killing bacteria, but many experiments have proven that the use of sub-lethal concentrations of antibiotics will contribute to reducing the adherence of different bacterial species to the surfaces, which will contribute significantly to reducing the virulence of bacteria to cause the infection[7-8].

A quinolone antibiotic called ofloxacin is effective in treating a variety of bacterial infections, such as urinary tract infections. It is a wide-spectrum antibiotic against different bacteria including Enterobacteriaceae and *P. aeruginosa*[9]. Previous studies highlighted the role of sub-inhibitory concentrations of ofloxacin in reducing the biofilm formation of *P. aeruginosa* to abiotic surfaces, but no previous studies showed the role of these concentrations on the adhesion of *P. aeruginosa* to epithelial cells (biotic surfaces)[10-11].

***Corresponding author:**
*Ayaid Khadem Zgair Email: ayaid.zgair@sc.uobaghdad.edu.iq

Therefore, the current study has contributed to filling the knowledge gap related to the effect of the sub-inhibitory concentration of ofloxacin on the adhesion of *P. aeruginosa* to human epithelial cells (HECs) as a model of biotic surface and contributing to confirming the published information about the effect of these concentrations on biofilm formation of *P. aeruginosa* to abiotic surfaces (polystyrene).

2. MATERIALS AND METHODS

2.1. *P. aeruginosa* isolation and identification

Hundred and ten urine samples were collected aseptically from patients suffering from urinary tract infections (UTI) (Baghdad Teaching Hospital, Iraq). All individuals have given consent to participate in the study. One milliliter from each urine sample was placed in 9 ml of *Pseudomonas* Asparagine broth enrichment medium (Himedia, India) and incubated for 48 h at 37°C. 100 µl of bacterial growth was re-inculcated onto asparagine agar plates containing (1.5 % agar, Himedia, India) and incubated at 37°C for 18 h[12]. The bacterial species were identified by using VITEK 2 DensiCheck instrument, fluorescence system (bioMe´rieux) (ID-GNB card)[13].

2.2. *Ofloxacin* Minimum inhibition concentrations (MICs)

The standard broth micro-dilution technique of Wiegand et al. (2008) was followed to determine the MICs of Ofloxacin against ten clinical isolates of *P. aeruginosa* (Pa1-Pa10). Briefly, Ofloxacin (Mylan, UK) powder was prepared in a stock concentration of 1mg/ml by dissolving them in sterile distilled water. The suspension of antibiotic was sterile by filtration (Millipore filter 0.2 µ). Double-fold dilutions (150 µl) were prepared in the microtiter plate by sterile Muller Hinton broth (MHB, HiMedia, India) (4, 8, 16, 32, 64, 128, 256, 512 µg/ml). Overnight growth of *P. aeruginosa* isolates was washed three times with sterile phosphate buffer saline (PBS, 0.1 M, pH 7.2), and the number of bacteria was adjusted to 10^6 c.f.u. /ml, then 5 µl of bacterial growth was added to each well. The plates were mixed gently. Three technical controls were made, first, MHB inoculated with bacterial isolates, second, only sterile MHB, and third, different double dilutions of antibiotics. MICs were checked post 18 h of incubation at 37°C[14].

2.3. Effect of Sub-MICs of Ofloxacin on Biofilm Formation

The standard method of Zgair and Chhibber, 2011 was followed. Briefly, two hundred microliters of sterile Tryptic soy broth (TSB) were added to the wells of flat-bottom polystyrene cell culture plates. The overnight growth of *P. aeruginosa* isolates (Pa1-Pa10) was washed three times with sterile PBS (0.1 M, pH 7.2), and the number of bacteria was adjusted to 10^6 c.f.u. /ml, then 5 µl of these bacterial suspensions were added to each well. The plates and incubated at 37°C for 18 h. The media were dispensed, and non-adherent bacterial cells were removed by washing them five times with distilled water. The quantity of biofilm formation was measured by the spectrophotometric method. In this method, the biomass of biofilm of different bacterial isolates was dried and fixed by incubating at 60°C for 30 min. Then, 250 µl of Hucker crystal violet (0.4%) was added to each well and incubated for 5 min at 21°C. After washing the plates five times with distilled water and drying them for 30 min at 37°C., 250 µl of acetone:ethanol (30:70) was added to each well. The absorbance of each well was read at a wavelength of 570 nm using BioTek 800 microplate reader (USA)[15].

The effect of sub-MICs of antibiotics on the biofilm formation of the *P. aeruginosa* isolate that produces the highest level of biofilm was evaluated using similar method of biofilm formation was followed but instead of TSB, double fold dilutions of sub-MICs (prepared in TSB) of ofloxacin were used (0.5×MIC, 0.25×MIC, 0.125×MIC, and 0.06×MIC). The tests were performed in triplicate and that were for each isolates and their positive control (bacterial isolates without antibiotic) and negative control (antibiotic without bacteria). The decreasing in the biofilm formation in comparing with control (biofilm production without ofloxacin) referring to the effect of sub-MIC of ofloxacin on biofilm formation.

2.4. Effect of sub-MICs of Ofloxacin on adhesion of *P. aeruginosa* to HECs

The standard method of Ali and Zgair, 2022 was followed to prepare HECs *in vitro*[16]. The method of Zgair and Chhibber[15] was followed to perform the adhesion of clinical isolates of *P. aeruginosa* that produced the highest level of biofilm to HECs *in vitro*. Briefly, in each well of 24 well microtiter plate (Thermo Scientific™, USA), 1×10^5 HECs were suspended in Dulbecco's modified Eagle's medium (D-MEM) containing 10% fetal calf serum, 10 mM L-glutamine and 100 µl of *P. aeruginosa* (5×10^7 c.f.u./ml). The plates were washed three times with PBS (0.1 M, pH, 7.2) after incubating for one hour at 37°C. The HECs were lysed with PBS-0.5% Triton×100 (Sigma-Aldrich), diluted tenfold, and plated on nutrient agar to count the adhered bacteria.

To study the effect of different concentrations of ofloxacin (0.5×MIC, 0.25×MIC, 0.125×MIC, and 0.06×MIC) on the adhesion of *P. aeruginosa* (produced the highest level of biofilm) to HECs. The colonies of bacteria were grown onto Mueller-Hinton agar (MHA) and suspended in TSB. The standard number of bacteria (1×10^7 c.f.u./ml)

was prepared in Mueller-Hinton broth and treated for 18 h at 37°C with the different concentrations of ofloxacin (0.5×MIC, 0.25×MIC, 0.125×MIC, and 0.06×MIC). The bacteria suspensions were washed 3 times with PBS (0.1 M, pH, 7.2) by centrifugation at 10,000 g for 5 min. The bacterial cells were re-suspended in TSB to check the ability of treated bacteria with ofloxacin to adhere to HECs. The untreated bacteria with ofloxacin was considered a control.

2.5. Scanning Electron Microscope

The method of Gomes LC, Mergulhão, (2017) was followed to stain the biofilm smear on the glass slides before treating with a sub-inhibitory concentration of antibiotic (0.25×MIX of ofloxacin) and after treating with ofloxacin to examine the effect of a sub-MICs concentration of ofloxacin on biofilm production under the scanning electron microscope (Axi ChemiSEM-ThermoFisher Scientific)[17].

2.6. Statistical analysis

The statistical analysis and graphs were done by using Origin 8 software. The data were expressed as means±SE. The differences were evaluated by using ANOVA test. The relationship between biofilm formation in terms of optical density at 570 nm and ofloxacin susceptibility in terms of MIC was calculated by using Pearson's correlation coefficient and a value of $P<0.05$ was considered to be statistically significant.

3. RESULTS

3.1. Isolation and Identification

In the current study, ten isolates of *P. aeruginosa* were isolated from 110 urine samples collected from patients suffering from UTIs. Gram stain and biochemical tests were used to identify the bacterial species. The VITIK 2 Technology proved that the ten isolates were *P. aeruginosa*.

3.2. Ofloxacin MICs

In the current study, the MIC of ofloxacin against ten clinical isolates of *P. aeruginosa* was measured. The highest MICs of ofloxacin were seen against Pa1 and Pa6 (64 µg/ml), while the lowest MICs of ofloxacin were seen against Pa 2 and Pa9 (16 µg/ml) (Figure 1a).

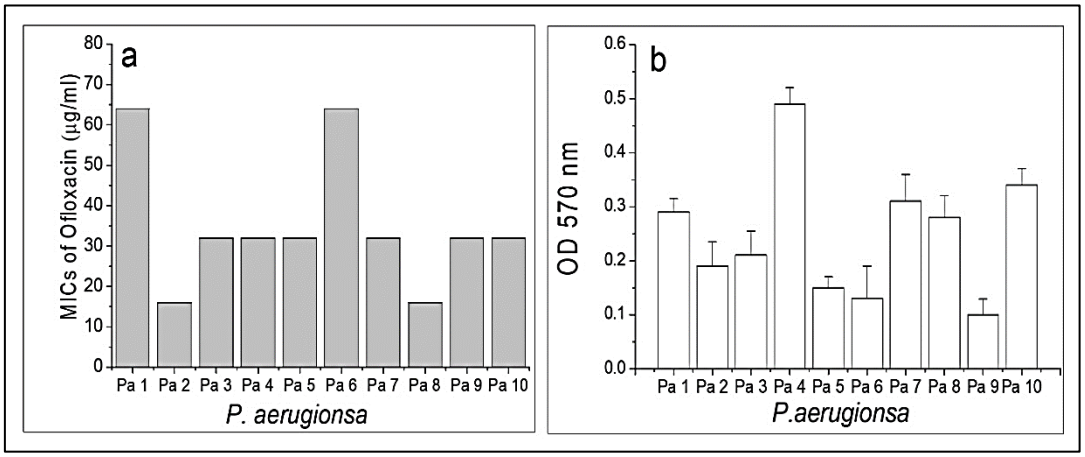

Figure 1. a, minimum inhibitory concentration of ofloxacin against three clinical isolates of *P. aeruginosa* (Pa1, Pa2, Pa3, Pa4, Pa5, Pa6, Pa7, Pa8, Pa9, and Pa10) that were isolated from the urine samples collected from patients suffering from UTI. b, Biofilm formation of the ten clinical isolates of *P. aeruginosa* to polystyrene microtiter plates.

3.3. Biofilm formation

Figure 1b shows the biofilm production of the ten *P. aeruginosa* isolates. The highest level of biofilm was produced by Pa4 (OD, 0.49) followed by Pa10 (OD, 0.34), while the lowest biofilm mass was formed by Pa 9 (OD, 0.1). The present study showed that all isolates have the ability to produce biofilm but the isolate Pa4 has the highest ability to form a biofilm mass thus, this isolate will be used for further experiments to evaluate the effect of sub-inhibitory concentration of ofloxacin in decreasing the adhesion and biofilm formation to biotic and abiotic surfaces *in vitro*.

3.4. The relationship between biofilm and ofloxacin susceptibility

There is no relation between the MICs values of ofloxacin against 10 *P. aeruginosa* isolates and biofilm formation of the same isolates of *P. aeruginosa* (Figure 2). The current study proved that there is no effect of biofilm formation of *P. aeruginosa* and the susceptibility of these bacterial isolates to ofloxacin.

3.5. Effect of sub-MICs of ofloxacin on biofilm

It was found that all the concentrations of ofloxacin

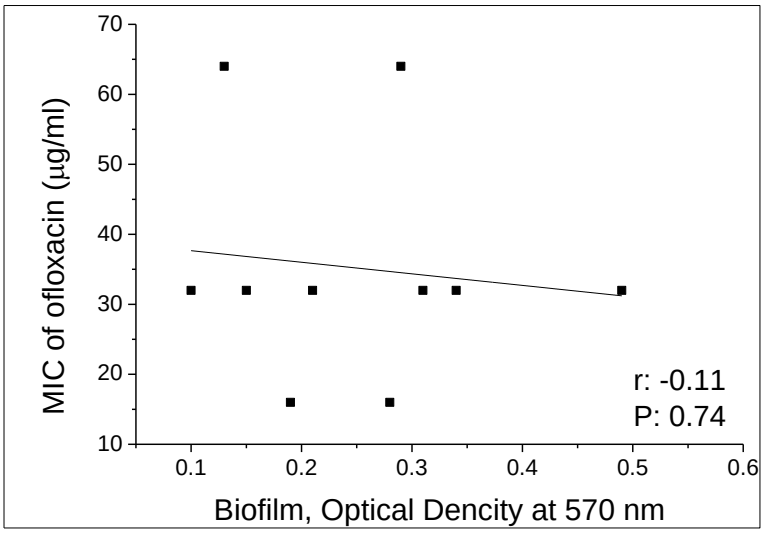

Figure 2. Relationship between MICs of ofloxacin against 10 clinical isolates of *P. aeruginosa* (Pa1, Pa2, Pa3, Pa4, Pa5, Pa6, Pa7, Pa8, Pa9, and Pa10) and biofilm formation of the same isolates of *P. aeruginosa* to the polystyrene microtiter plate. *P*>0.05.

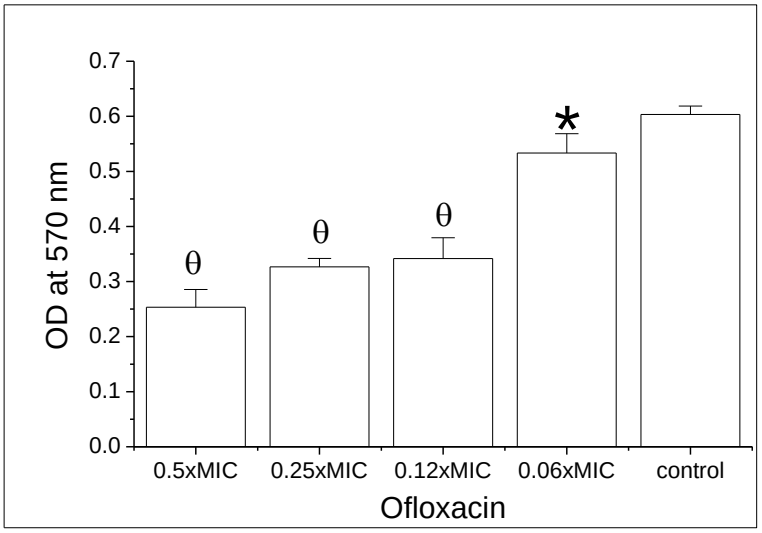

Figure 3. The effect of sub-inhibitory concentrations of ofloxacin (0.5×MIC, 0.25×MIC, 0.12×MIC, and 0.06×MIC) on biofilm formation by *P. aeruginosa* (Pa4). All concentrations of ofloxacin reduced the biofilm formation by Pa4 signifi-cantly. MIC, minimum inhibitory concentration; **P*<0.05, Θ, *P*<0.001.

(0.5×MIC, 0.25×MIC, 0.125×MIC, and 0.06×MIC) reduced the biofilm of Pa4 to polystyrene microtiter plates significantly (*P*<0.05) and the decrease in biofilm formation was in ofloxacin concentrations dependent manner. Whereas, the highest decrease in biofilm formation was observed when treating bacteria with 0.5×MIC of ofloxacin, while the lowest decrease in biofilm formation was found when treating bacteria with 0.06×MIC of ofloxacin (Figure 3).

The electron microscope images of the biofilm formation by Pa4 to polystyrene plates with and without treatment with ofloxacin (0.25×MIC) was shown in Figure 4. The images of biofilm formed by Pa4 (non-treated with 0.25×MIC of ofloxacin) showed the mature form of biofilm. Where the biofilm clumps formed by Pa4 can be observed protruding from the surface (polystyrene), forming mushroom-like shapes (Figure 4a and Figure 4b). The two images (Figure 3c and Figure 3d) show the formation of the biofilm by Pa4 when treated to 0.25×MIC of ofloxacin. Where the image shows the poor formation of the biofilm, which is characterized by low quantity, in addition to not protruding biofilm from the surface. This indicates that the treatment of *P. aeruginosa* with 0.25×MIC of ofloxacin contributed to decreasing the formation of biofilm and preventing biofilm from reaching to mature stage.

3.6. Effect of sub-MICs of ofloxacin on adhesion to HECs

In the current study, epithelial cells isolated from the human mouth were used as a model for biotic surfaces that were used to evaluate the adhesion of *P. aeruginosa* (Pa4) to biotic surfaces (HECs) and to estimate the role

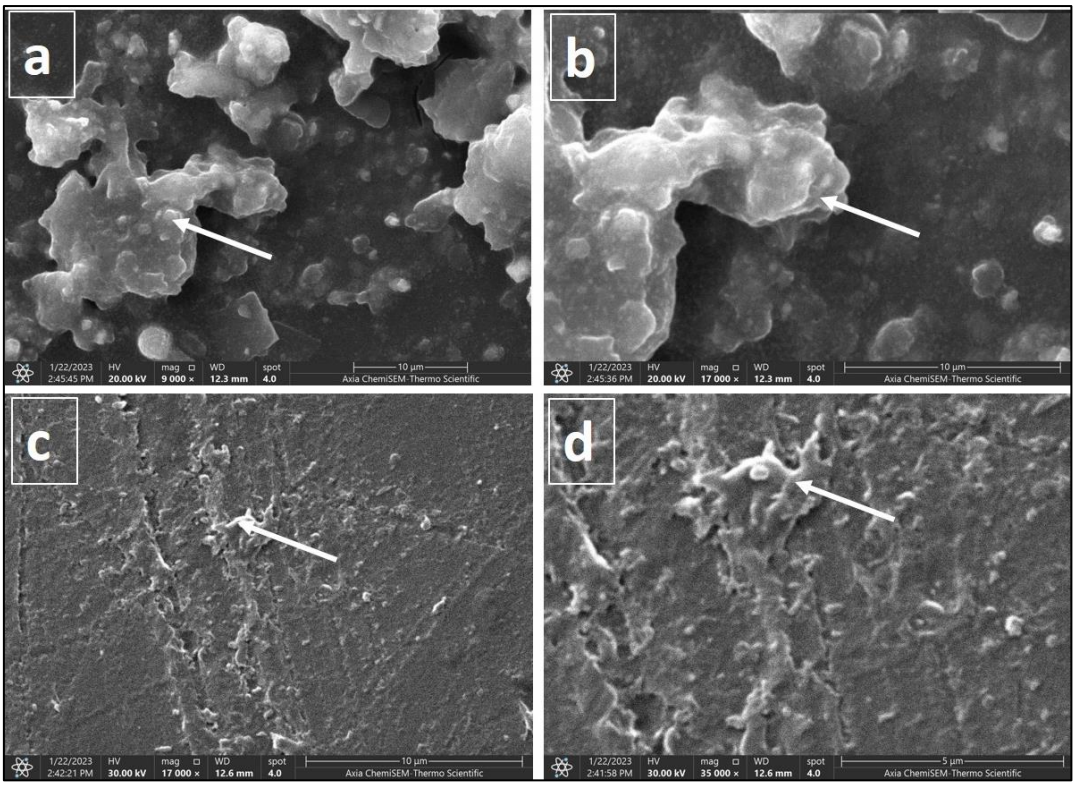

Figure 4. Scanning electron microscopy image of *P. aeruginosa* (Pa4) 36-h biofilm before treating with ofloxacin (a & b). c &d, SEM image of Pa4 36-h biofilm after treating with MICx0.25 ofloxacin.

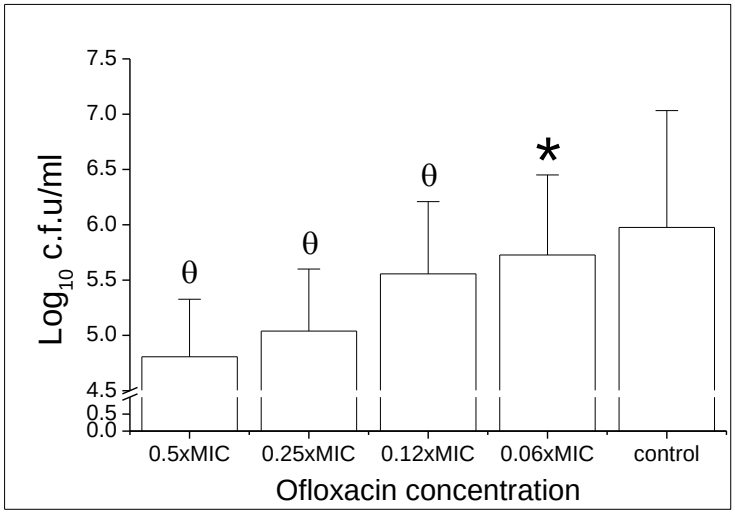

Figure 5. The effect of sub-inhibitory concentrations of ofloxacin (0.5×MIC, 0.25×MIC, 0.12×MIC, and 0.06×MIC) on *P. aeruginosa* (Pa4) adhesion to human epithelial cells (HECs) *in vitro*. All concentrations of ofloxacin reduced the number of adhered bacteria (c.f.u./ml) to HECs significantly. MIC, minimum inhibitory concentration; **P*<0.01, Θ, *P*<0.001.

of sub-MIC of ofloxacin in adhesion of Pa4 to HECs *in vitro*. It was observed that all the concentrations of ofloxacin (0.5×MIC, 0.25×MIC, 0.125×MIC, and 0.06×MIC) reduced the adhesion of Pa4 to HECs and the decrease in adhesion was in a concentration-dependent manner. Whereas, the highest decrease in bacterial adhesion was observed when treating bacteria with 0.5×MIC of ofloxacin (*P*<0.001), while the lowest decrease in biofilm formation was found when treating bacteria with 0.06× MIC of ofloxacin (*P*<0.01) (Figure 5).

The electron microscope images of the adhesion of Pa4 to HECs with and without ofloxacin (MIC×0.25) was shown in Figure 6. The images (Figure 6a and Figure 6b) show a moderate number of bacteria (treated with 0.25× MIC of ofloxacin) attached to HECs. While a heavy number of bacteria (non-treated with 0.25×MIC of ofloxacin) were seen attached to HECs (Figure 6c). This indicates that the treatment of *P. aeruginosa* with 0.25×MIC of ofloxacin reduces the adhesion of *P. aeruginosa* to biotic surfaces. The other concentrations gave similar results.

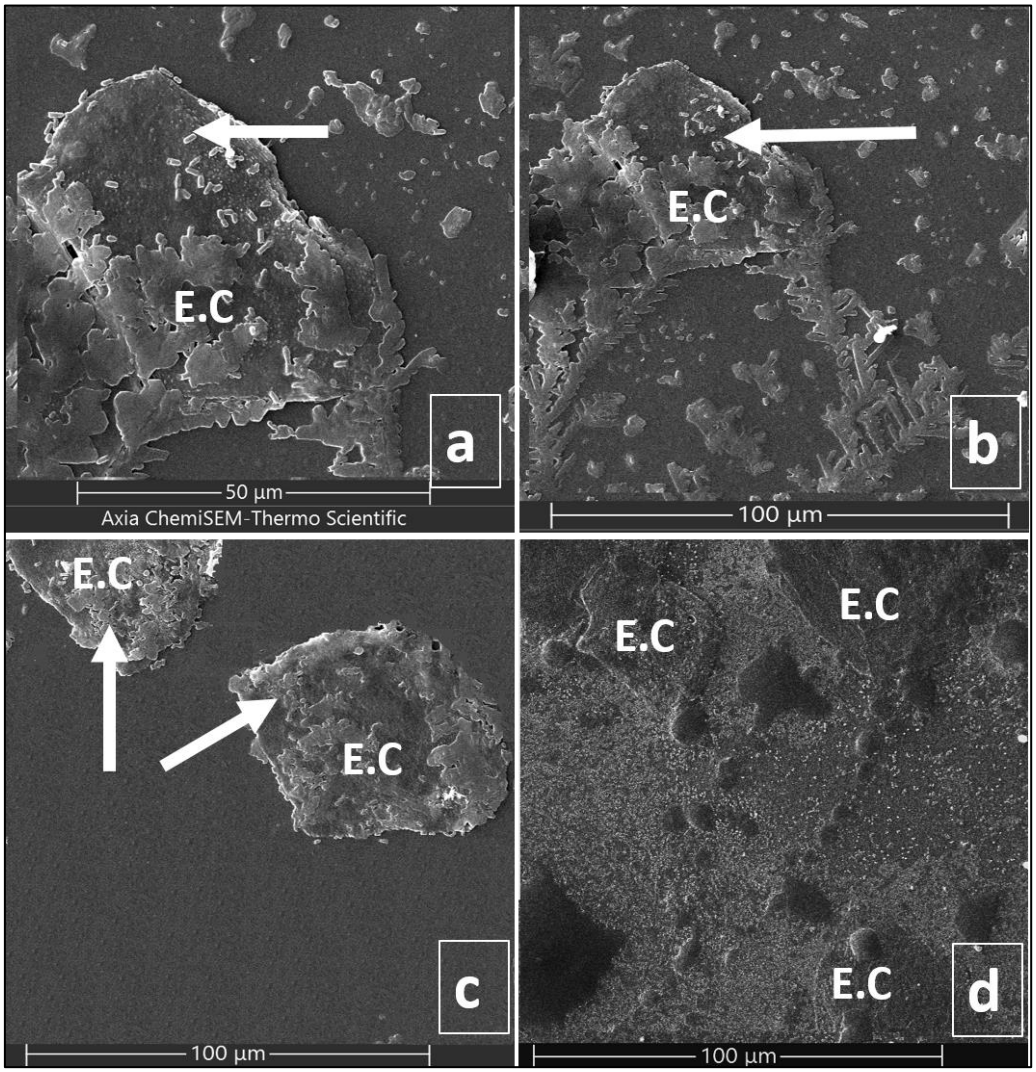

Figure 6. Scanning electron microscopy image of *P. aeruginosa* (Pa4) that adhered to human epithelial cells (HECs) post 2 h of incubation. Images a and b show a moderate number of Pa4 (pretreated with ofloxacin (0.25×MIC) adhered to HECs. Image c, a heavy number of Pa4 (non-pretreated with ofloxacin) adhered to HECs *in vitro* (control). Image d, HECs (non-pretreated with ofloxacin and bacteria). E.C., epithelial cell; white arrow, attached bacteria.

4. DISCUSSION

The formation of biofilms and adherence to host tissues is important for the establishment and persistence of many bacterial infections[18]. Biofilms have a significant role in bacterial virulence by protecting bacteria from host immune defenses and antibiotic treatment[19-20]. Bacterial adhesion is the first step in the establishment of an infection. Once attached, bacteria start to produce and release extracellular polymeric substances (EPS), which form a protective and adhesive matrix that helps to stabilize and reinforce the bacterial community[21]. Bacterial adhesion is the first step to penetrate the host tissue and cause infection[22]. Strategies aimed at disrupting bacterial adhesion and biofilm formation could prevent infectious diseases.

The current study showed that the effect of sub-inhibitory concentrations (sub-MICs) of ofloxacin reduced the biofilm formation of *P. aeruginosa* (Pa4) (isolated from urine and produced the highest level of biofilm *in vitro) in vitro* to polystyrene microtiter plates (abiotic surfaces), this finding was in line with previous studies of Yassien et al. (1995) and Masadeh et al. (2019)[10-11]. Moreover, the present study is the pioneer study showing the effect of sub-MICs of ofloxacin on reducing the *P. aeruginosa* adhesion to HECs *in vitro* significantly. The use of sub-inhibitory (sub-MICs) concentrations of ofloxacin has clearly affected the architectural structure of the biofilm formed by *P. aeruginosa* (Pa4) *in vitro*, it was observed that the use sub-MICs concentrations of ofloxacin restrict the biofilm maturation (Figure 4).

Bacteria within the biofilm (especially in the maturation stage) can become increasingly resistant to antibiotics by blocking the penetration of antimicrobial agents, preventing them from reaching the bacterial cells, the EPS can sequester and deactivate antimicrobial agents, and bacteria within the biofilm can enter a dormant state in which they become less metabolically active and therefore less susceptible to antibiotics[23]. Furthermore, biofilms can provide a physical barrier that protects

bacteria from host immune defenses, making it more difficult for the immune system to clear the infection[24]. Overall, the increased resistance of bacteria within biofilms to antibiotics presents a significant challenge in treating bacterial infections[25]. The reduction of the development of biofilm to the mature stage will reduce the chance of the bacteria causing infectious diseases by *P. aeruginosa*. Similarly, reducing bacterial adhesion to epithelial cells by using the sub-MIC concentrations of ofloxacin can be an effective strategy for preventing or reducing the severity of bacterial infections. The synergistic effect of different antibiotics and ofloxacin against *P. aeruginosa* growing in a biofilm was investigated by previous investigation. Kumon et al. (1995) found that the synergestic effect of fosfomycin and ofloxacin against *P. aeruginosa* growing in a biofilm[26]. Shao et al. (2012) found the synergetic effect of phytoanticipin derivative, sodium houttuyfonate and levofloxacin against biofilm formation by *P. aeruginosa*[27]. The strategy of using different materials with ofloxacin may increase the potential effect of the antibiotic against biofilm formation and producing bacterial infection. The synergistic effect of low concentrations of H2O2 and ofloxacin is evaluated in our labs. That is why we recommend using the other materials besides the ofloxacin as a synergistic material to improve the anti-biofilm effect of ofloxacin.

5. CONCLUSION

The current study proved the ability of *P. aeruginosa* to produce biofilm invitro and have high ability to adhere to abiotic surface (polystyrene). It also found that the sub-inhibitory concentrations of ofloxacin reduces the biofilm formation of *P. aeruginosa* into polystyrene and adhesion to biotic surfaces (HECs) *in vitro*.

Conflict of Interest

The authors declare that they have no conflict of interests.

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Ethical approval

The current study was conducted following approval from the human ethical committee of the Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq (Reference No 118, Date:02, Feb. 2022).

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Author contribution

LAAA: Conducted the research experiments and writing the manuscript.
AKZ: Conducted the research experiments, data interpretation, statistical analysis, writing the manuscript and revise the manuscript.

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