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Research Article

Effect of sub-minimum inhibitory concentrations of ceftriaxone on the *Pseudomonas aeruginosa* adhesion to human oral mucosal epithelial cells and biofilm formation to polystyrene *in vitro*

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ABSTRACT

The adhesion of *Pseudomonas aeruginosa* to human oral mucosal epithelial cells (OMECs) and the formation of biofilms on artificial materials are crucial in bacterial oral infections. The role of sub-inhibitory concentrations of ceftriaxone on the *P. aeruginosa* adhesion to human OMECs and biofilm formation onto polystyrene is not clear. The study aims to explore the role of sub-inhibitory ceftriaxone concentrations in *P. aeruginosa* adhesion to human OMECs and biofilm formation to polystyrene. Nineteen isolates of *P. aeruginosa* were obtained from infected wounds. Minimum inhibitory concentrations (MICs) of ceftriaxone against 19 isolates of *P. aeruginosa* (Pa1-Pa19) and biofilm formation were determined for all isolates. Epithelial cells were isolated from the mucous layer of the healthy volunteers' mouths. The effect of pre-treating Pa2, Pa6, and Pa10 (the highest biofilm-forming isolates) with sub-minimum inhibitory concentrations (sub-MICs) of ceftriaxone (0.5xMIC, 0.25xMIC, 0.125xMIC, and 0.06xMIC) on biofilm formation on polystyrene and adhesion to human (OMECs) was evaluated. The ceftriaxone MICs against all *P. aeruginosa* isolates were variable (311.6 ± 437.3 µg/ml, they ranged from 15.62 to 2000 µg/ml). All isolates form variable biofilm amounts (0.35±0.1 OD^{570nm}). All used sub-MICs of ceftriaxone reduced biofilm formation and adhesion to human (OMECs) by Pa2, Pa6, and Pa10 significantly ($P<0.05$) as compared to control (biofilm formation and adhesion of Pa2, Pa6, and Pa10 without antibiotic stress). The effect of sub-MICs on biofilm formation to polystyrene and adhesion to humans (OMECs) was highly variable according to *P. aeruginosa* isolate and concentration of sub-MIC. The study showed that ceftriaxone sub-MICs decrease *P. aeruginosa* biofilm formation to polystyrene and adhesion to human OMECs in isolates isolates-dependent manner and not a concentration-dependent manner.

Keywords:
Adhesion, Biofilm, Ceftriaxone, Human OMECs, MICs, Sub-MICs

1. INTRODUCTION

Gram-negative bacteria especially clinical isolates of *Pseudomonas aeruginosa* are a significant clinical challenge due to the presence of both intrinsic and acquired antibiotic resistance.[1,2] The emergence of antibiotic-resistant gram-negative pathogens, such as *P. aeruginosa*, in hospital-acquired infections has made treatment increasingly difficult, as antibiotic resistance has alarmingly increased in recent years.[3] It is an opportunistic pathogen that can cause infections in various organisms, including plants, invertebrates, and vertebrates.[4] It leads to cause infections in the eyes, ears, toes, and severe burn wounds, especially in immune-compromised patients. Notably, it is recognized as a major pathogen in healthcare settings, particularly in cases of (ventilator-associated) pneumonia and chronic lung infections in cystic fibrosis patients.[5,6]

The development of antibiotic-resistant organisms is driven by the indiscriminate, inappropriate, and excessive use of antibiotics.[7] One of the major challenges in regulating antibiotic use is that the entire population

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receives antibiotics at a specific concentration, often lower than the therapeutic dosage, which may be insufficient to inhibit bacterial growth. This phenomenon has been described as "sub-inhibitory," although "sub-minimum inhibitory concentration (MIC)" may also be applicable as some growth inhibition can still occur.[8,9]

In the absence of new antibiotics becoming available in clinical practice shortly, researchers have begun exploring the use of anti-virulence drugs.[2] A notable consequence of increased resistance to antimicrobials is that bacteria are often exposed to concentrations below their MIC. However, even at sub-inhibitory concentrations, antibiotics are likely to impact bacterial physiology and virulence.[10] The antibiotics reduce biofilm formation and bacterial adhesion to biotic and abiotic surfaces. The extent to which an antibiotic's bactericidal effect is due to its direct target effect or its broader physiological impact has been a subject of considerable debate over the past decade.[11-14]

Human oral mucosal epithelial cells (OMECs) play a crucial role in the interaction with bacteria and the immune system in the oral cavity. Human OMECs form a barrier that separates the underlying tissues from their environment and consist of two layers: the surface stratified squamous epithelium and the deeper lamina propria.[15] Human OMECs have various functions, including, protection from chemical, microbial, and physical challenges, [15] immunological roles,[16,17] autonomous immunity,[18] and influence on periodontal pathogens.[15]

Several previous studies have provided insights into the effects of sub-inhibitory concentrations of antibiotics on the adhesion of *P. aeruginosa* biotic and abiotic surfaces and form biofilms. Gupta, et al. (2016) also found that the sub-inhibitory concentrations of ciprofloxacin reduced the biofilm formation by *P. aeruginosa* on the surfaces.[19] Al-Mutalib and Zgair, (2023) investigated the negative effect of sub-inhibitory concentrations of ofloxacin and rifampicin on biofilm formation and adhesion of *P. aeruginosa* to different types of surfaces *in vitro*.[20,21] A previous study by Hoffman et al. (2005) showed that sub-inhibitory levels of aminoglycosides could induce bacterial biofilm formation by *P. aeruginosa*.[22] Onaolapo and Salami investigated the impact of sub-inhibitory concentrations of ceftriaxone on the adhesion of *P. aeruginosa* to inert surfaces, such as catheters, plastic, and glass, and they found that the ability to adhere varied depending on the bacterial growth phase.[23] However, there is no previous study highlighted the effect of sub-inhibitory concentrations of ceftriaxone on the adhesion of *P. aeruginosa* to human OMECs and biofilm formation onto polystyrene.

The present study aims to highlight the effect of sub-inhibitory concentrations of ceftriaxone on the adhesion of *P. aeruginosa* to human OMECs isolated from human mouth cavities and cultured *in vitro* and the biofilm formation of *P. aeruginosa* to polystyrene microtiter plates.

2. MATERIALS AND METHODS

1.1. *P. aeruginosa* isolation and identification

A hundred wound-infected samples (wound swabs) were obtained aseptically from 100 patients suffering from infected wounds at different sites of the body. The sterile wood swabs (SARSTEDT AG & Co. KG, Germany) were used to sample a representative area of the wound by moving the swab across the wound surface in a zigzag motion, at the same time as rotating it between the fingers. The samples were collected from Baghdad Teaching Hospital, and Al-Yarmouk Teaching Hospital, Baghdad, Iraq. All cohorts had given consent to participate in the study. The collected samples were inoculated immediately onto MacConkey agar (Himedia, India), blood agar (Himedia, India), and cetrimide agar (Himedia, India), and incubated for 48 h at 37ºC. Oxidase and catalase identification test was done on the lactose non-fermenting colonies on McConkey agar and grew on cetrimide agar. The bacterial cells were examined after staining with the gram stain (Himedia, India). The bacterial species were identified by using the VITEK 2 DensiCheck instrument, fluorescence system (bioMe´rieux) (ID-GNB card).[24,25]

1.2. Preparation of bacterial standard inoculum

The nineteen identified isolates of *P. aeruginosa* isolates (Pa1-Pa19) were grown in Nutrient broth (Himedia, India) at 37 ºC (18 h). The bacterial cell pellets were collected by centrifugation (10000 g for 10 min at 4ºC) (Beckman Coulter centrifuges, USA). The collected pellets were washed three times with phosphate buffer saline [PBS (0.01 M, pH 7.2)]. The final bacterial counts were adjusted to 10^8 c.f.u/ml (that corresponded to 0.25 optical density at 600 nm) by either Muller Hinton broth (MHB) (Himedia, India) for the experiment of minimum inhibition concentrations (MICs) measurement or Tryptic Soya broth (TSB) (Himedia, India), for the experiment of bacterial adhesion and biofilm formation.

1.3. Minimum inhibition concentrations (MICs)

The microtiter method which is dependent on the serial dilution of antibiotics into the wells of the microtiter plate was followed to identify the lowest concentration of ceftriaxone that inhibits bacterial growth. In this experiment, the MICs of ceftriaxone were checked against nineteen clinical isolates of

P. aeruginosa (Pa1 to Pa19) that were isolated from infected wounds. Briefly, ceftriaxone (Pharma International Co, Amman, Jordan) powder was prepared in a stock concentration of 1mg/ml by dissolving it in sterile MHB (HiMedia, India) and sterilized using Millipore filter 0.2µ (Biosharp life science, Chana). Double-fold dilutions (150 µl) were prepared in the microtiter plate by sterile MHB (1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8, 3.9 µg/ml). Five microliters of standard inoculum of *P. aeruginosa* isolates (10^7 c.f.u./ml) were added to each well. The plates were mixed gently. In this experiment, three controls were made, the 1st one was 150 µl of MHB inoculated with $5x10^6$ c.f.u./ml of *P. aeruginosa* isolates; the 2nd control was 150 µl of sterile MHB, while the 3rd control was the dilutions of ceftriaxone that corresponding to the sterile dilution of test wells. MICs were checked post 18 h of incubation at 37°C.[20,26]

1.4. Isolation of human oral mucosal epithelial cells (OMECs)

The human OMECs were isolated from four healthy volunteers (2 males and 2 females, with an age range of 35 to 47 years). The samples were isolated from the oral mucosa by gently scraping the inner surface of the mouth using sterile woody sticks. The collected mucus was subsequently rinsed with PBS (0.1 N, pH, 7.2) and supplemented with 600 U/mL penicillin and 600 µg/mL streptomycin (Sigma-Aldrich). The suspension of human OMECs was incubated for 1 h at 37 ºC. The suspension of OMECs was washed five times with PBS (0.1 M, pH 7.2) using a centrifuge (500 g at 4 ºC for 10 min) to remove the antibiotics. Finally, the number of human OMECs was adjusted to 10^5 cells/ml using Dulbecco's modified Eagle's medium (D-MEM) (Sigma-Aldrich) containing 10% fetal calf serum (Sigma-Aldrich), and 10 mM L–glutamine. The trypan blue stain (HiMedia, India) was used to check the number of viable cells. The viability of human OMECs prepared was 91 %.[17,27]

1.5. Adhesion of *P. aeruginosa* to human OMECs

The previous modified methods were followed to measure the number of bacterial cells that adhered to human OMECs *in vitro*.[20, 21] Briefly, 100 µl of $5x10^5$ cells/ml of human OMECs that were suspended in Dulbecco's modified Eagle's medium (D-MEM) containing 10% fetal calf serum, 10 mM L–glutamine, 100 µl of *P. aeruginosa* (10^8 c.f.u./ml, prepared in TSB), and 0.8 µl of sterile MHB were put into endotoxin-free micro-centrifuge tubes (2 ml tube, NEST Scientific USA). The tubes were washed three times with PBS (0.1 M, pH, 7.2) after incubating for one hour at 37°C (to remove non-adherent bacteria). From the final volume of 1 ml, 0.5 ml was lysed with 0.5 ml of PBS–0.5% Triton×100 (Sigma-Aldrich), diluted tenfold and plated on nutrient agar to count the number of adhered bacteria. Another 0.5 ml was added to glass slides to prepare smears and after drying, the smears were stained with Leishman's stain. The slides were examined using a light microscope (CH-Olympus, Japan), and the visual representations were recorded by employing a smartphone camera (Honor90/Pro, China) above the eyepiece of the microscope.[20, 21]

1.6. Effect of ceftriaxone on *P. aeruginosa* adhesion to human OMECs

To investigate the effect of various concentrations of ceftriaxone (0.5xMIC, 0.25xMIC, 0.125xMIC, 0.06xMIC, and 0.03xMIC) on the adhesion of three isolates of *P. aeruginosa* with the highest adhesion rates (from 19 isolates of *P. aeruginosa*) to human OMECs *in vitro*. Two methods were used. First, the viable adhered bacteria to human OMECs were checked using the plate count method. Second, the total adhered bacteria to human OMECs was measured by counting the number of *P. aeruginosa* adhered to each human OMEC (using a light microscope).

In the first method, double-fold dilutions (800 µl) of ceftriaxone (0.5xMIC, 0.25xMIC, 0.125xMIC, and 0.06xMIC) were made by MHB and put into endotoxin-free micro-centrifuge tubes (2 ml tube, NEST Scientific USA). A hundred microliters of standard bacterial inoculum (10^8 c.f.u/ml), and 100 µl of standard inoculum of *P. aeruginosa* (10^8 c.f.u./ml, prepared in TSB) of three isolates that gave the highest rate of adhesion to human OMECs. The tubes were incubated for 18 h at 37 ºC. The human OMECs were washed 3 times with PBS (0.1 M, pH, 7.2) by centrifugation at 1000g for 5 min (to remove non-adherent bacteria). From the final volume of 1 ml, 0.5 ml was lysed with 0.5 ml of PBS–0.5% Triton×100 (Sigma-Aldrich). The serial tenfold dilutions were made with sterile PBS (0.1 m; pH, 7.2) and 100 µl of each dilution was applied using glass spreader onto on nutrient agar to count the number of adhered bacteria. In the second method, another 0.5 ml was used to prepare epithelial cell smears. After drying, the smears were stained with Leishman's stain.[17] The slides were examined under a light microscope (CH-Olympus, Japan), and the visual representations were recorded by employing a smartphone camera above the eyepiece of the microscope. The results were compared with the number of bacteria (untreated with ceftriaxone) that adhered to human OMECs (control). The experiments were done in triplicate.[21]

1.7. Effect of sub-MICs of ceftriaxone on biofilm formation

Two hundred microliters of sterile Tryptic soy broth (TSB) (HiMedia, India) were added into the wells of flat-bottom polystyrene cell culture microtiter plates (Sigma-Aldrich). Five microliters of standard inoculums ($5x10^6$ c.f.u./ml) of *P. aeruginosa* isolates (Pa1- Pa19) were added to each well. The standard inoculums of *P. aeruginosa* were prepared by washing the overnight growth of *P. aeruginosa* (in nutrient broth at 37 °C) three times with sterile phosphate buffer saline (PBS, 0.1 M, pH 7.2), and the number of bacteria was adjusted to $5x10^6$ c.f.u. /ml by sterile TSB. The plates were then incubated at 37°C for 24 h. Subsequently, the media were dispensed, and non-adherent bacterial cells were removed through five washes with sterile distilled water. The biomass of biofilm of different bacterial isolates was dried and fixed at 60 °C for 30 min (Oven, Memmert, Germany). Following this, 250 µl of Hucker crystal violet (0.4%) was added to each well and allowed to incubate for 5 minutes at room temperature. After five additional washes with distilled water and a drying period of 30 minutes at 37°C (Incubator, Memmert, Germany), 250 µl of acetone: ethanol (30:70) was added to each well. The absorbance of each well was measured at a wavelength of 570 nm using the BioTek 800 microplate reader (USA).[21]

To evaluate the impact of sub-MICs of antibiotics on the biofilm formation of the *P. aeruginosa* isolates that exhibited the highest level of biofilm production (Pa2, Pa6, and Pa10), a similar technique used for measuring the biofilm formation was employed. However, instead of TSB, double-fold dilutions of sub-MICs that were prepared in TSB of ceftriaxone (0.5xMIC, 0.25xMIC, 0.125xMIC, 0.06xMIC, and 0.03xMIC) were used. The tests were carried out in triplicate for each isolate. Positive control (bacterial isolates without antibiotic), negative control (antibiotic without bacteria), and technical control of TSB only were used. The change in biofilm formation, in comparison to the control (positive control), was indicative of the impact of sub-MICs of ceftriaxone on biofilm formation.[21]

1.8. Statistical analyses

The statistical analysis was performed and the graphs were created using Origin v. 8 software (OriginLab, Nothampton, USA). The data were expressed as means ± standard error (M ±SE). The differences were evaluated using a Student's t-test and one-way analysis of variance (ANOVA). The relationship was assessed using Pearson's correlation coefficient. A value of $P < 0.05$ was considered statistically significant.

2. RESULTS

2.1. Isolation and identification of *P. aeruginosa*

In the current study, 19 isolates of *P. aeruginosa* were isolated and identified from 100 wound swabs taken from infected wounds of indoor patients. The isolates were identified using phenotypic characteristics of the bacterial cells and their colonies on different selective and differential media. The VITIK technology was also used to confirm the identification of the isolates. The results confirmed the identification of the nineteen isolates of *P. aeruginosa* (Pa1-Pa19)**.**

2.2. MICs of ceftriaxone and biofilm formation

The MICs of ceftriaxone against 19 isolates of *P. aeruginosa* were measured. The highest MIC of ceftriaxone was seen in the case of Pa7 isolate (2000 µg/ml) followed by Pa6, Pa 10, and Pa 18 (500 µg/ml). While the lowest ceftriaxone was seen against Pa17 (15.62 µg/ml). The results showed that the susceptibility of *P. aeruginosa* to ceftriaxone was highly variable (15.6 - 2000 µg/ml; 311.6 ± 437.3 µg/ml) because of the

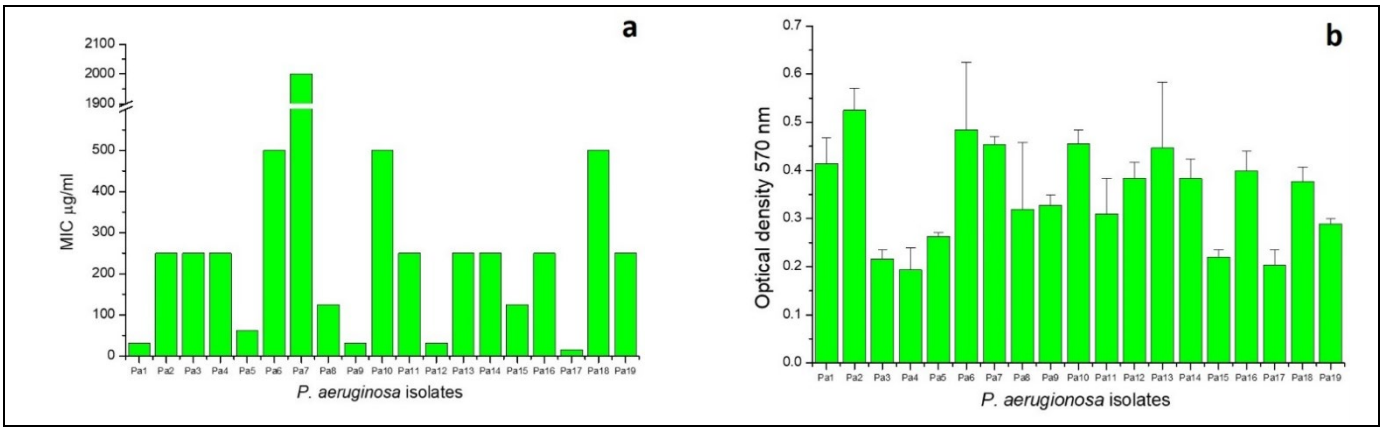

Figure 1. Minimum inhibitory concentrations (MICs) of ceftriaxone against 19 isolates of *P. aeruginosa* (Pa1 to Pa19) isolated from infected wound (a). Biofilm formation of 19 clinical isolates of *P. aeruginosa* (b).

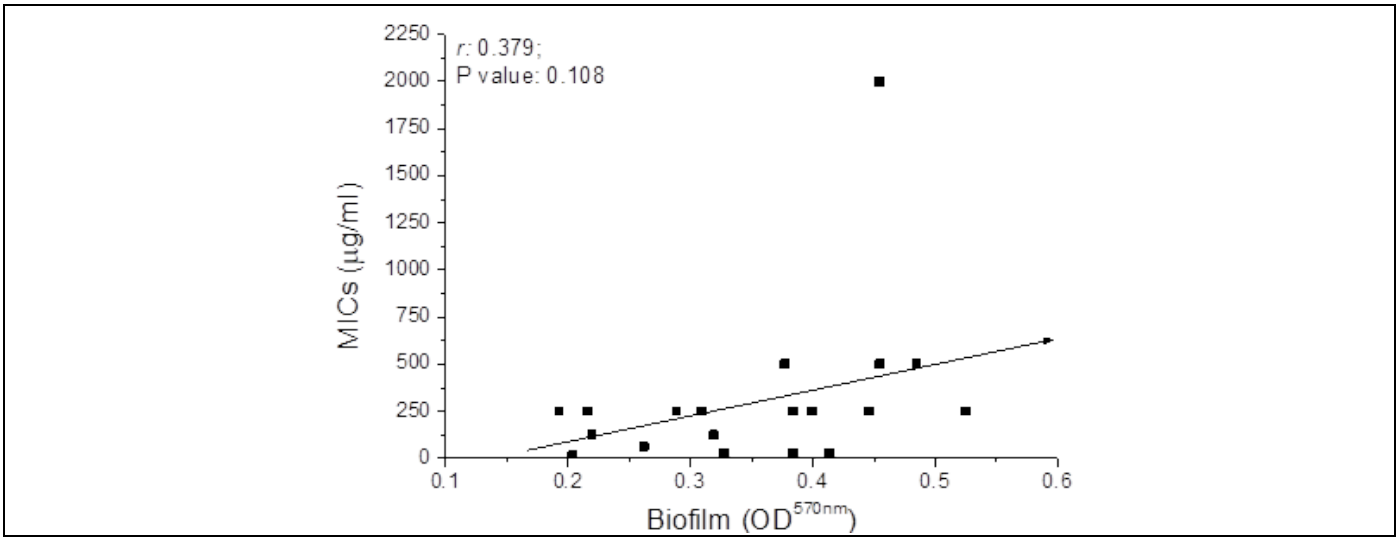

Figure. 2. The correlation between MICs of ceftriaxone and biofilm formation of 19 clinical isolates of *P. aeruginosa* isolated from infected wounds. Pearson correlation coefficient (*r*): + 0.379; P: 0.108 (P>0.05).

high level of standard deviation (Figure 1a). Figure 1b shows the biofilm formation of 19 isolates of *P. aeruginosa* (Pa1 to Pa19), the results showed that the highest biofilm formation was produced by Pa2 (OD^{570nm}, 0.525) followed by Pa6 (OD^{570nm}, 0.484) and Pa10 (OD^{570nm}, 0.454). The lowest biofilm formation was seen in the case of Pa4 (OD^{570nm}, 0.193). It was also observed that the biofilm formation of all *P. aeruginosa* isolates was variable (0.35 ±0.1).

The Pearson correlation coefficient (*r*) was used to check the relationship between the susceptibility to ceftriaxone (MICs) against 19 isolates of *P. aeruginosa* and biofilm formation. The results showed no relationship between the biofilm formation and susceptibility of bacteria to ceftriaxone (*r*,+ 0.379; *P*, 0.108). The current results undermine the role of bacteria's ability to form biofilm and their susceptibility to *P. aeruginosa* to ceftriaxone (Figure 2).

2.3. Effect of ceftriaxone on biofilm formation

In the current study, the effect of sub-inhibitory concentrations of ceftriaxone was evaluated on three isolates of *P. aeruginosa* that showed the highest biofilm formation in the polystyrene microtiter plate (Pa2, Pa6, and Pa10). Figure 3 shows that sub-inhibitory concentration (0.5 x MIC of ceftriaxone) exhibited the highest inhibitory effect on biofilm formed by the three isolates of *P. aeruginosa* (Pa2, Pa6, and Pa10) (P<0.01). Exposing to 0.25xMIC and 0.125xMIC reduced the biofilm formed by the three isolates moderately. The study showed that the effect of sub-inhibitory concentrations of ceftriaxone on biofilm formation was variable and cannot be considered as a concentration of ceftriaxone-dependent manure and also it was variable dependent on the isolate of *P. aeruginosa*.

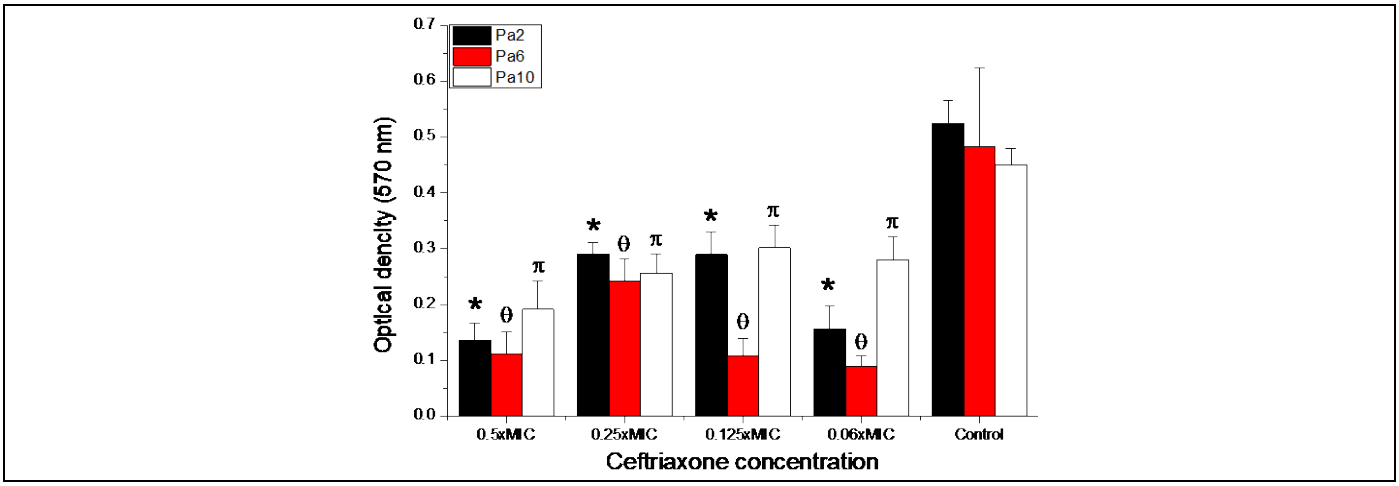

Figure 3. Effect of sub-inhibitory concentrations (0.5xMIC, 0.25xMIC, 0.125xMIC, 0.06xMIC, and 0.03xMIC of ceftriaxone) on biofilm formation by three isolates of *P. aeruginosa* (Pa2, Pa6, and Pa10). *, a significant difference from biofilm formation by Pa2 (without antibiotic, control); Θ, a significant difference from biofilm formation by Pa6 (without antibiotic, control); π, a significant difference from biofilm formation by Pa10 (without antibiotic, control). P<0.05 is considered a significant difference.

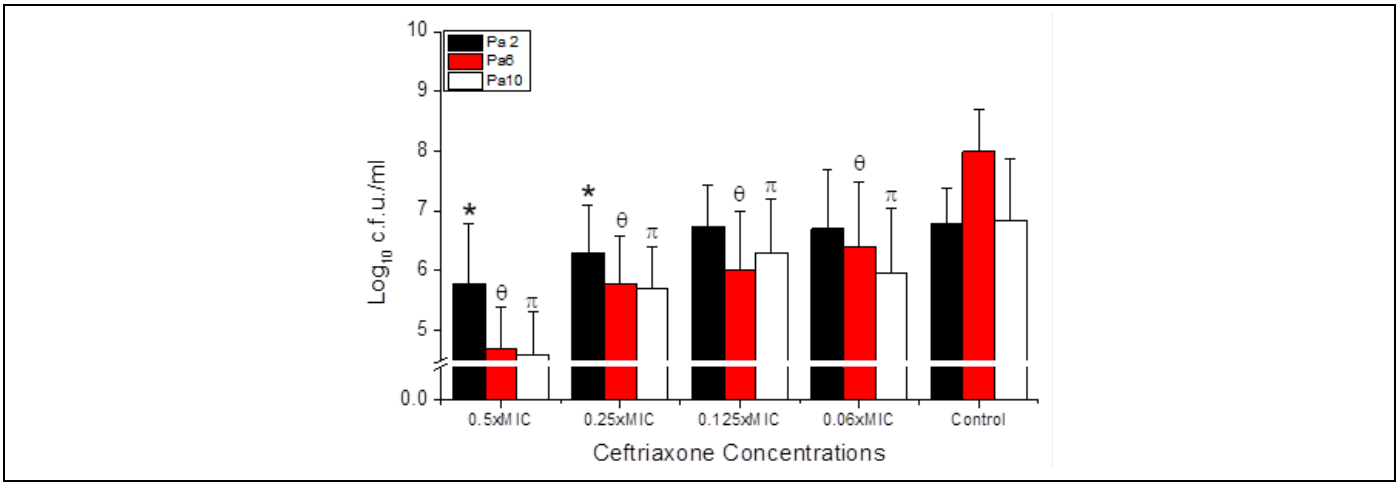

Figure 4. Viable bacterial count of *P. aeruginosa* (Pa2, Pa6, and Pa10) adhered to human OMECs post-exposure to different sub-inhibitory concentrations of ceftriaxone (0.5xMIC, 0.25xMIC, 0.125xMIC, and 0.06xMIC) the results were compared with control (Viable bacterial count of *P. aeruginosa* (Pa2, Pa6, and Pa10) adhered to human OMECs post-exposure to PBS). *, significant difference from adhesion of control (Pa2 exposed to PBS); Θ, significant difference from adhesion of control (Pa6 exposed to PBS); π, significant difference from adhesion of control (Pa10 exposed to PBS). P<0.05 is considered a significant difference.

2.4. Effect of ceftriaxone on adhesion to human OMECs

The adhesion of three isolates of *P. aeruginosa* (Pa2, Pa6, and Pa10) to human OMECs (biotic surface) post-exposure to the sub-inhibitory concentrations of ceftriaxone (0.5xMIC, 0.25xMIC, 0.125xMIC, and 0.06xMIC) was evaluated. The viable bacterial count method (plate count method) was used to estimate the number of live bacteria that adhered to the human OMECs (Figure 4). The results were compared with the viable bacterial count of *P. aeruginosa* isolates (P a2, Pa6, and Pa10 exposed to PBS) that adhered to human OMECs. In the case of isolate Pa2, the results showed that the highest significant decrease (P<0.001) in the adhesion of the bacterial cells to epithelial cells was observed when exposing the bacteria cells to 0.5xMIC, followed by the exposing to 0.025xMIC (P<0.01). No significant statistical decrease was observed in the number of living cells adhered to epithelial cells when the bacterial cells of this isolate were exposed to 0.12xMIC and 0.06xMIC of ceftriaxone (P>0.05). In the case of the two isolates Pa6 and PA10, it was found that the highest significant decrease in the number of adhered bacteria to the surface of epithelial cells (human OMECs) was found when they were exposed to 0.5xMIC of ceftriaxone followed by the exposure to 0.25xMIC of ceftriaxone, while the number of live bacteria adhered to the surface of epithelial cells increased when they were exposed to 0.125xMIC and 0.06xMIC but the number of adhered bacteria remained significantly low as compared with control. The present study showed that the effect of sub-inhibitory concentrations of ceftriaxone on the adhesion of *P. aeruginosa* to epithelial cells is variable and dependent on the isolate of bacteria.

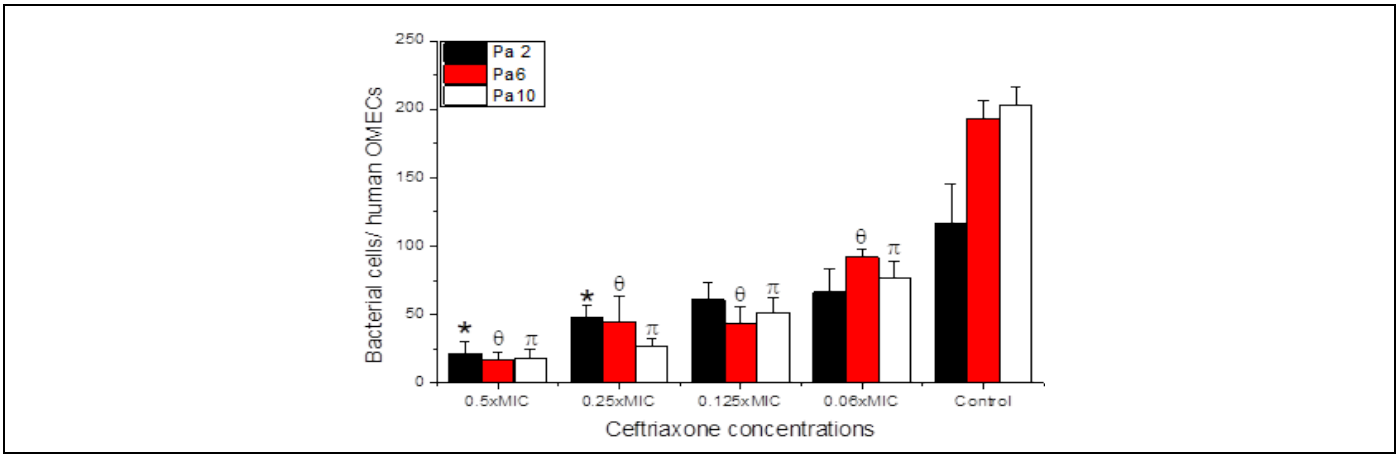

Figure 5. The mean number of *P. aeruginosa* (Pa2, Pa6, and Pa10) cells adhered to each human OMEC post-exposure to different sub-inhibitory concentrations of ceftriaxone (0.5xMIC, 0.25xMIC, 0.125xMIC, and 0.06xMIC) the results were compared with control (Viable bacterial count of *P. aeruginosa* (Pa2, Pa6, and Pa10) adhered to human OMECs post-exposure to PBS). *, significant difference from adhesion of control (Pa2 exposed to PBS); Θ, significant difference from adhesion of control (Pa6 exposed to PBS); π, significant difference from adhesion of control (Pa10 exposed to PBS). P<0.05 is considered a significant difference.

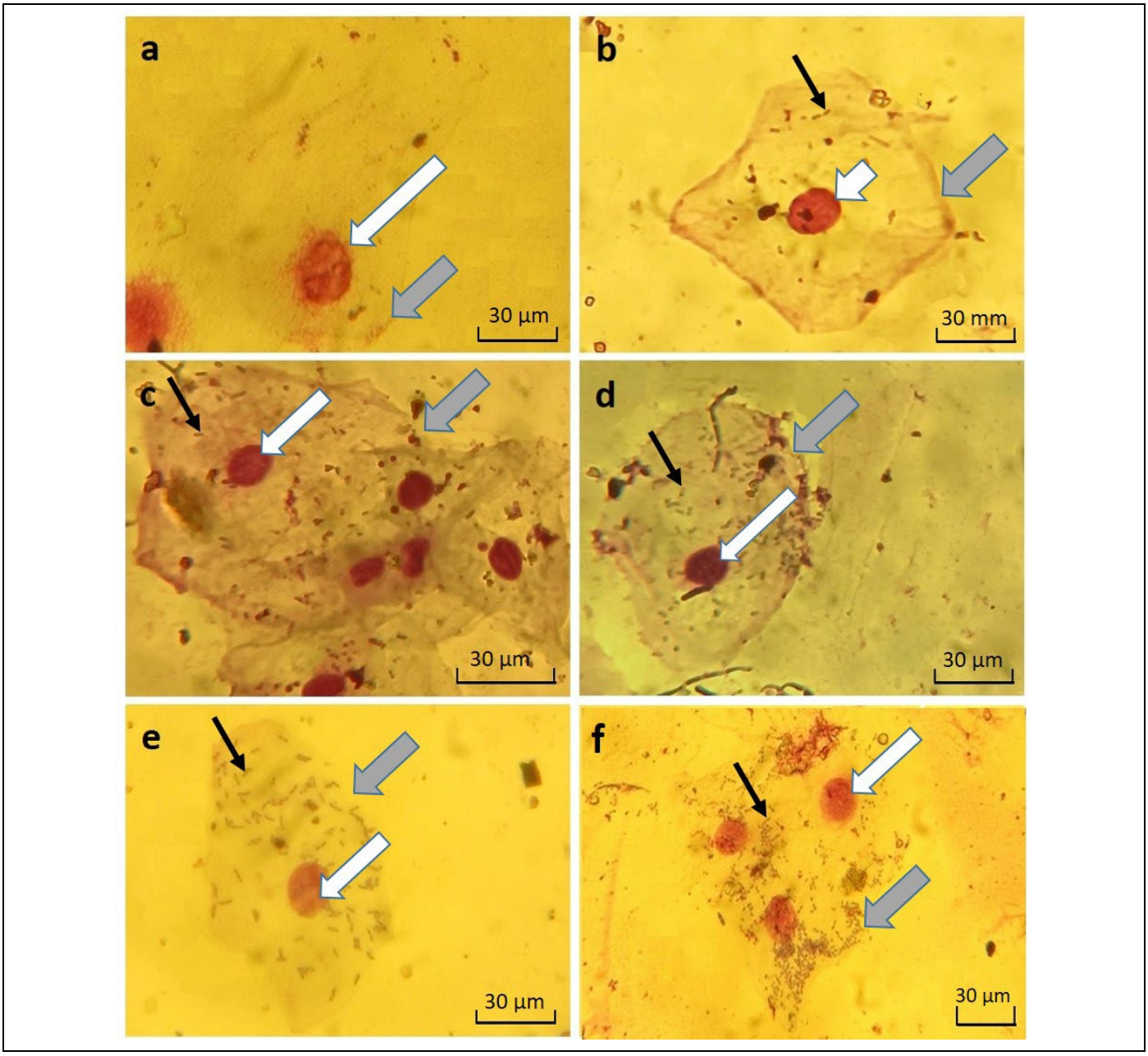

Figure 6. Photomicrographs of human OMEC stained with Leishman's stain and examined under light microscope. The epithelial cells were attached with *P. aeruginosa* (Pa6) cells that were pre-treated with different sub-inhibitory concentrations of ceftriaxone. a, human OMEC; b, human OMEC attached with Pa6 pre-treated with 0.5xMIC of ceftriaxone; c, , human OMEC attached with Pa6 pre-treated with 0.25xMIC of ceftriaxone; d, , human OMEC attached with Pa6 pre-treated with 0.125xMIC of ceftriaxone; e, human OMEC attached with Pa6 pre-treated with 0.06xMIC of ceftriaxone; f, human OMEC attached with Pa2 pre-treated with PBS. Black arrows point to the attached bacteria; the gray arrows point to the cell membrane of OMEC; white arrows point to nucleus of OMEC.

Another technique of calculating the number of total bacterial cells adhered to the surface of human OMECs was also used after staining them with Leishman's stain and examining them under a microscope. The results showed that the lowest significant inhibition ($P<0.01$) of adhesion of *P. aeruginosa* (Pa2, Pa6, and Pa10) was found when the bacteria cells exposed to 0.5xMIC of ceftriaxone followed by exposure to 0.25xMIC of ceftriaxone. The lowest inhibition of bacterial adhesion was observed when the bacterial cells (pa2, Pa6, and Pa10) were exposed to 0.06xMIC of ceftriaxone ($P<0.05$) (Figure 5).

The results of the current study were supported by the microphotographs of epithelial cells that were attached to *P. aeruginosa* (Pa2) (pre-treated with different concentrations of ceftriaxone). The results showed the lowest number of adhered bacteria (Pa2) was seen in the case of pre-treated with 0.5xMIC of ceftriaxone (Figure 6b). Moderate numbers of adhered bacteria were seen in the case of pre-treated bacteria

with 0.25xMIC, 0.125xMIC, and 0.06xMIC of ceftriaxone (Figure6 c, d, and e). The highest number of adhered bacteria was seen in the epithelial cells (human OMECs) that were attached to *P. aeruginosa* cells which were pre-treated with PBS (Figure 6f). Figure 6a showed human OMEC that was not exposed to *P. aeruginosa*.

3. DISCUSSION

The adhesion of opportunistic pathogenic bacteria to the biotic surface represents the first step in the formation of biofilms or the invasion of host cells, which protect the bacteria from the host immune system and facilitate chronic infection.[29] Adhesion of *P. aeruginosa* to mucosal epithelial cells is a crucial step in infection, particularly in mouth infections.[30] The adhesion of bacteria to host cells is a complex process that involves various factors and mechanisms. Understanding these interactions is essential for developing effective strategies to prevent and control bacterial infections.

In the current study, *P. aeruginosa* isolates were isolated from infected wounds. The susceptibility of these isolates to ceftriaxone, biofilm formation, and adherence to oral epithelial cells (human OMECs) *in vitro* were evaluated. The response of *P. aeruginosa* to ceftriaxone, as well as biofilm form (to the abiotic surface) and adhesion to human OMECs, was variable depending on the isolates. The study also showed that the effect of the sub-inhibitory concentrations of ceftriaxone on biofilm formation to polystyrene and adhesion to epithelial cells was also variable and could not depend on the sub-inhibitory concentration of ceftriaxone. This confirms that the sub-inhibitory concentrations of ceftriaxone reduce the chances of *P. aeruginosa* to adhere and biofilm formation to biotic and abiotic surfaces, but differently depending on the type of isolates. Therefore, ceftriaxone may be reducing the chances of *P. aeruginosa* adhering to mucous membranes as well as artificial surfaces, which is why, this antibiotic can work as a prophylactic agent in preventing oral infection by *P. aeruginosa*. The limitation of the present study was related to the number of isolates. Thus, the outcome requires further study with a high number of isolates. The researchers from our lab are currently studying the effect of sub-inhibitory concentrations of this antibiotic on the ability of *P. aeruginosa* bacteria to adhere to materials used in dental fillings.

The relationship between the adhesion of *P. aeruginosa* to human epithelial cells and its biofilm formation on polystyrene surfaces is one of mutual reinforcement, with common mechanisms such as adhesion factors, EPS production, and quorum sensing.[31] The adhesion is crucial for biofilm formation and development and that enhances bacterial adhesion and survival. The effect of ceftriaxone on this relationship can be described as the effect of this antibiotic on peptidoglycan synthesis (that effect is limited),[32] reduction of the bacterial population, interfering with synthesis or expression of adhesins, inducing alterations in adhesion structures, and alteration of bacterial surface properties.[33] These mechanisms collectively disrupt the processes essential for biofilm development and epithelial cell adhesion, enhancing the antibiotic's effectiveness in treating and preventing infections caused by *P. aeruginosa*.

The previous studies reported the role of ceftriaxone in bacterial adhesion and biofilm formation. Studies showed that ceftriaxone affects biofilm-forming bacteria, including *Escherichia coli* and Methicillin-resistant *Staphylococcus aureus* (MRSA).[34, 35] Another study reported that ceftriaxone alone did not eradicate mature biofilms in the case of *Enterococcus faecalis*. Overall, ceftriaxone has shown potential in affecting bacterial adhesion and biofilm formation, but further research is needed to fully understand its role in these processes. Onaolapo and Salami, (1995) investigated the effect of sub-inhibitory concentrations of ceftriaxone on the adhesion of *P. aeruginosa* to inert surfaces, such as catheters, plastic, and glass, and they found that the ability to adhere varied depending on the bacterial growth phase.[23] However, there is no previous study highlighted the effect of sub-inhibitory concentrations of ceftriaxone on the adhesion of *P. aeruginosa* to human OMECs and biofilm formation onto polystyrene.

The mechanism of the effect of ceftriaxone on bacterial adhesion and biofilm formation is not fully understood, but previous studies suggested that sub-inhibitory concentrations of ceftriaxone can induce morphological alterations and polysaccharide intercellular adhesion (PIA)-independent biofilm formation in *S. aureus*.[36] The chemical nature of the biofilm matrix produced by sub-MICs of ceftriaxone in *S. aureus* is non-PIA dependent and the sub-inhibitory concentration of ceftriaxone produce morphological changes in the bacterial cells that may affect the bacterial adhesion and biofilm formation.[36] These findings suggest that sub-inhibitory concentrations of ceftriaxone impact bacterial adhesion and biofilm formation, but the exact mechanisms are not yet fully understood and may vary depending on the bacterial species and conditions. The present study was carried out to help fill the gap of knowledge regarding the effect of sub-inhibitory concentrations of ceftriaxone on biofilm formation and adhesion to epithelial cells.

It can be concluded that the adhesion and biofilm formation of *P. aeruginosa* to biotic (human OMECs) and abiotic (polystyrene) surfaces reduce post-treating with sub-MIC of ceftriaxone and the effect of this

antibiotic on biofilm formation and adhesion was bacterial isolate-dependent manner (*P. aeruginosa*).

Conflict of interest
The authors declare that they have no conflict of interests.

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Ethics approval
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