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Effect of subinhibitory doses of rifaximin on in vitro *Pseudomonas aeruginosa* adherence and biofilm formation to biotic and abiotic surface models

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Abstract

Background. The adhesion of *Pseudomonas aeruginosa* to biotic and abiotic surfaces is responsible for the persistence and development of bacterial infection.

Objectives. To fill the gap in the knowledge regarding the relationship between rifaximin susceptibility and biofilm formation, and to investigate the effect of subinhibitory doses of rifaximin on the adhesion and biofilm formation.

Materials and methods. A total of 10 isolates of *P. aeruginosa* were obtained from 110 urine samples of urinary tract infection (UTI) patients. Biofilm formation on polystyrene microtiter plates, minimum inhibitory concentrations (MICs) of rifaximin against the 10 isolates of *P. aeruginosa* (Pa1–Pa10), the effect of sub-MICs of rifaximin (0.5 × MIC, 0.25 × MIC, 0.125 × MIC, and 0.06 × MIC) on biofilm formation by the Pa4 isolate to polystyrene microtiter plates, and the adhesion to human epithelial cells (HECs) in vitro were evaluated.

Results. The MICs of rifaximin against 10 isolates ranged from 62.5 µg/mL to 1000 µg/mL. The Pa4 isolate produced the highest level of biofilm formation, while the MIC of Pa4 was 125 µg/mL. There was no correlation between bacterial susceptibility to rifaximin and biofilm formation ($r: -0.016$; $p > 0.05$). Sub-MIC doses of rifaximin significantly reduced the biofilm formation on abiotic surfaces, while only 0.5 × MIC, 0.25 × MIC and 0.12 × MIC of rifaximin reduced the adhesion to HECs significantly ($p < 0.05$) in a dose-dependent manner.

Conclusions. This pioneering study demonstrated the negative effect of sub-MIC doses of rifaximin on biofilm formation and adhesion to abiotic and biotic surfaces in vitro.

Key words: adhesion, biofilm formation, *Pseudomonas aeruginosa*, rifaximin, subinhibitory doses

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Background

Pseudomonas aeruginosa is a common bacterium that can cause a range of infections in humans, including pneumonia, urinary tract infections (UTIs) and bloodstream infections.[1] One of the unique characteristics of *P. aeruginosa* is its ability to adhere to and form biofilms on biotic and abiotic surfaces. A biofilm is a complex community of microorganisms that adhere to surfaces and are surrounded by a protective matrix of extracellular polymeric substances (EPS). Several factors may affect the ability of *P. aeruginosa* to form biofilms, such as quorum sensing and flagella.[2] Biofilms are difficult to eradicate and are associated with chronic infections that are often resistant to antibiotics. The biofilms cause complications in infections which lead to serious issues and difficulties treating *P. aeruginosa* infection.[3]

The first step in *P. aeruginosa* infection is the adherence of the bacterium to the host human epithelial cells (HECs).[4] Adherence is a critical step in *P. aeruginosa* infection, as it allows the bacterium to establish a foothold in the host and initiate the infection process. The mechanisms by which *P. aeruginosa* adheres to epithelial cells are complex and involve multiple bacterial factors and host cell receptors. Once *P. aeruginosa* has adhered to host epithelial cells, it can initiate the infection process by secreting virulence factors such as toxins and proteases.[5] These factors can cause tissue damage, impair host immune responses, and promote bacterial survival and growth.[6]

Treatment of *P. aeruginosa* biofilm infections can be challenging and may require a combination of antibiotic therapy, removal of infected devices, and surgical debridement.[7] Antibiotics are the most important strategy for treating bacterial infections, and increasing resistance to antibiotics represents the biggest challenge for treating bacterial infectious diseases nowadays.[7] The use of antibiotics is limited to not only killing bacteria, but many experiments have proven that the use of sublethal doses of antibiotics reduces the ability of different types of bacteria to adhere to surfaces, which contributes significantly to reducing the virulence of bacteria.[7,8] Subinhibitory antibiotic concentrations can also lead to decreased biofilm formation by *P. aeruginos*a. Several studies have reported that certain antibiotics can impair bacterial attachment to surfaces and biofilm formation,[9] but there is no previous study that highlighted the impact of a subinhibitory concentration of rifaximin on biofilm formation.

Rifaximin is a broad-spectrum antibiotic used to treat a variety of infections, including traveler's diarrhea, hepatic encephalopathy and other infectious cases.[10,11] It works by inhibiting bacterial RNA synthesis, which ultimately leads to bacterial cell death.[12] Previous studies did not evaluate the role of rifaximin in reducing the ability of *P. aeruginosa* to adhere to surfaces and form biofilm. Therefore, our current pioneering study highlighted the effect of subinhibitory doses of rifaximin on the ability of *P. aeruginosa* to adhere to HECs (biotic surface model) and form a biofilm on polystyrene (abiotic surface model).

Materials and methods

Ethical approval

The current study was conducted after obtaining the approval from the human ethical committee of the Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq (Reference No. 609, date: April 14, 2022).

Isolation and identification of bacteria

Urine samples were collected aseptically from 110 patients suffering from UTIs and treated at Baghdad Teaching Hospital (Baghdad, Iraq). The average age of the patients was 42.3 ±11.2 years (59 years for females and 51 years for males). All patients did not receive antibiotic treatment 72 h before the date of sample collection and gave consent to participate in the study. Briefly, 500 µL of urine sample was placed in 4.5 mL of Pseudomonas Asparagine Broth medium (HiMedia, Mumbai, India). The containers were incubated for 48 h at 37°C with vigorous shaking at 210 rpm. Then, 50 µL of bacterial suspension was streaked onto asparagine agar plates (1.5% agar; HiMedia) and incubated at 37°C until colonies developed.[13] A VITEK 2 DensiCheck instrument and fluorescence system (bioMérieux, Marcy-l'Étoile, France) (ID-GNB card) were used to identify the isolates of *P. aeruginosa*.[14]

Rifaximin minimum inhibitory concentrations (MICs)

The standard broth micro-dilution technique described by Wiegand et al. was followed to determine the MICs of rifaximin against 10 clinical isolates of *P. aeruginosa* (Pa1–Pa10).[15] Briefly, a 1 mg/mL stock concentration of rifaximin (Mylan, Potters Bar, UK) was prepared by dissolving the powder in sterile distilled water. Double-fold dilutions (100 µL) were prepared in the microtiter plate with sterile Mueller–Hinton broth (MHB; HiMedia). After overnight growth, the *P. aeruginosa* isolates were washed 3 times with sterile phosphate-buffered saline (PBS; 0.1 M, pH 7.2) using centrifugation at 10,000 rpm for 10 min (High-Speed Centrifuge, Avanti JXN-26; Beckman Coulter, Brea, USA), and the number of bacteria was adjusted to 10^6 CFU/mL using the spectrophotometric method (double-beam spectrophotometer model SP-MUV8000T; Bioevopeak, Jinan, China) at 600 nm; then, 5 µL of the bacterial solution was added to each well. The plates were gently mixed. Three technical controls were made: MHB inoculated with bacterial isolates, sterile MHB and different double dilutions of antibiotics. The MICs were

determined after overnight incubation at 37°C and were defined as the lowest antibiotic concentrations completely inhibiting growth.[15]

Biofilm formation

The protocol of Zgair and Chhibber was followed.[16] Briefly, 200 µL of sterile tryptic soy broth (TSB) was added to the wells of flat-bottom polystyrene tissue culture plates. Then, 5 µL of overnight growth of *P. aeruginosa* (Pa1–Pa10) was washed 3 times with sterile normal saline using centrifugation at 10,000 rpm for 10 min (High-Speed Centrifuge, Avanti JXN-26; Beckman Coulter). The number of bacteria was adjusted to 10^6 CFU/mL using the spectrophotometric method (double-beam spectrophotometer model SP-MUV8000T; Bioevopeak) at 600 nm. Then, 5 µL of adjusted bacterial suspension was added to each well, and the plates were incubated at 37°C for 18 h. The media were discarded, and non-adherent bacterial cells were removed by washing them 5 times with normal, sterile saline. The quantity of biofilm formation was measured with the spectrophotometric method. The resultant biofilms of different *P. aeruginosa* isolates were dried and fixed by incubating at 65°C for 35 min. Then, 200 µL of Hucker crystal violet (0.4%) was added to each well and incubated for 6 min at 21°C. The plates were washed 4 times with distilled water and dried for 30 min at 37°C. Then, 200 µL of acetone:ethanol (30:70) was added to each well. The absorbency of each well was measured at a wavelength of 570 nm using a microplate reader (BioTek 800 TS; BioTek, Winooski, USA).[16] The experiment was repeated 3 times.

Effect of sub-MICs of rifaximin on biofilm formation

To determine the effect of sub-MICs of antibiotics on the biofilm formation of the *P. aeruginosa* isolate that produces the highest level of biofilm, a similar method of biofilm formation was followed. However, instead of TSB, double-fold dilutions of sub-MICs of rifaximin were used (0.5 × MIC, 0.25 × MIC, 0.125 × MIC, and 0.06 × MIC). Tests were performed in triplicate.

Adherence of *P. aeruginosa* to HECs

The standard method of Ali and Zgair was followed to prepare HECs in vitro.[17] The volunteers from whom the HECs were collected did not take any antibiotics 3 days before the HEC sample collection. The method of Zgair and Chhibber[16] was followed to evaluate the adhesion of clinical isolates of *P. aeruginosa* that produced the highest level of biofilm to HECs in vitro. Briefly, in each well of a 24-well tissue culture microtiter plate (Thermo Fisher Scientific, Waltham, USA), 1×10^5 HECs were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 10 mM of L–glutamine. Then, 100 µL of *P. aeruginosa* (5×10^7 CFU/mL) was added to each well. The plates were incubated for 1 h at 37°C and then washed 3 times with PBS (0.1 M, pH 7.2). The HECs were washed 3 times with PBS (0.1 M, pH 7) by centrifugation (1000 rpm, 10 min, 4°C). The HEC pellets were lysed with PBS containing 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, USA), diluted 10-fold, and plated on nutrient agar to estimate the number of bacteria adhering to HECs.

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

To study the effect of different concentrations of rifaximin (0.5 × MIC, 0.25 × MIC, 0.125 × MIC, and 0.06 × MIC) on the ability of *P. aeruginosa* (the isolate that produced the highest level of biofilm) to adhere to HECs, the colonies of bacteria that grew on Mueller–Hinton agar (MHA) were suspended in TSB (HiMedia). The bacterial number was adjusted to 1×10^7 with MHB (HiMedia) that was prepared in different concentrations of rifaximin (0.5 × MIC, 0.25 × MIC, 0.125 × MIC, and 0.06 × MIC). The broths were incubated for 18 h at 37°C. The bacterial cells were washed 3 times with normal, sterile saline (10,000 g for 10 min). The final number of bacteria was adjusted to 1×10^7 CFU/mL with TSB. A similar procedure to investigate the adherence of *P. aeruginosa* to HECs was followed in order to determine the ability of bacteria treated with different concentrations of rifaximin to adhere to HECs in vitro. The results were compared with the ability of bacteria (untreated with rifaximin) to adhere to HECs (control). The experiments were performed in triplicate.

Leishman's stain

Smears of HECs were prepared and dried at 37°C. The standard method of Sareen et al.[18] was followed to stain 3 groups of HECs: HECs that were untreated with bacteria and rifaximin, HECs that were treated with *P. aeruginosa* only, and HECs that were treated with *P. aeruginosa* and different concentrations of rifaximin (0.5 × MIC, 0.25 × MIC, 0.12 × MIX, and 0.06 × MIC). The slides were examined under a light microscope (Lomo Mikned 2; Lomo, Sankt Petersburg, Russia), and the images were captured using a smartphone camera over the microscope eyepiece.

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 assesed using Pearson's correlation coefficient. A value of $p < 0.05$ was considered statistically significant.

Results

Isolation and identification

Ten isolates of *P. aeruginosa* were obtained 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 (bioMérieux) proved that the isolates were *P. aeruginosa* (Pa1–Pa10).

Rifaximin MICs and biofilm formation

In the current study, the MIC of rifaximin against 10 clinical isolates of *P. aeruginosa* (Pa1–Pa10) was measured. The highest MIC of rifaximin was observed against Pa10 (1000 µg/mL), while the lowest MIC of rifaximin was seen against Pa8 (62.5 µg/mL) (Fig. 1A).

Figure 1B shows the biofilm formation of the 10 clinical isolates of *P. aeruginosa* that were obtained from urine samples. The highest level of biofilm was produced by Pa4 (optical density (OD): 0.49 ±0.029), followed by Pa10 (OD: 0.34 ±0.021), while the lowest biofilm mass was formed by Pa9 (OD: 0.11 ±0.019). The present study showed that all isolates can produce biofilm, but Pa4 produced the highest level of biofilm mass. Thus, this isolate was used for further experiments aimed at evaluating the role of a subinhibitory dose of rifaximin in reducing biofilm formation and bacterial adherence in vitro.

Figure 2 shows that there is no relationship between the values of MICs of rifaximin against 10 clinical isolates of *P. aeruginosa* and biofilm formation of the same isolates. The present study proved that there is no relationship between the ability of clinical isolates of *P. aeruginosa* to form biofilm and the susceptibility of these bacterial isolates to rifaximin.

Effect of sub-MICs of rifaximin on biofilm formation

The effect of sub-MICs of rifaximin on biofilm formation by *P. aeruginosa* (Pa4) was also evaluated. It was found that all the concentrations used (0.5 × MIC, 0.25 × MIC, 0.125 × MIC, and 0.06 × MIC) reduced the ability of Pa4 to form a biofilm on polystyrene microtiter plates, and the decrease in biofilm formation was shown in a concentration-dependent manner. Whereas the highest decrease in biofilm formation was observed when treating bacteria with 0.5 × MIC of rifaximin (0.207 ±0.032), the lowest decrease in biofilm formation was found when treating bacteria with 0.06 × MIC of rifaximin (0.403 ±0.035) (Fig. 3).

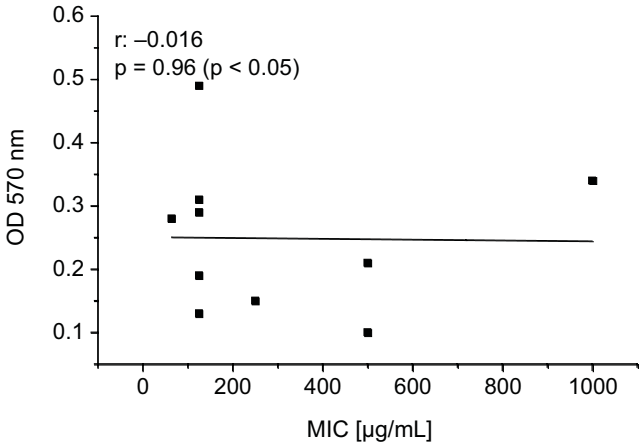

Fig. 2. Relationship between minimum inhibitory concentrations (MICs) of rifaximin against 10 clinical isolates of *Psedomonas aeruginosa* (Pa1–Pa10) and biofilm formation of the same isolates of *P. aeruginosa* (p > 0.05)

OD – optical density.

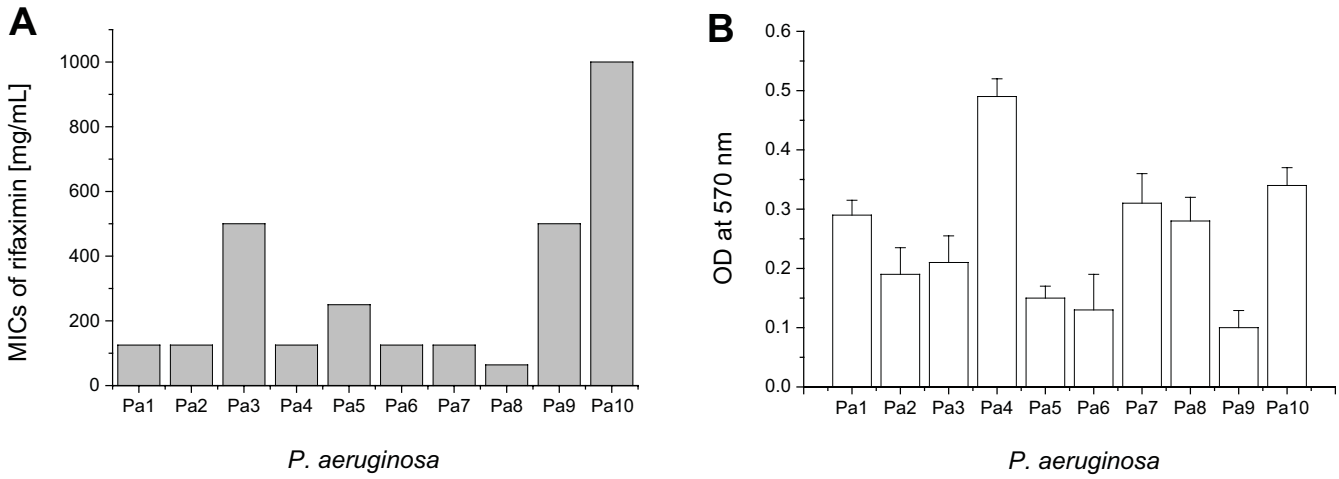

Fig. 1. A. Minimum inhibitory concentration (MIC) of rifaximin against 10 clinical isolates of *Psedomonas aeruginosa* (Pa1–Pa10) obtained from the urine samples collected from patients suffering from urinary tract infections (UTIs); B. Biofilm formation of the same 10 clinical isolates of *P. aeruginosa* to polystyrene microtiter plates

OD – optical density.

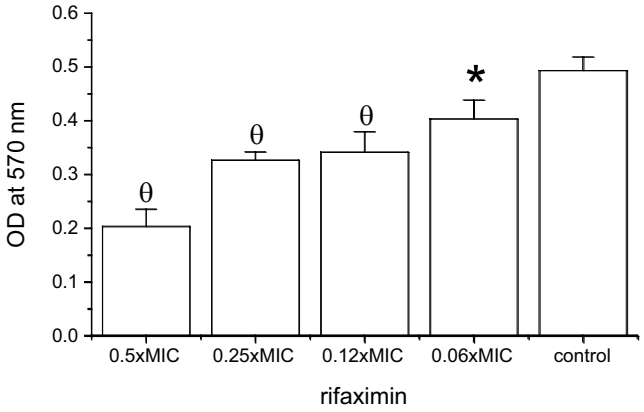

Fig. 3. Effect of subinhibitory doses of rifaximin (0.5 × minimum inhibitory concentration (MIC), 0.25 × MIC, 0.12 × MIC, and 0.06 × MIC) on biofilm formation by *Psedomonas aeruginosa* (Pa4). All concentrations of rifaximin significantly reduced the biofilm formation by Pa4

* $p < 0.05$; θ – $p < 0.001$; OD – optical density.

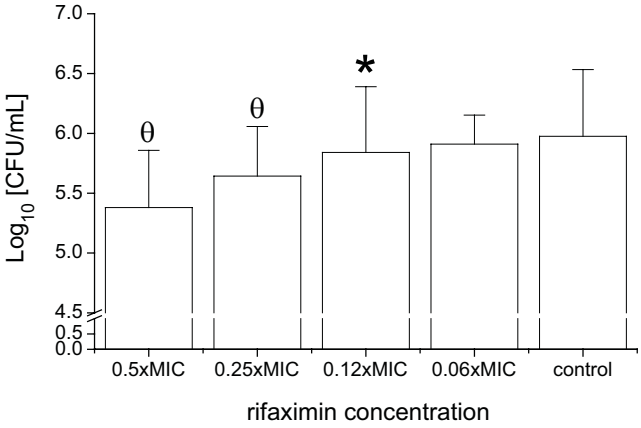

Fig. 4. Effect of subinhibitory doses of rifaximin (0.5 × minimum inhibitory concentration (MIC), 0.25 × MIC, 0.12 × MIC, and 0.06 × MIC) on *Psedomonas aeruginosa* (Pa4) adhesion to human epithelial cells (HECs) in vitro. All concentrations of rifaximin significantly reduced the number of adhered bacteria (CFU/mL) to HECs

* $p < 0.05$; θ – $p < 0.001$; OD – optical density.

Effect of sub-MICs of rifaximin 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 utilized to evaluate the adhesion of *P. aeruginosa* (Pa4) to biotic surfaces (HECs) and to estimate the effect of sub-MICs of rifaximin on the adhesion of *P. aeruginosa* (Pa4) to HECs in vitro. The 0.5 × MIC, 0.25 × MIC and 0.125 × MIC concentrations of rifaximin significantly reduced the adhesion of Pa4 to HECs ($p < 0.05$), while no significant decrease in the adhesion of bacteria to HECs was observed when the bacteria were treated with the 0.06 × MIC of rifaximin ($p > 0.05$). The decrease in adhesion was detected in a rifaximin concentration-dependent manner. While the highest significant decrease in bacterial adhesion was observed when treating bacteria with 0.5 × MIC of rifaximin ($p < 0.001$), the lowest significant decrease in bacterial adhesion was found when treating bacteria with 0.12 × MIC of rifaximin ($p < 0.05$) (Fig. 4).

The light microscopy images of the Pa4 adhesion to HECs with and without exposure to rifaximin (0.5 × MIC, 0.25 × MIC, 0.12 × MIC, and 0.06 × MIC) are shown in Fig. 5. Figure 5A shows a low number of bacteria (pretreated with 0.5 × MIC of rifaximin) attached to HECs that prove the 0.5 × MIC of rifaximin has a pronounced effect on the adhesion of Pa4 to HECs. However, pretreating Pa4 with 0.25 × MIC and 0.12 × MIC resulted in a moderate effect on the Pa4 attachment to the HECs (Fig. 5B,C). Figure 5D shows that the high number of bacteria pretreated Pa4 with 0.06 × MIC that attached to HECs, and this finding is similar to the attachment of Pa4 (untreated with rifaximin) to HECs (Fig. 5E) that proved there was no significant effect of 0.06 × MIC of rifaximin on the ability of Pa4 to attach to HECs. Figure 5F shows the HECs that were not exposed to the Pa4 (control). The present results showed the negative effect of rifaximin on the ability of *P. aeruginosa* to attach to HECs in vitro (biotic surface model).

Discussion

Biofilm formation is a process by which microorganisms, such as bacteria, adhere to surfaces and form a protective community of cells encased in a matrix of EPS. This matrix provides a protective barrier for the microorganisms, making it difficult for antibiotics and the immune system to reach and eliminate the bacterial cells.[19] The adhesion to HECs is an important step in the establishment of many infections, since HECs are the first line of defense against invading microorganisms and serve as a physical barrier to infection. Many bacterial pathogens have developed mechanisms to adhere to and invade HECs, allowing them to establish an infection and evade the immune system. The process of adhesion to the epithelial cells represents the first step for the bacteria to activate several mechanisms (secretion of some lytic enzymes such as protease) that enable them to penetrate this barrier.[20]

The ability of *P. aeruginosa* to form biofilms and adhere to HECs is a major factor in its capacity to cause chronic infections.[21] The present study has shown that all clinical isolates of *P. aeruginosa* can produce biofilm in vitro, and that was not related to rifaximin susceptibility. Moreover, it was highlighted for the first time that the subinhibitory concentrations (sub-MICs) of rifaximin reduced the ability of *P. aeruginosa* (Pa4) to produce the biofilm (in vitro) to polystyrene microtiter plates (abiotic surfaces) and adhesion to biotic surfaces (HECs) significantly (except 0.06 × MIC). The absence of a relationship between the ability of *P. aeruginosa* to form a biofilm and its response to the rifaximin indicates that *P. aeruginosa*

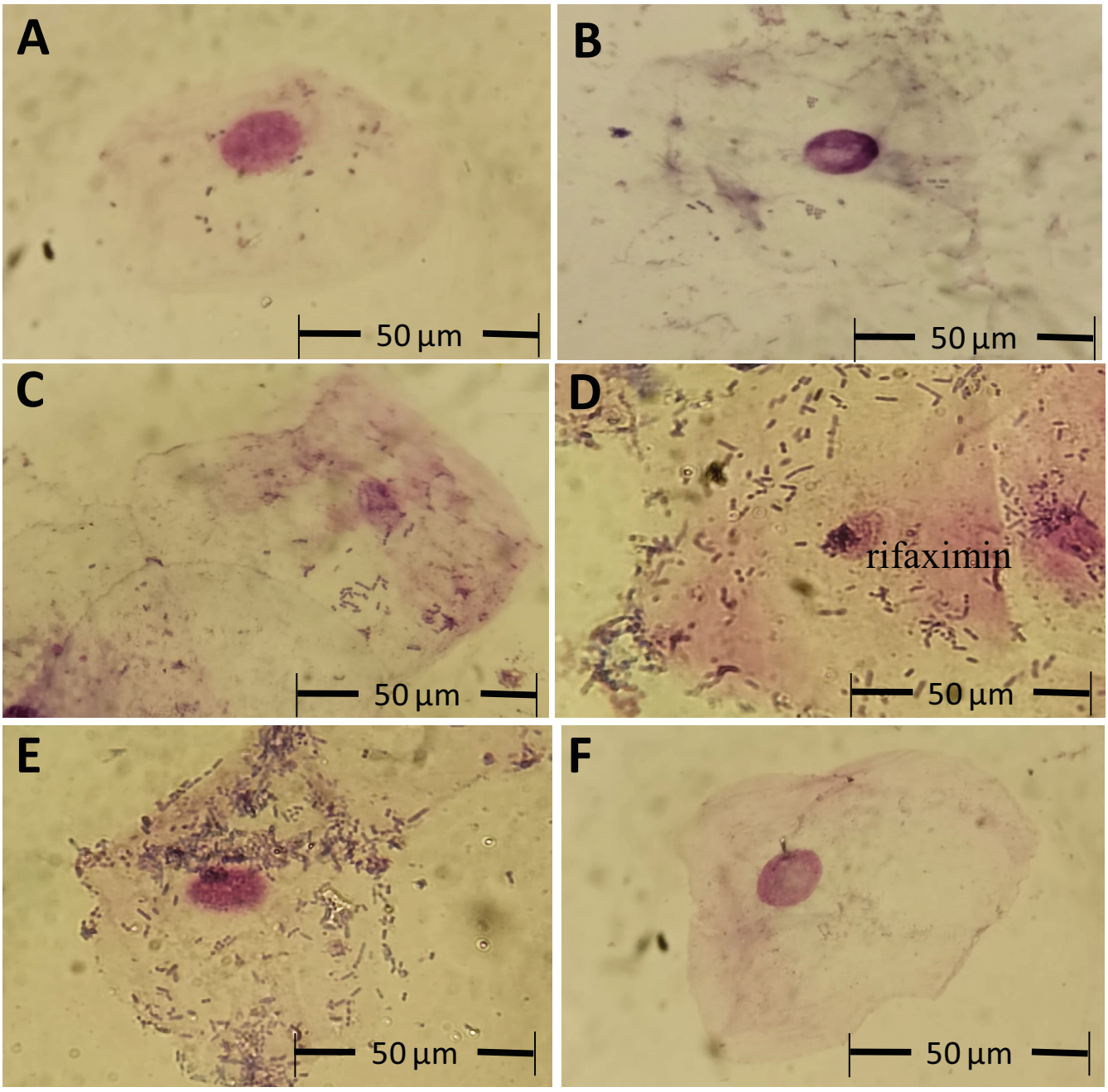

Fig. 5. Light microscopy images of Leishman-stained slides depicting adhesion of *Psedomonas aeruginosa* (Pa4) (pretreated with rifaximin) to human epithelial cells (HECs) after 2 h of incubation. A. Adhesion of Pa4 pre-treated with 0.5 × minimum inhibitory concentration (MIC) of rifaximin to HEC; B. Adhesion of Pa4 pretreated with 0.25 × MIC of rifaximin to HEC; C. Adhesion of Pa4 pretreated with 0.12 × MIC of rifaximin to HEC; D. Adhesion of Pa4 pretreated with 0.06 × MIC of rifaximin to HEC; E. Adhesion of Pa4 without rifaximin treatment to HEC; F. HECs alone

depends on other mechanisms rather than biofilm formation to resist this antibiotic.

The production of biofilm makes the bacteria more resistant to antibiotics by blocking the penetration of antimicrobial agents, thereby preventing them from reaching the bacterial cells. The EPS can sequester and deactivate antimicrobial agents. Bacteria within the biofilm can enter a dormant state in which they become less metabolically active and, therefore, less susceptible to antibiotics.[22] 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, which poses a serious challenge to public health.[23] 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 HECs by using the sub-MIC concentrations of rifaximin can be an effective strategy for preventing or reducing the severity of bacterial infections.

Overprescribing of treatments with antibiotics contributes to the creation of new strains of bacteria that are resistant to antibiotics,[24] so the use of effective low doses will have a positive economic and health impact if the doses can reduce the predominance of bacteria on adhesion and

biofilm formation. Moreover, this will help build a new treatment strategy to decrease bacterial infectious diseases by reducing the ability of pathogenic bacteria to adhere and form a biofilm on biotic and abiotic surfaces. However, such a strategy would require further study and development. Therefore, the strategy proposed in this study, which includes the use of low doses of antibiotics to reduce the ability of bacteria to adhere to biotic and abiotic surfaces and form a biofilm, is considered a promising strategy to prevent *P. aeruginosa* from causing infectious diseases.

Conclusions

The current study showed for the first time that there is no relationship between the susceptibility of *P. aeruginosa* to rifaximin and the ability of the bacteria to form a biofilm. Moreover, this is the first study that showed the role of subinhibitory doses of rifaximin in reducing the ability of *P. aeruginosa* to form the biofilm on abiotic surfaces (polystyrene) and to adhere to biotic surfaces (HECs) in vitro. This study contributes to developing a new strategy for the treatment of bacterial infections.

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