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Research Article

Comparative Adhesion of *Pseudomonas aeruginosa* to Human Oral Mucosal Epithelial Cells and Polystyrene Surfaces

Marwa M. Talib[1] , **Jenan A. Ghafil*[1]**
[1] Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.

Abstract

Background: The adhesion of bacteria to different surfaces reflects their ability to cause infectious diseases. The distinction between the *Pseudomonas aeruginosa* adhesion to biotic and abiotic surfaces is not clear in the literature.

Objectives: To shed light on the extent of similarities and differences between *P. aeruginosa types* in terms of their ability to adhere to different surfaces.

Methods: Ten isolates of *P. aeruginosa* were isolated from 100 wound and burn swabs. The samples were collected from Baghdad Teaching Hospital, and Al-Yarmouk Teaching Hospital, Baghdad, Iraq (from September 1st to December 24th 2023). The isolates were identified using biochemical and phenotypic tests in addition to VITIK-II technology. The susceptibility to antibiotics was estimated using the disc diffusion method. The microtiter-plate assay was used to measure the biofilm formation. The Human oral mucosal epithelial cells (OMECs) were used to estimate the adhesion of *P. aeruginosa* isolates. Plate and direct bacterial count were used for counting the bacteria adhered to human OMECs.

Results: Norfloxacin showed the highest antibacterial effect, while all isolates were resistant to Amoxicillin and Cefixime. Of all isolates that formed biofilm, P. aeruginosa 2 (Pa2) formed the highest biofilm, followed by Pa6, while the lowest biofilm formation was seen in Pa4. Pa6 showed the highest ability to attach to OMECs followed by Pa2.

Conclusion: The adherence and biofilm formation of *P. aeruginosa* isolates that are resistant to most antibiotics depend on the type of surface to which they adhere.

Keywords: Anti-Bacterial Agents; Antibiotics Resistant; Biofilm; *Pseudomonas aeruginosa;* Pseudomonas Infection

Introduction

Bacterial isolates that cause infectious diseases and are resistant to a wide spectrum of antibiotics, as well as overcoming high concentrations of detergents are considered a serious challenge facing doctors in curing hospital bacterial infections (1). *Pseudomonas aeruginosa* bacteria is the bacterial species that causes nosocomial infections due to its resistance to antibiotics in addition to detergents. *P. aeruginosa* is known to be the causative agent of different infectious diseases, affecting wounds and burns. These infections pose a challenge for professionals tasked with managing cases of *P. aeruginosa-infected* wounds and burns (2). Antimicrobial resistance has an impact on health and economic outcomes because it increases the chance of bacterial infectious disease outbreaks which would negatively impact the health services, ultimately increasing the cost of health care (3).

Human oral mucosal epithelial cells (OMECs) are believed to be a major set of players interacting with bacteria and immune systems (4). In addition, they are an important biological barrier that prevents microorganisms from entering deep tissues and defends the host against chemicals. The adhesion of bacteria to human epithelial cells is critical for bacterial invasiveness of the host (5).

The bacterial cells can attach to a wide range of biotic as well as abiotic surfaces. Different surfaces tend to prompt different responses from the bacterial cells to create the interaction between the bacterial cells and surfaces, yielding the attachment of bacteria to these surfaces (6). As a result, bacteria express appendages and produce adhesion proteins that are directed toward specific ligands or chemical characteristics on the surface (7). Adhesion may be affected by the physicochemical properties of the surface, such as charge, hydrophobic balance, and mechanical strength (6). Bacterial adhesion involves a complex combination of environmental, bacterial and material traits (8). Specific bacterial appendages, non-specific interactions (e.g. van der Waals forces and electrostatic interactions), and surface mechanical induction contribute to adhesion (9). Understanding the mechanisms of bacterial adhesion to surfaces is crucial for addressing biofilm formation, biofouling, and the development of antimicrobial surface technologies. The current study aims to clarify whether *P. aeruginosa* isolates adhesion to biotic surfaces is similar to their adhesion to abiotic surfaces.

Corresponding *Author:*
genan.atiyah@sc.uobaghdad.edu.iq

Materials and methods

Isolation and Identification: One hundred wound swabs were obtained aseptically from patients suffering from infected wounds. The samples were collected from Baghdad Teaching Hospital, and Al-Yarmouk Teaching Hospital, Baghdad, Iraq (from September 1st to December 24th 2023). The patients had given their written consent to participate in the study. They had not received antibiotics 3 days prior to the sample collection. The swabs were cultured immediately onto the appropriate media (MacConkey agar, blood agar, and Cetrimide agar (Himedia, India)). The Petri dishes were incubated for 48 hours at 37ºC. Biochemical tests such as oxidase and catalase tests were done. The morphological features of bacterial cells were determined by staining with Gram stain. The VITEK 2 DensiCheck instrument, fluorescence system (bioMe´rieux) (ID-GNB card) was used to identify the isolates (10)

Preparation of bacterial standard inoculum: The identified isolates of *P. aeruginosa* (Pa1-Pa10) were grown in Nutrient broth (Himedia, India) at 37ºC for 24 hours. The bacterial cells were washed three times with phosphate buffer saline (PBS (0.01 M, pH 7.1). The final bacterial counts (10^8 c CFU/ml) were prepared in either Muller Hinton Agar (MHA) for the experiment of disc diffusion method measurement or Tryptic Soya broth (TSB) for the experiment of bacterial adhesion and biofilm formation (11).

Disc Diffusion Method: This method was implemented for antimicrobial susceptibility testing. Briefly, standard inoculums of bacterial isolates of *P. aeruginosa* (10^8 CFU/ml) were spread onto Mueller-Hinton agar (MHA) plates. The plates were used for the sensitivity test. Standard commercial antibiotic discs (six discs were put on each plate). The standard antibiotic discs were Ticarcillin (TCC), Cefixime (CFM), Vancomycin (VAN), Erythromycin (ERY), Oxacillin (OXS), Bacitracin (BAC), Amikacin (AMK), Trimethoprim (TMP), Meropenem (MER), Cefamandole (FAM), Novobiocin (NOV), Imipenem (IPM), Amoxicillin (AMX), Cefadroxil (CFR), Levofloxacin (LEV), streptomycin (STR), Norfloxacin (NOR) for all isolates of *P. aeruginosa*. The plate was then incubated for 18 hours at 37°C. The scale was used to measure the inhibition zones (12, 13). The results were interpreted as resistance (R), Intermediate (I), and sensitive (S) according to the CLSI guideline (13).

Isolation of human oral mucosal epithelial cells (OMECs): The human OMECs were isolated from four healthy volunteers (2 males and 2 females, aged 35 to 47 years). They were isolated from the oral mucosa by gently scraping the inner surface of the mouth using sterile woody sticks. The standard method of Ali & Zgair was followed to prepare the standard number of human OMECs (10^5 cells/ml) in sterile PBS (0.01 M, pH 7.1). The trypan blue stain was used to check the number of viable cells. The viability of human OMECs prepared was 91% (4).

Bacterial adhesion to Human OMECs: The method of Al-Mutalib and Zgair, (2023) was followed to measure the number of bacterial cells that adhered to human OMECs *in vitro* (14). Briefly, 100 µl of $5x10^5$ cells/ml of human OMECs that were suspended in PBS (0.01 M, pH 7.1), 10 µl of 10^8 CFU./ml of bacterial cells prepared in TSB, and 90 µl of sterile MHB were put into endotoxin-free micro-centrifuge tubes (2 ml tube, NEST Scientific USA). The tubes were washed four times with the PBS after incubating them for 2 hours at 37°C to remove non-adherent bacteria. 100 µl of the final volume of 200 µl was lysed with 100 µl of PBS−0.5% Triton×100 (Sigma-Aldrich), diluted tenfold and plated onto nutrient agar plates to enumerate the viable bacteria that adhered onto OMECs. The remaining 100 µl was smeared onto glass slides and stained with Leishman's stain. The slides were examined using a light microscope (CH-Olympus, Japan), and images were taken by a smartphone camera above the eyepiece of the microscope. The number of adhered bacteria to one human OMEC calculated by the average of the number of adhered bacteria on 20 human OMEC at different places on the stained slide.

Biofilm Formation: Two hundred µl of sterile Tryptic soy broth (TSB) (HiMedia, India), and 5 µl of 10^8 CFU/ml (corresponding to the 0.26 at 600 nm) of *P. aeruginosa* isolates (cultured previously into TSB) were added into the sterile flat-bottom polystyrene microtiter plates wells (Sigma-Aldrich). The plates were then incubated at 37°C for 24 hours. Subsequently, the non-attached bacterial cells were removed by aspirating the TSB, and then the wells were washed five times with sterile distilled water. The formed biofilm was dried and fixed at 61ºC for 35 minutes. Following this, 220 µl of Hucker crystal violet (0.45%) was added to each well and incubated for 10 minutes at room temperature. After five washes with distilled water and a drying period of 35 minutes at 37°C (Incubator, Memmert, Germany), 220 µl of acetone: ethanol (30:70) was added to the wells. The absorbance was measured at a wavelength of 570 nm using the BioTek 800 microplate reader (Agilent, USA) (14).

Statistical analyses
Origin v. 8 software (OriginLab, Nothampton, USA) was used to do the statistical analysis. The values were expressed as means ± standard error (M ±SE). The Student t-test was used to calculate the group difference. The Pearson's correlation coefficient was used to explore the correlations between variables. A value of *P* less than 0.05 was considered statistically significant.

Results

Bacterial isolates: In the present study ten isolates were isolated and identified (Pa1-Pa10). These isolates were used in the further experiments of the present study.

Antimicrobial susceptibility test: In the present study, the inhibitory zone made by different antibiotic discs against *P. aeruginosa* clinical isolates (Pa1-Pa10) is shown in Table 1. The highest antibacterial effect was seen with Norfloxacin (12 - 35 mm, only Pa 1 was resistant to this antibiotic). All isolates were

resistant to Amoxicillin and Cefixime. Only one isolate was sensitive to Ticarcillin, Erythromycin, Oxacillin, Amikacin, Trimethoprim, Imipenem, and Cefadroxil. There is a high level of resistance to traditional antibiotics.

Table 1: The diameter of the inhibition zone (mm) around the different antibiotic discs for *P. aeruginosa* isolates (Pa1 – Pa10)

Isolates	TCC	CFM	VAN	ERY	OXS	BAC	AMK	TMP	MER	FAM	NOV	IPM	AMX	CFR	LEV	STR	NOR
Pa1	0.0 (R)	0.0 (R)	18 (S)	0.0 (R)	0.0 (R)	8 (I)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	25 (S)	0.0 (R)
Pa2	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	8 (I)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	12 (S)
Pa3	0.0 (R)	0.0 (R)	20 (S)	0.0 (R)	0.0 (R)	9 (S)	24 (S)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	8 (I)	16 (S)	15 (S)	30 (S)
Pa4	0.0 (R)	0.0 (R)	19 (S)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	30 (S)	18 (S)	33 (S)
Pa5	0.0 (R)	0.0 (R)	23 (S)	0.0 (R)	0.0 (R)	8 (I)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	10 (S)	0.0 (R)	0.0 (R)	0.0 (R)	30 (S)	19 (S)	30 (S)
Pa6	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	13 (S)	0.0 (R)	0.0 (R)	0.0 (R)	32 (S)	0.0 (R)	0.0 (R)	20 (S)	16 (S)	27 (S)
Pa7	0.0 (R)	0.0 (R)	0.0 (R)	15 (S)	20 (S)	13 (S)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	21 (S)	0.0 (R)	0.0 (R)	0.0 (R)	24 (S)	18 (S)	35 (S)
Pa8	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	11 (S)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	21 (S)	17 (S)	30 (S)
Pa9	0.0 (R)	0.0 (R)	19 (S)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	19 (S)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	18 (S)	0.0 (R)	27 (S)
Pa10	10 (S)	0.0 (R)	7 (I)	0.0 (R)	0.0 (R)	10 (S)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	8 (I)	0.0 (R)	0.0 (R)	0.0 (R)	21 (S)	16 (S)	25 (S)

S: sensitive; I: Intermediate; R: resistant

Biofilm formation: All isolates were able to form a biofilm. The highest biofilm production was found in isolate Pa2, followed by Pa6, while the lowest biofilm formation was found in isolate Pa4 (Figuer1).
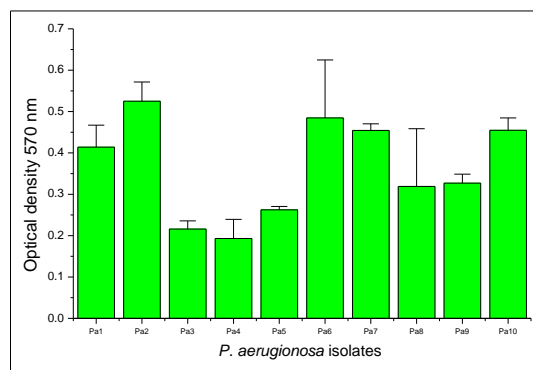

Figure 1: The average biofilm formation of *P. aeruginosa* isolates form infected wounds

Adherence of *P. aeruginosa* to Human OMECs: The adhesion of three isolates of *P. aeruginosa* (Pa2, Pa6, and Pa10, which showed the highest ability to form biofilm) to human OMECs (biotic surface) was tested. Figure 2 shows that the maximum bacterial adherence was seen in isolate Pa6 ($P<0.05$) as compared with Pa 2 and Pa10. A slight difference was seen between the number of bacterial adhesions of Pa 2 and Pa 10 (Figure 2a). When the Leishman's stain technique was used for calculating the number of total bacterial cells adhered to the surface of human OMECs, the highest number of adhered bacteria was seen in isolate Pa 6 followed by Pa10 ($P<0.05$).
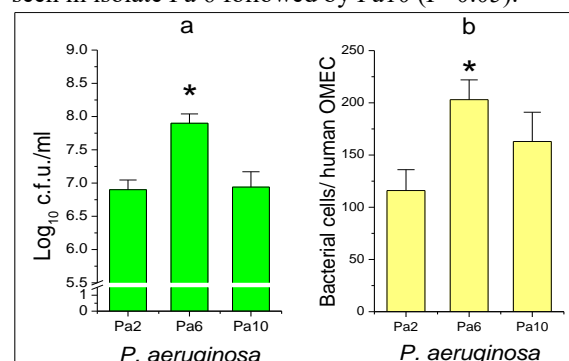
Figure 2: Viable *P. aeruginosa* count (c.f.u./ml) that adhered to human OMECs calculated using plate count method (a)

The asterisk indicates the significant difference from Pa2 and Pa10: The microphotographs of isolate Pa2 attached to the OMEC is shown in Figure 3a. The epithelial cell (Squamous epithelium, OMEC, not exposed to bacterial cells and treated with PBS), appear as large flattened cell with cytoplasm and a small and ellipsoid nucleus. The highest number of adhered *P. aeruginosa* to human OMECs is shown in Figure (3)
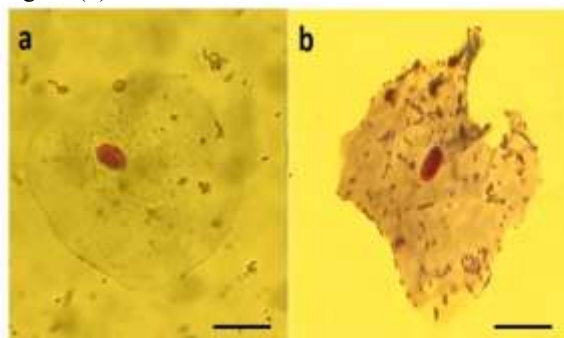

Figure (3): Photomicrographs of human OMEC stained with Leishman's stain and examined under light microscope. a: Human OMEC not treated with *P. aeruginosa*. b: Human OMEC treated with *P. aeruginosa* showing the bacteria adhered to the epithelial surface. The scale bar is 40 µm.

Discussion

The attachment of opportunistic pathogenic bacteria to biotic surfaces such as epithelial cells or abiotic surfaces such as medical plastic devices represents the initial stage in the establishment of biofilms or the invasion of host cells (15). These occasions offer to safeguard the bacteria from the host body's immune system and induce persistent infection. The adhesion of bacterial cells to the mucosal epithelial cells is an essential stage in the procedure of infection, especially in situations of dental infections. The adhesion of bacterial cells to host cells is a diverse procedure that incorporates different mechanisms and factors. An extensive understanding of these communications is essential to designing reliable methods for treating bacterial infections (5).

In the current study, the *P. aeruginosa* isolates showed resistance to a wide spectrum of antibiotics that are routinely used in treating the wound bacterial infection. The study found that *P. aeruginosa* isolates adhered to human OMECs and to polystyrene microtiter plates with high efficiency. The highest bacterial adhesion and formation of a biofilm on polystyrene was seen in the case of Pa 2, while this isolate did not show the highest ability to adhere to living surfaces (human OMECs). The studies addressing the same topic are scanty in the available literature. Previous studies have shown the biofilm formation of *P. aeruginosa* to polystyrene microtiter plate and the adhesion of *P. aeruginosa* to different kinds of human epithelial cells (but not to oral mucosal epithelial cells) (16, 17). However, there was no previous studies addressing the ability of the same isolates of *P. aeruginosa* to adhere to human OMEC and to form biofilm to polystyrene, which endorses the novelty of the current study.

P. aeruginosa appendages, such as flagella, pili, and fimbriae, are involved in their adherence to different types of surfaces (18). These appendages assist in the adhesion of *P. aeruginosa* to semi-solid surface areas along with the change from a motile state of bacteria to biofilm form (19). The appendages serve as added-cellular frameworks that aid in the preliminary accessory of the bacteria adherence to biotic surfaces (20). Additionally, the appendages add to the development of bacteria, like supplying mechanical security and mediating microbial adhesion to surface areas. The bacterial matrix, which consists of polysaccharides, develops a natural three-dimensional network that links and immobilizes microbial cells to form the network of microenvironments of biofilm (20). The role of appendages in adherence to various types of surfaces is not completely comprehended, but the appendages are understood to be crucial for the development as well as determination of *P. aeruginosa* biofilm. The current research showed that the capability of the isolates to adhere differs depending on the surface areas, and they are not just as reliable in adhesion to abiotic as well as biotic surface areas, which validates that the adhesion varies depending on the type of surface areas to which they adhere.

Conclusion

P. aeruginosa isolated from infected wounds showed a high resistance to antibiotics, with adherence to abiotic surface and forming biofilms on polystyrene and adherence to OMECs. The adherence and biofilm formation of *P. aeruginosa* isolates that are resistant to most antibiotics depend on the type of surface to which they adhere.

Authors' contribution

Marwa M. Talib: Conceptualization; Data curation; Investigation; Methodology; Project administration; Roles/Writing -original draft; Supervision; Validation.

Jenan A. Ghafil: Roles/Writing -original draft; Visualization; Writing -review & editing; Formal analysis; Resources.

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الإلتصاق المقارن لبكتيريا *Pseudomonas aeruginosa* بالخلايا الظهارية المخاطية للفم البشري وأسطح البوليسترين

مروة محمد طالب¹ ، جنان أحمد غافل¹*

¹قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق.

الخلاصة

الخلفية: إن إلتصاق البكتيريا بالأسطح الحية وغير الحية يعكس قدرتها على التسبب في الأمراض المعدية. إن التمييز بين قدرة بكتيريا *Pseudomonas aeruginosa* على الالتصاق بالأسطح الحية وغير الحية ليس واضحا بشكل تام في الأدبيات الطبية.

الأهداف: تهدف الدراسة الحالية إلى تسليط الضوء على مدى أوجه التشابه والإختلاف بين أنواع *P. aeruginosa* من حيث قدرتها على الإلتصاق بالأسطح الحية وغير الحية.

المنهجية: تم عزل عشر عزلات من بكتيريا *P. aeruginosa* من 100 مسحة من الجروح. تم تشخيص العزلات باستخدام الإختبارات الكيموحيوية والمظهرية بالإضافة إلى تقنية VITIK-II. وتم تقدير الإستجابة للمضادات الحيوية باستخدام طريقة إنتشار القرص. تم استخدام صفائح المعيارية الدقيقة-الطيفي لقياس تكوين الأغشية الحيوية على ألواح الصفائح الدقيقة البوليسترينية. تم استخدام الظهارة المخاطية للفم البشري (OMECs) لتقييم إلتصاق عزلات *P. aeruginosa* وتم استخدام عدد البكتريا على الأطباق بعد تخفيفها والعد البكتيري الكلي المباشر لحساب البكتيريا الملتصقة بـ OMECs في المختبر.

النتائج: أظهر النورفلوكساسين أعلى تأثير مضاد للجراثيم بينما لوحظ أقل تأثير مضاد للجراثيم عند استخدام أموكسيسيلين وسيفيكسيم (جميع العزلات كانت مقاومة). شكلت جميع العزلات من Pa2 أعلى غشاء حيوي يليه Pa6، في حين شوهد أقل تكوين للبيوفيلم في حالة Pa4. بينما أظهر Pa 6 أعلى قدرة على الإرتباط بـ OMECs البشرية، يليه Pa2.

الإستنتاج: أن عزلات *P. aeruginosa* مقاومة لمعظم المضادات الحيوية وأن قدرتها على الإلتصاق وتكوين الأغشية الحيوية تعتمد على نوع السطح.

الكلمات المفتاحية: الإلتصاق، الحساسية للمضادات الحيوية، تكوين الأغشية الحيوية،]، الزائفة الزنجارية (*Pseudomonas aeruginosa*).