Investigating the Biofilm Formation of Some Pathogenic Bacterial Isolates

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Abstract

A total of 35 bacterial isolates were collected from Al-Kadhimiya Teaching Hospital between December 2015 and March 2016, sourced from various clinical cases. The isolates were cultured on blood and MacConkey media to facilitate bacterial isolation and identification, , which were further confirmed using microscopic and biochemical tests.

Eight bacterial genera were identified with diferent numbers of isolates: ***Pseudomonas aeruginosa*** (6 isolates), ***Enterobacter* sp**. (6 isolates), ***Acinetobacter baumannii*** (2 isolates), ***Staphylococcus aureus*** (7 isolates), ***Staphylococcus epidermidis*** (1 isolate), and ***Escherichia coli*** (8 isolates**).** ***Escherichia coli* type**1 (1 isolate) ***Klebsiella pneumoniae*** (4 isolate). Antibiotic resistance testing revealed significant resistance among the isolates, with all exhibiting resistance to Carbenicillin and Cloxacillin. Notably, only one isolate demonstrated resistance to Imipenem, which was the most effective antibiotic in this study. Biofilm formation, a critical factor contributing to bacterial pathogenicity and resistance to host defenses and antibiotics, was also evaluated using the Congo Red agar test. All isolates of *Klebsiella pneumoniae* and *Enterobacter sp.*, also, 6 of the 7 isolates of *Staphylococcus aureus*, and, 5 of the 8 isolates of *Escherichia coli* demonstrated positive biofilm formation

Keywords: Bacterial isolates, Antibiotic sensitivity, Biofilm formation and Clinical samples

التحري عن الأغشية الحيوية لبعض العزلات البكتيرية المسببة للأمراض

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الخلاصة

جمعت ٣٥ عزلة بكتيرية من مستشفى الكاظمية التعليمي خلال الفترة (كانون اول 2015 - اذار ٢٠١٦) من حالات مرضية متنوعة. زرعت العزلات على اوساط الدم ووسط *MacConkey* لغرض تسهيل عزل وتشخيص البكتيريا ، واستكملت الفحوصات باستخدام الاختبارات الميكروسكوبية والكيميائية الحيوية. حددت ثمانية أجناس بكتيرية ضمت عدد من العزلات وهي *Pseudomonas aeruginosa* (٦ عزلات)، و *Enterobacter* sp. (٦ عزلات)، و *Acinetobacter baumannii* (عزلتين)، و *Staphylococcus aureus* (٧ عزلات)، و *Staphylococcus epidermidis* (عزلة واحدة)، و *Escherichia coli*. (8) عزلات و *Escherichia coli* type 1 ( ). (عزلة واحدة) (*Klebsiella pneumoniae* (٤ عزلات) . أظهرت نتائج اختبارات مقاومة المضادات الحيوية مقاومة ملحوظة بين العزلات، حيث أظهرت جميعها مقاومة للمضادين *Carbenicillin* و *Cloxacillin* . وأن عزلة واحدة فقط أظهرت مقاومة لعقار *Imipenem*، الذي كان الأكثر فعالية في الدراسة. درست قدرة العزلات على تكوين الأغشية الحيوية، نظرًا لأهميتها في زيادة ضراوة البكتيريا ومقاومتها لآليات الدفاع في الجسم وللمضادات الحيوية. أظهرت نتائج اختبار الكونغو الأحمر أن جميع عزلات *Klebsiella pneumoniae* و*Enterobacter sp.*، وستة من اصل سبعة عزلات من *Staphylococcus aureus*، بالاضافة الى خمسة من اصل ثمان عزلات من *Escherichia coli* كانت إيجابية لتكوين الأغشية الحيوية.

الكلمات المفتاحية: العزلات البكتيرية، حساسية المضادات الحيوية ،تكوين الغشاء الحيوي و عينات سريرية.

Introduction

Biofilm is an aggregation of microscopic organisms with their extracellular secretions. This aggregation takes many forms, either together in the form of aggregates, or they stick to a surface, such as their adhesion to the surface of a rock, to the surface of teeth, or to the surface of a pond, in tiny spaces called interfaces. (Donlan and Costerton, 2002, Zhao *et al.*, 2023). Biofilms consist of a single species or multiple species, and the microorganisms within a biofilm differ phenotypically from their members in suspension despite being genetically similar.Cells within a biofilm respond to the diffusion gradient of nutrients and metabolic products, direct their metabolism according to their functional location within the biofilm community, and participate in cell-cell communication (Cvitkovitch *et al.*, 2003, Mirghani *et al.*, 2022).Changes occur in the chemical and physical properties of the biofilm, as well as in the density of its microorganisms, depending on the following variables: First: The degree to which the biofilm accepts nutrients (Nutrient uptake). Second: The degree of movement of compounds through the biofilm, gases, and antimicrobial agents (Muthu, 2006, Sharma *et al*., 2023).

One of the most important features that distinguish a biomembrane from suspended cells is that its cells are embedded in a dense floor of extracellular polymeric materials (Extracellular Polymeric Substances (EPS) Matrix). The components of EPS vary depending on the organism present and the environmental conditions. Under appropriate conditions, cells affect The surrounding environment chemically and physically secretes specific biological macromolecules, and this environment, in turn, harbors components of non-living origin such as: non-specific biomolecules of litter derived from cell decomposition (Sutherland, 2001, Kaur and Dey, 2023).

There is a close relationship between the ability of bacteria to form a biofilm and their ability to cause disease and cause chronic inflammation. The bacteria that form the biofilm have a greater ability to colonize and reside in the patient’s body and become less sensitive to treatment with antibiotics(Zhao *et al.*, 2023).

Proteins known as Biofilm-Aassociated Protiens, or "BAP," are produced by bacteria in response to infection. The presence of these proteins (BAP) promotes the development of the biofilm linked to the germ's prolonged survival in the patient's body. The infected body reacts to these proteins immunologically, and antibodies are detected in response to them (Cucarella, *et al*., 2004, Zafer *et al.*, 2024).

Materials and Methods

Preparing the Cultivation Media (Blood Agar Media)

Blood agar was prepared, then left to cool to 50 degrees, and 7% of human

blood was added to it, then poured into sterile petri dishes and left to cool (Collee, *et al.*, 1996).

Congo Red Agar Medium

It was prepared by dissolving 37 grams of brain heart infusion medium, 50 grams of sucrose, and 15 grams of agar in 900 milliliters of distilled water, sterilized with an autoclave, and left to cool to 60 degrees Celsius, then mixed with the cango red dye solution (prepared by dissolving 0.8 grams of cango red dye in 100 milliliters of distilled, autoclaved water, pour into sterilized dishes and leave to cool (Freeman *et al.*, 1989).

Study design and specimen collection

The study was conducted from period from December 2015 to March 2016 in Baghdad / Iraq a total of 35 samples. All clinical specimen were collected from participantes attending to Al-Kadhimiya Teaching Hospital. Clean catch midstream urine specimens were obtained, using sterial disposatable class(5 mm) to avoid contamination. Blood samples were taken from pationt aspetically in blood culture tubles in addition wound nasd speciment samples were taken from sterid cottan swabs. Bacterial isolates were grown on blood agar and MacConkey agar and incubated at 37°C for 24 hours

Diagnosis of Isolates

Agricultural Characteristics

Bacterial isolates were initially identified based on the phenotypic characteristics of the colonies' shape, texture, and color.

Diagnosis with the ready-made kit: Vitek2 compact ACT

The Vitech device is considered one of the best devices to identify types of bacteria within a short period and very accurately. It includes diagnosis using (Card). The device determines the type of bacteria by conducting biochemical tests automatically in the device without the need to conduct any external tests. It identifies this device detects gram-positive and gram-negative bacteria, in addition to examining the sensitivity to antibiotics. It only recognizes live cells. The size of the device is small and the number of samples it can accommodate in one session is 30 (30 identification cards and 30 sensitivity cards).

Testing for Bacterial Susceptibility to Antibiotics

Young colonies were transferred to heart and brain broth and incubated for two hours at 37°C to obtain a turbidity standard equal to the turbidity of a Macfarland tube (0.5 turbidity standard), which gives a cell count approximately (1.5 * 108 cells/ml), taken from the bacterial culture with a cotton swab. Spread well and evenly on Mueller-Hinton agar and leave for 10 minutes, then the antibiotic tablets were placed using sterile forceps and pressed slightly to ensure contact between the agar and the tablets. The plates were incubated for 24 hours at a temperature of 37 degrees Celsius, then the results

were recorded by measuring the diameter of the inhibition zone formed around each tablet in millimeters if it appeared.

Detection of Biofilm Production with the Congo Red Test

The isolates were grown on congo red medium and left in the incubator at 37°C for 24 hours. The ability of the isolates to form a biofilm was examined by forming a black precipitate. while the colonies appeared in a pink or red color indicating that it is not productive for biofilms

Results and Discussion

The results of this study reveals that 35 bacterial isolates were collected from Al-Kadhimiya Teaching Hospital in Baghdad from different patients samples which included the bacterial isolates listed in Table 1 bellow:

Cultivation and Diagnosis of Isolates

The isolated samples were grown on stimulant and differentiation media, in addition to microscopic tests and examination with ready-made kits. *Pseudomonas aeruginosa* appeared to be a gram-negative bacillus, and its colonies were characterized by being flat and irregular in shape. They gave off an odor resembling that of fermenting grapes while producing a bluish-green dye. These bacteria were completely hemolytic on blood media, but in MacConkey medium their colonies were pale because they were not fermented for lactose.While both *Staphylococcus aureus* and *Staphylococcus epidermids* were characterized as being gram-positive in the form of clusters under the microscope, they did not grow on MacConkey medium because it contains bile salts that inhibit gram-positive bacteria. As for its colonies, *Staphylococcus aureus* was

Table1: Isolates from different speciment

No.	Bacterial isolates	Type of speciment	Frequency	Biofilm format
1	*Enterobacter sp.*	*Urine*	**4**	+
2	*Enterobacter sp.*	*wound*	**2**	+
3	*Escherichia coli*	*Urine sore swab*	**5**	+
4	*Escherichia coli*	*bed*	**3**	-
5	*Escherichia.coli type1*	*Urine*	**1**	-
6	*Pseudomonas aeruginosa*	*Ear sweb*	**2**	-
7	*Pseudomonas aeruginosa*	*wound*	**3**	-
8	*Pseudomonas aeruginosa*	*blood*	**1**	-
9	*Klebsiella pneumoniae*	*Urine*	**2**	+
10	*Klebsiella pneumoniae*	*Seputum swab*	**2**	+
11	*Staphylococcus aureus*	*Nasal Swab*	**2**	+
12	*Staphylococcus aureus*	*blood*	***2***	+
13	*Staphylococcus aureus*	*wound*	***3***	-
14	*Staphylococcus epidermids*	*wound*	***1***	-
15	*Acinetobacter baumannii*	*Seputum swab*	***2***	-

characterized as being medium to large, smooth, and highly pigment-forming. It was golden in color and was surrounded by an area of complete decomposition on the blood medium, It is characterized by being a fermenter of manthol sugar, so the color of the manthol medium turns yellow. These bacteria are distinguished by being a fermentation of manthol sugar, so the color of the mantol medium turns yellow. While the colonies of *Staphylococcus epidermids* are characterized by being small, white, that do not form pigments, are non-hemolytic, and are not fermented for the sugar mannitol, so they do not turn the color of the mannitol medium yellow. As for the colonies of *E.coli*, they are fermented for the sugar lactose. They are flat, dry, pink and are short, Gram-negative bacilli. As for the *E.coli* type1 bacteria, they are characterized by the same characteristics, except that their colonies appear pale in color and are characterized by the fact that their cells are equipped with cilia when examined under an electron microscope. *Klebsiella pneumoniae* is gram negative, its colonies are pink and mucous on the MacConkey medium. As for the colonies of *Acinetobacter baumannii* bacteria, they were relatively large, white, mucous in color on the MacConkey medium, and negative for Gram stain.

Policy Screening of Antibiotics

The importance of antibiotic susceptibility testing comes to investigating the extent of resistance of bacterial isolates to antibiotics. Detailed testing of the isolated bacterial isolates was performed for a number of antibiotics, as shown in Table 2.

The sensitivity to a number of antibiotics was tested, distributed among the beta-lactam group antibiotics, which included Cefoxitin, Ceftriaxone, and Imipenem, and from the amino glycoside group Amikacin, Gentamicin, and the macrolides Nitrofurantoin, and from the penicillin group Cloxacillin, Aztreonam, Amoxicillinl clavulanic acid, and Carbenicillin. All bacterial isolates showed resistance to Carbenicillin and Cloxacillin, while they varied in their resistance to the rest of the antibiotics, with very low resistance rates, as shown in Table1 While the most effective antibiotic was Imipenem, as only one isolate showed resistance to this antibiotic.

Amonng 35 bacteria isolates from Patogenic bacteria. Only (54.3%) isolates were biofilm produces as illustrated by Congo Red agar

Table 2 Percentage amount of Biofilm production

Biofilm production	Congo Red agar %
Strong positive	**28.6**
Modeurat positive	**14.3**
Week positive	**11.4**
Negative	**45.7**

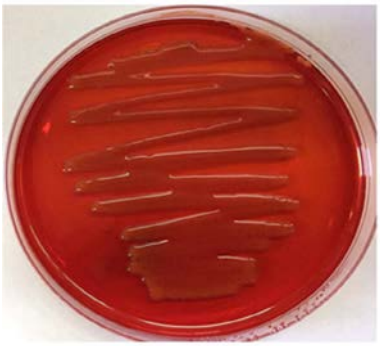
Biofilm Investigation

The ability of bacterial isolates to form biofilm was investigated by Congo Red Agar method. It was observed that the isolates forming the biofilm were colored black indicating the productivity

of the biofilms, while the colonies appeared in a pink or red color indicating that is not producing and biofilms

The results were illustrated in Fig.1 for forming biofilm:

negative bacteria isolate



positive bacteria isolate

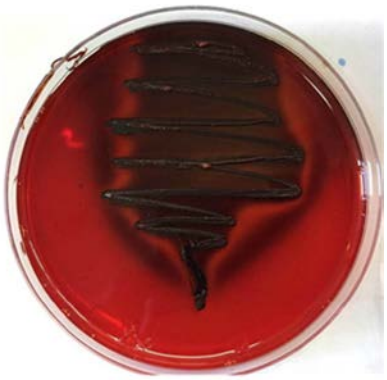


Figure1: The results of the Congo red test. *Pseudomonas aeruginosa* was negative for this test while *Staphylococcus aureus* bacteria formed a black precipitate indicating biofilm formation .

Table 3: Sensitivity of bacterial isolates to antibiotics.

Antibiotic / Bacterial Isolation		Nitrofurantoin	Carbencillin	Cefixime	Aztreonam	Imipenem	Amikacin	Cloxacillin	Ceftriaxone	amoxicillin-clavulanate
1	*Klebsiella pneumoniae* 1	R	R	R	R	S	R	R	R	R
2	*Klebsiella pneumoniae* 2	R	R	R	R	S	R	R	R	R
3	*Klebsiella pneumoniae* 3	R	R	R	R	S	R	R	R	R
4	*Klebsiella pneumoniae* 4	S	R	R	R	S	R	R	S	S
5	*Staphylococcus aureus*1	S	R	R	R	S	S	R	S	S
6	*Staphylococcus aureus*2	R	R	S	R	S	R	R	S	R
7	*Staphylococcus aureus*3	R	R	R	R	S	R	R	S	R
8	*Staphylococcus aureus*4	R	R	R	R	S	R	R	S	R
9	*Staphylococcus aureus*5	R	R	R	R	S	S	R	R	R
10	*Staphylococcus aureus*6	R	R	R	R	S	R	R	R	R
11	*Staphylococcus aureus*7	R	R	R	R	R	R	R	R	S
12	*Staphylococcus epidermids*	S	R	R	R	S	R	R	S	S
13	*Enterobacter sp*1	R	R	R	R	S	S	R	R	R
14	*Enterobacter sp.*2	R	R	R	R	S	R	R	R	R
15	*Enterobacter sp.*3	R	R	S	R	S	S	R	R	R
16	*Enterobacter sp.*4	R	R	R	R	S	S	R	R	S
17	*Enterobacter sp.*5	R	R	R	R	S	S	R	R	R
18	*Enterobacter sp.*6	R	R	R	R	S	R	R	R	R
19	*Escherichia coli*1	R	R	R	R	S	R	R	R	R
20	*Escherichia coli*2	R	R	R	R	S	R	R	R	R

Table 3: Sensitivity of bacterial isolates to antibiotics.

	Antibiotic / ***Bacterial Isolation***	**Nitrofurantoin**	**Carbencillin**	**Cefixime**	**Aztreonam**	**Imipenem**	**Amikacin**	**Cloxacillin**	**Ceftriaxone**	**amoxicillin-clavulanate**
21	*Escherichia coli*3	R	R	R	R	S	R	R	R	R
22	*Escherichia coli*4	R	R	R	R	S	S	R	R	S
23	*Escherichia coli*5	R*	R	R	R	S**	S	R	R	R
24	*Escherichia coli*6	R	R	R	R	S	R	R	R	R
25	*Escherichia coli*7	R	R	R	R	S	R	R	R	R
26	*Escherichia coli*8	R	R	R	S	S	S	R	R	R
27	*E-coli type 1*	R	R	R	R	S	R	R	R	R
28	*Pseudomonas aeruginosa*1	R	R	R	R	S	R	R	R	R
29	*Pseudomonas aeruginosa* 2	R	R	R	R	S	S	R	R	R
30	*Pseudomonas aeruginosa*3	R	R	S	R	S	S	R	R	R
31	*Pseudomonas aeruginosa*4	R	R	R	R	S	R	R	R	R
32	*Pseudomonas aeruginosa*5	R	R	R	R	S	R	R	R	R
33	*Pseudomonas aeruginosa*6	R	R	R	R	S	R	R	R	R
34	*1Acinetobacter baumannii*	R	R	R	S	S	S	R	R	R

*R= Resistance

**S= Sensitive

By observing the results of biofilm production, it was found that some species belonging to the same gene differ in their ability to produce biofilm, as Arvanati *et al.* (1994) stated that the chemical composition of the biofilm differs between species of bacteria in general and even between species belonging to the same family, as each bacteria has its own structure, and peptides may interfere with these membranes and cause disruption in their structure.

The results also showed that some amino acids showed inhibitory activity on the formation of the bacterial biosphere (Muzaffar *et al*; 1997, Warraich *et al.*, 2020).These results were similar to the results of Al-Ukaili *et al.* (2014), where their results showed 90% of bacterial isolates producing biofilm and 10% not producing.

By observing the results of biofilm production by the bacteria under study, we notice that most of the isolates were biofilm producers.

By observing the results of Table 3 of antibiotics resistance and comparing it to the results of Table 2 of biofilm production and the results of Table 1, the study observed a clear association between biofilm formation and increased bacterial resistance to antibiotics. Biofilm-producing bacteria exhibited reduced sensitivity to antibiotics, which makes it resistant and also causes infections that are difficult to treat (Costerton *et al*., 1999; Otoole and Mah, 2011, Shree *et al*., 2023).

These findings underscore the clinical challenge posed by biofilms, as their protective matrix enhances bacterial survival under antibiotic pressure. Future research should explore anti-biofilm therapies to improve treatment outcomes.

Conclusions

The current study finding the following:

1- Antibiotic resistance was detected in the majority of bacterial isolates.

2- All isolates exhibited complete resistance to both Cloxacillin and Carbenicillin, underscoring the limited efficacy of these antibiotics in treating infections caused by the tested bacteria.

3- Despite Imipenem being the most effective antibiotic tested, demonstrating potent activity against most bacterial isolates, resistance was still observed in one isolate.

4- This finding emphasizes the need for continuous monitoring and judicious use of antibiotics to prevent the emergence and spread of resistant strains.

5- The results underscore the importance of integrating biofilm detection and antibiotic susceptibility profiling in the clinical management of infections.

6- By understanding the interplay between biofilm formation and antibiotic resistance, clinicians can devise more effective treatment strategies, potentially incorporating biofilm-disrupting agents or combination therapies to enhance the efficacy of antibiotics and combat resistant infections

References

Anwar, H. ; Dasgupta, M.K. and Costerton, J.W.(1990). Testing the Susceptibility of Bacteria in Biofilms to Antimicrobial Agents. *Antimicrob. Agents Chemother.* 34, 2043-2046 .

Anwar, H.; Dasgupta, M.K.; Lam, K. and Costerton, J.W. (1989) Tobramycin Resistance of Mucoid *Pseudomonas aeruginosa* Biofilm Growth under Iron Limitation. *Antimicrob. Agents Chemother.* 24, 647-655.

Arvanati, A., N. K. Karamanos., G. Dimitracopoulos and E. D. Anastassiou.1994. Isolation and Characterization of A Novel 20 kDa Sulfated Polysaccharide from the Extracellular Slime Layer of *Staphylococcus epidermidis*. Arch. *Biochem. Biophys*. 308:432–438.

Bavington, C. and Page, C. (2005) Stopping Bacterial Adhesion: A Novel Approach to Treating Infections. *Respiration.* 72, 335-344.

Beloin, C. ; Valle , J. ; Latour-Lambert, P. ; Faure, P. ; Kzreminski, M. ; Balestrino, D. ;Haagensen, J. ; Molin, S. ; Prensier, G. ;Arbeille, B. and Ghigo, G.(2004) Global Impact of Mature Biofilm Lifestyle on *Escherichia coli* K12 Gene. *Respiration.* 71, 433-463

Costerton, J.W. (1988) Structure and Plasticity at Various Organization Levels in the Bacterial Cell. *Can. J. Microbiol.* 34, 513-521.

Costerton, J.W. ; Stewart, P.S. and Greenberg, E.P. (1999). Bacterial Biofilms: A Common Cause of Persistent Infections. *Science* 284, 1318-1322.

Cucarella , C . ; Tormo ,M. A. ; Ubed , C. ; Trotonda , M .P. ; Monzon , M. ; Peris , C. ; Amorena , B. ;Lasa , I . and Penades , J.R. (2004) Role of Biofilm-Associated Protein Bap in the Pathogenesis of Bovin *Staphylococcusaureas .infect. Immun.* 72 (4) , 2158 – 2177.

Cucarella, C. ; Tormo, M.A. ; Ubed, C. ; Trotonda, M.P. ; Monzon, M. ; Peris, C. ; Amorena, B. ; Lasa, I. and Penades, J.R. (2004) Role of Biofilm-Associated Protein Bap in the Pathogenesis of Bovine *Staphylococcus aureus*. *Infect. Immun.* 72(4) , 2177-2158.

Cvitkovitch, D.G. ; Li, Y.H. and Ellen, R.P. (2003). Quorum Sensing and Biofilm Formation in Streptococcal Infections. *J.Clin. Invest.* 112, 1626-1632.

Dankert, J. ; Hogt, A.H. and Feijen, J. (1986). Biomedical Polymers : Bacterial Adhesion , Colonization, and Infection. *CRC Critical Reviews in Biocompatibility* 2 , 219-301.

Davey, M.E. and Otoole, G.A. (2000). Microbial Biofilms: From Ecology to Molecular genetics. *Microbiol. Mol. Biol. Rev.* 64, 847-867.

Deka, N. (2014). Comparison of Tissue Culture plate method, Tube Method and Congo Red Agar Method for the Detection of Biofilm Formation by Coagulase Negative *Staphylococcus* Isolated from Non-clinical Isolates. *Int.J.Curr.Microbiol.App.Sci.* 3(10), 810-815.

Donlan , R. M. and Costerton , J. W.Biofilms (2002). Survival Mechanisms of Clinically Relevant Microorganisms. *Clin .microbial .Rev.* 15 , 167- 193.

Ford, T.E. (1999). Microbiological Safety of Drinking Water: United State and Global perspectives. Environ. *Health Prespect.* 107, 191-206.

Freeman, D.J. ; Falkiner, F.R. and Keane, C. T. (1989). New Method for Detecting Slime Production by Coagulase Negative Staphylococci. *J. Clin. Pathol.* 42,872-874.

Gottenbos, B. ; Henny, C. ; Van der Mei and Henk, J.B.(1999). Models for Studying Initial Adhesion and Surface Growth in Biofilm Formation on Surfaces. *Methods in Enzymology*. 310,523-33.

Haussler, S. ; Ziegler, I. ; Lottel, A. ; Gotz, F. ; Rohde, M. ; Whemhohner, D. ; Saravanamutha, S. ; Tummler, B. and Steinmetz, I. (2003). Highly Adherent Small-Colony Variants of *Pseudomonas aeruginosa* in Cystic Fibrosis Lung Infection. *J. Med. Microbiol.* 52, 295-301.

Harvey, J., Keenan, K. P. and Gilmour, A.(2007) Assessing Biofilm Formation by *Listeria monocytogenes* Strains. *Food Microbio.* 24(4), 380–392.

Justice, S.S. ; Hung, C. ; Theriot, J.A. ; Fletcher,D.A. ; Anderson, G.G. ; Footer, M.J. and Hultgren, J.(2004) Differentiation and Development Pathways of Uropathgenic *Escherichia coli* Urinary Tract Pathogenesis. *Proc. Natl. Acad. Sci.* USA 101:1333-1338

Kaplan, J.B. ; Meyenhofer, M.F. and Fine, D.H. (2003). Biofilm Growth and Detachment of *Actinobacillus actinomycetemcomitans*. *J. Bacteriol.* 185,1399-1404.

Kaur, N, Dey P. (2023). Bacterial Exopolysaccharides as Emerging Bioactive Macromolecules: from Fundamentals to Applications, Research in Microbiology,174, (4).

Keren, I. ; Kaldalu, N. ; Spoering, A. ; Wang, Y. and Lewis, K. (2004). Persister Cells and Tolerance to Antimicrobials. *FEMS Microbiol. Lett.* 230, 13-18.

Khoury, A.E. ; Lam, K. ; Ellis, B. and Costerton, J.W. (1992). Prevention and Control of Bacterial Infections Associated with Medical Devices. ASAIO J. 38: M174-M178.

Kristich, C.J. ; Li, Y.H. ; Cvitkovitch, D.G. and Dunny, G.M. (2004). ESP-Independent Biofilm Formation by *Enterococcus faecalis* . *J. Bacteriol.* 186 ,154-163.

Mah, T. F., and G. A. O'Toole (2001) Mechanisms of Biofilm Resistance to Antimicrobial Agents. *Trends Microbiol*. 9, 34–39.

Mendelman, P.M. ; Smith, A.L. ; Levy, J. ; Wber, A. ; Ramsey, B. and Davis R.L. (1985). Aminoglycoside Penetration, Inactivation, and Efficacy in Cystic Fibrosis Sputum. *Am. Rev. Respir. Dis.* 132, 761-765.

Mirghani R, Saba T, Khaliq H, Mitchell J, Do L, Chambi L, Diaz K, Kennedy T, Alkassab K, Huynh T, Elmi M, Martinez Sawan S, Rijal G.(2022). Biofilms: Formation, Drug Resistance and Alternatives to Conventional Approaches. AIMS Microbiol. 8(3), 239-277.

Muzaffr, H., H. Mathias., V.E. Christof., P.R. Francoise and P. Gerog.1997. A140-Kilodalton Extracellular Protein is Essential for the

of *Staphylococcus epidermidis* Strains on Surface. *Infection and immunity* . 65(2):519–524.

Nickel, J.C. ; Heaton, J. ; Morales, A. and Costerton, J.W. (1986). Bacterial Biofilm in Persistent Penile Prosthesis – Associated Infection. *J. Urol.* 135, 586-588.

Sauer, K. ; Camper, A. K. ; Ehrich, G.D. ; Costerton, J.W. and Davies, D.G. (2002). *Pseudomonas aeruginosa* Displays Multiple Phenotypes During Development As A Biofilm. *J. Bacteriol.* 184, 135-138.

Sharma S, Mohler J, Mahajan SD, Schwartz SA, Bruggemann L, Aalinkeel R.(2023). Microbial Biofilm: A Review on Formation, Infection, Antibiotic Resistance, Control Measures, and Innovative Treatment. Microorganisms.11(6), 1614.

Shree, P, Singh, C.K., Sodhi, K. S., Surya, J.N., Singh, D. K.(2023). Biofilms: Understanding The Structure And Contribution Towards Bacterial Resistance in Antibiotics, Medicine in Microecology, 16.

Singh, P.K. ; Schaefer, A.L. ; Parsek, M.R. ; Moninger, T.O. ; Welsh, M.J. and Greenberg, E.P. (2000). Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 407: 762-764.

Spoering, A.L. and Lewis, K. (2001). Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J. Bacteriol.* 183: 6746-6751.

Stewart, P.S. (2002) Mechanisms of antibiotic resistance in bacterial biofilms. *Int. J. Med. Microbiol.* 292: 107-113.

Stewart, P.S. and Costerton, J.W. (2001). Antibiotic resistance of bacteria in biofilms. *Lancet.* 358: 135-138.

Sufya, N. ; Allison, D.G. and Gilbert, P. (2003). Clonal variation in maximum specific growth rate and susceptibility towards antimicrobials. *J. Appl. Microbiol.* 95: 1261-1267.

Sutherland, I. (2001). Biofilm exopolysaccharides : A Strong and Sticky Framework. *Microbiol.* 147, 3-9.

Toledo-Arana, A. ; Valle, J. ; Solano, C. ; Arrizubieta, M.J. ; Cucarella, C. ; Lamata, M. ; Amorena , B. ; Lieva, J. ;Penddes, J.R. and Losa, I. (2001). The Enterococcal Surface Protein, Esp, is Involved in *Enterococcus Faecalis* Biofilm Formation. *Appl. Environ. Microbiol.* 67, 4538-4545.

Van Oss, C.J. (1994). Polar or Lewis-base interactions. In: *Interfacial forces in aqueous media* . (Van Oss, C.J. ed.) Marcel Dekker, New York. P.18-46.

Vieira, M.G. ; Melo, L.F. and Pinheiro, M.M. (1993). Biofilm formation : Hydrodynamic Effects On Internal Diffusion and Structure. *Biofouling* 7, 67-80.

Walters, M.C. ; Roe, F. ; Bugnicourt, A. ; Franklin, M.J. and Stewart, P.S. (2003). Contributions of Antibiotic Penetration, Oxygen Limitation, and Low Metabolic Activity to Tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob. Agents Chemother.* 47, 317-323.

Warraich A.A, Mohammed A.R, Perrie Y, Hussain M, Gibson H, Rahman A. (2020). Evaluation of Anti-Biofilm Activity of Acidic Amino Acids and Synergy with Ciprofloxacin on Staphylococcus Aureus Biofilms. Sci Rep. 10 (1), 9021. doi: 10.1038/s41598-020-66082-x.

Zafer, M.M., Mohamed, G.A., Ibrahim, S.R.M. (2024). Biofilm-Mediated Infections by Multidrug-Resistant Microbes: A Comprehensive Exploration and Forward Perspectives. *Arch Microbiol* . 206, 101 https://doi.org/10.1007/s00203-023-03826-z

Zhao A, Sun J, Liu Y.(2023). Understanding bacterial biofilms: From Definition to Treatment Strategies. Front Cell Infect Microbiol. 13:1137947. doi: 10.3389/fcimb.2023.1137947