ISSN (Online) = 2522-6754 ISSN (Print) = 2522-6746
WORLD JOURNAL OF BIOLOGY AND BIOTECHNOLOGY
Review Manuscript www.sciplatform.com

Biofilm formation and its regulation by extracellular appendages in *Pseudomonas aeruginosa*

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Authors' Contribution	**Ramzan, A. and T. Rehman** conducted the review of literature, **M. Sohail** organized the references **M. Imran and U. Qaisar** wrote and reviewed the manuscript.
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Review Proccess: Peer review	
Digital Object Identifier (DOI) Number:	**https://doi.org/10.33865/wjb.007.03.0516**

ABSTRACT

Pseudomonas aeruginosa is an infectious rod like bacterium which is equipped with a unique quorum-sensing system to sense the surrounding environment and communicate with bacterial cells in vicinity through excreting small molecules, called autoinducers. Quorum sensing system and autoinducer production are cellular density dependent mechanisms which are responsible for cell-cell signalling and regulating biological processes in this bacterium. *P. aeruginosa* is equipped with an arsenal of virulence factors and tools to protect it from toxins and host immune response which facilitate its survival in a wide range of habitats and environments. The most important virulence factor is biofilm which comprises of sessile community of bacteria covered with a matrix of polysaccharides and extracellular proteins offering enhanced resistance to antibiotics and host immune system. *P. aeruginosa* produces biofilms at a variety of infection sites in patients, on medical instruments and medical tools inside the patient body. It is very hard to eradicate and inhibit the formation of biofilms in immunocompromised patients. In this article, we described the stages of biofilm formation along with the role of various genetic and biochemical factors required for its formation. Special emphasis was given to extra-cellular appendages (flagella, pili and fimbriae) for their role in attachment of *P. aeruginosa* to the semi-solid serfaces and converting motile bacteria to sessile mode of life (biofilm). Moreover, polysaccharide matix formation and its resistance towards antibiotics is discussed. This review article can help in devising gene therapy strategies against biofilm formation and its eradication in nosocomial infections caused by drug resistant bacteria.

Keywords: Antibiotic resistance; biofilm; *Pseudomonas aeruginosa*; polysaccharides; quorum sensing.

INTRODUCTION: *Pseudomonas aeruginosa*, an opportunistic pathogen is gram-negative, non-fermenting and motile bacterium which causes nosocomial infections (Gale *et al.*, 2015). This ubiquitous bacterium is involved in lethal diseases in immune-deficient individuals which include patients with surgical or burn wounds, cancer, post-surgery, and patients affected with human immunodeficiency virus (HIV) (Driscoll *et al.*, 2007; Gomila *et al.*, 2018). This pathogenic bacterium induces serious diseases in humans' e.g. Urinary tract infections, cystic fibrosis, gastrointestinal infections, otitis media and bacteremia etc. Sometimes these diseases result in high morbidity and mortality rates. When life expectancy of immune-deficient people increased in several countries in 21st century, *Pseudomonas sp*. significantly increased hospital infections. This multifaceted bacterium grows in diverse environmental conditions i.e., aerobic, and non-aerobic conditions (Schurek *et al.*, 2012). The *P. aeruginosa* was considered as lethal bacterium in 2017 and was recorded as priority pathogen by World Health Organization for Research and production of new antibiotics for its inhibition (WHO 2017). Mortality rate by *P. aeruginosa* is extremely high due to adaptability and intrinsic resistance to antibiotics (Pang *et al.*, 2019). Biofilm confers 1000 times higher resistance than planktonic cells of the same bacterial strain (Garrett, 2015).

The *Pseudomonas aeruginosa,* is non-fermenting bacterium which is present everywhere on earth. *P. aeruginosa* is a member of ill-famed class of ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa,* and *Enterobacter species*), which are eminent cause of nosocomial infections all over the world (Mulani *et al.*, 2019). Due to the dynamic nature of *P. aeruginosa,* it can grow in a variety of habitats including hospital instruments and other nooks and corners of hospital. It can flourish in hospital rooms, medical instruments, showers and sinks from 6 h. to 6 months and is major cause of spreading nosocomial infections. Persistent usage of implanted medical tools, which include prosthesis, catheter and pacemaker have significantly increased the rate of biofilm associated diseases (Donlan and Costerton, 2002; Schurek *et al.*, 2012; Xu *et al.*, 2017). A study on 8247 patients infected with nosocomial diseases was done in Greece which showed that *P. aeruginosa* was the second major pathogen isolated from these patients. Approximately 16% infections were caused by this bacterium (Kritsotakis *et al.*, 2017).

The *Pseudomonas aeruginosa* is responsible for both acute and chronic infections. Infection which progresses immediately is termed as acute infection whereas the infection with slower and persistent growth rate is known as chronic infection (Stewart and Costerton, 2001). Chronic infections include infections caused by resistance, persistence and biofilms. biofilms are produced by colonization of bacteria on urinary catheters, ventilators, infected burn wounds and in chronic pneumonia in cystic fibrosis (CF) patients (Stewart and Costerton, 2001; Hammond *et al.*, 2010; Xu *et al.*, 2017). These biofilms become resistant to host immune system as well as resistant to antibiotics and become cause of chronic infections.

OBJECTIVES: Comprehensive information on the mechanism of quorum sensing and biofilm formation in *P. aeruginosa* can help in paving the way towards inhibition of infection in hospital environment. Moreover, the genetic regulation of network of

genes expressed during virulence can lead us to gene therapy against this bacterium.

Mechanisms of drug resistance: Two types of drug resistance mechanisms are seen in *P. aeruginosa*, innate resistance and acquired resistance. In innate resistance, the expression of efflux pumps followed by modulation in permeability of membrane is involved while in acquired resistance, genes related to efflux pump, prion encoding genes, gene related to penicillin binding protein and β lactamase are mutated due to environmental interaction and exposure to antibiotics (Botelho *et al.*, 2019). In *P. aeruginosa* there are four well-characterized efflux systems known as MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM where each pump have a preference for a specific substrate (Aeschlimann, 2003).

Misapplication and ill-use of antibiotics are responsible for establishing resistance in bacterial strains (Aloush *et al.*, 2006). Antibiotics can also kill beneficial bacteria that may have defensive role averse to pathogenic bacteria (Rasamiravaka *et al.*, 2015). Specific properties like resistance to antibiotics and host defence are exhibited by biofilms which help them to survive in harsh environment (Iftikhar *et al.*, 2020). As drug resistance has increased in different bacteria like *Escherichia coli*, *K. pneumoniae*, *A. baumanni etc* but extreme drug resistance is revealed by *P. aeruginosa*. Although, pathogen show susceptibility towards antibiotics in laboratory experiments, but in actual cases, these antibiotics are unable to cure diseases caused by these pathogens. Impaired antibiotic diffusion, antibiotic efflux, genetic ability of some pathogens to survive against antibiotics are main causes of establishing resistance. Phenomenon that aids in proffering resistance towards antibiotics are horizontal gene transfer and de novo mutations which lead to cause elevated minimal inhibitory concentrations (MIC) (Pang *et al.*, 2019). Optimal amounts of antimicrobial substances like fluoroquinolones, aminoglycosides and carbapenems destroy planktonic cells of *P. aeruginosa*. But when concentration of antimicrobials is below sub-inhibitory concentrations, it causes establishment of resistance. Multidrug resistance is seen when optimized quantity of antimicrobials is applied and disease is not cured (Mulani *et al.*, 2019).

Biofilms need large concentrations of antibiotics as compared to planktonic cells. Tobramycin is an antibiotic that kills planktonic cells up to 99% whereas disruption of biofilm was only 25%. Likewise, ciprofloxacin showed exceptional bactericidal activity in *P. aeruginosa* planktonic cells (MIC = 0.25 mg/L), but very minute effect was observed in biofilms (Khalid *et al.*, 2018; Kamble and Pardesi, 2021). Several theories described the mechanism of resistance development in biofilms against antibiotics.

Biofilm formation and resistance against antibiotics: In *P. aeruginosa*, exocellular appendages like flagella and pili are involved in the attachment of bacterial cells on solid and semi-solid surfaces. Flagella are responsible for swimming movement of pathogen towards its desired surface for attachment whereas these bacteria move over surface by twitch motility. The twitching motility of this bacterium is facilitated by type IV pili and fimbriae (Rashid and Kornberg, 2000; Davey *et al.*, 2003; Qaisar *et al.*, 2013). Attachment of planktonic cells leads to formation of micro-colonies over that surface and then after maturation of these colonies an organized structure is seen which is termed as biofilm. Exocellular polysaccharide like alginate and rhamnolipid are produced by these pathogens. These compounds protect them from severe environmental stresses provide physical resistance and antibiotic resistance (Hentzer *et al.*, 2001; Stewart and Costerton, 2001). Antibiotics are not able to treat several infections caused by *P. aeruginosa* biofilms due to enhanced resistance and ability to disperse bacteria in the surrounding environment. Antibiotics can also kill advantageous bacteria that may play defensive role averse to pathogenic bacteria (Rasamiravaka *et al.*, 2015). Misapplication and ill-use of antibiotics are responsible for establishing resistance in these bacterial strains. However, molecular mechanism by which they survive in the presence of antibiotic is still unknown (Allison *et al.*, 2011). According to different theories, limited diffusion, expression of drug-metabolizing enzymes and growth with obtuse rate are not sufficient to explain this resistance phenomenon (Pang *et al.*, 2019). Notion of resistance was associated with biofilms which is state of bacteria with modified gene expression. Reduction in virulence of bacteria can be achieved by inhibiting microbial stages of biofilm formation.

Mechanism of biofilm formation: The *P. aeruginosa* is considered as model organism for biofilm research. Bacteria's sessile mode of life is dependent on different factors. The factors playing major role in biofilm formation are extracellular appendages which include pili, flagella and fimbriae (Qaisar *et al.*, 2013). Colonies are protected by the exopolysaccharides and lipopolysaccharides (Friedman and Kolter, 2004; Leid *et al.*, 2005). They form covering around biofilms and grant them protection from external environment. Research has shown that in case of *P. aeruginosa*, attachment to surface is arbitrated to several adhesins, especially flagella, pili and fimbriae (Burrows, 2012). Two kinds of pili are seen in *P. aeruginosa*. Type 1 pili are short and fine and found all over on bacterium body and is associated with adhesion of bacteria on surface. Whereas type IV pili are less in number and few millimeters long and are responsible for twitching motility on different surfaces. Besides these, bacteria possess another organelle known as flagellum which is not only responsible for swimming of bacteria to different places, but it also helps in anchoring of bacteria to different surfaces. These organelles are involved in synthesis of biofilm in *P. aeruginosa* (Høiby *et al.*, 2011). Type IV pili are associated to two kind of motility which include twitching motility and swarming motility. In twitching motility, type IV pili structures are involved in monotonous movement of extension and retraction. This type of motility is often seen in media having average viscosities. Whereas swarming motility is dendritic style movement which is often observed in availability of specific nitrogen and carbon. This type of movement is also seen in media having low viscosities. Swarming motility is caused by type IV pili, flagella, and rhamnolipid surfactants (Davey *et al.*, 2003; Asif *et al.*, 2019). Both swarming and twitching mortalities are related to biofilm development in *P. aeruginosa* (Otton *et al.*, 2017).

The initiation of biofilm development starts by attachment of bacteria to solid and semi-solid surface which is achieved by several factors like adhesins and appendages and the need of factor for attachment is related to kind of surface for attachment and other atmospheric conditions. As flagella is locomotory organ, it helps in movement of bacteria from one place to other. Fimbrial gene clusters are associated with gathering of exocellular appendages and they are scrimped in

gram negative bacteria. Fimbriae is involved in performing different functions. They facilitate the adhesion of bacteria on surfaces or on host tissues and they cover the antigens and prevent the contact of antigens to host immune system (Mikkelsen *et al.*, 2011; Qaisar *et al.*, 2013). Three clusters of fimbrial genes termed *cupA1-cupA5*, *cupB1-cupB6* and *cupC1-C3* were identified in *P. aeruginosa* (Vallet *et al.*, 2001). *CupA* gene cluster is regulated by *cgrABC* genes while expression of cupB and cupC gene clusters is controlled by the Roc system (regulator of cup). *CupB* and *cupC* cluster expressions were investigated and found that they are co-regulated by the Roc locus (Kulasekara *et al.*, 2005). After attachment to the solid surfaces, maturation of these micro-colonies occur by encapsulation of these colonies in a glycocalyx matrix that contain aqueous channels. These channels are used as signaling pathways and are involved in movement of nutrients, waste materials and oxygen. It was reported that N-acyl homoserine lactone-dependent quorum sensing is involved in the maturation of biofilm.

Biofilm formation related genes: Gene clusters, known as cup which stands for chaperone-usher pathways were observed in *P. aeruginosa* and were responsible for development of fimbriae. Fimbriae has different structure from type IV pili. Chaperone usher pathway is involved in attachment of fimbrial submits that are present in periplasm and is also required for establishment of pili from pilin subunits. Chaperone- pilin complex is formed which is conveyed to usher protein. Usher protein is responsible for creating a pore in extracellular membrane. When usher-protein complex passes through membrane the pilin subunits get separated from chaperone and attach to fibrils. CupA gene cluster is needed for attachment of bacterial cells on the surfaces and are involved in biofilm formation and this gene cluster does not need type IV pili for attachment. Other gene clusters like cupB and cupC do not have role in biofilm initiation (Vallet *et al.*, 2001) but in their absence maturation of biofilm is disturbed (Qaisar *et al.*, 2013). The pslC cluster plays a role in cell-cell and/or cell-surface interaction in biofilm formation. It is present in pathogenic as well as non-pathogenic strains.

Flagellar hook-associated protein 1 flgK is involved in motility of bacterium to find suitable surface and help in initial attachment of bacteria to that surface. Study showed that, mutants with defective flagellum in *P. aeruginosa* were failed in attachment with the walls of microtiter plate. *motA* and *motY* are genes that are responsible for flagellar rotation. The need of flagellar rotation exhibits that flagellum has main role in motility of bacterium not in attachment stage in biofilm development of *P. aeruginosa* (Vallet *et al.*, 2001). Chaperones which are present in periplasm can be differentiated on basis of morphology and physiology. The difference between these chaperones is in length of loop. Longer loop length is seen in Longer F1 G1 Loop (FGL) family which is approximately 20 amino acids and are related to assembly of atypical and nonfimbrial adhesions. Smaller F1 G1 Loop (FGS) family has smaller loop and associated with assembly of fimbriae (Vallet *et al.*, 2001).

Type IV fimbrial precursor PilA is localised to the outer membrane vesicle and is associated with production of pili which is involved in twitching motility and has important role in biofilm formation (Toyofuku *et al.*, 2012). Different monomers of PilA join and form a filament. Product of many associated genes is needed for this monomer production and pilin formation. Pilus formation is controlled by three genes which include pilB, pilC, and pilY1. Mutated strains with non-functional pilus genes showed that without pili, attachment of bacteria to Polyvinyl chloride (PVC) was very poor. Monolayer was seen on plastic, but bacteria were unable to develop biofilm in these mutated strains (Vallet *et al.*, 2001).

Shearing stress severely affects the adherence of *P. aeruginosa* cells to the biotic and abiotic surfaces. Mutant strains including, ΔflgK which cannot encode for flagellar hook protein, ΔPilA mutant which has non-functional type 1 pili, bacteria with non-operative type IV pili (ΔPilC) and bacteria which were unable to producing extracellular polysaccharides (ΔPelA) were tested for their adherence under shear stress. In all mutant strains, adherence of cells was reduced significantly by shear stress. In mutants with defective FlgK and type IV pili, number of adhering cells was reduced two folds when compared with wild strain. In mutant with defective type 1 pili, number of adhering cells was very close to wild type. In mutants with defective polysaccharides matrix, adhering cells were more than adhering cells of wild type bacterium at the same time. For mutants with type IV and type 1 pili, when shear stress increased, time of residence also increased. And in type IV pili defective mutant, residence time was enhanced (Lecuyer *et al.*, 2011).

Genetic regulation of biofilm formation: In *P. aeruginosa*, biofilm regulation is done by two component system which include kinase and response regulator. Kinases are usually bound with membranes but sometimes present in cytosol, they sense temperature, pH and availability of different nutrients, poisonous chemicals and draw out responses that help organism to survive in such non-favorable conditions (Mikkelsen *et al.*, 2011). Two component system (TCS) deals with steps involved in biofilm production. It directs the congregation of appendages like cup fimbriae and type IV pili and deals with production of exopolysaccharides. Extracellular appendages determine the attachment of bacteria to surface whereas, exopolysaccharide and lipopolysaccharide complex is needed at the end of biofilm production. It creates covering around bacterial growth and gives and organized structure to bacterial biofilm. TCS system controls the development of biofilm by modification of transcriptional profile (Qaisar *et al.*, 2013).

Biofilm formation and its dissemination to other places is a phenomenon which is controlled at genetic level. Currently, cyclic diguanosine monophosphate (c-di-GMP), quorum sensing (QS), and small RNAs (sRNAs) are known as important regulators of biofilm in gram negative bacteria. The QS is cell-to-cell communication system which is regulated by population density of bacteria. Virulence of pathogen is related to its population density, QS signaling substances manages synthesis of virulence factors according to population density of *P. aeruginosa* (Diggle *et al.*, 2007). When population density is decreased, activation of virulence genes is prohibited because host may identify it and can activate its immune system against bacterial populations. When population densities become larger than threshold level signals are induced by QS system which cause activation of virulence genes. Due to this system, bacteria get enough time for their establishment in host cells. Biofilm formation and swarming motility is influenced by virulence

factors according to availability of extracellular polymeric compounds, oxygen, and density of matrix (Kruczek *et al.*, 2014; Asif *et al.*, 2019). Genes which are regulated by QS comprise 10% of bacterial genome. QS is associated with development and dissemination of biofilms but not associated with early attachment stages. Production of necessary substances i.e LecA and LecB lectins, pyoverdine and pyochelin siderophores which are associated to biofilm are formed by QS system. Pyoverdine and pyochelin are involved in iron metabolism. Reduced and enhanced amount of iron influence biofilm production (Wolska *et al.*, 2016).

The *P. aeruginosa* QS rely on 3 precise and specific signaling systems which include the lasI/lasR, rhlI/rhlR, and pqs/mvfR systems. Autoinducer molecules, such as 3OC12-homoserine lactone (C12-HSL) and N-butyryl-L-homoserine lactone (C4HSL) are present which give collective response to extracellular signals. In the las system, autoinducer C12-HSL activates transcription factor lasR which further induces lasI expression and activates synthesis of exotoxin A, the LasA protease and the LasB elastase (Diggle *et al.*, 2007). Likewise, expression of rhlI is enhanced by autoinducer C4-HSL, as C4-HSL binds with rhlR and triggers synthesis of LasB elastase, rhamnolipids, pyocyanin, and cytotoxic lectins in a controlled manner. These compounds are associated with production and development of biofilms and has important role in virulence. rhl system regulation is done at the transcriptional and posttranscriptional levels by lasR/C12-HSL complex. Due to absence of QS in humans, it can be considered as specific antibacterial target and novel antibiotics can be formed which target QS system and associated virulence factors.

In *P. aeruginosa,* five components are associated to "basic c-di-GMP signaling module" (Wolska *et al.*, 2016). When c-di-GMPs amount becomes high, it triggers production of adhesins and matrix polysaccharides which reduce movement of bacteria and help in attachment of bacteria to different surfaces and leads to formation of Biofilms. When amount of c-di-GMP reduces, production of adhesins and matrix polysaccharides is down regulated which enhance motility of bacteria which help in dispersal of biofilms.

CONCLUSION: Due to limitations exhibited by antibiotics in treatment of infectious diseases and inability of these antibiotics to treat infections related to biofilms, efficient novel or alternative antimicrobial agents are needed. Anti-microbial peptides are biologically active small proteins and have significant role in host defense system. So, they can be utilized in field of medicine as they positively increase the immune system of host and are involved in disturbance of plasma membrane of pathogen and in biofilm disruption and inhibition.

ACKNOWLEDGMENTS: The current study was conducted under the project number NRPU-6750 funded by Higher Education Commission of Pakistan.

CONFLICT OF INTEREST: Authors have no conflict of interest.

REFERENCES:

Aeschlimann, J. R., 2003. The role of multidrug efflux pumps in the antibiotic resistance of pseudomonas aeruginosa and other gram-negative bacteria: Insights from the society of infectious diseases pharmacists. Pharmacotherapy: The Journal of human pharmacology and drug therapy, 23(7): 916-924.

Allison, K. R., M. P. Brynildsen and J. J. Collins, 2011. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature, 473(7346): 216-220.

Aloush, V., S. Navon-Venezia, Y. Seigman-Igra, S. Cabili and Y. Carmeli, 2006. Multidrug-resistant *Pseudomonas aeruginosa*: Risk factors and clinical impact. Antimicrobial agents and chemotherapy, 50(1): 43-48.

Asif, A., A. Iftikhar, A. Hamood, J. A. Colmer-Hamood and U. Qaisar, 2019. Isonitrile-functionalized tyrosine modulates swarming motility and quorum sensing in *Pseudomonas aeruginosa*. Microbial pathogenesis, 127: 288-295.

Botelho, J., F. Grosso and L. Peixe, 2019. Antibiotic resistance in *Pseudomonas aeruginosa*–mechanisms, epidemiology and evolution. Drug resistance updates, 44: 100640.

Burrows, L. L., 2012. Pseudomonas aeruginosa twitching motility: Type iv pili in action. Annual review of microbiology, 66(1): 493-520.

Davey, M. E., N. C. Caiazza and G. A. O'Toole, 2003. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* pao1. Journal of bacteriology, 185(3): 1027-1036.

Diggle, S. P., S. Matthijs, V. J. Wright, M. P. Fletcher, S. R. Chhabra, I. L. Lamont, X. Kong, R. C. Hider, P. Cornelis and M. Cámara, 2007. The pseudomonas aeruginosa 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. Chemistry & biology, 14(1): 87-96.

Donlan, R. M. and J. W. Costerton, 2002. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clinical microbiology reviews, 15(2): 167-193.

Driscoll, J. A., S. L. Brody and M. H. Kollef, 2007. The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. Drugs, 67(3): 351-368.

Friedman, L. and R. Kolter, 2004. Genes involved in matrix formation in *Pseudomonas aeruginosa* pa14 biofilms. Molecular microbiology, 51(3): 675-690.

Gale, M. J., M. S. Maritato, Y. L. Chen, S. S. Abdulateef and J. E. Ruiz, 2015. Pseudomonas aeruginosa causing inflammatory mass of the nasopharynx in an immunocompromised hiv infected patient: A mimic of malignancy. IDCases, 2(2): 40-43.

Garrett, W. S., 2015. Cancer and the microbiota. Science, 348(6230): 80-86.

Gomila, A., E. Shaw, J. Carratalà, L. Leibovici, C. Tebé, I. Wiegand, L. Vallejo-Torres, J. M. Vigo, S. Morris, M. Stoddart, S. Grier, C. Vank, N. Cuperus, L. Van den Heuvel, N. Eliakim-Raz, C. Vuong, A. MacGowan, I. Addy and M. Pujol, 2018. Predictive factors for multidrug-resistant gram-negative bacteria among hospitalised patients with complicated urinary tract infections. Antimicrob resist infect control, 7: 111.

Hammond, A., J. Dertien, J. A. Colmer-Hamood, J. A. Griswold and A. N. Hamood, 2010. Serum inhibits p. Aeruginosa biofilm formation on plastic surfaces and intravenous catheters. Journal of surgical research, 159(2): 735-746.

Hentzer, M., G. M. Teitzel, G. J. Balzer, A. Heydorn, S. Molin, M. Givskov and M. R. Parsek, 2001. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. Journal of bacteriology, 183(18): 5395-5401.

Høiby, N., O. Ciofu, H. K. Johansen, Z. j. Song, C. Moser, P. Ø. Jensen, S. Molin, M. Givskov, T. Tolker-Nielsen and T. Bjarnsholt, 2011. The clinical impact of bacterial biofilms. International journal of oral science, 3(2): 55-65.

Iftikhar, A., A. Asif, A. Manzoor, M. Azeem, G. Sarwar, N. Rashid and U. Qaisar, 2020. Mutation in pvcabcd operon of *Pseudomonas aeruginosa* modulates mexef-oprn efflux system

and hence resistance to chloramphenicol and ciprofloxacin. Microbial pathogenesis, 149: 104491.
Kamble, E. and K. Pardesi, 2021. Antibiotic tolerance in biofilm and stationary-phase planktonic cells of *Staphylococcus aureus*. Microbial drug resistance, 27(1): 3-12.
Khalid, R., Q. Jaffar, A. Tayyeb and U. Qaisar, 2018. Peganum harmalapeptides (phamp) impede bacterial growth and biofilm formation in burn and surgical wound pathogens. Pakistan journal of pharmical science, 31(6): 2597-2605.
Kritsotakis, E. I., F. Kontopidou, E. Astrinaki, M. Roumbelaki, E. Ioannidou and A. Gikas, 2017. Prevalence, incidence burden, and clinical impact of healthcare-associated infections and antimicrobial resistance: A national prevalent cohort study in acute care hospitals in greece. Infection and drug resistance, 10: 317.
Kruczek, C., U. Qaisar, J. A. Colmer-Hamood and A. N. Hamood, 2014. Serum influences the expression of pseudomonas aeruginosa quorum-sensing genes and qs-controlled virulence genes during early and late stages of growth. Microbiology open, 3(1): 64-79.
Kulasekara, H. D., I. Ventre, B. R. Kulasekara, A. Lazdunski, A. Filloux and S. Lory, 2005. A novel two-component system controls the expression of *Pseudomonas aeruginosa* fimbrial cup genes. Molecular microbiology, 55(2): 368-380.
Lecuyer, S., R. Rusconi, Y. Shen, A. Forsyth, H. Vlamakis, R. Kolter and H. A. Stone, 2011. Shear stress increases the residence time of adhesion of pseudomonas aeruginosa. Biophysical journal, 100(2): 341-350.
Leid, J. G., C. J. Willson, M. E. Shirtliff, D. J. Hassett, M. R. Parsek and A. K. Jeffers, 2005. The exopolysaccharide alginate protects pseudomonas aeruginosa biofilm bacteria from ifn-γ-mediated macrophage killing. The journal of immunology, 175(11): 7512-7518.
Mikkelsen, H., M. Sivaneson and A. Filloux, 2011. Key two-component regulatory systems that control biofilm formation in *Pseudomonas aeruginosa*. Environmental microbiology, 13(7): 1666-1681.
Mulani, M. S., E. E. Kamble, S. N. Kumkar, M. S. Tawre and K. R. Pardesi, 2019. Emerging strategies to combat eskape pathogens in the era of antimicrobial resistance: A review. Front microbiol, 10: 539.
Otton, L. M., M. da Silva Campos, K. L. Meneghetti and G. Corção, 2017. Influence of twitching and swarming motilities on biofilm formation in *Pseudomonas* strains. Archives of microbiology, 199(5): 677-682.
Pang, Z., R. Raudonis, B. R. Glick, T.-J. Lin and Z. Cheng, 2019. Antibiotic resistance in *Pseudomonas aeruginosa*: Mechanisms and alternative therapeutic strategies. Biotechnology advances, 37(1): 177-192.
Qaisar, U., L. Luo, C. L. Haley, S. F. Brady, N. L. Carty, J. A. Colmer-Hamood and A. N. Hamood, 2013. The pvc operon regulates the expression of the *Pseudomonas aeruginosa* fimbrial chaperone/usher pathway (cup) genes. PloS one, 8(4): e62735.
Qaisar, U., L. M. Luo, C. L. Haley, S. F. Brady, N. L. Carty, J. A. Colmer-Hamood and A. N. Hamood, 2013. The PVC operon regulates the expression of the *Pseudomonas aeruginosa* fimbrial chaperone/usher pathway (cup) genes. PloS one, 8(4).
Rasamiravaka, T., Q. Labtani, P. Duez and M. El Jaziri, 2015. The formation of biofilms by *Pseudomonas aeruginosa*: A review of the natural and synthetic compounds interfering with control mechanisms. BioMed research international, 2015:759348
Rashid, M. H. and A. Kornberg, 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. Proceedings of the national academy of sciences, 97(9): 4885-4890.
Schurek, K. N., E. Breidenstein and R. E. Hancock, 2012. *Pseudomonas aeruginosa*: A persistent pathogen in cystic fibrosis and hospital-associated infections. In: Antibiotic discovery and development. Springer: pp: 679-715.
Stewart, P. S. and J. W. Costerton, 2001. Antibiotic resistance of bacteria in biofilms. Lancet, 358(9276): 135-138.
Toyofuku, M., B. Roschitzki, K. Riedel and L. Eberl, 2012. Identification of proteins associated with the pseudomonas aeruginosa biofilm extracellular matrix. Journal of proteome research, 11(10): 4906-4915.
Vallet, I., J. W. Olson, S. Lory, A. Lazdunski and A. Filloux, 2001. The chaperone/usher pathways of *Pseudomonas aeruginosa*: Identification of fimbrial gene clusters (cup) and their involvement in biofilm formation. Proceedings of the national academy of sciences, 98(12): 6911-6916.
Wolska, K. I., A. M. Grudniak, Z. Rudnicka and K. Markowska, 2016. Genetic control of bacterial biofilms. Journal of applied genetics, 57(2): 225-238.
Xu, Y., L. H. Larsen, J. Lorenzen, L. Hall-Stoodley, J. Kikhney, A. Moter and T. R. Thomsen, 2017. Microbiological diagnosis of device-related biofilm infections. Apmis, 125(4): 289-303.