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Author's Affiliation:
1. Department of microbiology, Faculty of Allied Health Sciences, SGT, University, Gurugram-122505, Haryana – India
2. Microbial and Pharmaceutical Biotechnology Laboratory (MPBL), Department of Pharmacognosy & 8 Phytochemistry, School of Pharmaceutical Education & Research, Jamia Hamdard, New Delhi-110062 – India
3. Department of Physiotherapy, College of Applied Medical Sciences, University of Ha'il, Ha'il P.O. Box 2440 – Saudi Arabia
4. Department of Clinical Nutrition, College of Applied Medical Sciences, University of Ha'il, Ha'il P.O. Box 2440 – Saudi Arabia
5. Al Rayan Private College of Health Sciences and Nursing, PO Box 167, Al Madinah Al Munawarah, 41411 – Saudi Arabia

***Corresponding Author:**
Syed Amir Ashraf
Email: amirashrafy2007@gmail.com

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Pseudomonas aeruginosa: A Current update on biofilm formation, immune response and antibiotic resistance

Neha Rawat[1] , Sunita Sheoran[1*], Mukesh Sharma[1] , Jalaluddin Khan[2] , SD. Shahanawaz[3], Amir Mahgoub Awadelkareem[4] , Angum M.M. Ibrahim[5] , Syed Amir Ashraf[4*]

Abstract

Pseudomonas aeruginosa is a Gram-negative bacillus found ubiquitously in nature and is known to cause life-threatening infections. A prime example of an opportunistic pathogen, *P. aeruginosa* is responsible for infections in environmental, industrial, and hospital settings. It has been classified as one of the "superbugs" involved in nosocomial infections and is a member of the ESKAPE pathogen group. Its virulent nature makes it a potent causative organism in both device-associated infections (such as catheter-associated bloodstream and urinary tract infections) and non-device-related infections (such as cystic fibrosis, otitis media, keratitis, and ventilator-associated pneumonia). Despite the use of various antimicrobial agents against *P. aeruginosa,* complications from hospital-acquired infections persist. Multiple studies have demonstrated, *P. aeruginosa* readily forms biofilms during prolonged infections, making treatment more challenging. This can be attributed to the fact that antibiotics are less effective against microbial biofilms. *P. aeruginosa* possesses several virulence factors, including lipopolysaccharides, extracellular polysaccharides (EPS), and toxin secretion systems such as the type III secretion system (T3SS), which evade host immunity and compete with other bacteria. The synergistic effect of these factors, along with biofilm formation, protects the pathogen from host immune defenses and reduces the efficacy of antimicrobial agents. This review provides a conceptual framework for understanding the association between microbial biofilms and host immune responses. Additionally, it emphasizes the critical need to address *P. aeruginosa* biofilms to improve patient outcomes and reduce hospital-acquired infections.

Introduction

Each single organism that resides on earth strives for its own existence. In this context Darwin's theory of "survival of the fittest" could be strongly imposed. In simple terms, the one with a novel mechanism to protect itself can easily manage to escape the unfavourable conditions around them in order to continue the survival. If any organism failed to do so, its existence would only be history. Microorganisms exhibit diverse mechanisms to survive harsh environments, and some adjust themselves accordingly. Additionally, certain pathogenic microorganisms protect themselves by developing biofilms. Within biofilm communities, bacteria are in close proximity and interact with one another through signal molecules; this process is known as quorum sensing [1]. Bacteria reside in biofilms as polymicrobial communities, enclosed inside a matrix composed of extracellular polymeric substances (EPS) along with extracellular DNA, cellular debris, and other components [2]. All these compounds forming the biofilm matrix are secreted by the plethora of microorganisms. As defined by Donlan et al. (2008), a biofilm is "a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface" [3]. Biofilms protect the microbial colonies within them from unfavorable conditions, such as the effects of antibacterial agents and host immune responses [4]. Biofilm communities increase the chronicity of an infection, and prolonging its duration prompts the pathogen to form sessile communities [2]. Additionally, it has been reported in patients with cystic fibrosis that there is a weakened host immune response and reduced effectiveness of antibacterial agents against persistent biofilms, unlike acute infections caused by the pathogen's planktonic form (figure 1: a and b). This may be attributed to biofilms serving as optimal niches for the horizontal transfer of genes conferring multidrug resistance via plasmid exchange and conjugation [4].

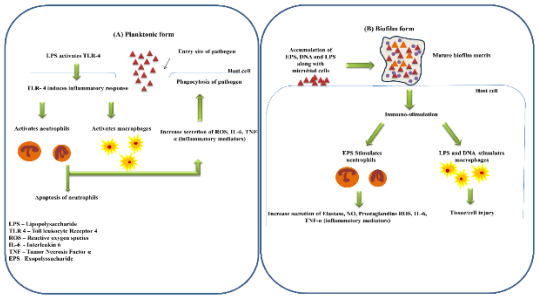

Figure 1: An overview of host immune response against a) Planktonic form of P. aeruginosa:

Pseudomonas aeruginosa in the planktonic state triggers TLR-mediated host inflammation through LPS, recruiting neutrophils and macrophages; neutrophils release ROS, IL-6, and TNF, undergo apoptosis, and are subsequently cleared by macrophages. **b) Biofilm form:** Biofilm (EPS, eDNA, LPS) delays immune activation; activation of neutrophils only after mature biofilm formation; leads to release of ROS and proteases while macrophages clear bacteria with potential collateral tissue injury.

Microbial biofilm infections elicit pronounced inflammatory responses that often progress to chronic conditions, thereby extending the duration of infection and complicating therapeutic interventions [6]. Despite the substantial recruitment of neutrophils to the site of infection, biofilms demonstrate significant resistance to immune clearance [7]. The increased resistance observed in biofilm-associated infections is attributed to the diminished capacity of phagocytes to effectively target and eliminate pathogens protected within the biofilm matrix [4]. Furthermore, the antagonistic interactions between host immune responses and established biofilms often result in collateral damage to surrounding tissues [4]. This biofilm-associated pathology is exemplified by *Pseudomonas aeruginosa*, an opportunistic, biofilm-forming pathogen that predominantly infects immunocompromised individuals, represents a common cause of healthcare-associated infections, and frequently exhibits resistance to multiple antimicrobial agents [8].

Infections caused by *Pseudomonas aeruginosa* biofilms are difficult to treat and are associated with high morbidity and mortality [9]. These biofilms form in a variety of clinical contexts, including cystic fibrosis, wound or burn infections, ventilator-associated pneumonia, keratitis, and otitis media [2]. Device-related infections are also common, such as catheter-associated urinary tract infections (CAUTI), endotracheal tube infections, catheter-related bloodstream infections (CBSI), and infections associated with intrauterine devices (IUDs) [5]. During biofilm development, bacterial colonies undergo a phenotypic transition from a non-mucoid to a mucoid state, a process largely driven by hydrogen peroxide released from surrounding polymorphonuclear neutrophils (PMNs), which contributes to the progression of infection toward a chronic phase [7]. Consequently, the presence of *P. aeruginosa* in biofilm form is a hallmark of chronic lung infection [8]. Bacterial biofilms employ multiple strategies to evade the host immune response, including interference with humoral immunity, secretion of toxins that hinder pathogen recognition, and modulation of the inflammatory activity of recruited myeloid-derived suppressor cells (MDSCs), macrophages, and neutrophils [10]. Chronic biofilm infections often result from an inadequate immune response, and effective treatment typically requires

physical removal of infected tissues or medical implants, sometimes necessitating surgical intervention [11]. This review aims to highlight the mechanisms by which the host immune system responds to persistent *P. aeruginosa* biofilm infections and how immune cells combat virulence factors that specifically promote biofilm formation.

Methodology

Literature Strategy and Selection Criteria

A comprehensive literature search was performed through Google Scholar, PubMed and ScienceDirect. Search terms included "biofilm," "immune response," "*Pseudomonas aeruginosa*," "medical devices," "device-related infection," "immune cells," and "antimicrobial peptide." Search string used was ("*Pseudomonas aeruginosa*" AND biofilm*) AND ("immune cell*" OR "host immune response" OR macrophage* OR neutrophil* OR monocyte* OR "dendritic cell*" OR leukocyte* OR phagocyte* OR "immune system") AND (role OR function OR interaction* OR mechanism* OR response OR contribution). Inclusion criteria consist of those review and research articles that were relevant to *P. aeruginosa* biofilm and interaction with host immune cells, and full text articles published in English. Conference proceedings and case studies were excluded. The study selection method followed the PRISMA 2020 recommendations. Database searches yielded 180 entries. Following the removal of 30 duplicates, 150 records were screened using titles and abstracts. At this point, 41 records were removed due to their lack of relevance to the review topic. Following that, 109 reports were requested for full-text retrieval, 12 of which could not be obtained. Ninety-seven reports were evaluated for eligibility, with 17 being eliminated because they did not match the inclusion criteria. Finally, 80 peer-reviewed research articles and review papers were included (Figure 2).

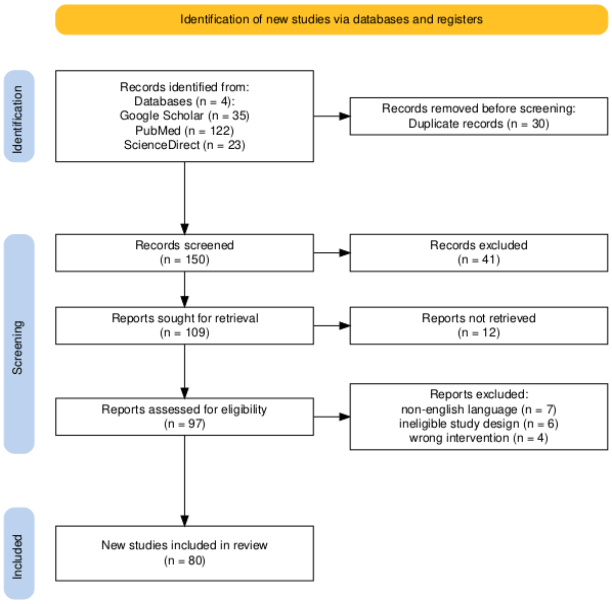

Figure 2: PRISMA 2020 flow diagram of study selection process

Discussion

Clinical manifestations by *Pseudomonas aeruginosa* biofilm

Biofilm easily adheres to biotic or abiotic surfaces that make them to colonize indwelling medical devices also [5]. Therefore, the microbial biofilm of *P. aeruginosa* causes several device- related and non-device- related infections (Figure 3).

Non-device-related infections

Patients with severe lung infections frequently develop cystic fibrosis as a result of *Pseudomonas aeruginosa*, and the development of biofilms makes the chronic illness more susceptible. At the initial stage, *P. aeruginosa* develops biofilm in paranasal sinuses, which marks the beginning of its colonization, causing infection in lungs one after the other until it transforms to the chronic stage. *P. aeruginosa* adheres to the adjacent mucosal surfaces, a process recognized by polymorphonuclear leukocytes (PMNs) [7]. The two most significant initial responders in the innate immune system are neutrophils and macrophages, and to be more precise, macrophages are the principal defense system against invasive pathogens because of their extended lifespans [12]. The infection causes inflammation, tissue damage, abnormal lung function, and hindrance in air passages. The infection induces inflammation, tissue damage, impaired pulmonary function, and obstruction of the airways. This pathological state leads to a reduction in glutathione levels within the cystic fibrosis airways, resulting in increased oxidative stress that promotes the formation of more robust sessile microbial communities [13]. The

secretion of alginate serves as a critical biomarker for the diagnosis of biofilm-associated infections, alongside the detection of serum IgG antibodies [4]. The pronounced mucoid phenotype results from the excessive secretion of alginate by the biofilm. Several studies have reported the presence of IgA antibodies in saliva and mucosal secretions, which correlate with the presence of biofilm in cystic fibrosis patients [6].

Another clinical picture is otitis media, which is the inflammation and infection in the cavity of the middle ear. The physiology of the eustachian tube among the pediatric population makes it more prone to ear infections such as otitis media, as the tube is much shorter and wider, which promotes the spread of pathogenic bacteria [14]. Several studies have confirmed that pathogenesis of otitis media is majorly due to biofilm formation in both fluid and the mucosal layer of the middle ear [15, 16]. Biofilm is also associated with cholesteatoma, an infection that not only involves the entrapment of keratinized squamous epithelial tissues in the mastoid process but also in the middle ear, causing high inflammation leading to drainage in the ear as a relapsing infection [17]. Inflammations of the middle ear and mastoid cavity characterize chronic suppurative otitis media (CSOM), also known as chronic otitis media [15]. The disease's characteristic presentation is persistent or chronic otorrhea over the course of two to six weeks due to a perforated tympanic membrane [14]. The chronicity and recurrent characterization of CSOM are because biofilm adhering to the middle ear acts as a source of bacterial dissemination [16].

Pathogens not only infect the wounded or burnt surface tissues, but the deeper tissues also house the biofilm, resulting in chronic inflammation and failure in healing [18]. A burn represents the most severe medical condition since it removes the protective outer layer of skin, resulting in an eschar that serves as a colony for many pathogenic microbes, including *Pseudomonas aeruginosa* [19]. With fewer therapeutic choices available for effective treatments and less penetration of immune agents to the site of infection, burn injuries are the primary global public health issue [18, 19, 20]. The involvement of microbial biofilm has serious consequences in increasing the chronicity of these infections, e.g., venous leg ulcers and pressure ulcers [18]. According to a study in India, the prevalence rate of *P. aeruginosa* in burn patients came out to be 32.1%, out of which major isolates formed biofilm [20].

Device-related infections

Artificial devices provide abiotic surface for the adhesion of microbial biofilm. When medical devices such as endotracheal tube (ETT), catheters (urinary, vascular, peritoneal, etc), orthopedic implants and artificial joints are insert in patients, a conditioning film is formed by the accumulation of host proteins [5]. Soon this layer matures into a biofilm that progress the disease from acute to chronic state. Planktonic pathogens get disperse from biofilm easily and disseminate all over to spread the infection [21]. Antibiotic agents are more effective on planktonic organisms than the entire biofilm. However, it has been reported by Alhede M et al. (2014) that the biofilm can only eradicate on removal of the medical devices [22]. Donlan et al. (2008) also mentioned that the use of urinary catheter elevates the chances of catheter associated infections by 10% every day [3]. The term catheter derives from the ancient Greek kathienai, which can be translated as 'to thrust into' or 'to send down', and describes a medical device used to drain fluid from a body cavity [3].

Pseudomonas aeruginosa is involved majorly in lower respiratory tract infections (LRTIs) leading to an increased mortality among the ICU patients due to its nosocomial spread [21]. Mechanical ventilation (MV) often used at ICUs maintains proper gaseous exchange for therapeutic support but during ventilator associated pneumonia (VAP) the ventilation is provided mechanically to the patients having weak respiratory weakness making LRTIs more severe [23]. Biofilm formed on the surface of tracheal tube worsens the scenario and elevates the its pathogenesis of VAP. The increased pathogenicity of VAP is because of biofilm which hinders the therapeutic treatment like antimicrobial agents as well as recognition by host immune defense system [24]. This leads to an elevated mortality and morbidity rate among ICU patients as observed by Rodrigues ME et al. (2017) [24]. Hence, decrease in these sources may favor reduction in ETT biofilm formation and eventually prevent VAP [23].

Intrauterine devices has been associated with 75% of reproductive tract infections [25]. *P. aeruginosa* also have a significant involvement in IUDs infection which can be proved. Urethral catheters especially Foley's catheter have been employed by clinical therapies since ages [26]. The bacteria populations that cause CAUTIs (catheter-associated urinary tract infections) often form biofilm and adhere directly to the catheter surface. This is possible due to the accumulation microbial colonies of surface-attached cells encased in an extracellular polymeric matrix that they manufacture themselves [3]. With nearly thirty million urinary catheters implanted annually in the USA alone, along with nearly a hundred purchased annually, urinary catheters are currently the most widely used medical device in the world [26].

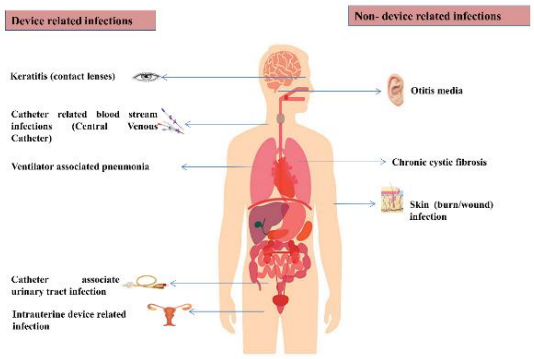

Figure 3: Infections caused by Pseudomonas aeruginosa biofilm: P. aeruginosa cause infections like cystic fibrosis, otitis media, wound/burn infections (non- device-related infections) and keratitis, catheter related blood stream infections (CRBSI), Ventilator associated pneumonia (VAP), Catheter associated urinary tract infection (CAUTI) and Intrauterine devices infection (IUDs) (Adapted from Tuon, F. F., Dantas, L. R., Suss, P. H., & Tasca Ribeiro, V. S. (2022). Pathogenesis of the P. aeruginosa Biofilm: A Review. Pathogens, 11(3), 300. https://doi.org/10.3390/pathogens11030300 [5]).

Functional virulence factors in biofilm

The discovery of a variety of microorganisms' virulence factors lead to the characteristics required to initiate an infectious cycle that engage directly with host cells to sets off the host's immune response. Furthermore, pathogenic bacteria can evade immune responses to persist in harsh environments and propagate to various diseases [4]. Similarly there are several other virulent factors constituting the Pseudomonas biofilm that shields the pathogen from various classes of antibiotics and induces host immune response. Some of the virulence factors in biofilm and their action on host immune cells are presented in table 1.

Exopolysaccharide (Alginate)

Extrapolysaccharide (EPS) is the main element responsible for the virulence of microbial biofilm. The planktonic microorganisms releases signals and communicate each other secreting the constituents of EPS. Alginate is the extracellular component vividly observed in both planktonic and biofilm stages of bacteria commonly found in mucoid strain of *Pseudomonas aeruginosa* [5,11]. It possess multiple functions against host immune response such as-

I) Chemotaxis by PMNs
II) Inhibiting complement actions
III) Reducing neutrophil or macrophages activity
IV) Protect against protozoa and antibacterial agents

Lipopolysaccharides

Lipopolysaccharide (LPS) is a significant part of Gram-negative bacteria's outer membrane and is crucial to the interaction between the pathogen and the host's innate immune system [27]. It is a significant microbe-associated molecular pattern (MAMP) that, in mammals, can activate the host innate immune defense by triggering cascades of signal, which inevitably result in the release of proinflammatory cytokines. Hydroxylation of secondary fatty acid chains induces important steric hindrance modifications in lipid A molecules. Usually hexa-acylated lipid A triggers strong inflammatory reactions when it binds to monocytes, dendritic cells, and macrophages via toll-like receptor-4 [28]. This and other polarity and hydrophobic properties change the molecular conformation of the LPS molecules and, as a consequence, their anchoring conditions in the external bacterial membrane. These alterations to the lipid by reducing the host's inflammatory reactions, an acylation pattern offers defense against the host's natural defenses. When LPS molecules are released, the same consequences apply to

Virulence factors	Mechanism of action	Interaction to immune cells	References
LPS	Activate signal transduction cascades; induce a more pronounced inflammatory response	Neutrophils, activates pro-inflammatory cytokines; Enhancement of M1 macrophages	[27]
Alginate	Scavenge radical oxygen species; Reduces phagocytosis	Elicit the oxidative-burst response from approaching PMNs; reducing phagocytosis by macrophages and PMNs causes hyper immune response in host (IgG and IgA antibody release)	[38]
Exotoxin A	Inhibits protein biosynthesis by catalysing the transfer of ADP ribosylation to the EF-2 to inactivate the EF-2	Inhibit Phagocytosis; Reduce the number of PMNLs	[39,40]
Pyocyanin	Severity of disease; Tissue damage; Impair organ function; Release reactive oxygen species	Induce apoptosis of neutrophils	[41]
Pyoverdin	Provides iron to the bacteria; synthesizes virulence factors such as exotoxin A and PrpL protease; acts as signaling molecule; maintains secretion of other molecules	Scavenge iron from host proteins	[42]
Exoenzyme S	Injury of host cells; cell death ADP- ribosyl transferase activity which inhibits protein synthesis and cause apoptosis; Antiphagocytic factor	Unable macrophages and neutrophils to uptake *P. aeruginosa* for phagocytosis; triggers Neutrophil apoptosis	[43]
Extracellular DNA	Provides viscoelasticity to the biofilm; acidifies the biofilm and develops *P. aeruginosa* resistance to aminoglycosides	eDNA released from necrotic PMN cell death protects the biofilm	[37]
Phospholipase C	Hydrolyzes phosphatidylcholine of host cell and lung surfactant	Elevate cytokines and chemokines; Cytotoxicity to macrophages; Suppress respiratory burst activity neutrophils	[44]

Table 1: Virulence factors, their mechanism of action within biofilm and interaction with host immune cells

their interaction with receptors, which can also be modified [29]. The increase in TNF-alpha and IL-6 is, however, more modest with LPS than with whole bacteria, suggesting the possible role of other bacterial molecules (such as flagella, peptidoglycan, lipoproteins or bacterial DNA) and other innate immune receptors in the increased inflammatory response of human monocytes [28]. In an experimental observation performed on mice by Berclaz et al. (2017), *P. aeruginosa* LPS-induced TLR-4 activation with the aid of GM-CSF and the transcription factor PU was able to cause NF-kB activation, the release of proinflammatory cytokines and chemokines, and the recruitment of neutrophils [30].

Type III secretion system

The type III secretion system (T3SS) is significant in the activation of the NF-kB signaling pathway, which stimulates the genes responsible for proinflammation. Some components of T3SS are recognized by TLR and bind PAMP to these TLRs, initiating the activation of NF-kB and inducing an immune response [30]. T3SS transports and secretes four different exotoxins (ExoS, ExoT, ExoU, and ExoY) from bacteria to their host cells through a needle-shaped complex. These are termed as effector proteins pierced into the cells initiating the host tissue damage [31]. Exoenzymes of T3SS ExoU and ExoS lead to the inhibition of caspase-1 activation mediated by phospholipase A2 and ADP-ribosyltransferase activities, respectively [31]. ExoU leads to deterioration of host tissues through the phospholipase as well as cytolytic actions. The exoenzyme S consists of ADP-ribosyl transferase expression that impedes synthesis of protein of the host cell and eventually brings about apoptosis [28]. While a lower expression of ADP-ribosyl transferase activity has been found in ExoT. Furthermore, ExoY also possesses adenylate cyclase activity. Exoenzymes assist *P. aeruginosa* in the adversity of inflammatory responses. They also cause destruction in the actin cytoskeleton and endothelial cell membrane that worsens the infection [30].

Thus, T3SS is functionally important in bacterial cell lysis as well as damage of the invading tissue and has four components:

i. Needle-like complex – It is a hollow cylindrical part of the T3SS complex that attaches to the pores of host cell membrane and forms a passage to transfer secreted proteins into the host cell membrane. It has been demonstrated that PscC protein, which exists on *P. aeruginosa's* outer membrane, is crucial for effector transportation and mutations in PscC cause bacterial pathogenicity to be reduced as well as the release of T3SS exotoxin [30].

ii. Translocation apparatus – The pores in the host cell membrane are formed by translocation apparatus proteins [32].

iii. Regulatory proteins – Regulatory protein such as cAMP receptor protein binds tightly to a specific DNA sequence in the promoters of a subset of bacterial genes, modulating their transcription. It is also known as catabolite gene activator protein (CAP) [32].

iv. Chaperones – They are present in the cytoplasm of bacteria and stores the effector proteins before they are secreted into the host cell membrane [32].

Extracellular DNA production

When quorum sensing signals are produced within *P. aeruginosa* colonies, they induce the secretion of extracellular DNA (eDNA), which is phenotypically similar to chromosomal DNA [33, 34]. The production of eDNA is likely regulated by quorum sensing systems, including Las, Rhl-acyl homoserine lactone, and Pseudomonas quinolone signalling (PQS). As noted by Allesen-Holm et al. (2006), flagella and type IV pili also induce lysis of cells and release of eDNA [33]. Das et al. (2014) demonstrated that additional virulence factors, such as pyocyanin and phenazine, in conjunction with oxidative stress, contribute to the induction of cell lysis, subsequently facilitating the release of extracellular DNA (eDNA) [35]. Whitchurch et al. (2002) were the first to report the prominent presence of extracellular DNA (eDNA) within the matrix of *P. aeruginosa* biofilms, highlighting its essential role in maintaining the structural integrity of the biofilm [36]. Furthermore, multiple studies have demonstrated that the adhesion of *P. aeruginosa* and subsequent biofilm development is significantly compromised when eDNA is degraded by DNase I, an enzyme that hydrolyzes the phosphodiester bonds linking nucleotides within DNA molecules [37]. Extracellular DNA serves as a vital source of carbon, nitrogen, and phosphorus for bacterial growth and contributes to biofilm resilience by enhancing matrix viscosity, thereby protecting bacteria from shear stress [34]. Additionally, eDNA facilitates horizontal gene transfer, promoting the dissemination of genes associated with antimicrobial resistance and virulence [37].

Immune response against *P. aeruginosa* biofilm

The inception of the war between the pathogen and host immunity ushers the innate immunity to function. The immune response operates through non-clonal cellular and humoral systems. As a matter of fact, this is the first immunity that comes into action since the initial contact with the pathogen [12]. Furthermore, biomaterials also trigger a series of host actions that differ from typical wound healing. When a wound or medical device is implanted, the first injury upsets the

body's homeostatic processes, which initiates the healing process [11]. The traditional approach to the host's response to biomaterials involves five phases: blood-material interactions, chronic and acute inflammation, the response of the foreign body, and fibrous entrapment [5]. Cellular and humoral responses are generalized forms of defense that respond even in the absence of contact with a pathogen. In response to *P. aeruginosa* biofilm, the complement system, dendritic cells, NK cells, neutrophils, and macrophages are the principal elements of the innate immune response. It has been observed that immune responses include neutrophil accumulation, respiratory burst, penetration, phagocytosis, cytokine production, and biofilm bacteria eradication [4]. Furthermore, higher bacterial aggregation in *P. aeruginosa* cultures lead to more respiratory bursts from neutrophils and cytokine release from macrophages [46]. Similarly, early lung samples from mice exposed to *P. aeruginosa* biofilm have shown that a substantial inflammatory neutrophil build-up in the airways is an essential component of the innate immune response [11].

Activation of Innate Immune Cells and Adaptive Immune Cells

Neutrophils

Biofilm formation activates the innate immune system, leading to the recruitment of its primary defenders, neutrophils, around the microbial biofilm. Evidence from the literature demonstrates the accumulation of neutrophils surrounding biofilms [22]. Trøstrup *et al.* (2014) similarly observed neutrophil aggregation at biofilm-associated infection sites in chronic wounds of experimental mice [47]. In addition, biofilm-derived detergent-like compounds can directly induce cytotoxicity in immune effector cells. For instance, rhamnolipids are toxic to neutrophils and surrounding host tissues, thereby facilitating *P. aeruginosa* biofilm development and the formation of biofilm channels [22]. Furthermore, chemokines like IL-8 are released upon the recognition of PAMP by macrophages and epithelial cells that facilitate the recruitment of neutrophils. Following opsonization by C3, immunoglobulins, and surfactant proteins, neutrophils recognize *P. aeruginosa* and initiate phagocytosis [47]. With the help of nitric oxide, reactive oxygen species, and proteolytic enzymes, neutrophils consume the pathogen inside phagolysosomes. Subsequently, neutrophils migrate into the infected tissues through several mechanisms, including selectin-mediated rolling, integrin-dependent adhesion to the vascular endothelium, and chemokine-mediated transmigration [48]. Lipopolysaccharide (LPS) and soluble innate immune mediators such as TNF-α, IL-8, leukotriene B4, and platelet-activating factor further amplify the neutrophil response [22]. The infected airways during lung infection by *P. aeruginosa* induce a significant neutrophil recruitment. The high susceptibility of neutropenic mice to *P. aeruginosa* infection indicates that neutrophils are essential for the effective and unequivocal clearance of this bacterium during acute lung infection [49]. LPS, immunological complexes, or alginate in CF airways are examples of virulence agents that activate neutrophils during lung infections. Numerous investigations have shown that there is a strong concentration of neutrophils at the location of biofilm not only in lung infection but also in biopsies from chronic wounds [50]. Neutrophil serine proteases are key enzymes involved in bacterial clearance, as demonstrated in mice deficient in neutrophil elastase (NE) [49]. These proteases—including NE, cathepsin G, and proteinase 3—target both host and bacterial proteins, which can exacerbate tissue inflammation during bacterial infection. NE specifically degrades the *oprF* gene product, a virulence factor of the *P. aeruginosa* biofilm, thereby disrupting the integrity of the bacterial outer membrane and leading to bacterial death [49]. These serine proteases are inhibited by Spi6 (serine protease inhibitor 6) and SerpinB1 [51]. A significant negative effect of the bulk recruitment of neutrophils has been observed, as they promote cell necrosis, which destroys the host tissue. Neutrophils release specific proteases and NETs (neutrophil extracellular traps) that increase tissue damage and inflammation [51]. Despite the adverse effect on the planktonic stage of the pathogen, neutrophils are unable to kill biofilm [47]. Therefore, virulent components of the *P. aeruginosa* biofilm, such as rhamnolipids, contribute to the persistence of infection by inducing host cell necrosis and killing neutrophils [50].

Macrophages

Owing to their extended lifespan, macrophages constitute a major effector population in the early line of defense against invasive infections [4]. As key players in both innate and adaptive immunity, macrophages eliminate pathogens and remove cellular debris resulting from tissue injury—a process known as efferocytosis [12]. Based on their activation state, macrophages are broadly categorized into two phenotypes: M1 (proinflammatory) and M2 (anti-inflammatory) [9]. M1 macrophages are primarily responsible for microbial killing and are characterized by elevated expression of inducible nitric oxide synthase (iNOS), whereas M2 macrophages contribute to tissue repair and remodeling following phagocytosis and are marked by high levels of arginase-1 (Arg1) [12]. Depending on the local microenvironment and stimuli, macrophages can thus function as either pathogen-eliminating or tissue-healing cells [4].

High expression of inducible nitric oxide synthase (iNOS) indicates the presence of M1 macrophages, whereas elevated levels of arginase-1 (Arg1) are characteristic of M2 macrophages [12]. In *P. aeruginosa* infection, lipopolysaccharide (LPS) and flagellin stimulate alveolar macrophages via Toll-like receptors TLR4 and TLR5, inducing the secretion of keratinocyte-derived chemokine (KC) and proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [52]. Through this mechanism, macrophages contribute to neutrophil recruitment to the infection site. Additionally, *P. aeruginosa* transmits type III secretion system (T3SS)-associated signals that activate caspase-1 in alveolar macrophages, leading to the release of interleukin-1β (IL-1β). Airway epithelial cells sense IL-1β and, in turn, increase the production of chemokines that further promote neutrophil recruitment [52].

Macrophages play a central role in host defense through pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPs) [52]. Among these, TLR2 and TLR4—expressed on the macrophage surface—recognize *P. aeruginosa* LPS via adaptor proteins such as MyD88, initiating inflammatory signaling cascades in the host [53]. Ciornei *et al.* (2010) reported that structurally modified LPS from biofilm-forming *P. aeruginosa* strains enhanced inflammatory cytokine production in human monocytes, an effect not observed in murine macrophages [54]. Moreover, extracellular substances derived from *P. aeruginosa* biofilms can indirectly stimulate macrophages and neutrophils in the absence of direct bacterial contact, owing to high local concentrations of PAMPs within the biofilm matrix—levels typically much lower in bacterial culture supernatants.

Dendritic cells

Dendritic cells (DCs) are professional antigen-presenting cells that bridge the innate and adaptive immune systems. Their role in the pulmonary response to *P. aeruginosa* infection has primarily been studied in post-sepsis models. During the initial encounter with pathogens, DCs play a pivotal role in initiating adaptive immune responses [9]. They can stimulate surface IgA-positive B cells through the secretion of proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF), both members of the TNF family [55]. Immature DCs, located in peripheral tissues, are highly efficient at antigen uptake and are particularly abundant at mucosal surfaces and within secondary lymphoid organs. Upon successful antigen capture and cytokine regulation, these cells mature into potent antigen-processing and antigen-presenting cells [48]. Evidence from cytokine profiling of peripheral blood mononuclear cells (PBMCs) in individuals with chronic cystic fibrosis (CF) infection suggests that *P. aeruginosa* outer membrane protein (OMP) and lipopolysaccharide (LPS) serve as antigenic epitopes within the biofilm, capable of eliciting T-helper cell–mediated immunity [4].

Role of MyD88 in host immune response

Myeloid differentiation primary response protein (MyD88) is a prime component of TLR pathway that is responsible for host immune response. It mediates the adhesion of TLR -2 and TLR-4 to virulent factor (LPS) of *P.aeruginosa* and stimulates the onset of host defense [53]. Via inflammasomes, PRR signaling initiates the activation of interleukin-converting enzymes such as ICE-1 that results in proteolytic cleavage giving rise to the pro-inflammatory cytokine IL-1β from pro-IL-1b (a precursor protein), mediated by MyD88–NF-κB [56]. A study by Skerrett et al. (2004) demonstrated that MyD88-induced responses are essential for shielding the lungs from *P. aeruginosa* infections, with MyD88-deficient animals having a heightened susceptibility to the pathogen [57].

Epithelial cells

The skin serves as the body's first line defense against infections in terms of immunity. The epithelium is essential for immunological protection during lung infection offering physical protection against bacterial invasion through the network of cell–cell contacts, including tight junctions. *P. aeruginosa* infection cannot develop in the upper respiratory tract due to mucociliary clearance [4,58]. Consequently, any type of epithelial deformation increases an individual's susceptibility to infection. This can be majorly observed in intubated patients. Epithelial cells release lactoferrin, which inhibits the formation of biofilm and reduces bacterial motility, preventing the bacteria from adhering to one another and forming microcolonies [58]. Another defense mechanism used by epithelial cells to combat the *P. aeruginosa* biofilm is the surfactant protein called SPLUNC 1 (Short palate, lung, and nasal epithelium clone). The respiratory epithelium secretes splunc-1 and possesses antimicrobial properties by inhibiting biofilm [4]. Cell surface receptors such as asialo-ganglioside M1 (asialo GM1) and Toll-like receptors, can identify *P. aeruginosa* and activate signal transduction pathways, resulting in the generation of inflammatory cytokines and chemokines. Epithelial cells also produce paraoxonases (PONs), which deactivate AHL QS molecules and so limit biofilm development [59].

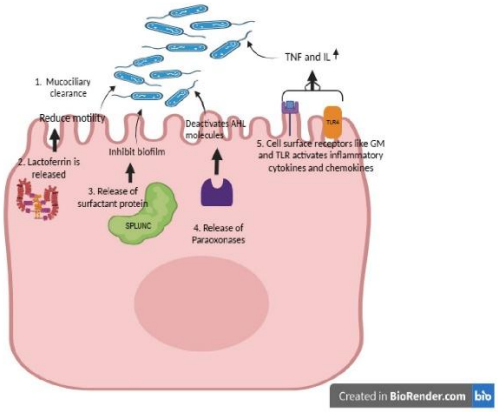

Figure 4: Epithelial defense mechanism during P. aeruginosa attack: 1) Mucociliary clearance of adhering planktonic cells by mucus secretion. 2) Lactoferrin inhibiting bacterial cells communication by reducing motility. 3) Surfactant protein (SPLUNC) inhibit biofilm formation. 4) Release of paraoxanases that deactivate QS signals. 5) Cell surface receptors (GM,TLR) release inflammatory mediators. (Created in https://BioRender.com)

Complement System

The complement system is prominent in the host's immune response against bacterial infections. The complement system opsonizes the bacteria, which facilitates immune cells' ability to phagocytose the bacteria [60]. As observed in the mouse model's lungs, *P. aeruginosa* infection was profound due to the lack of the C5aR receptor, despite enormous neutrophil recruitment [61]. A complement system executes its function in three ways: classical, lectin, and alternative. These three pathways release C3 and C5 upon activation. Following the attachment of the antibodies subclasses IgG1, IgG2, or IgG3 to antigen, immunological complexes (ICs) are formed, leading to the attachment of C1qr2s2 to the Fc region of these antibodies, starting the classical route [60]. *P. aeruginosa* usually finds multiple ways to evade the complement system. One of them involves using protease enzymes to separate the contents of the complement. These proteases may be derived by active neutrophils or microorganisms. The complement system is mostly inactivated by elastases and alkaline proteases [62]. Opsonization is inhibited via O-acetylation and O-glycosylation of alginate and type-IV pili respectively along with Pel can inhibit the lectin pathway [63]. The complement system is an important effector mechanism of the innate immune system, capable of direct bacterial killing through the assembly of the membrane attack complex (MAC; C5b-C9) on the bacterial cell envelope [60]. Forming of the C3b fragments triggers the membrane attack complex (MAC).

Antimicrobial peptides

Antimicrobial peptides (AMPs) are cationic-anionic or amphipathic weapons that play a crucial function in generating immune responses against a wide variety of pathogens [64]. AMPs have been acknowledged as potentially effective substitutes for traditional antibiotics because of their broad spectrum of targets and non-specific mode of action, which lessens the likelihood of resistance formation [65]. AMPs have demonstrated potent anti-biofilm efficacy against both clinically isolated and multidrug-resistant bacterial biofilm in a way that they can obstruct the first bacterial adherence to surfaces during the early phases of biofilm formation by killing or promote microbial detachment, which can dissolve established biofilm [66]. Certain synthetic AMPs rapidly dissolve pre-established *P. aeruginosa* biofilm. The quick disintegration of cells contained in biofilm may suggest that biofilm degradation occurs by rupturing bacterial membranes, though the exact mechanism is yet unknown [64]. Several researchers have used antibiotics and AMPs as combination strategies to elevate the anti-biofilm activity of AMPs. As presented in table-2.

AMPs	Mechanism	References
Esculentin Esc (1-21)	Downregulation of virulence genes obstructing motility; Increases susceptibility of *P. aeruginosa* to antibiotics; Decreases expression of MexAB-OprM efflux pump	[64]
LL-37	Increases twitching motility; Reduces the functional genes of biofilm; Affect the QS system	[67]
Peptide 1018	Decrease intracellular (p) PpGpp; Escape the stringent response; Decrease the spot promoter function	[65]
RN3(5-17P22-36)	Degradation of cell membrane by making it permeable and depolarize	[68]
L-K6L9	Disrupt the matrix of biofilm	[66]
(CSA)-13	Causes permeability of cell membrane making biofilm easily penetrable	[69]
Cathelicidic LL-37 and indolicidin	Downregulation of the transcription of the Las and Rhl systems	[70]

Table 2: Anti-biofilm activity of antimicrobial peptides and their probable mechanism of action

Novel Approaches to anti-biofilm therapies

Emerging multiple drug resistance is a common yet unsolved threat to clinical strategies. Biofilm is an adaptive mechanism of resistance, which, along with MDR, causes severity in infections. Antibiotics alone are not efficient to eliminate the biofilm due to antimicrobial resistance [11]. Therefore, the generation of new therapies that will synergistically function with antibiotics, enhancing their efficacy, is an immediate need. For instance, a QS inhibitor such as QSI4 inhibits pyocyanin pigment when combined with an antibiotic and the synergistic effect of Mycobacterial Pseudomonas Quinolone signal Dioxygenase Aqdc with N-AHL lactonase QsdA [71]. Nanoparticles also have anti-biofilm activities against *P. aeruginosa* biofilm by directly acting upon biofilm or functioning as a carriage

for antibiotics as they ease the penetration within biofilm. As observed in cystic fibrosis patients, where PGA nanoparticles carried ciprofloxacin or minocycline and silver-loaded polyphosphoester, they are effective against *P. aeruginosa* [72, 73].

Photothermal and photodynamic therapies possess anti-biofilm features specifically against *P. aeruginosa,* such as gold nanorods with hydrogel and toluidine blue with silica nanoparticles [74, 75]. Although these therapies have some side effects too [11]. Among the recent technologies, bacteriophage therapy has evolved as a potential method in biofilm treatment, facilitating permeability to antibiotics by disrupting the biofilm matrix [76]. Phage triggers the production of polysaccharide depolymerases, which are potent in the degradation of carbohydrates at the host-pathogen interface [77]. As mentioned above, antimicrobial peptides also possess anti-biofilm characteristics through perforation of the bacterial cell membrane and prevent bacterial adherence [78]. With changing global trends, more natural extracts are being explored due to fewer side effects. A study showed that extracts of *Triumfetta welwitschii* have the potential to decrease the extracellular DNA of *the P. aeruginosa* biofilm matrix [79]. Monoclonal antibodies have also shown effective treatment against in vivo biofilm models. Furthermore, eradication of biofilm is better understood on in vivo models, but due to very few studies in this area, this area is yet to be explored [80].

Conclusions

Microbial biofilms are hazardous to the environment as they harbor pathogens and shield them from unfavorable conditions by providing resistance to factors such as exposure to antimicrobial agents. *Pseudomonas aeruginosa* causes numerous opportunistic infections, and the presence of biofilms in these infections worsens the situation by prolonging treatment, increasing the economic burden, decreasing patients' quality of life, and ultimately elevating both morbidity and mortality rates. *P. aeruginosa* is supported by several virulence factors, including EPS, LPS, pyocyanin and pyoverdine pigments, T3SS, and eDNA, which favor biofilm production at the site of infection. The T3SS allows *P. aeruginosa* to inject effector molecules, such as exotoxins, into the cytoplasm of host target cells. Upon activation of the inflammasome, these factors are recognized by receptors in the host immune system. Immune cells such as dendritic cells, neutrophils, NK cells, and macrophages play a pivotal role in host immune responses, including neutrophil accumulation, respiratory burst, phagocytosis, and cytokine production. The complement system, along with other key components such as MyD88 and cyclic-di-GMP, plays a crucial role in countering Pseudomonas virulence factors by mediating pathogen adhesion to TLRs and reducing biofilm dispersal. Epithelial cells also possess their own defense mechanisms against biofilms, including lactoferrin secretion, surfactant protein (SPLUNC-1), paraoxonases, and mucociliary clearance. This internal combat between host immune responses and biofilm-associated virulence factors can lead to significant tissue injury, contributing to the pathophysiology of infection. Although antibiofilm agents are widely used, therapeutic failure often occurs due to the pathogen's resistance to common anti-pseudomonal drugs. Antimicrobial peptides have demonstrated strong activity against biofilms and should therefore be used synergistically with other antibiofilm agents, particularly natural extracts, to develop novel therapeutic strategies. This review provides insights into the fundamental mechanisms of host immune responses to biofilm infections. We conclude that since infections are more prevalent among immunocompromised populations, strategies targeting virulence factors and enhancing host immune cell activity should be prioritized. Implementing natural antimicrobial agents that act on virulence factors responsible for biofilm formation, while simultaneously boosting immune cell function, represents a promising approach to improving treatment outcomes.

Author contributions

Author contributions Sunita Sheoran : Conceptualization, methodology, formal analysis, investigation, data curation, writing—original draft, supervision. Syed Amir Ashraf: Conceptualization formal analysis, investigation, data curation, writing—review and editing. Neha Rawat: formal analysis, investigation, data curation, writing—review and editing. Mukesh Sharma: writing—original draft, writing—review and editing. Jalaluddin Khan: methodology, formal analysis, investigation, data curation, writing—original draft, writing—review and editing. SD. Shahanawaz: formal analysis, investigation, data curation, writing—review and editing. Amir Mahgoub Awadelkareem: methodology, formal analysis, investigation, data curation. Angum M. M. Ibrahim: writing—original draft, writing—review and editing. All authors reviewed, edited, and approved the final version of the manuscript for submission.

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