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BACTERIAL BIOFILM: A REVIEW ON FORMATION, CLINICAL IMPORTANCE, DETECTION AND CONTROL MEASURES

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Abstract

The rise in global antibiotic resistance is a significant concern, further intensified by the increasing prevalence of organisms that form biofilms. Biofilms are highly organised communities of microorganisms, including bacteria and fungi, comprising a mixture of strains forming a slimy extracellular matrix of extracellular polymeric substances. They could be found on biotic and abiotic surfaces. Infections associated with biofilms have been reported to be linked to treatment failure, increased cost of treatment and prolonged hospital stay and increased morbidity and mortality. The biofilm serves as a protective shield for organisms in the matrix, making them highly resistant to antibiotics, disinfectants, mechanical fluctuations, and immune response. This review aims to describe the process of its formation, clinical implications, methods of detection and preventive strategies.

Keywords: Antibiotic resistance, bacterial biofilm, clinical implications, biofilm detection

1.Introduction

A significant contributor to the global antibiotic resistance challenge is the ability of bacteria to form biofilms [1]. Antimicrobial resistance (AMR) has emerged as a chronic public health challenge, with forecasts by the WHO revealing that 10 million deaths will occur annually by 2050 [2]. Bacterial biofilm formation presents significant implications and challenges in the field of antimicrobial therapy as it protects the bacteria from antibiotics and the immune response [3]. As a result of drug resistance, antibiotics and other antimicrobial medicines become ineffective, and infections become difficult or impossible to treat, increasing the risk of disease spread, severe illness, disability, and death [4]. The first description of biofilms was made by the 17th-century microbiologist Anton Von Leeuwenhoek after observing bacterial aggregates from the scrapings of plaque on his teeth [5]. Biofilms are highly organised communities of microorganisms, including bacteria and fungi, comprising a mixture of strains that form a slimy extracellular matrix known as biofilm. Biofilms have osmotic barrier properties and are resistant to antimicrobial agents [6,7,8]. They represent a predominant form of microbial life ubiquitous in natural ecosystems. The formation of biofilms significantly enhances the survival efficiency of bacteria, rendering them 1000 times more resistant to pressures such as antibiotics, disinfectants, mechanical fluctuations, and physical scavenging compared to their planktonic counterparts [9,10]. Medical devices, such as catheters and cardiovascular devices, are prone to colonisation by bacterial biofilms, posing serious threats to patient health and compromising device function, ultimately leading to treatment failures [11,12].

The understanding that bacterial biofilms contribute to antimicrobial resistance and disease pathogenesis has led to heightened efforts to identify diseases associated with biofilms and the development of novel agents to eradicate them.

2.Biofilm Structure and Composition

The matrix in which microorganisms are encased comprises extracellular polymeric substances (EPS), typically consisting of polysaccharides, proteins or peptides, lipids, and deoxyribonucleic acids (DNA) (Figure 1). The presence of Ca_2+ or Mg_2+ forms supportive cross-bridges between polymers of the biofilm architecture, allowing them to grow to thicknesses of up to 300µm, while the hydroxyl groups on the polysaccharide increase mechanical strength by interacting with each other [13,14,15]. Water forms over 70%

of the biofilm by weight, allowing for the transportation of nutrients and waste [16]. These biofilm components facilitate the coherence of cells and cell surface attachment, maintain osmotic pressure and offer protection to the bacteria against host defence [17,18,19].

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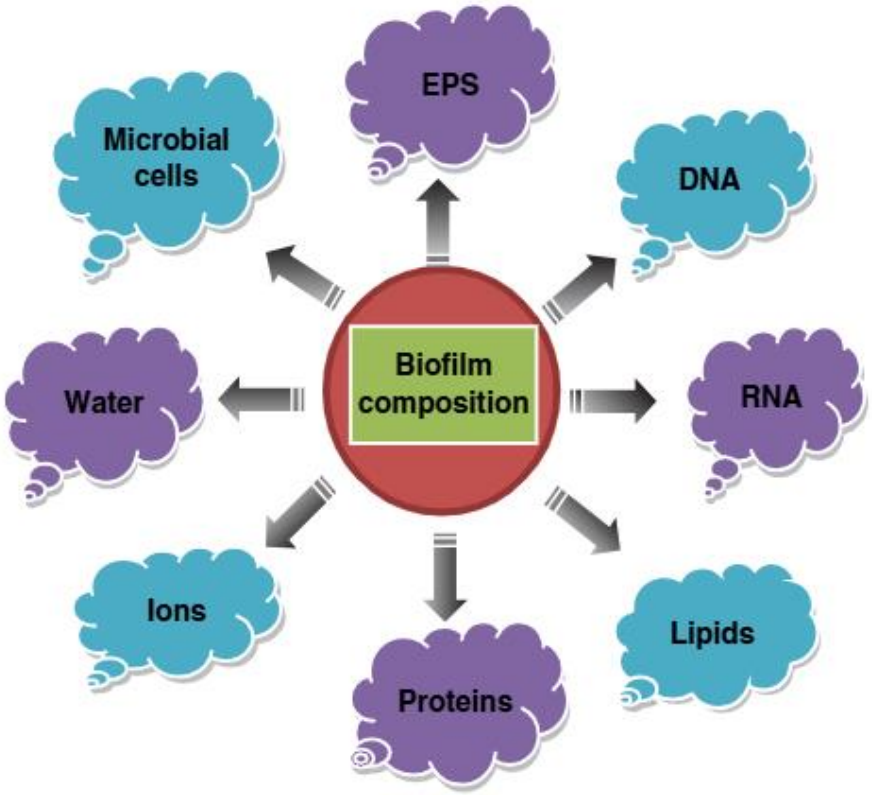

Figure 1: Schematic overview of a mature biofilm composition [20].

3.Mechanism of Biofilm Formation

Biofilm formation by bacteria is a survival strategy resulting from environmental challenges such as ultraviolet radiation, limited nutrients, extreme pH levels, high salinity, temperature extremes, pressure increase and the presence of antimicrobial substances [21]. Five main stages have been identified in the formation: initial cellular attachment, formation of microcolonies, biofilm maturation, and finally its dispersion (Figure 2)[7].

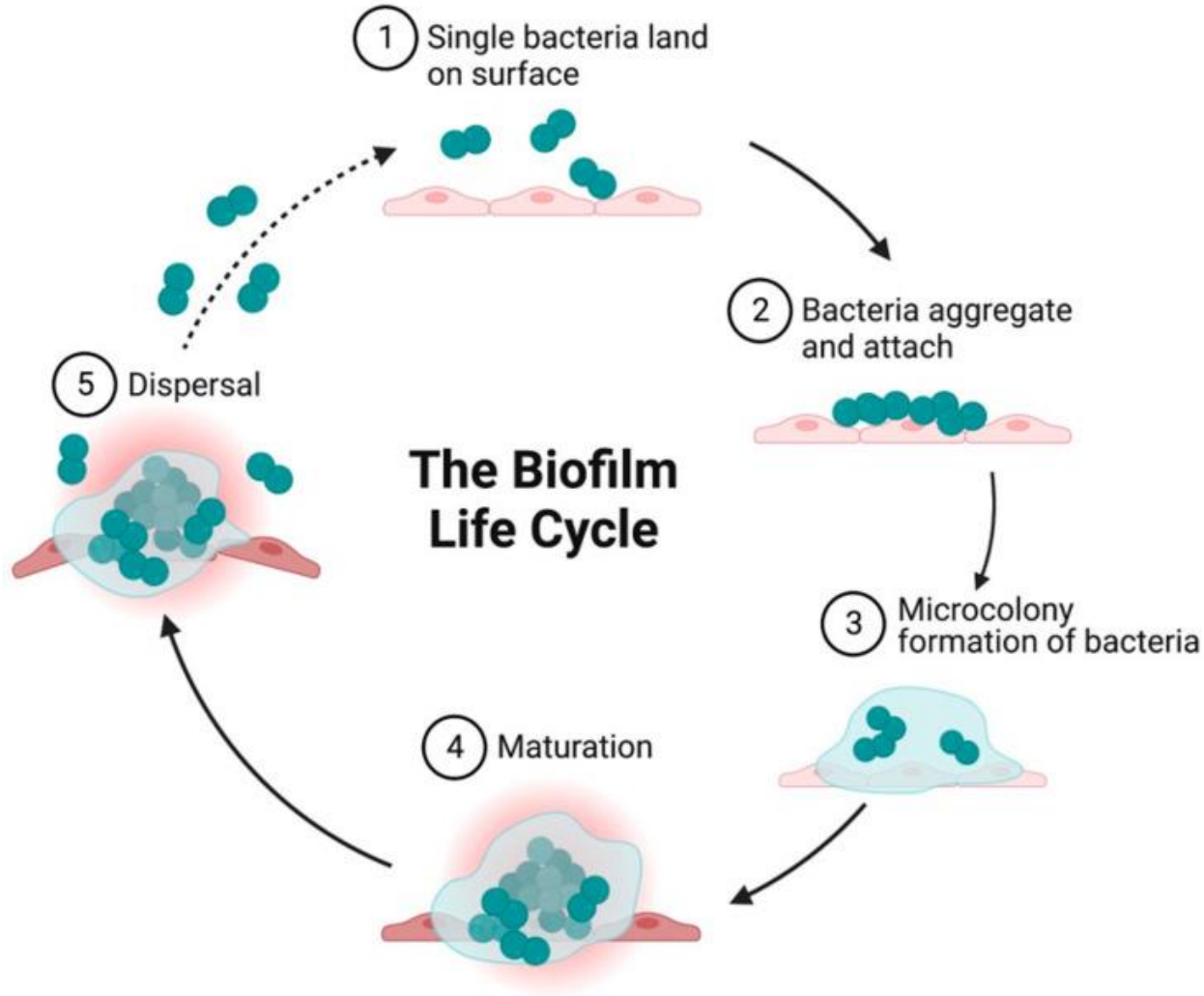

Figure 2. Biofilm Formation Life Cycle [15].

3.1 Cellular Attachment

The attachment of bacteria onto a surface is the initial step of biofilm formation. For an organism to bind to a surface, they have to overcome the repulsive forces caused by the negatively charged bacterial membrane and the surface [23]. A hydrophobic surface, such as plastic, has a reduced force of repulsion as compared to a hydrophilic surface, for example, glass and metal. Attachment is achieved by the presence of flagella and pili on the bacterial membrane [24]. The attachment phase involving the structural support is called a reversible attachment. The binding is reversible as bacteria are only poorly bound to a surface and can leave the surface at this stage [25].

3.2 Microcolony Formation

After bacterial cells have attached irreversibly to a surface, they divide and produce extracellular polymeric substances (EPS). The production of EPS leads to the formation of a biofilm matrix, a 'shelter' where all the attached cells reside [6]. EPS is involved in the adhesion of cells to surfaces, contributing to permanent attachment. Recently, it was found that matrix proteins of a biofilm possess adhesin-like properties that mediate cellular attachment [26]. Usually, a biofilm contains more than one type of micro-community and involves coordination between them. This coordination is important for substrate exchange, the distribution of essential metabolic products, and the excretion of harmful substances [15].

3.3 Biofilm Maturation

Cellular division with continuous production of EPS leads to the formation of an early biofilm, which matures after some time and ultimately becomes a three-dimensional (3-D) structure. The 3-D structure is contributed to by the EPS (produced by the embedded cells), they are also responsible for maintaining this architecture [15]. The maturation process involves cell-to-cell communication, in which cells embedded in the biofilm release signalling molecules called auto-inducers to facilitate quorum sensing [27]. Besides that, a mature biofilm also contains water channels in the matrix acting as a circulatory system. The functions of these channels include nutrient distribution and the removal of waste products [7].

3.4 Detachment of Biofilm

Following maturation, biofilms undergo a process known as dispersion. At this stage, some cells leave the biofilm and return to

their planktonic lifestyle. As these cells return to their free-floating form, they are now able to attach to a new surface, and the cycle starts all over again [28]. Cells can either detach from the biofilm actively or passively. Passive dispersal of a biofilm is mediated by mechanical forces or external forces such as abrasion, fluid shear, and solid shear [7].

3.5 Quorum Sensing (QS)

This is the regulation of gene expression in response to differences in cell population densities. The processes of QS and biofilm formation are interdependent. When the QS gene is activated, it causes the biofilm to develop and then coordinates its maturation and breakdown. The phenomenon of QS is feasible only when a specific volume has a minimal quantity of bacteria. The amount of auto-inducer signalling molecules secreted by the bacteria in a micro-colony can be used to determine the number of bacteria in each volume [15,29].

3.Implications of Microbial Biofilm Formation

Biofilm is a critical problem in the medical sector, as it is formed on medical implants within human tissue and is involved in a multitude of serious chronic infections [30]. Common bacteria involved in severe biofilm-associated infections include pathogens of the "ESKAPE" group (*Enterococcus spp*., *Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella spp., Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter spp*.), which cause a variety of infections [31,32]. There are three ways by which bacterial biofilms have been implicated: a) Reduced Antimicrobial Efficacy, b) Increased Resistance and c) Persistent Infections. The most common microorganisms for medical device-associated infections are *Staphylococcus aureus* and *Staphylococcus epidermidis*. Multi-resistant gram-negative bacteria (e.g., *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Acinetobacter baumannii*) can also cause medical device-associated infections in complex hospital settings [33]. In addition, microorganisms can adhere to various tissue surfaces in the body (e.g., skin, connective tissue, intestinal mucosa, vascular endothelium, oral cavity, airway, bone tissue, and vagina), which in turn can cause non-device (tissue) -associated biofilm infections and lead to various diseases [34,35]. Within biofilms, bacteria adapt to low oxygen levels and nutrient scarcity by altering their metabolism, gene expression, and protein production. This can result in a decreased metabolic rate and slower cell division [36]. As a result, biofilm-related

diseases tend to be persistent infections that progress slowly, are infrequently resolved by the immune system, and show inconsistent responses to antimicrobial therapies [37].

5. Laboratory Detection of Biofilm-Forming Bacteria

In the laboratory, analysis of these samples is difficult since the microorganisms in the biofilm adhere to each other and the surface, forming a microbial aggregate. Therefore, sonication is generally performed using sound energy to agitate particles in a solution, removing the aggregated and adhered bacteria from biomaterial surfaces, allowing for analysis [38,39].

5.1 Fluorescence in Situ Hybridisation (FISH)

This method consists of the binding of short fluorescence-labelled oligonucleotides, which can bind to the specific ribosomal RNA of the target organisms. It has the advantage of allowing the analysis of the sample without prior treatment, easily identifying microbial aggregates; the samples are analysed via microscopy [40,41].

5.2 Electronic Microscopy

This approach allows the visualisation of microbial aggregates after sonication or FISH. Scanning electron microscopy (SEM), transmission electron microscopy (TEM), and confocal laser scanning microscopy (CLSM) are the most used tools in biofilm visualisation [42,43]. The high resolution of this microscopy allows analysis of all biofilm structures, facilitating direct detection and visualisation of the biofilm where it was formed, such as in a catheter [44,45].

5.3 Polymerase Chain Reaction (PCR)

This approach detects biofilm-forming pathogens directly in clinical samples. Compared to methods that involve culture, DNA-based analyses are more sensitive in detecting microbes, including unculturable cells and samples with mixed species. It is based on the amplification of specific regions, providing high specificity and sensitivity in the identification of genes involved in biofilm formation, after the sonication process [46]. Some examples of developed PCR methods are real-time PCR, multiplex PCR and reverse transcriptase PCR. These techniques can be used to identify clinical biofilm-forming isolates [47].

5.4 Colourimetric Assays

This is the most widely used method and is commonly applied in studies related to the development and susceptibility of biofilms to

drugs as well as the quantification of specific biofilm structures[15].

5.4.1 Crystal Violet Staining (Agar Plate and Tube Methods)

Agar Plate Method

Isolates from fresh agar plates are inoculated in 5 ml of Trypticase soy broth and incubated at 37°C for 24 hours. The cultures are then diluted 1:100 with fresh medium. Individual wells of 96-well flat-bottom polystyrene plates are then filled with 0.2 ml aliquots of the diluted culture. The plates are then incubated at 37°C for 24 hours, and after the incubation, the contents are removed from the plates by tapping gently.

Plates are then washed twice with 0.2 ml of phosphate buffer saline (pH 7.2) and incubated at 37°C for an hour. The plates are stained with 0.2 ml of 0.1% crystal violet for 10 minutes. Excess stain is removed by washing twice with deionised water, and the plates are kept for drying. 200 µl of 33% glacial acetic acid is added to the wells (Figure 3). The optical density (OD) of the isolates is determined using an ELISA auto reader at a wavelength of 570 nm. The average optical density (OD) values of each test strain and negative control are calculated, and the final OD values of a test strain are obtained by subtracting the OD cutoff (ODc) value of the negative control from the average OD value of the test strain. Biofilm formation is classified into Strong, moderate and weak or non-biofilm producers [48,49].

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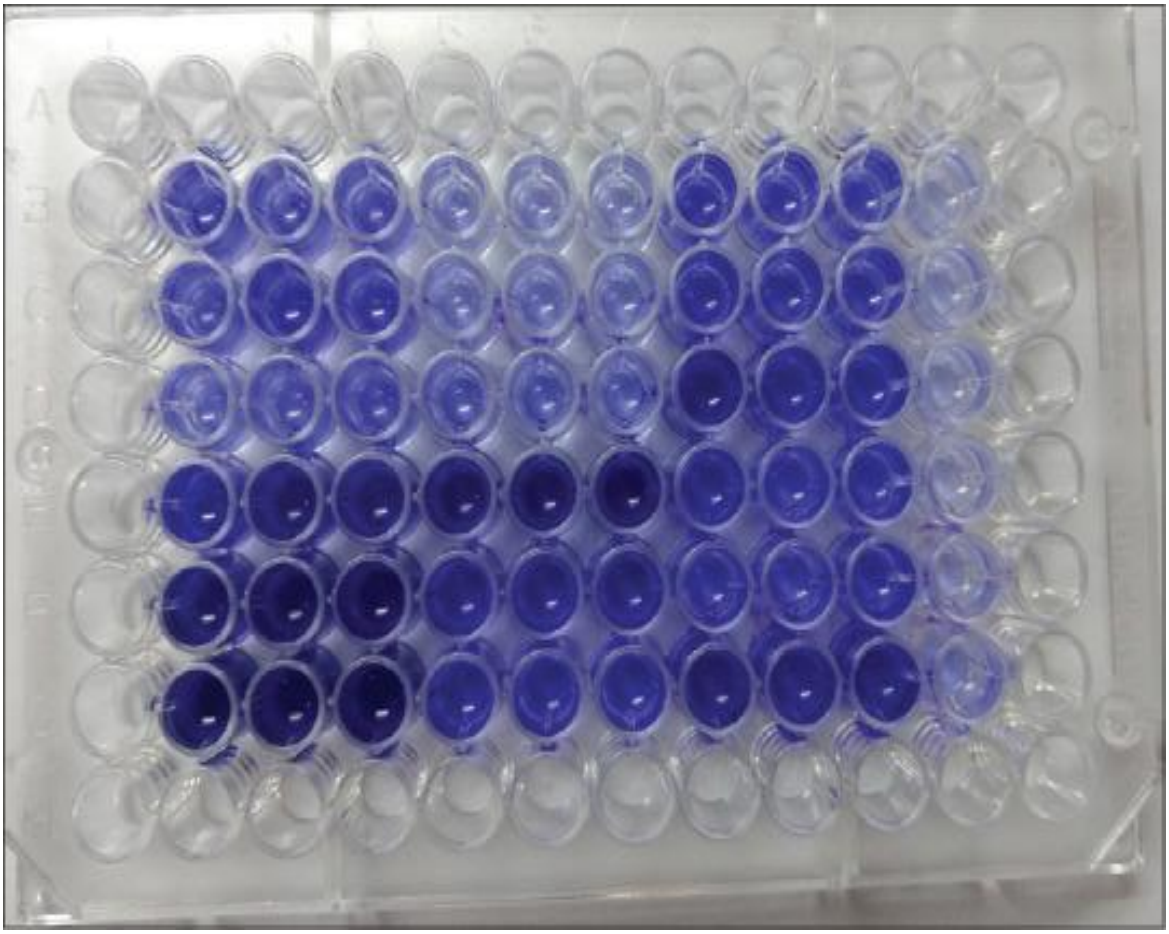

Figure 3. Moderate biofilm formation in a 96-well plate: a top-down view of *Escherichia coli* growth stained with 0.1% crystal violet [47].

Tube Method

In this method, 5 ml of Trypticase soy broth is inoculated with a loopful of organisms and incubated for 24 hours at 37 °C. The tubes are then decanted and washed with phosphate buffer saline (pH 7.2) and allowed to air dry. Crystal violet (0.1%) is used to stain for 10 minutes and washed with deionised water. The tubes are then left to air dry in an inverted position. Biofilm production is considered when there is a formation of a visible layer on the walls and at the base of the tube. While the formation of a ring at the interface of the liquid medium is an indication that the organism is non-biofilm producing. It is scored as negative, weak positive, moderate positive, and strong positive (Figure 4)[47]

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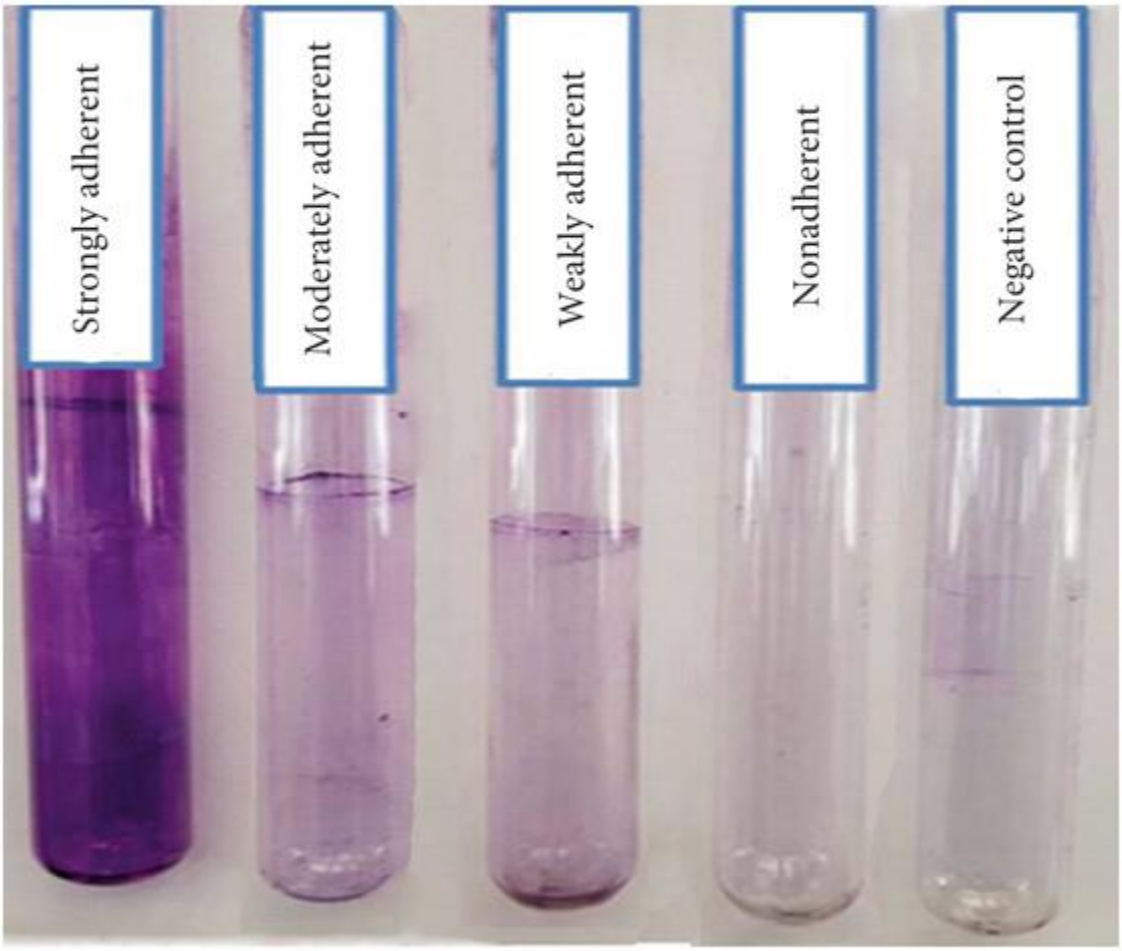

Figure 4. Test tube method showing *Pseudomonas aeruginosa* biofilm production on the inner sides of test tubes stained with 0.1% crystal violet[50].

5.4.2 Congo Red Agar Test

This agar allows the differentiation of the colonies in two colours, with black indicating strong biofilm production and pale-red indicating no biofilm production[51]. Special media such as Brain Heart Infusion (BHI) agar with Sucrose and Congo red in the following composition: BHI agar-52 g/L; sucrose-36 g/L; agar-10 g/L; Congo red-0.8 g/L can be used. It is prepared as a concentrated solution and autoclaved, then added to the medium when the agar is cooled to 55°C and poured into Petri dishes. Plates are then inoculated with test organisms and incubated for 24–48 hours at 37°C. Black colonies with dry crystalline morphology are considered positive for biofilm-producing organisms, while weak or non-biofilm-producing organisms appear pink in colour (Figure 5)[47,52].

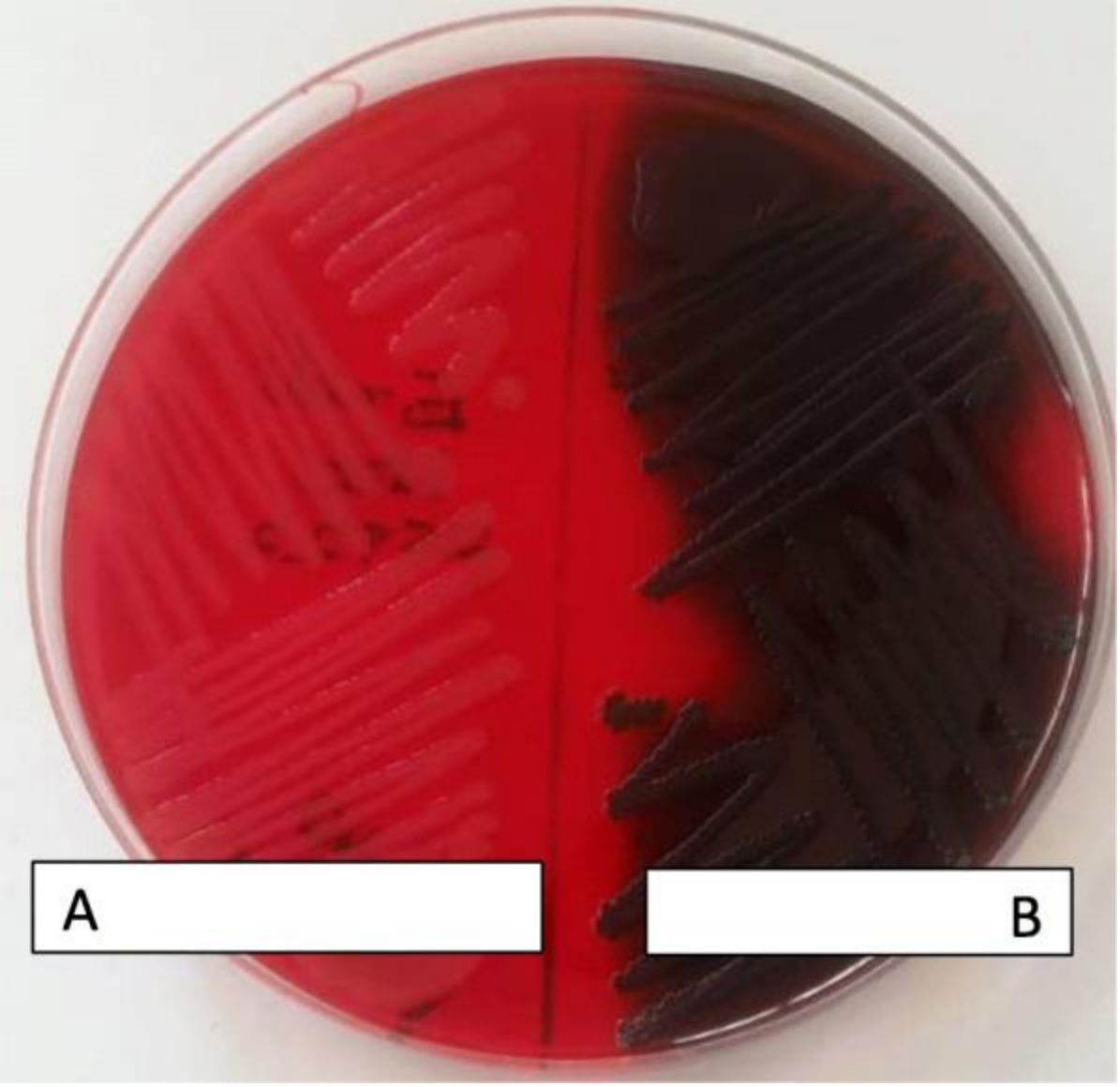

Figure 5. *Escherichia coli* biofilm negative (A) and positive colonies (B) on Congo Red Agar[51].

5.4.3 Biofilm Assay Kit

This is an assay kit that allows for the detection and quantification of biofilms. The assay kits allow for the formation of biofilms on the pegs attached to the plate's lid and make it possible to avoid peeling off biofilm, which occurs during the process of formation (Figure 6). In the first step, the amount of biofilm is measured after staining the live or dead microorganisms with crystal violet by targeting the extracellular polysaccharides at 590 nm.

Secondly, the metabolic activity of the live organisms within the biofilm is measured at 480 nm after staining with warm toluidine blue solution[53, 54].

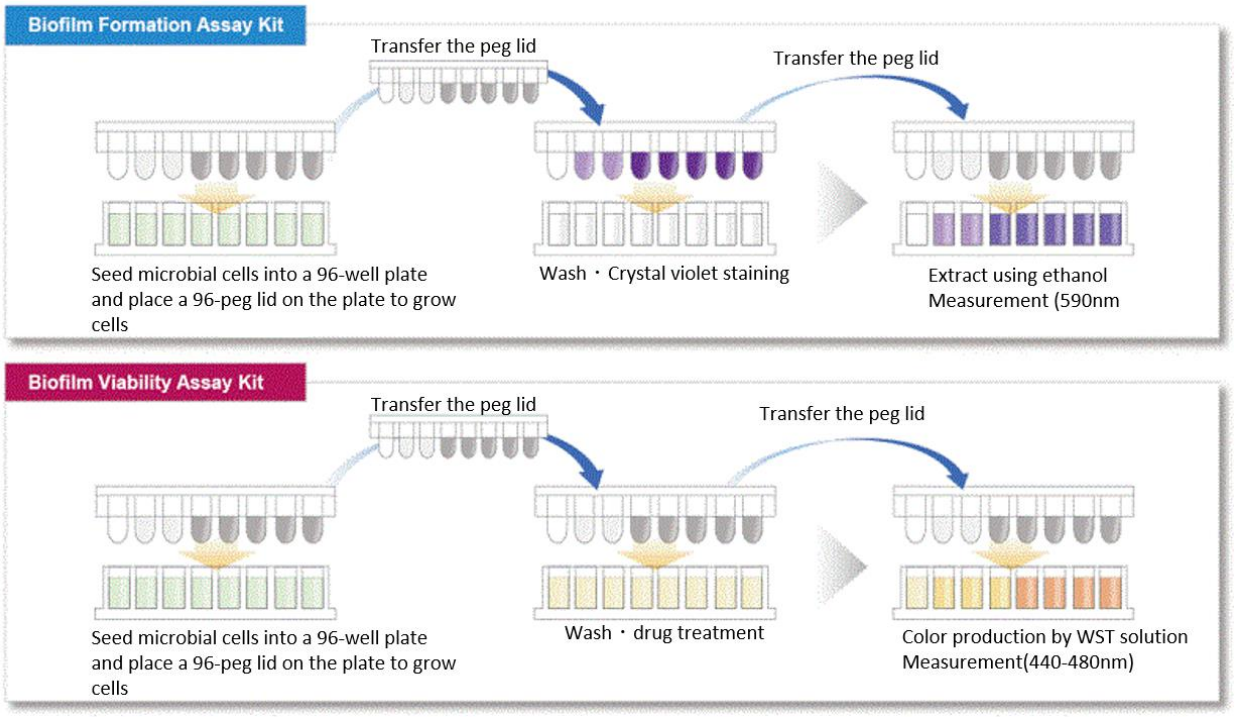

Figure 6. Procedures for Biofilm Formation Assay and Biofilm Viability Assay[54].

6. Strategies to Combat Biofilm Formation

6.1 Targeting Biofilm-Specific Mechanisms

Due to the growing bacterial resistance to antibiotics that have been overused, the search for alternative antimicrobial therapies is on the increase. One of them is quorum quenching (QQ), which disrupts microbial communication[55,56]. QQ-driving molecules can decrease or even completely inhibit the production of virulence factors, including biofilm formation. For example, Kaempferol and Naringenin obtained from citrus plants can inhibit quorum sensing [57,58].

6.2 The Use of Biofilm-disrupting Agents and Enzymes

Azithromycin has been shown to reduce biofilm formation by Pseudomonas aeruginosa and inhibit QS-regulated virulence factors, includin autoinducer production, pyocyanin production, and swarming. Azithromycin downregulates the expression of *gacA*, which mediates the switch between the motile and biofilm lifestyles of *Pseudomonas aeruginosa* [59,60].

6.3 Nanotechnology-based Antimicrobials

Nanoparticles (NPs) are being increasingly utilised as an alternative to antibiotics for targeting bacteria. Their application in treating bacterial infections shows promise, especially in areas such as antibacterial coatings for implants, medicinal materials for infection prevention and wound healing, antibiotic delivery systems, microbial diagnostics, and antibacterial vaccines [61,62].

6.4 Combination Therapies and Adjunctive Treatments

Due to the resistance of biofilms to antibiotic treatments, combination therapy with different medications has been employed to eradicate biofilms[63]. An inhaled Fosfomycin/tobramycin combination antibiotic strategy has been effective against Gram-negative as well as Gram-positive organisms. Along with antibiotics, the use of natural or synthetic adjuvants shows promising effects in treating biofilm infections. Fluoroquinolone activity on biofilms is disrupted by low oxygen tension in the matrix, a strategy known as hyperbaric oxygen treatment that can enhance the efficacy of antibiotics if well optimised. Adjuvants such as Ethylenediaminetetraacetic acid (EDTA), Sodium Dodecyl Sulphate (SDS), and chlorhexidine act by killing non-growing microbial cells aggregated on the inner layer of the biofilm [64].

A new therapeutic approach describes the application of stimulus-responsive therapy along with drug combinations. This experimentation made use of phototherapy; the two main techniques employed in light-induced therapy are photodynamic therapy (PDT) and photothermal therapy (PTT) [65]. Light-induced eradication has a 99-100% efficiency rate. Some of the light-induced therapies are PDT and Ampicillin used against *Escherichia coli* K12-MG 1655 processed on a carbon dot platform, PTT and Ciprofloxacin were effective against *Escherichia coli*, *Staphylococcus aureus* processed on hydrogel, and ferulic acid/sulfur dioxide against *Enterococcus cloacae* MTCC 509 [66].

6.5 Wound Debridement

The exceedingly high incidence of false-negative cultures for bacteria in a biofilm state leads to missed diagnoses of wound infection. The use of topical and parenteral antimicrobial therapy without wound debridement has had a limited impact on decreasing biofilm infection, which remains a major problem in wound care. However, a combination of topical antimicrobial agents alongside wound debridement has been shown to reduce the effect of biofilm-forming bacteria [67].

6.6 Water Treatment

Biofilms develop as a survival mechanism for bacteria in aquatic environments. Ozone effectively targets the extracellular polysaccharides of bacterial colonies on surfaces, rapidly cleaving through the biofilm's structure and breaking it down into harmless microscopic fragments. Its efficacy stems from ozone being a strong oxidant, present in much larger concentrations compared to common disinfectants like chlorine. This method has primarily been used for water purification [68]. Other physical methods include the use of heat, light, sonification and electromagnetic energy [69,70].

4.Conclusion

It is estimated that about 70% of all human microbial infections are due to biofilms and lead to various diseases, including non-healing chronic wounds, endocarditis, periodontitis, cystic rhinosinusitis, fibrosis, meningitis, osteomyelitis, kidney infections, prosthesis, and implantable device-related infections. The bacteria within biofilms exhibit significant heterogeneity with complex properties. As a result, the diagnosis and treatment of infections associated with biofilms remains a considerable hurdle in healthcare, with treatment strategies for such

infections still in their infancy. The tube and plate method for the demonstration of biofilm-forming organisms, as well as the Congo red method, are simple that are cost cost-effective and can be performed with little training. Microbial biofilms pose a significant challenge to antimicrobial therapy due to their unique biological characteristics that confer resilience against conventional treatments. The implications of biofilm-associated infections underscore the need for innovative diagnostic procedures and implementation of therapeutic strategies that target biofilm formation and enhance the efficacy of existing antimicrobial agents.

Author contributions

Conceptualisation by I.I and O.M., review of literature and writing M.S. J., O.M. and I.S., drafting of original manuscript I.I., U.R.U and O.U.D., supervision O.M., U.D. and E.T. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest

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