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Isolation of bacteriophages from sewage sample and studying their effect on MDR *pseudomonas aeruginosa* biofilms:

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ABSTRACT:
Multi-Drug Resistant (MDR) *Pseudomonas aeruginosa* is one of the most important bacterial pathogens that causes infection with a high mortality rate due to resistance to different antibiotics. This bacterium prompts extensive tissue damage with varying factors of virulence, and its biofilm production causes chronic and antibiotic-resistant infections. Therefore, due to the non-applicability of antibiotics for the destruction of *P. aeruginosa* biofilm, alternative approaches have been considered by researchers, and phage therapy is one of these new therapeutic solutions. Bacteriophages can be used to eradicate *P. aeruginosa* biofilm by destroying the extracellular matrix, increasing the permeability of antibiotics into the inner layer of biofilm, and inhibiting its formation by stopping the quorum-sensing activity. Furthermore, the combined use of bacteriophages and other compounds with anti-biofilm properties such as nanoparticles, enzymes, and natural products can be of more interest because they invade the biofilm by various mechanisms and can be more effective than the one used alone. On the other hand, the use of bacteriophages for biofilm destruction has some limitations such as limited host range, high-density biofilm, sub-populate phage resistance in biofilm, and inhibition of phage infection via quorum sensing in biofilm. Therefore, in this review, we specifically discuss the use of phage therapy for inhibition of *P. aeruginosa* biofilm in clinical and in vitro studies to identify different aspects of this treatment for broader use.

INTRODUCTION:

In most natural, clinical and industrial settings, bacteria often grow attached to surfaces in communities known as biofilms. (*Arne Heydorn et'al*). Biofilms can be defined as communities of microorganisms attached to a surface. It is clear that microorganisms undergo profound changes during their transition from planktonic (free-swimming) organisms to cells that are part of a complex, surface-attached community. These changes are reflected in the new phenotypic characteristics developed by biofilm bacteria and occur in response to a variety of environmental signals *(O'toole et'al).* Organisms often adapt to such environmental signals by altering their gene expression and general physiology including increased resistance to antibiotics. One of the ways in which microbial communities adjust to environmental changes is by changing the structural organization of the biofilm *(A. Heydorn and et'al).* Biofilms may form on living or non-living surfaces and can be prevalent in natural, industrial, and hospital settings. The microbial cells growing in a biofilm

are physiologically distinct from planktonic cells of the same organism, which, by contrast, are single-cells that may float or swim in a liquid medium. Biofilms can form on the teeth of most animals as dental plaque, where they may cause tooth decay and gum disease.

The formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. The first colonist bacteria of a biofilm may adhere to the surface initially by the weak Van der Waals forces and hydrophobic effects. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures such as pili. During surface colonization bacteria cells are able to communicate using quorum sensing (QS) products such as N-acyl homoserine lactone (AHL). Once colonization has begun, the biofilm grows by a combination of cell division and recruitment. Polysaccharide matrices typically enclose bacterial biofilms. In addition to the polysaccharides, these matrices may also contain material from the surrounding environment, including but not limited to minerals, soil particles, and blood components, such as erythrocytes and fibrin. The process of biofilm formation is summarized by five major stages of biofilm development.

1. Initial attachment
2. Irreversible attachment
3. Maturation I
4. Maturation II
5. Dispersion

Biofilms can be found almost anywhere and may impact human health both positively and negatively. One example of a positive effect includes the biofilms of commensal bacteria such as *Staphylococcus epidermidis*, which can impede the colonisation of potentially pathogenic bacteria through the stimulation of host-cell immune defences and the prevention of adhesion. However, biofilms are more often associated with many pathogenic forms of human diseases and plant infections.

Antibiotics have been the cornerstone of the clinical management of bacterial infections since their discovery in the early part of the last century. Eight decades later, their widespread, often indiscriminate use, has resulted in an overall reduction in their effectiveness, with reports of multidrug-resistant bacteria now commonplace. Increasing reliance on indwelling medical devices, which are inherently susceptible to biofilm-mediated infections, has contributed to unacceptably high rates of nosocomial infections, placing a strain on healthcare budgets *(Louise Carson and et'al)*. Biofilm associated pathogenic diseases to humans and animals are prevalent and are becoming a challenging task for cure by medical drugs. Moreover, the prevalence of antimicrobials in targeting bacteria may randomly cause the risk pathogenic antimicrobial resistance and immune response *(Dakshinamurthy Sasikala and et'al)*. Notably, the occurrence of antibiotics resistivity is often attributed to the release of a protective and adhesive matrix as a result of microbial cell aggregations. Profoundly, this may prevent the antibacterial to penetrate into the mature biofilm bacterial surfaces, rather than nutrient deficient, and extend to regulate the further proliferation of microbial cells (*(Dakshinamurthy Sasikala and et'al)*. Antibiotic resistance genes encoding for bacterial resistance to common antibiotics, including β-lactams, aminoglycosides, chloramphenicols, and tetracycline, are posing a major threat to current medical treatment of common diseases, and these genes now appear to be abundant in the environment. The spread of antibiotic resistance genes carries a unique danger in that many antibiotics have diminishing efficacy against common infections, particularly the difficult-to-treat nosocomial infections caused by

the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.).

Pseudomonas aeruginosa is Gram-negative bacterium, one of the leading cause of drug-resistant nosocomial infections in developing countries. P. aeruginosa is a Gram-negative bacterium and is usually present in diverse environmental habitats ranging from living to non-living surfaces *(M. Adnan, et'al.)*. Pseudomonas aeruginosa is an opportunistic human pathogen that causes major problems in a number of clinical settings, particularly in patients with burns or other wounds, patients who have indwelling medical devices, and cystic fibrosis (CF) sufferers. Patients with CF are prone to respiratory infections caused by mucoid strains of P. aeruginosa, and these strains are recalcitrant to treatment with antibiotics *(Hanlon Et'al.).* Pathogenic strains of this Gram-negative bacillus frequently cause pneumonia in hospitalized patients. There are also many case reports available of a community-acquired pneumonia (CAP) in healthy people caused by this bacterium. A very high mortality rate is reported from P. aeruginosa in form of acute pneumonia in immuno-compromised patients. It triggers chronic inflammation in cystic fibrosis patients and may leads to complete dysfunction of the lungs *(M. Adnan, et'al.)*.

According to U.S. Centers for Disease Control and Prevention report, it is estimated that approximately 51,000 healthcare-associated infections caused by *P. aeruginosa* occur in the United States each year, and 13% of these infections are multidrug-resistant (MDR), with roughly 400 deaths per year attributed to such infections. The main mechanisms of these resistances are low antibiotic permeability of the outer membrane, chromosomally encoded AmpC, and drug efflux via multi-drug efflux (Mex) systems. In addition to intrinsic resistance, *P. aeruginosa* has different mechanisms for resistance to various antibiotics, such as horizontal gene transfer and mutation-driven resistance. Mobile genetic elements such as transposons, resistance islands, prophages, integrons, and plasmids can accommodate antibiotic resistance genes and transmit them to *P. aeruginosa*, causing MDR bacteria. For example, aminoglycoside-modifying enzymes are transported to *P. aeruginosa* via mobile genetic elements and reduce the binding affinity of the antibiotic to its target site, which is the 30_S ribosomal subunit. Therefore, it causes resistance to aminoglycosides. Furthermore, the random mutation frequency differs between antibiotics with resistance frequencies ranging from 10^6 to 10^9 for individual antibiotics. The rate of mutation can increase in some situations, such as the presence of DNA-damaging agents or within growth in a biofilm . *Pseudomonas aeruginosa* can bind to various surfaces and form biofilms leading to chronic infections by increasing resistance to antibiotics, disinfectants, various irradiation treatments, environmental conditions, and the immune system Biofilms are approximately 10 to 1000 times more resistant to antibiotics than planktonic cells due to the lack of antibiotic penetration into the complex polysaccharide matrix (glycocalyx) of biofilms. Thus, biofilms and the inherent and acquired antibiotic resistance mechanism of *P. aeruginosa* have increased the prevalence of MDR strains in recent years with virtually no fully effective antibiotics available to stop this bacterium.

So, researchers are looking for new ways to inhibit *P. aeruginosa* biofilms. Phage therapy is one of the important methods to inhibit *P.* aeruginosa biofilm [18]. Bacteriophages are viruses that invade bacteria; they were discovered almost a century ago and are divided into two lytic (virulent phages) and temperate categories depending on their life cycle. The lytic life cycle is where phages infect and rapidly kill their infected host cells, thereby shaping bacterial population dynamics and occasionally assisting in their long-term evolution via generalized transduction. The lysogenic life cycle in contrast, is where phages instead of directly killing their hosts, integrate into their host genome, or exist as plasmids within their

host cell. This lysogenic life cycle can be stable for thousands of generations and the bacteriophage may alter the phenotype of the bacterium by expressing genes that are not expressed in the usual course of infection in a process known as lysogenic conversion. lytic bacteriophages are often a matter of interest for therapeutic purposes because they lead to the killing of their bacterial host cell rapidly.On the other hand, temperate phages generally integrate their genome into the host chromosome or sometimes keep it as a plasmid, which is transmitted to the daughter cells by cell division. Using antibiotics has always been a good solution for the treatment of bacterial infections due to their inexpensive cost and extreme effectiveness on various bacterial agents. After World War II, the widespread effective use of antibiotics diminished the interest of different societies in using bacteriophages. Nevertheless, over the years, for various reasons such as overuse and misuse of broad-spectrum antibiotics, bacterial resistance to the existing antibiotics increased, and MDR strains dramatically expanded worldwide. This situation forced scientists to think about reusing bacteriophages instead of antibiotics to treat bacterial infections

Isolation of bacteriophages from sewage sample and studying their effect on MDR *pseudomonas aeruginosa* biofilms.

P. aeruginosa is one of the main causes of hospital acquired infections worldwide mostly in developing countries. P. aeruginosa also has high capacity of developing biofilms which show resistant to commonly used antibiotics and is the cause of about up to 80% of human infections. Biofilm is formed on both biotic and abiotic surfaces and can provide a strong defense mechanism to bacteria due to its unique structure. Biofilm cannot be eradicated by disinfectants and biocides but phages have demonstrated to damage biofilm by disintegrating its structural components. Bacteriophages thus offer very specific options in the control of bacterial infections which cannot be cured through the empirical therapy of antibiotics. The worldwide abundance of bacteriophages and their prevalence make them ideal candidates to be used as antimicrobial agents. Phages are highly specific in their actions and can kill specific strains of bacteria. The high specificity of the phage-host relationship has led to the need of isolate new phages with the ability to control and kill multi-drug resistant P. aeruginosa strains.

Many studies have indicated that bacteriophages are one of the most promising weapons for the elimination of in vitro *P. aeruginosa* biofilms; for example, In the study by *M. Adnan, et'al*, the aim of their study was to isolate and characterize a bacteriophage against P. aeruginosa with MDR and biofilm ability. A bacteriophage MA-1 with moderate host range was isolated from waste water. The phage was considerable heat and pH stable. Electron microscopy revealed that phage MA-1 belongs to Myoviridae family. Its genome was dsDNA (≈50 kb), coding for eighteen different proteins (ranging from 12 to 250 KDa). P. aeruginosa-2949 log growth phase was significantly reduced by phage MA-1 (2.5 × 103 CFU/ml) as compared to control (without phage). Phage MA-1 also showed significant reductions of 2.0, 2.5 and 3.2 folds in 24, 48, and 74 h old biofilms after 6 h treatment with phage respectively as compared to control. It was concluded from this study that phage MA-1 has capability of killing P. aeruginosa planktonic cells and biofilm, but for complete eradication cocktail will more effective to avoid resistance.

Another study by Fong et'al studied, the activity of a phage cocktail in eradicating biofilms of ex vivo P.aeruginosa isolates from CRS patients. They used P. aeruginosa isolates from CRS patients with and without cystic fibrosis (CF) across three continents were multi-locus sequence typed and tested for antibiotic resistance. Biofilms grown in vitro were treated with a cocktail of four phages (CT-PA). Biofilm biomass was measured after 24 and 48 h, using a

crystal violet assay. Phage titrations were performed to confirm replication of the phages. A linear mixed effects model was applied to assess the effects of treatment, time, CF status, and multidrug resistance on the biomass of the biofilm. CT-PA bacteriophage cocktail displayed suitable anti-biofilm activity in vitro. It had a broad host range in the 45 isolates tested, with 89% of isolates susceptible. The use of a cocktail as opposed to individual phages increased the host range significantly, with only 53–73% of isolates being susceptible to each of the four phages individually. The lytic effect of CT-PA on planktonic bacteria translated well to a reduction in biofilm, consistent with previous reports showing efficacy of bacteriophage to reduce biofilm in vitro and in vivo. A single dose of phages is able to significantly reduce biofilms formed in vitro by a range of P.aeruginosa isolates from CRS patients. This represents an exciting potential and novel targeted treatment for P. aeruginosa biofilm infections and multidrug resistant bacteria.

In 2017, in a study, researchers isolated bacteriophage AZ1 and tested its anti-biofilm activity against MDR *P. aeruginosa.* They identified isolated, and characterized a lytic bacteriophage against the multiple-drug resistant clinical strain of Pseudomonas aeruginosa-2995 and to determine the phage efficacy against the bacterial planktonic cells and the biofilm. Wastewater was used to isolate a bacteriophage. The phage was characterized with Transmission electron microscopy (TEM). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was used to identify the expressed proteins. Bacteria were cultured in both suspension and biofilm to check and compare their susceptibility to phage lytic action. The activity of the phage (determined as AZ1) was determined against P. aeruginosa2995 in both planktonic cells and the biofilm. A bacteriophage, designated as AZ1, was isolated from waste water showing a narrow host range. AZ1 was characterized by TEM and could be identified as an isolate in the family Siphoviridae. Seventeen structural proteins ranging from about 12 to 110 kDa were found through SDS-PAGE analysis. Its genome was confirmed as dsDNA with a length of approx. 50 kb. The log-phase growth of P. aeruginosa-2995 was significantly reduced after treatment with AZ1 (4.50 ×108 to 2.1×103 CFU/ml) as compared to control. Furthermore, phage AZ1 significantly reduced 48 hours old biofilm biomass about 3-fold as compared to control. Pseudomonas aeruginosa is a ubiquitous free-living opportunistic human pathogen characterized by high antibiotic tolerance and tendency for biofilm formation. The phage, identified in this study, AZ1, showed promising activity in the destruction of both planktonic cells and biofilm of P. aeruginosa-2995. However, complete eradication may require a combination of phages.

Kwiatek et al. investigated the effects of two bacteriophages MAG1 and MAG4, and their capability to control carbapenem-resistant *P. aeruginosa* in planktonic and biofilm models. It was found that each phage individually affected approximately 50% of *P. aeruginosa* isolates, but when they were used as a cocktail, the anti-biofilm property was increased to 72.9%. Although MAG4 effectively reduced biofilm shortly after the treatment, MAG1 affected biofilm after a more extended period. This study also reported that bacteriophages can utilize three different mechanisms for the eradication of biofilms, including lysis biofilm-forming bacteria by typical phage infection (lysis from within), production of extracellular polymeric substance (EPS) depolymerase, and "lysis from without" that does not need for phage gene expression after absorption. It was also suggested that YefM antitoxin of the bacterial toxin-antitoxin system as a MAG1-encoded homolog might increase the effectiveness of MAG1 over MAG4. In another experimental study, it was reported that ΦKMV, ΦPA2, ΦPaer4, and ΦE2005 phages, either individually or as a cocktail, were capable of destroying biofilm of MDR *P. aeruginosa* isolates in a dose-dependent manner in 24-h assays. In this study, the phage cocktail was not active against two isolates after biofilm formation because of the high production of alginate that its accumulation inhibits phage anti-biofilm activity during 24 h. However, in the conditions

with pre-existing biofilm formation, the phages affected these two resistant isolates and eliminated the alginate, which was produced immediately after infection.

In another study, the effects of bacteriophages vB-Pa4 and vB-Pa5 on the formation and development of MDR *P. aeruginosa* biofilms were investigated, and the results suggested that bacteriophages almost prevented biofilm formation and also pre-formed biofilms were partially destroyed by phage. Ahiwale et al., in an in vitro study, investigated the management of biofilm produced by antibiotics resistant *P. aeruginosa* using native BVPaP-3 phage. It was found that T7-like lytic phage (BVPaP-3) could inhibit the biofilm formation (three logs) of hospital isolates of *P. aeruginosa*. Also, it was able to disperse pre-made biofilms of all isolates after 24 h. Furthermore, bacteriophage PA1Ø was tested against *P. aeruginosa* biofilm, and it was found that the bacteriophage had lytic properties and required bacterial type IV pili to infect *P. aeruginosa* isolates. Phage PA1Ø had bactericidal activity against a wide range of bacteria (both Gram-positive and Gram-negative), and it was able to eradicate biofilm. This phage can also be introduced as an antimicrobial agent for the treatment of biofilm-associated mixed infections of *Staphylococcus aureus* and *P. aeruginosa*. Due to the probable production of lytic phage enzymes, the mechanism of phage antibacterial action against Gram-positive bacteria may be different from that of *P. aeruginosa*. For example, endolysin can degrade the cell wall of Gram-positive bacteria by destroying peptidoglycan

CONCLUSION:

Biofilm is one of the leading causes of antibiotic resistance and chronic infections. Because of the inefficacy of antibiotics to inhibit bacterial biofilm, new strategies are needed to combat it. Recent studies have identified phage therapy as one of the effective methods for the destruction of *P. aeruginosa* biofilm. As noted above, there are still limitations to the widespread use of phage therapy, and a focus is needed to address these issues in future studies. The use of new strategies to enhance the efficacy of bacteriophages on the biofilm of *P. aeruginosa* is helpful. Furthermore, it is recommended that future studies use phage therapy to prevent chronic infections caused by *P. aeruginosa* biofilm so that hopefully it paves the way for more using this therapeutic approach.

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