# POTENTIAL USE OF VIBRIOPHAGE IN ASSOCIATION WITH ANTIBIOTIC TO TREAT THE *Vibrio cholerae* BIOFILM

By
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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology

Department of Mathematics and Natural Sciences
BRAC University
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It is hereby declared that

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3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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The thesis titled "**POTENTIAL USE OF VIBRIOPHAGE IN ASSOCIATION WITH ANTIBIOTIC TO TREAT THE *Vibrio cholerae* BIOFILM**" submitted by Saria Farheen (19176003), of Spring, 2019 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology on 3rd August 2021.

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Ethics Statement

The entire research work has no conflict of interest.  No human and animal model was used in this experiment.

# *Abstract*

In cholera prevalent areas, *Vibrio cholerae* in the aqueous environment can exist in a cell aggregated form covered by an extra polysaccharide matrix called Biofilm. These type of cells are named as VBNC (viable but non culturable cell) and CVEC (conditionally viable environmental cell). The cells in the biofilm can persist in a wide range of environmental conditions by remaining metabolically dormant and can resuscitate into planktonic cells leading to a cholera outbreak. So it is imperative to treat these biofilms. It has been found that antibiotic treatment is not effective in degrading the biofilm and also can lead to the dissemination of antibiotic resistance genes. So as a safe alternative, this study has shown the use of a bacteriophage to degrade the pathogenic *V. cholerae* biofilm. This study also shows the synergistic effect of antibiotics and bacteriophages on the elimination of toxigenic *Vibrio* biofilms.



Keywords: Bacteriophage, *V.cholerae,* Biofilm, Antibiotic.

*Dedicated to my parents*

# Acknowledgement

This dissertation is very special to me since I have studied meticulously to choose this topic of research. It may have been a small step but a great leap towards fulfilling my research passion. I needed help and encouragement during repeated failures in conducting the experiments. Fortunately there were a number of people around me who provided the needed supports. Firstly, I would like to thank my parents and the Almighty for their constant moral support during my research work.

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Before concluding, I would like to share that this thesis study could not be completed due to the ongoing corona lockdown and so few works were left pending. I hope to continue the undone works upon return of the pre-corona condition. I am still hopeful about the cessation of corona pandemic and may we stay safe to fulfill our long cherished works and contribute to the betterment of the humankind and the environment.

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# *Chapter 1: Introduction*

## 1.1: Background of the study

*Vibrio cholerae* is a gram-negative bacillus that occurs widely in the aquatic environment. Toxigenic *Vibrio* species infects humans to cause the deadly Cholera disease. Cholera, a waterborne disease, is estimated to affect 3–5 million people worldwide annually and poses a major healthcare burden in endemic and epidemic areas (Enserink, 2010; R et al., 2012). *Vibrio* species are found to flourish in diverse environments including the human intestine, rivers, lakes, estuaries, and the ocean (K. K & PI, 2003). In the aquatic environment, *V. cholerae* can form conditionally viable biofilm-like aggregates through quorum sensing pathways. Quorum sensing enables the cells to communicate by releasing small chemical molecules as a signaling process to other cells and result in biofilm formation (Flemming et al., n.d.). Biofilms are formed of cells enclosed in an extracellular polymeric substance (EPS) matrix and are differentiated from their suspended cells by reduced growth rates and the up and down-regulation of specific genes (RM, 2002). The cells forming the biofilm can enter into a dormant form known as 'Conditionally Viable Environmental Cells (CVEC)' and Viable But Non-culturable Cells (VBNC), which resist cultivation by conventional techniques (Faruque et al., 2006). These cells can become active upon various ecological conditions and multiply to increase planktonic cells. The increased concentration of the *Vibrio* planktonic cells is known to initiate cholera epidemics (Naser et al., 2017; *Presence of Vibrios in Surface Water and Their Relation with Cholera in a Community - PubMed*, n.d.).

Biofilms are known to cause over 80% of bacterial infections in humans (S. M, 2011). Different approaches have been adopted to combat the biofilm formation such as inhibition of quorum sensing pathway, alteration of membrane permeabilization, cleavage of peptidoglycan, antibiotic treatment etc. (Roy et al., 2018). It has been shown that biofilm cells are 100 to 1,000 times more resistant to antimicrobial agents than planktonic bacterial cells (JW et al., 1999). Many different types of antibiotics have been tested against bacterial biofilms and in almost every case bacteria in the biofilm are found to be less sensitive to killing than the same strain when grown in free aqueous suspension (MC et al., 2000). *Vibrio cholerae* is susceptible to a number of antibiotics belonging to β-lactam, Tetracycline, Rifampicin, Macrolides, Aminoglycosides, Quinolones, etc. Kanamycin is such an aminoglycoside bacteriocidal antibiotic. Kanamycin is isolated from the bacterium

*Streptomyces kanamyceticus* and its most commonly used form is kanamycin sulfate. Most of the *V.cholerae* species have been shown sensitive to the kanamycin at a concentration of 44±1 (~50)µg/ml (*(PDF) Antibiotic Sensitivity and MIC of Vibrio Species Isolated from Diseased Penaeus Monodon (Fab)*, n.d.). However, the 'persister' cells in the *Vibrio* biofilm can inhibit kanamycin when added in a range from normal to greater concentration (PS, 2002a). Resistance to aminoglycosides may occur based on several mechanisms: enzymatic modification, increased efflux, decreased permeability etc. (Y et al., 2016). The 'persister' cells can lead to the regrowth of the biofilm after antibiotic treatment (F. M et al., 2011).

Researches have shown that kanamycin when used from sub-inhibitory to 10×MIC (Minimum Inhibitory Concentration), was not efficient for the complete removal of many biofilms (Gilbert et al., 2002; Margarida Pereira et al., 2012). For this reason, Kanamycin suspension at a concentration from 100-1000 µg/ml has been used in this study to see the susceptibility pattern of the *Vibrio* cells in the biofilm.

As mentioned earlier that a biofilm is a form of bacterial persistence in water that can deliver a large dose of pathogenic *Vibrio cholera*e, so there is an increasing demand for effective biofilm treatment. Such an approach of biofilm treatment requires methods that are safer and more effective than antibiotic treatment. One such approach can be the use of *Vibrio* specific bacteriophages in the aquatic dwellings of the *Vibrio cholerae* biofilm (Naser et al., 2017). Bacteriophages are naturally occurring viruses that infect bacteria. They are unaffected by antibiotic resistance and unlike many antibiotics, they can target bacteria within biofilms (Harper et al., 2014; *Use of Bacteriophages to Control Biofilms*, n.d.). They can either coexist with their host by inserting themselves into the bacterial genome (lysogenic) or destroy those (lytic) (*Use of Bacteriophages to Control Biofilms*, n.d.). It is known that many bacteriophage genomes contain genes for enzymes capable of breaking down elements of the biofilm matrix (Y. J et al., 2014; PG et al., 2004). Likewise, Vibriophages are also seen to degrade the biofilm matrix and release an increased number of planktonic bacteria in the aquatic condition (Naser et al., 2017). JSF7 is such a Vibriophage, which is found to be active on the biofilms of both phage-susceptible and phage-resistant *Vibrio cholerae*. Four *Vibrio cholerae* strains which were chosen for this experiment are not susceptible to JSF7. The *Vibrio* biofilms formed by the four strains showed resistance against the antibiotic treatment but were degraded by JSF 7 phage to release the planktonic cells.

This research could be used to develop a phage-mediated cholera control technique. The findings of this research can be studied further 'in vivo', to observe its efficacy in controlling the dissemination of *V. cholerae* in the aquatic environment. Moreover, the study suggests using an innocuous alternative to antibiotics in regulating biofilm formation. This in turn also contributes to limiting the spread of antibiotic resistance among the bacterial species. Besides, this study also produces a new path of research to see the combined effect of antibiotics and bacteriophages on the degradation of toxigenic *Vibrio* biofilm. Since JSF7 can act on both phage-resistant and phage-susceptible cells, so this property of JSF7 can also be further studied to treat biofilms formed by other bacterial species such as *Salmonella,* Shiga-Toxin producing *E.coli* (STEC), etc.

## 1.2: Objective:

The main purpose of this study is to observe how bacteriophages can be employed to degrade the potential *V. cholerae* biofilms instead of using antibiotics.

## 1.3: Specific Aims:

1. To observe the capacity of an antibiotic in degrading the *V.cholerae* biofilm
2. To study the effect of the antibiotic in dispersing cells from the biofilm
3. To observe the degradation of *V.cholerae* biofilm using the bacteriophage treatment
4. To determine the effect of bacteriophage on the planktonic cells in the biofilm
5. To study the effect of combined treatment of antibiotic and phage on the biofilm

# *Chapter 2: Literature Review*

## 2.1: Biofilm

Bacteria when exist as individual organisms are considered to live in a 'planktonic state'. Microorganisms can naturally accumulate on a wide variety of surfaces and form sessile and sedentary communities. These surfaces can range from household and industrial pipes, biomaterials such as contact lenses, medical devices including implants and urinary catheters, to plant and animal tissues. These mono- or poly-microbial aggregates surrounded by a matrix of extracellular polymeric substance (EPS) are called biofilm which may consist of diverse communities of bacteria and fungi. Biofilm present unique problems for antibiotics and biocides, due to the nature of the extracellular matrix and the presence of metabolically inactive 'persister' cells (Harper et al., 2014).

The close proximity of the microorganisms enables substrate exchange, distribution of metabolic products and removal of toxic end products so that the different species can support each other. Biofilm formation is an important adaptation and survival strategy commonly employed by bacteria. The structure of biofilm communities can protect the bacteria within them from attack by antimicrobials, shear forces and the immune system (Flemming et al., n.d.).

The presence of different conditions including gaseous, nutrient stratifications etc. leads cell to different states and exist within the biofilm. Studies have established that many species of bacteria monitor their cell-population densities through the exchange of chemical signaling molecules called autoinducers. The autoinducers accumulate extracellularly and trigger alterations in behavior at high population densities. This phenomenon is referred to as quorum sensing. Quorum sensing regulates many processes by making the bacterial population act in a coordinated manner rather than as individuals (and & Bassler, 2003; Z. J et al., 2002; WC et al., 1994).

Biofilm formation can be divided into five stages: Initial reversible attachment (1), irreversible attachment (2-3), maturation (4) and dispersion (5) as shown in Figure 1.

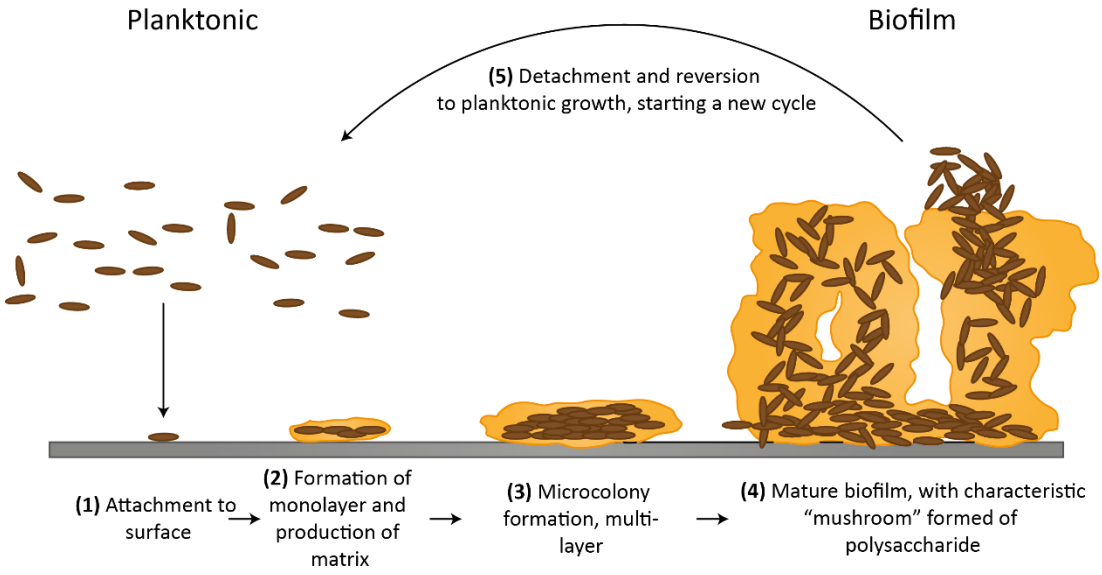



**Figure 1:** ***Schematic representation of a biofilm formation.*** *The formation begins with a reversible attachment of the planktonic cells (brown ovals) followed by the adhesion to the surface (grey)* ***(1).*** *The bacteria then form a monolayer and irreversibly attach by producing an extracellular matrix* ***(2).*** *Next, a microcolony is formed where multilayers appear* ***(3).*** *During later stages, the biofilm is mature, forming characteristic "mushroom" structures due to polysaccharides (4). Finally, some cells start to detach and the biofilm (shown in yellow) will disperse* ***(5).*** (Vasudevan, 2014)

The initial contact of the moving planktonic bacteria with the surface is the starting point, which is still reversible at this stage. The bacteria will then start to form a monolayer and will produce an extracellular matrix for protection. The EPS matrix consists of extracellular polysaccharides, structural proteins, cell debris and nucleic acids. The initial steps of the matrix formation are dominated by extracellular DNA (eDNA), whereas polysaccharides and structural proteins take over later on. In these stages, the formation of microcolonies takes place, which exhibit significant growth and cell-cell communication as quorum sensing. The biofilm grows in a three-dimensional manner and the attachment is now irreversible. In the last stage, some cells of the mature biofilm start to detach and disperse into the environment as planktonic cells again to potentially start a new cycle of biofilm formation.(Lewandowski, 2000)

2.2: *Vibrio cholerae* Biofilm:

*Vibrio cholerae* can switch between motile and biofilm lifestyles. Two chemical signaling systems, quorum sensing (QS) and 3′,5′-cyclic diguanylic acid (c-di-GMP), are known to reciprocally control biofilm formation in *Vibrio cholerae* (CM et al., 2008).

Quorum Sensing (QS) is a mechanism through which bacteria can communicate among themselves through regulation of gene expression in compliance with population density via the help of signaling molecules (*Quorum Sensing | Biology | Britannica*, n.d.). Bacteria uses QS for a variety of purposes including biofilm formation, bioluminescence, competence, swarming, sporulation, motility and virulence (Kaur et al., 2018).

Quorum Sensing works through autoinducers that allows bacteria to synchronize the behavior of the population (CM & BL, 2005). *V. cholerae* produces two Autoinducers (AIs) and responds to them by using parallel phosphorelay signaling systems. In the low-cell density state i.e., when AIs levels are low, the autoinducer receptors function as kinases and funnel phosphate to the response regulator, LuxO (Fig. 2). LuxO∼P activates the expression of four genes encoding the Qrr small regulatory RNAs (sRNAs) (DH et al., 2004; Tu & Bassler, 2007).

The Qrr sRNAs destabilize the mRNA encoding a major regulator of Quorum Sensing, HapR. This relay culminates in the expression of low-cell density specific genes, including genes required for biofilm formation and virulence factor production (H. BK & BL, 2003; Z. J & JJ, 2003; Zhu et al., 2002), shown in Fig. 2, left side.

When the cell density increases, the AIs bind their cognate receptors and switch the receptors to phosphatases. Phosphatase activity leads to dephosphorylation of LuxO and termination of qrr expression. The mRNA encoding HapR is stabilized and HapR protein is produced. HapR is a DNA-binding transcription factor that initiates a program of gene expression that switches the cells from the individual, low-cell-density state to the high-cell-density state (Fig. 2, right side). When HapR is produced, biofilm and virulence genes are repressed, which promotes dispersal of *V. cholerae* (Z. J & JJ, 2003; Zhu et al., 2002).

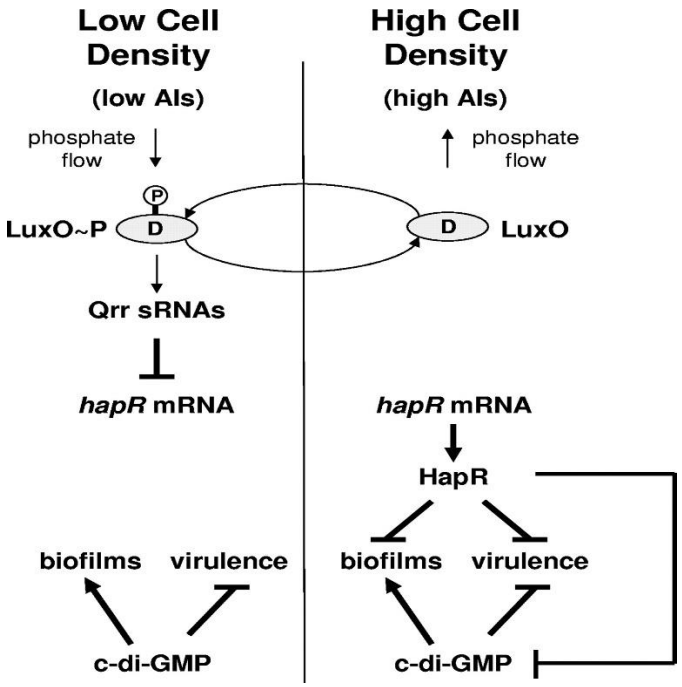



**Figure 2**: ***Simplified model of the interaction between QS and c-di-GMP in the regulation of gene expression in V. cholerae***. *When concentrations of AIs are low (left side), the response regulator LuxO is phosphorylated, resulting in expression of multiple genes encoding the Qrr sRNAs that repress translation of the master transcriptional regulator, HapR. When concentrations of AIs are high (right side), LuxO is dephosphorylated which leads to termination of qrr expression. In the absence of the Qrr sRNAs, HapR is produced. HapR represses both biofilm formation and virulence factor expression. Like HapR, c-di-GMP also represses virulence factor expression, but unlike HapR, c-di-GMP activates biofilm formation. Here, HapR is shown to repress biofilm formation both directly (via control of vpsT) and indirectly by reducing the levels of c-di-GMP.* (CM et al., 2008)

The intracellular second messenger molecule c-di-GMP regulates the transition from a motile lifestyle to a sessile state in numerous bacteria (PA & S, 2007; Ryan et al., 2006). Proteins containing domains with GGDEF motifs synthesize c-di-GMP by the cyclization of two GTP molecules and the loss of two pyrophosphate moieties. Degradation of c-di-GMP is carried out by proteins containing domains with EAL or HD-GYP motifs. *V. cholerae* possesses 62 genes that encode proteins with domains involved in governing c-di-GMP levels (MY, 2004). In *V. cholerae*, high levels of c-di-GMP enhance biofilm formation and repress virulence factor expression and motility, while low levels of c-di-GMP repress biofilm formation and induce virulence factor expression and motility (AD & A, 2004; S et al., 2006; Tischler & Camilli, 2005).

2.2.1: *Vibrio cholerae* in CVEC and VBNC form:

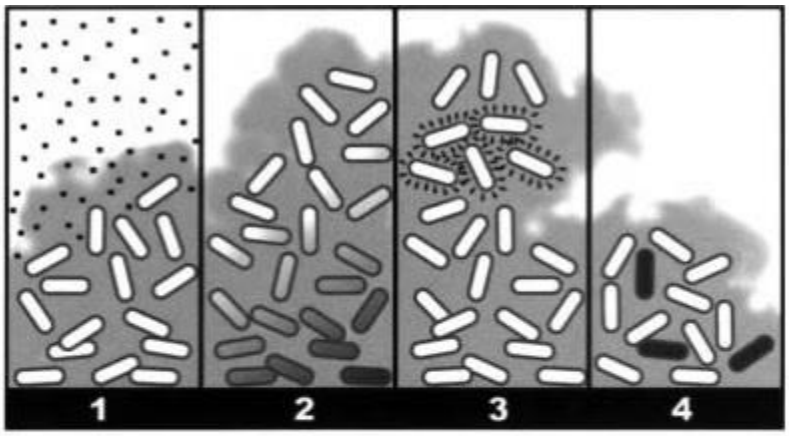
The cells in biofilms often enter an inactive form called CVEC and VBNC, where the cells may be viable in reduced form but are usually non-cultural on media (JD, 2010; Nilsson et al., 1991; *The Significance and Detection of VBNC Microorganisms | American Pharmaceutical Review - The Review of American Pharmaceutical Business & Technology*, n.d.).
Factors which induce CVEC formation in *V. cholerae* includes extremes in temperature, salinity and nutrient deprivation (KU et al., 2006; Mishra et al., 2012). This CVEC state is important in cholera epidemiology where virulence and colonization traits were actively expressed in CVEC incubated in freshwater microcosms. Upon various conditions, such as temperature upshift or an increase in nutrients, may lead in resuscitation of cells from CVEC state. However, these cells are capable of resuscitation for a limited time and eventually lose the ability to resuscitate over time (S. M et al., 2010; N et al., 2004).

It has been hypothesized in a number of studies that CVECs resuscitate in a stochastic manner rather than in response to environmental parameters (Epstein, 1993). The authors claim that some cells in CVEC state will randomly revive from dormancy and if conditions are favorable, they will grow. These revived cells can be compared to "scouts" inspecting environmental conditions (Buerger et al., 2012). In unfavorable conditions, the scouts will die and cause the loss of only a small fraction of the population. However, if conditions are favorable, then the genetic pool is amplified and maintained.

2.3: Mechanisms of antibiotic resistance in biofilm:

A great deal is known about the genetic and molecular basis of antibiotic resistance in bacteria. Some common mechanisms include target mutations, low cell permeability, efflux pumps and modifying enzymes etc. However, these methods do not seem to be at the root of reduced antimicrobial susceptibility in biofilms. This is because, studies showed that bacteria lacking protective mutations or plasmids, become less susceptible to antibiotics when grown in the biofilm state (Seifi et al., 2016).

Antibiotic sensitivity is usually quickly restored when bacteria are dispersed from a biofilm. This rapid reversal of resistance upon dispersion from a biofilm suggests an adaptive resistance mechanism rather than a genetic alteration. The natural protection given by a biofilm may provide a breeding ground for spontaneous mutants. Moreover, the close spatial proximity of bacterial cells within a biofilm has been speculated to accelerate plasmid transfer (H. M & S, 1999). A number of the possible mechanisms involved in antibiotic susceptibility of the biofilms are discussed as follows:




Four hypothesized biofilm resistance mechanisms. *1* – The antibiotic (squares) penetrates slowly or incompletely; *2* – a concentration gradient of a metabolic substrate or product leads to zones of slow or non-growing bacteria (shaded cells); *3* – an adaptive stress response is expressed by some of the cells (marked cells); *4* – a small fraction of the cells differentiate into a highly protected persister state (dark cells).

**Figure 3:** *Mechanisms of antibiotic resistance in biofilm* (PS, 2002b)

2.3.1: Antibiotic penetration of the biofilm:

Biofilms are mostly water and so the solutes of antibiotics are supposed to diffuse readily in the biofilm matrix. Measurements of effective diffusion coefficients of solutes in biofilm coincides with about 40 percent of the respective diffusion coefficient in pure water (Nichols et al., 1988).The physical mobility of antibiotics in biofilm does not ensure that the antibiotic will penetrate the biofilm. If the antibiotic is inactivated by reaction or sequestered by binding, then its delivery to the depths of the film can be retarded. Such a reaction-diffusion interaction is sufficient to prevent a penicillin antibiotic from penetrating a biofilm formed by a beta-lactamase-positive

bacterium (H. M & S, 1999). There is some evidence suggesting that binding of the positively charged aminoglycosides such as Kanamycin, to negatively charged biofilm matrix retards penetration of these agents (CA et al., 1988a, 1988b, 1988c).

Significant limitations to biofilm penetration have only been reported for beta-lactams and aminoglycosides and not for other antibiotics. If an antibiotic slowly penetrates the biofilm, then enzymes that inactivate the antibiotic are responsible for the impermeability in the biofilm. Such enzymes include beta-lactamases, aminoglycoside-modifying enzymes, chloramphenicol acetyltransferases etc. (PS, 2002b).

2.3.2: Altered microenvironment and slow growth:

It has been shown that killing by many antibiotics is growth-dependent (Reid, 1999). For instance, penicillin only kill the growing bacteria (E et al., 1986). Since most antibiotics target some type of macromolecular synthesis, so these agents would not have much effect on bacteria in which macromolecular synthesis is arrested. Over the past decade, researchers have studied to directly visualize patterns of bacterial growth and activity in biofilms using fluorescent probes and reporter genes(S. C et al., 1999; EJ et al., 1996; KD et al., 2000).

Wimpenny and Kinniment have studied that within biofilms, microgradients occur in the concentration of key metabolic substrates and products (*Microbial Extracellular Polymeric Substances: Characterization, Structure ... - Google Books*, n.d.). Because of these chemical gradients, biofilms include slow-growing or stationary phase cells. Even in single-species biofilms, the bacterial population includes both rapidly growing and metabolically inactive cells.

Bacteria in non-growing zones of a biofilm are uniquely well positioned to survive antimicrobial challenge and are less susceptible than a biofilm in which all of the bacteria grow at a uniform intermediate rate (MR et al., 1988a; Reid, 1999).

Factors other than slow growth may contribute to antibiotic resistance in biofilms. The same chemical gradients that lead to growth limitation in biofilms can alter antibiotic potency. For example, oxygen availability alone is known to modulate action of the aminoglycosides (MR et al., 1988b). Bacteria in an anaerobic region of a biofilm may be differentially protected from these antibiotics, even if they are capable of fermentative growth. Gradients in pH may similarly impact antibiotic efficacy negatively (Retsema et al., 1991). If reduced antibiotic susceptibility in biofilms

depends on metabolically inactive or slow-growing bacteria, then genes products which are involved in switching bacterial metabolism pathways, would be essential for the biofilm defense. These may include genes required for the formation of multicellular structures. The establishment of nutrient-limited zones in biofilms depends on cell aggregates reaching a certain critical dimension. Hentzer et al., described three *P. aeruginosa* mutants with reduced susceptibility to antibiotics (H. M et al., 2001). The first mutant overproduces the extracellular polymer alginate, which makes biofilms thicker than the wild type. Biofilms formed by this mucoid mutant are less suspectible to tobramycin than the wild type. The second mutant is affected in the stationary-phase sigma factor rpoS. This mutant also makes biofilms that are thicker than the wild-type strain and these biofilms are also less sensitive to tobramycin (W. M et al., 2001). The third mutant has a lesion in gacA, part of a two component regulatory system required for normal biofilm development (MD et al., 2001). Biofilms of the gacA mutant fail to form mature structures and are slightly more susceptible to several antibiotics (H. M et al., 2001).

2.3.3: Adaptive Responses:

Bacteria are equipped with a host of stress responses that allow them to cope with environmental fluctuations, such as abrupt temperature changes, oxidative stress, low water activity, DNA damage etc. Many of these stress responses have been characterized in molecular and genetic detail using planktonic bacteria (M. A, 1991; G & JA, 1999). These protective responses may be deployed in biofilms. RpoS, a sigma factor expressed in Gram-negative bacteria as they enter stationary phase, has been detected in biofilms of *P. aeruginosa* (KD et al., 2001). Studies of antimicrobial susceptibility of biofilms formed by rpoS mutants have shown to fail in protecting biofilms (W. M et al., 2001). The constitutive expression of multi-drug efflux pumps in biofilms may contribute to resistance. Using DNA microarrays, it has been reported that biofilms of *P. aeruginosa* challenged with tobramycin were able to transcribe the gene for an efflux pump (W. M et al., 2001). Stress responses may be induced in biofilm by environmental challenge, just as they are in suspended bacteria. However, biofilm embedded cells are known to respond better to an antimicrobial challenge that may found to overwhelm the planktonic cells. For example, *P. aeruginosa* in a biofilm are able to activate katB, an inducible catalase gene, in response to treatment with 50 mM hydrogen peroxide (JG et al., 1999). Peroxide treatment of the same strain

of bacteria in the planktonic state resulted in no catalase expression, since the free-floating cells were overwhelmed by the antimicrobial effects of the hydrogen peroxide before the stress response could be activated.

2.3.4: Persisters

Bacteria in biofilms not only evade killing by antibiotics but they also resist chemical disinfectants, such as chlorine bleach, glutaraldehyde etc. The presence of a subpopulation of persisters in the biofilm may account for the observed broad resistance. Persisters may constitute a relatively small fraction of the population but these few cells have entered a highly protected state (L. K, 2001; PS & JW, 2001). The difference between planktonic and biofilm communitities is that the frequency of persisters is much higher in the biofilm population. Data in support of the persister hypothesis include measurements of biphasic biofilm killing in which most of the population is rapidly killed but a fraction of the cells are unaffected even by prolonged antibiotic treatment (B. A et al., 2000; T et al., 1999). The fact that bacteria can develop reduced susceptibility even in very thin biofilms can be explained by persisters (JR et al., 1998; WL et al., 2000). Genes that contribute to the persister state may include those encoding regulatory circuits that determine the entry and exit from this state as well as specific protective responses.

2.4: Bacteriophages:

A bacteriophage or phage, is a virus that infects bacteria. Like other types of viruses, bacteriophages vary a lot in their shape and genetic material. Phage genomes can consist of either DNA or RNA and can contain as few as four genes or as many as several hundred (H & RW, 2002).

The capsid of a bacteriophage can be icosahedral, filamentous or head-tail in shape. The head-tail structure seems to be unique to phages and their close relatives (Dimmock et al., 2016; Pietilä et al., 2013).

There are two different cycles that bacteriophages may use to infect their bacterial hosts:

- **The lytic cycle:** The phage infects a bacterium and takeovers the bacterial genetic material to make lots of phages. The phages then kills the cell and release to infect further cells.
- **The lysogenic cycle:** The phage infects a bacterium and inserts its DNA into the bacterial chromosome. The inserted phage DNA is called a prophage which can be copied and passed on along with the cell's own DNA. Under the right conditions, the prophage can

become active and come back out of the bacterial chromosome. The prophage then triggers to initiate the lytic cycle.

The steps involved in the infection process are collectively called the lifecycle of the phage. The phage decides to enter into either of the lifecycle depending on a number of factors. One important factor is the number of phages infecting the cell at once. Larger numbers of co-infecting phages make it more likely that the infection will use the lysogenic cycle. This strategy may help prevent the phages from wiping out their bacterial hosts when the phage-to-host ratio gets too high (Abedon et al., 2011). However, it has been found that environmental stress such as, temperature, pH etc. may trigger the phage to enter into a lytic cycle from the lysogenic state. Conversely, a small fraction of the prophages in a population can spontaneously enter the lytic cycle, even without these external cues (Baron, 1996). (*Reece, Urry, Cain, Wasserman, Minorsky & Jackson, Campbell Biology, Books a La Carte Edition | Pearson*, n.d.)

2.4.1: Vibriophage: JSF 7

The bacteriophage that infects the *Vibrio* species are commonly called Vibriophages. The environmental surveillance system in Bangladesh has generated data of various phage isolates from the aquatic environment, which are shown in the following table below:

**Table 1: Lytic vibriophages isolated from surface water and cholera patients in Bangladesh** (SM & JJ, 2012)

| Phage designation | Primary host strains | Alternative host strains | Plaque type | Isolation of lysogens |
|---|---|---|---|---|
| JSF-1 | *V. cholerae* O1 | Not found | Clear | - |
| JSF-2 | *V. cholerae* O1 | Not found | Turbid | + |
| JSF-3 | *V. cholerae* O139 | Not found | Clear | + |
| JSF-4 | *V. cholerae* O1 | Not found | Clear | + |
| JSF-5 | *V. cholerae* O1 | Not found | Clear | - |
| JSF-6 | *V. cholerae* O1 | *V. cholerae* non-O1 non-O139 | Clear | - |
| JSF-7 | *V. cholerae* O1 | *V. cholerae* O141 strain V50; *V. cholerae* O139 strain AI1853 | Clear on O1 strain; Clear/ turbid on non-O1 strains | + |
| JSF-8 | *V. cholerae* O1 | *V. cholerae* non-O1 non-O139 strains 3565, 3548; *V. mimicus* strains 957V1621, 778V1349, and 1016V1721 | Clear on O1 strain; Clear/turbid on non-O1 strains | + |
| JSF-9 | *V. cholerae* O1 | *V. cholerae* O141 strain V50; non-O1 strains 79, 3565, 3548; *V. mimicus* strains 957V1621, 778V1349, and 1016V1721 | Clear | - |
| JSF10 | *V. cholerae* O1 | *V. cholerae* O139 strain Arg-3 *V. cholerae* O141 strains V46 and V47 | Clear | - |
| JSF-11 | *V. cholerae* O1 | Not found | Clear | - |
| JSF12 | *V. cholerae* O1 | *V. cholerae* non-O1 strains 79; *V. mimicus* strains 957V1621, 1016V1721 | Clear | - |
| JSF-13 | *V. cholerae* O1 | Not found | Clear | - |
| JSF-14 | *V. cholerae* O1 | Not found | Clear | - |
| JSF-15 | *V. cholerae* O1 | *V. cholerae* O141 strain V50; non-O1 strain 79, *V. mimicus* strains 957V1621, and 778V1349 | Clear | - |
| JSF 16 | *V. cholerae* O1 | *V. cholerae* O141 strain V50 | Clear/turbid | + |

Among these phages, JSF7 will be discussed here. JSF7 has double stranded DNA and has linenage with the viruses: Duplodnaviria, Heunggongvirae, Uroviricota, Caudoviricetes, Caudovirales, Autographiviridae, Tawavirus and Vibrio virus. The host specificity of this phages includes specifically *V. cholerae* O1 strains but it has been found to nonspecifically lyse the *V. cholerae* O139 strains as well. JSF7 is capable of dispersing the biofilms formed by both *V. cholerae* O1 and O139 strains. Electron microscopic examination revealed that the phage has isomeric head and a contractile tail **(Figure 4)**. The phage genome consists of 46.31 Kbp nucleotides and 49 open reading frames (ORFs) (Naser et al., 2017). The ORFs of JSF7 are found to encode lipase and polysaccharide- degrading enzyme. These enzymes are considered to be involved in biofilm degradation.

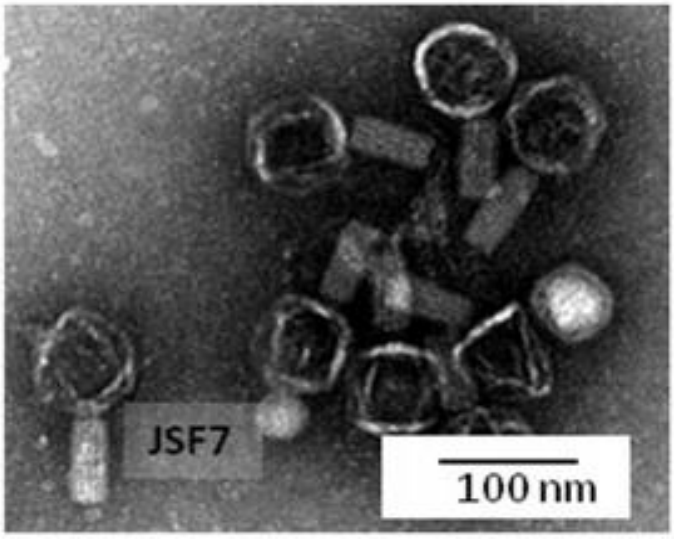



**Figure 4**: *Electron micrograph showing the morphology of JSF7 phages.* (Naser et al., 2017)

Based on the morphology, it is found that JSF7 belongs to Myoviridae family (*Bacteriophage Taxonomy in 1987 - PubMed*, n.d.). This phage remains stable at temperature below 37˚C and the stability decreases with rise of temperature. The phages are found to rapidly inactivate at temperatures above 45˚ C. The phage also remain mostly infectious (65% to 98%) at pH ranging from 6.0 to 9.0. Phage particles remain stable and infectious for more than 4 weeks when they were stored at room temperature in Phage buffer (or SM buffer) (Naser et al., 2017).

2.5: Bacteriophage:  A safer substitute of antibiotics:

Before antibiotics were discovered, there was considerable research on bacteriophages as a treatment for human bacterial diseases. Bacteriophages attack only their host bacteria and not human cells. So they are potentially good candidates to treat bacterial diseases in humans.

After antibiotics were discovered, the phage approach was largely abandoned in many parts of the world. However, phages continued to be used for medical purposes in a number of countries, including Russia, Georgia, and Poland, where they remain in use today (Abedon et al., 2011).

However, extensive researches on "phage approach" are continued since antibiotic-resistant bacteria has become a global health problem. In this study, the use of vibriophages instead of the antibiotics are shown as a safe alternative in degrading pathogenic *Vibrio cholerae* biofilm.

The interaction between the host bacteria and the lytic phages occurs in six different steps as shown in Figure 5. The adsorption of the bacteriophage and release of the new phage progeny play a key role in the bacteriophage infection process. When host bacteria are included in a biofilm, the biofilm matrix can constitute a first physical barrier to the phage.

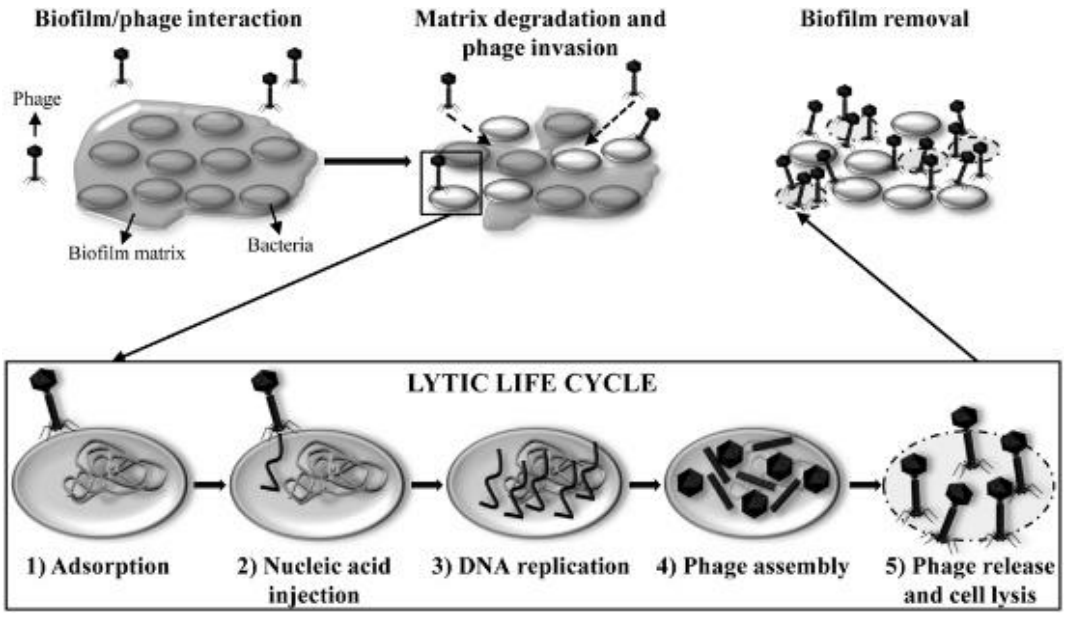



**Figure 5:** *Lytic life cycle of phages inside a biofilm. (1) Adsorption of the phage particle onto the host bacterial cell surface. Tail fibers bind to specific receptors on the cell surface. (2) Injection of the nucleic acid into the cytoplasm of the host bacterium. (3) Replication of the phage genome in multiple copies. Phage early genes are expressed to*

*regulate the host metabolic machinery to be subjected to phage propagation. (4) Formation of new phage particles by expression of the phage late genes and assembly of the phage heads and tails, packaging of the nucleic acid inside the heads and maturation of the virions. (5) Lysis of the host bacterium and release of the new phage progeny ready to infect other cells in the biofilm and start a new cycle.* (Gutiérrez et al., 2016)

To solve this problem, some phages possess depolymerases which are specific hydrolytic enzymes that invade the biofilm matrix and allow infection of new bacteria as shown in Figure 6(A) (Latka et al., 2017). Moreover, some phages are provided with lytic enzymes which are named VAPGHs, important in the first step of the infection cycle (Figure 6B). Their activity produces a small hole in the cell wall through which phage genetic material reaches the cytoplasm (M. M & IJ, 2004). Recently, these proteins have also been proposed as new antimicrobials due to their lytic activity (L et al., 2013).

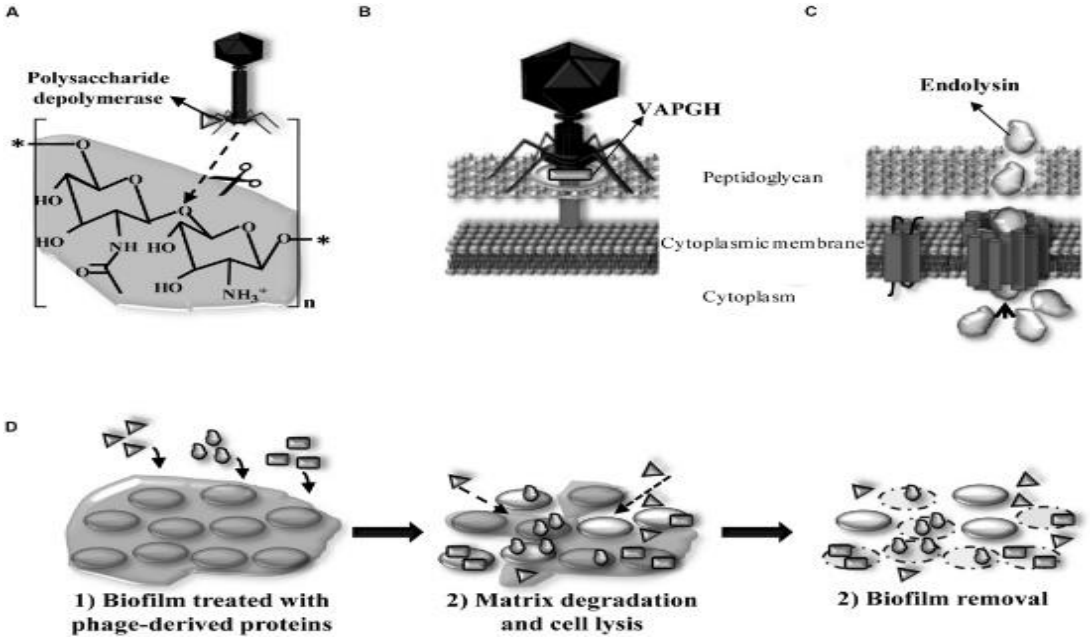



**Figure 6:** *Mechanisms of 'Bacteriophage' actions in biofilm (A) Location of exopolysaccharide depolymerase degrading β-(1,6) bonds of the biofilm extracellular matrix (PIA/PNAG) of staphylococcal species in the phage particle and mode of action. (B) Location of virion-associated peptidoglycan hydrolase (VAPGH) at the phage particle and its role in the infection process. (C) Structure of Gram-positive bacteria cell wall and role of the endolysin during the bacterial lysis. (D) Activity of phage derived proteins when added exogenously and their application as anti-biofilm agents degrading polysaccharidic matrices (polysaccharide depolymerases) and lysing bacteria (VAPGHs and endolysins).* (Gutiérrez et al., 2016)

Double-stranded phages encode lytic proteins named endolysins, which disrupts the cell wall and lyse the host bacteria at the last step of the lytic infection cycle (Figure 6C). In Gram-positive bacteria, endolysins are able to degrade the peptidoglycan when they are added from outside the cell, which gives them an antimicrobial activity (VA, 2008). In Gram-negative bacteria, peptidoglycan is protected by the outer membrane and so these bacteria become insensitive to endolysins. However, research efforts made into endolysin applications against Gram-negative pathogens. Such studies discovered Artilysins that combine a polycationic peptide, able to penetrate the outer membrane with an endolysin. This renders a protein with high bactericidal activity against Gram-negative pathogens (Briers et al., 2014).

So to conclude, it is clear that the bacteriophages and phage-derived proteins could be used as effective means of removing pathogenic biofilms that are responsible to arise epidemics and cause food contamination on industrial levels.

2.6: Different means of phage applications against biofilm:

Bacteriophage treatment has been proposed as one of the effective methods for controlling bacterial biofilms (JJ & RM, 2006). Phage have been used since the early 20th century to treat bacterial infections, especially in Eastern Europe and have been shown to decrease biofilm formation (CR et al., 2003; JJ & RM, 2006). Phage have also been modified to extend their natural host range. Phage-based treatments include phage therapy involving single or multiple phage cocktails, phage-derived enzymes, phages in combination with antibiotics and genetically modified phages (DP et al., 2017). In this section, some of the main applications of phages and their by-products for the removal of biofilms are discussed (Figure 7).

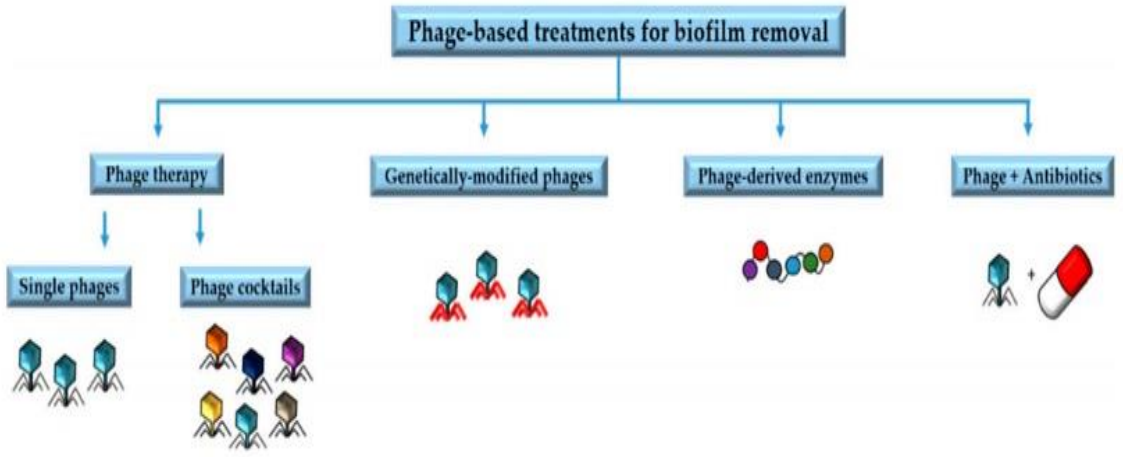



**Figure 7**: *Phage-based treatments for biofilm removal* (Ahiwale et al., 2011; F.-G. C & P, 2020; JJ & RM, 2006)

2.6.1. Phage Therapy

Since phages can actively penetrate and disturb biofilms in nature, they can be used to improve treatments against biofilms (P & J, 2018). Phage-based therapies focus on lytic phages because they destroy their bacterial hosts and also because they lack integrases and other enzymes involved in horizontal gene transfer (JM et al., 2016). In order to design phage-based methods to remove biofilms, it is important to know the specific characteristics of the phages that may play a role in their penetration, diffusion and propagation through the biofilm. For example, penetration of the biofilm is often less efficient for larger phages (H. J et al., 2010). Phages encoding EPS-degrading enzymes are of particular interest against biofilms. Depolymerases are enzymes encoded by phages that degrade EPS matrix components and improve phage penetration (C. A et al., 2011). Another source of EPS-degrading enzymes, are the bacteria found inside the biofilm under stress conditions. Stress can be triggered by phage infection, facilitating increased penetration and dissemination of phages within the community. This has been demonstrated in *Pseudomonas aeruginosa* biofilms, where phage infection was found to reduce the viscosity of biofilms by bacterial enzymes (H. J et al., 2010). Phages are also proved to be effective against oral biofilms that cause infections such as caries, periodontal and peri-implant disease, including *Enterococcus faecalis, Fusobacterium nucleatum* and *Streptococcus spp*. (SP et al., 2017). Antibiotics are usually broad-spectrum stable chemical compounds, while phages are very specific and evolving entities. Moreover, their specificity is an advantage, as it reduces off-target damage and restricts

the development of resistance to target-specific bacteria (M. AM et al., 2016). In addition, phages are evolving entities that can counteract bacterial resistance. However, specificity is also a limitation because it requires great efforts in terms of phage bioprospecting. Furthermore, specificity means that the bacterial pathogen has to be identified at species or even strain level before treatment is administered and so is a problem in rapid response against acute infections. This issue can be addressed by phage cocktails. Biofilms are often multi-species communities, which means that phage cocktails can contribute to disrupting biofilms more efficiently (C. BK et al., 2013). Another interesting aspect of phage cocktails is that they can prevent the emergence of phage resistant bacteria (O. AM & M, 2013; C. BK & ST, 2012). The phages within a cocktail can also interact synergistically, increasing lytic activity (S. M et al., 2014). However, antagonistic interactions between phages could be also possible. Recent studies support the use of at least two cocktails against bacterial biofilms in vivo, especially for multi-species biofilms (Khalifa et al., 2018). Some phage-based products already on the market have been proposed as promising tools to remove biofilms, such as commercially available phage-based products against *Listeria* sp. or *E. coli* with bactericidal effects that are interesting for biofilm prevention (Ferriol-González & Domingo-Calap, 2020).

2.6.2: Phage-Derived Enzymes:

Some enzymes encoded with phages may be useful for treating bacterial infections and biofilms (B et al., 2018). These enzymes or enzybiotics derived from phages can be used as an alternative to antibiotics for human and animal health. There are two main types of phage degradation enzymes- lysins and depolymerases, useful in the removal of biofilms:

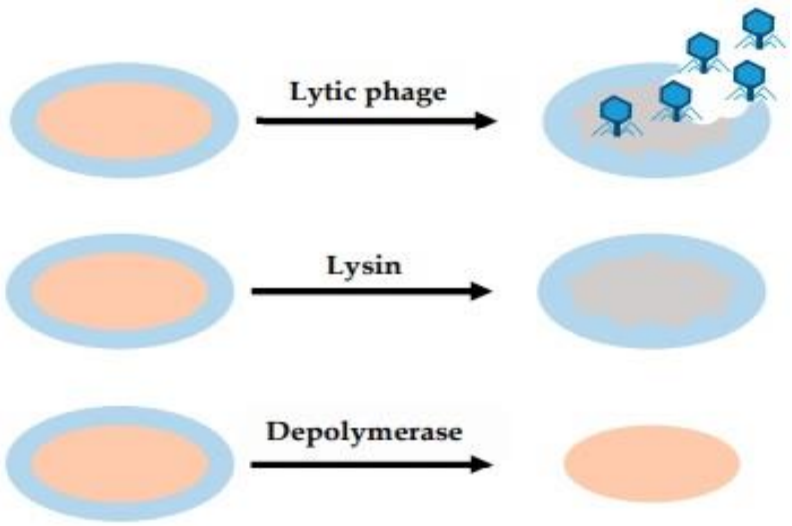



**Figure 8:** ***Differences between the actions of lytic phages: lysins and depolymerases.*** *Lytic phages provide antibacterial effect, degrading cell wall and EPS. Lysins provide a bactericidal effect, disrupting cell walls when they establish contact with their target. Depolymerases degrade EPS* (Ahiwale et al., 2011)

Lysins:

Lysins are peptidoglycan hydrolases that have a bactericidal effect on susceptible bacteria. They break peptidoglycan bonds, degrading the bacterial cell wall and biofilm structure (U et al., 2018; Vázquez et al., 2018). Lysins can be present as soluble enzymes during the phage cycle or in phage tails as virion-associated lysins, acting as receptor to degrade the cell wall and inject the phage genomic material (Vázquez et al., 2018). Depending on the peptidoglycan bonds they break, lysins are classified into different categories. Glycosidases (or glycoside hydrolases) and endopeptidases. Glycosidase breaks glycosidic bonds in complex sugars present in certain cell-wall glycopeptides. Endopeptidases are proteolytic peptidases that break peptide bonds in non-terminal amino acids (L. A et al., 2017).

Lysins have found to exhibit thermostability, high ionic tolerance and synergistic activity with antibiotics and other lysins (Ahiwale et al., 2011). In addition, lysins can be engineered to modify their target specificity and improve killing activity (Nelson et al., 2001). An interesting feature of lysin is that their activity is independent of the bacterial physiological state (R & E, 2004). For this it has been proved that lysins can destroy persistent bacteria within biofilms, even at low metabolic rates (Vázquez et al., 2017).

Depolymerases:

Depolymerases are enzymes derived from phages that facilitate the early stages of phage infection by degrading the extracellular substances of encapsulated bacteria. They are capable of degrading the chains of capsular polysaccharides, exopolysaccharides and O-polysaccharides from lipopolysaccharides and peptidoglycan. All these substances are important components of the biofilm matrix. Therefore, depolymerase activity is particularly interesting in the removal of biofilms, as it alters the EPS matrix and decreases bacterial virulence. Depolymerases are especially remarkable for treating human or animal infections caused by biofilms. They can enhance the action of the immune system against bacteria by degrading the EPS matrix and allowing immune cells to access the bacteria in the biofilm (B et al., 2018). Depolymerases have been tested against biofilms formed by different bacterial species. Depolymerase Dpo7, of vB_SepiS-phiPLA7 phage, was shown to reduce *Staphylococcus* sp. biofilm biomass by 53%–85% among 67% of the bacterial strains tested (Gutiérrez et al., 2015). Lastly, lysins and depolymerases are also good anti-biofilm agents in combination. For instance, lysin LysK and depolymerase DA7 have been tested in combination against *Staphylococcus aureus* biofilms in static and dynamic models. These enzymes showed a synergistic behavior and significantly reduced the number of viable cells in the biofilm (NMC et al., 2018).

### 2.6.3. Genetically Modified Phages:

Penetration and diffusion of phages through the EPS-matrix is mandatory to eliminate biofilms using phage-based treatments. However, there are a number of phages that do not encode these specific enzymes. In this case, phages can be genetically modified to produce enzymes that degrade the EPS-matrix, facilitating the removal of biofilms (RM, 2009). For example, a modified T7 E. coli phage has been designed to express a hydrolase which when released to the extracellular matrix, result in biofilm degradation. Phages can also be designed to selectively kill antibiotic resistant bacteria. Usually, lytic phages are used to destroy bacteria but temperate phages can also be used to deliver programmable DNA nucleases associated with CRISPR to reverse antibiotic resistance. This system can selectively destroy plasmids that confer antibiotic resistance (DJ et al., 2019).

### 2.6.4. Phages in Combination with Antibiotics

A sub-lethal dose of antibiotics can stimulate phage virulence under certain conditions. This phenomenon is known as phage-antibiotic synergy (PAS). The idea of combining phage therapy and antibiotics comes from the understanding that by using two different selective pressures we can obtain more efficacy than by using each separately (M. AM et al., 2016; T.-B. C & ME, 2016). Combination therapy of phages and antibiotics on *E. coli* biofilms has been tested using T4 phages and tobramycin, which strongly reduced antibiotic-resistant bacteria (LB et al., 2014). The combination of phage-derived enzymes with antibiotics, can increase the antibacterial effect by facilitating the access of antibiotics to the bacteria within the biofilm (B et al., 2018). However, the combination therapy has some drawbacks as well such as, emergence of double-resistant bacteria (Pena-Miller et al., 2013). It may turn into a case where antibiotics could potentially interfere with bacterial metabolism and prevent in phage infection to bacteria. For these reasons, the effects of combination therapy should be tested to avoid incompatibilities (ST, 2019; Tagliaferri et al., 2019).

# *Chapter 3: Materials and Methods*

## 3.1: Bacterial strains, antibiotic and Phage:

The entire research was carried out in the Environmental Life Science Laboratory of BRAC University using four *Vibrio cholerae* strains (033, WT 324, WT 333, WT 334) and one *Vibrio* specific phage JSF7. The Vibrio species were already isolated from the cholera patients previously and were stored for different experimental purposes at the Laboratory. The Vibriophage JSF7 was originally isolated from the environmental water samples and stored in the laboratory at $4^0$C. The Vibrio species were grown in Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar for confirming their presence and subsequently cultured on Luria Bertani (LA) agar on a regular basis to use them as working stocks. Additionally, one *Vibrio cholerae* strain (WT-346) which is a specific host of the phage JSF7 was used for the purification and amplification of the phage. Furthermore, the antibiotic Kanamycin (Kanamycinsulfate, ROTH) was chosen to check its activity on *Vibrio* biofilm. This particular antibiotic was chosen because the vibrio strains are susceptible to the antibiotic Kanamycin.

## 3.2: Preparation of Antibiotic Stock solution:

1gm of Kanamycin powder was added to 100ml of distilled water. This produces the stock solution of kanamycin which was next filter sterilized using 0.22µm syringe filters and stored at -$20^O$C. For this study, Kanamycin solution with concentration ranging from 100-1000 µg/ml was used and was prepared using the stock kanamycin solution.

## 3.3: Identification of the phage JSF7 from the stored sample:

Phage specific host WT 346 was streaked in LA plate and incubated overnight at 37ºC. Two to three isolated colonies from the overnight grown plate was added to 3ml of freshly prepared Luria Bertani broth (LB). The suspension was then placed in shaker incubator for 2 hours at 37ºC. This produces a young culture or logarithmic phase cells of WT 346 strain. After incubations, 500µl of the logarithmic-phase cells of WT 346 was added to 3ml of soft agar (Luria Bertani broth containing 0.8% Bactoagar, Difco) and overlaid on the freshly prepared LA plates. The laboratory stored phage was filtered through 0.22µm pore size filters to make them free from

bacterial contamination. 10-50 µl of the pure phage solution was inoculated on the WT 346 overlaid LA plates and incubated overnight at 37ºC. This procedure was done for confirming the presence of the phage in the stored suspension. The presence could be confirmed if a clear plaque is seen on the bacterial lawn LA plate.

3.4: Enrichment of the pure phage JSF7:

Upon phage confirmation, further procedures were carried out in order to enrich the phage solution. The first step includes picking a single discrete plaque from the bacterial lawn LA plate, using sterile pipette tip and placing it in Phage or SM buffer (100 mM NaCl, 8.1 mM MgSO4, 0.05 mM Tris-Cl [pH 7.5]). The pipette tip was cut to widen the diameter and the cut plaque was dispersed in the SM buffer by gently pulling off the micropipette. The suspended plaque was vigorously vortexed for 5 minutes to release the phages from the plaque agar. The suspension was then centrifuged at 4000 rpm for 5 minutes at $25^{O}$C. This results the agar to settle as pellets and the phages remain suspended within the supernatant. The supernatant next is collected in a fresh micro-centrifuge tube and chloroform in the volume one-third of that of the SM buffer was added. The solution was gently vortexed to allow mixing and then stored at $4^{O}$C. During storage, the chloroform evaporates from the solution and leaves the pure phages within the clear suspension.

For enrichment of the pure phage, the host bacteria WT 346 was streaked on LA plate and incubated overnight at $37^{0}$C. A few colonies from overnight grown WT 346 was inoculated in 3 ml of LB and incubated for 1.5-2 hours in the shaker incubator at $37^{O}$C. After incubation, the turbidity of the bacterial solution was checked since slight turbidity ensure logarithmic phase cells. 100µl of the pure phage solution was added to the young culture of WT 346 and incubated for 4-6 hours at $37^{O}$C in the shaker incubator. During this time, the phages infect the host bacterial cells and increase their number. After incubation, the solution was centrifuged at 13000 rpm for 5 minutes. This separates the bacterial cells as pellets and the enriched phages suspend in the supernatant. The supernatant was then collected in a fresh borosilicate vial and filter sterilized using 0.22-micron syringe filter. This yielded a clear suspension of phages which is completely free from any bacterial cells. This phage solution was enriched a couple of times using the same procedure to get the desired phage titer. The enriched phage stock was always stored at $4^{O}$C.

3.5: Phage Titer determination:

The phages were enriched a couple of times to ensure a proper phage titer that is essential to perform the study. For this purpose, the enriched phage solution was diluted 10 folds starting from $10^{-1}$ upto $10^{-8}$. The phage solution of a definite dilution factor was taken in separate vial and labelled properly. The titer of the phage was quantified using the Soft Agar Plaque Assay. The first step is to add a single colony of overnight grown WT 346 into 3ml of LB in a vial as mentioned in section 3.3. Then it was incubated for 2 hours inside the shaker incubator at $37^{O}$C which yields young culture of the host bacteria. After incubation, 300-µl of young culture, 5ml of soft agar (Luria Bertani agar containing 0.7% Bactoagar, Difco) and 10µl phage of a certain dilution were mixed and poured into LA plates and allowed to dry for 15 minutes. Separate plates for phage of a specific dilution should be used. The dried plates were then incubated overnight at $37^{O}$C. After incubation, single plaques were observed and counted to determine the titer using the Plaque Forming Unit (PFU) formula. Each plaque is considered one PFU and the titer (PFU/ml) is calculated using the following formula:

$$\frac{\text{PFU X Dilution factor}}{\text{Volume phage lysate in (ml)}}$$

3.6: Biofilm Preparation:

For the preparation of biofilms, four *Vibrio cholerae* strains were cultured overnight at $37^{0}$C on LA plates. Next, single colonies of *Vibrio cholerae* strains were inoculated on fresh Luria Bertani Broth (LB) and grown for 3 hours at $37^{0}$C in the shaker incubator. Atfer incubation, the suspension was diluted 100 folds in LB to get an optical density of 0.6 at 540nm. 500µl of this suspension was then distributed in different borosilicate vials and allowed to stand for 48 hours at room temperature.

To visualize the formed biofilms, 1% Coomassie Brilliant Blue solution was prepared which could be used for staining. The biofilms were kept submerged with Coomassie Brilliant Blue for 30 minutes. Afterwards, the tubes were rinsed with .9% saline solution a couple of times in order to remove any non-adherent dye.

3.7: Antibiotic Treatment on Biofilm:

The bacterial suspension which were allowed to stand for 2days for biofilm preparation (as mentioned in section 3.6) were next used for antibiotic treatment. After 2 days, the cell suspension in vials was discarded and the vials containing the biofilm was filled with 200µl of fresh LB solution. To this LB suspension, Kanamycin solution having concentration from 100-1000µg/ml was used to submerge the biofilm. This kanamycin treated biofilm was allowed to stand for 48 hours at room temperature. During this time, 100µl of the suspension was taken from the vials each day and spread plated on LA media to get the biofilm-dispersed bacteria or planktonic cell count. The cell count [colony forming unit (CFU)/ml] was calculated using the formula:

$$\frac{\text{CFU}}{\text{Volume(ml)}} \times \text{Reciprocal of dilution factor}$$

3.8: Bacteriophage treatment on Biofilm:

After biofilm formation in 48 hours, the vials were taken to treat biofilms with the enriched phage JSF7 solution. The cell suspension was removed from the vials and the vials containing the biofilm was filled with 200µl of fresh LB solution. The biofilms within the vials were then submerged under the phage solution and kept standing for 48 hours at room temperature. During this time, 100 µl of the suspension was taken from these vials and spread plated on LA agar to quantify the planktonic cells using the formula as mentioned in section 3.7.

3.9: Combination therapy of phage and antibiotic on biofilm:

The effect of combination therapy of phage and antibiotic was also studied in this experiment. This treatment also followed the procedure as mentioned above. The vials with biofilm were taken and the suspension were removed to fill the vials with 200µl of the LB solution. Next, a mixture of phage and antibiotic solution was used to submerge the biofilm. The solution contained Kanamycin at a concentration of 100 µg/ml and Phage JSF7 having concentration of $10^{10}$ PFU/ml. The vials were next allowed to stand for 24 hours at room temperature. During this time, the planktonic cell count could not be recorded due to the corona led lockdowns.

# *Chapter 4: Results*

## 4.1. Identification of the phage JSF7 from the stored sample:

The phage JSF7 when tested with the specific host WT 346 using the soft agar overlay method, showed the presence of clear plaque. The picture below supports the data of confirmation of the phage JSF7 in the stored sample.

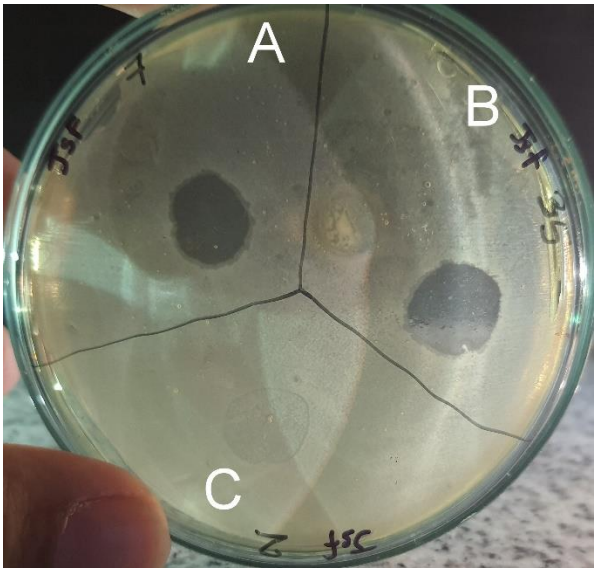



**Figure 9:** Checking the presence of Phage JSF7 through plaques in the stored sample shown in the Figure as **"A". "B"** and **"C"** are plaques checked for JSF 35 and JSF2 respectively, which are not used in the study

## 4.2. Phage Titer determination:

The phage upon enrichment for a couple of times showed increased PFU/ml. The table and pictures are provided below which shows the presence of isolated plaques at different dilution factor of the enriched phage.

Table 2: Increase in Phage titer over the times of enrichment

| Number of Enrichment | Phage titer (PFU/ml)<br>Dilution factor: | |
|---|---|---|
| First time (1x) | $10^{-4}$ | 9.00E+05 |
| Second time (2x) | $10^{-4}$ | 3.20E+06 |
| Third time (3x) | $10^{-5}$ | 1.20E+08 |
| | $10^{-6}$ | 5.00E+08 |
| | $10^{-7}$ | 3.00E+09 |
| | $10^{-8}$ | 1.00E+10 |

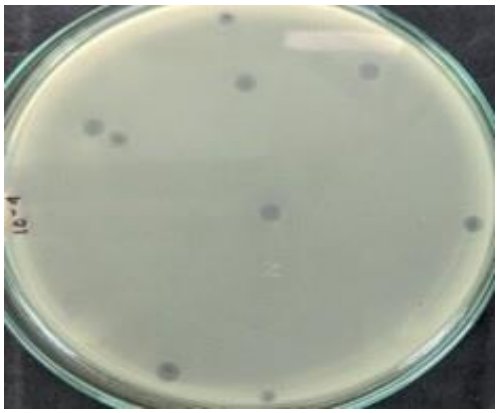


Figure 10: Phage concentration at $10^{-4}$ on first enrichment method

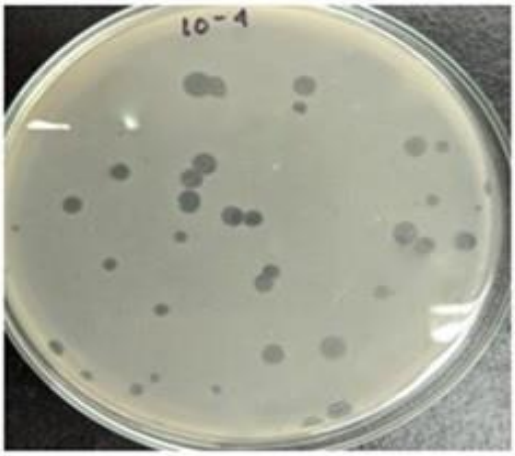



Figure 11: Phage concentration at $10^{-4}$ on second round of enrichment

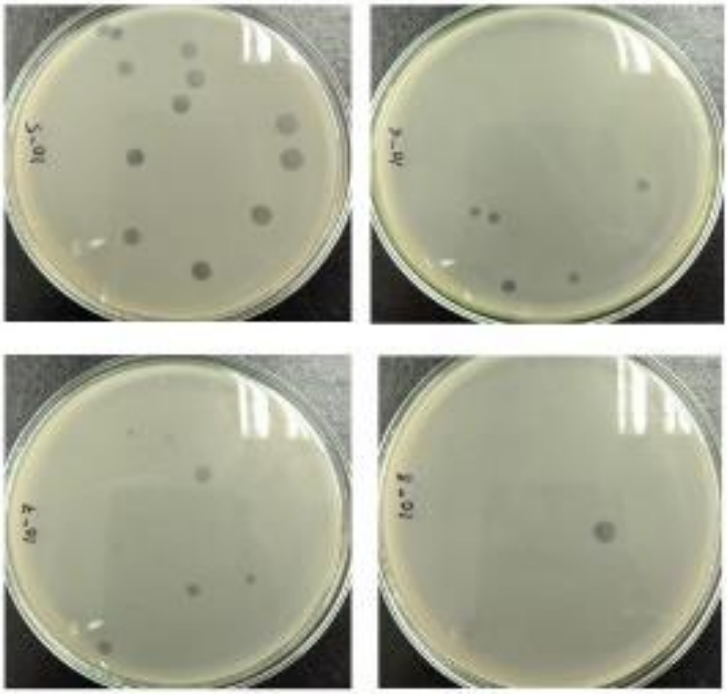


Figure 12: Phage titer at different dilution factors on final round of enrichment

4.3. Biofilm formation:

The strains of *V.cholerae* were allowed to stand at room temperature for 48 hours to form biofilm. All the strains were able to form biofilm which were seen vividly after staining with Coomassie Brilliant Blue. The pictures are provided below and this biofilms were next used as controls for comparing with those treated with antibiotic and phage.

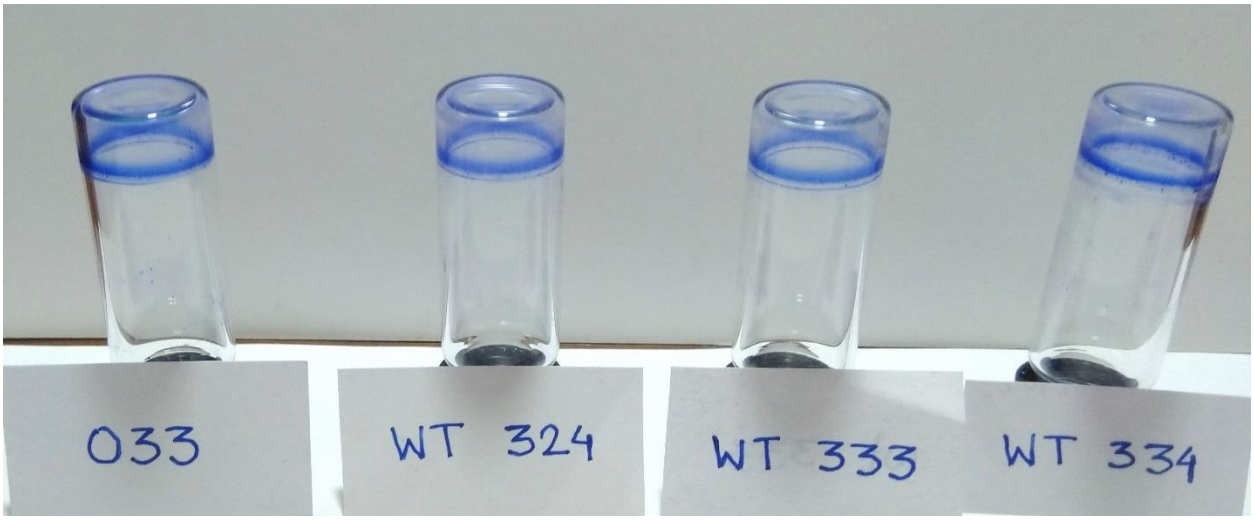



**Figure 13:** Biofilms formed by four strains of *V.cholerae*

4.4. Antibiotic Treatment on Biofilm:

The vials after biofilm formation, were treated with antibiotics in the process as mentioned in the section 3.7. The antibiotic treatment on the biofilms of the four strains, were observed for kanamycin solutions at concentration ranging from 100-1000µg/ml and in all cases, biofilms were unharmed by the action of kanamycin. The pictures below shows that kanamycin treatment at 500µg/ml concentration were unable to completely degrade the *Vibrio* biofilms.

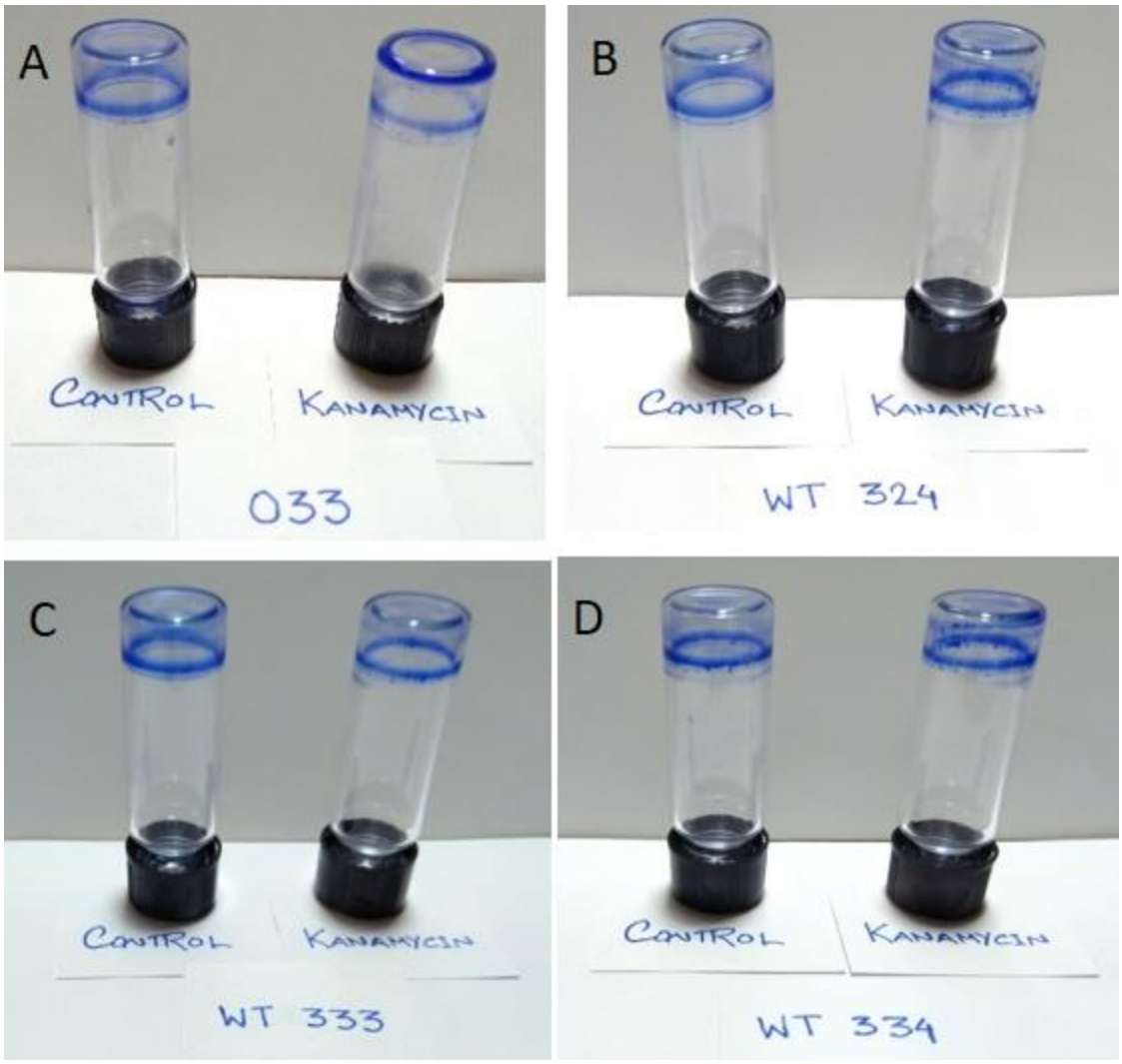



**Figure 14:** Kanamycin treatment on Biofilms of *V.cholerae.* The treatment brings no noticeable change on the formed biofilms

4.5. Viable cell count during the kanamycin treatment:

The planktonic cell count during the treatment was recorded two times in two days. The count was found to decrease on second day than the first day. The impermeability of the kanamycin to the biofilm may have failed to disperse the planktonic cells from the biofilm and this resulted in a reduced CFU/ml for each strains.

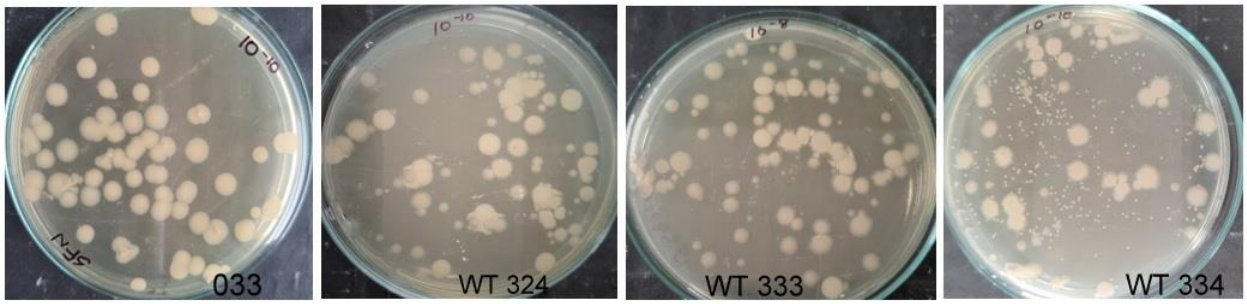


Figure 15: Viable cell count on first day of the antibiotic treatment on biofilm

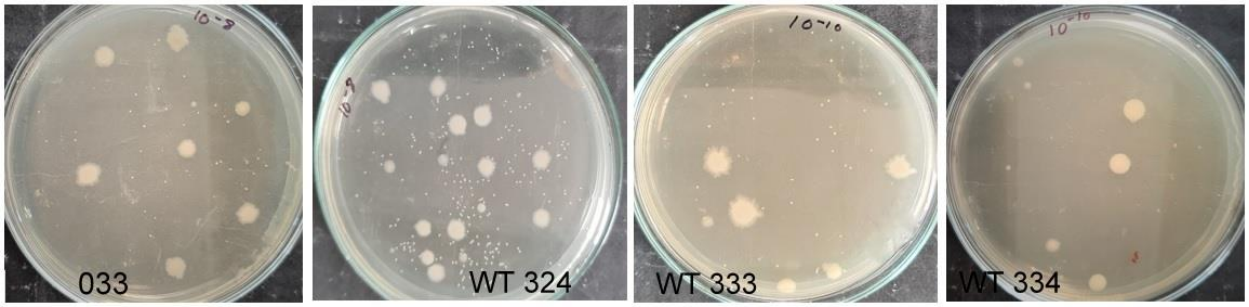


Figure 16: Viable cell count on second day of the antibiotic treatment on biofilm

Table 3: Viable cell count on dispersion from biofilm under the action of Kanamycin (500µg/ml)

| *Vibrio cholerae* Strains | Bacterial Cell count (CFU/ml) | |
|---|---|---|
| | Day 1 | Day 2 |
| 033 | 5.50E+09 | 7.00E+06 |
| WT 324 | 4.60E+09 | 1.30E+07 |
| WT 333 | 5.80E+09 | 5.00E+07 |
| WT 334 | 3.50E+09 | 4.00E+07 |

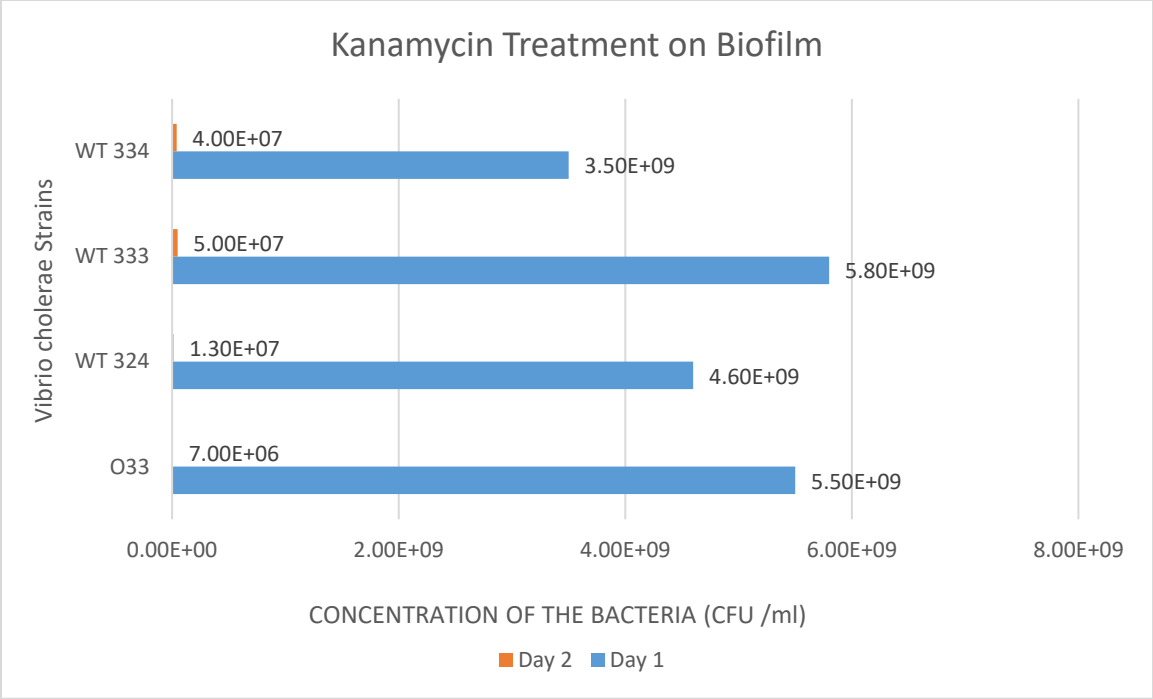



Figure 17: Graphical Representation of the decrease in cell dispersed from biofilms

4.6: Bacteriophage treatment on Biofilm:

The biofilms formed after two days of incubation, were treated with the highly concentrated phage solution of $10^{10}$ PFU/ml. In each of the four strains, the biofilms after the phage treatment were observed to be thinner than the width observed in the control biofilms.

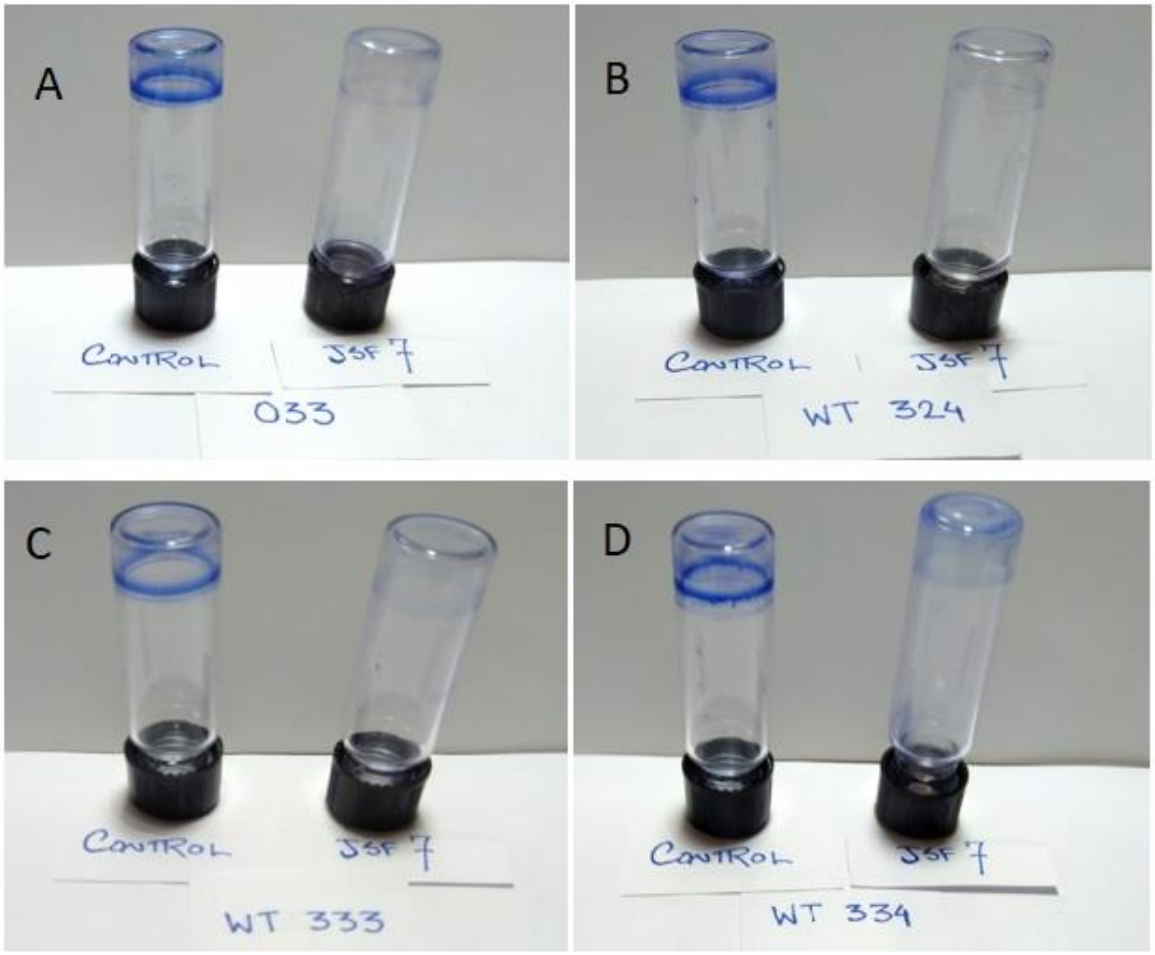



**Figure 18:** JSF 7 treatment on the biofilms of *V.cholerae*. The treatment causes noticeable degradation of the formed biofilms.

4.7: Planktonic cell count during the phage treatment:

During the two days treatment of the biofilms with phage, the planktonic cell count of each of the four strains were taken into account. It was seen that cell count increased over the matter of time. The CFU/ml of the strains were more on the second day than that of the first day. This data supports the idea that the phage was able to properly disperse the cells from the biofilm which increased the planktonic cell number over time.

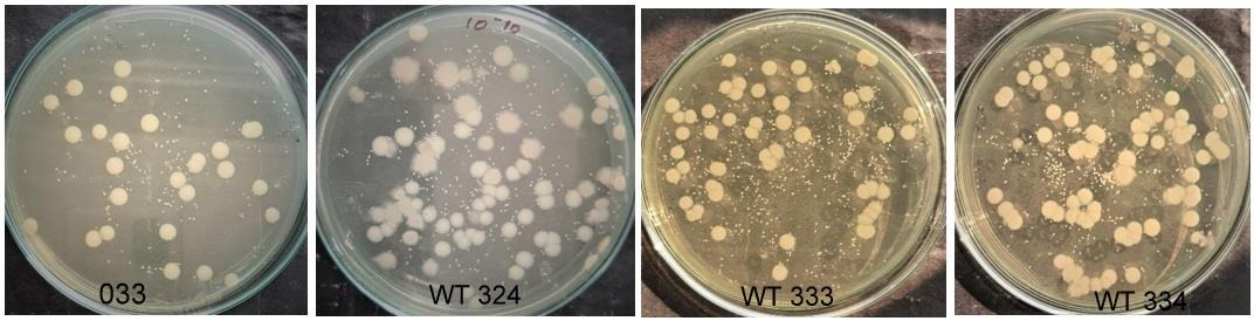


Figure 19: Planktonic cell count on first day of the Phage treatment on biofilm

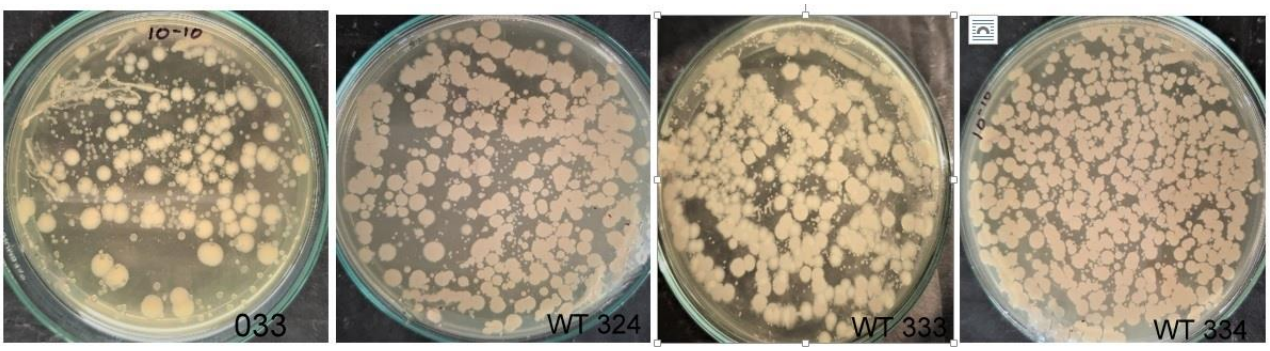


Figure 20: Planktonic cell count on second day of the Phage treatment on biofilm

Table 4: Planktonic cell count on dispersion from biofilm under the action of JSF 7 Phage

| *Vibrio cholerae* Strains | Planktonic Bacterial Cell count (CFU/ml) | |
|---|---|---|
| | Day 1 | Day 2 |
| 033 | 2.60E+07 | 8.90E+09 |
| WT 324 | 7.30E+07 | 1.68E+10 |
| WT 333 | 5.10E+08 | 2.17E+10 |
| WT 334 | 6.70E+07 | 3.33E+10 |

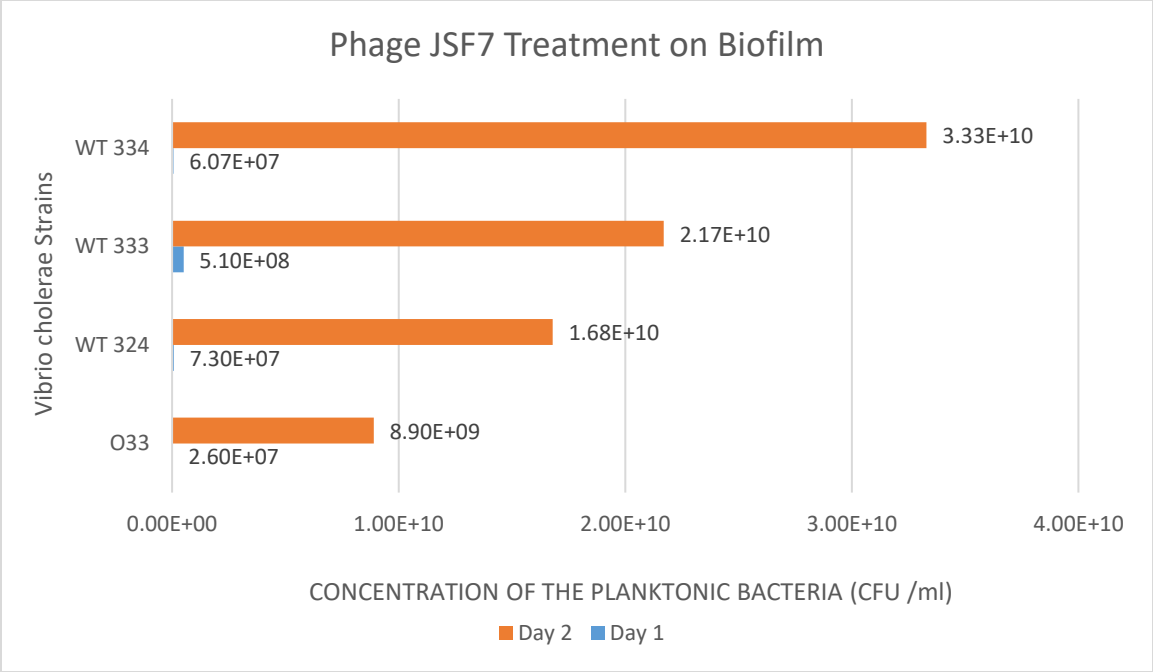



Figure 21: Graphical Representation of the increase in planktonic cell dispersal from biofilms

4.8. Combination therapy of Phage-Kanamycin on Biofilms:

After biofilm formation, the biofilms of the four strains were treated with the combination therapy of phage and antibiotic. For this therapy, concentration of kanamycin used was 100µg/ml and that of phage solution was $10^{10}$ PFU/ml. The biofilms of the each four strains were completely removed by this treatment. However, due to sudden spike in corona cases in Bangladesh, closure of the laboratory was announced and this hindered in taking the planktonic cell count.

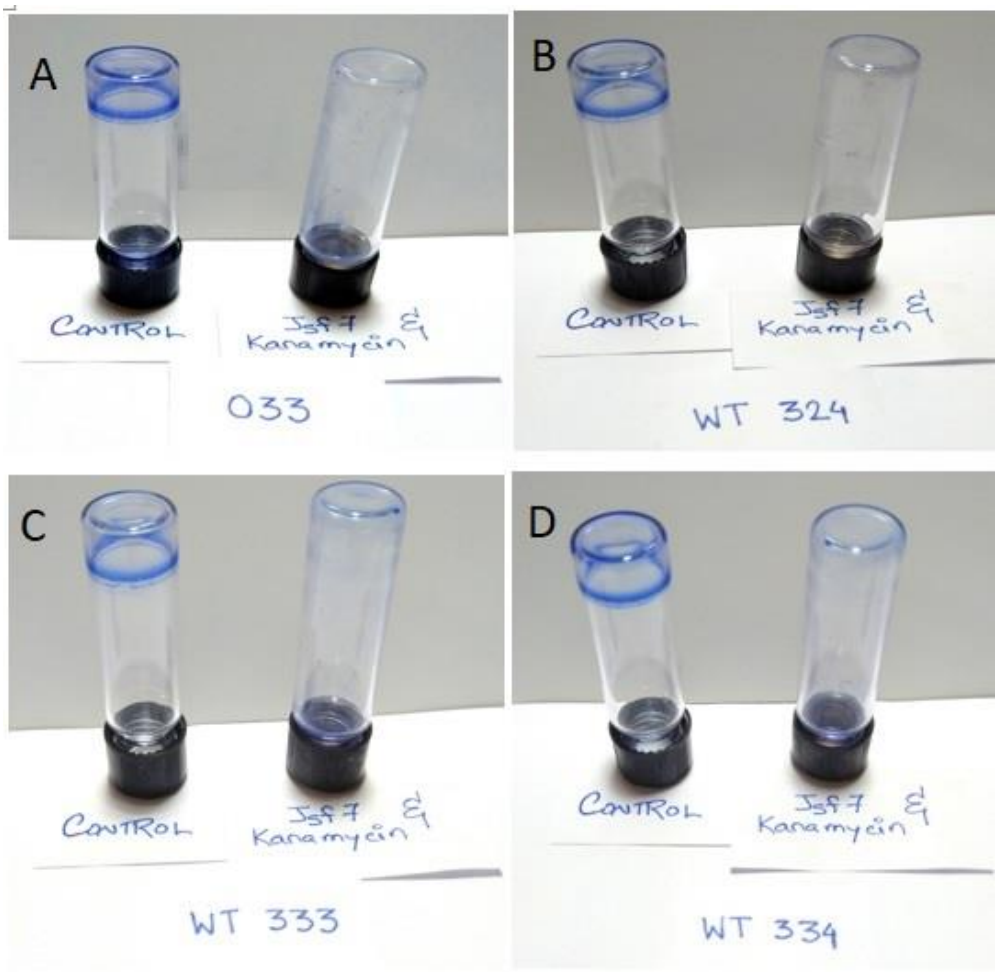



**Figure 22:** Combination therapy of Phage-Kanamycin on biofilms of *V.cholerae.* The treatment causes almost complete degradation of the formed biofilms.

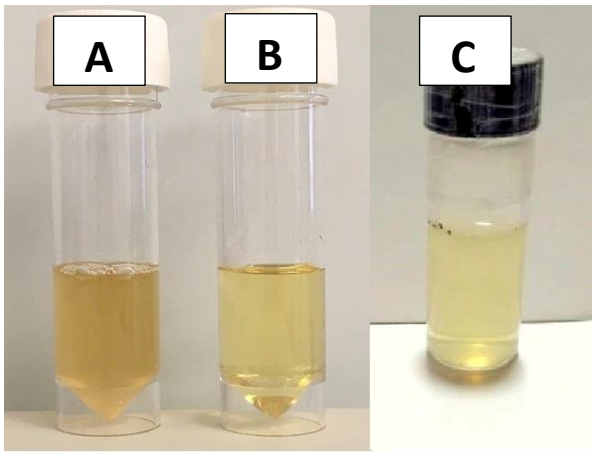



**Figure 23:** Bacterial Growth on LB broth shown in "A". Fresh LB broth Shown in "B". LB suspension after treatment as shown in "C"

# *Chapter 5: Discussion*

Cholera epidemics can become a major public health concern for the national and international health community because of the persistence of the causative *Vibrio* species through biofilm formation. To mitigate this challenge, the use of vaccines, antibiotics, and proper sanitation practices are advised. It is known that antibiotic resistance case is amplified when antibiotics are used to treat bacteria that form the biofilms (I. A et al., 2009). Thus, the treatment of biofilm-associated infections with the existing approved therapies remains a significant medical challenge. However, in the present study, the bacteriophage treatment can be used as a significant remedy of biofilm formation by pathogenic *V. cholera.*

It is shown in studies that the aquatic environment in the cholera prevalent areas harbors a mixture of *V. cholerae* specific and non-specific phages (SM & JJ, 2012). This mixture including the phages that can kill bacteria in biofilm as well as those that can disperse the planktonic bacteria from the biofilm (Naser et al., 2017). Using the data from this study, I have used four *V.cholerae* strains which are not host cells for the phage JSF7 and tried to observe the phage's mechanism of action against the non-specific bacterial biofilms. The phage JSF7 was enriched to a final titer of $10^{10}$ PFU/ml and applied on the four *V.cholerae* biofilms to observe its effect. In all the strains, upon phage treatment, the biofilms were found to be thinner than the controls. Unfortunately, due to the corona pandemic, the time of performing the study was constricted which didn't allow me to measure the biofilm. However, it was observed from the increasing planktonic cell count that the phage JSF7 was able to penetrate the biofilm matrix and disperse the cells from the biofilm to the LB suspension. Hence this concludes that the phage JSF7 does not kill the bacteria within the biofilm, rather disperse them from the biofilm. So, it is believed that if the treatment was carried on for more than 48hours then the phage might have been able to disperse all the cells from the biofilm, thus completely degrade the biofilm. Therefore, the use of this phage can play a remarkable role in degrading the *V.cholerae* biofilms. However, the dispersed planktonic cells from the biofilms can pose threat as well and so to combat this situation, antibiotic solution can be used to kill the free bacteria dispersed from the biofilm.

In this study, the antibiotic-kanamycin when used at very high strength (≥500µg/ml), had shown no noteworthy changes in the biofilm formed in all of the four *V.cholerae* strains. So, using the antibiotic solution alone, in preventing the *V.cholerae* biofilms cannot be considered a suitable

choice. As mentioned earlier, when an antibiotic solution was used at strength 10XMIC, were shown unable to penetrate and degrade the biofilm. In fact, antimicrobial concentrations necessary to inhibit bacterial biofilms can be up to 100-1000 times higher than those needed to inhibit the same bacteria grew planktonically (Gilbert et al., 2002). In contrast to the results of JSF7, the viable cell count decreased over time when treated with Kanamycin. This suggests that the Kanamycin was not able to penetrate as well as disperse the planktonic cells from the biofilm. Besides, from the size of the biofilms when compared with the controls, it seems that they were unaffected by the antibiotic treatment. The increased resistance to antibiotic by *V.cholerae* strains within biofilm may have been achieved through any of the following mechanisms as discussed earlier: efflux pumps, cellular impermeability, enzymes that confer resistance and natural evolutionary mutations etc. Furthermore, studies have shown that the resistance genes can be transferred from one to another bacterial cell within the biofilm and show pervasive antibiotic resistance in a network (LM et al., 2007). These cells in the biofilm, when released as planktonic cells may still retain the antibiotic resistant genes and consequently confer the resistant gene among other species in the environment. Simões *et al.* have reported in a number of studies that the species association can increase biofilm resistance and resilience to antimicrobial exposure (S. M et al., 2009; Simões et al., 2010). So, considering all these effects of the antibiotic treatment, this paper infers that other efficient therapeutic strategies instead of the antibiotic must be developed to treat the pathogenic biofilms.

The combination therapy of antibiotic-phage used in this study, has proved to be an effective means of removing the cholera biofilms which are grown ‘in vitro’. Kanamycin and Phage JSF7 when used combinedly, showed almost the complete removal of the bacterial biofilm. Although cell count of the bacteria dispersed from the biofilm was not taken in the present study due to the time restriction following the corona pandemic. But, it can be anticipated that the phage JSF7 penetrated the biofilm and dispersed the planktonic cells while the Kanamycin in the solution was able to kill the planktonic bacteria suspended in the solution. The suspension was observed to be much clear in comparison to the control which predicts the killing of the planktonic cells by the antibiotic. Also, the biofilm formed was seen to almost completely disappear during this combined therapy. However, further studies need to be performed on this therapeutic method in order to derive an authentic explanation behind the complete removal of the cholera biofilm.

Better clearance of bacterial cells and reduced evolvement of phage or antibiotic resistance are the major advantages of this combined therapy. Positive interactions between phages and antibiotics give hope that combined treatments will also be successful against the worst case of pandrug-resistant "super bugs" (AP et al., 2012). However, drawbacks of the combined therapy have also been discussed in many studies (ST, 2019). The negative interactions among antimicrobials have been shown to interfere with the phage's mechanism of action on the biofilms. Nevertheless, these problems have been reported to be only transient and the phages are not further disturbed by the presence of the antibiotic at a later treatment stage (T.-B. C et al., 2018). Combination therapies might greatly benefit from the careful choice of dosing and from the time points at which antibiotic is administered. In future studies, particular attention should be given to the sequential application, where the antibiotic needs to be introduced after the phages have already started to tackle the bacteria (Chaudhry et al., 2017).

In order to achieve even more improvements with phage-antibiotic combinations, the use of bio-engineered phages as adjuvants for antibiotics sounds promising (Lu & Collins, 2009). Although, bio-engineered phages may become broadly used in the future, yet progress with natural phages can also be made meanwhile by using the findings from the present study.

## *Chapter 6: Conclusion and Future directions*

The findings from this study can provide great insights into indicating an effective method of treating *V.cholerae* biofilms. The methods from this paper can be studied 'in vivo' to check its efficiency in the control of biofilm formation in an aqueous environment. The antibiotic treatment to biofilm may result in a number of complications such as dissemination of antibiotic resistance genes, interference of antibiotic dose on phage action, mutation of the planktonic cells to become 'persister' cells etc. So this paper shows the use of Vibriophage JSF7 as a substitute to antibiotic in successfully degrading *V.cholerae* biofilm and dispersing the planktonic cells. Moreover, the understanding of the effect of Kanamycin and Phage JSF7 on the biofilm might also help in the improved application of these tools. The degrading or dispersing effect of JSF7 to any non- specific cells also proves that this phage treatment can be studied to control other pathogenic biofilms such as *Salmonella,* STEC etc. This study also discusses the effective use of phage- antibiotic therapy, in killing the toxigenic *V.cholerae* biofilms. Although, due to certain restrictions further studies on this particular field were not being done, yet it can be hoped that the method is studied further to yield better results. So, to conclude it can be said that the study suggests useful information in using a condign alternative to antibiotics in regulating biofilm formation.

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# Appendix – I

**Instruments used:**

| | |
|---|---|
| Autoclave | Wisd Laboratory Instruments<br>Made in Korea |
| Electronic Balance | Model: WTB 200<br>RADWAG Wagi ELEktroniczne |
| Incubator | Model: DSI 3000 Digisystem Laboratory Instruments Inc.<br>Made in Taiwan |
| Microcentrifuge | Model: MC-12<br>Benchmark Scientific |
| Refrigerated microcentrifuge | Model: ScanSpeed 1730R<br>Labogene |
| Shaking Incubator | Model: JSSI-1000C JS RESEARCH INC.<br>Made in Rep. of Korea |
| Syringe filter | MS® MCE Syringe Filter<br>Membrane Solutions, LLC |
| Vortex Mixer | Model: VM-2000 Digisystem Laboratory Instruments Inc.<br>Made in Taiwan |
| Water Bath WiseBath® | Wisd Laboratory Instruments DAIHAN Scientific Co.,<br>Made in Korea |