Degrading *Vibrio cholerae* Biofilms with Bacteriophage and Potential Combined Effect of Phage and Antibiotic on Biofilms

By

Md Rockybul Hasan
20376002

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

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
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.
4. I have acknowledged all main sources of help.

Student's Full Name & Signature:

Md Rockybul Hasan
20376002

Approval

The thesis titled "Degrading *Vibrio cholerae* Biofilms with Bacteriophage and Potential Combined Effect of Phage and Antibiotic on Biofilms" submitted by **Md Rockybul Hasan** (20376002) of Fall, 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology on 10th November 2022.

Examining Committee:

Supervisor:
(Member)

_____

Iftekhar Bin Naser, PhD
Associate Professor, MNS Department
Brac University

Program Director:
(Member)

_____

Munima Haque, PhD
Associate Professor, MNS Department
Brac University

External Expert Examiner:
(Member)

_____

Md. Aminul Haque, PhD
Assistant Professor, Department of Pharmacy
Brac University

Departmental Head:
(Chair)

_____

A F M Yusuf Haider, PhD
Professor and Chairperson, MNS Department
Brac University

Ethics Statement

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

Abstract

Vibrio cholerae is a gram-negative coccobacillus known as the causative agent of cholera- a gastrointestinal disease endemic to developing and underdeveloped countries, Bangladesh being one of them. The ability to form biofilms made it very difficult for many broad host range antibiotics to penetrate through them and destroy them completely. The cells in the biofilm can persist in a wide range of environmental conditions by remaining metabolically dormant and able to resuscitate into planktonic cells after getting suitable opportunities which can lead to a cholera outbreak. Antibiotics have been used to breakdown biofilms, however the attempts remained unsuccessful in most cases even if the concentration is a few times higher compared to the planktonic counterpart of the same bacteria. In this study, bacteriophage therapy was introduced as an alternative to antibiotic treatment for degrading *V. cholerae* biofilms. Combined treatment of phage and antibiotic (kanamycin) was performed to check the effectiveness in degrading biofilms and killing the planktonic bacteria at the same time. In both cases of single phage and combination treatment there were evidence of decreasing the biofilm layers of *Vibrio* strains but not when treated with kanamycin alone. Shiga Toxin producing *E. coli* (STEC) was used to screen the phage's ability to target hosts other than *Vibrio* strains, which turned out to be ineffective. There is potential scope to use combined phage and antibiotic therapy in regulating biofilm formation, but further investigation and risk evaluation is required.

Key words: *Vibrio cholerae*; Biofilms; Bacteriophage; Antibiotic

Dedication

Dedicated to my family and friends
for their love and support…

Acknowledgement

The completion of my postgraduate thesis would not have been possible without the constant guidance of Almighty Allah.

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List of Acronyms

CVEC	Conditionally Viable Environmental Cells
VBNC	Viable But Non-culturable Cells
QS	Quorum sensing
EPS	Extracellular Polymeric Substance
OD	Optical Density
LA	Luria Agar
LB	Luria/Lysogeny Broth
DLA	Double Layer Agar
PFU	Plaque Forming Unit
CFU	Colony Forming Unit
RPM	Revolutions Per Minute
ml	millilitre
µl	microlitre
µg	microgram
µm	micrometer

Chapter 1

Introduction

1.1 Background of the Study

Vibrio cholerae is a widespread motile gram-negative comma-shaped rod (Richard A. Finkelstein, 1996) found in the aquatic environment especially in brackish water (Daboul et al., 2020; Grant et al., 2015). Some of the *Vibrio* species are toxigenic and infects human resulting to a deadly disease named Cholera. According to World Health Organization, this water born disease is responsible for 21000 to 143000 deaths and infects 1.3 to 4.0 million people every year worldwide (WHO, 2022). The source of *V. cholerae* is different types of watery environment including human intestine, lakes, rivers, and the ocean (Kierek & Watnick, 2003). Capacity of biofilm formation is well documented for *V. cholerae*, both in laboratory conditions and in natural habitats (Faruque & Albert, 1998; Flemming & Wingender, 2010). In a watery environment this bacterium mostly resides in a dormant form known as conditionally viable environmental cells (CVEC) (Naser et al., 2019). This form of *V. cholerae* is not culturable in a conventional bacteriological media, so they are called viable but non-culturable cells (VBNC) (Cottingham et al., 2003; Yoshifumi, 2011). Sometimes these dormant cells become active naturally and act as active planktonic cells upon receiving appropriate ecological factors and contribute to cholera epidemics (Bari et al., 2013; Naser et al., 2017; Jemielita et al., 2018).

Some research suggests that bacterial biofilms are responsible for causing up to 80% infections in human (Jamal et al., 2018; Kumar et al., 2013; Römling & Balsalobre, 2012). To mitigate the biofilm formation various approaches are tried out such as antibiotic treatment, quorum sensing pathway inhibition, alteration of membrane permeabilization, inhibition by polysaccharides and many more (Roy et al., 2018). Many studies have provided evidence that

bacteria in biofilms are more resistant to antibiotic compared to their planktonic form (Herrmann et al., 2010), up to 1000 times more resistant to antibiotics which make them very stable (Hall & Mah, 2017; Lewis, 2001). *V. cholerae* is susceptible to several antibiotics of various groups such as ampicillin, ciprofloxacin, erythromycin, tetracycline, kanamycin etc. In this study, kanamycin was used alone as well as in combination with bacteriophage to treat biofilms. Kanamycin sulfate is the most used form of it. In a study conducted by Chandrakala et al (2014) it has been shown that many strains of *V. cholerae* have shown sensitivity to this antibiotic at a concentration of 44 ±1 µg/ml. However, several studies suggest that kanamycin is unable to kill the 'persister' cell in many biofilms at regular to a few times high concentration (Margarida Pereira et al., 2012; Shih & Huang, 2002).

Demand for effective treatment of *V. cholerae* biofilm is increasing gradually as in many cases antibiotic is not very helpful. Bacteriophage could be a potential option here in eradicating biofilms and releasing planktonic cells (Naser et al., 2017). Bacteriophages or phages are bacterial viruses that can recognize and infect host bacterial cells. As virus infectivity requires binding to a specific receptor, phages are specific for a small host range and have no ability to infect the human cell (Jensen et al., 2015). Unlike most of the antibiotics, many phages are able to remove biofilms completely (Harper et al., 2014; Tian et al., 2021). Similarly, there is evidence of removing *V. cholerae* biofilms by using a *Vibrio* specific phage which leaves a lot of planktonic cells in the aquatic environment after breaking the biofilm matrix. Vibriophage JSF7 is such a phage that can be active against biofilms for both host (susceptible) and non-host (resistant) *V. cholerae* strains (Naser et al., 2017). Sometimes a single phage is not enough to remove the biofilm as well as to kill the planktonic bacteria derived from biofilm degradation. Combining phage with antibiotic have shown promising results in several experiments in combating bacterial biofilms (Liu et al., 2020; Segall et al., 2019). So JSF7 can be combined with antibiotic to check out if this mixture can degrade biofilms and kill the

planktonic bacteria at the same time. As JSF7 has shown biofilm removing activity in *V. cholerae* strains previously which are resistant to this phage (Naser et al., 2017), non-Vibrio strains, for example, STEC biofilm can be treated with this phage to see if the phage has the property to breakdown a biofilm other than host organism.

1.2 Objective

The objective of this study is to degrade *Vibrio cholerae* and *Escherichia coli* biofilms with a specific bacteriophage and check the effect of combined treatment of this phage and an antibiotic.

1.3 Specific Aims

1. To observe the capacity of a *Vibrio* phage to degrade biofilm
2. To observe the effect of antibiotic on dispersing planktonic cells from biofilm
3. To observe the effect of phage and antibiotic combination therapy on biofilm

Chapter 2

Literature Review

2.1 *Vibrio cholerae*

Filippo Pacini identified *Vibrio cholerae* in 1854 and Robert Koch discovered it again independently in 1884 (Lippi & Gotuzzo, 2014). *V. cholerae* is Gram-negative, highly motile, facultative anaerobe and comma-shaped bacteria belonging to the Vibrionaceae family. The bacteria naturally live in brackish or saltwater (Daboul et al., 2020). There are more than 200 serogroups of *V. cholerae* which includes pathogenic and non-pathogenic strains (P. Lu et al., 2022; Ramamurthy et al., 2019). Among these, serogroup O1 and O139 are mainly known as toxigenic. For O1, serotype Inaba and Ogawa and two biotypes classical and El Tor cause the cholera disease (Chowdhury et al., 2017; Somboonwit et al., 2017).

2.2 Biofilm

2.21 Bacterial Biofilm

Biofilm is a microbial community that live in biotic or abiotic surfaces comprising of an extracellular polymeric substance (EPS) which is secreted by the microbes themselves (Amankwah et al., 2021;Chang et al., 2022). Biofilms consist of one or more species of bacterial cells and attach to surfaces such as plant and animal tissues, medical devices, pipes, food, industrial equipment and many more (Jamal et al., 2018;González et al., 2017;Harper et al., 2014). Exopolysaccharides are the main components in EPS matrix of a biofilm, but also contain secreted proteins, nucleic acids, lipids, and some other minor components. Moreover, the matrix also contains some enzymes which help them extract nutrients by acting as an external digestive system (Chang et al., 2022; Jamal et al., 2018).

Formation of biofilm is an important adaptation and survival strategy for many bacteria if not all. When under environmental stresses such as limited nutrition, UV radiation, extreme pH or temperature, high salt concentration or in pressure with antimicrobial agents- bacteria tend to form biofilms to ensure their survival. The biofilm structure can save a bacterium from attack by various bactericidal or antibacterial agents, shear forces and our immune system (Aparna & Yadav, 2008; Galié et al., 2018; Jefferson, 2004).

2.22 Bacterial Biofilm Formation

The formation of biofilm is a complex and cooperative group process which involves chemical communication processes and occur in five steps mainly: (i) surface sensing (ii) attachment, (iii) EPS excretion, (iv) maturation and (v) dispersion as shown in the figure **2.22**, below:

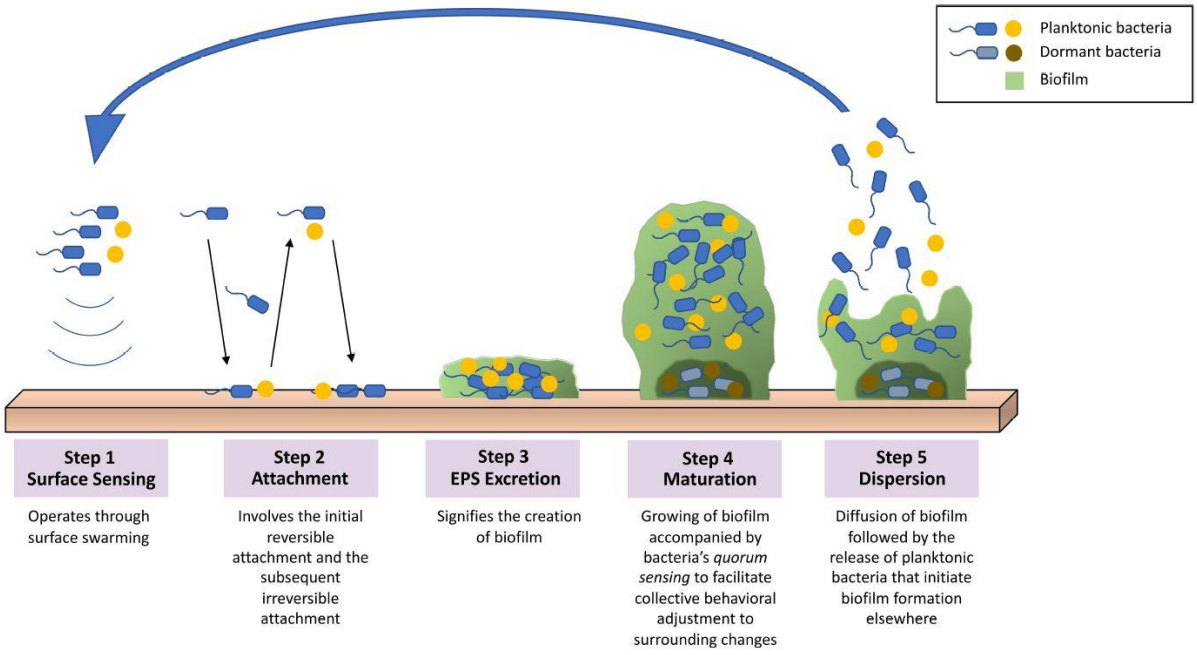


Figure 2.22: Schematic presentation of how biofilm forms (Chang et al., 2022).

The formation begins with surface sensing operated by surface swarming (1). The attachment step involves initial reversible attachment and subsequent irreversible attachment (2). Eps excretion step signifies the creation of biofilm (3). Next, maturation of biofilm occurs by quorum sensing which is involved to simplify collective behavioral adjustments to surrounding

changes (4). Finally, dispersion of mature biofilm structure and releasing planktonic cells which can initiate forming biofilm elsewhere (5).

The initial stage of the biofilm formation begins with the surface sensing operated by the planktonic bacteria's flagella that facilitates such signaling through surface swarming (Chang et al., 2022). Next step is the temporary attachment of the free-living bacteria to the surface is known as reversible attachment which causes a weak adhesion and then irreversible attachment, causes a permanent adhesion that results in strong attachment of the bacteria to the surface (Moormeier & Bayles, 2017; Renner & Weibel, 2011). Then, cells start to divide and multiply to form microcolonies, and formation of the EPS matrix fixes the initial adhesion. Microcolonies are protected from various stresses by EPS matrix. Chemical communication between and within the cell is necessary for the coordination of different bacteria in the biofilm. Here, quorum sensing, the mechanism of cell-cell communication, plays its role by synchronizing cell expression in response to population cell density (Lappin-Scott et al., 2014). With the synthesis of EPS matrix from the major structural components i.e., polysaccharides, protein, and environmental DNA (eDNA), the formation of microcolonies leads to the generation of thin biofilm starting the maturation phase. Structural changes as well as many gene expression changes occur through signal molecules of biofilm maturation process (McCall et al., 2018; Nassar et al., 2021; Toyofuku et al., 2016). The final step of biofilm formation is dispersal of biofilm structure and possibly making a new cycle of biofilm formation by switching the cells form sessile mode to planktonic form (Chang et al., 2022; Solano et al., 2014).

2.23 *Vibrio cholerae* Biofilm

Vibrio cholerae can stay in the environment in both motile and biofilm state. The global transcriptional profile of *V. cholerae* cells differs in the planktonic, monolayer, and mature

biofilm phases. The down-regulation of motility gene expression and the activation of genes necessary for the creation of the biofilm extracellular matrix are significant in the shift from a planktonic to a biofilm lifestyle (Moorthy & Watnick, 2005). The transition between biofilm formation and planktonic cell in *V. cholerae* is mainly controlled by two chemical signaling systems- 1) quorum sensing (QS) and 2) 3',5'-cyclic diguanylic acid (c-di-GMP). QS works through autoinducers which bacteria use to synchronize the behavior of their population (Waters et al., 2008). Two autoinducers (AIs) are produced by *V. cholerae* which are CAI-1 and AI-2. When cell density is low (AIs are low), the receptors of autoinducers act as kinases and pass phosphate to LuxO, the response regulator. LuxO~P then activate genes that are responsible for encoding the Qrr small regulatory RNAs (sRNAs) which destabilize the mRNA encoding HapR, a major regulator of QS (Lenz et al., 2004). Here, AphA, the low cell density master regulator, activates expression of genes which are needed for biofilm formation as well as to show pathogenicity (Papenfort et al., 2017). When *V. cholerae* cells becomes high in density, the AIs accumulate, bind their cognate receptors, and converts them to phosphatase from kinase leading to dephosphorylation of LuxO and cessation of *qrr* expression. Now, the mRNA coding HapR is stabilized leading to HapR production. Biofilm and virulence genes are repressed due to HapR and presumably disperse the *V. cholerae* cells from host to environment (Miller et al., 2002; Papenfort et al., 2017).

The second messenger c-di-GMP also plays role in switching between *V. cholerae* planktonic and biofilm phase is being controlled by intracellular concentration of (Beyhan et al., 2008; Lim et al., 2006). Three significant regulators recognize the intracellular level of c-di-GMP: FlrA, σ54-dependent activator, is necessary for the expression of flagellar motility (Srivastava et al., 2013). Enhancing of the c-di-GMP pool, promotion of dimerization, and activation of VpsT to induce biofilm formation are done with the help of Five membrane-bound DGC (CdgA, H, L, K, and M) (Shikuma et al., 2012). The biofilm matrix also includes proteins and

extracellular DNA, which helps to maintain the biofilms' structural integrity (Okshevsky & Meyer, 2015).

2.24 Antibiotic Resistance Mechanism of Biofilm

Biofilm communities share different resistance mechanisms compared to planktonic bacterium’s target mutations, low cell permeability, efflux pumps, modifying enzymes of drug or protein neutralization (Walsh., 2000; Kumar et al., 2013). When bacteria have fewer protective mutations, lack of plasmids or other genetic elements which carrying resistant genes becomes more resistant when grow in the biofilm (Anderl et al., 2000). When planktonic bacteria are dispersed from a biofilm, antibiotic sensitivity restored again rapidly, and this quick reversal of resistance proposes an adaptive resistance mechanism instead of a genetic alteration (Stewart, 2002). A biofilm can provide a better breeding ground for spontaneous mutants and the close spatial proximity of bacterial cells within a biofilm accelerate plasmid transfer (Hausner & Wuertz, 1999). The antibiotic delivery to the depths of the film can be slow down if it is inactivated by reaction or sequestered by binding as it diffuses into the biofilm. Beta-lactamases, enzymes that alter aminoglycosides, and enzymes that acetylate chloramphenicol are a few examples of these enzymes. For instance, penicillins only eradicate developing germs (Tuomanen et al., 1986). Since the majority of antibiotics aim to inhibit some form of macromolecular synthesis, it is unlikely that these medications will have a significant impact on bacteria whose macromolecular synthesis is inhibited. Bacteria in an anaerobic region of a biofilm may be differentially protected from these antibiotics, even if they are capable of fermentative growth. Studies on the antimicrobial sensitivity of rpoS mutant biofilms show no evidence of this gene's function in defending biofilms (Cochran et al., 2000; Greenberg et al., 2001). Bacteria in biofilms not only evade killing by antibiotics, but they also resist chemical disinfectants, such as chlorine bleach and glutaraldehyde. Though persisters may constitute a relatively small fraction of the population but few cells of these entered a

highly protected, perhaps spore-like, state. Genes encoding regulatory circuits that control the state's entry and exit as well as certain defensive responses may be among those that contribute to the persister state. In *E. coli*, genes for high-level persistence (hip) have been identified (Lewis, 2001).

2.3 Bacteriophages

2.3.1 Background

Bacteriophages or phages are bacterial viruses, which are intracellular parasites, composed of a DNA or RNA molecule encapsulated by a protein structure (White & Orlova, 2016). Metagenomics have estimated the number of phage species in nature to be at 10^{31} which is more than the number of bacteria at 10^{29} (Strange et al., 2021). They are classified according to their genetic material (ss versus ds; DNA versus RNA) and their genomic sizes range from 4kb upto 600kb (Brüssow & Hendrix, 2002). While the head-tail structure is innately unique to phages, their capsids can be icosahedral, filamentous or head-tail in shape (Dimmock et al., 2016; Pietilä et al., 2013).

2.3.2 Life Cycle of Bacteriophage

Bacteriophages have two different life cycles i.e., lytic (virulent) life cycle and lysogenic (temperate) life cycle. Upon discovery of bacteriophages, it was thought as a lethal agent for bacteria. But it was only in 1951 and thereafter, when bacteriophage λ was discovered, scientists suggested that phage can be in virulent or temperate life cycle (Casjens & Hendrix, 2015). However, to use as therapeutic and control bacterial infections only lytic life cycle can be utilized. There are two steps which are common in both the life cycle- absorption of phage and penetration of genetic material. When lytic phages infect the host bacteria, they share their genetic information with the bacteria. Here, viral nucleic acids and proteins production take

place. Releasing of phage progeny occur after observing the assembly and packaging of phages. After that several phage enzymes such as lysins, murein or holins aided to burst the virion in the extracellular surroundings from the host cell (Wittebole et al., 2014). In lysogenic life cycle, on the other hand, phage inserts its viral genome into the bacterial chromosomes, the prophage, where it replicates as a part of the bacterial chromosome. This incorporated viral genome is transmitted vertically to bacterial progenies with the bacterial genome until the lytic cycle is induced (Wittebole et al., 2014). Generally, lysogenic phages are stable, but sometimes they may help to initiate the lytic cycle. Little (2005) showed that a λ phage, which is a temperate phage, can proliferate by both lytic and lysogenic cycle. Prophage has significant roles in impersonation of the pathogenicity of several bacteria. A group of researchers showed that prophages are responsible for a notable number of genetic variations in two closely related bacterial strains (Ferretti et al., 2001).

2.3.3 Vibriophage: JSF7

Vibriophages are those bacteriophages that commonly infect *Vibrio* species. Many researchers have reported various *Vibrio* phages in Bangladesh mainly isolated from the aquatic environments. Faruque & Mekalanos, 2012 published a list of *Vibrio* phages isolated from surface water and cholerae patients from Bangladesh, shown in a table below:

Table 2.3.3: Lytic vibriophages isolated from surface water and cholerae patients in Bangladesh (Faruque & Mekalanos, 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 the phages mentioned in the table above, JSF7 is the phage of interest for this study. Naser et al., 2017 have done a thorough study about the characteristics of this phage. JSF7 is a double stranded DNA phage and has lineage with the Duplodnaviria, Heunggongvirae, Uroviricota, Caudoviricites, Caudovirales, Autographiviridae, Tawavirus and Vibrio Virus. This phage specifically hosts for *V. cholerae* O1 strains, but it has been found to degrade both *V. cholerae* O1 the *V. cholerae* O139 biofilms. The phage showed isometric head and a contractile tail in electron microscopic study. The genome size of JSF7 is 46.31Kbp and it has 49 open reading frames (ORFs). These ORFs of the phage were found to encode lipase and polysaccharide enzymes which are presumed to be involved in biofilm degradation. The phage morphology showed that JSF7 belongs to the Myoviridae family. Temperature tests showed that this phage is stable at 37℃ and below but decreases its stability when temperature goes above this. 65% to 98% of the phages remain infectious at pH 6.0 to 9.0. When stored in phage buffer or SM buffer in room temperature, JSF7 remain infectious for more than 4 weeks (Naser et al., 2017).

2.4 Different Means of Phage Application Against Biofilm

2.4.1 Phage Therapy

Phages can be used as antibacterial agents and this what we know as phage therapy. Phages can recognize, bind and multiply inside bacterial cell which leads to lysis of the host cell (Burrowes et al., 2011). Phage therapy was introduced almost a century ago, but the discovery of antibiotics against broad spectrum of bacteria almost demolished the use of phages. In recent times, the interests in phage therapy have been renewed due to the emergence of multidrug resistance bacteria (Gordillo Altamirano & Barr, 2019; Lin et al., 2017). Phage based therapies mainly focus on lytic phages as they have ability to destroy their bacterial hosts as well as they lack some enzymes like integrases in horizontal gene transfer (Tinoco et al., 2016). In case of biofilms, EPS-degrading enzymes encode by phages are the main interests (Hu et al., 2010). The bacteria found inside the biofilm could be another source of EPS-degrading enzymes when they are in stressed condition. These type of stress could be triggered by phage infections, facilitating increased penetration of the biofilm and dissemination of the phages inside the biofilm (Hu et al., 2010). Doolittle et al (1995) showed the first case of phage treatment to eradicate the biofilm caused by *E. coli*. Since then, many studies have been conducted which evidently suggests that bacteriophage therapy could be a successful pathway in controlling bacterial biofilms (Parasion et al., 2014; Phee et al., 2013; Son et al., 2010).

2.4.2 Phage Derived Enzymes

Infection of bacteriophages is specific to a particular serotype of bacteria only. The accompanying microflora of a target bacterium host remains unharmed. Their high specificity makes phages and phage derived enzymes promising anti-bacterial agents. They also help override the problem of uprisal of antibiotic resistant bacteria, which is now a global health crisis (Knecht et al., 2020). To negate the physical obstruction imposed by biofilms, hydrolytic

enzymes come in play. Bacterial cell surface consists of polysaccharides such as capsular polysaccharide (CPS), exopolysaccharide (EPS) or lipopolysaccharide (LPS). Their function aids biofilm production, virulence and phage interaction. Recognition of these specific ligands by the tail fiber or tail spike proteins (TSPs) is integral for adsorption. The phage depolymerase enzyme appears as TSPs. After binding with the complimentary ligands (CPS, EPS or LPS), polysaccharide repeating units are cleaved off and the phage can reach the cell wall to inject its DNA (Knecht et al., 2020).

Another kind of phage encoded lytic enzyme that locally degrade the peptidoglycan of bacterial cell wall during infection is the Virion-associated peptidoglycan hydrolases (VAPGHs). They technically drill a small hole in the cell wall through which the phage genetic material reaches into the cytoplasm. This disruption of bacterial cell wall occurs prior to phage production and is caused by a high number of phages adsorbed onto the cell surface (Rodríguez-Rubio et al., 2012).

Bacteriophage endolysins are also peptidoglycan degrading proteins that allow the phage to escape from the bacterial during the phage lytic cycle. Endolysins degrade peptidoglycans with glycosidase, amidase, endopeptidase or lytic transglycosylase activities (Nelson et al., 2012)**.** Endolysins are produced by double stranded phages and work best on gram positive bacteria upon being added exogenously. Peptidoglycans in gram negative bacteria is protected by outer membrane thus making them irresponsive towards endolysin activity. Recent studies have emphasized on the effects of artilysins in combination with endolysin on such bacterial cells. The duo, in combination has been proved to have superior bactericidal activity (Briers et al., 2014).

2.4.3 Phages in Combination with Antibiotics

Adding antibiotics to bacteriophages can give unpredictable results when interact with bacteria as the reaction could be synergistic, antagonist, neutral or additive (Himmelweit, 1945; Oechslin et al., 2017; Shlezinger et al., 2019). Using phage or antibiotic alone could be less effective compared to using a combination of these two. The possible advantages of using the joint approach might enhance the suppression of bacteria, weaken the capacity of bacteria to become resistant against bacteriophages and/ or antibiotics, and make powerful penetration into biofilms (Li et al., 2021). In a study in 2015, it has been shown that addition of gentamycin into phages removes *Staphylococcus aureus* strains and similar phenomenon was noticed for vancomycin and tetracycline (Ali et al., 2015). In another study Torres-barceló & Hochberg (2016) showed that using combined treatment of phages and antibiotics significantly control the bacteria rather than using them separately. There are many other evidence in controlling or eradicating bacteria with intrinsic resistance to antibiotics by using bacteriophages in combination with various types of antibiotics (Grygorcewicz et al., 2020; Kamal & Dennis, 2015). However, as mentioned earlier, the combination therapy might give antagonistic effects or help to emerge resistant bacteria (Torres-barceló & Hochberg, 2016). So, the effects of these kind of combination therapy should be tested before implementing it in outside lab environment to avoid any undesired outcome (Abedon, 2019; Tagliaferri et al., 2019).

Chapter 3

Materials and Methods

3.1 Bacterial Strains

There are a total of six bacterial strains used in this study. Among them five are *Vibrio cholerae* strains namely 031, 1877, WT324, WT346 and WT406 and another strain is Shiga toxin-producing *E. coli* (STEC).

3.2 Antibiotic

The antibiotic Kanamycin was selected to see its activity on *Vibrio* and STEC biofilms. This antibiotic was used because it is one of the antibiotics that is susceptible to both Vibrio and STEC strains.

3.3 Bacteriophage

JSF7 is the bacteriophage that was used in this study and this phage was isolated previously from environmental sample and stored at 4℃ in the Biochemistry and Environmental Microbiology laboratory of Brac University. *V. cholerae* strain WT346 is the host of JSF7 phage.

3.4 Reagents, Chemicals and Instruments

The reagents and chemicals that were used in this experiment are listed as in the **Table 3.4a,** below:

Table 3.4 a: Chemical and Reagents used in the experiments

Chemicals and Reagents	**Manufacturer/Brand**
Ethanol	Emsure
Spirit	Sigma-Aldrich
Glacial Acetic Acid	Sigma-Aldrich

Crystal Violet	Sigma-Aldrich
Tryptone	Himedia
Yeast extract	Oxoid
NaCl	Emsure
Bacto-Agar	Himedia
TCBS	Himedia
Luria Broth	Himedia
MacConkey	Himedia
Muller Hinton Agar	Himedia

The instruments/laboratory apparatus that were used in this experiment are listed as in the **Table 3.4b,** below:

Table 3.4 b: A list of the instruments/laboratory apparatus

Instruments/apparatus	**Brand/Model**
General incubator	Incucell
Shaking incubator	JSR
Ultra centrifuge machine	TOMY MX-307
Autoclave machine	TOMY ES-315
Laminar	Haier biomedical
Refrigerator	Samsung
Elisa machine	Thermofisher multiskan ex
Vortex	DIGISYTEM VM-2000
Water bath	WiseBath
Spirit lamp	N.A.
Micropipette	Eppendorf
Micropipette tips	NEST
Petri dishes	N.A.
Conical flask	SCHOTT Duran®
Screw-capped bottles	SCHOTT Duran®
Polypropylene screw-capped tubes	Falcon
Glass test tubes	Pyrex
Borosilicate vials with cap	Pconlab

Microcentrifuge tubes (1.5ml and 2.0ml)	Eppendorf
Syringe (5ml and 10ml)	JMI
0.22µ filter	Pconlab
Inoculation loop	N.A.
Inoculation needle	N. A.
Cotton swab	Dearon
Parafilm tape	Bemis Company, Inc

3.5 Overview of the Methodology

Overview of the methodology that has been used in this study, shown below:

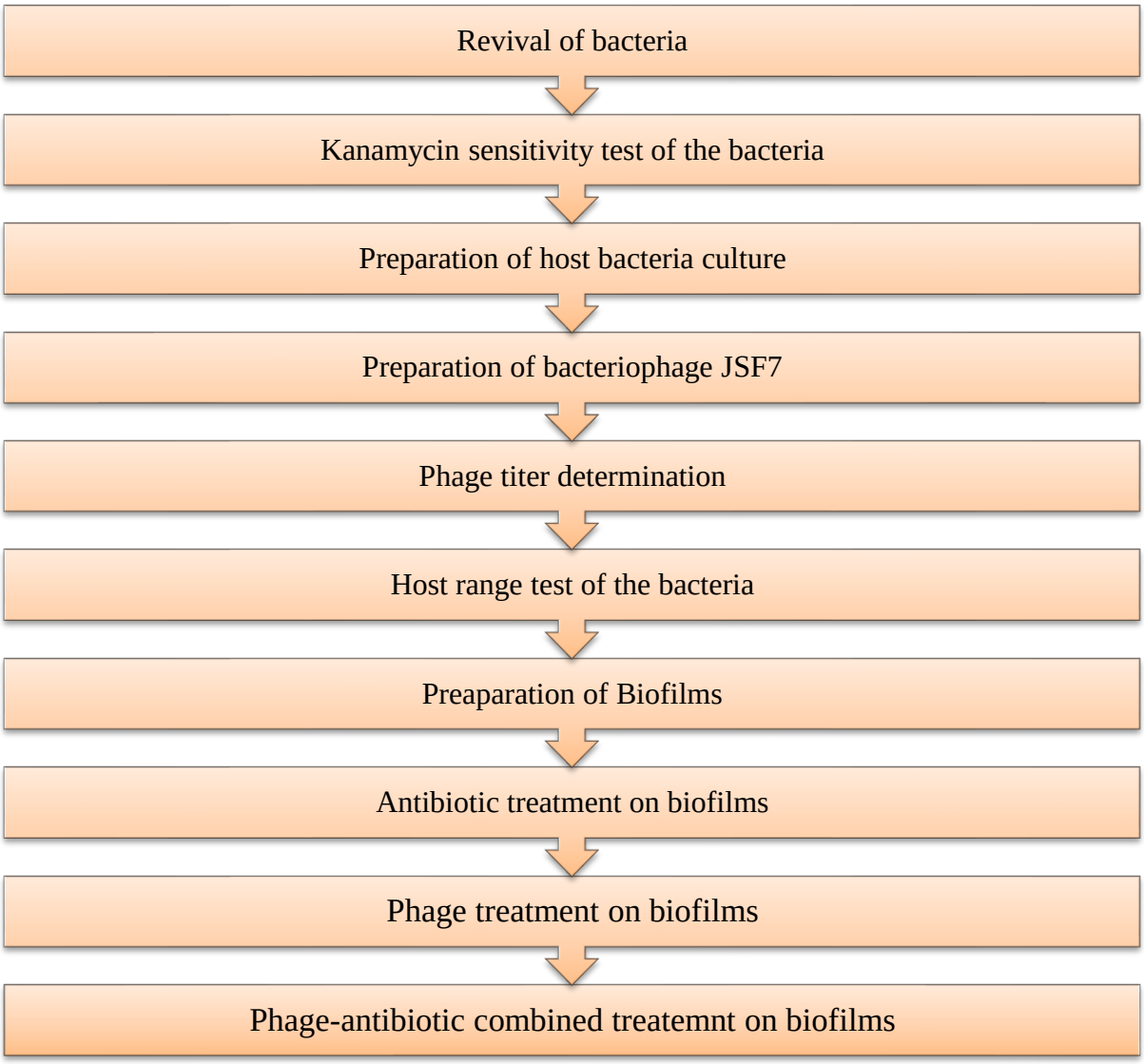


Figure 3.5 1: Overview of the methodology

3.6 Methodology

3.61 Revival of Bacteria

The *Vibrio* strains were isolated from cholerae patients previously and stored at the Biochemistry and Environmental Microbiology Laboratory of Brac university. The *Vibrio* strains were revived on LA plates from T1N1/LA stock culture and later their presence was confirmed on Thiosulfate Citrate Bile Salt Sucrose (TCBS) media. STEC was also collected from the same lab and confirmed its presence on the MacConkey agar media. Throughout the experiment all the bacterial species were grown on LA plates.

3.62 Preparation of Antibiotic Stock Solution

The antibiotic stock solution was prepared by adding 1g of Kanamycin powder to 100 ml of distilled water. This was then filter sterilized by using 0.22 µ syringe filters and stored at -20℃ to use later. In this study, 50µg/ml of kanamycin concentration from the stock solution was used for the biofilm treatment purpose.

3.63 Kanamycin Sensitivity Test

To check out if the bacteria are resistant to kanamycin, susceptibility test was done by Kirby-Bauer disc diffusion method. Briefly, bacterial inoculum was prepared by picking up a few isolated colonies from a freshly streaked bacterial plate and mixed well into saline solution at 0.5 McFarland standard. Then a sterile swab was dipped into the inoculum tube. The swab was rotated and gently pressed at the side of the tube to remove extra fluid, if there was any. After that the swab was used to streak on a dry Muller Hinton Agar (MHA) plate and made sure an even distribution. Following that, the plate was allowed at room temperature for 3-5 minutes to be dried out completely. Now, kanamycin discs (K 30µg, oxoid) were placed on the surface

of the agar plate by using a sterile forceps. The plates were then inverted and kept at 37℃ for 16-18 hours. All the experiments were done in triplicates.

In addition to disk diffusion method above, kanamycin sensitivity was also checked on LA plate supplemented with 50µg/ml kanamycin solution. For this, LA was prepared, autoclaved and then kanamycin solution was added when the liquid agar is at ~50-55℃. The antibiotic supplemented agar was then poured on plates, waited to be solidified and stored at 4℃. To check the kanamycin sensitivity of bacterial strains, a single isolated colony from a freshly streaked bacterial plate was taken and streaked on the kanamycin supplemented agar plate, and left for overnight incubation at 37℃. This experiment was done three times to check the consistency of the result.

3.64 Preparation of Host Bacteria Culture

The host bacteria culture of WT346 for phage JSF7 enrichment or titer count was prepared by inoculating a single colony in 5 ml LB broth by shaking at 37℃.

3.65 Preparation of Bacteriophage JSF7

3.651 Reviving JSF7 phage from the Stored Sample

JSf7 specific host WT346 was streaked on LA plate and incubated at 37℃ for 16-18 hrs. Then 2-3 colonies were inoculated in 3 ml freshly prepared LB and placed in a shaker incubator at 37℃ for two hours which will produce logarithmic phase cells of the host bacteria. 300µl of this host bacteria culture was added to 3.5ml of soft LA (LB medium containing 0.6% of bacteriological agar) and overlayed on a LA plate and kept for around 30 minutes to solidify. The stored JSF7 phage was syringe filtered with 0.22µ syringe filter to make it free from any bacterial contamination. After that 10-20µl of phage lysate was inoculated on this LA plate and

incubated for 18-24hr at 37°C. The presence of the bacteriophage was confirmed based on the formation of clear plaque on the LA plate after incubation.

3.652 Enrichment of JSF7

After confirming the presence of bacteriophage, the next procedure was to enrich the phage. At first the phage plaque was prepared to go for enrichment process. A single discrete phage plaque was picked up from the LA plate lawned with the host bacteria by using a micropipette tip and placed in SM buffer (100mM NaCl; 8.1mM $MgSO_4$, 0.05 mM Trish-Cl [pH 7.5]) in a sterile microcentrifuge tube. The suspended plaque was vortexed vigorously for five minutes for releasing the phages from the agar plaque. Then the suspension was centrifuged at 5000 rpm for 5 mins at 24°C. In a new microcentrifuge tube, the supernatant was collected, and chloroform was added around one third of the supernatant volume. Then this solution was mixed properly by gentle vortex and stored at 4°C for later usage. During this time the chloroform evaporated from the solution and left clear phage solution in the tube.

For the enrichment process, the host bacteria WT346 was streaked on a LA plate and left for overnight incubation at 37°C. A few colonies were inoculated in 3 ml LB broth and were put in shaker incubator for 1.5-2 hours (120-150) rpm; 37°C). When the solution is slightly turbid, it is thought to be in a logarithmic phase and 100µl of the JSF7 pure phage solution was added to this host culture. The incubation time was 4-6 hours at 37°C at 120-150 rpm. After incubation, the mixture solution was centrifuged at 14000 rpm at 4°C for 10 minutes which separates the bacterial cells as pellet. Then, the supernatant containing bacteriophages was filtered through 0.22µ syringe filter and stored in a sterile polypropylene screw-caped tube at 4°C. The enrichment process was repeated a couple of times to get high titer phage.

3.653 Phage Titer Determination by Agar Overlay Assay

The phage titer of JSF7 was determined by following the double agar overlay assay. Briefly, the enriched phage solution was diluted in LB broth as 10-fold serial dilution stared from 10^{-1} up to 10^{-8}. Soft agar was prepared beforehand using LB broth containing 0.6% bacteriological agar. Host bacteria (WT346) culture was prepared as described in **3.64**. Then 300µl of young host bacteria and 100µl of diluted phage lysate was added in a test tube containing 3ml of soft agar. After mixing gently, the solution was poured on to a LA plate and allowed to dry for a few minutes. After making sure the plates were dry, they were inverted and left for overnight incubation at 37℃. The next step was to observe the plaque formation. Single plaque formation was observed, and phage titer was counted using the plaque forming unit (pfu/ml) following the formula below:

$$Pfu/ml = \frac{Plaques\ counted \times Dilution\ factor}{Volume\ of\ lysate\ plate\ (ml)}$$

3.654 Host Specificity Test

Host range test of JSF7 was done by following spot test assay using the selected six bacteria stated in **3.1**. Bacterial cultures were prepared as described in **3.64**. Then Double layer agar essay was performed as stated in **3.653** except without any phage, just bacterial culture was added to the soft agar before pouring it to LA plates. The plates were then allowed to solidify for a few minutes and 10µl of phage lysate was spotted in each plate. When the plates were fully dry, they were left for incubation at 37℃ for 16-18 hrs.

3.66 Biofilm

3.661 Biofilm Preparation

Biofilm preparation was done by sub-culturing all six strains of bacteria overnight at 37℃ on LA plates. After that single colony of each strain was inoculated in LB broth and left for

overnight incubation at 37℃. Now, 500µl of this suspension was added to 9.5ml of fresh LB media and put in a shaker incubator for 2-3 hours at 37℃. Then, 500µl bacterial suspension was poured in different borosilicate vials. These vials were kept at room temperature for 48 hours to form biofilm. For visualizing biofilm, 0.1% crystal violet solution was prepared and used for staining the biofilm. After discarding the bacterial culture solution and washing the vials with saline water, the biofilms were submerged in crystal violet solution for 15-30 minutes. Then, the tubes were rinsed with saline water for removing the non-adherent dye. For each strain, multiple glass vials were prepared for biofilm formation.

3.662 Antibiotic Treatment on Biofilms

Kanamycin treatment was performed to a set of all six biofilms formed in the section **3.661**. After forming the biofilms for two days, the cell suspension was discarded, and the vial was washed three times with saline water. Then 1.25ml of fresh LB and 0.75ml of kanamycin (50µg/ml) solution was added to each vial and kept at room temperature for 30 hours. During the treatment process, 100 µl of suspension from each vial was taken at 8 hours, 24 hours and 30 hours interval, and serially diluted for spread plating on LA plates. All the experiments were done in triplicates. The biofilm dispersed cells or planktonic cell count (colony forming unit/ml) was performed by using the formula below:

$$Cfu/ml = \frac{Number\ of\ coloines \times Dilution\ factor}{Volume\ of\ culture\ plated\ (ml)}$$

3.663 Bacteriophage Treatment on Biofilms

Bacteriophage treatment was done by the same way as antibiotic treatment described in section **3.662**. Briefly, 1.25ml fresh LB and 0.75ml JSF7 phage lysate (2.0 x 10^8 pfu/ml) was added to the biofilm containing vials to be treated and allowed to stand for 30 hours at room

temperature. The planktonic cell count and OD reading were taken by the same way as the antibiotic treatment method mentioned in **3.662**. Each experiment was done in triplicates.

3.664 Antibiotic and Phage Combined Therapy on Biofilms

After conducting individual antibiotic and phage treatment, the combined therapy of JSF7 and kanamycin was performed. The treatment was similar like the antibiotic and phage treatment above. Here, 0.5ml of LB was taken in a biofilm containing vial followed by adding 0.75ml kanamycin and 0.75ml JSF7. The vials were then kept at room temperature for 30 hours. Similar like section **3.662**, planktonic cell count and OD count were taken at specific time intervals.

3.7 Dissolving the Treated Biofilms in Glacial Acetic Acid and Taking the Optical Density Reading

After treating the biofilms with kanamycin, JSF7 and combined treatment of phage and kanamycin, all the borosilicate glass vials were washed with saline water. The vials were then dried by inverting them on tissue papers. After that all the vials were stained with 0.1% crystal violet and washed with saline water after 15-20 minutes. Then 33% glacial acetic acid was used to dissolve the stained biofilms for 15 minutes. Optical density (OD) was checked by taking 200µl of the suspension of each vial in a microtiter plate by using a Multiskan ELISA machine at 620nm. All the readings were taken three times.

Chapter 4

Results

4.1 Kanamycin Sensitivity Test

Both in disc diffusion method and on LA plate supplemented with kanamycin, *Vibrio cholerae* strain 1877 showed resistance. The rest five bacteria were susceptible to kanamycin.

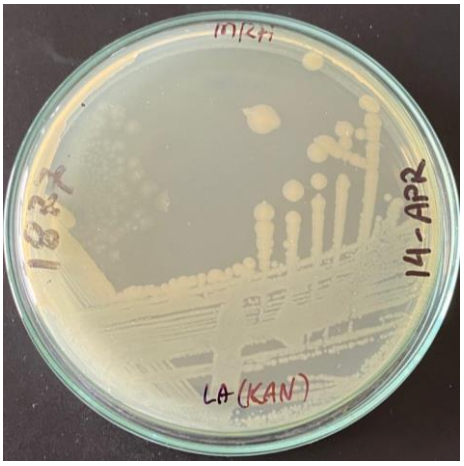


Figure 4. 1: Kanamycin Resistant strain 1877

4.2 Reviving JSF7 Phage from the Stored Sample

JSF7 showed clear plaques on its host WT346 while performed double layer agar assay from the stored sample. This means the phage is still in viable condition and can be used for further experiments.

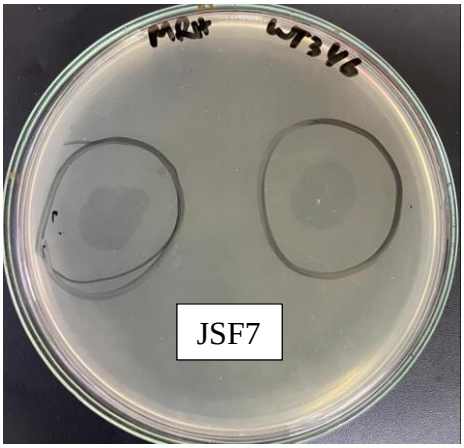


Figure 4. 2: The presence of the phage JSF7 from the stored sample

4.3 Phage Titer Determination after Enrichments

The bacteriophage was enriched several times to get high number of phage plaques. The phage showed increased titer with more enrichment. In the below table, the titer of enriched phage is shown.

Table 4.3 1: Phage titer after enrichments

Number of enrichments	Number of plaques	Dilution factor	Phage titer (pfu/ml)
First time	28	10^{-4}	2.8×10^{6}
Second time	26	10^{-6}	2.6×10^{8}
Third time	44	10^{-7}	4.4×10^{9}

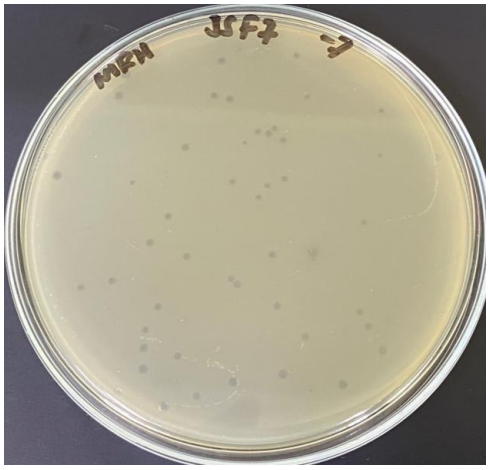


Figure 4. 3: Phage concentration at 10^{-7} on third round of enrichment

4.4 Formation of Biofilms

All the selected bacterial strains were able to form biofilms which were observed after staining the borosilicate vials with crystal violet solution. The pictures of borosilicate vials containing biofilms are provided below, which was later used as controls to compare with those treated with Kanamycin and JSF7. The blue ring in each vial indicates the formation of biofilm.

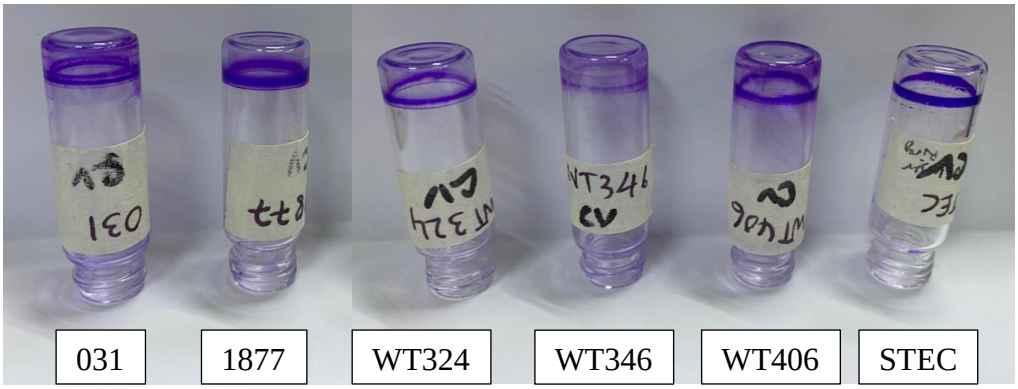


Figure 4. 4 Biofilm formation by various bacterial strains

4.5 Antibiotic Treatment on Biofilm

Biofilms of all six bacterial strains were unharmed by the kanamycin treatment. The control biofilm and the kanamycin treated biofilm looks almost same after staining them with crystal violet. No degradation of the biofilm ring is observed. The pictures below show the control vials and kanamycin treated vials after staining with crystal violet.

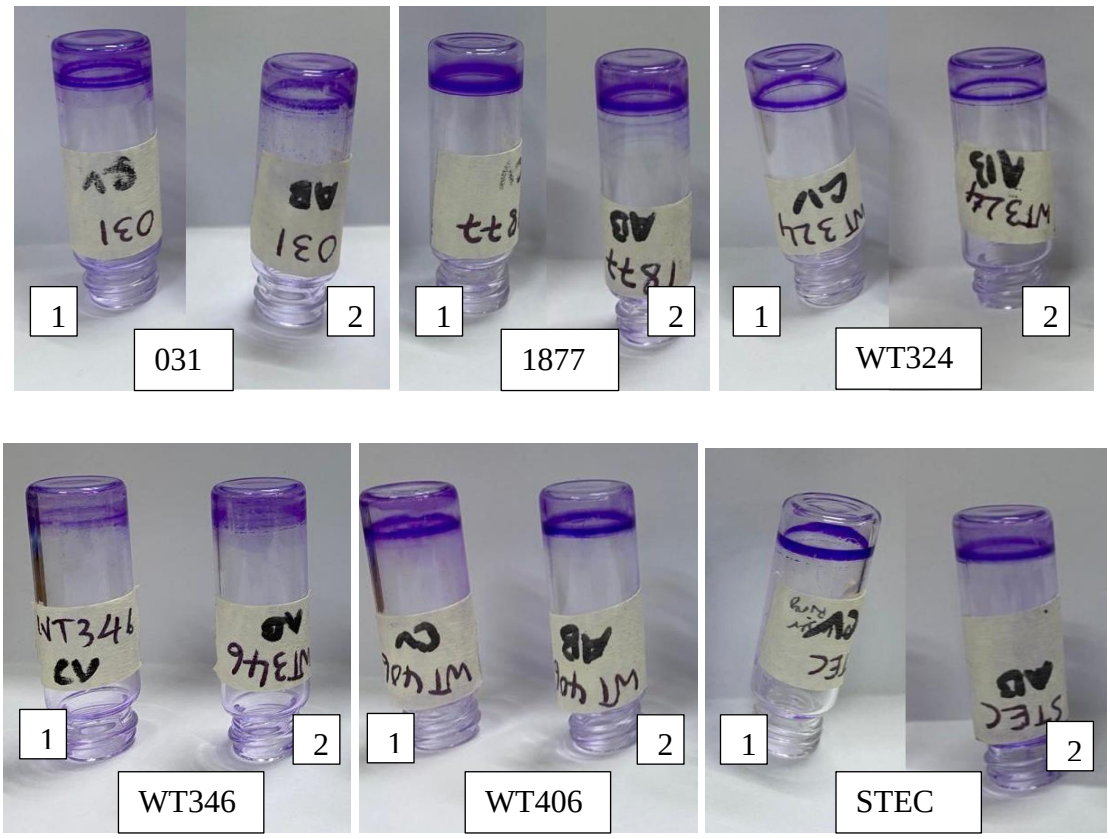


Figure 4. 5: Kanamycin treatment on bacterial biofilms

Here, number 1 and 2 indicate the control biofilm and kanamycin treated biofilm respectively.

4.6 Viable Cell Count and OD Reading During Antibiotic Treatment

The planktonic cell count during the kanamycin treatment was recorded at 8 hours, 24 hours, and 30 hours. There is no significant increase in the CFU/ml over time as kanamycin may have failed to release the planktonic cells from the biofilms. The data set is shown in **Appendix A**.

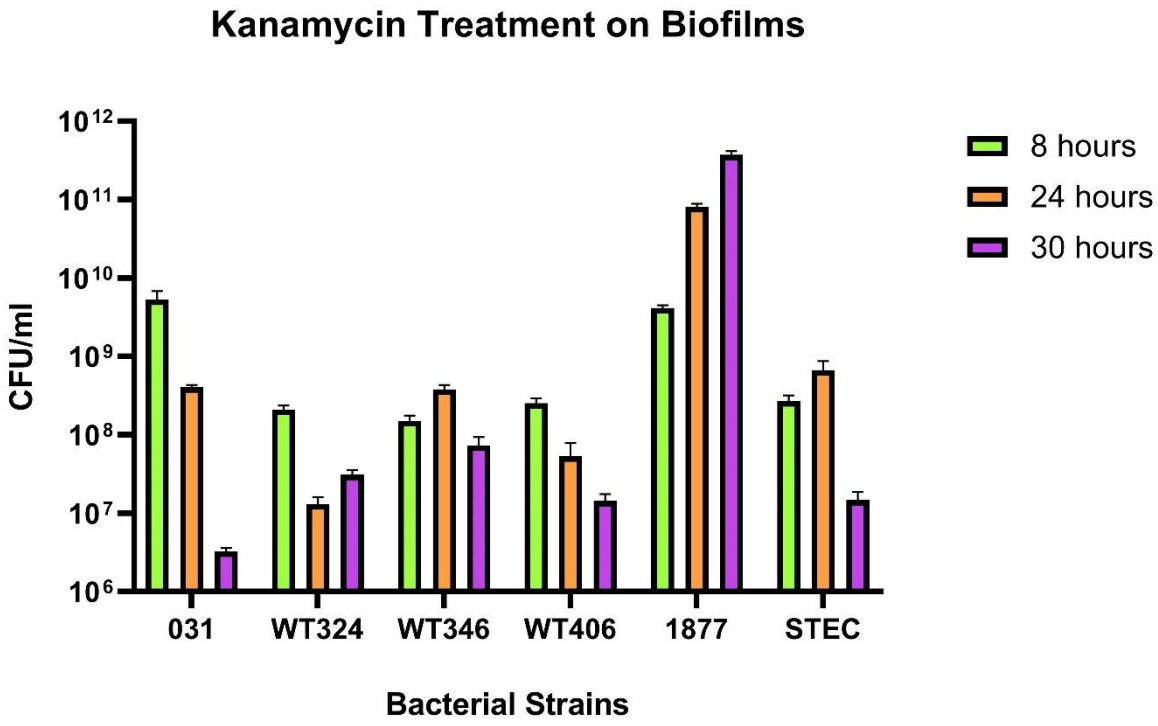


Figure 4.6 a: Viable cell count upon treating biofilms with kanamycin.

Cell count shows that kanamycin resistant strain 1877 has high cell densities with the increase of time. The rest of the strains showed downfall of the CFU/ml with time.

OD Result: Absorbance of the kanamycin treated bacteria at 620nm after dissolving them in 33% glacial acetic acid is shown in the figure below:

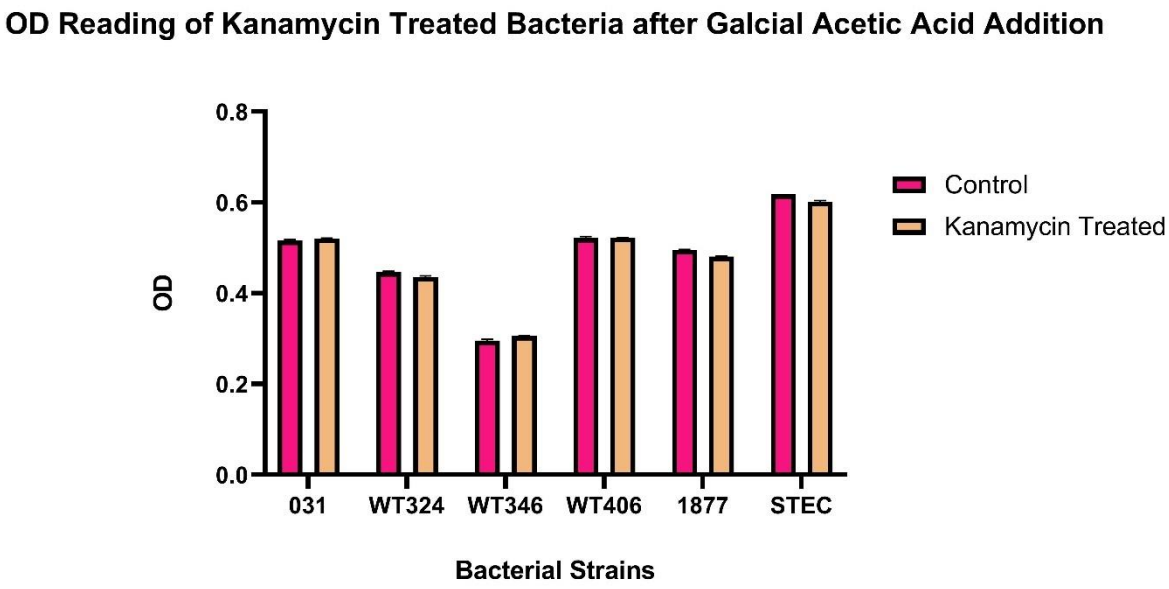
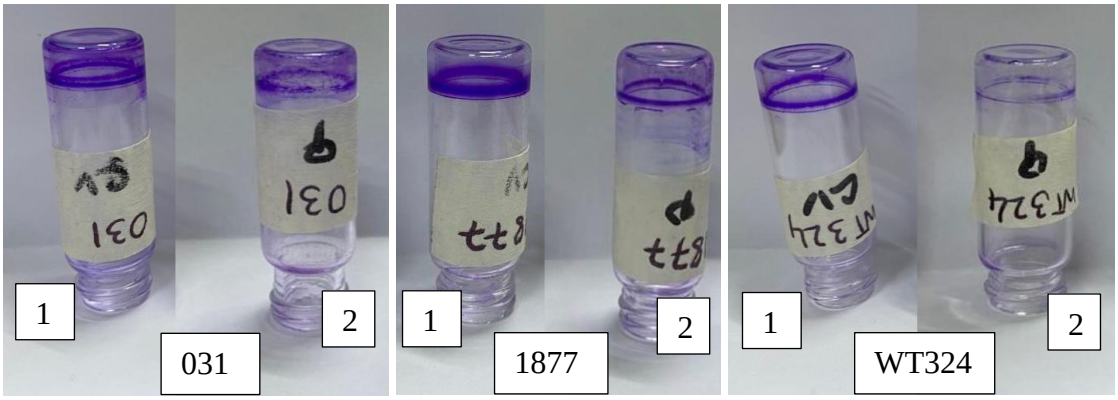


Figure 4.6 b: OD reading of kanamycin treated bacteria after dissolving in glacial acetic acid

After adding glacial acetic acid to the kanamycin treated biofilms, it is clearly shown that there is not much significant difference between the control biofilms and the treated biofilms.

4.7 Bacteriophage Treatment on Biofilm

After treating with JSF7, the biofilm containing borosilicate vials were observed for changes in the biofilm ring. Except STEC, the rest strains showed somewhat thinner rings when stained with crystal violet, compared to the control biofilms.



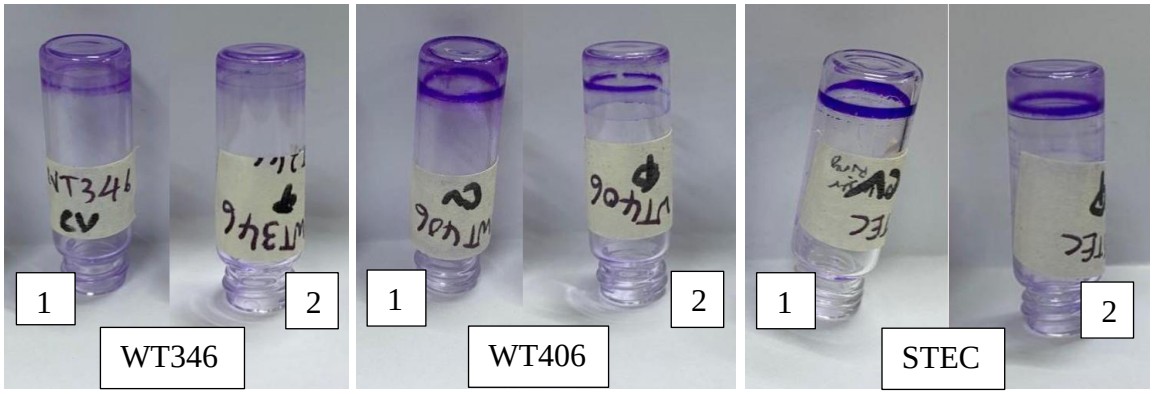


Figure 4.7 a: Treatment of JSF7 to the biofilms

Here, number 1 indicates control biofilm and number 2 means kanamycin treated biofilm. This treatment shows noticeable degradation of the *Vibrio* biofilms only as the treated biofilm rings are thinner than the control rings. No degradation was observed between JSF7 treated STEC biofilm and its control biofilm.

4.8 Planktonic Cell Count During the JSF7 Treatment

During the phage treatment of biofilms, planktonic cell counts of all the strains taken after specific time interval. All the *Vibrio* strains showed increased CFU/ml with time, but STEC did not show any significant increase at the end of the treatment compared to starting time. This data supports the idea of *Vibrio* cell dispersion over time upon JSF7 treatment, as the cell numbers were increased. The data set is shown in **Appendix B**.

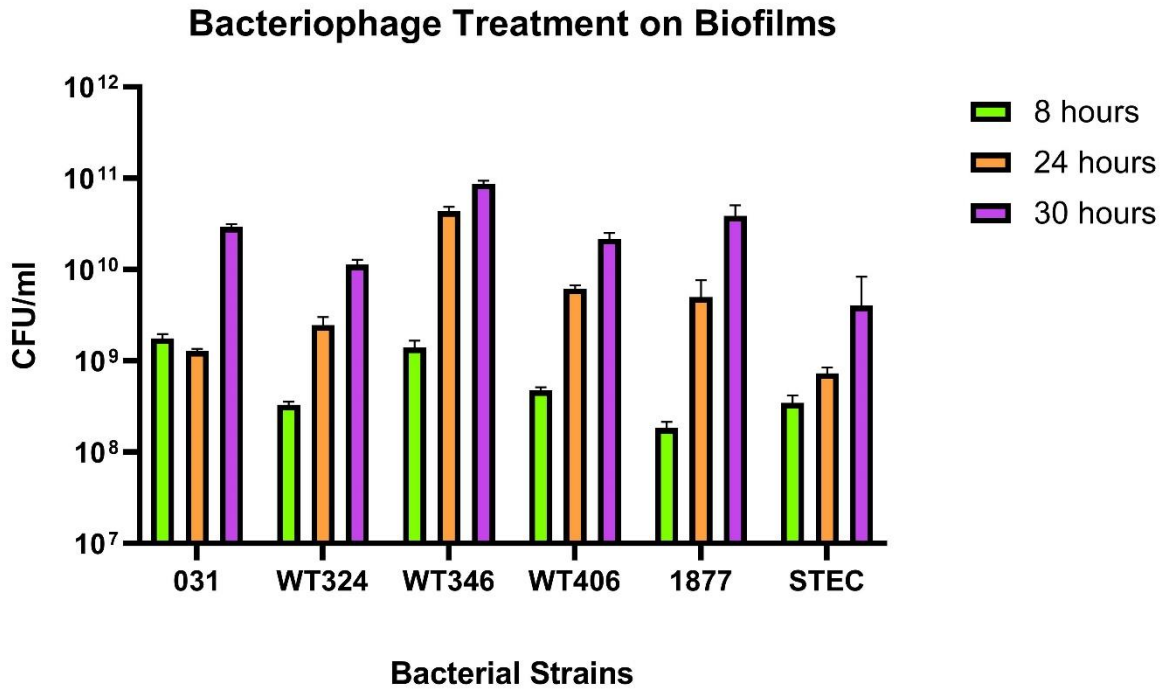


Figure 4.8 a: Viable cell count upon treating biofilms with JSF7

Here, planktonic cell count showed increased CFU/ml with time for all the strains, but STEC did not show much increasement compared to the *Vibrio cholerae* strains.

OD Result: Absorbance of the JSF7 treated bacteria at 620nm after dissolving them in 33% glacial acetic acid is shown in the figure below:

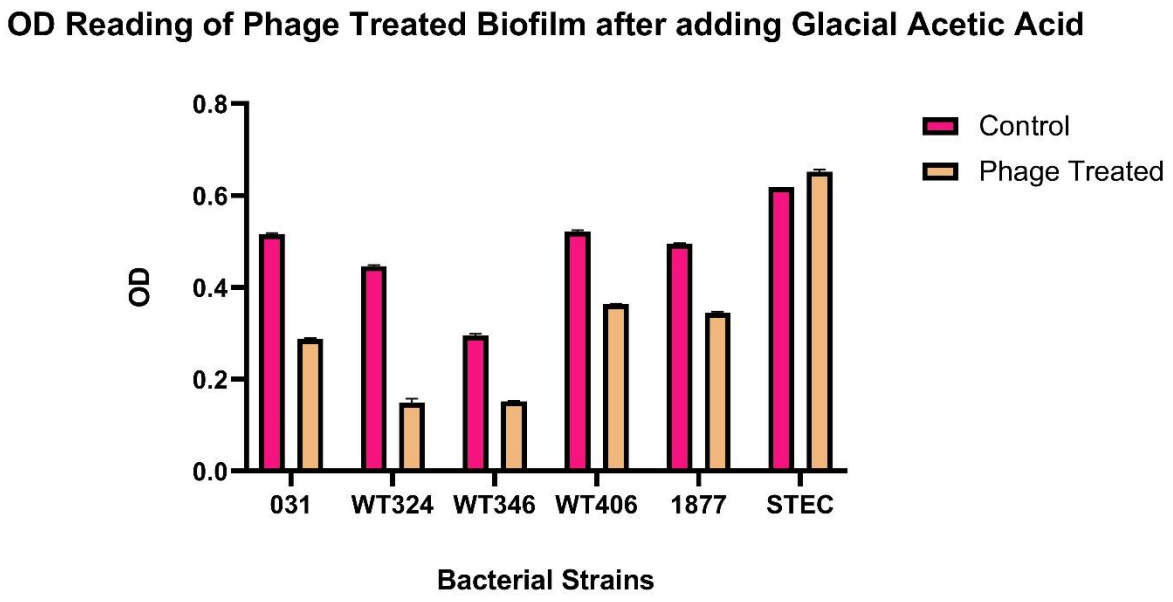
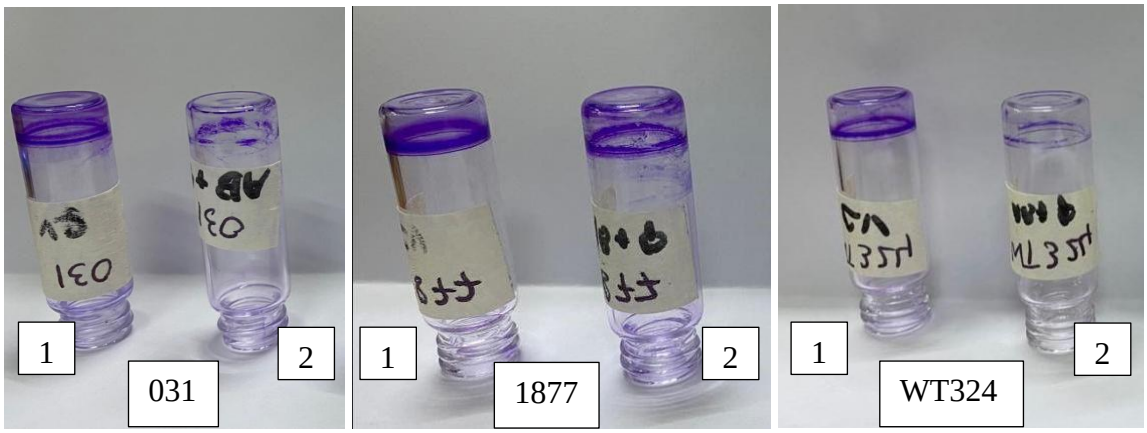


Figure 4.8 b: OD count of JSF7 treated biofilms after dissolving in glacial acetic acid

The figure shows no significant difference on the OD count of STEC. All the *Vibrio* strains has lower OD for the phage treated biofilms compared to control biofilms suggesting that the biofilms became thinner after phage treatment.

4.91 JSF7-Kanamycin Combined Therapy on Biofilms

After forming the biofilms, they were treated with combination therapy of JSF7 and kanamycin. All the *Vibrio* biofilm rings were degraded significantly after this treatment, but STEC showed no noticeable changes in the ring.



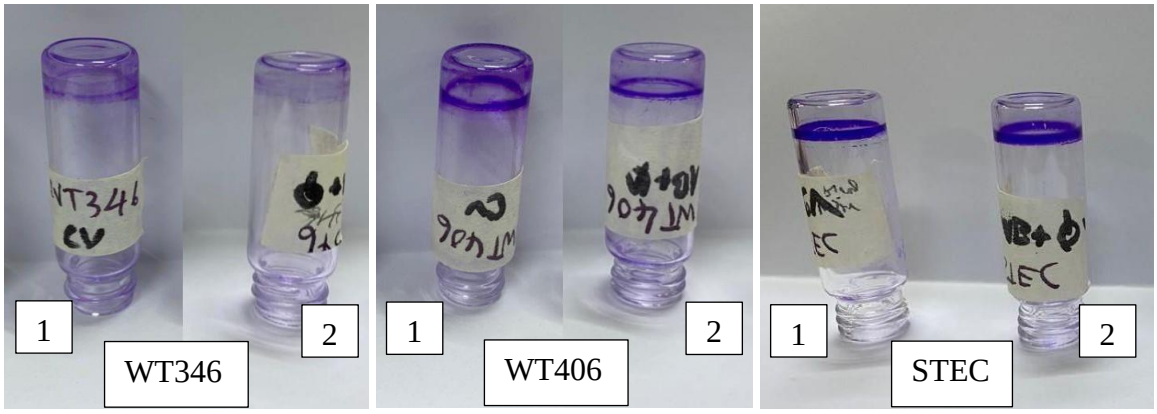


Figure 4.9 1: JSF7 and kanamycin combined therapy

On the above figure number 1 and number 2 indicate control vial and treated vial respectively. 031, WT324 and WT346 show almost complete lysis of biofilms, while 1877 and WT406 show somewhat reduced biofilms. STEC does not show any visible changes in the phage-kanamycin treated biofilm compared to the control biofilm.

4.92 Planktonic Cell Count During the Combined JSF7-Kanamycin Treatment

During the combined treatment of biofilms, planktonic cell counts were not high like the phage treatment for all the strains. This is probably because when the bacteriophage releases planktonic cells from a *Vibrio* strain (except 1877), kanamycin kills those cells. Hence, there is increased CFU/ml for 1877, as this strain of *Vibrio* is kanamycin resistant. The data set is shown in **Appendix C**.

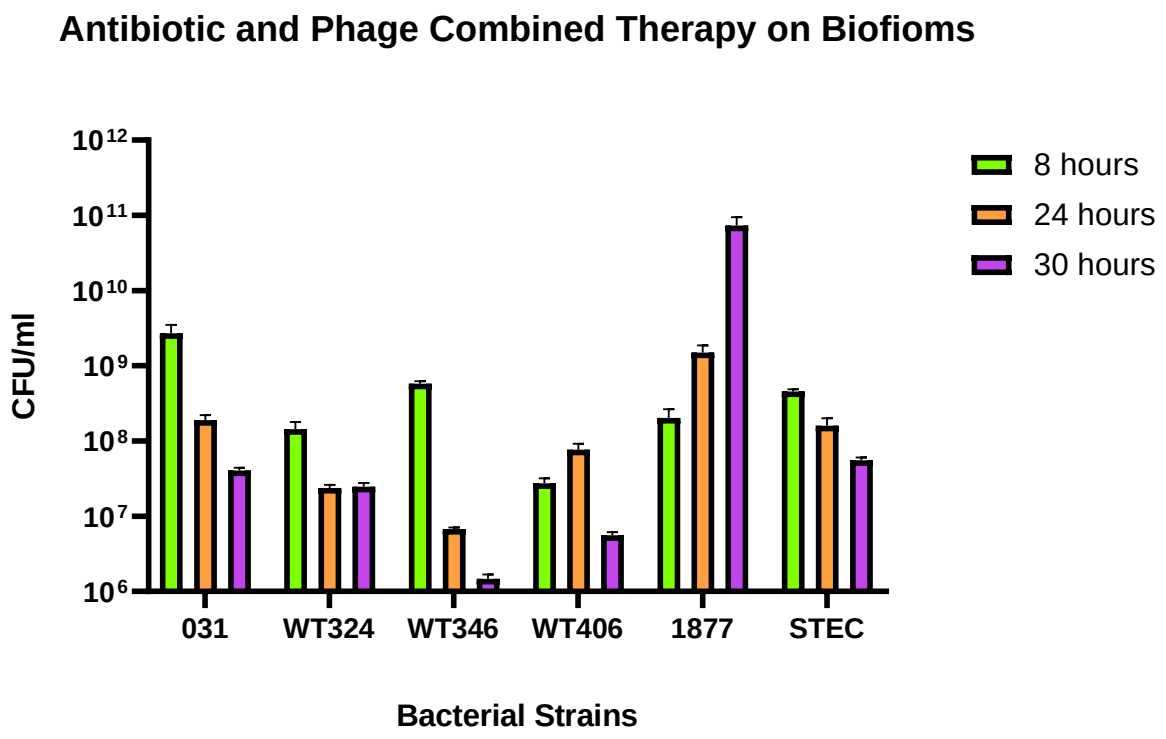


Figure 4.9 2: Viable cell count upon treating biofilms with JSF7+kanamycin.

The above figure shows significant improvements in lowering the planktonic cell count for all the strains except the kanamycin resistant bacteria 1877 after treating with phage and antibiotic combination therapy in comparison between start and end time. As kanamycin could not kill the free bacteria in 1877, the CFU/ml of planktonic cell count is higher compared to the rest of the strains.

OD Result: Absorbance of the kanamycin and JSF7 combined treated bacteria at 620nm after dissolving them in 33% glacial acetic acid is shown in the figure below:

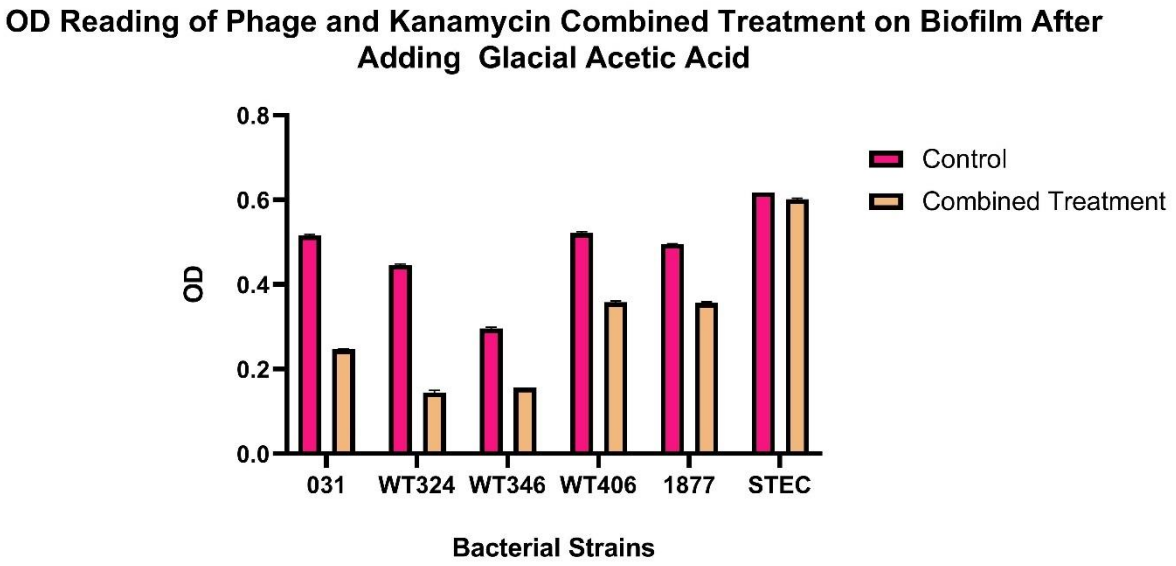


Figure 4.9 3: OD count of JSF7 and kanamycin combined treated biofilms after dissolving in glacial acetic acid

Above figure indicates that all the *Vibrio cholerae* strains has thinner biofilms after treating with both JSF7 and kanamycin. But this combination therapy had no role in degrading STEC biofilms.

Chapter 5

Discussion

Cholera epidemics is a major public health concern for many countries worldwide (Maheshwari et al., 2011) including Bangladesh because of the persistence of the causative *Vibrio* species through biofilm formation (Sultana et al., 2018). In order to overcome this challenge proper sanitation practices, use of medications i.e., antibiotics and vaccination can help (Gabutti et al., 2020). Resistance to various antibiotics is a common phenomenon for many *V. cholerae* strains especially when they form biofilms. Hence, it is a great medical challenge to the existing therapeutic strategies to mitigate the biofilm associated infections. This study sheds light on a possible way out of this problem by using bacteriophage therapy to eradicate bacterial biofilms and then using antibiotic to kill the free-living bacterial cells.

Studies suggest that the aquatic environment where *V. Cholerae* live in harbors a mixture of bacteriophages- both specific and non-specific to *Vibrio* sp. (Faruque & Mekalanos, 2012). Naser et al (2017) showed that this mixture contains phages that can kill bacteria as well as those phages that are able to disperse planktonic bacteria from the biofilms. In this study, total five *V. cholerae* strains and a STEC were used, *Vibrio* strain WT346 was the host of the bacteriophage JSF7, and rest were nonspecific to the phage. After treating all the bacterial strains with JSF7 (titer= 2.0 x 10^8), the biofilms of all the *Vibrio* strains found to be thinner in the borosilicate vials compared to the control biofilms. There was no significant difference between the control biofilm and STEC biofilm. The planktonic cell count increased greatly upon treating the *Vibrio* strains with JSF7 phage which indicates that this phage is probably releasing the planktonic cells from biofilms. In case of STEC, the free cell count was not very high as *Vibrio* which means the phage was possibly unable to release planktonic bacteria from biofilm. For further confirmation, control biofilms and phage treated biofilms were stained with 0.1% crystal violet and dissolved into 33% glacial acetic acid solution followed by saline wash.

Then, their absorbance was measured at 620nm in microtiter plates. Generally, 570nm is ideal to measure absorbance for crystal violet stained biofilms, but our lab has limited options available at present. So, the closest available value which is 620nm was chosen and subsequent absorbance tests were performed. The results showed that phage treated *Vibrio* biofilms had less optical density value in comparison to the control biofilms, suggesting that biofilms were harmed and became less thick upon phage treatment, except for STEC. Here, JSF7 were able to degrade both susceptible and resistant biofilms of the *Vibrio cholerae* strains. Therefore, it is possible to degrade *Vibrio cholerae* biofilms with phage treatment and treating for longer hours might even eradicate the whole biofilm. But there are still concerns regarding planktonic bacteria released from biofilms, as they can pose great threat to public health (Naser et al., 2017). Here, antibiotics can come forward to combat this situation by killing the free bacteria dispersed from biofilms.

The antibiotic kanamycin was used in this study to kill the free-living bacteria released from biofilms by JSF7. The concentration of kanamycin was used as 50µg/ml as MIC as many previous studies used around this value for *Vibrio cholerae*. When kanamycin was used to treat biofilms, there were no significant difference between the control and treated biofilms. This means antibiotic alone cannot disperse planktonic cells from the biofilms. As opposed to the JSF7 treatment, the planktonic cell count decreased over time when treating with kanamycin. The optical density result after dissolving the crystal violet stained biofilms with glacial acetic acid showed no noteworthy difference between antibiotic treated and untreated biofilms. This evidence suggests that the kanamycin is unable to penetrate the biofilms hence cannot release and kill the planktonic cells. So, antibiotic alone might not be a suitable option for treating *Vibrio cholerae* biofilms.

Combining two or more conventional antimicrobials could lead the way in more effective biofilm degradation. Adding phage and antibiotic together may show better therapeutic results

in combating planktonic bacteria as well as biofilms (Knezevic et al., 2013; Nouraldin et al., 2016). The next approach involved combined therapy of phage and antibiotics to treat *Vibrio cholerae* biofilms and showed promising results. JSF7 and kanamycin combined treatment lessen the biofilm ring remarkably inside the borosilicate vials. The OD reading suggests the same conclusion as the absorbance were much lower compared to the control strains of *Vibrio Cholerae*. But similar like phage treatment or kanamycin treatment individually, combined treatment did not play any role in degrading STEC biofilm as well. While taking planktonic cell counts for the *Vibrio* strains, the CFU/ml decreased over time suggesting that the free-living cells released by JSF7 from biofilms are killed or inhibited by kanamycin. The only kanamycin resistant strain used in this study (1877) showed higher CFU/ml with time indicates that JSF7 probably released the planktonic cells by degrading biofilm, but kanamycin could not kill them. So, phage and antibiotic combined treatment could be effective if the *Vibrio* cells are not resistant to antibiotics. However, further studies are required to understand the concentration of each phage and antibiotic as well as the order effects of them to get the optimum outcome in overcoming the biofilm related health issues.

Both combined treatment and individual JSF7 treatment could not harm the STEC biofilm suggesting that this JSF7 phage might be specific for *Vibrio cholerae* biofilms only, in this case. Although further investigation could be done with other non-*Vibrio* spp. like *Shigella*, *Salmonella*, different spp. of *E. coli* etc.

As resistance to antibiotic is a serious concern nowadays, finding alternatives have become very crucial. So, for some cases, after releasing biofilms with phage treatment, multiple antibiotics could be used to kill the planktonic bacteria. Another way is to breakdown the biofilm with suitable phages, and then using *Vibrio cholerae* susceptible phages to kill the free cells as shown by Naser et al., 2017. To get the most out of combination therapy, the dose of the phage and antibiotic is very important. Another major factor could be the order effect of

phage and antibiotic (Chaudhry et al., 2017). Using bioengineered phages as adjuvants for antibiotics could be something promising while performing the phage-antibiotic combination therapy (Gordillo Altamirano & Barr, 2019; T. K. Lu & Collins, 2009).

Chapter 6

Conclusions and Future Directions

Vibrio cholerae biofilms can be treated with specific bacteriophages while in combination with a broad-spectrum antibiotic might have a greater effect as this study findings suggest. Antibiotics alone have limited effectiveness in breaking biofilms and pose several complications, for example, dissemination of antibiotic resistant genes, mutation of the planktonic cells to become persister cells and many more. So, this experiment was to show Vibriophage JSF7 as an alternative to antibiotics to degrade *V. cholerae* biofilms successfully by dispersing planktonic cells. In addition, there is possible scope for antibiotics in combating planktonic cells dispersed from biofilms after phage therapy. Other than *Vibrio* strains, STEC biofilm was tested for JSF7 in this study which did not show any positive data in degrading biofilms, but there is scope for more experiments with other non-*Vibrio* spp. As this experiment shows significant findings which indicate a possible effective method to treat *V. cholerae* biofilms, further study including in-vivo experiments can be done to understand the process better and implement it for the great benefits of mankind.

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Appendices

Appendix A: Kanamycin treatment on biofilms. Average of three independent studies.

Strains	8 hours	24 hours	30 hours
031	5.3E+09	4.1E+08	3.27E+06
WT324	2.1E+08	1.3E+07	3.10E+07
WT346	1.5E+08	3.8E+08	7.33E+07
WT406	2.5E+08	5.3E+07	1.43E+07
1877	4.1E+09	8.1E+10	3.73E+11
STEC	2.7E+08	6.7E+08	1.47E+07

Appendix B: JSF7 treatment on biofilms. Average of three independent studies.

Strains	8 hours	24 hours	30 hours
031	1.77E+09	1.29E+09	2.93E+10
WT324	3.3E+08	2.47E+09	1.13E+10
WT346	1.4E+09	4.37E+10	8.73E+10
WT406	4.77E+08	6.17E+09	2.17E+10
1877	1.83E+08	5E+09	3.87E+10
STEC	3.47E+08	7.33E+08	4.03E+09

Appendix C: Kanamycin and JSF7 combined treatment on biofilms. Average of three independent studies.

Strains	8 hours	24 hours	30 hours
031	2.7E+09	1.9E+08	4.1E+07
WT324	1.4E+08	2.4E+07	2.5E+07

WT346	5.8E+08	1.47E+06	5.57E+06
WT406	2.7E+07	7.7E+07	5.56E+06
1877	2E+08	1.5E+09	7.3E+10
STEC	4.6E+08	1.6E+08	5.6E+07