THE POTENTIALITY OF BACTERIOPHAGE ON BIOFILM TREATMENT

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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
of Bachelor of Science in Biotechnology

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It is hereby declared that

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Abstract

Bacteria can exist both in a planktonic state and within a biofilm. The bacteria remain inert within biofilm. Due to their extracellular matrix, problem arises for the effectivity of antibiotics and biocides. However, bacteriophages are found effective on targeting this common form of bacterial growth and they have enzymes to degrade the extracellular matrix. This review discusses about basic knowledge about bacteriophages and the effectivity of bacteriophages against bacterial biofilm formed by *Salmonella spp*, *Listeria monocytogenes*, *Vibrio cholera*, *Enterococcus faecalis, Streptococcus agalactiae, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.*

Bacteriophage can eradicate Multi-Drug Resistant (MDR) *Pseudomonas aeruginosa, Enterococcus faecalis biofilm,* and *eradicate S. agalactiae* through the destruction of the extracellular matrix thereby allowing antibiotics to enter into the inner layer of the biofilm and using the quorum-sensing activity restricts the formation of biofilms. In addition to that, the combined effect of bacteriophage with other compounds having anti-biofilm capability such as nanoparticles, enzymes and natural products can more effectively invade the biofilms than each of them applied alone.

It was found through various methodology that the phage KTN4 immediately degraded the biofilms of *Pseudomonas aeruginosa* upon its application. Furthermore, strong antibacterial capability has been shown by the KTN4 phages and the significant inhibition of toxigenic agent pyocyanin and pyoverdin produced by the *P. aeruginosa* proves the ability of these phages to be used in *Pseudomonas aeruginosa* biofilm treatment.

The phage KP34 alone and in combination with ciprofloxacin have showed significant decrease in the *K. pneumoniae* biofilm biomass and also the bacteria itself.

Test results of the bacteriophage Sb-1 combined with several other antibiotic against 10 antibiotic resistant strains of *Staphylococcus aureus* was obtained. It was found that the best choice for creating a therapy of antibiotic with phage Sb-1 as an adjunctive agent would be the combination of Sb-1 with daptomycin either applied simultaneously or staggered manner.

On the other hand, the use of bacteriophages for biofilm destruction has some limitations such as limited host range, high-density biofilm, sub-populate phage resistance in biofilm, and inhibition of phage infection via quorum sensing in biofilm.

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Chapter 1

Introduction

Bacteriophages are a gathering of infections broadly appropriated in nature whose life cycle is carefully connected with the bacterial cell. They are known as bacterial parasites since they do not have the cell structure and compound frameworks fundamental for food take-up, protein synthesis or development of new particles, and as deficient creatures can just replicate in a live cell.
Bacteriophages were discoverd by Twort (1915) as unidentified particles that inhibits bacterial development, however in 1917 D'Herelle was the first to isolate and charecterize phages, and he additionally built up the principal phage treatment against fowl typhoid actuated by *Salmonella Gallinarum* in chickens. Positive aftereffects of the utilization of bacteriophages in battling bacterial contaminations have added to the improvement of research on the potential utilization of infections that demolish microorganisms in treatment of sicknesses in both human and creatures.

1.1 Life Cycle Of Bacteriophage

Two sorts of activity are distinguished against the bacterial cell: lytic activity, which is normal for destructive phages, and lysogenic action, including joining of the genetic material of the bacteriophage with the bacterial chromosome and replication as a feature of the bacterial DNA, bringing about the presence of a prophage.

The lytic cycle of bacteriophages comprises of adsorption, which includes adhesion to the bacterial cell, and binding of phage proteins to pre-recognized receptors on the bacterial cell surface, for example, teichoic and lipoteichoic acid for Gram-positive or LPS for Gram-negative microbes. The penetration includes crack of the cell wall by the bacteriophage proteins and infiltration of the hereditary material into the host cell. Next is the eclipse stage, including replication of nucleic acid and proteins comprising the structural portion of the capsid, while replication of the bacterial DNA is restrained. This is trailed by the formation and development of the bacteriophage, lysis of the bacterial cell and the arrival of daughter phages equipped for contaminating different cells. Examples of bacteriophages going through the lytic cycle are phages T1 and T4. The lysogenic cycle includes direct coordination of genetic material with the bacterial chromosome, incorporation with the host genome and arrangement of the prophage. The replication of the bacteriophage is blocked and its genome enters a condition of inertness. This state can be interfered with immediately or because of the presence of daylight, UV radiation, alkylating factors, or certain anti-microbial, for example, mitomycin C. Instances of bacteriophages with a lysogenic cycle

incorporate λ *Escherichia coli*; Mu, with movement against *E. coli*, *Salmonella*, *Citrobacter* and *Erwinia*; MM1 *S. pneumoniae*; and φ11 *S. aureus.*

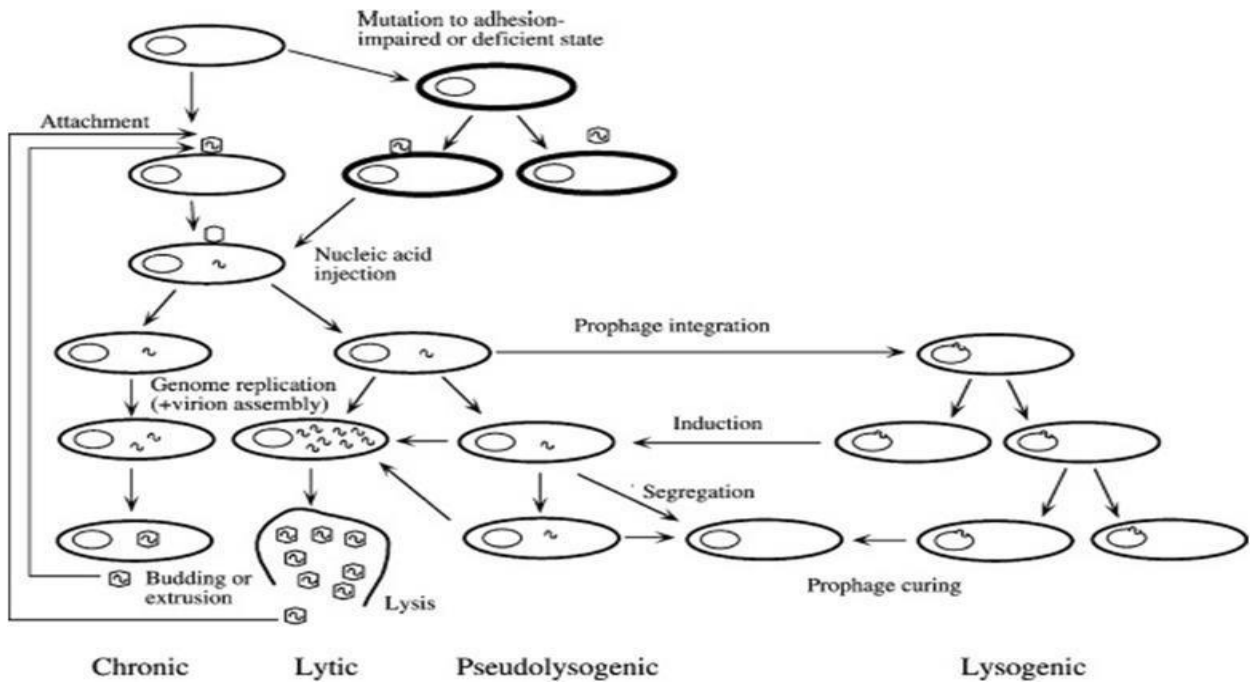


Figure 1.1 11Life Cycle Of Bacteriophage

1.2 History Of Phage Therapy

Despite the fact that utilizing bacterial viruses therapeutically against bacterial infections has came forward on the development of multidrug-safe microbes, the practice has been around for almost a century. Since the underlying perceptions of phage-induced bacterial lysis, the organic idea of phage and their therapeutical value, has been controversial. Frederick Twort first depicted the characteristic zone of lysis related with phage infection in 1915, yet it was Felix d'Herelle who distinguished the source of this phenomena, ascribed the plaques to bacterial infections, and coined the expression "bacteriophage" (in a real sense "bacteria eater"). It was additionally d'Herelle who considered the plan to utilize phages therapeutically and is responsible for the principal archived clinical utilization of phage in 1919 at the Hôpital des Enfants-Malades in Paris where phages were

effectively used to treat 4 pediatric cases of bacterial looseness of the bowels. Regardless of a few effective preliminaries, d'Herelle's initial analyses were famous for being ineffectively controlled and his exploration was vivaciously questioned by established researchers. All things considered, d'Herelle kept on continuing phage treatment with the treatment of dysentery, cholera, and the bubonic plague in the mid twentieth century with a progression of phage treatment focuses and commercial phage production plants all through Europe and India. One 1931 trail of phage treatment as a treatment for cholera in the Punjab locale of India included an accomplice of 118 control subjects and 73 trial subjects who got phage treatment; d'Herelle noticed a 90% decrease in mortality with 74 deadly results in the control group and just 5 in the experimental group.

Alongside d'Herelle, a few different business people attempted to popularize phage production in Brazil and the United States with phage arrangements for Staphylococcus, Streptococcus, E. coli, and other bacterial microbes. These arrangements were transported all through the world to willing clinicians yet treatment was met with blended achievement; this absence of reliability, in huge part, added to the inclination for anti-microbial in western medication.

Numerous mistakes were made during these early preliminaries of phage treatment and most can be credited to a poor comprehension of the biological idea of phages. Simple refinement and capacity conventions brought about low titers of active phage and contamination from bacterial antigens, and phages that needed infectivity for the target bacteria were utilized for treatment. Moreover, conveyance of phage to the site of disease was cofounded by the clinical constraints of the day. For instance, the function of the patient's

natural immune response in eliminating active phage and lessening the viability of phage treatment was just noticed as of late as a possibly jumbling physiological instrument. Thus, phage treatment was generally excused by the greater part of western medication after the presentation of drug anti-infection agents in the 1940's. The exemption for this is in the previous Soviet Union and Eastern Europe where clinical phage treatment has been utilized widely to treat anti-microbial safe contaminations brought about by a scope of irresistible microorganisms, for example, *Staphylococcus*, *Pseudomonas*, *Klebsiella*, and *E. coli.*

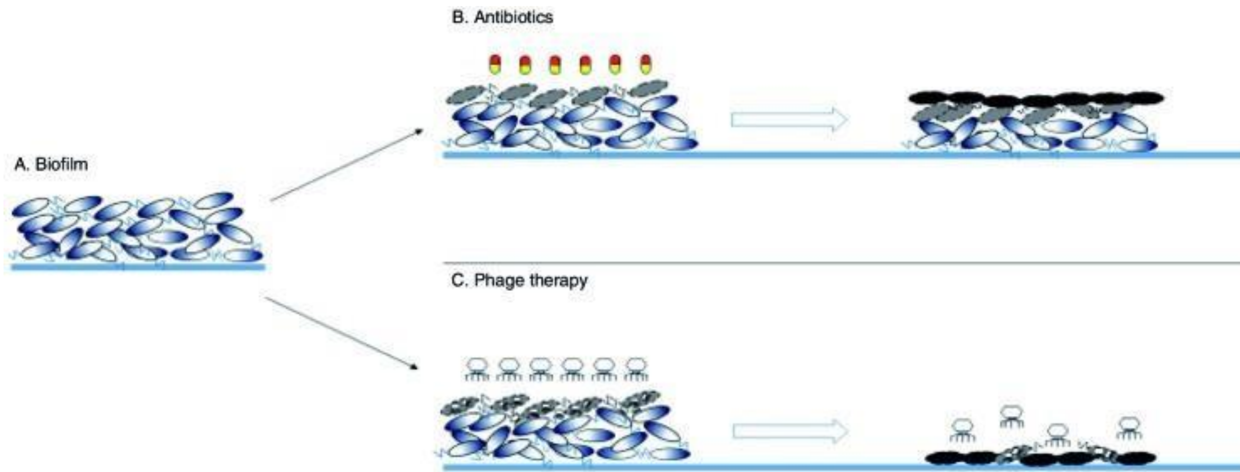
1.3 Phage Therapy Benifits

Phage therapy offers various benefits over antibiotics. They are: -

1. **High specificity**: The phages target specific pathogenic bacterium and cause no damage to the commensal microbiome of the body.
2. **Ease of isolation**: Phages are bacterium-dependent and hence are found where their target bacteria are present.
3. **Possibility for clinical improvement**: With the development of biological science and genetic engineering, the chance of transforming phages accumulated within the past decades.
4. '**Single shot**': Phage multiplication happens in correlation with the expansion of the microorganism. Hence theoretically, only one shot is required
5. **No residual**: Once the infective bacteria are fully eradicated from the host cell due to phage-induced lysis, as a result of the phages will now not grow while not the microorganism host, it's expected that they're going to be shed from the cell with none damage to the cell
6. **Biofilms destruction**: Biofilm destruction by phages is far a lot of efficiency when compared with antibiotics. The phages can infect the bacterium present within the higher layer of the biofilm and replicate to create new phages, which might penetrate the inner

layers and infect the remaining bacteria. the continual replication of the phages and subsequent infection and killing of biofilm bacteria cause their eradication.

Figure 1.2 1 ***Graphical Demonstration Between Antibiotics And Phage Therapy***



Chapter 2

Use Of Bacteriophage Against Salmonella Present In Chicken Slaughterhouse Surface

2.1 Inception Of The Topic

Foodborne illnesses refers a significant danger to public health around the world. Pathogenic bacteria can live as biofilm inside the food industry, giving a lasting source of contamination. Most of the examples utilized were delegated as weak biofilm makers, with serovars Enteritidis and Heidelberg demonstrating the most noteworthy recurrence of biofilm development. Glass and tempered steel surfaces fundamentally preferred biofilm development at 60 and 36 h of incubation individually, however the polyvinyl chloride surface didn't support biofilm creation, proposing that the sort of material may interfere with production. The bacteriophage pool action period focused on 3 h, however treatment of 9 h on glass surface biofilms was better than different medicines since it influenced the biggest number of tests. These outcomes proposes that some surface types and Salmonella serotypes may advance biofilm arrangement and demonstrate bacteriophages as a choice to control biofilms. However, further examinations are needed to demonstrate the adequacy and safety of bacteriophage treatment as an option in the antimicrobial control in the processing plants.

The salmonellosis is one of the most challenging Food Borne Illness and comprises a significant public health issue in many nations. One reason for food contamination is the adhesion of bacteria to processing surfaces, where the presence of organic residues in the processing environment and refrigerating equipment can give conditions to the development and persistence of coordinated communities called biofilms, which may contain pathogenic bacterial organisms and go about as a source of cross-contamination.

2.2 Some Common Strategies And Their Drawbacks

The majority of the pathogenic bacteria related with FBD can cling to and structure biofilms on an variety of materials, for example, plastic, metal, glass, wood, and staples and under practically all conditions in food handling plants. An illustration of this relationship was spoken to by a populace of microbes in chicken remains ready to shape biofilms on tempered steel surfaces with various completions.

Common strategies among the several tools used for combating microbial adhesion and biofilm formation are the regular application of cleaning and disinfection procedures in industrial equipment and in the products themselves; but such procedures are not fully effective against biofilms and can induce the selection of resistant phenotypes. Once formed, biofilms are extremely difficult to remove, as is the case of ***S. Typhimurium*** biofilms formed on different surfaces that exhibited resistance to the action of sodium hypochlorite.

2.3 Experiments With Bacteriophages And Their Results

Bacteriophages, obligate parasites of bacteria have promising highlights for controlling FBD- related microbes, and are viewed as viable in repressing biofilm arrangement and dispersion of develop biofilms. They present a more accurate methodology contrasted with conventional techniques utilized in safety and food preservation. In this research, the investigation is aimed to assess the activity of a mix of bacteriophages for controlling Salmonella spp. biofilm on 3 unique surfaces: tempered steel, glass, and polyvinyl chloride (PVC), all basic in the food business, and furthermore to describe the arrangement of the biofilm on the surfaces previously mentioned.

The present examination assessed whether bacteria of the sort Salmonella spp. may specially form to specific surfaces commonly found in the food industry – in particular hardened steel, glass, and PVC, by noticing the development of the arrangement of these biofilms at 8 explicit occasions – and particularly whether the mix of bacteriophages of demonstrated lytic activity can wipe out or control the biofilm arrangement by these microbes previously mentioned.

Strains of *S. Enteritidis, S. Typhimurium, S. Heidelberg, S. Mbandaka, S. Senftenberg*, and *S. Kentucky* were analyzed, adding up to 123 examples. It was seen that 47% of the examples had the option to form biofilms on a polystyrene microplate, and that the serotypes Enteritidis and Heidelberg introduced a fundamentally higher number of samples forming biofilm. Among the 58 examples chose for biofilm arrangement on surfaces, 57 of them were weakly formed, while just one example of the serovar Heidelberg got the moderate order for its biofilm forming capacity. In any case, different investigations have demonstrated Salmonella spp. tests with weak, moderate, and strong appraisals for biofilm formation. The results acquired in Experiments demonstrated glass and stainless steel materials fundamentally preferring biofilm development by Salmonella spp. Although both have supported the creation of the biofilm, glass introduced the most noteworthy creation recurrence at 24, 48, 60, 72, and 84 h of incubation, while the

stainless steel demonstrated positive biofilm arrangement at 36, 48, and 84 h. Although numerous examinations propose the creation of biofilm by some serotypes of Salmonella spp. on glass surfaces, hardened steel, and PVC, our outcomes indicated no critical biofilm development on the PVC surface examples. These outcomes don't permit us to represent why there was no biofilm development in this specific region, however numerous examinations show that such factors as the finish, roughness, hydrophobic interactions, physical and chemical stability and so on, altogether interfere with the adhesion of cells and subsequently with biofilm arrangement. From the information acquired in experiments, a selection of tests ready to imitate the equivalent biofilm-development results concerning the surfaces and incubating time for the completion of the subsequent analysis was chosen; however just 34% of these examples demonstrated similar outcomes in three replicates, which recommends an irregularity in the biofilm creation design in ***Salmonella spp.*** utilized in this investigation.

2.4 Discussion

The use of a bacteriophage pool repressed biofilm development of Salmonella spp. on the 3 surfaces utilized in this investigation, thinking its time of activity at 3 hours. Biofilms adhering to the glass were resistant to 6 hours of treatment with the bacteriophage pool, yet at 9 h of activity, bacteriophages had begun to diminish the biofilm. The reduction of bacteriophage activity on Salmonella tests was additionally seen by Andreatti Filho et al, who assessed the capacity of bacteriophages isolated from poultry and a sewage treatment plant to diminish S. Enteritidis in vitro. The authors saw that the bacteriophages diminished recovery of Salmonella tests in 1.5 to 5 logs. Comparative outcomes were introduced by Hosseinidoust et al, who announced that the blend of two known bacteriophages (PRD1 and P22) had the option to control biofilm arrangement of S. Typhimurium, with the resistance diminishing the recurrence in 1/100, while the utilization of bacteriophages PRD1 and P22 independently had the option to diminish the biofilm levels just for 12 h and 24 h, separately. Moreover, there was a period in which the combination of bacteriophages is not, at this point powerful in controlling the biofilm, whose activity was reestablished at 72 h of treatment. Among the theories recommended by the authors to clarify the nonappearance of activity right now, is the presence of a momentary resistance where the bacteria have gotten resistant to support the selection of bacteriophages ready to adjust to this change, producing a specific bit of advantage and expanded range of bacteriophage activity, and thus brings about the profit of activity for the biofilm. In any case, another

clarification of a similar occasion would be the delivery and aggregation of a bacterial extracellular DNA, delivered at the hour of lysis interceded by bacteriophages, substances that would be liable for the expansion in biofilm levels in the period. It should be likewise viewed as that common biofilms once in a while are of a solitary life form, making the treatment with a solitary bacteriophage or a pool of bacteriophages diverse in a multi-organism matrix, which typically happens in a handling plant. The current examination shows the significance of a particular methodology according to the contact surface with the food and the microorganism associated with the pollution, as both are fit for interfering in the permanence of bacteria in industrial environment through the biofilm formation. Consequently, bacteriophage treatment presents an elective technique for a more explicit and hence safer methodology, however its utilization actually requires further investigation to explain its association with the microorganism and the environment so it turns into a commercially viable tools.

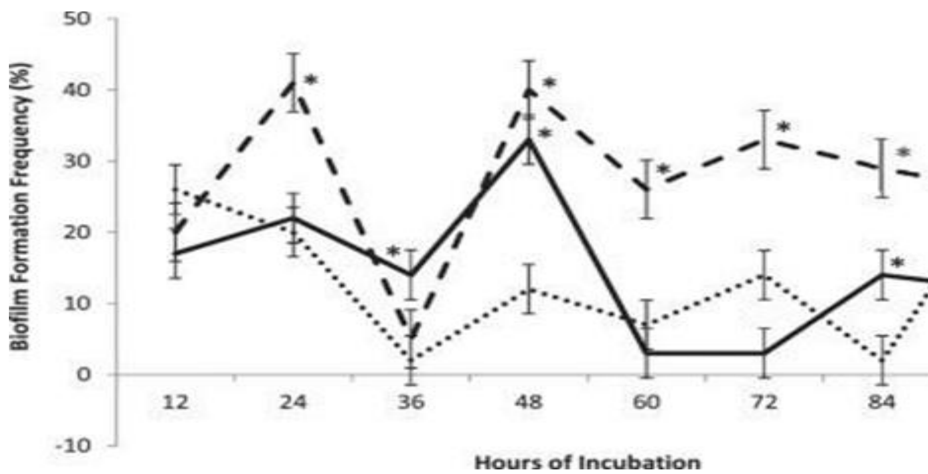


Figure 2.4 1 ***Frequencies of biofilm formation by*** **Salmonella** ***spp. on stainless steel, glass, and PVC surfaces. The results are the mean of triplicates.***

Chapter 3

Bacteriophage as a Weapon Against Biofilm In Food Industry

3.1 Inception of the Topic

Food Safety is a significant issue for health specialists and enterprises because of the impact in health and financial losses brought about by the food contamination. Regardless of the execution of Good Manufacturing Practices (GMP) and Hazard Analysis Critical Control Point (HACCPs) in food businesses, in 2014 the European Food Safety Authority (EFSA) detailed a sum of 5,251 foodborne outbreaks bringing about 6,438 hospitalizations. In the United States, 866 foodborne outbreaks were accounted for in 2014, bringing about 714 hospitalizations.

Food is regularly polluted during handling and packaging through contact with equipment surfaces. Of note, defilement with hemolytic bacteria (Staphylococcus aureus and Streptococcus agalactiae) was recognized in hands, hand-contact and food-contact surfaces in foodservice settings ; the presence of coliforms in washing water and mechanical facilities are associated with the low microbiological nature of tomatoes or the outstanding occurrence of S. aureus and other pathogenic microbes on food industry surfaces in Spain are a portion of the incredible number of announced models.

3.2 Effects Of Biofilm In Food Industry

Formation of Biofilms has remarkable effects in industries, specifically in processing of foods, with a negative effect on sanitation and the resulting financial losses. In such manner, further investigations about biofilm improvement and dismantling have been performed for significant pathogenic microbes, for example, *S. aureus* and *Listeria monocytogenes* . Various biofilm control systems have been proposed yet the issue stays unsolved, presumably due to the complexity of these structures, which contain the two cells and extracellular substances. Preferably, a biofilm expulsion system should have the option to get inside the biofilm structure and dispose of proficiently all the grid segments and the microscopic organisms. In food industry, there are various variable conditions, for example, temperature, pH, oxygen and accessibility of nutrients, and types of surfaces, which can balance development of biofilm. Surface properties, for

example, electrostatic charges, hydrophobicity, and roughness impact biofilm improvement in certain species. For example, hydrophilic surfaces are all the more immediately colonized by ***L. monocytogenes***, while S. aureus have not indicated any contrasts among hydrophobic and hydrophilic surfaces, and ***Salmonella*** has a higher capacity to hold fast to certain materials utilized in food-contact surfaces like Teflon, trailed by stainless steel, glass, Buna-N rubber, and polyurethane. At times, biofilm maintenance is more influenced by the roughness of surfaces than by the substance organization. Different parts of food conditions, for example, NaCl also influence the increase of adhesion of ***L. monocytogenes*** to surfaces, despite the fact that it is affected by temperature and nutrients too , and even by the presence of different microscopic organisms in the food-proccessing environment.

3.3 Factors Of Biofilm Formation And Inhibition

Factors related to food processing environment, variably affects development of biofilms. Bacteria sense these elements through refined intracellular and extracellular signaling networks bringing about a negative or positive reaction. For example, nutrient constraint prompts Salmonella enterica serovar Typhimurium to formation of biofilm though V. cholerae needs a supplement rich environment to build up a biofilm structure. Likewise, in S. aureus an expansion in biofilm arrangement was seen in a nutrient rich growth media and at high incubating temperatures. Optional metabolites, for example, anti-toxins may likewise instigate biofilm development. Another model was as of late detailed by Nesse et al. (2014), where conceivably human-pathogenic E. coli from the ovine supply can frame biofilms under conditions utilized in the food creation chain [on various surfaces, for example, stainless steel, glass, and polystyrene and at temperatures pertinent for food creation and taking care of (12, 20, and 37°C)]. Of note, for most microscopic organisms, impediment of inorganic atoms, for example, iron and inorganic phosphate inhibitory affects biofilm arrangement and high osmolality hinders by and large, biofilm development, despite the fact that this impact is unmistakably subject to the osmolyte.

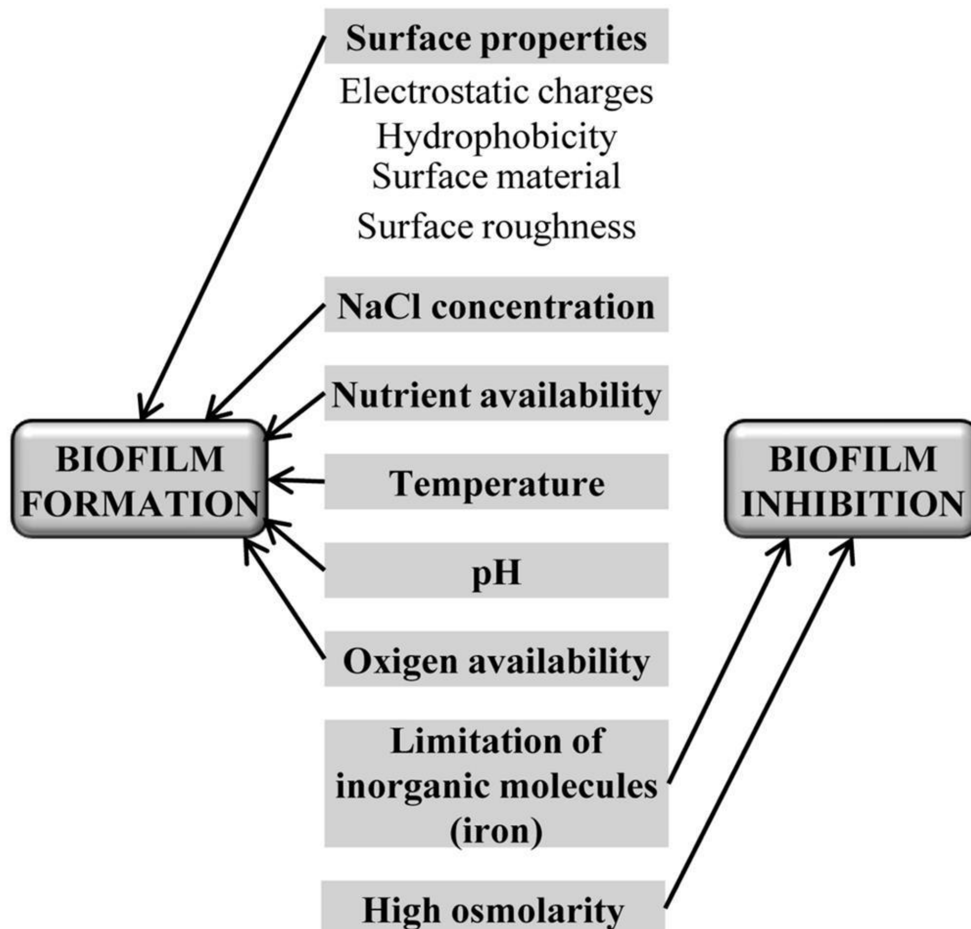


Figure 3.3 1 ***Factors Of Biofilm Formation And Biofilm Inhibition***

3.4 Studies And Researches On Controlling Biofilm Formation By Phage And Phage Protein

Number of studies on biofilms preformed in research centers affirm the capability of phages in biofilm expulsion. For biofilms shaped by pathogenic microbes related to food industry, there is proof of effective evacuation in various conditions and utilizing materials like those found in food- contact surfaces. With respect to, three phages LiMN4L, LiMN4p, and LiMN17 contaminating L. monocytogenes were tested, independently and as a cocktail, against 7-day biofilms formed by a combination of three strains on stainless steel coupons (104 cfu/cm2), recently covered with a fish broth layer that recreated seafood processing offices. Medicines with the single phages (109 pfu/ml) diminished adhered bacterial cells to up 3 log units, while treatment with the phage mixed drink decreased cell checks to imperceptible levels after 75 min . Additionally, a treatment with phage P100 (109 pfu/ml) diminished biofilms formed by L. monocytogenes strains in 3.5–5.4 log/cm2, independent of the serotype, development conditions and biofilm level. In spite of the adequacy of bacteriophages to decrease L. monocytogenes biofilms, there is proof that total expulsion

isn't constantly accomplished. By utilizing epifluorescence microscopy, L. monocytogenes was checked after treatment with phage P100 (108 pfu/ml) and, despite the fact that disaggregation of biofilms could be seen after 8 h, feasible cells were as yet present up to 48 h later, demonstrating that other disinfection techniques should be utilized in blend with phages.

Staphylococcus aureus is another significant foodborne pathogen with the capacity to form biofilms on various surface's materials. Staphylococcal phage K and a combination of subsidiary phages with more extensive host ranges were utilized to successfully prevent S. aureus biofilm formation over 48 h of incubation. It was likewise indicated that the expulsion of bacteria by the phage cocktail (109 pfu/ml) was time-subordinate, with the most noteworthy decrease happening after 72 h at 37°C. A comparative outcome was gotten utilizing phage K joined with another staphylococcal phage, DRA88 (MOI 10), to treat set up biofilms delivered by three

S. aureus isolates, which were fundamentally diminished after 4 h and totally eliminated after 48 h at 37°C. Other staphylococcal phages, for example, ISP, Romulus, and Remus applied separately at 109 phages for every polystyrene stake had the option to degrade by 37.8, 34.4, and 60.4%, individually, a S. aureus PS47 biofilm after 24 h . Comparable outcomes were gotten after the utilization of phages phiIPLA-RODI, phiIPLA- C1C, and a combination of the two phages, against biofilms shaped by S. aureus where a decrease by around 2 log units was accomplished after 8 h of treatment at 37°C. Now and again, notwithstanding, it was additionally important to join phages with different antimicrobials to expand their adequacy. Hence, treatment of 1-day-old biofilms framed by ***S. aureus*** D43 strain with phage SAP-26 diminished live microorganisms by about 28% while a synergistic impact with rifampicin permitted a decrease of about 65%.

Sharma et al. (2005) evaluates that the lytic bacteriophage KH1 (7.7 log pfu/ml) against stainless steel coupons containing E. coli O157:H7 biofilms (2.6 log cfu/coupon). These were treated for 4 days at 4°C and a decrease of 1.2 log units per coupon was noticed. Better outcomes were gotten while treating E. coli O157:H7 biofilms preformed on different materials ordinarily utilized in food preparing surfaces (tempered steel, earthen ware tile, and high thickness polyethylene), since a decrease to imperceptible levels was seen after 1 h of treatment at 23°C with a phage blend named BEC8. The utilization of a phage combination to eliminate biofilms shaped on cutting edges used to gather spinach was additionally illustrated, a decrease of 4.5 log units of the reasonable cells of E. coli O157:H7 being accomplished after 2 h of phage treatment.

As it was recently detailed, a blend of T4 bacteriophage and cefotaxime altogether upgraded the annihilation of E. coli biofilms when contrasted with treatment with phage alone.

Phage lytic proteins are alternate option for eliminating bacterial biofilms in food-related environments. Endolysin from phage phi11 (10 μg/well) had the option to eliminate biofilms formed by S. aureus strains on polystyrene surfaces after 2 h at 37°C . Likewise, endolysin SAL-2 from bacteriophage SAP-2 killed S. aureus biofilms utilizing 15 μg/well (Son et al., 2010). Recently, Gutiérrez et al. (2014) indicated that endolysin LysH5 (0.15 μM) can eliminate staphylococcal biofilms after treatment of 12 h at 37°C and even to lyse persisted cells. Designed endolysins, by cancellation or shuffling areas, have likewise been effectively utilized as against biofilm specialists. For example, peptidase CHAPk (31.25 μg/ml), got from the staphylococcal endolysin LysK, had the option to totally forestall biofilm development. This protein additionally eliminated staphylococcal biofilms after treatment of 4 h at 37°C. Also, the base fixation (6.2–50 mg/l) of ClyH, a staphylococcal fanciful lysin, needed for S. aureus biofilm destruction was lower than that required when anti- toxins were utilized. This protein contains the synergist area of endolysin Ply187 and the cell divider restricting space of phiNM3 lysin. Concerning framed by Gram- negative bacteria, expulsion of these structures by utilizing endolysins needs an extra part to disestablish the external film. Biofilms formed by S. enterica serovar Typhimurium were treated with endolysin Lys68 (2 μM), which decreased by 1 log unit the suitable cells in preformed biofilms after 2 h of hatching within the sight of external membrane permeabilizer.

Phage or phage protein	Scope of application	Bacteria	Efficacy of the treatment	Reference
Phages LiMN4L, LiMN4p, and LiMN17	Stainless steel	*L. monocytogenes*	Phage cocktail reduced biofilm cell counts to undetectable levels after 75 min	Ganegama-Arachchi et al., 2013
Phage P100	Stainless steel	*L. monocytogenes*	Reduction in the cell counts from 3.5 to 5.4 log units/cm^2	Soni and Nannapaneni, 2010
Phage P100	Stainless steel	*L. monocytogenes*	Reduction of the biofilm cell counts to undetectable levels after 48 h	Montañez-Izquierdo et al., 2012
Phage K and phage derivatives	Polystyrene	*S. aureus*	Complete elimination of the biomass after 72 h of incubation. Complete inhibition of biofilm formation was achieved when co-culturing phage mixture and bacteria	Kelly et al., 2012
Phage K and DRA88	Polystyrene	*S. aureus*	Complete elimination of the biomass after 48 h of treatment	Alves et al., 2014
Phages ISP, Romulus, and Remus	Polystyrene	*S. aureus*	Biofilm reduction of 37.8, 34.4, and 60.4% after 24 h treatment when using phages ISP, Romulus, and Remus, respectively	Vandersteegen et al., 2013
Phages philPLA-RODI and philPLA-C1C	Polystyrene	*S. aureus*	Reduction by 2 log units/well was achieved after 8 h of treatment	Gutiérrez et al., 2015b
Phage SAP-26	Polystyrene	*S. aureus*	Reduction of bacteria about 28% after phage treatment, while a synergistic effect with rifampicin allows a reduction of about 65%	Rahman et al., 2011
Phage CP8 and CP30	Glass	*C. jejuni*	Reduction in the biofilm cell counts of 1–3 log units/cm^2	Siringan et al., 2011
Phage KH1	Stainless steel	*E. coli* O157:H7	Reduction of 1.2 log units per coupon after 4 days treatment at 4°C	Sharma et al., 2005
BEC8 (phage mixture)	Stainless steel, ceramic tile, and high density polyethylene	*E. coli* O157:H7	Reduction of the biofilm cell counts to undetectable levels after 1 h of treatment at 37, 23, and 12°C	Viazis et al., 2011
Phage mixture	Spinach harvester blade	*E. coli* O157:H7	Reduction of biofilm cell counts by 4.5 log units per blade after 2 h of treatment	Patel et al., 2011
Phage T4	Polystyrene	*E. coli* O157:H7	Complete elimination of the biomass after phage treatment combined with cefotaxime	Ryan et al., 2012
Endolysin from phage phi11	Polystyrene	*S. aureus*	Complete elimination of the biomass after 2 h of treatment at 37°C	Sass and Bierbaum, 2007
Endolysin SAL-2	Polystyrene	*S. aureus*	Reduction of the biomass after 2 h of treatment at 37°C	Son et al., 2010
Endolysin LysH5	Polystyrene	*S. aureus*	Reduction of biofilm cell counts by 1–3 log units after 3 h of treatment	Gutiérrez et al., 2014
Domain CHAP$_K$ derived from endolysin LysK	Polystyrene	*S. aureus*	Complete elimination of the biomass after 4 h of incubation Complete inhibition of biofilm formation was achieved	Fenton et al., 2013
Chimeric lysin ClyH	Polystyrene	*S. aureus*	Reduction of the biomass in more than 60% after 30 min of treatment	Yang et al., 2014
Endolysin Lys68	Polystyrene	*S.* Typhimurium	Reduction of biofilm cell counts by 1 log unit after 2 h of treatment in the presence of outer membrane permeabilizers	Oliveira et al., 2014
Exopolysaccharide depolymerase Dpo7	Polystyrene	*S. aureus*	Degradation of 30% of the polysaccharidic matrix of the biofilm	Gutiérrez et al., 2015a

Figure 3.3 2 ***Application Of Phage And Phage Protein On Biofilm Removal***

As a whole, these outcomes demonstrated an observable capability of phages and phage-derived proteins, however, without a doubt extra investigations are important to move this information to the food business. For example, use of these anti-biofilm mixes would be feasible as long as their application can be executed as a feature of the standard cycles of cleaning in the mechanical offices. Consequently, the investigation of cooperative energy/enmity with disinfectants and the adequacy at temperatures normally utilized in the business could be important. It should be likewise seen the scant information accessible about the utilization of phages and phage lytic proteins against blended biofilms framed by various strains from a few animal varieties in food mechanical surfaces. This hole should be filled in to go further into the control of bacterial biofilms.

Chapter 4

Environmental Bacteriophage Active On Biofilms And Planktonic Forms Of Toxigenic Vibrio Cholerae: Potential Revelance In Cholera Epidemiology

4.1 Inception Of The Topic

In cholera-endemic regions, toxigenic Vibrio cholerae continue in the aquatic environment generally in a biofilm-related state in which the microscopic organisms stay embedded in an exopolysaccharide matrix. The biofilm-related cells frequently go into dormant form as conditionally viable environment cells (CVEC), which oppose development on routine bacteriological media. In any case, these cells can normally revive into the active planktonic structure through different mechanism, multiply, and cause epidemics of cholera. This investigation was led to consider potential impacts of natural bacteriophages on the predominance and distribution of the pathogen between the biofilm associated state, and the planktonic form.

4.2 Phenotypic and genetic characteristics of JSF3, JSF4 and JSF7 phages

Phages JSF3, JSF4 and JSF7 were at first separated from various samples of river water in Dhaka, Bangladesh. The host particularity of these phages was analyzed by utilizing a board of strains having a place with various animal types or serogroups. Just V. cholerae O1 strains were susceptible to JSF4 and JSF7 phages, though the JSF3 phage was explicit for V. cholerae O139 strains. JSF7 was discovered to be capable for scattering biofilms formed by both V. cholerae O1 and O139 strains. Each of the three phages created away from with a distance across of ~ 1 mm on a grass of their separate host bacteria.

Characteristics	Designation of Phages		
	JSF3	JSF4	JSF7
Morphology	**Isometric head with short non-contractile tail**	**Isometric head with long non-contractile tail**	**Isometric head with long contractile tail**
Family	*Podoviridae*	*Siphoviridae*	*Myoviridae*
Head diameter	58.3 ± 4.0	62.3 ± 2.5	58.3 ± 4.0
Tail length	10.8 ± 2.0	86.9 ± 3.3	55.9 ± 2.5
Tail width	-	15.5 ± 1.7	24.4 ± 0.8
Nucleic acid	Double stranded DNA	Double stranded DNA	Double stranded DNA
Genome size	69Kb	124Kb	46Kb
GC percentage	37.81	37.08	48.42
Host- specificity	*V. cholerae* O139	*V. cholerae* O1, El Tor	*V. cholerae* O1, El Tor
Dispersion of biofilms of *V. cholerae* O1 and O139	Negative	Negative	Positive

https://doi.org/10.1371/journal.pone.0180838.t001

Figure 4.2 1 ***Phenotypic And Genotypic Charecteristics Of Phages***

Electron microscopic examination reveals that all three phages had isomeric heads and whereas JSF3 had a short tail, JSF4 had a long non-contractile tail, and JSF7 had a contractile tail (Fig 1). Based on the morphology, JSF3 belongs to the family Podoviridae, though JSF4 belongs to Siphoviridae and JSF7 belongs to Myoviridae family.

All three phages were stable at temperature underneath 37°C. The steadiness diminished with rise of temperature and more than 80% of phages were quickly inactivated at temperatures over 45°C. The phages moreover remained for the most part irresistible (65% to 98%) at pH extending from 6.0 to 9.0. (S1 Fig). Phage particles were steady and remained irresistible for more than 4 weeks when they were put away at room temperature in SM buffer (100 mM NaCl, 8.1 mM MgSO4, 0.05 mM Tris-Cl [pH 7.5], 0.01% gelatin).

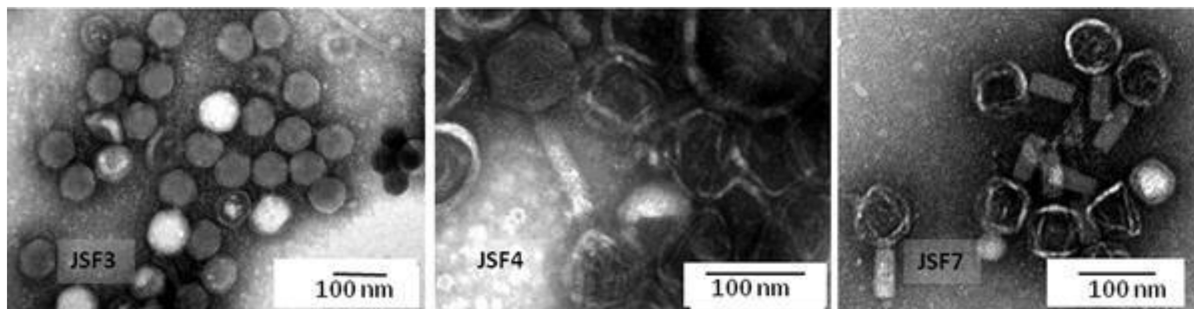


Figure 4.2 2 ***Electron micrograph showing the morphology of three different V. cholerae specific phages***

4.3 Dispersion Of Biofilm Associated V.cholerae Cells By JSF3, JSF4 And JSF7

The JSF7 phage was found to disrupt biofilm matrices of both V. cholerae O1 and O139 and cause scattering of the biofilm related cells, recommending that the method does not require infection of the bacterial cells by the phage. Expansion of 1.5 x 107 pfu of the phage into borosilicate vials with biofilms joined on the internal surface, caused recognizable biofilm corruption by 2h and diminished biofilms biomass by 50% inside 6h. The scattering of biofilms was related with rise in free bacterial cell number within the medium (Fig 2). In any case, the observed cell number of the phage-susceptible strain C6706 within the watery stage was lower than those of the phage-resistant strains MO1220 and V51 utilized in this consider, showing that a extent of the vulnerable cells were apparently slaughtered by the phage after their scattering from the biofilm matrix.

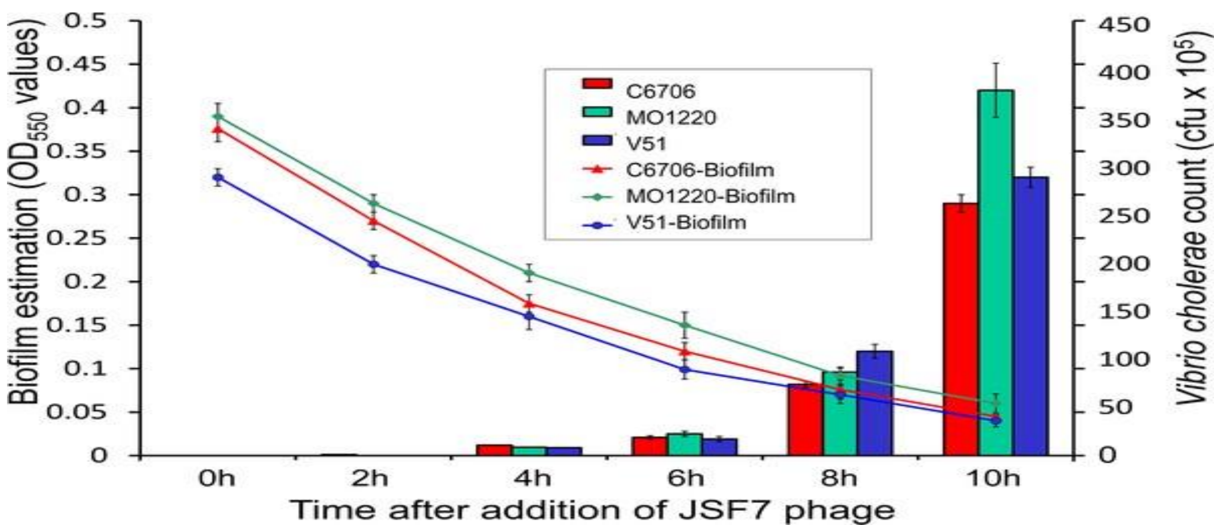


Figure 4.3 1 ***Degradation of biofilms by JSF7 phage and concomitant increase in corresponding V. cholerae count.***

Other than JSF7, the other two lytic phages included in this study, to be specific JSF3 and JSF4 were moreover tried for their action on biofilms, but these two phages did not appear any biofilm- degrading action (data not appeared). Phages JSF3 and JSF4 were moreover utilized to test the vulnerability of biofilm-associated V. cholerae to these phages, as compared to that of the planktonic form of the same microscopic organisms. As appeared in Fig, whereas the planktonic cells were attacked by the phages at high rate, the biofilm-associated cells were for the most part safe to these lytic phages that don't have biofilm-degrading movement. The contrast in survival rate between biofilm related cells and comparing planktonic cells was clear after 2h of hatching with the phage, and after 5h of incubated the distinction

between the survival of biofilm related cells as compared to planktonic cells was exceedingly noteworthy both for phage JSF4 (p = 0.0090) and for JSF3 (p = 0.0066). Surprisingly, V. cholerae cells separated from biofilms by physical disturbance kept up the upgraded resistance phenotype, and remained essentially more phage-resistant than new societies of the microscopic organisms (p = 0.01 for JSF4 and p = 0.007 for JSF3).

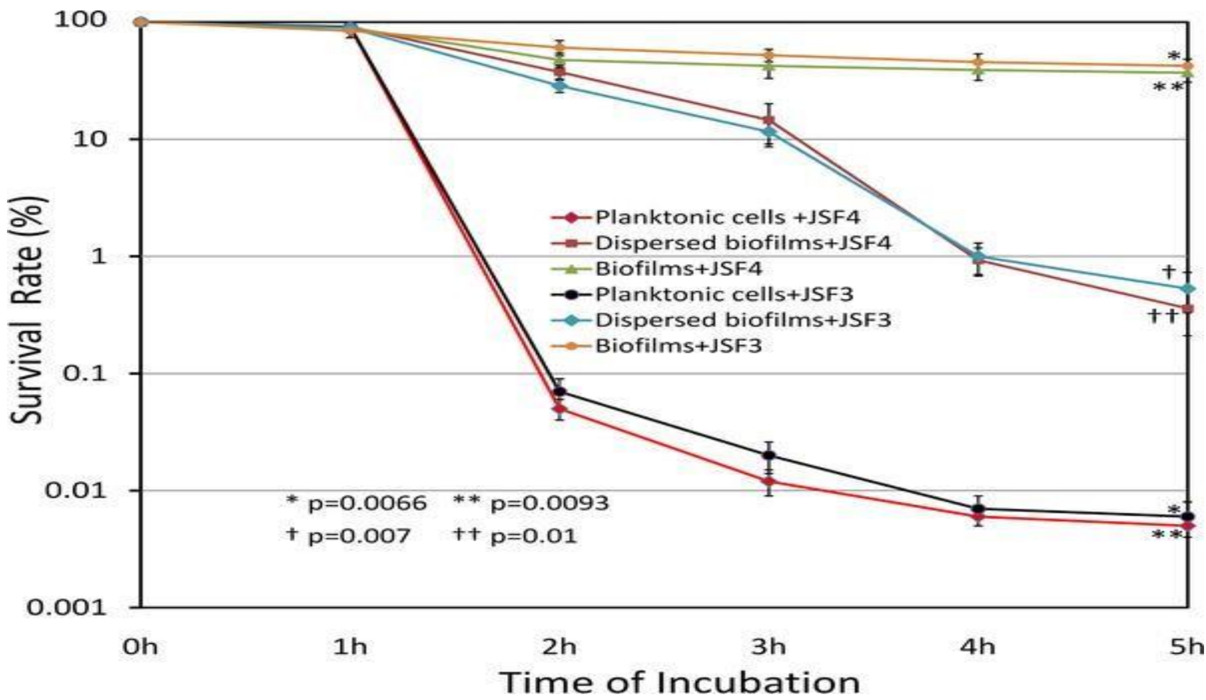


Figure 4.3 2 ***Resistance of biofilm-associated Vibrio cholerae to lytic phages JSF3 and JSF4.***

4.3.1 Results And Effectivity Of Phages

Three diverse phages, JSF3, JSF4 and JSF7 were immunized completely different combination as well as independently into jar containing pre-formed biofilms (on 22mm × 22 mm cover slips) and filtered stream water. The pH extend of the water was found to be 7.5 to 8.0, and the salinity was 0.4 to 0.5 parts per thousand (ppt). The flasks were incubated at room temperature, and viable cell count and phage checks within the aqueous stage were checked. We found that phage JSF7 was able to disperse biofilm-associated cells and in this way increment the concentration of planktonic cells within the aqueous stage. For V. cholerae O139 strain MO1220, which was safe to lysis by JSF7 phage this increment was more than 106 overlap in 6h, though for V. cholerae O1 strain C6706 which was helpless to lysis by JSF7 the residual increment in cell number was around 75 folds. The increment in cell tallies within the watery stage was steady with the diminishment in biofilm network held on the cover slips, as tested by crystal violet staining.

As mentioned over, the observed concentration of dispersed cells within the aqueous stage was essentially lower when the biofilm strain was vulnerable to the phage as compared to a strain which was safe ($p = 0.0034$), showing that a extent of the susceptible cells were killed by the phage after their scattering from the biofilm matrix. This presumption was further backed by an observed rise in phage titer demonstrating enhancement of the phage utilizing vulnerable have bacteria. When biofilms of V. cholerae were exposed to JSF3 and JSF4 phages within the absence of JSF7 phage there was no noteworthy change within the checks of V. cholerae cells within the fluid stage or the thickness of the biofilm network. Be that as it may when a combination of phages counting JSF7 were utilized on biofilms of a phage vulnerable V. cholerae, the biofilm strain discharged from the network were nearly totally dispensed with. For case, when biofilms of strain C6706 was treated with phage JSF4 and JSF7 together, as it were almost 5 cfu/ml of the bacteria survived the phage treatment. When all three phages were utilized at the same time to treat biofilms of either strain C6706 or MO1220, as it were a mean cfu of 2.7 x 10 to 3.2 x 10 cells/ml survived after 6h of incubation. Essentially, exceptionally no survival rates of the microscopic organisms were watched when blended biofilms of V. cholerae O1 and O139 strains C6706 and MO1220 were exposed to all three phages.

V. cholerae strains (serogroup) used to prepare biofilms	Phages used	Various parameters measured after 6h of incubation with or without adding phage				
		Colony count without phage (cfu/ml)	Colony count with phage (cfu/ml)	Phage count (pfu/ml)	Biofilm (OD_{550}) without phage	Biofilm (OD_{550}) with phage
C6706 (O1, El Tor)	JSF3	$0.5 \times 10^2 \pm 0.4 \times 10$	$0.9 \times 10^2 \pm 1.1 \times 10$	$7.5 \times 10^6 \pm 2.0 \times 10^5$	0.45 ± 0.05	0.42 ± 0.05
MO1220 (O139)	JSF3	$1.2 \times 10^2 \pm 2.0 \times 10$	$0.3 \times 10^2 \pm 0.3 \times 10$	$1.5 \times 10^7 \pm 1.5 \times 10^6$	0.51 ± 0.04	0.5 ± 0.05
C6706 (O1, El Tor)	JSF4	$1.5 \times 10^2 \pm 1.7 \times 10$	$0.6 \times 10^2 \pm 0.4 \times 10$	$1.4 \times 10^7 \pm 2.0 \times 10^6$	0.41 ± 0.03	0.39 ± 0.04
MO1220 (O139)	JSF4	$1.2 \times 10 \pm 1.5 \times 10$	$1.5 \times 10^2 \pm 1.7 \times 10$	$1.0 \times 10^7 \pm 5.0 \times 10^5$	0.51 ± 0.08	0.48 ± 0.08
C6706 (O1, El Tor)	JSF7	$0.75 \times 10^2 \pm 0.4 \times 10$	$5.2 \times 10^3 \pm 6.6 \times 10^2$	$7.3 \times 10^8 \pm 2.0 \times 10^7$	0.48 ± 0.05	0.15 ± 0.02
		*Fold increase in cfu = 70.09 ± 12.69				
MO1220 (O139)	JSF7	$1.2 \times 10^2 \pm 1.7 \times 10$	$7.5 \times 10^8 \pm 6.2 \times 10^7$	$1.4 \times 10^7 \pm 1.1 \times 10^6$	0.55 ± 0.1	0.21 ± 0.03
		*Fold increase in cfu = 6298701 ± 650777				
C6706 (O1, El Tor)	JSF3, JSF4	$1.5 \times 10^2 \pm 0.5 \times 10$	$0.9 \times 10 \pm 0.05 \times 10$	JSF3 $1.1 \times 10^7 \pm 2.0 \times 10^6$ JSF4 $8.2 \times 10^7 \pm 1.3 \times 10^7$	0.51 ± 0.02	0.48 ± 0.01
MO1220 (O139)	JSF3, JSF4	$1.2 \times 10^2 \pm 1.5 \times 10$	$0.14 \times 10^2 \pm 0.1 \times 10$	JSF3 $9.0 \times 10^7 \pm 1.2 \times 10^7$ JSF4 $1.2 \times 10^7 \pm 2.6 \times 10^6$	0.48 ± 0.03	0.47 ± 0.03
C6706 (O1, El Tor)	JSF3, JSF7	$0.5 \times 10^2 \pm 0.3 \times 10$	$2.2 \times 10^2 \pm 1.7 \times 10$	JSF3 $1.2 \times 10^7 \pm 3.0 \times 10^6$ JSF7 $6.2 \times 10^8 \pm 1.1 \times 10^8$	0.48 ± 0.02	0.11 ± 0.02
MO1220 (O139)	JSF3, JSF7	$8.1 \times 10 \pm 0.7 \times 10$	$1.4 \times 10^2 \pm 2.0 \times 10$	JSF3 $5.4 \times 10^8 \pm 8.0 \times 10^7$ JSF7 $1.1 \times 10^7 \pm 1.1 \times 10^6$	0.46 ± 0.06	0.20 ± 0.01
C6706 (O1, El Tor)	JSF4, JSF7	$0.5 \times 10^2 \pm 0.3 \times 10$	$0.5 \times 10 \pm 0.1 \times 10$	JSF4 $8.4 \times 10^7 \pm 8.0 \times 10^6$ JSF7 $7.4 \times 10^7 \pm 1.0 \times 10^7$	0.52 ± 0.05	0.12 ± 0.02
MO1220 (O139)	JSF4, JSF7	$1.1 \times 10^2 \pm 0.5 \times 10$	$6.5 \times 10^8 \pm 1.0 \times 10^7$	JSF4 $1.0 \times 10^7 \pm 1.5 \times 10^6$ JSF7 $1.1 \times 10^7 \pm 1.2 \times 10^6$	0.47 ± 0.04	0.22 ± 0.02
C6706 (El Tor)	JSF3, JSF4, JSF7	$0.5 \times 10^2 \pm 0.3 \times 10$	$0.32 \times 10^2 \pm 0.6 \times 10$	JSF3 $1.0 \times 10^6 \pm 1.5 \times 10^5$ JSF4 $6.6 \times 10^8 \pm 1.2 \times 10^8$ JSF7 $5.9 \times 10^8 \pm 3.2 \times 10^7$	0.43 ± 0.04	0.11 ± 0.01
MO1220 (O139)	JSF3, JSF4, JSF7	$3.4 \times 10 \pm 0.4 \times 10$	$2.7 \times 10 \pm 0.4 \times 10$	JSF3 $5.6 \times 10^8 \pm 1.1 \times 10^8$ JSF4 $1.1 \times 10^7 \pm 1.1 \times 10^6$ JSF7 $1.0 \times 10^7 \pm 1.5 \times 10^6$	0.47 ± 0.05	0.23 ± 0.04
Mixed biofilm of C6706 (O1, El Tor) and MO1220 (O139)	JSF3, JSF4, JSF7	C6706 $3.6 \times 10 \pm 0.1 \times 10$ MO1220 $4.5 \times 10 \pm 0.4 \times 10$	C6706 $2.9 \times 10 \pm 0.8 \times 10$ MO1220 $3.2 \times 10 \pm 0.4 \times 10$	JSF3 $6.3 \times 10^8 \pm 1.1 \times 10^8$ JSF4 $6.9 \times 10^8 \pm 1.2 \times 10^8$ JSF7 $6.2 \times 10^8 \pm 4.6 \times 10^7$	0.46 ± 0.05	0.12 ± 0.02

The initial titer of different phages used were 1.5×10^7 pfu/ml. The El Tor biotype *V. cholerae* O1 strain C6707 was susceptible to both JSF4 and JSF7, whereas the *V. cholerae* O139 strain was only susceptible to JSF3. Phage JSF7 could disperse biofilms of both these *V. cholerae* strains. Figures represent mean values and standard deviations of three independent observations.

*For JSF7 phage and C6706 derived biofilms, the observed increase in ratio of C6706 cells in the aqueous phase with and without phage was significantly lower compared to that for JSF7 phage and biofilms of strain MO1220 (($p = 0.0034$).

https://doi.org/10.1371/journal.pone.0180838.t002

Fig 4.3.3: Activity Of Diverse Phages On V.cholera O1 or O139

4.4 Discussion And Conclusion

A variety of other variables too contribute and control the predominance of V. cholerae and the event of cholera episodes. For illustration, bacterial cell density-dependent quality expression named “quorum sensing” which is controlled by signal molecules called auto inducers (AIs) moreover causes scattering of biofilms. AIs moreover revive torpid natural cells of V. cholerae into effectively partitioning cells. Interests, we moreover appeared as of late that AIs improve resistance of V. cholerae to phages. Improved infectivity of V. cholerae amid a cholera plague has been recommended to be a result of inherent "hyper infectivity" of cells shed in stools of cholera casualties as well as the nearness of biofilm-like clumps of cells which

permits the conveyance of the pathogen at a tall dosage to be able to contaminate a potential casualty. On the other hand, lytic phages have been recommended to diminish the infectivity of V. cholerae by balancing the desired irresistible dosage. In contrast, results of this study propose that the JSF7 phage can moreover cause dispersion of biofilms, driving to abundance and spread of effectively isolating V. cholerae cells in water, which could be a hazard figure for the event of cholera outbreaks. In this way a combination of different components fine tune the predominance and infectivity of V. cholerae in water, of which phages are critical operators both in deceasing as well as expanding the predominance of the pathogen. In summary, other than conceivable application of a blend of phages to treat water sullied with V. cholerae to diminish transmission, the comes about of this consider give curiously refinements to our understanding of the potential part of phages within the environment and the study of disease transmission of cholera.

Chapter 05

Bacteriophage therapy against *Pseudomonas aeruginosa* biofilm

5.1 Introduction

Pseudomonas aeruginosa is a gram-negative Bacilli and one in every of the most opportunist microorganisms that have a number one role in nosocomial, acute, and chronic infections. Infection with this pathogen ends up in diseases with a high death rate in patients diagnosed with cystic fibrosis, cancer, severe burns, and upset patients. This bacterium can survive on water, completely different surfaces, and medical devices by exploiting its powerful binding factors similar to flagella, pili, and biofilms. *P. aeruginosa* has shown high intrinsic resistance to a spread of antibiotics, as well as beta-lactams, fluoroquinolones, and aminoglycosides, which ends in important morbidity and mortality rates. *Pseudomonas aeruginosa* can bind to numerous surfaces and kind biofilms resulting in chronic infections by increasing resistance to antibiotics, disinfectants, varied irradiation treatments, environmental conditions, and therefore the immune system.

Phage therapy is one of the necessary strategies to inhibit *P.* aeruginosa biofilm. Bacteriophages are viruses that invade bacteria; they were discovered nearly a century past and are divided into 2 lytic (virulent phages) and temperate classes betting on their life cycle. Once attaching to their host, the lytic phages inject their genetic materials into the host body and replicate at the side of the host cell DNA, then disperse via the host lysis to repeat the infection cycle for different hosts.

Bacteriophages that specifically target *Pseudomonas* genus were initially discovered within the middle of the twentieth century, and because of the nice role of this organism in healthcare facility infections and high antibiotic resistance, using bacteriophages to inhibit *P. aeruginosa* has been extremely regarded. the utilization of two or a lot of bacteriophage mixtures with completely different host ranges in a very single suspension as a bacteriophage cocktail is sometimes more practical for inhibiting microorganism infections. Virus cocktail causes better reduction of bacterial density and improve bacteriophages' efficiency, and additionally in vitro studies have shown that bacteriophage cocktail end in the next reduction in *P. aeruginosa* infections.

5.2 Phage therapy for inhibition of MDR *P. aeruginosa* biofilm: in vitro studies

Under *in vitro* conditions bacteriophages showed good effectivity against *P. aeruginosa* biofilms. There are different studies where the researchers found that many types of bacteriophages are capable to reduce *P. aeruginosa* biofilm. The different studies are: -

Virus M-1 was isolated from wastewater in order to inhibit the biofilm produced by MDR isolates of *P. aeruginosa.* From the results obtained it was found that the bacteriophage diminished the expansion rate of *P. aeruginosa* and reduced biofilms after 6 h of treatment. a very important purpose mentioned during this study was that this bacteriophage with the aid of its enzymes was capable of breaking down alginate polymers. In addition to that, it could degrade 20 days old biofilm formed by *P. aeruginosa.* Bacteriophage may destroy biofilms indirectly by killing microorganisms before attaching, or after colonizing the surface.

PB1-like, phiKZ-like, and LUZ24-like viruses against MDR *P. aeruginosa* under variable growth conditions; the results indicated that every phage alone was able to suppress being and biofilm kind of MDR isolates. In case of the eliminating the planktonic forms, phiKZ-like viruses were the most effective. For degrading the biofilms of antibiotic-resistant isolates, LUZ24-like phages showed the most potent results.

The phiKZ-like viruses were the most potent phages within the suppression of planktonic form. Besides, LUZ24-like phage was the most effective phage to destroy the biofilm of antibiotic-resistant isolates.

To examine the degradation of biofilms of *P. aeruginosa* bacteriophages Pa193, Pa204, Pa222, and Pa223 was obtained from chronic rhinosinusitis patients and it was found that one dose of those bacteriophages alone and in cocktail considerably diminished the speed of biofilm production once 24 and 48 h of treatment. Though single phages reduced 53–73% of the biofilms of the isolates, the efficaciousness of the phage cocktail on the biofilms increased by 89%

Although there are some more phages which are AZ1, MAG1 & MAG4(cocktail), {ΦKMV, ΦPA2, ΦPaer4, ΦE2005} (cocktail), vB-Pa4 & vB-Pa5(cocktail), PA1Ø also tested for against *P. aeruginosa* biofilm and those are capable to inhibit the biofilm of *P. aeruginosa.*

Based on the on top of studies, it is complete that the identification of the latest phages will be superb various antimicrobial agents for the treatment of MDR *P. aeruginosa* biofilm and should even eradicate the infections caused by MDR *P. aeruginosa* mixed with microorganism in vitro. The results of in vitro investigations can facilitate extending the appliance of phages against MDR *P. aeruginosa* nosocomial infections. The use of phage cocktails can increase anti-biofilm performance and additionally prevent resistance to bacteriophages.

Using phage therapy to inhibit the biofilm of different strains of *P. aeruginosa*

First author and year	**Species**	**Type of phage**	**Experimental results**
Liyuan Mi (2019)	*P. aeruginosa* 1193	Lytic IME180 phage depolymerase	This phage enzyme degraded *P. aeruginosa* exopolysaccharide, enhanced bactericidal activity mediated by serum complement proteins in vitro, and disrupt the bacterial biofilm
Yangyijun Guo (2019)	*P. aeruginosa* PAO1	vB_PaeM_SCUT-S1 and vB_PaeM_SCUT-S2	These two phages inhibited the growth of bacterium at low multiplicity of infection levels, had good performance both on preventing biofilm formation and eradicating preformed biofilms
Tomasz Olszak (2017)	*P. aeruginosa* PAO1	O-specific polysaccharide lyase from the phage LKA1	This enzyme reduced *P. aeruginosa* virulence, sensitized this bacterium to serum complement activity, and caused biofilm degradation

Muafia Shafique (2017)	A hospital isolate of *P. aeruginosa*	JHP	This phage reduced biofilm biomass from 2 to 4.5 logs (60–90%) and reduced bacterial load that highlights its potential to prevent biofilm formation from indwelling medical devices
Diana R. Alves (2016)	*P. aeruginosa* PAO1	A cocktail of six specific phage	After 4 h of biofilm contact with the phage suspension (MOI 10), more than 95% of biofilm biomass was eliminated, and 48 h after adding the phage cocktail in the flow biofilm model, the biofilm was dispersed
Katarzyna Danis-Wlodarczyk (2015)	*P. aeruginosa* PAO1	Bacteriophages KTN6 and KT28	Both of these bacteriophages reduced colony-forming units (70–90%) in 24 h to 72 h *P. aeruginosa* PAO1 biofilm cultures, reduced the secretion of pyocyanin, and pyoverdin, and increased diffusion rate through the biofilm matrix

Table 5.2 1 Studies using phage therapy to inhibit the biofilm of different strains of P. aeruginosa

5.3 Phage therapy for inhibition of MDR *P. aeruginosa* biofilm: in vivo studies

As like in vitro studies, various studies have shown advances in using phages against MDR *P. aeruginosa,* which causes chronic inflammation media, cystic fibrosis, and burn wounds; however, these studies are restricted to pre-clinical evaluations. The phages have been examined in animal models to find out the protection and effectiveness of phages to counteract each major gram-positive and gram-negative clinical pathogen. The *P. aeruginosa* was particularly important because it had high antibiotic resistance, it caused high mortality rates and it could produce high concentration of biofilms in nosocomial infections.

Two novel bacteriophages was used in mouse models of acute respiratory disease, which were infected with drug-resistant (XDR) *P. aeruginosa* in vitro, in vivo and in silico. They were BΦ-R1836 and BΦ-R656. Each phage exhibited potent restrictive activity and lysed XDR *P. aeruginosa* strains isolated from pneumonia patients. For *in vivo* studies, 2 models were also prepared by the researchers. From the results it was found that in *Galleria mellonella* larvae the phages BΦ-R656 and BΦ-R1836 could destroy XDR *P. aeruginosa* strains and acute pneumonia mice models.

The results demonstrated that BΦ-R656 and BΦ-R1836 eliminated XDR *P. aeruginosa* strains in *Galleria mellonella* larvae and acute pneumonia mice models. These phages were capable of eliminating the host XDR *P. aeruginosa* biofilms extensively.

To overcome the chronic respiratory organ infection caused by *P. aeruginosa* the phage PELP20 was examined in a mice model. The outcomes from these results were that the phage successfully reduced the biofilms by 3-log phages and the phage contained antimicrobial activity against *P. aeruginosa.* This means that phage PELP20 will kill the biofilm-related microorganism present within the lungs of CF patients.

Phage ΦPan70, a temperate phage, was accustomed control MDR *P. aeruginosa* infection in being, biofilm, and mouse burn models. The numerous results showed that the phage affected each planktonic and biofilm cell and considerably reduced the microorganism population. A fascinating finding was that the phage increased the burned mice's survival rate from 80% to 100%.

To prevent the *P. aeruginosa* biofilm in murine lungs two bacteriophages were evaluated. They were ΦMR299-2 and ΦNH-4. It demonstrated that these two bacteriophages killed all clinical isolates (mucoid and non-mucoid isolates). The phage cocktail was resulted in killing mucoid and non-mucoid strains growing within the mucoviscidosis cartilaginous tube animal tissue CFBE41o-cell line. Also, the phage cocktail showed a deadly effect on *P. aeruginosa* in murine lungs, and this bacterium was effectively cleared from the lungs once six hours.

For the reduction of *P. aeruginosa* biofilms the effect of the bacteriophage cocktail containing PYO2, DEV, E215, E217, PAK_P1, and PAK_P4 in acute respiratory tract infection model in mice and pathology in wax moth (Galleria mellonella) larvae was studied. The results showed that these bacteriophages alone may lyse *P. aeruginosa* in each organism and biofilm forms. The phage cocktail was conjointly found to be effective on the MDR and mucoid composition of *P. aeruginosa* isolates.

Bacteriophages alone and together effectively control XDR and MDR *P. aeruginosa* infections in the organism and biofilms forms within the animal models. Therefore, human infections that are Bacteriophages alone and together effectively control XDR and MDR *P. aeruginosa* infections in the organism and biofilms forms within the animal models. Therefore, human infections that are related to the *P. aeruginosa* biofilm, significantly XDR and MDR infections, love respiratory organ and wound infections, are associated with therapeutic problems, and that they are often the potential future targets of bacteriophage therapy. Because the results of the in vitro studies show, the use of bacteriophage cocktails in animal models has shown higher performance, therefore it's suggested that it's used more in future studies.

5.4 Use of combination therapy of antibiotics and bacteriophages to inhibit *P. aeruginosa* biofilm

Applying a mixture of various substances with antibiotics to extend their effectiveness on MDR bacterium has received a lot of attention. Furthermore, the combination of medical aid can even help destroy biofilms. There are several studies of bacteriophage and antibiotics both therapies to inhibit biofilm. They are: -

To inhibit the biofilm from *P. aeruginosa* combined application of the bacteriophage named vB_PaM_EPA1 and antibiotic.

Phage PEV20 and antibiotic were used to inhibit the biofilm of *P. aeruginosa* isolated from wound and cystic fibrosis patients. The results showed that combined antibiotic virus treatment increased biofilm demolition compared with single ciprofloxacin treatment. This phenomenon is often a result of the low penetration of this antibiotic into the biofilm and bacterium on the inner layers of biofilm, which show high antibiotic resistance due to low metabolic activity.

To degrade various strains of *P. aeruginosa* isolates a combined application of the bacteriophage Pa1 containing ATCC 12,175-B1, Pa2 containing ATCC 14,203-B1 and ciprofloxacin was evaluated. The results showed that bacteriophages had a higher restrictive impact on biofilm than antibiotics, and once used in combination, they performed better than applying antibiotics alone.

To eliminate *P. aeruginosa* PAO1 biofilm a combination of PB-1 phage and antibiotic drug was used. The results from this study showed that the combined application was not that much useful compared to the application of antibiotics alone. Although, the combined application of PB-1 and tobramycin showed an exceedingly 60% and 99% crease in tobramycin and phage resistant cells, respectively, compared to the use of tobramycin or phage alone.

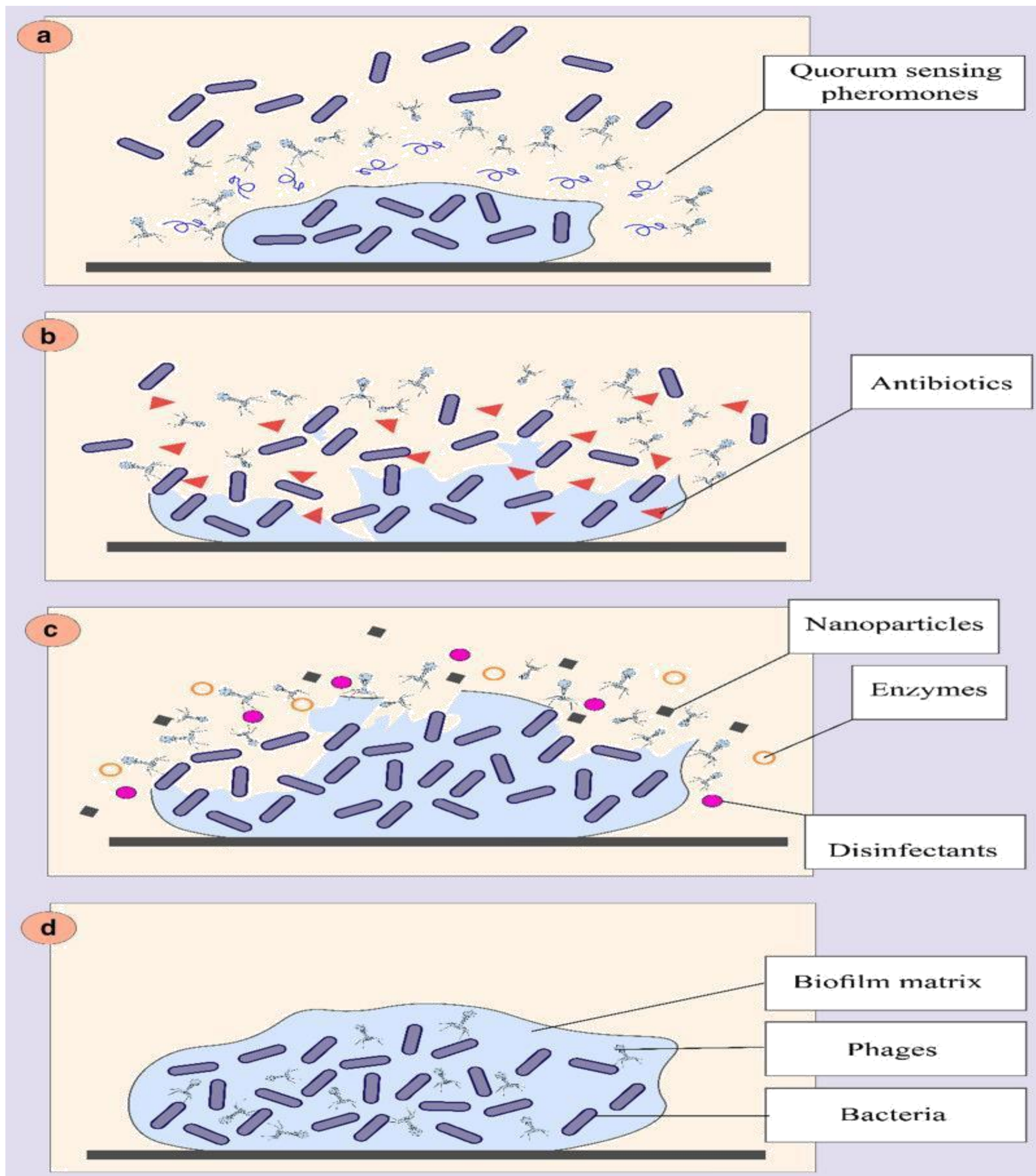


*Figure 5.4 1 Anti-biofilm mechanisms of bacteriophages. **A**) Bacteriophages inhibit biofilm formation by inhibiting quorum sensing and reducing cellular communication. **B**) Combined treatments with sequential application of phage and antibiotics have a killing efficacy on* P. aeruginosa *biofilm. **C**) Combined use of bacteriophages with molecules with anti-biofilm properties can help biofilm destruction. **D**) Bacteriophages can penetrate the inner layers of the biofilm through the biofilm void spaces without destroying the external matrix and replicate in the deeper-layer of biofilm*

Chapter 06

Phage therapy against *Enterococcus faecalis* in dental root canals

E. faecalis is a commensal gram-positive facultative anaerobic microorganism inhabiting the gastrointestinal tract of humans and varied animals, however is additionally found in environments like soil and water. *E. faecalis* is one in every of the most frequently isolated species from hospital-associated infections; it causes endocarditis, bacteremia, tract infections, meningitis, and alternative fatal forms of systemic and native infection in humans. *E. faecalis* has evolved within the past few decades to become one of the most difficult bacteria to eliminate. As mentioned above, in root canals it's hidden from the system and antibiotics. Varied antiseptic and antibiotic materials are used for intracanal microorganism eradication, which includes hydrated oxide or antibiotic pastes to boost microorganism control before root canal sealing.

6.1 Phage against *E. faecalis*

For combatting VRE *E. faecalis* infections, phages are isolated and tested for their effectiveness by many researchers. Most of those phages belong to the *Myoviridae* or the *Siphoviridae* families of caudated phages.

Phages of *E. faecalis*	Lytic/lysogenic phage	Accession number	Family
phiEF24C	Lytic	AP009390.1	*Myoviridae*
ECP3	Lytic	KJ801817.1	*Myoviridae*
IME-EF1	Lytic	KF1920531	*Siphoviridae*
SAP6		JF731128.1	*Siphoviridae*
BC611		AB712291.1	*Siphoviridae*
EfaCPT1		JX193904.1	*Siphoviridae*
EFDG1	Lytic	KP339049.1	*Myoviridae*
EFLK1	Lytic	KR049063.1	*Myoviridae*
Q69			
Phi4D			*Myoviridae*
IME_EF3	Lytic	KF728385	*Siphoviridae*
EFRM31	Lytic	GU815339	*Siphoviridae*
EFRM42	Lytic		*Siphoviridae*
EFRM54	Lytic		*Siphoviridae*
PhiFL1A	Lysogenic	GQ478081	*Siphoviridae*
PhiFL1B	Lysogenic	GQ478082	*Siphoviridae*
PhiFL1C	Lysogenic	GQ478083	*Siphoviridae*

Figure 6 .1 1 Phages isolated against E. faecalis*, their accession numbers, and the family they belong to. This indicates the huge possibility of using these phages for phage therapy in the future.*

<u>6.2 Phage therapy against *E. faecalis* biofilm</u>

Apart from being bacterium eradication, a more difficult and relevant part of *E. faecalis* infections is eliminating its biofilms. Therefore far, among the *E. faecalis* described phages, only EFDG1 was tested on *E. faecalis* biofilms. EFDG1, isolated from sewage water, was efficient in nearly eliminating a 2-week-old *E. faecalis* biofilm of ~100 µM thickness. The results showed that the bacteriophage treated samples caused biofilm biomass reduction among 7 days, whereas the untreated biofilms showed no reduction. Microorganism viable counts from the biofilm supported this notion by showing a five-log reduction compared with the untreated biofilms. Scanning microscopy revealed the destruction of the treated biofilm that sounds like clumps of distributed bacterium compared with the intact, untreated biofilm.

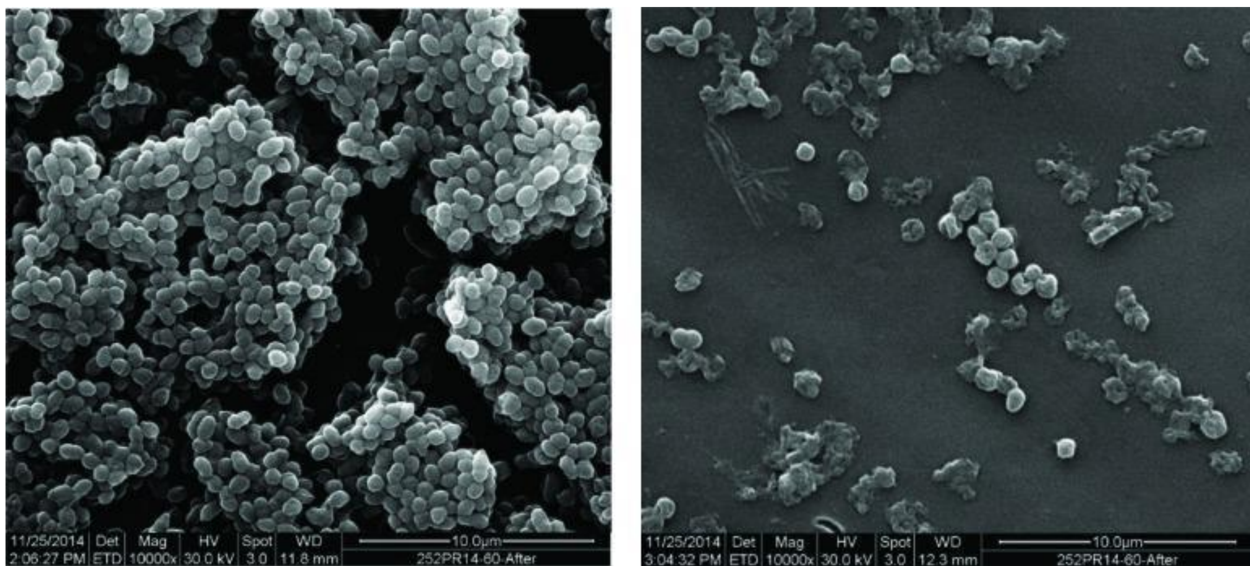


Figure 6.2 1 Scanning electron microscopy images of untreated and treated biofilms with the phage EFDG1. The image on the left shows a well-developed biofilm, while the right image is one with EFDG1 phage treatment. Both the biofilms are 2 weeks old.

The activity of EFDG1 was more tested in post-treated root canal infections using an *ex vivo* two-chamber microorganism escape model of human teeth. The bacteria that leaked from the root apex was measured and indicated that the obturated root canals subjected to EFDG1 irrigation caused dramatic reduction of eight logs in bacterial leakage compared with the standard sample. Confocal research pictures of horizontal root sections incontestable that live bacteria were evident within the dentinal tubules of the management group, whereas dead bacteria were seen in the phage-treated teeth.

In conclusion, considering all the accessible positive outcomes from the usage of phages against not only *E. faecalis* but also different microorganisms in biofilms, phage medical aid appears to be a tool against infectious biofilms. within the future, phages such as EFDG1 and other phages of *E. faecalis* like phiEF24C, IME-EF1, and EFLK1 is used either as cocktails or as combinations with antibiotics to combat VRE *E. faecalis* in dental biofilms.

Chapter 07

Recombinant of the Staphylococcal Bacteriophage Lysin $CHAP_k$ and Its Elimination against *Streptococcus agalactiae* Biofilms

7.1 Introduction

Streptococcus agalactiae of group b (GBS) is one of all the viral pathogens that are related to clinical/subclinical mastitis in bovine and infant infection and infectious disease in humans. Bovine mastitis is the primary health hazard, resulting in a severe reduction of milk production and the result of milk quality. It is responsible for vital economic losses within the dairy farm industry worldwide. The sequence varieties (STs) of *S. agalactiae* in bovine mainly belong to ST67, ST103, and ST568, which contain the virulence characteristics of biofilms formation capability, growth ability in milk, and adhesion ability, and might persist indefinitely within the mammary gland.

Lysins are peptidoglycan hydrolases made by bacteriophages. Among the various sources of bacteriophages, matters show specificity on the pathogen. lysins were active against streptococcus and staphylococci pathogens, inflicting membrane and general infection. Lysins quickly induce microorganism lysis and death by hydrolyzing valency bonds, which are essential for plasma membrane integrity and viability, and consequently develop very little resistance compared with antibiotics. $CHAP_k$, comprised only of the lytic domain, maybe a truncated derivative of native lysin of *Staphylococcus aureus*, a cysteine-and histidine-dependent amidohydrolase/peptidase that specifically cleaves coccus cell wall on the peptide linkage between D-alanine and the 1st glycine within the pentaglycine cross bridge. $CHAP_k$ exhibited sturdy activity against *S. aureus* and settled on the *S. aureus* biofilms. $CHAP_k$ also had medicine activity against streptococcus, which can be regarding the similar peptidoglycan cross-bridge of staphylococcal and streptococcal pathogens.

7.2 $CHAP_k$

$CHAP_k$ nucleotide sequence was with success inserted into the *Xho*I and *Nco*I sites of pET28a to construct the pET28a-$CHAP_k$ plasmid when being confirmed by DNA sequencing.

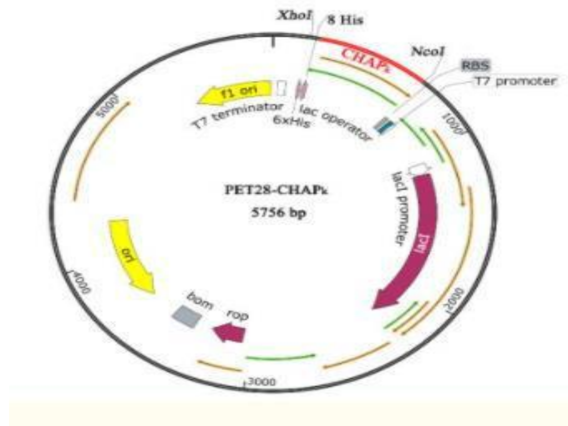


Figure 7.2 1 The schematic diagram of the pET28a-CHAP$_k$ expression vector.

7.3 SEM observation

Scanning electron microscope (SEM) was used to observe the changes in cell of *S. agalactiae* ATCC 13813 in terms of cell morphology, integrity, and cellular structure when treated with CHAPk or antibiotic drug. The untreated microorganism was ovoid and clustered together, displaying morphologic integrality, and also the cell surface was smooth. When treatment with vancomycin, wrinkles on the cell surface were observed, some cells were slightly deformed however, majority of the cells remained undamaged. On the other hand, the quantity of bacteria was significantly reduced, cells were ruptured and wrinkled after the application of CHAPk . This indicated that the vancomycin and CHAPk have absolutely different bactericidal mechanisms.

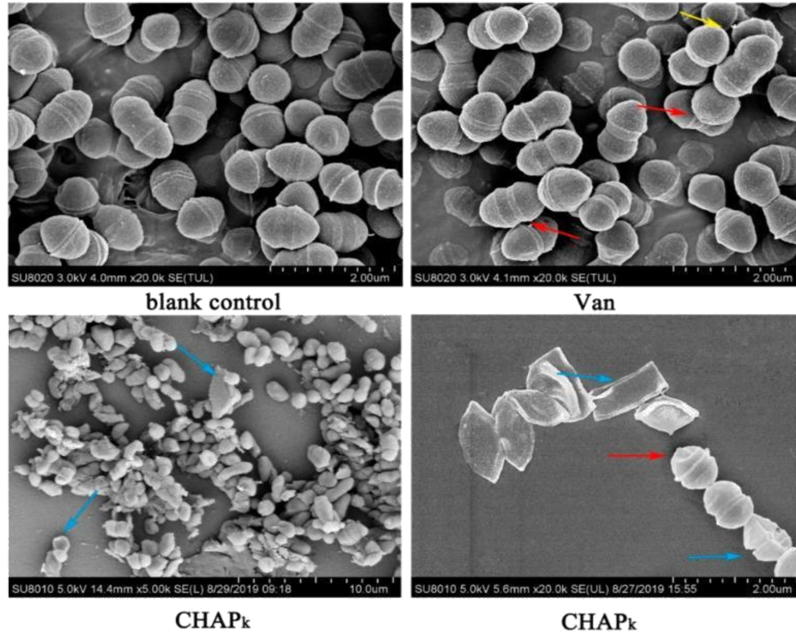


Figure 7.3 1 Scanning electron microscope observation. The magnification of the image is 20,000 (blank control), 20,000 (Van), 5000 (CHAP$_k$-left bottom), and 20,000 (CHAP$_k$-right bottom), respectively. Red arrows: Cell shrinkage; Yellow arrows: Vesicular bulge; Blue arrows: Cell rupture; Van: vancomycin.

7.4 The ability of CHAPk against biofilms and bacteria *S. agalactiae*

7.4.1 Biofilm Formation Capacity of *S. agalactiae* ATCC 13813

According to the analysis criteria of biofilms formation capability supported crystal violet staining method, the absorbance worth of *S. agalactiae* ATCC 13813 (0.361 ± 0.04) was four times above that of blank control (0.089 ± 0.01) at OD570 nm, which showed that *S. agalactiae* ATCC 13813 could be a strong biofilm-forming strain.

7.4.2 Effect of $CHAP_k$ on *S. agalactiae* ATCC 13813 Early and Mature Biofilms

Early biofilm indicated clear signs of cell-cell aggregation. The repressing impact of $CHAP_k$ on early biofilms, totally different concentrations of $CHAP_k$ were exposed to *S. agalactiae*. Once growth of microorganisms in early biofilms.

The growth of microorganisms in early biofilms was investigated by plate counting. *S. agalactiae* ATCC 13813 cells in early biofilms considerably declined once exposed to $CHAP_k$. When a concentration of 0.25–4 × MIC of $CHAP_k$ and antibiotic drug was used there was no difference in microorganism reduction between them. However at the concentrations of 8–32 × MIC, the bacterial treatment with 0.25–8 × MIC $CHAP_k$ or vancomycin, the biofilms were eliminated by 67.3–95% and 67.3–90.9%, respectively, whose inhibitory effect was showed in a concentration-dependent manner. This information advised that $CHAP_k$ has a potent inhibition ability to early biofilms that is comparable to vancomycin.

Peptidoglycan is further bound and fully distributed to form mature biofilms. Compared to the early biofilms, the mature biofilms are harder to remove. After applying to 0.5 × MIC vancomycin only 31% mature biofilms were eliminated. 62.3% mature biofilm was eradicated with 16 × MIC vancomycin application. The elimination rate of mature biofilms with 0.5-16 X MIC $CHAP_k$ concentration was 68.4-89.4%, which was greater than the vancomycin. $CHAP_k$ eradicated the mature biofilms in a concentration-dependent manner. Therefore it terms of eradicating biofilms, $CHAP_k$ was better capable than vancomycin.

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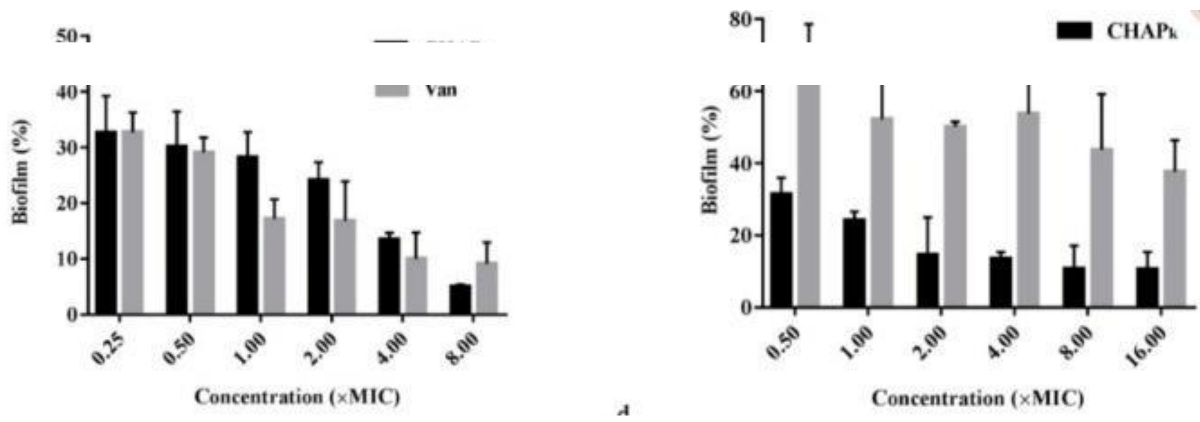


Figure 7.4.2 1 Effects of $CHAP_k$ on S. agalactiae *biofilms and bacteria in biofilms. (**a**) Inhibition of biofilms formation by $CHAP_k$. (**b**) Eradication of mature biofilms by $CHAP_k$.*

Using plate counting method, to examine the reduction caused by CHAPk was superior to it of vancomycin, and there were significant differences between CHAPk and vancomycin groups. CHAPk can result in kill bacteria in early biofilms of *S. agalactiae* in a concentration-dependent manner, which was following results of its repressing effect on biofilms in early stages.

The activity of CHAPk against bacterium in mature biofilms that were immune to antibiotic were evaluated. A concentration of 25 × MIC vancomycin was applied against mature biofilms of *S. agalactiae* ATCC 13813 for 24 hours which resulted in 10^8 CFU/mL to 10^3 CFU/mL reduction of biofilm encapsulated bacteria and none of the colonies were regrown. Within the mature biofilms the bacteria were significantly killed by 99% with 4 × MIC CHAPk in 24 h, which was superior to vancomycin. From the obtained results it can be concluded that CHAPk has the capability to eliminate vancomycin-resistant bacteria.

7.5 Observation of Biofilms by Confocal laser scanning microscopy (CLSM)

The inhibition and eradication effects of biofilms and internal microorganisms, the *S. agalactiae* ATCC 13813 cells were treated with SYTO9 and PI and discovered by CLSM, the thickness of biofilms shaped by untreated *S. agalactiae* in confocal dish reached 20.72 µm. Compared with the untreated group, the biofilms considerably became an agent (thickness of 11.28 µm) and dead bacteria inflated in the $CHAP_k$ treatment group, which was superior to the antibiotic treatment group, implying the strong activity of $CHAP_k$ against *S. agalactiae* and the biofilms.

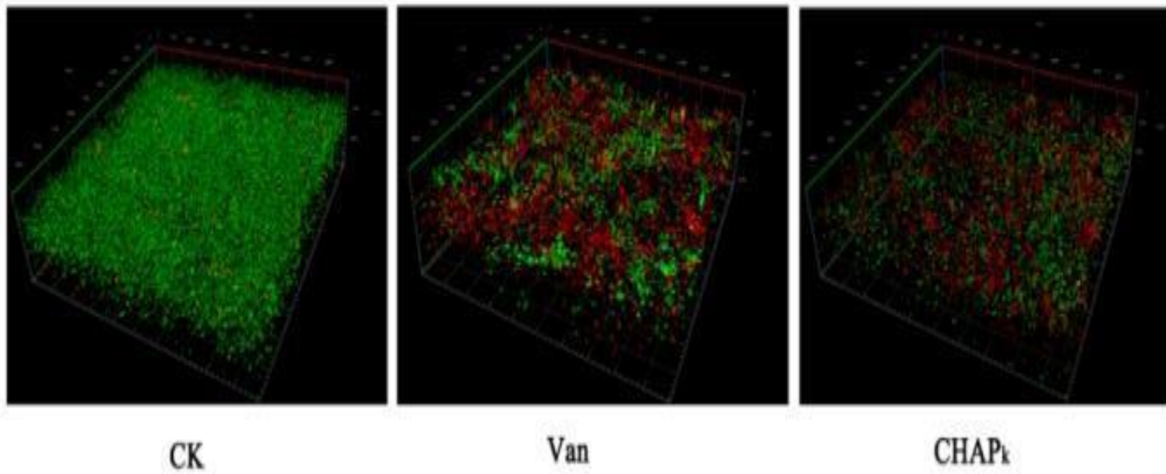


Figure 7.5 1 Observation of S. agalactiae *biofilms by CLSM.* S. agalactiae *was incubated with 8 × MIC $CHAP_k$ or vancomycin for 24 h; after removing planktonic bacteria, biofilms were stained with dyes and visualized by CLSM. Live cells are stained in green by SYTO9 and dead cells are stained in red by PI, CK: the untreated* S. aureus *biofilms. Van: vancomycin. All assays were performed in triplicate*

Chapter 8

Evaluation of Bacteriophage therapy in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram negative bacteria which is responsible for a number of opportunistic infections like open wounds, weakened immune system and chronic pulmonary conditions such a cystic fibrosis. *P. aeruginosa* can change according to its environment in terms of both phenotype and genotype and lives inside biofilms. Therefore multiple multidrug-resistant strains of *P. aeruginosa* are present and their treatment is difficult due to the absence of sufficient effective antibiotics. Hence, in search for an alternative strategy, bacteriophage therapy can be a possible solution.

Bacteriophage therapy has shown effective potential in treating infections caused by antibiotic resistant microbes when used simultaneously with antibiotics. A number of studies proved the effectiveness of Bacteriophage therapy in treating bacterial colony both *in vitro* and *in vivo* of cystic fibrosis patient. Phage therapy is particularly beneficial as they can eliminate the bacterial biofilms. They have been evolutionary developed to eradicate bacteria along with their biofilms through some specific enzymes like polysaccharides depolymerase and alginate lyase. Once these biofilms are degraded they can become accessible to antibiotic or host immune components.

To evaluate such potential, *Pseudomonas* phage KTN4 isolated from sewage has been examined for its biofilm inhibition capability using interferometry and profilometry. In addition to that, the antibacterial capability of this phage was examined with non CF and CF epithelial cells.

8.1 Evaluation of KTN4 phage activity on biofilms

The effectiveness of the phage KTN4 against biofilm was evaluated with various methods. This include:

1. Using Crystal violet assay to analyze effect of biofilm disruption by KTN4 and compare with anti-pseudomonal drug colistin
2. Analysis of pyocyanin and pyoverdin secretion in media by Spectrophotometry and fluorometry
3. Laser interferometry to analyze effect of biofilm disruption by KTN4
4. ZETA-20 non-contact optical profiler to analyze effect of biofilm disruption by KTN4

1. Using Crystal violet assay to analyze effect of biofilm disruption by KTN4 and compare with anti-pseudomonal drug colistin

Pseudomonas aeruginosa (PAO1) biofilm was grown on Nephrophane membrane and treated with colistin, KTN4, inactive KTN4, colistin + KTN4 and colistin + inactivate KTN4 separately at 24, 48 and 72 hours. The results are summarized below. The mass of the biofilm was then evaluated with Crystal Violet staining.

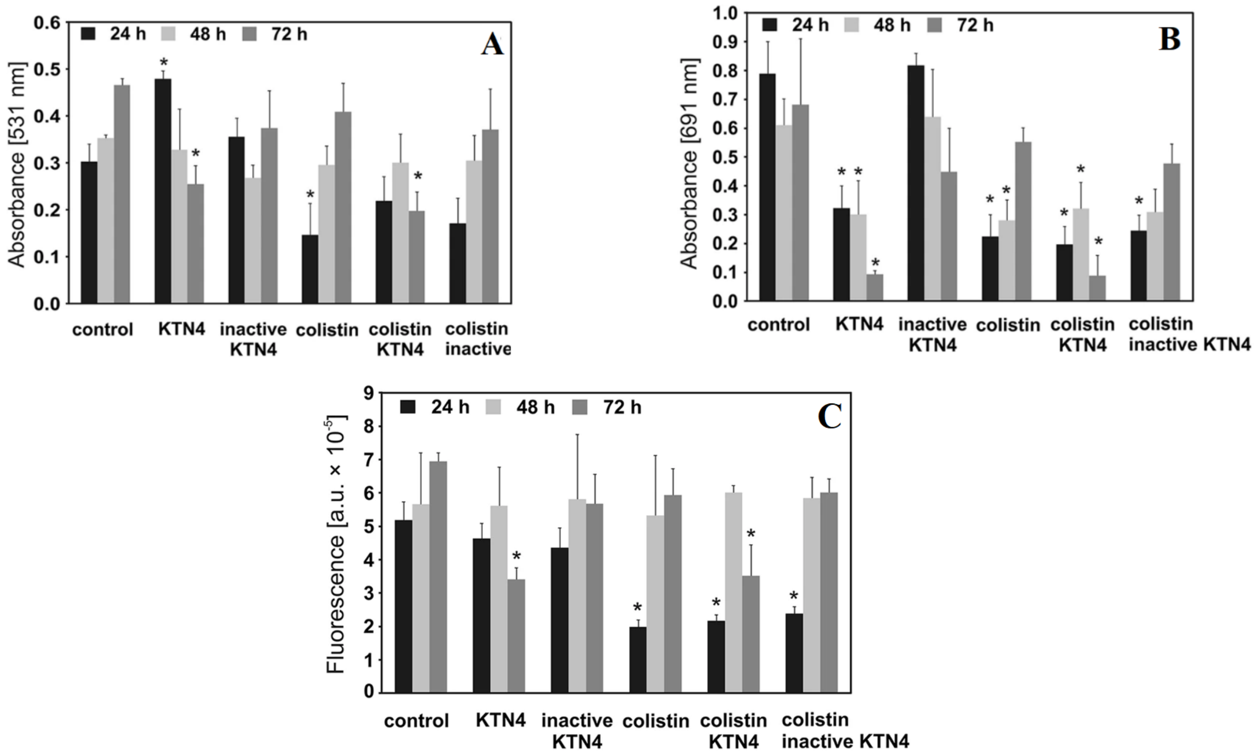


Figure 8.1 1 Anti-biofilm activity by KTN4 and colistin on Nephrophane membrane; (A) biomass evaluation by CV staining; (B) Pyocyanin level in growth medium; (D) fluorescence of pyoverdin in growth medium. Untreated biofilm was used as control.

As shown in Figure 8.1.1 (A) for the 74 hour, the active KTN4 + colistin showed significant biofilm biomass eradication with CV staining. For the 24 hour, colistin alone reduced the biofilm biomass notably. On the other hand intact KTN4 showed increase in biofilm biomass of 24 hour. The inactive KTN4 showed no change in biofilm biomass whether applied alone or combined with colistin.

The results retrieved from CV staining was not efficient. For the results obtained from intact KTN4, it was suspected that the phages propagated in early biofilms resulted in the production exopolysaccharide degrading enzyme which

caused negatively charged moieties to increase in the medium. Afterwards when CV staining was done these moieties were detected as increase in biofilm biomass.

2. Analysis of pyocyanin and pyoverdin secretion in media by Spectrophotometry and fluorometry

Due to the limitation of CV staining, other reliable method was used, which included measuring the quantity of pyocyanin and pyoverdin into the medium with the help of spectrophotometry and fluorometry. As shown Figure 8.1.1 (B) active KTN4, colistin, colistin + KTN4 all decreased the concentration of pyocyanin in the medium at 24, 48 and 72 hours. The active form of the KTN4 significantly reduced the concentration of pyocyanin whether applied alone or with colistin.

As shown in Figure 8.1.1 (C) The pyoverdin concentration was significantly reduced at 24 hour when colistin was applied and also when combined with KTN4. With active KTN4 was applied at 72 hours the pyoverdin concentration reduction was observed.

The integrated application of colistin and KTN4 did not show synergistic effect. The reason behind this can be due to the fact that colistin have its antibacterial effect by destabilizing the cell membrane of the bacteria which makes it difficult for the bacteriophage to propagate within the bacteria. Furthermore, the phage KTN4 recognizes IV type pili hence it does not increase or decrease the colistin activity.

3. Laser interferometry to analyze effect of biofilm disruption by KTN4

The laser interferometry was used to measure the quantitative changes in the biofilm matrix for low molecular compounds. For this purpose the diffusion of TSB medium through the biofilm was measured which was proportional to the degradation of the biofilm. The results obtained are summarized below.

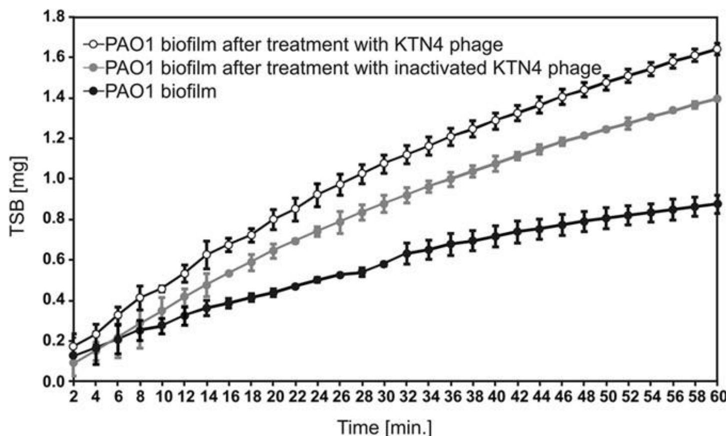


Figure 8.1 2 TSB medium diffusion through PAO1 biofilm treated with KTN4 phage analyzed with laser interferometry. Intact biofilm was used as control.

As shown in Figure 8.1.2, after treating the PAO1 biofilm with active and inactive KTN4 for 4 hours, the diffusion rate was significantly higher compared with intact biofilm. The increased diffusion rate of TSB media through the biofilm indicate the degradation of the biofilms.

4. ZETA-20 non-contact optical profiler to analyze effect of biofilm disruption by KTN4

ZETA-20 non-contact optical profiler is a 3D measurement technique. As shown in figure 3, the yellow colour on the Nephrophane membrane was due to the siderophores produced by the bacterial cells inside the PAO1 biofilm. Upon the application of the phage KTN4 the colour of the biofilm returned back to the native colour of the membrane. This proves that the phage restricted the PAO1 cells from producing siderophores.

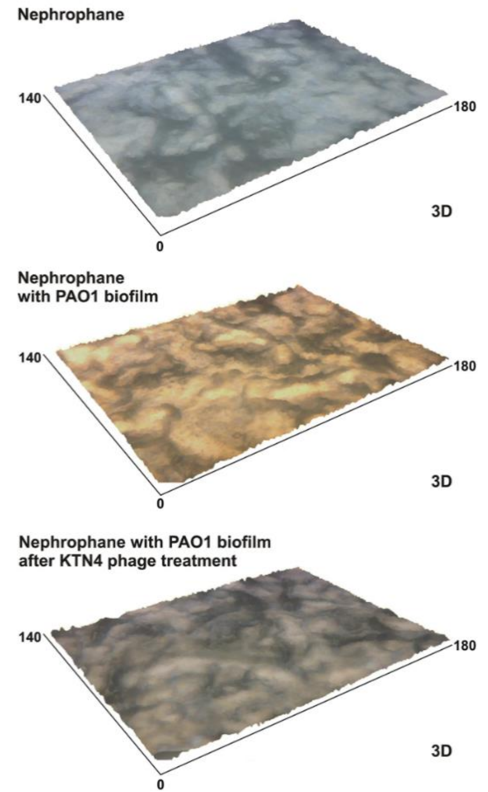
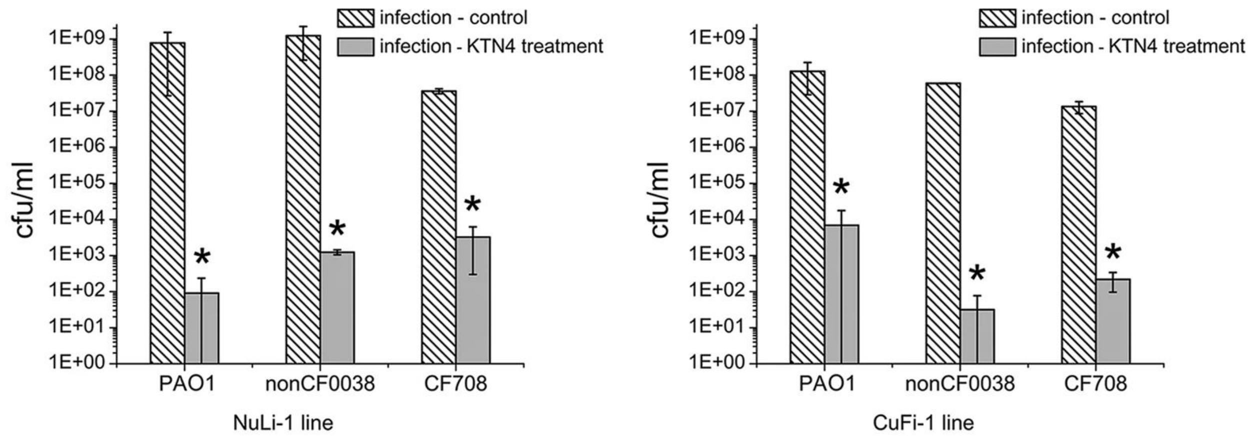


Figure 8.1 3 Nephrophane membrane analysis by ZET 20 instrument, PAO1 biofilm and PAO1biofilm after application KTN4 phage

8.2 Evaluation of KTN4 phage antibacterial efficacy

The antibacterial study of KTN4 was carried out using gentamicin exclusion assay on Airway Surface Liquid (ASL) infection model. For the experiment two cell lines were chosen, which were: 1. Nuli-1 (derived from bronchial epithelium of healthy human) and 2. Cufi-1 (derived from bronchial epithelium of Cystic Fibrosis patient). The strains of *P. aeruginosa* selected were: PAO1 reference strain, nonCF0038 and CF708. Initially all these strains were grown on both the cell lines on ASLs for 3 hours. Then after adding KTN4 phage, elimination of the bacterial

load was evaluated. As shown in Figure 8.2.1, CFU count of *P. aeruginosa* was significantly reduced for both the cell lines NuLi-1 and CuFi-1



8.2 1 Treatment of phage KTN4 to the P. aeruginosa infected NuLi-1 and CuFi-1 epithelial cells

Infection from *Pseudomonas aeruginosa* involve the formation of biofilms and application of the bacteriophage KTN4 to degrade this biofilm and hence prevent infection from *Pseudomonas aeruginosa* was thoroughly examined. It was found through various methodology that the phage KTN4 immediately degraded the biofilms upon its application. Furthermore, the strong antibacterial capability as shown by these phages and the significant inhibition of toxigenic agent pyocyanin and pyoverdin produced by the *P. aeruginosa* proves the ability of these phages to be used in *Pseudomonas aeruginosa* biofilm treatment.

Chapter 9

Anti-biofilm activity comparison between *Klebsiella* phage KP34, its recombinant depolymerase KP34p57 and Ciprofloxacin antibiotic

Klebsiella pneumoniae is a gram negative bacteria having a rod shaped structure and are encapsulated. They have become antibiotic resistant to almost all antibiotics mainly due to having a thick mucoid acidic capsules providing it the protection against detergents and antibiotics. Furthermore, they can efficiently form biofilms thereby having better protection from antibiotics from causing any harm to them. Bacteriophage can provide a solution for protection against *Klebsiella* as they diffuse inside the biofilms with the aid of the enzyme depolymerase. For such purpose, the effectivity of the bacteriophage KP34, its recombinant depolymerase enzyme KP34p57 and the

antibiotic ciprofloxacin have been tested separately and in a combined manner. The biofilm degradation result was obtained through colony count, Live/Dead BacLight Bacterial Viability method and crystal violet staining. The anti-biofilm activity of each of the agents was tested on the multidrug-resistant *K. pneumoniae* 77 strain at 24 hours, 48 hours and 72 hours of incubation.

9.1 Anti-biofilm activity of KP34 bacteriophage

Two concentration or titer of 10^9 PFU/ml and 10^6 PFU/ml of the bacteriophage KP34 was examined for anti-biofilm activity. As shown in figure 1a, 2a and 3a in violet bars, both the titter concentrations significantly reduced the colony count in between 2-3 logs. The highest reduction of colony (3.1 logs) was achieved by the 10^9 PFU/ml on 72 hours biofilm. (Figure 3a)

As shown in figure 1b. 2b and 3b (violet bars) the ratio of LIVE/DEAD cell reduced greatly from 86 to 99.7% for 24, 48 and 72 hours biofilm formation.

With crystal violet staining, (figure 1c, 2c, 3c) the 24 hours biofilm biomass was reduced by 94%, the 48 hours biofilm biomass was reduced by 60% and the 72 hours biofilm biomass was reduced by 67% when they were treated with 10^9 PFU/ml KP34 titer. With 10^6 PFU/ml of KP34 titter only 24 hours biofilm's biomass was reduced by 71% but in the 48 hours and 72 hours biofilm no notable reduction was observed.

9.2 The anti-biofilm activity of the phage depolymerase KP34p57

For examining the anti-biofilm activity of the depolymerase KP34p57, four concentration of the enzyme was used i.e. 7.5 ng/ml (corresponding to 1 minimal halo forming concentration or MHF), 75 ng/ml (10 MHF), 750 ng/ml (100 MHF) and 7500 ng/ml (1000 MHF). With colony count as shown in the figure 1a, 2a and 3a with green bars, no significant reduction was observed for any of the concentration of the depolymerase used.

The LIVE/DEAD cell ratio (figure 1b, 2b and 3b green bars) showed that with 1 MHF of depolymerase at 24hour biofilm reduced slightly by 14% and the 10 MHF concentration reduced the 72 hours biofilm by 12% only. On the other hand, with crystal violet staining the biofilm biomass increased upon applying different concentrations of the depolymerase (figure 1c, 2c and 3c green bars).

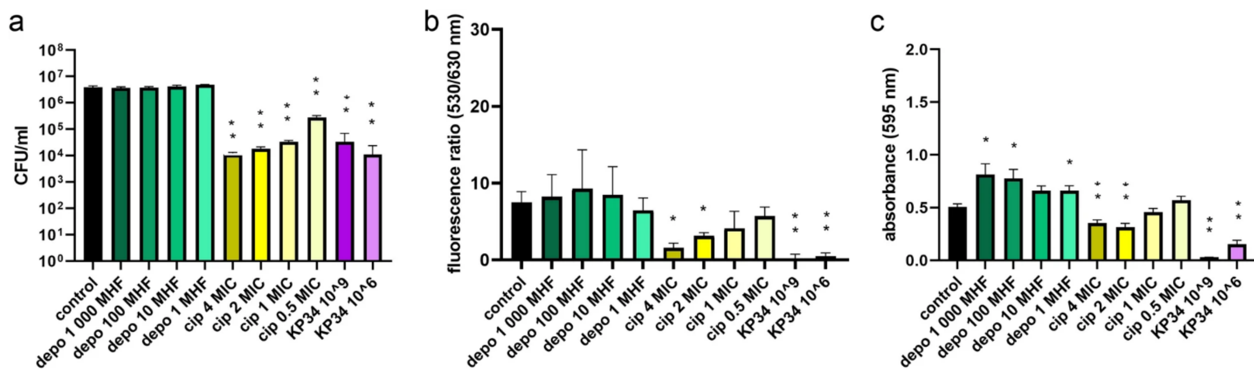
It was concluded that because the polymerase only degrades the capsule, the bacterial cell did not die or causes any change to the bacterial cell membrane.

9.3 The anti-biofilm activity of the ciprofloxacin

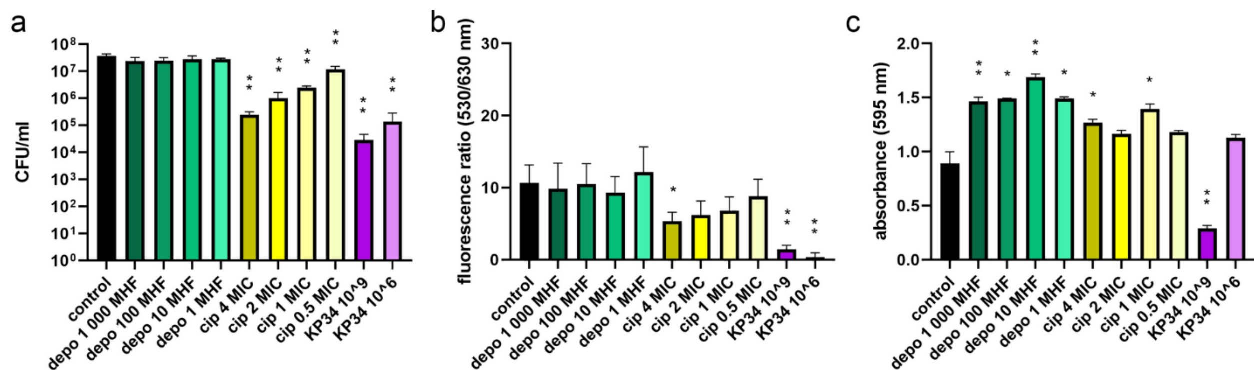
Four concentrations of ciprofloxacin was used to examine its anti-biofilm activity against *K. pneumoniae*. They were: 4 MIC (1 µg/ml), 2 MIC (0.5 µg/ml), 1 MIC (0.25 µg/ml) and 0.5 MIC (0.125 µg/ml). As shown in figure 9.1 a, 9.2 a and 9.3a with yellow bars significant reduction of colony was observed for all the concentrations. The lowest reduction was observed with 0.5 MIC on 48 hours and 72 hours biofilm of 0.5 log and 0.9 log respectively.

With the LIVE/DEAD cell count shown in figure 9.1b, 9.2b and 9.3b (yellow bars) 79% reduction was achieved at 24 hour biofilm. 29% reduction was achieved from 48 hours biofilm and 50% reduction was achieved from 72 hours biofilm by the 4 MIC ciprofloxacin.

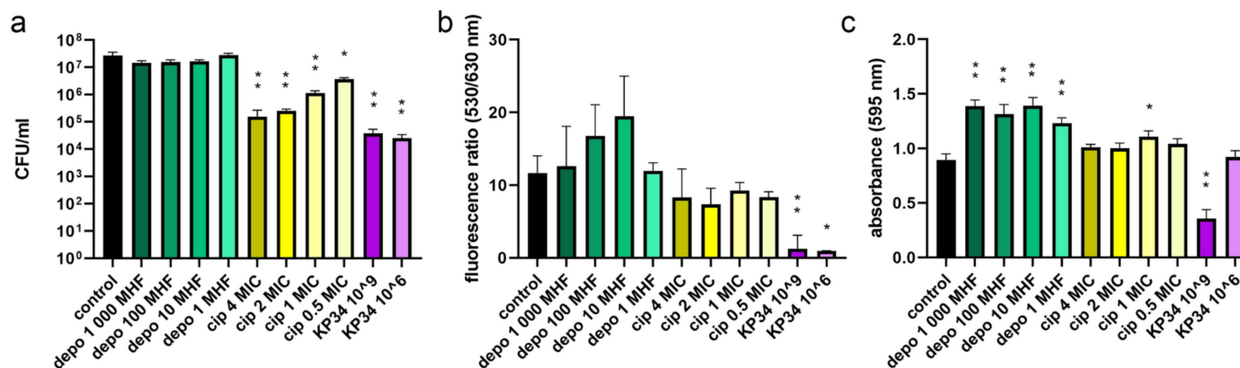
From crystal violet staining result (figure 9.1c, 9.2c and 9.3c yellow bars) biofilm was degraded only with 4 MIC, 2 MIC and 1 MIC of ciprofloxacin by 31%, 39% and 10% respectively for 24 hours biofilm. The 48 hours and 72 hours biofilm biomass increased with all four concentrations of ciprofloxacin.



9.1 1 The anti-biofilm activity of depolymerase, ciprofloxacin and the phage KP34 on K.Pneumniae 77 grown for 24 hours. Biofilm reduction was examined with 3 different methods: (a) Colony count (b) LIVE/DEAD BacLight Bacterial Viability kit and (c) Crystal violet staining



9.2 1 The anti-biofilm activity of depolymerase, ciprofloxacin and the phage KP34 on K.Pneumniae 77 grown for 48 hours. Biofilm reduction was examined with 3 different methods: (a) Colony count (b) LIVE/DEAD BacLight Bacterial Viability kit and (c) Crystal violet staining



9.3 1 The anti-biofilm activity of depolymerase, ciprofloxacin and the phage KP34 on K.Pneumniae 77 grown for 72 hours. Biofilm reduction was examined with 3 different methods: (a) Colony count (b) LIVE/DEAD BacLight Bacterial Viability kit and (c) Crystal violet staining

9.4 The Combined anti-biofilm activity

To examine the combined effect of the phage Kp34, depolymerase and ciprofloxacin 72 hours biofilm was used. The concentration the agents were: phages 10^8 PFU/ml, depolymerase 10 MHF and ciprofloxacin 4 MIC.

With the combination of the depolymerase and ciprofloxacin resulted in 2.2 logs of CFU reduction in cell count (Figure 9.3.1a green and yellowish striped bar), 50% reduction in LIVE/DEAD cell ratio ((Figure 9.3.1 b green and yellowish striped bar) and crystal violet staining showed an increase biofilm biomass (Figure 9.3.1 c green and yellowish striped bar).

The greatest reduction of biofilm biomass was obtained from the combination of ciprofloxacin plus the phage KP34, phage KP34 combined with the phage KP15 and both the phages KP34 and KP15 combined with ciprofloxacin. All these above mentioned combinations resulted in 4-5.7 logs of reduction for colony count, in LIVE/DEAD cell count 82-94% reduction and in crystal violet staining 34-53% reduction of biofilm biomass.

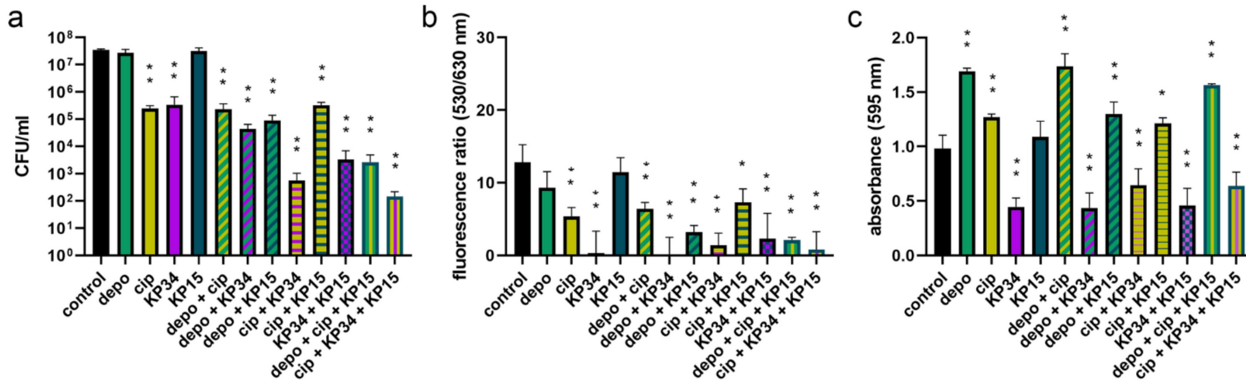


Figure 9.4 1 The anti-biofilm activity of depolymerase, ciprofloxacin and the phage KP34 on K.Pneumniae 77 grown for 72 hours. Biofilm reduction was examined with 3 different methods: (a) Colony count (b) LIVE/DEAD BacLight Bacterial Viability kit and (c) Crystal violet staining

Klebsiella pneumoniae is a multi-drug resistant bacteria that can infect skin, mucosal tissues and intestines. It has been termed as critical priority pathogen by the World Health Organization. The treatment with this *Klebsiella pneumoniae* is also difficult due to its wide range of antibiotic resistance and biofilm formation. With the findings from above, incorporating the phage KP34 to the treatment of *K. Pneumoniae* can have positive impact in preventing its infection. The phage KP34 alone and in combination with the phage KP15 and ciprofloxacin have showed significant decrease in the *K. pneumoniae* biofilm biomass and also the bacteria itself.

Chapter 10

Staphylococcal Bacteriophage Sb-1 as an adjunctive agent to antibiotics against Rifampin-Resistant *Staphylococcus aureus* biofilms

Staphylococcus aureus has been associated with various implant-associated infections like periprosthetic joint infection, fracture-related infections and spinal implant-associated infections (Y. C. Tong, S. Davis, Eichenberger, L. Holland, & G. Fowler, 2015). Along with various other antibiotics used to treat *S. aureus* rifampin has been popular as it can degrade the biofilm produced by the bacteria. However, it is concerning that the increased use of rifampin has led to the development of resistant *S. aureus.* Rifampin has shown no activity against the biofilm of the resistant strains either *in vitro* or *in vivo.* Therefore, there has been search for alternate antibiotics. However none of the antibiotics show *in vivo* activity against the *S. aureus* biofilms. To solve such problem bacteriophage can provide a solution. The *staphylococcal* bacteriophage Sb-1 has been previously used for anti-infective in human and

has been proved suitable for antimicrobial therapy. (Kutateladze & Admia, 2008). Therefore, the effectiveness of the phage Sb-1 has been assessed in combination with several antibiotics (vancomycin, daptomycin, fosfomycin, gentamycin, flucloxacillin, cefazolin and rifampin) either simultaneously or in a staggered manner against 10 strains of rifampin resistant *S. aureus* (RRSA), 4 strains of MRSA, 6 strains of MSSA and MRSA ATCC 4300 and MSSA ATCC 29213 strains.

10.1 Susceptibility of the bacteria against combined effect of conventional antibiotics and Sb-1

At first the bacterial strains were applied with the antibiotics and Sb-1 alone. The susceptibility of the bacteria was measured based on isothermal microcalorimetry, whereby recovered bacteria are detected by measuring growth-related heat production. Both the ATCC strains of MRSA and MSSA were susceptible to most antibiotics. Only the MRSA ATCC 43300 showed resistance against gentamycin. The 10 strains of RRSA was susceptible to all antibiotics. The MRSA 4 showed resistance to fosfomycin and for the 6 MSSA strains MSSA 5 showed resistance to gentamycin.

The exposure of the phage Sb-1 to both the biofilm of ATCC strains had a sharp effect on either strains. When the Sb-1 phage was applied against the strains of MRSA a significant reduction occurred but absolute inhibition of heat production was not obtained compared to the heat production by the control. Against the MSSA strains the activity of the Sb-1 phage was similar to the control. (Shown in Figure 10.1.1)

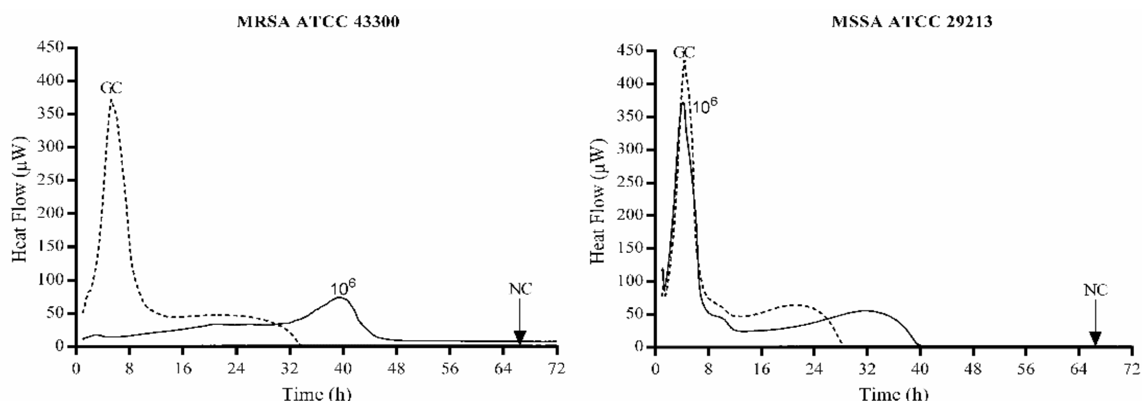


Figure 10.1 1 Microcalorimetry analysis of MRSA ATCC 43300 (right plot) and MSSA ATCC 29213 (left plot) biofilms treated with Sb-1 (10^6 PFU/ml). Each curve shows the heat produced by viable bacteria present in the biofilm after 24h of exposure to Sb-1 (continuous line) or no exposure (dotted line). GC, growth control; NC, negative control. Data of a representative experiment are reported.

10.2 Activity of the combined effect of phage Sb-1 and antibiotic against ATCC strains

With the combination of the phage and vancomycin or daptomycin the strongest synergistic effect was found against MRSA ATCC 43300 strain. With the combination of phage and gentamycin no synergistic effect was found. Against the MSSA ATCC 29213 no synergistic effect was found with the combination of the phage and any antibiotics. When the phage was applied against the MRSA ATCC 4300 biofilm 24hours prior to the addition of the antibiotics, more delayed and reduced heat was observed from the isothermal microcalorimetry in comparison to when the phage and the antibiotics were applied simultaneously.

10.3 Activity of the combined effect of phage Sb-1 and antibiotic against clinical rifampin-resistant *Staphylococcus aureus* strains

All the four strains of MRSA was observed to have been effected by synergistic effect of the Sb-1 and daptomycin combination when applied both simultaneously and in staggered manner. Three out of the four strains of MRSA was observed to have synergistic effect by the Sb-1 and gentamycin combination when applied in a staggered manner. Two out of the four strains were susceptible to the synergistic effect of fosfomycin and Sb-1 combined. With the Sb-1 and vancomycin combination no synergistic effect was observed.

Upon combined addition of Sb-1 and cefazolin or flucloxacillin in a staggered manner, synergistic effect was shown on four out of the six MSSA isolates. Against three out of the six strains of MSSA Sb-1 and flucloxacillin combination showed synergistic effect when applied simultaneously. Also, synergistic effect was shown by the Sb-

1 and gentamycin combination against three out of the six strains when applied simultaneously or staggered manner. Against MRSA or MSSA no synergism was found when Sb-1 and rifampin was added.

From the obtained result it can be seen that the best choice for creating a therapy of antibiotic with phage Sb-1 as an adjunctive agent would be the combination of Sb-1 with daptomycin either applied simultaneously or staggered manner, because it showed the highest activity against all MRSA biofilms. On the other hand no biofilm degrading activity was found with the combination of Sb-1 and vancomycin. Therefore, it can be said that it is possible to eradicate rifampin-resistant *S. aureus* biofilms *in vitro* with the combined effect of Sb-1 bacteriophage and antibiotics.

Chapter 11

Phage therapy limitation for inhibition of bacterial biofilm

Inhibition of bacterial biofilm by phage therapy there are some limitations. They are: -

Biofilm extracellular matrix limit the diffusion

The extracellular polymeric substance called EPS consists of 90 % of the biofilm mass and creates a three-dimension form of biofilm. EPS prevents the diffusion of the antimicrobial agents through the bacterium by covering bacteria cells. Furthermore, phages might have initial reversible interaction with some elements like capsule polysaccharide, teichoic acids, and lipopolysaccharides, however, cell wall components are necessary for irreversible attachment.

Narrow host range

The molecules on the surface of bacteria, similar to lipopolysaccharide and peptidoglycan components, outer membrane proteins, and teichoic acids, might be the attachment sites for the phage tail. Also, the host vary is decided by the specificity of phage receptors. Furthermore, some phages have a broad spectrum, whereas other phages have a narrow spectrum of host range. Narrow host range could be problematic, particularly in polymicrobial biofilms that are formed on medical devices.

Sub-populate phage resistance in biofilm

Becoming resistant to bacteriophages is critical for the survival of bacterium, and this happens by four completely different mechanisms. These mechanisms offer bacteria adoption to phages and build a phage resistance mutant.

There are reports concerning the rapid growth of phage resistance sub-populated when the first reduction of biofilm cells that were treated with phages.

Reduction in metabolic activity of biofilm bacterial cell

Since phage infection strictly depends on the expansion condition of its host, one of all the obstacles within the productive use of phage medical care against microorganism biofilm is that the reduction of metabolism. a microorganism that is a gift in biofilms are underneath nutrient-limited conditions, and that they grow slowly. On the opposite hand, phage infection relies on the resources of bacteria, which is directly relating to the physiological state, and it's expected that phage infection in organism bacteria is additional efficient than biofilm bacteria.

Inhibition of phage infection via Quorum sensing (QS) in biofilm

Many bacterium use QS as a communication system between cells with living thing chemical molecules referred to as auto-inducers. QS permits bacteria to coordinate organic phenomenon and density of the cell population. Furthermore, it's accepted that QS controls biofilm formation, growth, and dispersion, and there are suggestions for inhibiting biofilm formation by disabling QS.

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