PHAGE ACTIVITY AGAINST MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA* BIOFILM FORMATION ON MEDICAL IMPLANTS

By

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DECLARATION

I, the undersigned, declare that this thesis is my original work, has not been presented for a degree in this or any other university and that all sources of material used for the thesis have been fully acknowledged.

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APPROVAL SHEET FOR SUBMITTING FINAL THESIS

As members of the Board of Examination of the Final MSc. Thesis open defense, we certify that we have read and evaluated the thesis prepared by **Stephen Amankwah** under the title **Phage activity against multidrug-resistant *Pseudomonas aeruginosa* biofilm formation on medical implants** and recommend that, the thesis be accepted as fulfilling the thesis requirement for the Degree of Masters of Science in Medical Microbiology.

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ABSTRACT

Background: Phage therapy is reemerging due to the rise in antimicrobial resistance. Despite the growing interest in the use of bacteriophage (phage) for the prevention, control and removal of bacterial biofilms, limited scientific data exist on phage application to serve as dual purposes of preventing bacterial colonization and removing preformed biofilms on medical implant surfaces.

Objective: The study objective was to isolate, partially characterize and assess phages as potential antibiofilm agents to prevent and/or reduce multidrug-resistant (MDR) *Pseudomonas aeruginosa* clinical isolate biofilm from medical implant surfaces at Jimma Medical Center (JMC).

Methods: Seven well identified clinical strains of MDR *P. aeruginosa* were obtained from different specimens of various patients at JMC. Specific phages were isolated and characterized based on standard protocols. The phages were tested for their antibiofilm effects after coating the phage in preventing colonization as well as for their treatment effects in reducing preformed biofilms of MDR *P. aeruginosa* on catheter and endotracheal tube segments.

Results: Two *P. aeruginosa* specific phages (ΦJHS-PA1139 and ΦSMK-PA1139) were isolated from JMC compound sewage sources. The phages were partially characterized of being thermally stable up to 40ºC and viable between pH 4.0 and 11.0. The two phages tested against six clinical MDR strains of *P. aeruginosa* showed broad host ranges but not on other tested bacterial species. Both phages reduced MDR bacterial biofilms during screening step. The phage-coated segments showed 1.2 $\log_{10}$ up to 3.2 $\log_{10}$ inhibition relative to non-coated segments after 6 h of exposure to microbial load. In both phages, 6 h treatment of the segments with 10^6 PFU/mL yielded 1.0 $\log_{10}$ up to 1.6 $\log_{10}$ reductions for ΦJHS and 1.6 $\log_{10}$ up to 2.4 $\log_{10}$ reductions for ΦSMK.

Conclusion: The results of this study suggest that phages in this study have great potential for the development of surface coating agents for preventing MDR bacterial colonization of medical implants and biofilm removal agents in implant-associated infections.

Keywords: *Bacteriophage; Biofilm; Multidrug-Resistant P. aeruginosa; Catheter; Endotracheal tube.*

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ACRONYMS AND ABBREVIATIONS

AHL	N-acyl homoserine lactones
AI	Autoinducer
AIP	Autoinducer oligopeptides
AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
CA	Columbia agar
CAUTIs	Catheter-associated urinary tract infections
CDC	Centers for Disease Control and Prevention
c-di-GMP	cyclic-di-GMP
CFU	Colony forming units
CLABSIs	Central line-associated bloodstream infections
CRISPR	clustered regularly interspaced short palindromic repeats
DGC	diguanylate cyclase
DISARM	defense island system associated with restriction modification
eDNA	extracellular DNA
EPS	Extracellular Polymeric Substance
ET	Endotracheal tube
HAIs	Hospital-acquired Infections
IRB	Institutional Review Board
JMC	Jimma University Medical Center
MDR	multidrug-resistance
NB	Nutrient broth
OD	Optical density
PA	*Pseudomonas aeruginosa*
PBS	Phosphate buffered saline
PFU	Plaque forming units
QS	Quorum sensing
RM	restriction modification
RT	room temperature
SD	standard deviation
SM	saline magnesium
SSIs	Surgical site infections
TCS	two-component system
TSB	tryptic soy broth
VAP	Ventilator-associated pneumoniae

CHAPTER ONE

INTRODUCTION

1.1 Background

Bacteriophages (Phages), first identified and characterized independently by Frederick Twort in 1915, Felix d'Herelle in 1917 and earlier by Ernest Hankin and Nikolay Gamaleya in 1896 and 1898, respectively, are bacterial viruses that infect bacterial cells with high specificity (1). Phages are reckoned to be the most bountiful life forms on earth with numbers estimated to be 10 times more than their bacterial hosts (2). Two types of phages exist depending on the life cycle: virulent bacteriophages, which manifest lytic cycle by replicating their genome and subsequent release of assembled progeny phages causing abrupt cell destruction and lysis of the host cells; and temperate phages , which express lysogenic cycle by persisting as prophages within the genome of the host bacteria to achieve a co-existing, which may later undergo the lytic cycle following induction by environmental stimulus (3). As such, temperate phages are mostly avoided for direct use as therapeutics due to transduction of genetic material from one bacterial cell to another. Moreover, temperate phages may also transmit genes that increase the virulence and or antimicrobial resistance (AMR) of the host. A bacterial cell harboring a prophage within its genome may become immune to infection by the same or closely related phages (4,5). It has been suggested that the ability of lytic phages to replicate and rapidly destroy bacteria regardless of their AMR patterns make them the ideal candidates for use as agents in phage therapy and for destroying biofilm-forming bacteria (4).

Phage therapy was being practiced once globally before the advent of antibiotics. It has been reemerging recently in the world due primarily to the threat posed by increasing incidence of antibiotic-resistant bacteria coupled with the paucity of new antibacterial drugs invention (6). Similarly, the ineffectiveness of antibiotics on biofilms has brought about a growing scientific interest in phages as an alternative strategy in controlling and preventing biofilm formation (7). Indeed, the interaction of phages and biofilms as a subject of research in scientific publications has jumped up exponentially in the last decade (8–11). Reports from the majority of studies on application of phages and phage-encoded proteins as alternative approaches to control and prevent biofilms formed on medical devices are encouraging (12–14). In this regard, this study aimed to focus on phage-biofilm prevention and/or phage-biofilm removal on the surfaces of medical implants.

1.2 Problem Statement

Pseudomonas aeruginosa (*PA*), a ubiquitous, opportunistic and notorious biofilm-forming bacteria, causes a wide range of severe lifethreatening hospital-acquired infections (HAIs). These are associated with contamination of medical devices, equipment used in hospitals and other hard or liquid surfaces which act as reservoirs for biofilm-acquired infections. The ability of *PA* to remain viable on medical devices or grow in use-dilutions of disinfectants is unparalleled as survival advantage results presumably from its nutritional versatility, its unique outer membrane that constitutes an effective barrier to the passage of antimicrobials, and/or efflux systems (15). As a more frequently transferable bacteria in clinical settings, *PA* is responsible for high rates of morbidity and mortality due to its resistance to several antibiotics which is attributed to multidrug efflux pumps, extracellular polymeric substance (EPS) protected biofilm which makes its outer membrane impermeable to antibiotics, mutation and aquisition of resistance genes (16).

During 2015-2017, the National Healthcare Safety Network managed by the Centers for Disease Control and Prevention (CDC) reported that more than 28,000 *PA* were isolated from adult HAIs in the United States representing 8.0% of total pathogens isolated. Adult HAIs reported include device-associated: central line-associated bloodstream infections (CLABSIs), catheter-assocaited urinary tract infections (CAUTIs), ventilator-associated pneumoniae (VAP); and surgical site infections (SSIs). *P. aeruginosa* was among the 3 most frequently reported CAUTI and VAP pathogens in device-associated adult HAIs. In CAUTI, *PA* was reported from 22.6% of long-term acute-care hospitals, 15.4% from inpatient rehabilitation facilities, 13.2% from hospital oncology units and 12.8% from hospital wards and intensive care units. Likewsie, in VAP, *PA* was reported from 32.6% of long-term acute-care hospitals, 21.8% from hospital wards and 12.9% from hospital intensive care units (17). In Sub-Saharan Africa, inadequate comprehensive data exist on the burden of *PA* in HAIs due to lack of resources for surveillance system. However, studies available in Ethiopia reported 14.3-18.4% isolation rate of *PA* in HAIs (18,19).

On AMR pattern of device-associated HAIs, *PA* exhibits 26.2% resistance to fluoroquinolones (ciprofloxacin or levofloxacin), 20.7% resistance to carbapenems (imipenem, meropenem or doripenem), 20.3% resistance to extended-spectrum cephalosporins (cefipime or ceftazidime), 15.0% resistance to piperacillin or piperacillin/tazobactam and 14.4% resistance to aminoglycosides (amikacin, gentamicin or

tobramycin). In SSIs, *PA* exhibits 11.0%, 9.1%, 10.2%, 7.7% and 5.8% resistance to the antibiotic classes above in the same order respectively. The multidrug-resistant (MDR) pattern, operationalized as resistance to one agent in at least three of the different antibiotic classes above, showed that *PA* exhitbits 14.2% resistance in device-associated HAIs and 4.5% resistance in SSIs (17).

The potential threat of MDR *PA* biofilms on medical devices and implants has resulted in growing interest in the development of antimicrobial-coated biomaterial. The presence of antimicrobial materials such as metals, quaternary ammonium compounds, chlorhexidime, hydroxyapatite, nanoparticles, antibiotics, and antimicrobial peptides, have demonstrated decrease in biofilm formation on the device surfaces (20,21). However, these antimcrobial-coated biomaterials have been implicated in bacterial resistance in some studies (21–23). Novel strategies to prevent device-associated infections by material scientists, biologists and microbiologists are urgently needed. Hence, new approaches to control MDR *PA* on medical devices and implants should be investigated. For microbiologists, this include the use of bacteriophages.

Recently, new lytic bacteriophages able to control and also prevent colonization of endotracheal tube (ET)-associated *PA* biofilms were described (14,24). In addition, new lytic bacteriophage able to efficiently reduce number of viable *E. faecalis* cells in biofilms formed on foley silicone catheters was reported (13). Although promising, there are very limited reports on bacteriophage applicability to prevent bacterial colonization and to reduce preformed biofilms associated with medical implants. As one of the most frequently isolated pathogen in CAUTIs and VAP, the burden of *PA* in catheters and ET cannot be over-emphasized. It is thus essential to aim this study on phages that could serve dual purposes of preventing MDR *PA* colonization and reducing preformed biofilms on catheter and ET surfaces.

1.3 Significance of the Study

The study is to provide information on the effect of newly isolated bacteriophages on MDR *PA* in terms of bacterial colonization prevention and/or removing preformed biofilms on surfaces of medical implants in resource limited settings. This information will bring to view the presence of alternative antimicrobial agents from environmental sources and their applications on medical implants. Moreover, this information will be helpful to establish the

baseline data for further *in vitro* investigations on the prophylactic and therapeutic effects of bacteriophages on medical implants in resource limited settings. The findings of these i*n vitro* studies of potential therapeutic bacteriophages will be useful to implant-coating producing companies. This will ensure that only the most effective phages progress to *in vivo* studies and future preclinical and clinical based trials on their capability to lyse pathogens in planktonic and biofilm formations with wide host range coverage for the benefit of mankind. Finally, this study is to add onto the existing knowledge about phages against *PA* biofilms which have the potential of serving as alternative remedy against MDR strains to combat AMR.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Apart from their natural existence as discrete cells in planktonic form, most bacteria are capable of forming multiple cell structures called biofilms by adhering to abiotic and biotic surfaces. Bacterial biofilm formation is seen as one of the most paramount strategies of survival in biotic system which increases the virulence to be more pathogenic and consequently contributes to further resistance to antibiotics (25). Biofilms formed on human tissues and surfaces of medical implants are implicated in the pathogenesis of chronic bacterial infections such as urinary tract infections, pneumonia, orthopedic infections, otitis media & oral infections, wound infections, and cystic fibrosis among others (8,26). Bacterial biofilms, in spite of their beneficial use in other fields of interest such as for biological stages of wastewater treatment, waste reactors, numerous food production systems, and space travel (27), they have enormous negative implications on human health in medicine. In the search for effective alternative approaches to combat biofilm-forming bacteria and their associated infections, it is essential to discuss phages and bacterial biofilms.

2.2 The Bacterial Biofilm

2.2.1 The Genesis of Bacterial Biofilms

Biofilm is a complex integrated aggregate of bacterial cells associated with adherence to both biotic and abiotic surfaces. This microbial or bacterial community is encased in a matrix of hydrated EPS produced predominantly by the cells themselves within the biofilm structure (25). The EPS matrix consists of polysaccharides (40-95%), proteins (1-60%), lipids (1-40%), nucleic acids (extracellular DNA (eDNA) and RNA) (<1%), lipoproteins, enzymes, and inorganic components. Typically, 2-35% of the biofilm volume is composed of the microorganisms (less than 10% in bacterial biofilms) while the matrix accounts for about 90% of the total biofilm mass (28,29). Clearly, variation exists in the physical and chemical constituents of biofilm between different species of bacteria depending on the microorganisms type, stress level, nutrients availability, and host environment (30). As one of the critical steps in biofilm formation, the matrix provides structural support and protection

for the bacterial communities. Besides offering architectural stability and forming a defense shield from antimicrobial factors, the bacterial extracellular matrix plays alternative roles such as serving as signal targets and amplifiers, mediation of migration and colonization, capturing of cations, and exchange of genes (31). Pores and channels within the biofilm aid in the transport of nutrients, gases, water and other molecules within the matrix and between the biofilm and the environment. It is noteworthy that the main component of the matrix is water (up to 97%), which baths the architectural and functional components of the matrix (25). In effect, by forming biofilm, bacteria are able to adapt to the surrounding environment and also able to survive in hostile environmental conditions.

2.2.2 Bacterial Biofilm Formation

Biofilm formation is a complex cooperative group process which occurs in step-by-step processes and involves chemical communication within and between cells. This cell-to-cell communication system is coordinated by crosstalk of various signaling networks including two-component systems (TCS), diguanylate cyclase (DGC) systems and quorum sensing (QS) (8,32). The TCS, composed of histidine sensor kinase and response regulators, regulate signal transduction *via* phosphorylation or cyclic di-GMP (c-di-GMP), a secondary messenger, which permits DNA specific binding for modulation of gene expression. As a key signal network molecule synthesized by the DGC system, c-di-GMP level depends on membrane or cytoplasmic DGC and phosphodiesterase activities working solely or as part of TCS. Signal transduction by c-di-GMP involves allosteric modification of enzymes, interaction with transcription factor, riboswitch and participation in post-transcriptional and post-translational processes within the cell. These activities by c-di-GMP coordinate the transition of bacteria from planktonic to biofilm growth mode (32).

The QS system involves two main factors, the autoinducer (AI) and the receptor, which is based on population cell density or concentration. The AIs are small diffusible signaling molecules produced by the bacteria, which are detected by the receptors once a concentration threshold is achieved (33). Gram-negative and -positive bacteria secrete N-acyl homoserine lactones (AHL) and autoinducer oligopeptides (AIP) as signaling molecules, respectively, as well as AI-2 (16,33). The binding of the AIs to their cognate receptors activates specific gene expression including biofilm formation and antibiotic resistance among others (32). Biofilm formation occurs within and between species of bacteria as well as interkingdom signaling among plants, fungi, and host cells, suggesting that microorganisms in a biofilm interact by

self-talk, crosstalk and listen in (34). Biofilm formation proceeds in four stages: (i) adhesion (ii) microcolony formation (iii) maturation (iv) dispersion (Figure 1).

2.3 Burden of Biofilm Occurrence in Medical Field

Biofilm control and prevention is an enormous problem currently for the food industry, agricultural sector and the medical field. The widespread occurrence of bacterial biofilms in every habitat on earth (35), including biofilms on medical device surfaces and in human tissues, pose a major threat causing chronic infections. According to CDC, it is estimated that biofilms are responsible for over 65% of all chronic bacterial infections, while the National Institutes of Health estimates around 80% of microbial infections and over 60% of nosocomial infections (36). Biofilm formation on or within medical devices, implants, and prostheses, cause device-associated infections such as urinary tract infections, orthopedic infections, endocarditis, periodontitis, gingivitis, osteomyelitis, cystic fibrosis, pneumonia, and wound infections, notably by MDR bacteria (37).

Biofilm burden in medical field is exacerbated by antibiotics resistance of bacteria in biofilm communities contributing to persistent infections. With about 500-5000-fold increase in resistance to antibiotics compared with nomadic cells (38), bacterial biofilms formation has rendered conventional antibiotics ineffective and insufficient at eradicating biofilm-mediated infections (39). Furthermore, bacteria in biofilms are tolerant to antiseptic agents, germicides and the response of host immunity regardless of their location (33,39). The tolerance of bacterial biofilms to antimicrobials depends on several factors which stem from different intrinsic and acquired resistance mechanisms of bacteria. Notable among them is the generation of semi-dormant cells from the deepest biofilm regions with reduced metabolic activity called persister cells (33).

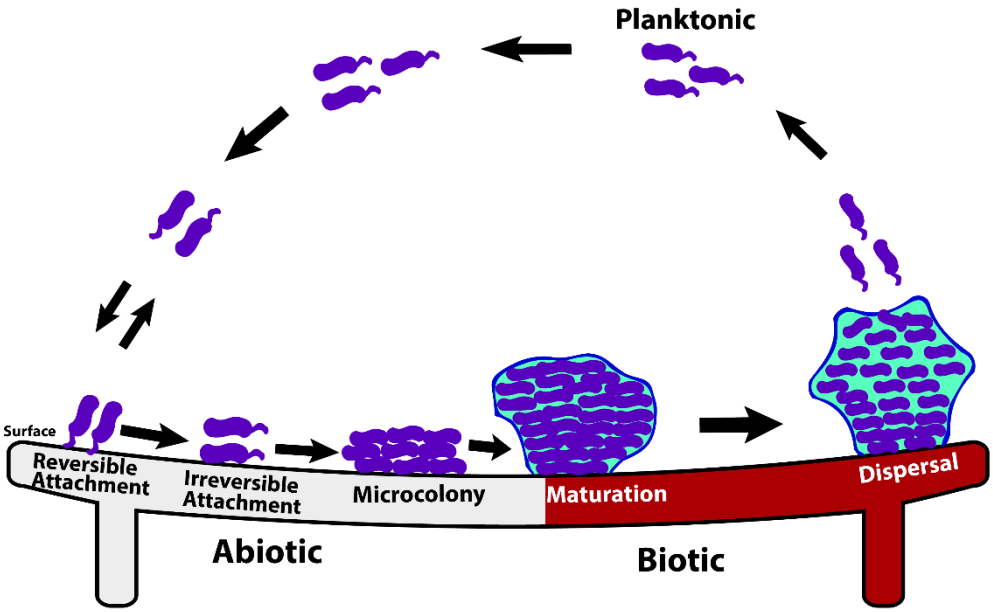

Figure 1. Schematic representation of biofilm formation stages. Formation of biofilm begins with reversible and irreversible adhesion of planktonic cells to the surface. Then, bacteria start to multiply and form micro-colonies which develop into the mature biofilm. In the last stage, bacterial cells multiply quickly, and start to detach and disperse. This process enables the immotile bacteria to convert to motile forms that can help to spread and colonize new surfaces (40).

As reported, resistance of bacteria in a biofilm may be due to (a) restriction of antibiotics diffusion by polymeric matrix, (b) interaction of the biofilm matrix with antibiotics that can retard and lower their activities, (c) action of the modifying enzymes such as β-lactamases or aminoglycoside adenylyl transferases, (d) alteration in metabolic activity inside the biofilm (chemical microenvironment), (e) genetic modifications of target cells or camouflaging the target sites, (f) slow growth rates of bacteria in which drugs are not effective, (g) generation of persister cells which are tolerant to different antibiotics, (h) multiple microbial species, (i) extrusion of antibiotics using efflux pumps, and (j) the age of the biofilm. Thus this multifactorial nature of bacterial biofilm formation and antimicrobial resistance impose great challenges for the use of conventional antibiofilm therapeutic strategies (28,33).

2.4 Bacteria–Phage Co-Interaction within the Biofilm

As natural enemies of bacteria, phages are perfectly adapted to infect biofilms using different mechanisms such as by degrading the extracellular matrix, penetrating the biofilm and infecting the bacteria (Figure 2). One of such mechanisms is the stimulation of the host bacteria to produce EPS-degrading enzymes. These host induced enzymes breakdown the rich polysaccharides and proteins within the extracellular matrix to facilitate phage penetration, replication, and elimination of the bacteria present in various metabolic states *via* lytic activity (41). In addition, phages can express enzymes with exopolysaccharide degrading activities (polysaccharide depolymerases) that degrade extracellular polymers by digesting the polysaccharide matrix and proteins in the biofilm that surrounds the bacteria as well as polysaccharide forming capsules and lipopolysaccharides. This process clears the bacterial protection barrier and then allows the entry of phage particles into the biofilm to replicate within the bacteria (42). High rates of phage replication occur given the high densities of bacteria in the biofilm structure. Phage induced bacterial lysis results in the release of progenies as they achieve local lysis of susceptible cells and as associated enzymes weaken the bacterial cell wall and degrade EPS within the biofilm. Lytic phages retain lytic activity against persister cells with reduced metabolic activity and the associated enzymes degrade EPS within the biofilm (43). Lysogenic phages can also integrate into the bacteria genome causing the bacteria to float naturally without adhering to surfaces to initiate the formation of mature biofilms (42).

Biofilms defensive mechanisms can resist phage infection by affecting phage adsorption, penetration, diffusion, and proliferation within the formed biofilms. Factors such as structure and thickness of the biofilm matrix, age of the biofilm, physiological heterogeneity within the biofilm, and the bacterial species or strains that form the biofilm in multispecies state, may limit phage infection and activity of the biofilm (44). Another interesting mechanism to prevent infection of phages is to specifically recognize the nucleic acids of the phages and destroy them. Bacteria use restriction modification (RM) system, defense island system associated with restriction modification (DISARM), prokaryote argonaute proteins and clustered regularly interspaced short palindromic repeats (CRISPR) – Cas9 to prevent phage infection (45). As a final barrier to phage infection, the bacteria can use an abortive infection system that leads to the death of the infected cell, preventing the spread of phages through the community (42).

To overcome these resistance mechanisms, phages have developed several strategies, however. Phages are equipped with specific enzymes such as hydrolases, endolysins and depolymerases to overcome the structure, thickness, composition, and age of the biofilm with associated matrix (46). Phages can diffuse through biofilm water channels and penetrate the inner biofilm layers (47). Phages can also adsorb reversibly to the appendices of motile bacteria to penetrate inside the biofilm (48). Phages can tackle persister cells through the release of intracellular material which triggers the metabolism of the persister cells for phage infection and replication (8). Strategies by which phages escape the bacterial immune systems include the potential escape from RM systems by lacking the endonuclease recognition site throughout the genome, acquisition of point mutations in the spacer sequence and production of anti-CRISPR protein which interferes with the system to escape the CRISPR/Cas9 system (49).

The interaction of phages with bacteria is often seen as an antagonistic co-evolutionary cycle. The presence of phages may contribute to active biofilm formation as eDNA release through phage-mediated cell lysis by prophages, is responsible for horizontal gene transfer, which triggers stringent response of stabilizing the biofilm matrix (50,51). Phage interaction with bacteria may cause changes in the biofilm matrix leading to enhanced biofilm adhesion, virulence, dispersion of biofilms, colony variation and antibiotic tolerance (52). Owing to the co-evolution mechanism, phages are thus seen as actively involved in biofilm formation either as promoting or destructing agents (53).

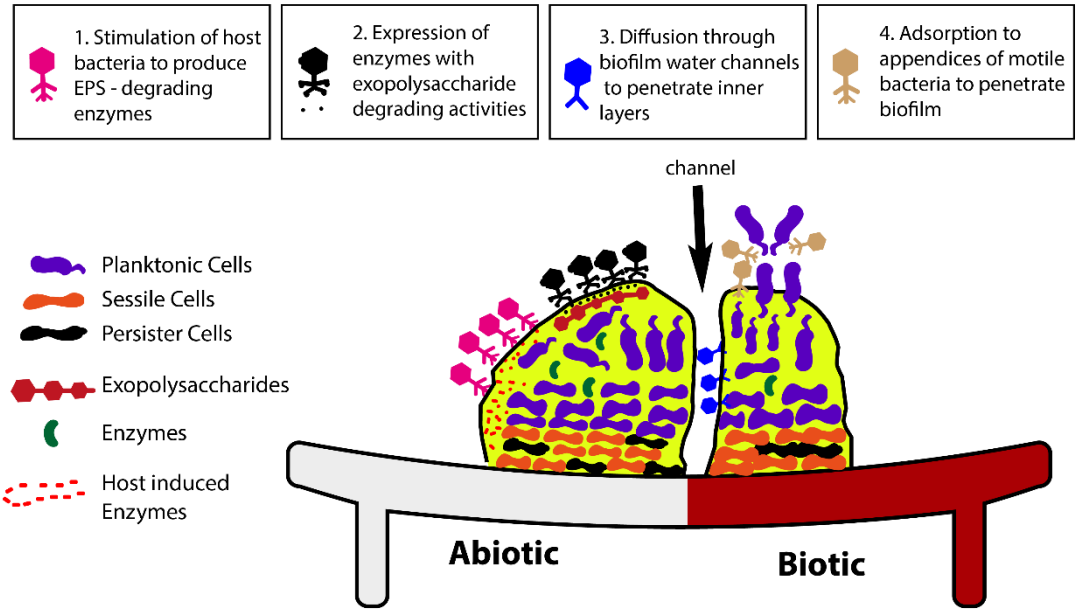

Figure 2. Schematic representation of phage mechanisms of biofilm destruction (40).

2.5 Application of Phages in Bacterial Biofilm Destruction

The tolerance of bacterial biofilms to antibiotics and host immunity has resulted in the search for alternative methods against bacteria in biofilms as well as antibiotic-resistant strains. Phages and phage-derived products have triggered scientists as the most important alternative to antibiotics in preventing and treating biofilms and associated infections. However, development of efficient phage-based treatments requires a deeper understanding of bacteria resistance to phages and co-evolutionary mechanisms between phages and bacteria, to minimize the likelihood emergence of resistance. Phage-based treatments for bacterial biofilm destruction includes the use of mono phages, phage cocktails, genetically engineered phages, and phage-derived enzyme. Some of the most recent application of phages and phage-derived products in bacterial biofilm destruction are summarized in this section.

Application of mono phage in bacterial biofilm destruction involves the use of naturally occurring strictly virulent or lytic phages that do not encode genes for virulence, toxins or AMR. In addition, the phage should not be able to mediate horizontal gene transfer or transduce infected bacterial cells. Single phages usually have narrow host range as they are

generally specific for a limited set of strains of the same bacterial species. Their applications as therapeutic and biocontrol agents in clinical, veterinary, environmental and food bacterial isolates are enormous and encouraging. Recently, phages PSTCR4 and PSTCR6, as part of 17 characterized novel phages, exhibited efficient reduction of well-established *Providencia stuartii* biofilms formed in catheter models. In addition, the phages showed killing capabilities in solid and liquid cultures in various patterns and levels of effectiveness. The phages, found to be free of virulence factors and resistance genes, suggest their potential as reliable therapeutic agents for phage therapy against *P. stuartii* biofilms in bacteriuric catheterized patients (54).

Phage therapy is commonly applied in the form of phage mixture or cocktail targeting either mono or several bacterial strains. In using phage cocktails, the simultaneous treatment targeting a variety of bacterial receptors with diverse antibacterial pathways results in an efficient decrease in bacterial burden, expand host range coverage, lysis potential, mitigate resistance or development of lysogenic strains. In a recent study, phage cocktail composed of four lytic phages, completely inhibited the growth of MDR *E. coli* and significantly prevented the development of biofilms compared with single phages. The phage mixture caused strong biomass reduction of biofilm and showed the highest biofilm inhibition up to nearly 87% (55). Due to their broad host range coverage, phage cocktails are effective on mixed-species biofilms. For example, phage cocktails AB-SA01 and AB-PA01 which target *S. aureus* and *PA*, respectively, when combined together, significantly reduced biofilm biomass in mixed-species biofilms, compared to the respective phage cocktail treatment (56).

Phages which lack enzyme-encoding genes are genetically engineered to express degradation enzymes for adsorption, penetration and diffusion of the phages through the EPS-matrix for biofilms destruction (57). For example, a modified $T7_{DspB}$ *E. coli* phage has been designed to express intracellularly a hydrolase that is released during infection as well as to the extracellular matrix enhancing biofilm degradation. Testing on *E. coli* biofilms showed the efficient expression of biofilm dispersing (DspB) enzyme during phage infection, causing biofilm degradation rate of about 99.997% and a decrease in the population of viable bacterial cells in the biofilm by 4.5 orders of magnitude, around 100 times higher than the efficiency of the parent T7 (58). An engineered T7 bacteriophage encoded with a lactonase enzyme when constructed, expressed the AiiA lactonase to effectively degrade AHLS from many bacteria. Addition of the engineered phage to mixed-species biofilms of *E. coli* and *PA*

inhibited biofilm formation (59). Some temperate phages with non-lytic features are genetically engineered into lytic phages with the production of endolysins useful for biofilm destruction and removal (60). Such manipulation of phage genetic material for effective destruction of bacterial biofilms from biotic or abiotic surfaces may become a feasible option of the 21st century.

Some enzymes encoded with phages maybe useful for treating bacterial infections and biofilms. Under current safety standards and regulations, the application of phage products is easier than use of the phage itself. In relation to this, two main types of phage degradation enzymes are useful in the removal of biofilms: lysins and depolymerases In a recent study on investigating the combination of a phage-derived lytic protein, CHAPSH3b, and the virulent bacteriophage phiIPLA-RODI, the results showed that synergy exist between both antimicrobials for the removal of *S. aureus* biofilms, with greater reductions in viable cell counts observed when phage and lysin were applied together compared to the individual treatments. Time-kill curves and confocal microscopy revealed that the fast antibacterial action of CHAPSH3b reduces the population up to 7 hours after initial exposure, which is subsequently followed by phage predation, limiting regrowth of the bacterial population (61). In a study involving Gram-negative bacteria, the endolysin of *A. baumannii* bacteriophage D2, Abtn-4, was found to have broad antimicrobial activity against MDR *S. aureus, PA, K. pneumoniae, Enterococcus* and *Salmonella* in the absence of outer membrane permeabilizers. Abtn-4 had the ability to reduce biofilm formation and showed antimicrobial activity against phage-resistant bacterial mutants (62). With their activities independent of the bacterial physiological state, lysins are relevant for biofilm removal especially phage-resistant bacteria.

Phage depolymerases are proteins encoded in the region of structural genes in a phage genome which recognize, bind, and digest the polysaccharide compounds of bacterial cell walls. EPSs are mainly responsible for the structural and functional integrity of bacterial biofilms and have an influence on their virulence. Recently, the enzymatic activity of a capsular polysaccharide depolymerase TSP of phage ϕAB6, to degrade *A. baumannii* biofilm, showed significant inhibition of biofilm formation and degradation of formed biofilms. Additionally, TSP inhibited the colonization of *A. baumannii* on the surface of Foley catheter sections, indicating that it can be used to prevent the adhesion of *A. baumannii* biofilms to medical device surfaces (63). In another study, recombinant Dep42, a putative tail fiber protein with depolymerase activity from bacteriophage SH-KP152226, showed specific enzymatic

activities in the depolymerization of the K47 capsule of *K. pneumoniae* and significantly inhibited biofilm formation and/or degrade formed biofilms (64).

Apart from their individual activities as antibiofilm agents, effective removal of biofilms can be achieved with a combination of lysin and depolymerase. In a study on investigating the efficacy of endolysin LysK and depolymerase DA7 against staphylococcal biofilms, in addition to LysK and DA7 removing static and dynamic biofilms from polystyrene and glass surfaces at low micromolar and nanomolar concentrations respectively, a combination of the enzymes significantly reduced viable cell counts compared to individual enzyme treatment (65). Besides their ease of application, phage enzymes can offer to serve as potential new candidates of antibiofilm agents and antimicrobial drugs (enzybiotics) more than live phages particularly in the advent of phage-resistant bacteria.

As part of preparations for the research thesis, this review represents excerpts of the full literature review published in *Nanotechnology, Science and Applications* journal with the title "Bacterial biofilm destruction: A focused review on the recent use of phage-based strategies with other antibiofilm agents". https://doi.org/10.2147/NSA.S325594

CHAPTER THREE

OBJECTIVES AND HYPOTHESIS

3.1 Hypothesis:

3.1.1 Null Hypothesis:

There would be no difference in *Pseudomonas aeruginosa* biofilm when challenged with specific bacteriophages.

3.1.2 Alternative Hypothesis:

There would be a difference in *Pseudomonas aeruginosa* biofilm when challenged with specific bacteriophages.

3.2 Objectives:

3.2.1 General Objective:

To isolate, characterize and investigate the antibiofilm potential of phages to prevent and/or reduce MDR *Pseudomonas aeruginosa* biofilm from medical implants at Jimma University.

3.2.2 Specific Objectives:

- To isolate bacteriophages specific against MDR *Pseudomonas aeruginosa.*
- To characterize the biophysical nature of the specific bacteriophages against MDR *Pseudomonas aeruginosa.*
- To assess the *in vitro* antibiofilm effect of the phages in preventing the colonization of MDR *Pseudomonas aeruginosa* on catheter and ET surfaces.
- To assess the *in vitro* antibiofilm effect of the phages in reducing preformed MDR *Pseudomonas aeruginosa* biofilm on catheter and ET surfaces.

CHAPTER FOUR

MATERIALS AND METHODS

4.1 Study Area, Design, and Period

The study was conducted in Jimma University, one of the leading universities in Ethiopia with over 47,000 student population. Geographically, the university is located in Jimma town which is 346 km southwest of Addis Ababa. The university has a medical center which is a teaching institution and referral center for over 20 million population with over 700 beds in Southwest Ethiopia. Jimma Medical Center (JMC) provides specialized health services including surgical, medical, gynecological, maternity, pediatric, ophthalmic, dental, clinical laboratory and other diagnostic departments. The clinical laboratory services include bacteriology laboratory focused on bacterial isolation and identification from clinical specimens and their antimicrobial susceptibility tests results, to generate data on AMR and MDR patterns of bacterial isolates. Currently, JMC provides services for about 20,000 inpatients and over 205,000 outpatient attendants per annum.

Laboratory-based experimental study was conducted at the medical microbiology laboratory of Jimma University from June to October, 2021 on clinical and environmental samples collected from the university medical center.

4.2 Bacterial Strains and Growth Conditions

The bacterial strains used in this study were obtained from JMC bacteriology laboratory. MDR *PA* isolates were recovered from different specimens of patients (Table 1). The bacterial strains were biochemically re-identified as *P.A* strains through different biochemical reactions (Appendix II). From pure cultures grown on Columbia agar (CA; bioMérieux, Marcy l'Etoile, France), bacteria inoculums were checked for their multidrug resistance property as depicted in Table 1. Furthermore, pure cultures of the bacteria were prepared and suspended in sterile 0.85% NaCl and kept in refrigerator at 4°C until use. Inoculums were routinely grown with agitation on nutrient broth (NB; Oxoid, Hampshire, UK) at 37°C for phage isolation tests. Biofilms were grown in tryptic soy broth (TSB; Oxoid) containing 1% glucose at 37°C. For all experiments, bacteria were grown on CA and as a maintenance medium Nutrient agar i.e., nutrient agar slant in tubes (Himedia, India).

Table 1. Bacterial isolates and their antimicrobial resistant patterns.

Isolates	Source	Antibiotic Resistance
PA 1095	wound abscess	AMP, AMC, CTX, CXM, TZP, TET
PA 1098	sputum	AMP, AMC, AMK, CRO, CXM, CHL, SXT, CN, MEM
PA 1139	wound abscess	AMP, AMC, AMK, CXM, CHL, SXT, CRO, CTX, MEM
PA 1280	pleural fluid	AMP, AMC, CFZ, CXM, CHL, SXT, TET
PA 1321	wound abscess	AMP, AMC, CFZ, CAZ, CTX, CXM, CHL, SXT,
PA 1329	wound abscess	AMP, AMC, CAZ, CRO, CTX, CXM, CHL, SXT, CN, TET, TOB
PA 1668	urine	AMP, AMK, CRO, CXM, CIP, CN, NIT, NOR

AMC, amoxicillin-clavulanic acid; AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CFZ, cefazolin; CHL, chloramphenicol; CIP, ciprofloxacin; CN, gentamicin; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; MEM, meropenem; NIT, nitrofurantoin; NOR, norfloxacin; SXT, sulfamethoxazole-trimethoprim; TET, tetracycline; TOB, tobramycin; TZP, piperacillin-tazobactam

4.3 Isolation of Bacteriophages from Hospital Sewage

4.3.1 Bacteriophage Isolation and Enrichment

Isolation of bacteriophages specific against *PA* was carried out from hospital sewage sources (JMC, Ethiopia) according to the standard enrichment protocol described earlier (66) with some modification. The clinical *PA* 1095, 1139, and 1329 strains were chosen at random and used as the host strains for phages isolation. Sewage samples were collected in sterile 500 mL containers from three different areas around the JMC compound and quickly transported to the medical microbiology laboratory for enrichment. Using 50 mL falcon tubes, sewage samples were centrifuged at 10,000×g, 4°C for 15 min, to remove particulate materials. The supernatants were filter sterilized through 0.45 μm membrane filter and mixed with equal volume (50 mL) of sterile double strength NB containing 2mM $MgCl_2$, alongside 5ml log phase grown *PA* host strains. After overnight aerobic incubation at 37°C with frequent agitation, the mixtures were centrifuged at 10,000×g, 4°C for 15 mins, filter sterilized through 0.45 μm membrane filter, and enriched for the second time with the same host strain to amplify the filtrate.

4.3.2 Spot Assay

The amplified filtrates obtained above were re-filtered through sterile membrane of pore size 0.45 μm and tested for phages activity following the spot assay as described elsewhere (6). Briefly, 100 μl of *PA* hosts inoculum was added to 5-7 ml molten soft agar and poured onto

CA plate surface. After solidification, 10 µl of amplified filtrates were spotted and plates were allowed to dry (absorb) at room temperature (RT) for a few minutes and incubated overnight at 37ºC. Positive spotted phage activities were purified by successive single plaque isolation until homogenous plaques were obtained according to the standard procedure described previously (67). In a 5 ml broth of fresh log phase *PA* host, one plaque from a plate was added and incubated at 37ºC under shaking condition alongside a control tube without the host strain until complete lysis occurred in the test preparation. Afterwards, tubes were centrifuged at 10,000×g, 4ºC for 15 minutes. Supernatants were chloroform treated and serially diluted for plaque assay. The procedure was repeated three times to ascertain the purity and activity of isolated phages.

4.4 Quantitative Assay of Bacteriophages

The number of phage particles or titers were estimated by the double agar overlay method as described before by counting plaque-forming units per milliliter (PFU/mL) (68). Serial dilutions of phage lysates (10 folds) were made in sterile saline magnesium (SM) buffer solution (100 mM NaCl, 25 mM Tris-HCl, 8 mM $MgSO_4$, pH 7.5). Then, 100 µl of phage suspension from each dilution mixed with 100 µl of host inoculum in a 5-7 ml molten soft agar was quickly poured on CA plate surface without creating air bubbles. After overnight incubation at 37°C aerobically, the number of plaques was counted and plates with 20–200 plaques were selected to determine the phage titer from two countable plates. Subsequently, plaques morphology and diameter were determined (68).

4.5 Determination of Phages Host Ranges

The host ranges of phages were determined following the standard spot test procedure described earlier (6) with undiluted phage stocks having a predetermined plaque count of 10^7 or 10^6 per milliliter phage lysates. All seven MDR *PA* isolates as well as ten clinically used American Type Culture Collection (ATCC) strains of *PA* and other species were used in this experiment as listed in Table 2. Briefly, 100 µL of each of the bacterium inoculum was mixed with 5-7 mL of molten soft agar and layered over CA plate. The phage lysates were serially diluted (10-folds) in SM buffer and 10 µL were spotted onto the solidified soft agar with the bacterium. Plates were left undisturbed until the drop got absorbed at RT. After overnight incubation aerobically at 37°C, bacterial sensitivity to phages were confirmed by the presence of zone of clearance at the sites of phage application. Positive spotted tests were assayed for

plaques to verify the lysis and sensitivity to the phages. The obtained results were differentiated as clear plaques or complete lysis (++), turbid plaque or partial lysis (+), or no plaque or no lysis (–).

4.6 External Factors Stability tests on Phages

Stability of phages at varying ranges of temperature and pH is important for their ability to act at various physiological or environmental conditions. The stability of phages to different physical and chemical factors, including temperature, pH and organic solvents were tested according to the protocols described earlier (69) with some modifications. All assays were performed in triplicates and plating was by double agar overlay procedure.

a) *Temperature stability assay:* Phage suspensions were diluted in SM buffer (1:9 dilution) and incubated for 1 h at different temperatures (15, RT, 37, 40, 50 and 90°C). Next, the phage suspension was withdrawn, 10-fold serially diluted in SM buffer and used for plating. After overnight incubation at 37°C, the percentage of viable phages able to lyse the host bacterial cells was estimated. Initial plaque count of phages kept at 4°C was taken as controls.

b) *pH stability assay:* Phage suspensions (100 µL) were diluted in SM buffer (900 µL) of different pH values (2.0, 4.0, 7.0, 10.0 and 12.0) with 1 M HCl or 1 M NaOH and incubated for 1 h at 37 °C. Next, 10-fold serial dilutions were prepared in SM buffer and used for plating. After overnight incubation at 37°C, the percentage of viable phages able to lyse host bacterial cells was calculated. Plaque count of phages incubated in SM buffer of pH 7.0 was taken as controls.

c) *Organic solvents tolerance:* The stability of phage particles was tested against three different organic solvents: ethanol, acetone and chloroform. A stock solution of phage lysate was added to chloroform, acetone, 96% and 48% ethanol. The mixture was incubated for 1.5 h at RT (chloroform, acetone and ethanol). Next, 10-fold serial dilutions in SM buffer were prepared and used for plating. After overnight incubation at 37°C, the percentage of viable phages able to lyse host bacterial cells was estimated. Phage particles incubated in SM buffer under the conditions described above were used as controls.

4.7 Biofilm Formation Assay

Quantitative assessment of MDR *PA* biofilm formation was performed according to the method described earlier (70) with some modification. Briefly, the concentrations of overnight cultures of *PA* were adjusted to that of the turbidity of a 0.5 McFarland standard. The suspensions were then diluted to 100-fold, containing approximately 10^6 colony forming units (CFU/mL) in TSB medium supplemented with 1% glucose. 200 µL of these were seeded into sterile flat-bottomed 96-well polystyrene micro plate (Greiner Bio-one CELLSTAR). *PA* static biofilms were grown at 37°C for 24 h without renewal of media. After incubation, the non-adherent cells were removed from the wells and washed twice with 200 µL of sterile phosphate buffered saline (PBS), pH 7.4. Biofilm was fixed with methanol for 15 min and it was removed, air dried, and stained with 220 µL of 0.1% crystal violet for 15 min at RT. Again, the wells were washed twice with PBS to remove excess stain and allowed to air-dry. The stained biofilms were solubilized with 220 µL of 96% ethanol for 15 min and optical density (O.D) of eluted stain was measured with a microtiter plate reader (Elisys Uno Human) at a wavelength of 630 nm. For quantitative assays, experiments were performed in triplicate wells. Sterile TSB medium in the wells left without the bacteria was used as negative control. The cut-off O.D for biofilm formation and its strength was calculated and defined as three standard deviations above the mean O.D of the negative control (70).

4.8 Screening of Lytic Activity of Phages on Biofilms

To test the lytic activity of phages in mature biofilms, static biofilms of MDR *PA* were cultured as described above. After incubation, biofilms were washed in PBS, pH 7.4 to remove planktonic cells and then proceeded to phage treatment. Two hundred microliters of phage lysates were added to each well to a final titer of 10^6 PFU per well, agitated at 120 rpm for 1 h and incubated for 6 h at 37°C. Control biofilms of MDR *PA* were treated with sterile TSB in place of the phage. Next, the mixture was removed and washed twice with 200 µL of PBS. The plate was fixed with methanol, and phage treated and untreated biofilms proceeded to crystal violet staining and measurement of O.D as described above. All assays were conducted in triplicate wells.

4.9 Assessment of Phage Activity on Biofilm Formation in Catheters and Endotracheal Tubes

PA 1098 and 1668 strains, isolated from sputum and urine, respectively, were selected for this experiment. The two *PA* strains were also adequately lysed by the isolated virulent bacteriophages. Sterile silicone Foley balloon catheters (Ramsons Int, Noida, India) and Endotracheal Tubes; ETs (Henso Ltd, Hangzhou, China) were prepared prior to experimentation as described earlier (13) with some modification. Briefly, with the use of sterile scissors, catheters and ETs from their packages were cut into 15-mm and 12-mm long segments, respectively, followed by cutting in half lengthwise to expose the interior surfaces of the tubes (Figure 3A-D). The segments were soaked in 70% ethanol followed by UV light irradiation for 2 h. The segments were then placed in sterile flat-bottomed 24-well polystyrene culture plates (Becton Dickinson Labware, NJ, USA) for pre- and post-treatment with phage lysates (Figure 3E-F).

i). **Pre-treatment experiments:** 1 mL of phage lysates containing 10^6 PFU/mL were added to the sterile segments, agitated at 120 rpm for 1 h and incubated for 6 h at 37°C to allow phage adsorption to catheter and ET surfaces. Afterwards, the suspension was removed and segments were washed with PBS, pH 7.4, to remove non-adhered phages. Control segments were covered with sterile TSB medium. Phage-coated and non-coated catheter and ET segments were covered with 1 mL of prepared overnight cultures of MDR *PA* in TSB, containing approximately 10^6 bacterial cells as described above. Plates were incubated at 37°C for 24 h with static-non-renewal conditions, for biofilm formation assessment. Phage-coating was operationalized as the adhesion of phage particles in a solution on the surface of catheter and ETs to form a thin film on the surfaces.

ii). **Post-treatment experiments:** Catheter and ET segments were covered with 1 mL of MDR *PA* cultures in TSB containing approximately 10^6 bacterial cells and incubated for 96 h at 37°C for biofilm formation, with renewal of half the volume of media every 24 h in order to mimic *in vivo* contamination conditions. Afterwards, the medium with planktonic bacterial cells was aspirated from each well containing segment and washed twice with PBS, pH 7.4. Segments were then treated with 1 mL of each of 10^2, 10^4 and 10^6 PFU/mL titer or 1 mL of sterile TSB (control), agitated for 1 h at 120 rpm and incubated for 6 h at 37°C.

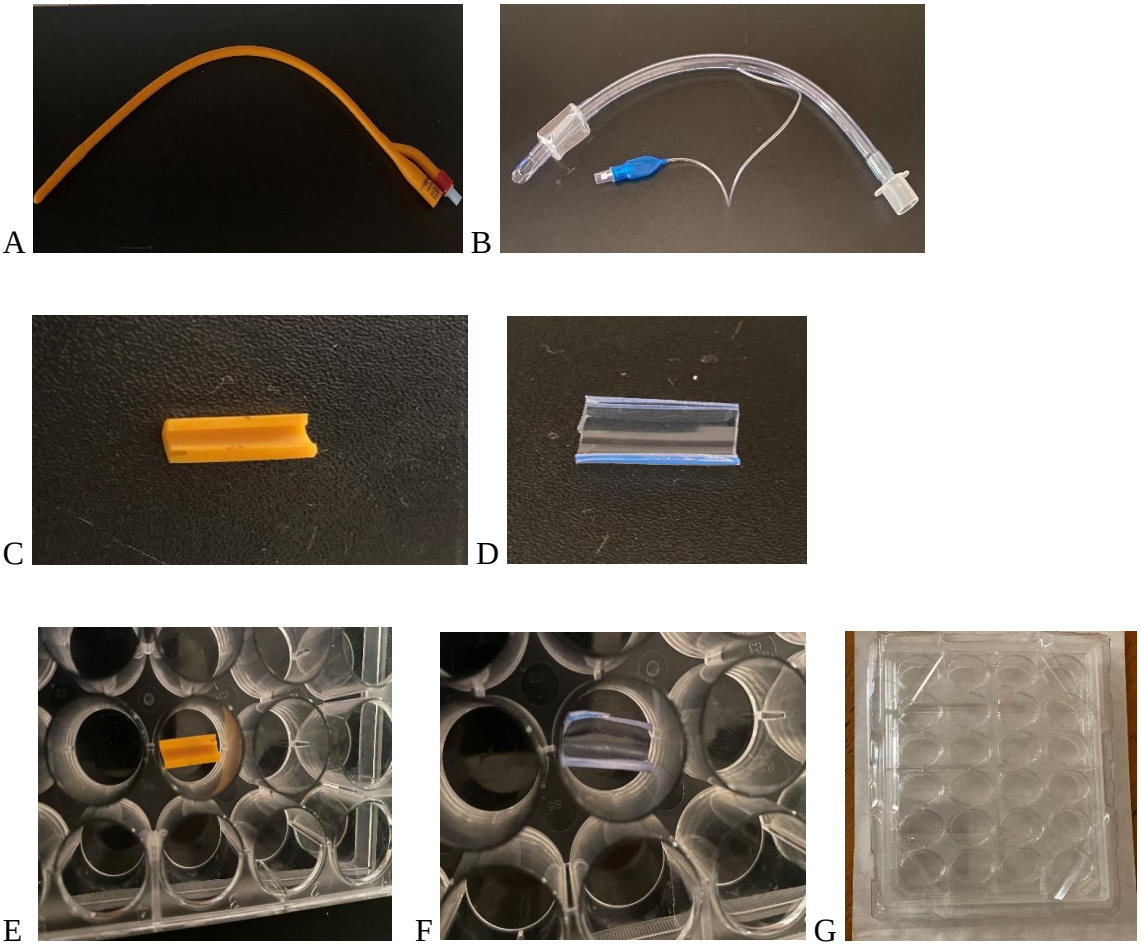

Figure 3. Medical implants used for biofilm experiment. (A) Foley balloon catheter. (B) Endotracheal tube. (C) 15-mm long catheter tube cut in half. (D) 12-mm long endotracheal tube cut in half. (E) Catheter segment and (F) Endotracheal tube segment placed in (G) sterile 24-well culture plate.

4.10 Recovery and Determination of Surface-Attached Bacteria Cells

After the required incubation time, liquid contents of the wells were removed, segments were washed twice with PBS and aseptically transferred to Eppendorf tubes containing 1 mL of 0.85% NaCl. The tubes were vortexed at maximum speed for 60 s to detach cells from the segments. Ten-fold serial dilutions were prepared in 0.85% NaCl and 100 µL of each dilution was spread onto CA plates. After overnight incubation at 37°C, number of viable recovered *PA* cells was estimated on the basis of counted colonies and expressed as $\log_{10}$CFU/mL.

4.11 Statistical Analysis

All experimental data were analyzed as mean ± standard deviation (SD) using GraphPad Prism version 9.0.0 for Windows (GraphPad software, San Diego, CA, USA). Statistically

significant differences between mean values of experimental samples and controls were performed using unpaired t test followed by Bonferroni-Dunn multiple comparisons test and were marked with asterisks when $p < 0.05$ (∗), $p < 0.01$ (∗∗), or $p < 0.001$ (∗∗∗).

4.12 Quality Assurance

Quality control measures were implemented throughout the experimental procedures. All media, reagents and consumables were checked for expiry date before use. All culture media were prepared according to the directions of the manufacturers and aseptic techniques were employed in all steps of the experiment. The ATCC strains were used as control strains for biochemical identification, drug susceptibility testing and host range determination.

4.13 Ethical Consideration

This experimental study was approved by the Institutional Review Board (IRB) of Jimma University Institute of Health with reference number JHRPGN/166/. Antimicrobial susceptibility tests results were obtained without patient names for confidentiality purposes. Bacterial and phage biofilm cultures were sterilized before disposal appropriately. All plates, test tubes and petri dishes were decontaminated before washing and sterilized for re-usage.

4.14 Dissemination Plan

The results of this study are to be disseminated to the School of Medical Laboratory Sciences and the Postgraduate Library of Jimma University. In addition to the literature review published online in *Nanotechnology, Science and Applications* journal (https://doi.org/10.2147/NSA.S325594), this study is to be disseminated and published in an international, reputable, peer-review journal.

CHAPTER FIVE

RESULTS

5.1 Isolation of Phages specific against *Pseudomonas aeruginosa*

Two *PA* bacteriophages were isolated from samples of hospital sewage after testing for the presence of phages that may infect MDR *PA* clinical strains. The phages were named ΦJHS-PA1139 and ΦSMK-PA1139 (hereafter, called ΦJHS and ΦSMK respectively) based on the source of the sewage (Jimma Hospital Sewage; sewage from Surgical, Medical and Kitchen block) and the host strain used for propagation (*PA* 1139). Waste water samples were collected from JMC in Ethiopia in June, 2021.

5.2 Plaques Morphology

Phages ΦJHS and ΦSMK were propagated using the host strain, MDR *PA* 1139 from clinical specimens. Subjecting the phage lysates to further analysis revealed that both phages formed clear plaques on the lawn of the host and produced complete lysis in moderate titers (1.0 – 6.0 x10^7 PFU/mL) for ΦJHS and (1.0 – 5.0 x10^6 PFU/mL) for ΦSMK. Plaques of ΦJHS (Figure 4A) and ΦSMK (Figure 4B) had average diameter of about 5 mm and 2 mm, respectively, on the lawn of the *PA* 1139 host. Such plaques morphology indicated that these viruses are lytic bacteriophages.

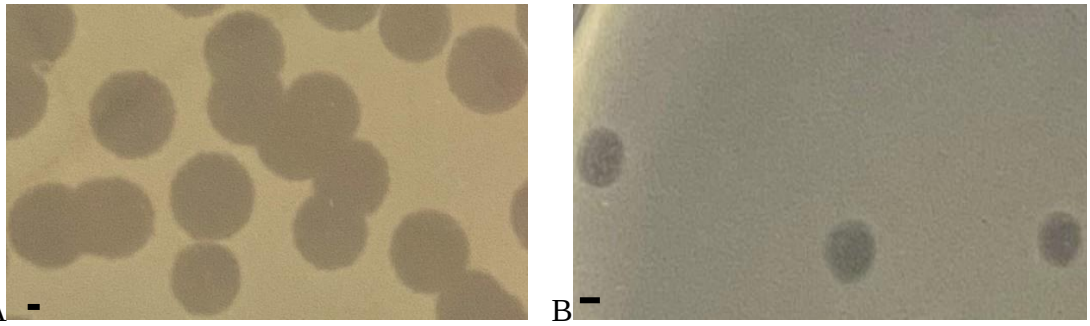

Figure 4. Plaques formed by bacteriophages (A) ΦJHS and (B) ΦSMK on the lawn of *PA* 1139 strain using double agar overlay method. The bar corresponds to 1 mm.

5.3 Host Ranges

The host ranges of phages ΦJHS and ΦSMK were tested with clinical *PA* strains as well as strains of other clinically relevant Gram-negative and Gram-positive species. Both phages exhibited broad host range against tested *PA* clinical strains from different patients. The

proportion of clear complete lysis was 75% (6/8) for ΦJHS and 50% (4/8) for ΦSMK. To verify positive spotted results, further spot tests in serial dilutions were performed to obtain plaques which were assayed in all cases. Compared to the host, the plaque sizes did not vary among the sensitive strains tested. No cross-sensitivity to non-*PA* strains such as *E. coli* ATCC 25922, *A. baumannii* ATCC 19606 and *S. aureus* ATCC 25923 was detected as appeared in Table 2.

Table 2. Lytic activity of phages ΦJHS and ΦSMK against tested bacterial strains.

Bacterial Strain	Phage Sensitivity	
	ΦJHS	**ΦSMK**
***Pseudomonas aeruginosa* 1095**	+	+
***Pseudomonas aeruginosa* 1098**	++	++
Pseudomonas aeruginosa* 1139	++	++
***Pseudomonas aeruginosa* 1280**	++	+
***Pseudomonas aeruginosa* 1321**	+	+
***Pseudomonas aeruginosa* 1329**	++	++
***Pseudomonas aeruginosa* 1668**	++	++
***Pseudomonas aeruginosa* ATCC 27853**	++	+
***Escherichia coli* ATCC 25922**	–	–
***Salmonella typhimurium* ATCC 13311**	–	–
***Proteus mirabilis* ATCC 35659**	–	–
***Klebsiella pneumoniae* ATCC 700603**	–	–
***Enterobacter cloacae* ATCC 13047**	–	–
***Acinetobacter baumannii* ATCC 19606**	–	–
***Shigella dysenteriae* ATCC 13313**	–	–
***Staphylococcus aureus* ATCC 25923**	–	–
***Staphylococcus saprophyticus* ATCC 15305**	–	–

(++), clear plaques or complete lysis; (+), turbid plaque or partial lysis; (–) no plaque or no lysis; (*), host

5.4 Stability of Phages ΦJHS and ΦSMK to External Factors

The stability of ΦJHS and ΦSMK to various physical and chemical factors, including different temperatures, pH conditions and organic solvents were tested. The virions of both phages appeared relatively stable to various temperatures and pH conditions, though the virions could not withstand extreme conditions (pH of 2.0 and 12.0 and temperature of 90°C). Stability to organic solvents varied depending on the nature of the tested solution. Notwithstanding, virions of both phages could not survive under acetone and 96% ethanol. (Table 3).

Table 3. Stability of phages ΦJHS and ΦSMK to external physical and chemical factors

External Factors	Time and Conditions of Incubation	Percentage Viability of Phages ± SD	
		ΦJHS	**ΦSMK**
Temperature			
4°C*	1 h	100 ± 0.0	100 ± 0.0
15°C	1 h	100 ± 7.5	100 ± 4.4
RT	1 h	100 ± 6.5	100 ± 3.2
37°C	1 h	100 ± 6.1	100 ± 6.5
40°C	1 h	100 ± 3.1	100 ± 5.5
50°C	1 h	76.9 ± 0.6	55.4 ± 2.5
90°C	1 h	0.0 ± 0.0	0.0 ± 0.0
pH			
pH 2.0	1 h; 37°C	0.0 ± 0.0	0.0 ± 0.0
pH 4.0	1 h; 37°C	38.0 ± 2.0	29.8 ± 0.6
pH 7.0*	1 h; 37°C	100.0 ± 0.0	100.0 ± 0.0
pH 10.0	1 h; 37°C	78.9 ± 3.1	68.1 ± 1.1
pH 12.0	1 h; 37°C	0.0 ± 0.0	0.0 ± 0.0
Organic solvent			
SM buffer*	1.5 h; RT	100 ± 0.0	100 ± 0.0
Chloroform	1.5 h; RT	82.0 ± 2.5	70.8 ± 1.5
Acetone	1.5 h; RT	0.0 ± 0.0	0.0 ± 0.0
96% Ethanol	1.5 h; RT	0.0 ± 0.0	0.0 ± 0.0
48% Ethanol	1.5 h; RT	90.0 ± 4.9	57.0 ± 2.1

(*), controls

5.5 MDR *Pseudomonas aeruginosa* Biofilm Formation

Biofilms of MDR *PA* were cultured for 24 h at static non-renewal conditions in 96-well culture plates and stained with crystal violet. The intensity of color generated by the biofilms with crystal violet (O.D measured at 630 nm) is a direct indication of the biomass formed. In general, all the MDR *PA* clinical isolates obtained from JMC were biofilm formers. Categorically, with the exception of *PA* 1095 and *PA* 1139 strains which exhibited weak and moderate biofilms respectively, all isolates were strong biofilm formers (Figure 3).

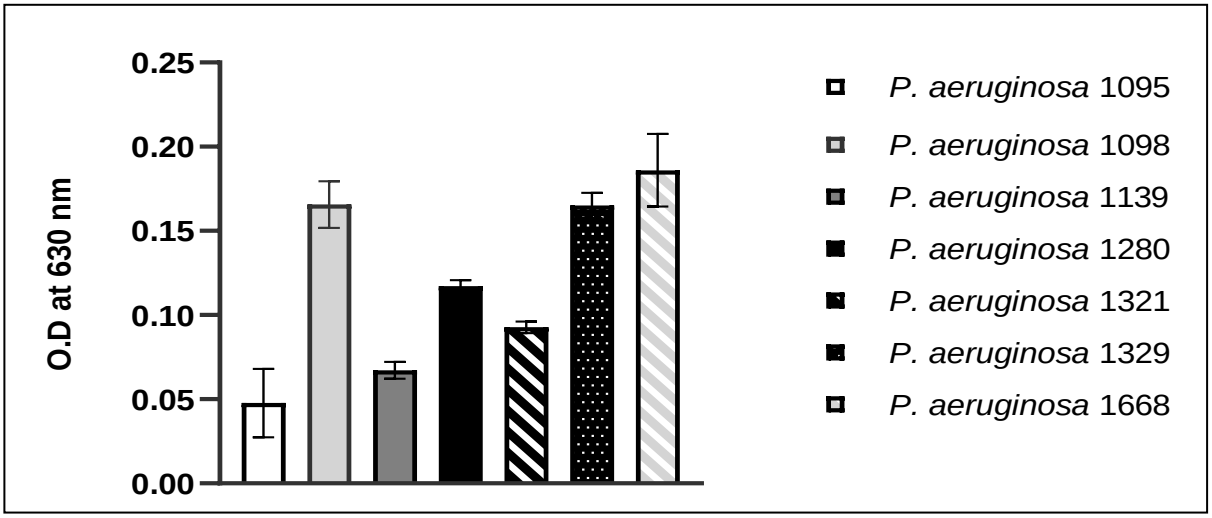

Figure 5. Biofilms formed by MDR *PA* clinical isolates cultured for 24 h at static non-renewal condition, as analyzed by crystal violet staining procedure and shown as O.D values measured at a wavelength of 630 nm. All assays were performed in triplicates. The values presented are mean ± SD from two readings of triplicate experiments (n = 6).

5.6 Screening of Phage Effect on MDR *Pseudomonas aeruginosa* Biofilms

The lytic activity of phages ΦJHS and ΦSMK on biofilms formed by clinical isolates of MDR *PA* were assessed. Twenty-four hour-old biofilms obtained under static non-renewal conditions were treated with the phages for 6 h. After incubation with the phages, biofilms were stained with crystal violet and the biomasses were determined by O.D measurement at 630 nm. As depicted in Figure 4, upon biofilm treatment with phages ΦJHS and ΦSMK, with the exception of *PA* 1095, biomasses of MDR *PA* 1098, 1139, 1280, 1321, 1329 and 1668 decreased significantly ($p < 0.001$, $n = 6$).

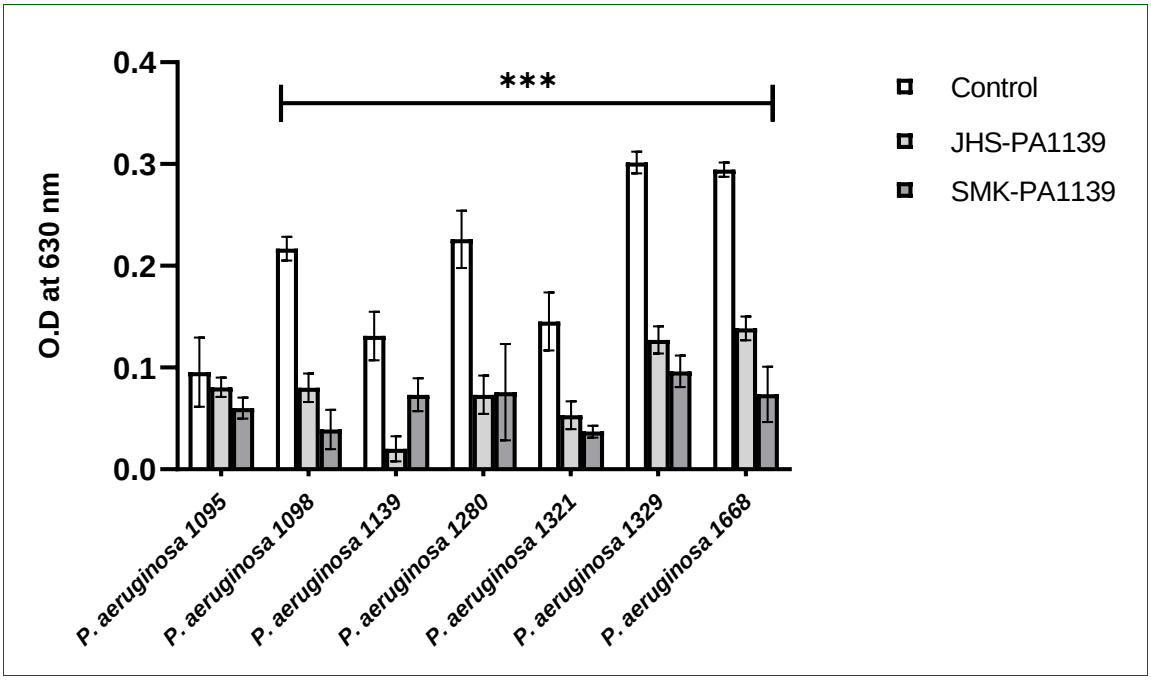

Figure 6. Lytic activity of bacteriophages on biofilms formed by MDR *PA* clinical isolates after 6 h treatment with phages ΦJHS-PA1139 and ΦSMK-PA1139, as analyzed by crystal violet staining procedure and shown as O.D values measured at a wavelength of 630 nm. The values presented are mean ± SD from two readings of triplicate experiments (*n*= 6). Statistically significant differences between control and analyzed samples are marked with asterisks (*p* < 0.001 (∗∗∗) in the multiple unpaired t-test).

5.7 Effect of Phage Coating on Biofilm Formation on Catheters and Endotracheal Tubes

The coating effect of phages ΦJHS and ΦSMK against MDR *PA* biofilm formation on catheters and ETs were assessed. Catheter and ET segments were first coated with ΦJHS and ΦSMK in a final titer of 10^6 PFU/mL for 6 h incubation before biofilm formation with static non-renewal conditions. Following 24 h incubation at 37°C, numbers of viable surface-attached bacterial cells were estimated.

Comparing the CFU values, microbial load on non-coated catheter and ET segments were similar among both strains. However, different microbial growth patterns were observed on phage-coated segments. Phage-coated segments showed statistically significant ≥ 1.2 $\log_{10}$ inhibition of microbial load compared to the controls (Figure 7). With both MDR strains, phage ΦSMK was the most efficient and achieved highest inhibition of 3.2 $\log_{10}$ ($p < 0.001$,

n = 6) (Figure 7B). With regards to phage ΦJHS, highest inhibition achieved in comparison to non-coated segments (control) was 2.2 $\log_{10}$ ($p < 0.001$, n = 6) (Figure 7B).

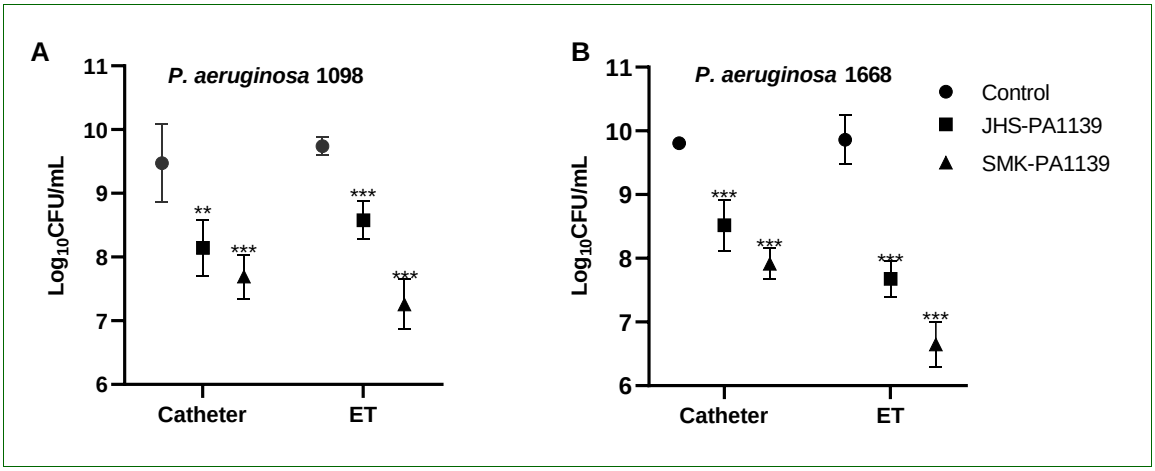

Figure 7. Microbial load inhibition of MDR *PA* 1098 (A) and 1668 (B) biofilm formation on non-coated and phage-coated catheter and ET segments, quantified as $\log_{10}$CFU/mL. The values presented are mean ± SD from two counts of triplicate experiments (n= 6). Statistically significant differences between control and analyzed samples are marked with asterisks ($p <$ 0.01 (∗∗), $p < 0.001$ (∗∗∗) in the multiple unpaired t-test).

5.8 Effect of Phage Treatment of Preformed Biofilms on Catheter and Endotracheal Tubes

The treatment effect of phages ΦJHS and ΦSMK on MDR *PA* biofilms formed on catheters and ETs were assessed. In an *in vitro* model, MDR *PA* biofilms were formed on catheter and ET segments for 96 h with renewal of media to mimic *in vivo* contamination conditions, followed by treatment with phages ΦJHS and ΦSMK at different titers of 10^2, 10^4 and 10^6 PFU/mL. Following 6 h incubation at 37°C, numbers of viable surface-attached bacterial cells were estimated.

Comparing the CFU values, treatment with lower titers resulted in a slight reduction of microbial load. At titer of 10^2 PFU/mL, statistically significant reduction in microbial load was achieved with phage ΦSMK with 0.4 (p = 0.03, n = 6) (Figure 8A) and 0.7 (p = 0.02, n = 6) (Figure 8B) $\log_{10}$ reduction relative to control experiment (phage-untreated segments). Both phages were however, effective at titers of 10^4 PFU/mL with statistically significant $\log_{10}$ reduction values. With the application of this titer, phage ΦJHS achieved > 0.8 $\log_{10}$ reduction in the number of viable bacteria cells. Treatment with phage ΦSMK however,

resulted in >1.0 log_{10} reduction of viable microbial load. The most efficient log_{10} reductions were achieved when phages were applied at titers of 10^6 PFU/mL relative to control experiment. Phage ΦSMK achieved the highest reduction and was therefore most efficient with 2.4 log_{10} reduction ($p < 0.001$, $n = 6$) (Figure 8B). With regards to phage ΦJHS, a 1.6 log_{10} reduction was observed as the highest log_{10} reduction value in comparison to control. The results presented indicate that phages ΦJHS and ΦSMK were efficient in reducing the numbers of viable MDR *PA* cells in biofilms formed on catheters and endotracheal tubes.

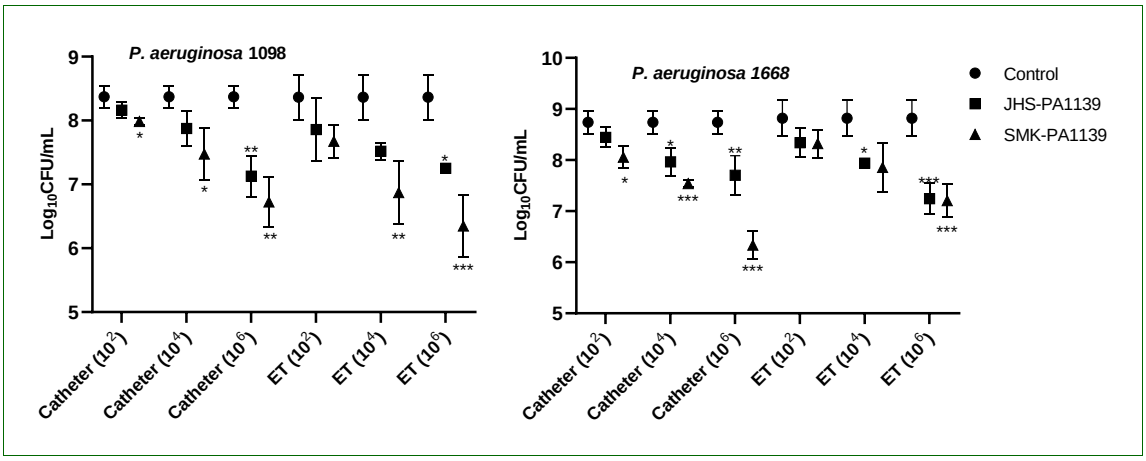

Figure 8. Microbial load reduction with 6 h phage treatment of MDR *PA* 1098 (A) and 1668 (B) biofilms formed on catheter and ET segments, quantified as log_{10}CFU/mL. The values presented are mean ± SD from two counts of triplicate experiments (n= 6). Statistically significant differences between control and analyzed samples are marked with asterisks ($p < 0.05$ (∗), $p < 0.01$ (∗∗), $p < 0.001$ (∗∗∗) in the multiple unpaired t-test).

CHAPTER SIX

DISCUSSION

In this study, two *PA* bacteriophages (ΦJHS and ΦSMK) were isolated from hospital sewage. With their abundance on earth, it is estimated that at least ten phages exist for each bacterial/archaeal cell, showing that phages existence spans from ocean and freshwater environments, to terrestrial and complex environments (71). The isolated phages showed similarity in their plaque morphologies by forming clear plaques on the lawn of the host strain, indicative that both phages are virulent or lytic bacteriophages. The isolated phages, however, showed differences in plaque size, indicative that two distinct bacteriophages infecting the same host *PA* were isolated (24,69).

Both ΦJHS and ΦSMK phages showed broad lytic spectra on clinical MDR *PA* strains as all *PA* strains tested were sensitive to both phages. Infection of seven MDR *PA* strains in comparison with the results of previously isolated *PA* bacteriophages (24), suggests that the isolated phages have the potential to be further developed as candidates for clinical phage therapy or as biocontrol agents on abiotic surfaces. Furthermore, the isolated phages have the potential to be formulated as phage cocktail to target a single bacterial species (*Pseudomonas aeruginosa*). Such a cocktail which targets only a single rather than multiple bacterial species, is described as generally emphasizing spectrum of phage activity breadth in its design, rather than necessarily emphasizing spectrum of phage activity depth (72).

Physiological factors such as pH and temperature play important roles in bacterial-phage interactions. In this study, the isolated phages were thermally stable at 15-40°C and displayed maximum viability at pH 7.0. These observations are in agreement with previous studies (24,73), indicating that the phages can be stored at RT particularly in resource limited settings. These observations also indicate that the phages can be applied with minimal denaturation on surfaces of inanimate objects and their infectivity could be maintained in human conditions as physiologic systems do not change abruptly (74).

The phages tested for organic chemical stability showed that they were not absolutely resistant to chloroform, an organic solvent traditionally used in bacteriophage isolation. The results revealed that 1.5 h exposure of the two phages to chloroform yielded between 70-82% of viable phage virions for infection, suggestive that the phages could be lipid enveloped

membrane-containing bacteriophages which are relatively underrepresented among identified phage isolates (75,76). This could possibly explain also the lower phage titers (10^6 – 10^7 PFU/mL) obtained when compared to other studies (≥ 10^9 PFU/mL) (24,74) as chloroform was continuously used throughout the phage purification processes in this study. Nonetheless, chloroform reduction of the viability of non-membrane-containing bacteriophages has been reported in some studies (69,73). The high similarity in the response of the two isolated phages to the various physical and chemical factors could result from their indistinct structures.

Assessing biofilm producing capabilities of the clinical MDR *PA* isolates revealed that all isolates were biofilm producers. Biofilm formation has been reported to be significantly higher in MDR *PA* clinical isolates due primarily to the presence of biofilm-related genes such as *psl*A in these isolates (77). In this study, 71.4% (5/7) of the isolates were strong biofilm formers and 14.3% (1/7) were moderate biofilm former and weak biofilm former each. On the basis of variation in the source of the isolates highlights the enormous burden of MDR *PA* in the clinical settings, particularly in device-associated infections which must be dealt with.

In the screening of the efficacy of the phages in reducing adherent biofilms of MDR *PA* under static conditions, significant reductions ($p < 0.001$) were observed in all the strains with the exception of one strain after 6 h of phage treatment. Such reductions in biomass were reported in a recent study with four mono phages infecting each of MDR *PA, S. aureus, K. pneumoniae* and *E. coli* (78). The authors reported that the phages were effective on the biofilms in static, dynamic with medium renewal and dynamic with nonrenewal conditions tested.

To maximize lytic activity of the phages in a resource limited setting, this study employed a combination of dynamic and static conditions in phage treatment of the biofilms. The experimental setup was first agitated to aid in the spread of the phages to adsorb onto the entire span of the biofilm, and then incubated statically to induce the release of progeny viruses to attach to the neighboring bacteria within the biofilm. Although phage ΦJHS presented a dominant advantage over phage ΦSMK in infecting the MDR *PA* strains, phage ΦSMK presented dominance in reducing biofilms formed by these strains. Consistent with the report of a previous study (24), one MDR *PA* isolate did not show statistically significant reduction in biofilm at the evaluated time and could be attributed to the development of phage resistance by bacteria which is inevitable.

A recent *in vitro* study reported the appearance of phage-resistant mutants which sheds more light on the fact that development of phage resistance by bacteria occur more frequently *in vitro* than *in vivo* (13). On the basis of the initial antibiofilm results, the hypotheses of applying the isolated phages in preventing MDR *PA* biofilm formation and removing preformed biofilms from catheter and ET surfaces are supported.

Since the development of phage therapy against *PA* appears to be crucial in establishing effective treatment of chronic infections caused by MDR strains of this pathogen (79,80) and biofilm formation on medical devices is one of the major challenges faced in the medical settings (81–83). This study sought to assess whether phage pre-treatment and post-treatment could efficiently inhibit and reduce MDR *PA* catheter and ET-associated biofilms respectively. Based on the results, the null hypothesis was rejected since there were statistically significant differences for MDR *PA* catheter and ET-associated biofilms.

In this study, to assess the phages potential in inhibiting biofilm formation, catheter and ET segments were coated with the isolated phages by physical adsorption (12,14). The results of the study indicated that the physical adsorption promoted phage immobilization on the segments as evidenced by the statistically significant differences between phage-coated segments and non-coated segments. The results showed that phage coating of catheter and ET inhibited bacteria colonization on these devices. Phage-coated segments showed a minimum of 1.2 $\log_{10}$ for 6 h coating, corresponding to > 90% inhibition of microbial load relative to non-coated segments. Inhibitory activities of the isolated phages on catheter segments remained similar in the two MDR *PA* 1098 and 1668 strains tested. These MDR strains were selected based on their source of isolation (sputum and urine) parallel with the medical implants used in this study. In addition, compared to the phage-isolation host strain (*PA* 1139) which formed moderate biofilm, *PA* 1098 and 1668 formed strong biofilms making them bacterial strains of choice for *in vitro* biofilm study. Phage ΦJHS achieved 1.3 $\log_{10}$ inhibition on catheter segments in both strains while phage ΦSMK achieved 1.8 $\log_{10}$ inhibition.

The activities of the phages were however different and dominant on ET segments. Phage ΦJHS exhibited a 1.2 $\log_{10}$ inhibition in *PA* 1098 strain and a 2.2 $\log_{10}$ (> 99%) inhibition in the *PA* 1668 strain. In the same order, phage ΦSMK achieved 2.5 $\log_{10}$ inhibition and 3.2 $\log_{10}$ (> 99.9%) inhibition respectively. The high $\log_{10}$ inhibition values achieved in ET segments could suggest that phages adsorb effectively on ET than catheters. Contrary to this

finding, reduced phage adsorption on ET was reported recently (14). With limited literature on phage immobilization on polyvinyl chloride ET surfaces, the increased phage adsorption on ET segments in this study could be attributed to the use of segments other than a whole tube in the physical adsorption process. Another possible explanation could be related to the combinatory dynamic-static conditions used in this study.

On the other hand, this study further assessed the potential of using bacteriophages as destructive agents in preformed biofilms on medical implants. The results showed that 6 h phage treatment efficiently reduced the number of viable bacterial cells, providing further evidence for their possible use in preventing bacterial colonization of medical implants and eradication of preformed biofilm on the implants. The interactions revealed that highest $\log_{10}$ reductions were observed with phage titers of 10^6 PFU/mL. Differences in efficiency were noted between the isolated phages against the two MDR *PA* strains, as well as between the different phage titers used. Of the isolated phages, phage ΦSMK produced significant 0.4 and 0.7 $\log_{10}$ reductions in the numbers of *PA* 1098 and *PA* 1668 respectively, on catheter segments at titer of 10^2 PFU/mL. The same titer however, did not result in statistically significant $\log_{10}$ reductions in both MDR strains on ET segments by phages ΦJHS and ΦSMK. With phage titers of 10^4 PFU/mL, phage ΦJHS was only efficient at reducing numbers of viable cells with 0.8 and 0.9 $\log_{10}$ reductions on catheter and ET segments colonized by *PA* 1668 strain respectively.

On the other hand, phage ΦSMK achieved 0.9 and 1.2 $\log_{10}$ (> 90%) reductions on catheter segments colonized by *PA* 1098 and *PA* 1668 respectively, and 1.5 $\log_{10}$ reduction on ET segments colonized by *PA* 1098 strain. These findings are in agreement with a previous study (13) which reported the efficient decrease of viable *E. faecalis* cells in biofilms formed on catheters when low phage titers were applied. Treatment with phage titers of 10^6 PFU/mL yielded highest $\log_{10}$ reductions in both phages with ≥ 1.0 $\log_{10}$ reduction for ΦJHS and ≥ 1.6 $\log_{10}$ reduction for ΦSMK. With 2.4 $\log_{10}$ reduction, phage ΦSMK achieved the highest $\log_{10}$ reduction value, further confirming its dominance over phage ΦJHS. The results further showed that increasing phage titer increased $\log_{10}$ reduction value, indicating that greatest significance of ≥ 3 $\log_{10}$ could be achieved with phage titers above or equal to 10^8 PFU/mL.

The results clearly show that mono phages alone can be used to either prevent *PA* colonization of medical implants or reduce the numbers of preformed biofilm on these implants. Besides using mono phages, positive results have been reported on the use of phage cocktail to prevent

ET colonization (14) by this bacterium. Outside the medical scope, effective use of mono phages or phage cocktail as biocontrol agents of *PA* in water (84) and phage combination with disinfectant to remove plastic-surface associated *PA* (85) have been recently reported. These studies highlight the urgent need to investigate new strategies to prevent, control or remove *PA* biofilms on surfaces.

This study has demonstrated that phages can serve dual purposes of coating surfaces to prevent bacterial colonization and reducing bacterial bioburden on surfaces. Most researches however, have been conducted on the latter, at the expense of the former, although the results of this study clearly indicate that phages are more effective at inhibiting bacterial colonization, than removing preformed biofilms. Though some issues remain in phage immobilization, future research must turn more attention on the use of phages as preventive agents rather than removal agents. As the saying goes, prevention is better than cure.

CHAPTER SEVEN

CONCLUSION AND RECOMMENDATION

7.1 Conclusion

The results of this study suggest that phages in this study have great potential for the development of phage-coated catheter and ETs and phage-biofilm removal from these implants. The encouraging results obtained with the inhibition and reduction of MDR *PA* biofilm formation on catheter and ET surfaces emphasize the potential of using phages for dual purposes of bacterial colonization prevention and bacterial biofilm removal. However, the use of mono phages alone is not enough to completely inhibit bacterial colonization or remove biofilms, suggesting that mono phages alone may be used as complementary strategies rather than alternative strategies in bacterial colonization prevention and biofilm removal. Furthermore, the results of this study depict that phage serves better as preventive agents than removal agents and therefore would require more efforts to enhance phage-coating and immobilization methods.

7.2 Recommendation

As a suggestion for future studies in the study setting, genome analysis of the phages can be done for a complete characterization of the phages. It is recommended also for future research to investigate the synergistic action of phages, phage cocktails or phage-derived proteins with other antimicrobials such as antibiotics, antimicrobial peptides, or nanoparticles in preventing bacterial colonization and removing preformed biofilms on the surfaces of catheters, ETs or other medical implants.

7.3 Limitations of the Study

Micrographs of the isolated phages could not be taken for full morphological analysis and classification of the phage virions due to unavailability of Electron microscope. As a result, phage cocktail was not developed for the antibiofilm assessment on catheter and endotracheal tube surfaces. Likewise, detail phage genome analysis for a complete characterization of the phages is recommended throughout the world but in this study, it was not planned and performed due to the ack of reagents and necessary equipment. Lack of biofilm reactor resulted in a change of biofilm formation from dynamic to static.

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APPENDIX I

REAGENTS AND MEDIA PREPARATION

Saline magnesium (SM) buffer:

NaCl	0.58 g
Tris-Base	0.606 g
$MgSO_4.7\ H_2O$	0.2 g
H_2O	100 mL
pH	7.5

Columbia agar (CA):

Columbia agar powder: 42.5 g

H_2O	1000 mL

Nutrient broth (NB):

Nutrient broth powder	1.3 g
H_2O	100 mL

Soft agar:

Nutrient broth powder	1.3 g
Bacteriological agar	0.75 g
H_2O	100 mL

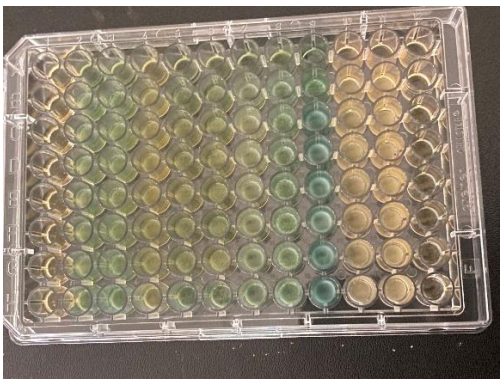
APPENDIX II

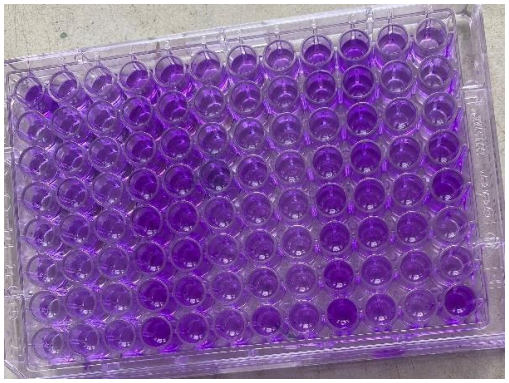
BIOCHEMICAL IDENTIFICATION

Strain	Oxidase	Catalase	Indole	Citrate	Urea	Gas	H_2S	Glucose	Lactose	Lysine	Accept/Reject
ATCC 27853	+	+	–	+	–	–	–	–	–	–	✓
1321	+	+	+	+	–	–	–	–	–	–	✓
1098	+	+	–	+	–	–	–	–	–	–	✓
1139	+	+	–	+	–	–	–	–	–	–	✓
1280	+	+	–	+	–	–	–	–	–	+	✓
1054	+	+	–	+	+	+	–	+	+	+	•
1668	+	+	–	+	–	–	–	–	–	–	✓
1329	+	+	–	+	–	–	–	–	–	+	✓
1776	+	+	–	–	+	+	–	+	+	+	•
1095	+	+	–	+	–	–	–	–	–	–	✓

APPENDIX III

BIOFILM FORMATION ASSAY AND OPTICAL DENSITY READINGS





Primary Filter=630; Differential Filter=None; vs. Stored Blank

		G05	0.1219; 0.1221
A01	0.0104; 0.0106	H05	0.1270; 0.1276
B01	0.0152; 0.0159	A06	0.1529; 0.1530
C01	0.0116; 0.0121	B06	0.1354; 0.1355
D01	0.0725; 0.0730	C06	0.0382; 0.0387
E01	0.0505; 0.0505	D06	0.0924; 0.0922
F01	0.0571; 0.0572	E06	0.0901; 0.0904
G01	0.0720; 0.0724	F06	0.0587; 0.0586
H01	0.1259; 0.1259	G06	0.0954; 0.0959
A02	0.0774; 0.0770	H06	0.0931; 0.0940
B02	0.0281; 0.0282	A07	0.0486; 0.0485
C02	0.0251; 0.0254	B07	0.0306; 0.0304
D02	0.0642; 0.0641	C07	0.0231; 0.0235
E02	0.0637; 0.0641	D07	0.1199; 0.1202
F02	0.0807; 0.0824	E07	0.1145; 0.1150
G02	0.0950; 0.0951	F07	0.0521; 0.0516
H02	0.0762; 0.0774	G07	0.0325; 0.0324
A03	0.0894; 0.0892	H07	0.1624; 0.1631
B03	0.0535; 0.0540	A08	0.0850; 0.0854
C03	0.0040; 0.0044	B08	0.2066; 0.2065
D03	0.0271; 0.0272	C08	0.1542; 0.1546
E03	0.0287; 0.0290	D08	0.2129; 0.2122
F03	0.0264; 0.0270	E08	0.1255; 0.1255
G03	0.0687; 0.0692	F08	0.1094; 0.1097
H03	0.1316; 0.1321	G08	0.1620; 0.1626
A04	0.0951; 0.0946	H08	0.2310; 0.2321
B04	0.0530; 0.0530	A09	0.1370; 0.1367
C04	0.0719; 0.0724	B09	0.1017; 0.1020
D04	0.0414; 0.0414	C09	0.0480; 0.0476
E04	0.0401; 0.0400	D09	0.0799; 0.0805
F04	0.0294; 0.0299	E09	0.0610; 0.0606
G04	0.0439; 0.0437	F09	0.2299; 0.2297
H04	0.0445; 0.0449	G09	0.2554; 0.2559
A05	0.0707; 0.0707	H09	0.1929; 0.1930
B05	0.1136; 0.1141	A10	0.1377; 0.1379
C05	0.0954; 0.0959	B10	0.1005; 0.1010
D05	0.0796; 0.0789	C10	0.1179; 0.1182
E05	0.1154; 0.1159	D10	0.1251; 0.1254
F05	0.1437; 0.1441	E10	0.1279; 0.1289

F10	0.1820; 0.1821	H11	0.3002; 0.3010
G10	0.0831; 0.0831	A12	0.2172; 0.2171
H10	0.1006; 0.1012	B12	0.2054; 0.2056
A11	0.1035; 0.1039	C12	0.2027; 0.2035
B11	0.3111; 0.3112	D12	0.2155; 0.2162
C11	0.3046; 0.3055	E12	0.1981; 0.1982
D11	0.2882; 0.2879	F12	0.2947; 0.2957
E11	0.0896; 0.0901	G12	0.2116; 0.2125
F11	0.0994; 0.0995	H12	0.2470; 0.2476
G11	0.0920; 0.0919		

APPENDIX IV

ETHICAL CLEARANCE APPROVAL FORM

Jimma University Institute of Health

Institutional Review Board

Ref.No JHRPGn/166/.

Date: 01/06/2021.

To: Mrs. STEPHEN AMANKWAH

Subject: Ethical Approval of Research Protocol

The IRB of Institute of Health has reviewed your research project titled **"Phage activity against multidrug – resistant pseudomonas aeruginosa biofilm formation on medical devices"**

Thus, this is to notify that this research protocol has presented to the IRB meets the ethical and Scientific standards outlined in national and international guidelines. Hence, we are pleased to inform you that your research protocol is ethically cleared.

We strongly recommend that any significant deviation from the methodological details indicated in the approved protocol must be communicated to the IRB before it has been implemented.

With Regards!

IRB chairperson
Million Tesfaye, PhD
Tel: +251917063744
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APPENDIX V

GRAPHPAD PRISM OUTPUT FOR MULTIPLE UNPAIRED t TESTS

Table Analyzed	Phage treatment of biofilms
Column A	Control
vs.	vs.
Column B	ΦJHS
Test details	
Test name	Unpaired t test with Welch correction
Variance assumption	Individual variance for each group
Multiple comparisons	Set P value threshold
Method	Bonferroni-Dunn method
Alpha	0.05
Number of tests performed	7
Number of rows omitted	0

	Below threshold?	P value	Mean of Control	Mean of ΦJHS	Difference	SE of difference	t ratio	df	Adjusted P Value
PA 1095	No	0.341125	0.09555	0.08063	0.01492	0.01438	1.037	5.773	>0.999999
PA 1098	Yes	<0.000001	0.2169	0.08017	0.1367	0.007438	18.38	9.693	<0.000001
PA 1139	Yes	0.000013	0.1310	0.02007	0.1110	0.01099	10.10	7.475	0.000089
PA 1280	Yes	0.000002	0.2261	0.07333	0.1528	0.01382	11.06	8.698	0.000014
PA 1321	Yes	0.000167	0.1452	0.05307	0.09217	0.01292	7.132	7.176	0.000172
PA 1329	Yes	<0.000001	0.3014	0.1272	0.1742	0.006958	25.04	9.577	<0.000001
PA 1668	Yes	<0.000001	0.2944	0.1386	0.1559	0.005592	27.87	8.220	<0.000001

Table Analyzed	Phage treatment of biofilms
Column A	Control
vs.	vs.
Column C	ΦSMK
Test details	
Test name	Unpaired t test with Welch correction
Variance assumption	Individual variance for each group
Multiple comparisons	Set P value threshold
Method	Bonferroni-Dunn method
Alpha	0.05
Number of tests performed	7
Number of rows omitted	0

	Below threshold?	P value	Mean of Control	Mean of ΦSMK	Difference	SE of difference	t ratio	df	Adjusted P Value
PA 1095	No	0.050616	0.09555	0.06013	0.03542	0.01447	2.448	5.899	0.354311
PA 1098	Yes	<0.000001	0.2169	0.03918	0.1777	0.009239	19.23	8.208	<0.000001
PA 1139	Yes	0.000913	0.1310	0.07328	0.05775	0.01178	4.901	8.768	0.000392
PA 1280	Yes	0.000143	0.2261	0.07583	0.1503	0.02249	6.684	8.150	0.000999
PA 1321	Yes	0.000177	0.1452	0.03703	0.1082	0.01189	9.099	5.405	0.000240
PA 1329	Yes	<0.000001	0.3014	0.09625	0.2052	0.007686	26.69	8.894	<0.000001
PA 1668	Yes	0.000002	0.2944	0.07367	0.2208	0.01151	19.18	5.670	0.000016

Table Analyzed	Transform of Phage-coating of PA1098
Column A	Control
vs.	vs.
Column B	ΦJHS
Test details	
Test name	Unpaired t test with Welch correction
Variance assumption	Individual variance for each group
Multiple comparisons	Set P value threshold
Method	Bonferroni-Dunn method
Alpha	0.05
Number of tests performed	2
Number of rows omitted	0

	Below threshold?	P value	Mean of Control	Mean of ΦJHS	Difference	SE of difference	t ratio	df	Adjusted P Value
Catheter	Yes	0.001869	9.471	8.143	1.328	0.3068	4.330	9.077	0.003737
ET	Yes	0.000053	9.742	8.578	1.163	0.1344	8.655	7.041	0.000106

Table Analyzed	Transform of Phage-coating of PA1098
Column A	Control
vs.	vs.
Column C	ΦSMK
Test details	
Test name	Unpaired t test with Welch correction
Variance assumption	Individual variance for each group
Multiple comparisons	Set P value threshold
Method	Bonferroni-Dunn method
Alpha	0.05
Number of tests performed	2
Number of rows omitted	0

	Below threshold?	P value	Mean of Control	Mean of ΦSMK	Difference	SE of difference	t ratio	df	Adjusted P Value
Catheter	Yes	0.000269	9.471	7.693	1.778	0.2865	6.204	7.923	0.000537
ET	Yes	0.000005	9.742	7.258	2.484	0.1698	14.63	6.220	0.000009

Table Analyzed	Transform of Phage-coating of PA1668
Column A	Control
vs.	vs.
Column B	ΦJHS
Test details	
Test name	Unpaired t test with Welch correction
Variance assumption	Individual variance for each group
Multiple comparisons	Set P value threshold
Method	Bonferroni-Dunn method
Alpha	0.05
Number of tests performed	2
Number of rows omitted	0

	Below threshold?	P value	Mean of Control	Mean of ΦJHS	Difference	SE of difference	t ratio	df	Adjusted P Value
Catheter	Yes	0.000395	9.808	8.518	1.290	0.1689	7.638	5.506	0.000789
ET	Yes	0.000001	9.861	7.677	2.184	0.1936	11.28	9.128	0.000002

Table Analyzed	Transform of Phage-coating of PA1668
Column A	Control
vs.	vs.
Column C	ΦSMK
Test details	
Test name	Unpaired t test with Welch correction
Variance assumption	Individual variance for each group
Multiple comparisons	Set P value threshold
Method	Bonferroni-Dunn method
Alpha	0.05
Number of tests performed	2
Number of rows omitted	0

	Below threshold?	P value	Mean of Control	Mean of ΦSMK	Difference	SE of difference	t ratio	df	Adjusted P Value
Catheter	Yes	<0.000001	9.808	7.915	1.892	0.1049	18.03	6.401	0.000002
ET	Yes	<0.000001	9.861	6.648	3.213	0.2128	15.10	9.930	<0.000001

Table Analyzed	Transform of Post-treatment PA1098
Column A	Control
vs.	vs.
Column B	ΦJHS
Test details	
Test name	Unpaired t test with Welch correction
Variance assumption	Individual variance for each group
Multiple comparisons	Set P value threshold
Method	Bonferroni-Dunn method
Alpha	0.05
Number of tests performed	6
Number of rows omitted	0

	Below threshold?	P value	Mean of Control	Mean of ΦJHS	Difference	SE of difference	t ratio	df	Adjusted P Value
Catheter (10^2)	No	0.065227	8.371	8.161	0.2104	0.09728	2.163	7.401	0.391361
Catheter (10^4)	No	0.011793	8.371	7.875	0.4958	0.1452	3.414	6.782	0.070757
Catheter (10^6)	Yes	0.001490	8.371	7.128	1.243	0.1773	7.012	4.419	0.008939
ET (10^2)	No	0.095039	8.363	7.860	0.5038	0.2656	1.897	7.867	0.570235
ET (10^4)	No	0.012875	8.363	7.517	0.8468	0.1842	4.598	3.597	0.077247
ET (10^6)	Yes	0.007262	8.363	7.252	1.112	0.1776	6.261	3.127	0.043574

Table Analyzed	Transform of Post-treatment PA1098
Column A	Control
vs.	vs.
Column C	ΦSMK
Test details	
Test name	Unpaired t test with Welch correction
Variance assumption	Individual variance for each group
Multiple comparisons	Set P value threshold
Method	Bonferroni-Dunn method
Alpha	0.05
Number of tests performed	6
Number of rows omitted	0

	Below threshold?	P value	Mean of Control	Mean of ΦSMK	Difference	SE of difference	t ratio	df	Adjusted P Value
Catheter (10^2)	Yes	0.005817	8.371	7.985	0.3861	0.08180	4.720	4.801	0.034905
Catheter (10^4)	Yes	0.001933	8.371	7.474	0.8972	0.1859	4.827	6.965	0.011599
Catheter (10^6)	Yes	0.001446	8.371	6.725	1.646	0.2090	7.879	3.971	0.008677
ET (10^2)	No	0.019388	8.363	7.676	0.6873	0.2047	3.358	5.128	0.116328
ET (10^4)	Yes	0.000536	8.363	6.873	1.490	0.2657	5.608	7.869	0.003216
ET (10^6)	Yes	0.000069	8.363	6.350	2.014	0.2650	7.599	7.859	0.000416

Table Analyzed	Transform of Post-treatment PA1668
Column A	Control
vs.	vs.
Column B	ΦJHS
Test details	
Test name	Unpaired t test with Welch correction
Variance assumption	Individual variance for each group
Multiple comparisons	Set P value threshold
Method	Bonferroni-Dunn method
Alpha	0.05
Number of tests performed	6
Number of rows omitted	0

	Below threshold?	P value	Mean of Control	Mean of ΦJHS	Difference	SE of difference	t ratio	df	Adjusted P Value
Catheter (10^2)	No	0.074778	8.738	8.445	0.2927	0.1393	2.102	6.804	0.448665
Catheter (10^4)	Yes	0.004350	8.738	7.966	0.7726	0.1687	4.579	5.672	0.026098
Catheter (10^6)	Yes	0.001664	8.738	7.700	1.038	0.1981	5.239	6.305	0.009987
ET (10^2)	No	0.045856	8.819	8.339	0.4803	0.2018	2.380	7.672	0.275138
ET (10^4)	Yes	0.004301	8.819	7.938	0.8814	0.1595	5.528	4.274	0.025806
ET (10^6)	Yes	0.000074	8.819	7.245	1.574	0.2099	7.500	7.893	0.000446

Table Analyzed	Transform of Post-treatment PA1668
Column A	Control
vs.	vs.
Column C	ΦSMK
Test details	
Test name	Unpaired t test with Welch correction
Variance assumption	Individual variance for each group
Multiple comparisons	Set P value threshold
Method	Bonferroni-Dunn method
Alpha	0.05
Number of tests performed	6
Number of rows omitted	0

	Below threshold?	P value	Mean of Control	Mean of ΦSMK	Difference	SE of difference	t ratio	df	Adjusted P Value
Catheter (10^2)	Yes	0.002501	8.738	8.055	0.6829	0.1446	4.723	6.616	0.015008
Catheter (10^4)	Yes	0.000074	8.738	7.538	1.200	0.1050	11.43	5.155	0.000444
Catheter (10^6)	Yes	0.000010	8.738	6.336	2.403	0.1669	14.39	5.736	0.000061
ET (10^2)	No	0.073555	8.819	8.324	0.4955	0.2227	2.225	5.303	0.441327
ET (10^4)	No	0.018689	8.819	7.857	0.9625	0.2885	3.336	5.339	0.112136
ET (10^6)	Yes	0.001561	8.819	7.207	1.612	0.2438	6.612	4.656	0.009363