Efficacy of Bacteriophage Treatment on *Pseudomonas aeruginosa* Biofilms

By:

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A thesis submitted in conformity with the requirements
for the degree of Master of Science

Faculty of Dentistry
University of Toronto

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2012

Abstract

Introduction: Bacterial viruses (phages) have been used successfully in the treatment of animal and human bacterial infections. This study examined the potential use of phage therapy against *Pseudomonas aeruginosa* strain PA14 biofilms in a root canal model. **Methods: Part 1:** 24 and 96h PA14 biofilms grown in microplates were treated with phages identified as possessing potential biofilm degrading activities and the post-treatment bacterial biomass was quantified using crystal violet staining. **Part 2:** 24 and 96h PA14 biofilms grown in prepared root canals of extracted human mandibular incisors were treated with phages identified with potential biofilm-degrading activities. Post-treatment intra-canal samples using paper points and round burs were taken to assess phage and bacterial counts. **Results: Part 1:** We identified two phages (JBD4 and JBD44a) with putative biofilm degrading activities. Treatment of PA14 biofilms with these phages produced a significant reduction in the mean percentage of biomass in 24h ($p<0.05$) and 96h ($p=0.08$) biofilms. **Part 2:** In 24 and 96h PA14 biofilms in a root canal model, no significant difference was found in the number of colony forming units after phage treatment ($p>0.05$). **Conclusion:** Phage application significantly reduced the biomass of 24 and 96h PA14 biofilms grown on microplates, but did not produce significant reduction of 24 or 96h PA14 biofilms grown in the extracted tooth model.

Acknowledgments

I would like to thank the members of my advisory committee, Dr. Bettina Basrani, Dr. Anil Kishen, and Dr. Alan Davidson. I would like to extend this thank you to include Dr. Karen Maxwell, who provided me with many hours of her time, a place to conduct my study, an endless amount of supplies, and laboratory instruction. This project would not have taken place without Dr. Maxwell's guidance. Dr. Amir Azarpazhoooh provided statistical assistance and I am truly thankful for all of the time he devoted to this project. The writing and editing of this manuscript was completed with the help of Dr. Calvin Torneck, a big thank you is indeed indicated for all of his time.

The technical help from Diane Bona, Senjuti Saha, Mostafa Fatehi Hassanabad, Nichole Cumby, and Joe Bondy-Denomy was greatly appreciated. Without the lessons from Diane on how to perform the necessary laboratory techniques to complete the project and the understanding of the science behind these techniques from Joe I would not have been able to complete this project. Thank you to these members of the Maxwell and Davidson laboratories for all of their time and efforts.

Last but not least, I would like to thank Dr. Brian Phee, husband extraordinaire. From helping me come up with the initial idea of the project to picking me up in the middle of the night from the lab, he has gone out of his way to be supportive.

This study was supported in part by grants from the Canadian Academy of Endodontics Endowment Fund, the American Association of Endodontists Foundation, and an Operating Grant from the Canadian Institutes for Health Research to K.L.M. (Fund No. MOP-6279).

Aly Phee, June 2012

Table of Contents

Abstract	ii
Acknowledgements	iii
Table of Contents	iv
1. Introduction	1
1.1 Primary Apical Periodontitis	1
1.2 Persistent Apical Periodontitis	2
1.3 Biofilm	3
1.4 Biofilm in Endodontics	4
1.5 <i>Pseudomonas aeruginosa</i>	6
1.6 <i>Pseudomonas aeruginosa</i> in Endodontics	7
1.7 Bacteriophage Therapy	8
1.8 Bacteriophage Therapy in Dentistry	10
2: Objectives and Hypothesis	
2.1 Objectives	11
2.1.1 General Objectives	11
2.1.2 Specific Aims	11
2.2 Hypothesis	12
3: Article	13
4: Discussion	31

5: Conclusions	38
6: References	39

1. Introduction

Bacteriophages (phages) are obligate parasitic viruses that adsorb to bacterial cell surfaces via their tail by binding to a membrane bound phage receptor (1). While this binding is at times strain specific, most phages have a broader spectrum and retain an ability to infect several strains within a species (2,3). Once bound to the bacterial cell receptor, phage DNA is injected into the host cell and can alter the bacterial DNA to disrupt its metabolism or cause its death (3,4). This ability makes phages potentially useful in the reduction of viable bacteria. This study was undertaken to investigate the potential use of phages in the management of endodontic infections, primarily persistent root canal infections dominated by the presence of *Pseudomonas aeruginosa*.

1.1 Primary Apical Periodontitis

Primary apical periodontitis is an inflammatory response of the periodontal ligament and surrounding bone to infection present in the root canal system (5,6). Bacteria and bacterial products in the root canal initially interact with resident and migrant host cells to initiate inflammation and destruction of the tissue present at anatomical sites along the dental root where blood vessels and nerves normally enter and exit the pulp space. The process then extends into the surrounding cancellous bone (7). While bacteria, can and often do, extend from the root canal into the inflamed tissue at these sites, the inflammation is mostly sustained by presence of bacteria in the root canal supported by an environment that is favorable to their growth. Successful treatment of root canal

infection requires physical reduction in the number of root canal bacteria and an alteration in the root canal environment to one that does not encourage bacterial colonization and survival (8,9). After treatment, an effective coronal seal to prevent the future ingress of bacteria must protect the new environment (10-13). Outcome studies that assess the efficacy of current treatment methods used in the management of root canal infections report a favorable response rate of 74-86% (14-17).

1.2 Persistent Apical Periodontitis

Persistent endodontic disease occurs when endopathic bacteria are not adequately controlled by the endodontic treatment or when new bacteria or other factors that promote disease are introduced into the root canal and periapical tissues during or after therapy. Unlike primary apical periodontitis, persistent disease can be caused not by just microorganisms, but also by cyst development in the inflamed apical tissue, and by the inadvertent introduction of foreign materials into the apical tissues during initial treatment (18-20). Microorganisms responsible for persistent disease can be those responsible for primary apical periodontitis that have survived the initial disinfection protocol and have adapted to the post treatment environment created in the root canal (primary persistence) (21), or new microorganisms that have gained access to, and colonized the root canal after initial treatment has been completed (secondary persistence) (11,12,20,22-25). Predisposing factors that lead to primary persistence include inadequate aseptic control, missed anatomy, inadequate instrumentation, and inadequate debridement (26-29). Those factors that lead to secondary persistence and

biofilm resilience include poor or broken coronal restorations, active caries, cracks, or poorly filled root canals (10-13,18,30,31).

The microbiota associated with persistent disease is markedly different from that of primary disease (32,33). Non-vital teeth with apical periodontitis and clinically intact crowns display a microbiota dominated (> 90%) by obligate anaerobes (34-37). Root canals exposed to saliva display a modest decrease in anaerobic bacteria (>70%) and an increase in those that are facultative anaerobes (38). Unlike primary AP, the microbiota associated with persistent apical periodontitis is dominated by facultative microorganisms, particularly Gram-positive rods and cocci (29,33). As a group these microorganisms display more resistance than those that are strictly anaerobic (33,34,39-57). Both primary and persistent infections can be a mono-infection caused by a variety of species that include *Pseudomonas*, *Actinomyces*, or *Enterococci* (58-65). Like teeth with primary root canal infections, teeth with persistent infections also can be treated by non-surgical and/or surgical methods with an expected healing rate of 77-80% (66,67) and 74-76% (68,69) respectively.

1.3 Biofilm

Biofilm is a surface-associated complex bacterial community encased in a hydrated extracellular matrix of exopolysaccharides (EPS), proteins, nucleic acid, and lipids (70). They are generally heterogeneous, structurally organized microbial communities loosely connected by water channels and voids (71). The EPS component of the biofilm accounts for 50 – 90% of its organic carbon content and is highly hydrated due to their ability to

incorporate water into its structure by hydrogen bonding. The hydration of the EPS protects the biofilm against desiccation (72), and affords the bacteria present in the biofilm sufficient environmental protection to achieve densities approximately 1000 fold greater than what would be achievable if they existed in planktonic form (73).

Biofilm formation begins when bacteria penetrate the hydrodynamic boundary of a compatible surface through the interplay of van der Waal's forces of electrostatic attraction and repulsion (74). These forces are general, long range, weak, and reversible. Binding to a surface becomes irreversible when stereo-chemical coupling between bacterial adhesion molecules and complementary surface receptors, occurs. Bacteria that possess a flagellum are able to overcome surface electrostatic repulsion faster than those that do not, and bacteria that manifest certain types and large numbers of adhesion molecules can also bind to a surface more efficiently than those that do not (74). Once attachment has been established, bacteria can aggregate and co-aggregate to increase the density and complexity of the biofilm. Some biofilm-forming bacteria, such as *P. aeruginosa*, exhibit a cell-to-cell molecular signaling mechanism (quorum sensing) that influences their adhesion potential. When signaling molecules reach a critical concentration, there is an increase in their expression of virulence and an enhancement in biofilm formation (74).

1.4 Biofilm in Endodontics

Ricucci and Siqueira (75) studied the prevalence of biofilm in endodontic disease and concluded that their incidence was consistent with the criteria necessary to classify apical

periodontitis as a “biofilm-induced disease”. They reported that biofilm was present in the apical third of the root canal in 77% of teeth examined, 80% in those that had not been endodontically treated, and 74% in those with root fillings. They also reported a positive relationship between the presence of biofilm and the radiographic size of the lesion. Other studies have reported the presence of biofilm at more diverse sites in root canals (76), in root canal ramifications and isthmuses (77), and on the exterior root surface (75). Their presence in teeth with persistent disease raised the question as to their possible role as an etiological factor in disease persistence.

Because of the potential of biofilm to cause disease is high, methods of root canal disinfection are continually being explored and assessed. Promising results in the disruption of biofilm have been reported with the use of photodynamic therapy (PDT) (78-81) and ultrasonic assisted irrigation using a wire or small endodontic file to propagate an energy wave (82,83). Root canal irrigants, new and old, also have been assessed in regards to their biofilm disrupting potential. Most of these assessments were *in vitro* that use constructed biofilm grown on the dentin obtained from extracted teeth (84,85), nitro-cellulose membranes (86,87), or membrane filter discs (88,89). These and other studies confirmed what had long been suspected, that sodium hypochlorite was effective in reducing bacterial mass, but required prolonged exposure (>30min) when commonly used dilutions (1% and 2.5%) were used. They noted that reduction is mass dependant upon factors including biofilm species, type, volume, and concentration of the irrigant, and duration of exposure (8,90-94).

1.5 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, a member of the Gamma Proteobacteria class of bacteria, is a motile, Gram negative, facultative, rod-shaped bacterium measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm in size (95,96). Its optimum temperature for growth is 37°C, but retains a growth potential at temperatures as high as 42°C. It is resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics. *P. aeruginosa* strains produce two types of soluble pigments, a fluorescent pigment pyoverdine, and a blue pigment, pyocyanin. The latter plays a role in its iron metabolism and is produced in abundance in low-iron content media (97).

Cell-surface polysaccharides produced by *P. aeruginosa* serve as a barrier between the cell wall and the environment, mediate host-pathogen interactions, and form structural components of its biofilm. Lipopolysaccharide (LPS) plays a key structural role in the outer membrane integrity of *P. aeruginosa* and acts as an important mediator of host-pathogen interactions during disease (98). *P. aeruginosa* is primarily a nosocomial pathogen and has been described as an opportunistic human pathogen that exploits a disruption in host defense to initiate infection (99). It often infects immune-compromised patients, like those with cystic fibrosis, cancer, or AIDS (100) and produces endocarditis, respiratory infections, bacteremia, septicemia, central nervous system infections, ear and eye infections, bone and joint infections, urinary tract infections, gastrointestinal infections, and skin and soft tissue infections (101).

P. aeruginosa is classified as a dangerous pathogen because it is resistant to therapeutic

doses of commonly used antibiotics. The resistance is attributed to the impermeability of its outer membrane to antibiotic penetration, its ability to form an exopolysaccharide-protected (EPS) biofilm (101), the presence of multidrug efflux pumps in the cytosol, and chromosomally encoded antibiotic resistance genes. In addition to these intrinsic resistance factors, *P. aeruginosa* can easily acquire resistance by mutation of its chromosomally encoded genes or through horizontal gene transfer of antibiotic resistance determinants from plasmids (98). Recent studies have shown that phenotypic resistance associated with biofilm formation and the emergence of small-colony variants also may be important factors in its development of resistance (98).

1.6 *Pseudomonas aeruginosa* in Endodontics

P. aeruginosa has been recovered from primary and persistent endodontic infections (63-65,102-107). Several studies have identified *P. aeruginosa* as a component of the root canal microbiota of primary endodontic infections (65,106,107). Fujii *et al.* found *P. aeruginosa* represented 6.8% of the bacterial isolates recovered from twenty teeth with persistent apical infections (102). More importantly they found that four of five teeth with a draining sinus contained *P. aeruginosa*, and that in two of the twenty samples, *P. aeruginosa* occurred as a mono-species infection. This finding, coupled with the proven ability of some species to form biofilm, has identified *P. aeruginosa* as a putative pathogen in persistent endodontic disease. Its persistence after conventional endodontic treatment (63-65,102-105) indicates that it also may express resistance to commonly used endodontic disinfection protocols. This concept was supported experimentally by

Raphael *et al.* who reported that irrigation with 5.25% sodium hypochlorite could not routinely eliminate *P. aeruginosa* from the root canal (108), and by Leonardo *et al.* who reported that growth of *P. aeruginosa* was not inhibited by several of the commonly used root canal sealers (109). A 48h intra-canal dressing of calcium hydroxide (110) and photodynamic therapy (PDT) (111) have reportedly been shown to be only partially effective in eliminating its presence. While the PDT study also reported only partial reduction in *P. aeruginosa* density, it reported that its degree of resistance was 100-1000 times that of *E. faecalis*, a microorganism that has been reported in several studies (112-114) of persistent endodontic disease (111).

1.7 Bacteriophage (phage) Therapy

Phage therapy is an alternative in the management of persistent bacterial infections. Phages are obligate parasitic viruses that infect and kill bacteria. Tailed phages are the most numerous group. They display a protein shell and have a head that contains nucleic acid for encoding information required to direct its reproduction within a host bacterium, and a tail that allows them to attach and interact with their host cell. They initially adsorb to the bacterial cell wall via their tail fibers. While some phages are highly virulent and cause death of the host cell once they infect it, others are less lethal and just incorporate their DNA into host cell DNA to alter its function (115), and many phages can do both. Advantages of phage therapy include continuous self-replication of the phage at the site of infection and a host-specificity that leaves other bacterial and body cells undisturbed (116). Some phages have depolymerases as tail or spike fibers and this enables them to

reach the bacterial cell wall when an EPS coating is present (117). Through their interaction with host cells, phages may cause biofilm disruption, lysis, and degradation of the EPS (70). Phage therapy is potentially broad in its application and can be particularly important in the treatment of bacterial infections that display multidrug resistance (118). It is currently being used in the food industry for the prevention of poultry infection (119), in the bacterial reduction of fresh produce (120), human cancer therapy (121), wound healing therapy (122), allergy prevention therapy (123), and in the control of opportunistic bacterial infections present in immuno-compromised mice (124).

One phage has shown an ability to effectively diffuse through *P. aeruginosa* EPS matrix (115) and retain an infection potential in the deeper layers of the biofilm by permeating the water channels in the biofilm core (125). This property makes this phage a useful tool in the management of deep infections. Cornelissen *et al.* have suggested that some phages are useful because EPS acts as their primary receptor where they induce a time and dose dependant degradation of the biofilm. This effect is independent of the bacterial strain and biofilm age (70). Other phages are effective because they induce a bacterial synthesis of enzymes that degrade polymers that occlude their cell wall (126). Glonti *et al.* for example, showed that infection of certain *P. aeruginosa* strains by phage PT-6 induced secretion of a hydrolyzing enzyme that degraded host EPS (127). A new phage created by Lu and Collins (128) was engineered to not only induce the expression of a biofilm-degrading enzyme, but also to destroy the host cell. The anti-biofilm potential of this phage proved to be significantly greater than that of the non-enzyme producing phage used as the experimental control. The ability to engineer a phage with specific properties

demonstrates the potential that phage therapy may have in biofilm management in the future (128).

Clinical trials in which phage therapy have been used are limited. Hawkins *et al.* (129) reported a veterinary clinical trial of phage treatment in the management of *P. aeruginosa* ear infections in the dog. They found that a topical phage mixture applied to the infected ear led to *P. aeruginosa* lysis and a resolution of the infection without apparent detrimental effects to the dog's health. In a randomized, double-blinded, placebo-controlled Phase I/II clinical trial, Wright *et al.* (130) showed efficacy and safety of phage treatment in the management of antibiotic resistant *P. aeruginosa* induced chronic otitis in 24 patients where all showed improvement subsequent to the therapy (130).

1.8 Phage Therapy in Dentistry

The use of phage therapy in dentistry is limited. Shultz suggested its use in oral surgery and exodontias as early as 1932 (131). The only support for this suggestion was the encouraging reports of its use in other medical fields at that time. More recently, Paisano *et al.* (132) demonstrated that phage therapy could be used to reduce *E. faecalis* biofilm grown on dentin *in vitro*. In 2009 Stevens *et al.* reported on the recovery of a lysogenic phage from an infected root canal (133). The significance of this finding was not discussed.

2. Objectives and Hypothesis

Interest in phage therapy, in particular the role that it may play in the management of endodontic infections, is limited at this time. However, in view of the reported *in vivo* efficacy in the management of *P. aeruginosa* infective chronic otitis, it may be an interesting and reasonable avenue to pursue (129,130). Phage therapy may be a beneficial alternative or adjunctive disinfection strategy for the elimination of apical periodontitis. To this end a two-part study was undertaken.

2.1 Objectives

2.1.1 General Objectives

This study was designed to use an *in vitro* microplate assay as well as extracted tooth models to test the efficacy of bacteriophage treatment against a constructed *P. aeruginosa* biofilm.

2.1.2 Specific Aims

- To establish microplate assay parameters by testing different phages, combinations of phages, and the phage concentrations to identify the most effective and reproducible method of reducing the biomass.
- To establish a reproducible extracted tooth model for growing biofilms and testing the efficacy of bacteriophage treatment against the bacterial biomass.

- To quantify post-treatment phage concentrations in both parts of the study and compare these values to the pre-treatment loading concentration.
- To identify the potential benefits and limitations of using phage addition for the purpose of reducing biomass.

2.2 Hypothesis

It was predicted that *P. aeruginosa* biomass would be reduced by the addition of preselected phages in a microplate assay and in extracted tooth models. Phage therapy would reduce a younger 24h biofilm more effectively than an older 96h biofilm. Post-treatment phage concentrations would be higher than the initial loading concentrations.

3. Article

Submitted for Publication: *May 18, 2012*

Title Page

Efficacy of bacteriophage treatment on *Pseudomonas aeruginosa* biofilms

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Acknowledgement: The authors deny any conflicts of interest.

This study was supported by grants from the American Association of Endodontists Foundation and the Canadian Academy of Endodontics Endowment Fund. This work was supported in part by an Operating Grant from the Canadian Institutes for Health Research to K.L.M. (Fund No. MOP-6279). The authors thank Dr. Calvin Torneck for his editing and Diane Bona for her technical assistance.

Abstract

Introduction: Bacterial viruses (phages) have been used successfully in the treatment of animal and human bacterial infections. This study examined the potential use of phage therapy against *Pseudomonas aeruginosa* strain PA14 biofilms in a root canal model.

Methods: Part 1: 24 and 96h PA14 biofilms grown in microplates were treated with phages identified as possessing potential biofilm degrading activities and the post-treatment bacterial biomass was quantified using crystal violet staining. **Part 2:** 24 and 96h PA14 biofilms grown in prepared root canals of extracted human mandibular incisors were treated with phages identified with potential biofilm-degrading activities. Post-treatment intra-canal samples using paper points and round burs were taken to assess phage and bacterial counts. **Results: Part 1:** We identified two phages (JBD4 and JBD44a) with putative biofilm degrading activities. Treatment of PA14 biofilms with these phages produced a significant reduction in the mean percentage of biomass in 24h ($p<0.05$) and 96h ($p=0.08$) biofilms. **Part 2:** In 24 and 96h PA14 biofilms in a root canal model, no significant difference was found in the number of colony forming units after phage treatment ($p>0.05$). **Conclusion:** Phage application significantly reduced the biomass of 24 and 96h PA14 biofilms grown on microplates, but did not produce significant reduction of 24 or 96h PA14 biofilms grown in the extracted tooth model.

Keywords: *Pseudomonas aeruginosa*, bacteriophage therapy, apical periodontitis, root canal

Introduction

Primary apical periodontitis is an inflammatory response of the periodontal ligament and surrounding bone to infection within the root canal system (1). Successful treatment of root canal infection requires physical reduction in the number of root canal bacteria and an alteration in the environment that discourages bacterial re-colonization and survival (2). Outcome studies that assess the efficacy of current treatment methods used in the management of root canal infections report a favorable healing rate of 68-85% (3). Persistent endodontic disease occurs when endopathic bacteria are not adequately controlled or when new microorganisms are introduced into the root canal and periapical

tissues, during or after initial treatment (4). Teeth with persistent infections can be treated non-surgically and/or surgically and have an expected healing rate of 80% (5) and 74% (6) respectively. A study of the prevalence of endodontic biofilm reported its presence in the apical third of the root canal system in 80% of untreated teeth with AP and in 74% of those with root fillings (7). This is consistent with the criteria necessary to classify apical periodontitis as a “biofilm-induced disease”.

A potential cause of endodontic infections is *Pseudomonas aeruginosa*, a Gram-negative, facultative, rod-shaped bacterium that belongs to the Gamma Proteobacteria class of bacteria (8). It has been recovered from primary and persistent endodontic infections (10-17) and in one study represented 6.8% of the bacterial isolates recovered from persistent apical infections (10). Notably, in that study *P. aeruginosa* was found in four of five teeth with a draining sinus, two of which appeared to be a mono-species infection. *P. aeruginosa* is resistant to high concentrations of salts, dyes, weak antiseptics, and many commonly used antibiotics (9). Its persistence after conventional endodontic treatment (10-15) also indicates that it may be resistant to commonly used endodontic disinfection protocols. Irrigation with 5.25% sodium hypochlorite did not routinely eliminate *P. aeruginosa* from the root canal (18), and growth was not inhibited by frequently used root canal sealers (19). A 48h intra-canal dressing of calcium hydroxide (20) and photodynamic therapy (PDT) (21) have also been shown to be ineffective in eliminating its presence. These findings, coupled with the ability of *P. aeruginosa* to form biofilms, are sufficient to designate *P. aeruginosa* as a possible cause of persistent endodontic disease.

A potent anti-biofilm strategy that is passive to host tissue is necessary in endodontics to augment the limited mechanical bacterial debridement and antiseptic agents used. Bacteriophages (phages) are viruses that can infect and kill bacteria. They have been used as a treatment alternative in the management of non-dental persistent bacterial infections (22). Advantages of phage therapy include continuous self-replication of the phage at the site of infection and a host-specificity that leaves the other bacterial and human cells undisturbed (23). Through their interaction with bacteria, phages can degrade the extracellular matrix of exopolysaccharides (EPS), proteins, lipids, and nucleic acids that

comprise the bacterial biofilm community, leading to disruption of the biofilm and subsequent lysis of the bacterial cells (24).

The clinical use of phage therapy has been explored in the management of *P. aeruginosa* ear infections in dogs (25). A topical phage mixture applied to the infected ear led to *P. aeruginosa* lysis and a resolution of the infection, without apparent detrimental effects to general health. In a 2009 randomized, double-blinded, placebo-controlled Phase I/II clinical trial the efficacy and safety of phage therapy was demonstrated for the management of antibiotic resistant *P. aeruginosa* induced chronic otitis in 24 human patients (26). Phage therapy studies are limited in dentistry. They have been shown to reduce *Enterococcus faecalis* biofilm grown on human dentin (27) and they have been recovered from lysogenic *E. faecalis* strains isolated from infected root canals (28). In view of its specific antibacterial potential, this study was undertaken to investigate the efficacy of phage therapy in reducing *P. aeruginosa* biofilms grown in microwell plates and extracted tooth models.

Materials and Methods

Part 1: Characterization of biofilm-degrading phages and microwell plate assay

Phage isolation and propagation

Eighty phages isolated from *P. aeruginosa* samples from both environmental and clinical sources were assessed for potential exopolysaccharide-degrading activity. Serial dilutions of the phages were titrated on *P. aeruginosa* strain UCBPP-PA14 (PA14) and the plaques were examined for halo production, indicating the potential presence of polysaccharide depolymerases. Two halo producing phages, JBD4 and JBD44a, isolated from strains ATCC 15524 and Env110BP, respectively, were identified as having potential biofilm-degrading activity. High titer lysates of these phages were prepared by mixing plaque purified phage with PA14 and top-plating in 0.7% lysogeny broth (LB) agar supplemented with 10mM MgSO₄.

Phages were collected from the plates by soaking in suspension medium (SM; 100 mM sodium chloride, 10 mM MgSO₄, 50 mM Tris-HCl, pH 7.5, 0.01% (w/v) gelatin), to

which DNase (5 µg/mL) and RNase (5 µg/mL) was added (29). The phage lysates were passed through a 0.45 µm filter and the phage particles were concentrated by precipitation using polyethylene glycol 8000 (16h stirring at 4°C) and centrifugation (11,000 rpm for 20 minutes). The phage pellets were re-suspended in SM and the phage were banded on two sequential cesium chloride-equilibrium gradients (50,000 rpm, 24h). The phage bands were extracted and dialyzed into SM before further characterization.

Biofilm treatment assay

PA14 biofilms were grown using a modified version of the Calgary Biofilm Device that was previously shown to grow reproducible *P. aeruginosa* biofilms (30). A saturated overnight culture of PA14 in 0.5X LB broth was adjusted to an optical density of 0.4 at 600 nm ($\sim 6 \times 10^8$ colony-forming units (CFU/mL)) and used for inoculation. 150 µL of 0.5X LB was placed in 95 wells of two 96-well flat bottom microtiter plates and 84 wells were seeded with 5 µL of the PA14 inoculum (NUNC; Rochester, NY). A transferable solid-phase (TSP) pin lid (NUNC; Rochester, NY) was placed into the microtiter plate and incubated with shaking at 37°C for 24 or 96 h to allow biofilm growth. The TSP pin lid was then removed, rinsed in sterile water, air dried for 30 seconds, and transferred to a 96-well plate in which the wells contained JBD4, JBD44a, or a combination of the two at a final concentration of $\sim 6 \times 10^6$ pfu/well in 0.5X LB. The plate was incubated overnight at 37°C.

The phages present in the wells following the overnight incubation with the biofilms were enumerated using the double agar overlay method (31). 125 µL of solution was removed from wells containing JBD4, JBD44a, or the combination, and was sterilized by the addition of 5 drops of chloroform. The samples were centrifuged at 10,000 rpm for 5 minutes to remove bacterial cell debris. Serial dilutions of the supernatant were plated to determine the number of plaque forming units (PFU) present in each well.

Quantification of biofilm remaining after phage treatment

Following incubation with the phages, the TSP pin lid was rinsed with sterile water to remove unattached cells and air dried for 5 minutes. The pins were then immersed in a

1% crystal violet solution for 1 minute followed by three 30-second rinses in sterile water. The crystal violet remaining adsorbed to the cell mass on the pins of the lid was solubilized by soaking the TSP pin lid in a 96-well plate containing 70% ethanol (1 hour, 25°C) and the absorbance was determined for each well. The solubilized crystal violet was used as a measurement of biomass remaining on the pins (30).

Part 2: *In vitro* biofilm assay in the root canal model

Specimen preparation

The Research Ethics Board of the University of Toronto approved the collection and use of extracted human teeth for experiments conducted in this study. Twenty-eight intact, non-carious, human mandibular incisors without visible evidence of cracks, maintained in phosphate buffered solution, were used in this study. Teeth were radiographed to confirm the presence of a single canal and then decoronated to create a standardized root length of 15 mm. Access into the pulp chambers was made with a high-speed bur under water coolant, and the working length was established with a 10 K-type file 1 mm short of the apical exit. The root canals were prepared with ProTaper (Dentsply Tulsa Dental Specialties; Tulsa, OK) rotary instruments to size F5 with copious (10mL) intermittent irrigation of 2.5% sodium hypochlorite. A longitudinal groove of 0.5 mm was prepared on the facial and lingual surfaces of the roots with a diamond disc (Brasseler USA; Savannah, GA) and the apex sealed with two coats of varnish. Specimens were then autoclaved in distilled water at 121°C for 20 minutes. They were dried with sterile cotton gauze and paper points immediately prior to use.

Biofilm treatment assay

The teeth were randomly assigned to 24 and 96h biofilm periods ($n=14$). One tooth in each group received 5 μ L of 0.5X LB (control), while the rest of the teeth were inoculated with 5 μ L PA14 ($\sim 5 \times 10^7$ CFU/mL). This concentration is comparable to that used in previously reported phage therapy trial of human dentin infected with *E. faecalis* (2×10^8 CFU/mL) (27). Each tooth was placed vertically in a well of a 96-well plate, covered with parafilm and incubated at 37°C in a shaking incubator. After 24h, three groups of 3

teeth each were treated with phages at a final concentration of $\sim 10^7$ PFU/tooth: (a) 5 μ L JBD4; (b) 5 μ L JBD44a; (c) 2.5 μ L of each JBD4 and JBD44a. Four teeth remained untreated with phage. The treatment microwell plate was incubated at 37°C and media replenishment was provided for the 96h growth at 48h. At the end of the incubation period a coarse paper point (Dentsply Tulsa Dental Specialties; Tulsa, OK) was used to extract the contents of each canal and transfer it to a sterile Eppendorf tube containing 1 mL 0.5X LB. In addition, a size 2 round bur was used to remove dentin shavings in the coronal third of the tooth (3 seconds at 3,200 rpm) and the bur was transferred to a sterile Eppendorf tube containing 1 mL 0.5X LB. The tubes were incubated at 48°C for 1h before being vortexed, diluted, spread plated on LB and incubated at 37°C for 16h to determine the CFUs. Post-treatment phage concentrations were determined by removing 100 μ L from the paper point and bur samples, sterilizing with chloroform, and plating serial dilutions. All experiments were performed in triplicate.

Scanning Electron Microscopy

To visually inspect the bacterial presence along the root canal, two random specimens from the positive control group were prepared for scanning electron microscopy (Amray 1830; San Jose, CA). The specimens were fixated by placement in 2.5% gluteraldehyde in 0.1M phosphate buffered solution for 24h. The specimens were then sectioned longitudinally with a razor blade and serially dehydrated in dilutions of ethanol. The specimens were mounted and sputter-coated with gold/palladium alloy before examination.

Statistical Analysis

Descriptive data and statistical analyses were performed utilizing the SPSS 17.0 software package (SPSS Inc. Chicago IL). One-way analysis of variance (ANOVA) with least significance difference (Fisher's LSD) post-hoc comparisons was conducted to compare the bacteriophage treatment of the samples within each group. Independent *t*-tests were also conducted on each group at the two different growth time intervals. All statistical analyses were interpreted at a 5% level of significance.

Results

Part 1: Characterization of the biofilm degrading activity of JBD4 and JBD44a

Analysis of the plaque morphology of 80 temperate phages isolated from induced *P. aeruginosa* strains led to the identification of two phages (JBD4 and JBD44a) that formed turbid plaques with halos, suggesting the presence of EPS-degrading activity. JBD4 and JBD44a were propagated twice on PA14 and banded on a cesium chloride gradient to ensure a pure phage stock. We examined these phages using negative stain transmission electron microscopy and discovered that both were members of the *Siphoviridae* family of phages (Fig. 1A). This class of phages possesses an icosahedral head containing a double stranded DNA genome attached to a long, non-contractile tail through which it interacts with the bacterial host cell.

The ability of phages JBD4 and JBD44a, alone and in combination, to degrade PA14 biofilms was characterized in 96-well microplates. 24h and 96h time points were chosen as previous studies revealed that 24h *P. aeruginosa* biofilms were susceptible to selected antibiotics, and that increased antibiotic resistance occurred in biofilms grown for greater than 48h (32-35). As expected, the untreated 96h samples contained significantly more biomass ($p<0.001$) than the 24h biofilms. The addition of phages JBD4 and JBD44a into wells containing pre-formed biofilms produced a significant reduction in the mean percentage of bacterial biomass in 24h ($p<0.05$) and 96h ($p<0.003$) samples (Fig. 2). There was no additional decrease in the bacterial biomass with treatment using the phages in combination as compared to using them individually in either time period ($p=0.08$).

To assess whether the phages were actively replicating during the course of the assay, phage titers were determined for a number of individual treatment wells following the 24h incubation period. As shown in Table 1, the titers of both JBD4 and JBD44a increased approximately 10-fold, indicating that the phages are infecting the cells, replicating within them, and being released back into the medium following cell lysis. There was no significant difference in the number of phages released when using JBD4 and JBD44a alone or in combination in the 24h or 96h biofilms.

Part 2: Phage biofilm degrading activity in the root canal model

After confirming that phages JBD4 and JBD44a can infect and kill PA14 cells growing as a biofilm in the microplate assay, their ability to eradicate PA14 biofilm in the more biologically relevant tooth model was assessed. The growth of biofilm on the tooth surface was examined in a 24h biofilm using scanning electron microscopy, which confirmed the presence of a layer of bacterial cells adhered to the dentin surface (Fig. 1B).

Analysis of both planktonic and biofilm-forming cells in the untreated samples in the root canal model revealed an increase in the number of CFUs in the 96h sample as compared to the 24h time point (Table 2). The 96h biofilm was determined to have a 5-fold increase in the number of cells adhered to the surface of the teeth as compared with the 24h biofilm. This is consistent with the findings of increased biomass in the 96h biofilms in the microplate assay. The addition of phages JBD4, JBD44a, or a combination of the two did not significantly decrease the number of planktonic or biofilm-adhered cells in the 24h or 96h samples (Table 2).

While there was no appreciable difference in cell counts following treatment with the phages, the number of phages present at the end of the incubation period was analyzed to assess their replication. Since JBD4 and JBD44a were isolated from bacterial cells in which they were maintained as lysogens, and they produce turbid plaques on PA14 suggesting the formation of lysogens, it is likely that they also formed lysogens in this assay. This would provide resistance to further phage infection to cells in which the phage lysogen had integrated and could confound the resistant bacterial cell count numbers. The number of phages present in the planktonic fraction of the root canal increased by 100- to 1000-fold over the course of the experiment (Table 3). This illustrates that the phages were actively infecting, replicating within, and lysing the bacterial population even though the bacterial counts did not decrease over the time course of the experiment. Further, the bur assay shows that while the 24h biofilm samples contained phages at approximately the same concentration as they were seeded into the assay, the 96h biofilms averaged 100- to 1000-fold more phages associated with them.

This is especially interesting to note as the number of bacterial cells in the 96h biofilm was only increased 5-fold above the 24h biofilm.

Discussion

Unlike endodontic management of primary infections, the treatment of persistent infection often can be more specific and directed towards the elimination of a lesser number of identifiable species (4). One species identified as a cause of persistent disease in endodontics is *P. aeruginosa* (10-15), a Gram-negative opportunistic pathogen capable of forming biofilms that are resistant to antibiotics. *Pseudomonas* phage therapy has been successfully used for the management of persistent antibiotic-resistant otitis infections in humans and dogs (25,26), as well as for the treatment of *E. faecalis* biofilms grown on dentin (27). For these reasons, we investigated the use of phage therapy for the treatment of *P. aeruginosa* infections in root canal models.

The phages used in this study, JBD4 and JBD44a, were initially selected for their ability to produce plaques with halos, which is suggestive of the ability to degrade exopolysaccharides, a component of the extracellular matrix present in biofilms. We assessed the efficacy of a combination of the two phages in addition to their individual use as previous clinical trials reported the success of phage mixtures in the management of human and animal *P. aeruginosa* otitis infections (25,26). In a microwell plate assay, bacteriophage treatment significantly reduced the biomass of 24h and 96h PA14 biofilms in the microplate assay, but complete bacterial elimination was not observed. In contrast, there was no significant reduction of viable bacterial counts of 24h or 96h PA14 biofilms in the extracted tooth model. Two sampling methods were used in the extracted tooth model. Paper point absorption was specifically chosen to recover planktonic bacteria and phages present within the root canal lumen (12-16,27) and round burs were used to recover bacteria and phages adherent to the root canal wall, as well as those that might be present in the root dentin. Disparity in biomass reduction between the microwell plate assay and the extracted tooth model may possibly be attributed to the inability of the phages to effectively reach all parts of the root canal anatomy and penetrate dentinal tubules.

While both JBD4 and JBD44a were able to decrease the biomass in the microwell plate assay, complete elimination of bacteria was never observed, and the combination of phages did not demonstrate a significant reduction as compared to the individual phages with the same final concentration. It was suspected that lysogens of these phages might provide resistance to further phage infection to their bacterial hosts, and characterization of lysogens of JBD4 and JBD44a in PA14 indicated this to be correct (data not shown). Thus, PA14 into which either phage inserts as a lysogen are resistant to both JBD4 and JBD44a. Whether this resistance is provided by immunity due to the phage repressor or another mechanism is unknown. If it is repressor-mediated immunity, it can be overcome by engineering a virulent mutant of the phage that lacks the ability to form lysogens. The ability of JBD4 and JBD44a to form lysogens confounds the results of this study as the cells that persist in the assays are expected to be a mixture of bacterial cells that have integrated either JBD4 or JBD44a as a lysogen, and bacterial mutants that are able to interfere with the phage life cycle, thereby preventing it from infecting or replicating. Additional studies using virulent mutants of these phages will be required to fully deconvolute these results.

Previous phage therapy studies that examined the post-treatment phage concentrations found them to be significantly higher than the initial concentrations (25,26,34). In this study the phages were also able to replicate, and the final titers from the extracted tooth model were 100- to 1000-fold higher than were input into the assay. The propagation of phages in the assay illustrated their ability to infect cells, replicate within them, and lyse them, even though there was no corresponding decrease in bacterial cell counts. The lack of decrease in biomass adhered to the dentin of the teeth, as evidenced by the cell counts from the bur samples, suggested that the phages might have difficulty effectively penetrating the *P. aeruginosa* biofilms formed within a root canal. This could result from difficulty in delivering effective phage concentration to the infected site. Improved phage administration and encouraging their diffusion through the root canal system will be required for them to be effective in this environment.

While phage therapy has been effective in the management of some infections, further research into the most effective phage or phages combination is required before it is

practical for the treatment of endodontic infections. The use of virulent phages that are unable to form lysogens is a necessary step, and engineering of such phages will be undertaken.

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Figure Legends

Figure 1. **A.** Negative stain transmission electron microscopy image of phages JBD4 and JBD44a. **B.** Scanning electron microscopy image of *P. aeruginosa* on the dentin surface of an inoculated root canal specimen.

Figure 2: Mean percentage of biomass removal as detected by a crystal violet assay for 24 and 96 hours of PA14 growth with untreated PA14 as 100%. *Significance $p < 0.05$ for all except 24h JBD4/JBD44a.

Table 1. Post-treatment phage concentrations in 24 and 96h PA14 biofilms grown on microwell plates. No significance was detected.

	Initial Phage Titer (PFU/mL)	24 h Biofilm Titer (PFU/mL)	96 h Biofilm Titer (PFU/mL)
JBD4	7.6×10^7	3.1×10^8	2.1×10^8
JBD44a	6.4×10^7	2.2×10^8	1.9×10^9
JBD4/JBD44a	7.0×10^7	4.0×10^8	7.0×10^8

Table 2. The viable bacterial counts after 24h and 96h of growth in extracted teeth models. No significance was detected..

	Paper Point Assay		Bur Assay	
	24h Bacterial Count (CFU/mL)	96h Bacterial Count (CFU/mL)	24h Bacterial Count (CFU/mL)	96h Bacterial Count (CFU/mL)
PA14 untreated	4.8×10^7	9.6×10^8	1.5×10^6	5.2×10^6
JBD4	1.7×10^8	6.8×10^8	1.3×10^6	1.2×10^7
JBD44a	4.8×10^7	9.8×10^8	1.2×10^6	8.8×10^6
JBD4/JBD44a	1.6×10^8	7.0×10^8	4.0×10^6	1.6×10^6

Table 3. The post-treatment phage concentrations in 24h and 96h PA14 biofilms grown in extracted teeth models. No significance was detected.

	Initial Titer (PFU/mL)	Paper Point Assay		Bur Assay	
		24h Titer (PFU/mL)	96h Titer (PFU/mL)	24h Titer (PFU/mL)	96h Titer (PFU/mL)
JBD4	2.6×10^7	1.8×10^{10}	2.4×10^{10}	9.4×10^7	9.5×10^{10}
JBD44a	2.4×10^7	3.2×10^9	4.3×10^9	7.0×10^7	1.0×10^{10}
JBD4/JBD44a	1.3×10^8	6.1×10^9	4.8×10^9	1.1×10^8	5.3×10^9

Figure 1. A.

Figure 1. B.

Figure 2.

4. Discussion

The human oral microbiome consists of over 800 identifiable species (134-136). Many of these have reportedly been found in the root canal and periapical tissues of teeth with endodontic infections (32,35,137-143). While most infections caused by these microorganisms can be readily eliminated by current endodontic protocols, there are instances when the infection persists. Bacteria that are present in a biofilm and resistant to current disinfection protocols (78-89) are one of the causes of persistent infection (144-146). The most significant evasion from the host defense system is the microbial arrangement in a biofilm (31) and it is also the most important survival mechanism for bacteria (21). Tronstad *et al.* observed periapical plaque in teeth refractory to endodontic treatment (146). Surgical intervention was performed and bacteria that were held together by an extracellular material were noted in 73% of the specimens. Bacterial biofilm may explain the ability to sustain disease even after conventional interventions. Noiri *et al.* also analyzed surgical specimens and 82% had extraradicular biofilms (145). Reduction of biofilm is therefore significant as it may increase the prognosis of endodontic treatment by limiting the microbial causes of disease.

Unlike endodontic management of primary infections, the treatment of persistent infection often can be more specific and directed towards the elimination of a lesser number of identifiable species (25,61). One species identified as a cause of persistent disease in endodontics is *Pseudomonas aeruginosa* (63-65,102-105). Additionally, *P. aeruginosa* being a Gram-negative opportunistic pathogen with excellent ability to form biofilm, will serve as an excellent model organism to assess the antimicrobial efficacy of

bacteriophages. For this reason and the reported limited, but modestly successful use of phage therapy in the management of antibiotic-resistant *P. aeruginosa* infections *in vivo* (129,130), this microorganism was selected as the target species in this study. A report by O'Toole *et al.* in 1986 (147) showed that *P. aeruginosa* biofilm having a dense monolayer of cells punctuated with numerous micro-colonies could be successfully grown at 25-37°C on polystyrene plastic in 8 hours. In this study, *P. aeruginosa* biofilm was grown on the pegs of polystyrene microplates, which provided a readily available substrate for the study.

P. aeruginosa biofilms grown for 24 and 96 hours were selected for use as substrates based upon a 2010 study published by Wolcott *et al.* (148) which showed 24h *P. aeruginosa* biofilms were susceptible to selected antibiotics and that increased resistance to antibiotic was typical of those that were grown for 48h or longer (147,149,150). Indirectly this also supported the use of the 96h biofilm, the second substrate used in this study. At twice the age of a 48h biofilm, it was felt that at 96h the biofilm would be representative of one where antibiotic therapy would have little chance for success, making phage therapy a reasonable conservative treatment option. The 96h biofilm was confirmed to be significantly different from the 24h biofilm. This conclusion was based on visual confirmation of differences in biofilm morphology and consistency as well as crystal violet adherence quantified using a plate reader. As a biofilm ages there is a proportionate increase in the ratio of dead to live cells within the biomass. This makes cells in the deeper layers more difficult to reach because phages bind equally to receptor sites on both live and dead cells. This effectively reduces their infection rate of viable cells (125). In addition, bacteria in older biofilms metabolize at a lower rate than those

that are younger ones, especially if the bacteria are present in the deeper layers where the availability of oxygen and other nutrients is limited. As phage infection and phage life cycle are both dependent upon the growth stage of the host cell (119,151), it would be expected that the more slowly metabolizing cells present in older biofilms would demonstrate an increased degree of phage resistance (152). Since the reduction in biomass of the 96h biofilm in this study was comparable to that of the 24h biofilm after phage treatment, consideration had to be given as to why the expected outcome did not occur. It was suspected that this resulted from a variance in which the 96h biofilm was grown, a variance that did not allow it to reach sufficient maturity to express the phage resistance expected. Another reason for the choice of a 24h treatment time was based on a report that even at 48h, release of endotoxin by lysed bacteria causes injury to the host cell (153). This may have affected the ability to recover viable bacteria post-treatment for phage efficacy quantification. Studies that had used successfully longer or multiple treatment times (129,130,132) may have used a variant phage. The 24h phage treatment time used in this study allowed for comparison with other research that also used this interaction period (37,39,40,42,132,149).

The choice of phages used in this study, JBD4 and JBD44a, was based upon our preliminary data that showed that these particular phages, selected from a collection of 80 isolated *P. aeruginosa* phages, had the potential for reducing *P. aeruginosa* biomass. The chosen phages resulted in halo formation during plaquing assays, indicating their possible ability reduce exopolysaccharide and to effectively reduce a bacterial biofilm. A combination, as well as use of single phages, was tested because previous clinical trials had reported the successful use of phage combination in the treatment of human and

animal *P. aeruginosa* infections (129,130). When the combination of phages proved to be less effective than the use of just one, it was theorized that they could share a common genomic sequence. Since no bacterial clearance by either phage had been detected in JBD4 or JBD44a *P. aeruginosa* lysogens, the infection by one had prevented infection by the other, proving this theory correct. Destruction of the matrix is an important aspect in phage selection because the matrix represents a physical impediment but also serves as a reservoir for proteolytic enzymes and endoglucanases that can cause phage inactivation. Phages that produce enzymes that disrupt the matrix are more successful at penetrating biofilm and reaching the deep cells of the biofilm at therapeutic concentrations (152). Disrupting the biofilm without harming dentin substrate would have the combined benefit of allowing increased antimicrobial penetration during endodontic disinfection.

A phage concentration of 1×10^5 pfu/mL had been used in the Wright study (130) and 2×10^8 pfu/mL concentration was used in the Paisano study (132). Our preliminary data showed that there was reproducibility of biomass reduction at the higher concentrations and as a result, an initial concentration of 2×10^7 pfu/mL of JBD4 and JBD44a was selected. It is possible that in the Wright study the gene segment coding for polysaccharide depolymerases, DNase, or protease enzymes carried by the phage could have been sufficiently different than the ones used in this study to account for the decrease in concentration that still allowed it to generate a similar therapeutic effect (152). Studies that examined the post-treatment phage concentrations (35,36,39) found a significantly higher phage concentration compared to the initial concentration. The lack of significance between the pre-treatment phage concentration and the post-treatment

concentrations in this study could have been a result of the sampling methods used and their ability to recover the phages.

One challenge identified with the microplate assay was determining the cause of crystal violet absorbance to the polystyrene pegs of the microplates. The negative charge of *P. aeruginosa* and the biofilm both bind to the crystal violet dye, hence the quantification of biomass during the microplate assay. Until the answer to the binding of the dye to polystyrene can be explained, this assay cannot effectively be used to determine phage efficacy. The use of polystyrene microplates was modeled after the Calgary biofilm device created by Ceri *et al.* in 1999 (154) that was developed to test the antibacterial susceptibility of biofilms in general. Growth curves demonstrated that biofilms of a predetermined size could consistently be formed on the device at specific time points with no significant differences on each of the 96 pegs. The use of a crystal violet assay to quantify biomass destruction was modeled after a microtitre plate assay created by Knezevic and Petrovic in 2008 (150). The assay used in the current study was modified to adjust for the biofilm growth on the pegs of the lid rather than the wells but was quantified in a similar manner with a plate reader. The assay in the current study used 70% ethanol to remove the stained biofilm, rather than the 95% ethanol recommended by O'Toole *et al.* (147) as the higher concentration of ethanol was found in preliminary experiments to have too high an evaporation rate. Preliminary experiments showed that the incubation time of 1h in the ethanol provided the most consistent results.

Several problems were encountered when phage therapy was assessed as a disinfection option for the root canal of *P. aeruginosa* infected extracted teeth. Because a lab pipette was used to place the phage suspension into the root canal, delivery of the phage to apical

third of the root canal could not be assured; however, preliminary work using scanning electron microscopy analysis of infected teeth showed *P. aeruginosa* existed throughout the length of the canal. The lack of evidence of consistent biofilm formation in the apical third of the root canal could explain the relative ineffectiveness of phage therapy in the tooth model. Other delivery systems should therefore be explored before the clinical application of phage therapy is to be tested. These could include, but not limited to, the use of sonic or ultrasonic vibration, a pre-fitted gutta percha used in a plunging action, or a lentulo spinner. The differences in the surfaces of the two test models may also explain the difference in the results between the two parts of the study. The microwell plate assay may provide a better adhesive surface for the attachment of biofilm compared to dentin as the polystyrene surface is hydrophobic in nature versus the hydrophilic surface of dentin.

One of the sampling methods used in this study was paper point absorption. This method was chosen based on previous studies (6,31,60,63,65,104,132,155) that used paper points as their main method to extract the contents inside the root canal system. The paper points were specifically chosen to address the planktonic bacteria within the root canal. Round burs were also used in an attempt to show the presence of both bacteria and the bacteriophages attached to the dentin surface as well as showing penetration inside the dentinal wall of the root canal. Further studies are needed to compare different sampling methods and identify a sampling method that adequately removed both planktonic cells and attached biofilm structure. Other methods of consideration include hand files, rotary files, and sonic or ultrasonic agitation combined with paper point sampling. Research is needed to determine the depth of penetration of the phages into dentin and their sustainability inside the dentin.

This study represents a fraction of the scope of research that needs to be done to understand the potential role of bacteriophages in endodontic therapy. Further investigation into the most effective phage, or combination of phages, against *P. aeruginosa* and other microorganisms that are also responsible for persistent endodontic infections is needed. Bacteriophage genetic engineering and characterizations require further research to determine the optimal treatment parameters against each type of endopathic bacteria. Methods of delivering the bacteriophages using different media into the root canal have to be assessed to optimize the distribution of the phages within the complex anatomy of the root canal system. More research is necessary to determine whether phage therapy will someday become a reasonable treatment choice for persistent endodontic disease.

5. Conclusions

It was concluded that addition of JBD4, JBD44a, and a combination of both phages significantly reduced the biomass of 24 and 96h *P. aeruginosa* biofilms grown on microwell plates. Phages were able to significantly reduce more 96h biomass compared to 24h.

Phage addition did not produce significant reduction of viable bacterial counts for 24 or 96h *P. aeruginosa* biofilms grown in extracted root canal models.

Post-treatment phage concentrations were higher than pre-treatment loading concentrations.

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