Efficacy of Bacteriophage Treatment on *Pseudomonas aeruginosa* Biofilms

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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: The 24-hour and 96-hour PA14 biofilms grown in microplates were treated with phages identified as possessing potential biofilm-degrading activities, and the post-treatment bacterial biomass was quantified by using crystal violet staining. Part 2: The 24-hour and 96-hour PA14 biofilms grown in prepared root canals of extracted human mandibular incisors were treated with phages identified with potential biofilm-degrading activities. Post-treatment intracanal samples were taken by using paper points and round burs to assess phage and bacterial counts. **Results:** Part 1: We identified 2 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 24-hour ($P < .05$) and 96-hour ($P = .08$) biofilms. Part 2: In 24-hour and 96-hour PA14 biofilms in a root canal model, no significant difference was found in the number of colony-forming units after phage treatment ($P > .05$). **Conclusions:** Phage application significantly reduced the biomass of 24-hour and 96-hour PA14 biofilms grown on microplates but did not produce significant reduction of 24-hour or 96-hour PA14 biofilms grown in the extracted tooth model. *(J Endod 2013;39:364–369)*

Key Words

Apical periodontitis, bacteriophage therapy, *Pseudomonas aeruginosa*, root canal

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Primary apical periodontitis is an inflammatory disease of the periodontal ligament and surrounding bone caused by infection present in 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 recolonization 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 bacteria or other factors that promote disease are introduced into the root canal and periapical tissues during or after initial treatment (4). Teeth with persistent infections can be treated nonsurgically and/or surgically and have an expected healing rate of 77% (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 apical periodontitis 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."

Pseudomonas aeruginosa, a gram-negative, facultative, rod-shaped bacterium that belongs to the Gammaproteobacteria class of bacteria, is frequently found in periodontal infections (8). It has been recovered from primary and persistent endodontic infections (9–16) and in 1 study represented 6.8% of the bacterial isolates recovered from persistent apical infections (9). Notably, in that study *P. aeruginosa* was found in 4 of 5 teeth with a draining sinus, 2 of which proved to be a monospecies infection. *P. aeruginosa* forms biofilms that are resistant to high concentrations of salts, dyes, weak antiseptics including chlorhexidine gluconate (17, 18), and many commonly used antibiotics (19). Its persistence after conventional endodontic treatment (9–14) also indicates that it may be resistant to commonly used endodontic disinfection protocols. Irrigation with 5.25% sodium hypochlorite was not able to routinely eliminate *P. aeruginosa* from the root canal (20), and growth was not inhibited by frequently used root canal sealers (21). A 48-hour intracanal dressing of calcium hydroxide (22) and photodynamic therapy (23) 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 compensate for the limitations of mechanical bacterial debridement and antiseptic properties of current root canal sealers. Bacteriophages (phages) are viruses that can infect and kill bacteria that have been used as a treatment alternative in the

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management of non-endodontic persistent bacterial infections (24). 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 (25). Through their interactions 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 (26).

The clinical use of phage therapy has been explored in the management of *P. aeruginosa* ear infections in dogs (27). 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-blind, placebo-controlled phase I/II clinical trial, the efficacy and safety of phage therapy were demonstrated for the management of antibiotic-resistant *P. aeruginosa*–induced chronic otitis in 24 human patients (28). Although phage therapy studies are limited in dentistry, phages have been recovered from *Enterococcus faecalis* strains isolated from infected root canals (29), and they have been shown to reduce *E. faecalis* biofilm grown on dentin (30). 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 an extracted tooth model.

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 EPS-degrading activity. Serial dilutions of the phages were titered on *P. aeruginosa* strain UCBPP-PA14 (PA14), and the plaques were examined for halo production, which indicated 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 10 mmol/L $MgSO_4$. Phages were collected from the plates by soaking in suspension medium (SM) (100 mmol/L sodium chloride, 10 mmol/L $MgSO_4$, 50 mmol/L Tris-HCl, pH 7.5, 0.01% (w/v) gelatin), to which DNAse (5 μg/mL) and RNAse (5 μg/mL) were added (31). The phage lysates were passed through a 0.45-μm filter, and the phage particles were concentrated by precipitation by using polyethylene glycol 8000 (16-hour stirring at 4°C) and centrifugation (11,000 rpm for 20 minutes). The phage pellets were resuspended in SM, and the phages were banded on 2 sequential cesium chloride equilibrium gradients (50,000 rpm, 24 hours). The phage bands were extracted and dialyzed into SM before further characterization.

Biofilm Treatment Assay. PA14 biofilms were grown by using a modified version of the Calgary Biofilm Device that was previously shown to grow reproducible *P. aeruginosa* biofilms (32). A saturated overnight culture of PA14 in 0.5X LB was adjusted to an optical density of 0.4 at 600 nm ($\sim$6 × 10^8 colony-forming units [CFU]/mL) and used for inoculation. One hundred fifty microliters 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) was placed into the microtiter plate and incubated with shaking at 37°C for 24 or 96 hours to allow biofilm growth. The TSP pin lid was then removed, rinsed in sterile water to remove unattached cells, air-dried for 30 seconds, and transferred to

a 96-well plate in which the wells contained JBD4, JBD44a, or a combination of the 2 at a final concentration of $\sim$6 × 106 plaque-forming units (PFU)/well in 0.5X LB. The plate was incubated overnight at 37°C.

The phages present in the wells after the overnight incubation with the biofilms were enumerated by using the double agar overlay method (33). One hundred twenty-five microliters 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 PFUs present in each well.

Quantification of Biofilm Remaining after Phage Treatment. After 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.

Part 2: *In Vitro* Biofilm Assay in the Root Canal Model
Specimen Preparation. The Office of Research Ethics University of Toronto approved the collection and use of extracted human teeth for experiments conducted in this study. Twenty-eight intact, noncarious, 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-file 1 mm short of the apical exit. The root canals were prepared with Pro-Taper (Dentsply, Tulsa, OK) rotary instruments to size F5 with copious (10 mL) 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 disk (Brasseler, Savannah, GA), and the apex was painted with 2 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 before use.

Biofilm Treatment Assay. The teeth were randomly assigned to 24-hour and 96-hour biofilm periods (n = 14). One tooth in each group received 5 μL 0.5X LB (control), and the rest of the teeth were inoculated with 5 μL PA14 ($\sim$5 × 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) (30). Each tooth was placed vertically in a well of a 96-well plate, covered with Parafilm (Pechiney Plastic Packaging Company, Chicago, IL), and incubated at 37°C in a shaking incubator. After 24 hours, 3 groups of 3 teeth each were treated with phages at a final concentration of $\sim$$10^7$ PFU/tooth: (1) 5 μL JBD4, (2) 5 μL JBD44a, and (3) 2.5 μL each of JBD4 and JBD44a. Four teeth remained untreated with phage. The treatment microwell plate was incubated at 37°C, and medium replenishment was provided for the 96-hour growth at 48 hours. At the end of the incubation period a sterile coarse paper point (Dentsply) was used to extract the contents of each canal and transfer it to an Eppendorf tube containing 1 mL 0.5X LB. A sterile size 2 round bur was used to remove dentin shavings in the coronal third of the tooth (3 seconds at 3200 rpm), and the bur was transferred to an Eppendorf tube containing 1 mL 0.5X LB. The tubes were incubated at 48°C for 1 hour before being vortexed, diluted, spread plated on LB, and incubated
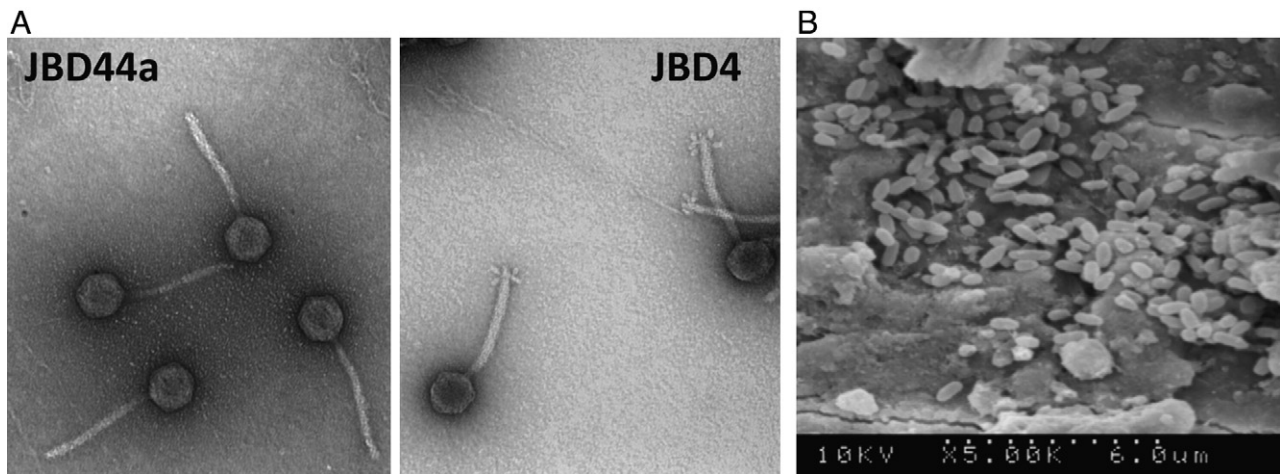
Figure 1. (A) Negative stain transmission electron microscopy image of phages JBD4 and JBD44a. (B) Scanning electron microscopy image of *P. aeruginosa* on the dentin surface of an inoculated root canal specimen.

at 37°C for 16 hours to determine the CFUs. Post-treatment phage concentrations were determined by removing 100 μL 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, 2 random specimens from the positive control group were prepared for scanning electron microscopy (Amray 1830; SEMTech Solutions, North Billerica, MA). The specimens were fixed by placement in 2.5% glutaraldehyde in 0.1 mol/L phosphate-buffered solution for 24 hours. The specimens were then sectioned longitudinally with a razor blade 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 by using the SPSS 17.0 software package (SPPS Inc, Chicago, IL). One-way analysis of variance with least significant difference (Fisher least significant difference) 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 2 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 2 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 by using negative stain transmission electron microscopy and discovered that both are members of the Siphoviridae family of phages (Fig. 1*A*). 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. The 24-hour and 96-hour time points were chosen because previous studies

revealed that 24-hour *P. aeruginosa* biofilms were susceptible to selected antibiotics, and that increased antibiotic resistance occurred in biofilms grown for more than 48 hours (34–37). As expected, the untreated 96-hour samples contained significantly more biomass ($P < .001$) than the 24-hour biofilms. The addition of phages JBD4 and JBD44a into wells containing preformed biofilms produced a significant reduction in the mean percentage of bacterial biomass in 24-hour ($P < .05$) and 96-hour ($P < .003$) samples (Fig. 2). There was no additional decrease in the bacterial biomass with treatment using the phages in combination as compared with using them individually ($P = .08$).

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 after the 24-hour incubation period. As shown in Table 1, the titers of both JBD4 and JBD44a increased approximately 10-fold, indicating that the phages were infecting the cells, replicating within them, and being released back into the medium after cell lysis.

There was no significant difference in the number of phages released when using JBD4 and JBD44a alone or in combination in the 24-hour or 96-hour 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
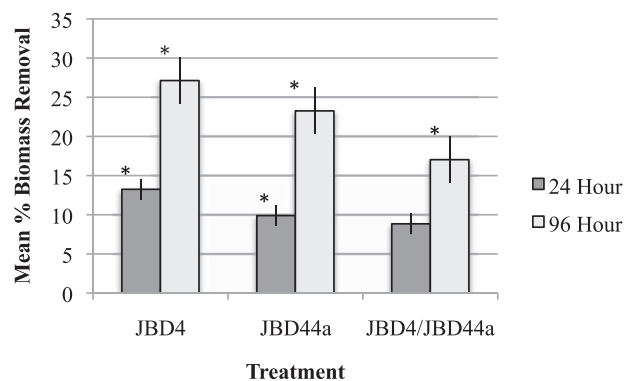

Figure 2. Mean percentage of biomass removal as measured by a crystal violet assay for 24 and 96 hours of PA14 growth. *Significance $P < .05$ for all except 24-hour JBD4/JBD44a.

TABLE 1. Post-treatment Phage Concentrations in 24-hour and 96-hour PA14 Biofilms Grown on Microwell Plates

	Initial phage titer (PFU/mL)	24-Hour biofilm titer (PFU/mL)	96-Hour 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

eradicate PA14 biofilm in the more biologically relevant tooth model was assessed. The extracted teeth were inoculated with PA14 ($\sim 5 \times 10^7$ CFU/mL) and incubated for 24 or 96 hours to allow biofilm formation. The growth of biofilm on the tooth surface was examined in a 24-hour biofilm by using scanning electron microscopy, which confirmed the presence of a layer of bacterial cells adhered to the dentin surface (Fig. 1B). After the biofilm growth incubation period, phages were added to the root canal and were incubated for an additional 24 hours. The analysis of bacterial counts and phage replication was performed on planktonic cells growing in the medium contained within each tooth canal by using paper points to sample and from the biofilms formed on the dentin by the use of a bur to remove dentin shavings containing any adhered cells and phages.

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 96-hour sample as compared with the 24-hour time point (Table 2). The 96-hour 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 24-hour biofilm. This is consistent with the findings of increased biomass in the 96-hour biofilms in the microplate assay. The addition of phages JBD4, JBD44a, or a combination of the 2 did not significantly decrease the number of planktonic or biofilm-adhered cells in the 24-hour or 96-hour samples (Table 2).

Although there was no appreciable difference in bacterial cell counts after treatment with the phages, the number of phages present at the end of the incubation period was analyzed to assess replication. Because 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. Both JBD4 and JBD44a were inoculated into the root canal at a concentration of $\sim 2 \times 10^7$ PFU/mL. The number of phages present in the planktonic fraction of the root canal increased by 100-fold to 1000-fold during 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 during the time course of the experiment. Furthermore, the bur assay showed that whereas the 24-hour biofilm samples contained phages at approximately the same concentration as they were seeded into the assay, the 96-hour biofilms averaged 50-fold to 1000-fold more phages associated with them. This is especially interesting to note because the number of bacterial cells in the 96-hour biofilm was only increased 5-fold to 10-fold above the 24-hour biofilm.

Discussion

Unlike endodontic management of primary infections, the treatment of persistent infection often can be more specific and directed toward the elimination of a lesser number of identifiable species (4). One species identified as a cause of persistent disease in endodontics is *P. aeruginosa* (9–14), 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 (30). Although many phage therapy reports have supplied anecdotal evidence of the efficacy of phage treatment (38), a recent phase I/II randomized, double-blind, placebo-controlled clinical trial examining the use of bacteriophage treatment for chronic otitis caused by antibiotic-resistant *P. aeruginosa* in humans determined that the bacterial counts were greatly reduced in the phage-treated group, and no adverse events were reported (27). The use of phage therapy has not been associated with a human immune response such as anaphylaxis (39), presumably because of the fact that phages exist in all environments to which people are exposed, including water, food, and within the normal human flora. In fact, LISTEX (Micreos Food Safety, Wageningen, The Netherlands), a phage therapy product used as a processing treatment to kill *Listeria* during the production of many food products including meat, cheese, fruit, and vegetables, has been approved by the U.S. Food and Drug Administration and Department of Agriculture and is currently in use in food manufacture. For these reasons, we investigated the use of phage therapy for the treatment of *P. aeruginosa* infections in a root canal model.

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 EPS, a component of the extracellular matrix present in biofilms. These 2 phages, which belong to the Caudovirales family, contain double-stranded DNA genomes packaged into an icosahedral protein capsid and a long, non-contractile tail through which they interact with the bacterial cell. We assessed the efficacy of a combination of the 2 phages in addition to their individual use, because previous clinical trials reported the success of phage mixtures in the management of human and animal *P. aeruginosa* otitis infections (27, 28). In part 1 of the current study, bacteriophage treatment significantly reduced the biomass of 24-hour and 96-hour PA14 biofilms in the microplate assay, but complete bacterial elimination was not observed. By contrast, there was no significant reduction of viable bacterial counts of 24-hour or 96-hour PA14 biofilms in the extracted tooth model in part 2 of the study. Two sampling methods were used in part 2 of this study. Paper point absorption was specifically chosen to recover planktonic bacteria and phages present within the root canal lumen (11–15, 30), and round burs were used to recover bacteria

TABLE 2. Viable Bacterial Counts after 24 and 96 Hours of Growth in Extracted Teeth Models

	Paper point assay		Bur assay	
	24-Hour bacterial count (CFU/mL)	96-Hour bacterial count (CFU/mL)	24-Hour bacterial count (CFU/mL)	96-Hour 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. Post-treatment Phage Concentrations in 24-hour and 96-hour PA14 Biofilms Grown in the Extracted Tooth Model

	Initial titer (PFU/mL)	Paper point assay		Bur assay	
		24-Hour titer (PFU/mL)	96-Hour titer (PFU/mL)	24-Hour titer (PFU/mL)	96-Hour 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

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.

Although 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 show a significant reduction as compared with the individual phages with the same final concentration. It was suspected that lysogens of these phages, in which the phage genome is integrated into and maintained within the host genome, might provide their bacterial host with resistance to additional phage infection. Characterization of lysogens of JBD4 and JBD44a in PA14 proved this correct (data not shown). Thus, PA14 into which either phage inserts as a lysogen is resistant to both JBD4 and JBD44a. Whether this resistance is provided by immunity caused by the phage repressor protein or some other 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 because 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. Studies using virulent mutants of these phages will be required to fully deconvolute these results. Phage and bacteria are continuously engaged in an evolutionary arms race in which bacteria mutate to resist infection by phages, and then the phage mutates to be able to infect these cells. Thus, the use of phage cocktails in which there are numerous phage strains that are able to infect the bacterial cell by using different membrane receptors may be required.

Previous phage therapy studies that examined the post-treatment phage concentrations found them to be significantly higher than the initial concentrations (27, 28, 36). In this study the phages were also able to replicate, and the final titers from the extracted tooth model were 50-fold to 1000-fold higher than were input into the assay. The propagation of phages in the assay illustrates that they were able 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 suggests that the phages may 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 or may be an inhibitory effect of dentin on phage action. Improved phage administration and encouraging their diffusion through the root canal system will be required for them to be effective in this environment. Although phage therapy has been effective in the management of some infections, further research into the most effective phage or phage 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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