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In vitro antimicrobial effect of bacteriophages on human dentin infected with *Enterococcus faecalis* ATCC 29212

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This study assessed the effect of bacteriophages on the viability of *Enterococcus faecalis*. Human dental roots were inoculated with a suspension of *E. faecalis* at three different multiplicities of infection − 0.1, 1.0 and 10.0. The phage lysate was able to significantly inhibit bacteria growth when incubated at the multiplicities of infection of 1.0, 10.0 and 0.1. The dental roots were also inoculated with bacteria for 6 days to allow bacterial penetration into the teeth tubules. Addition of the phage lysate to the roots following the 6-day incubation period led to a substantial reduction in bacteria viability. Phage therapy may be an important alternative for the treatment of root canal infections refractory to conventional endodontic therapy.

Key words: bacteriophage; endodontic infection; *Enterococcus faecalis*, phage; phage therapy

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One of the purposes of endodontic treatment is the disinfection of the root canal system. Leftovers of viable microorganisms in association with favorable growth conditions prevent the repair process and may cause treatment failure (13, 15, 17). Microorganisms that survive chemical–surgical proceedings may rapidly increase in number between treatment sessions, making the use of antimicrobial medications obligatory, especially in clinical situations refractory to habitual procedures or in endodontic retreatment (17).

Enterococcus faecalis has been recovered from several oral sites and exhibits a high level of resistance to a wide variety of antimicrobial agents (18, 27). In endodontic infections, *E. faecalis* can survive in the root canal as a single organism, without the support of other species (9, 15), and represents a problem in conservative root canal therapy (4, 12, 19).

In the last decades, the resistance of microorganisms to chemotherapeutic agents has been increasing. This situation makes the introduction of new therapeutic techniques compulsory and, in this context, a renewed interest in phage therapy has arisen.

The use of phages for the control of infection has some advantages. They can be administered in a single dose because they reproduce within the target bacteria and remain in the region while infection persists. In contrast, antibiotics require multiple doses with days or weeks of treatment (5). On the other hand, phages are highly specific for a bacteria species, and sometimes even for a bacteria strain, narrowing their spectrum of application.

The aim of the present study was to assess *in vitro* the antimicrobial effect of bacteriophages specific for *E. faecalis* ATCC 29212 from the root canals and from the dentinal tubules of human teeth.

Material and methods

Phage isolation

Bacteriophages were isolated by the phage enrichment method of Smith & Huggins (25). A sample was collected from the Pirajussara stream in the city of São Paulo, Brazil, and carried to the laboratory packed in ice. A volume of 100 ml of LB medium (Luria Bertani broth) and 100 ml of TB medium (Terrific broth) was added to 300 ml of the stream water. After 1 h of incubation at 37°C, 3 ml of an *E. faecalis* ATCC 29212 culture was added and grown for 16 h at 37°C without aeration. Then 500 µl of chloroform was added to 10 ml of culture and the mixture centrifuged for 10 min at 5,300 ×*g*. The supernatant was supplemented with 100 µl chloroform and stored at 4°C (crude lysate). Dilutions of the crude lysate were added to a lawn of *E. faecalis* on LB plates and incubated overnight at 37°C. A phage

plaque was isolated and used to prepare a new lysate, which was used in the subsequent experiments.

Titration of the phage lysate

A 100 µl culture of *E. faecalis* was added to 3 ml top agar and poured onto LB Petri dishes. After solidification, 10 µl aliquots of different dilutions of the phage lysate were applied to the surface of the culture medium. Following overnight incubation at 37°C, the presence of lysis plaques was assessed. The concentration of phages in the lysate, measured by the number of lysis plaques at a given dilution, is reported as PFU/ml (plaque-forming units/ml).

Bacterial strains and growth conditions

The *Enterococcus faecalis* ATCC 29212 strain stored in TSB (Tryptic Soy Broth; Difco Laboratories, Detroit, MI) supplemented with 40% glycerol at − 20°C was used. A 100 µl volume of the stored bacteria was transferred to 10 ml LB supplemented with 10 mM calcium chloride (16) and grown for 24 h at 37°C. The absorbance of the culture was adjusted to 0.14 at 546 nm, which corresponds to 1.75×10^8 colony-forming units (CFU)/ml.

Specimen preparation

We selected 20 human teeth with a single root with complete root formation, obtained from the tooth bank of the Discipline of Anatomy of the Methodist University of São Paulo. The study was approved by the Research Ethics Committee of the University. Steel disks were used to remove the crown of the teeth. The canals were filled with sterilized physiological saline and their content was removed with a #15 K file. The working length was determined by introducing a fine caliber K-type file with a silicon stop until its penetration guide reached the apical foramen. From this, 1 mm was subtracted, and chemical–surgical instrumentation was performed using K files and 50 ml sterilized physiological saline at each instrument change, up to a #45 file. The final irrigation was performed with 5 ml of EDTA-T solution followed by 100 ml physiological saline under ultrasound vibration. The outer surface of the roots was impermeabilized with instant cyanoacrylate adhesive. After instrumentation, the specimens were sterilized in an autoclave at 121°C for 20 min (1 psi) in flasks containing physiological saline, dried with previously sterilized gauze pads and absorbent paper points, and inserted into sterilized polypropylene tubes.

Specimen inoculation

The specimens were divided at random into four groups of five roots each. Three roots of each group were inoculated with bacteria in the presence of the phage lysate as described below (titration corresponding to 2×10^8 PFU/ml) and the other two roots were inoculated either with bacteria alone (positive control) or LB medium (negative control).

Group I was inoculated with bacteria and phages at a 1 : 1 ratio (multiplicity of infection [MOI] = 1). Group II and Group III contained bacteria and phages at an MOI of 10 and 0.1, respectively. The samples were homogenized and aliquots of 20 µl were inoculated into each canal. The inoculated teeth were kept at 37°C for 3 h. After 3 h, aliquots were removed from the lumen of the canals, serial diluted and seeded in triplicate on the surface of LB-plates. After overnight incubation, the number of colonies was counted.

Group IV consisted of four teeth that were inoculated with 20 µl of the bacterial culture and kept at 37°C for 24 h, while the fifth specimen was inoculated with LB alone (negative control). During the following 6 days, the five roots were inoculated daily with 10 µl LB to maintain appropriate conditions for penetration of bacteria into the dentinal tubules and its viability. The medium was withdrawn and the roots were wiped with sterilized paper points to remove the bacteria that remained in the lumen. Three roots were inoculated with the phage lysate, while the remaining two were inoculated with LB alone. After 24 h of incubation at 37°C, the contents of the canals were withdrawn, diluted and seeded in triplicate on LB-plates. After these procedures, the roots were reinoculated with LB and kept at 37°C for two additional days. After 24 and 48 h of incubation, aliquots were removed from the lumen of the canals, diluted and spread on LB-plates.

Results

The antimicrobial activity of phages on human dentin infected with *E. faecalis* was analyzed by comparing the number of CFU/ml of bacteria treated with the phage lysate at different MOIs (Tables 1 and 2).

In Groups I and II (MOI 1 and 10, respectively), after 3 h of incubation in the presence of phages, no bacterial growth was detected (Table 1). In Group III, where the MOI was 0.1, the number of bacteria CFU was reduced by 98% after a 3 h treatment in two of the roots. In the third root, no bacteria growth was observed.

In samples of Group IV, where the growth conditions of the bacteria favored their penetration in the dentinal tubules, no bacteria were observed after 24, 48 or 72 h of the treatment with the phage lysate, indicating that the phages were able to suppress bacteria growth in the tubules (Table 2).

Table 1. Viability of *Enterococcus faecalis* in the lumen of the canals following incubation in the presence of different multiplicities of infection of bacteriophages for 3 h at 37°C

Teeth	Group I/1 : 1 CFU/ml	Group II/1 : 10 CFU/ml	Group III/10 : 1 CFU/ml
1	0	0	4×10^6
2	0	0	4×10^6
3	0	0	0
Mean value	0	0	2.6×10^6
4 (positive control)	2.0×10^8	3×10^6	2.0×10^8
5 (negative control)	0	0	0

Table 2. Viability of *Enterococcus faecalis* in the dentinal tubules after treatment with bacteriophage lysate for 24, 48 and 72 h

	Teeth	CFU/ml after 24 h	CFU/ml after 48 h	CFU/ml after 72 h
Group IV	1	0	0	0
	2	0	0	0
	3	0	0	0
	4 (positive control)	2.6×10^8	1.6×10^8	5.5×10^7
	5 (negative control)	0	0	0

Discussion

Several studies have reported that enteric bacteria in monoinfection or associated with other bacteria can be the cause of persistent or secondary infections (6, 8, 11). Among them, Hancock et al. (13) have shown that persistent infections are often caused by *E. faecalis*. *E. faecalis* is a facultative anaerobic bacterium which resists conventional therapeutic procedures, persisting and multiplying in the root canal system (14, 21, 26). It has also been shown that these bacteria are present in endodontic infections associated with persistent periapical lesions (17, 26) and in patients that underwent endodontic treatment that failed (1, 20). The aim of the present study, therefore, was to find a bacteriophage that could successfully kill *E. faecalis* in the root canal as a first step to achieving an alternative treatment for *E. faecalis* infections.

The root canal system is normally cleaned and disinfected by mechanical instruments, flux and reflux of irrigating solutions and the bactericidal action of auxiliary chemical solutions (10, 27). Microorganisms are not routinely eliminated during disinfection of the root canal because of the difficulty of access to the internal part of dentinal tubules, and the microorganisms remain viable even after the systemic administration of antimicrobial agents (3, 18). Consequently, to disinfect the root canal system, intracanal medication or effective topical methods must be used (12, 18, 27). Calcium hydroxide is not recommended for the treatment of enterococci and yeasts since these microorganisms tolerate alkaline media. Also, in cases of recurrent treatment, microorganisms that survive the application of calcium hydroxide are frequently found, indicating that other alternative medication should be used (6, 17, 22).

Bacteriophages offer an alternative to chemotherapy. One of its main advantages of bacteriophages is their ability to replicate in the cell and to release large amounts of new viral particles capable of infecting other bacteria (2, 5). Thus, a single phage dose may be sufficient to control bacterial infection. In addition, the use of phage therapy would diminish the establishment of new antibiotic-resistant strains. Furthermore, phages are specific for a given bacterial species and therefore eliminate only the pathogen, causing no harm to the indigenous microbiota (2, 25). However, this phage-specificity can also be a disadvantage, since most phages would not be effective in cases of mixed infection (2, 5).

In the present study we isolated a bacteriophage that grows on the *E. faecalis* strain ATCC 29212. The phage was able to inhibit the bacteria growth after a short period of incubation in Groups I and II, and a significant reduction occurred in Group III, which presented a multiplicity of infection of 0.1 (one phage per 10 bacteria). Had the time of incubation been extended, it is possible that no bacteria growth would have been detected in any of the specimens analyzed.

Since there is a special concern about the elimination of bacteria from the internal part of the dentinal tubules, particularly in cases where conventional endodontic treatment is ineffective, alternative methods should be employed (27). Even when the root dentin was infected (Table 2, Group IV), bacteria were not found in the lumen of the canals following phage treatment, suggesting that the phages were able to penetrate the dentinal tubules and kill bacteria, and that the environmental conditions inside the root canal did not interfere with the bactericidal action of the phages (Table 1).

In 2001, Barrow (2) reported that phage therapy should be considered in cases of infection in which resistant bacteria are present, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecium* and *E. faecalis*. However, before phage therapy can be adopted as a therapeutic alternative in cases of recurrent treatment or of refractory infectious processes, additional *in vitro* investigations are needed. The spectrum of action of these microorganisms, the type of vehicle used and their toxicity must be studied, since there is a wide diversity in the morphologic and behavioral pattern of these viruses.

Phage therapy is practiced in Poland, albeit on a small scale. Studies performed in the 1980s reported on 550 cases of suppurative bacterial infections (empyemas, peritonitis, osteomyelitis) in humans, either chronic or resistant to the available antibiotics. The phages used by these investigators were able to cure approximately 90% of the cases (23, 24).

Though more studies involving phage therapy in animals and humans are necessary, we believe that in the near future phages will be a serious alternative for the treatment of bacterial diseases, both in combination with antibiotic treatment, in cases in which bacteria susceptible to antibiotics are present, or alone, for the elimination of bacteria resistant to antibiotics. Phage therapy might be specially helpful for the elimination of bacteria resistant to antibiotics, being a second front in the fight against multidrug-resistant bacteria.

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