Archives of Oral Biology 82 (2017) 166–170

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Antibacterial effect of genetically-engineered bacteriophage ϕEf11/ϕFL1C(Δ36)P^{nisA} on dentin infected with antibiotic-resistant *Enterococcus faecalis*

Justine Monnerat Tinoco[a,*], Nadia Liss[b], Hongming Zhang[b,c], Roni Nissan[b], Wanda Gordon[b], Eduardo Tinoco[d], Luciana Sassone[a], Roy Stevens[b,c]

[a] Department of Endodontology, School of Dentistry, State University of Rio de Janeiro, Rio de Janeiro, RJ 20551-030, Brazil
[b] Department of Endodontology, School of Dentistry, Temple University, Philadelphia, PA 19140, USA
[c] Laboratory of Oral Infectious Diseases, School of Dentistry, Temple University, Philadelphia, PA 19140, USA
[d] Department of Periodontology, School of Dentistry, State University of Rio de Janeiro, Rio de Janeiro, RJ 20551-030, Brazil

ARTICLE INFO

Keywords:
Antimicrobial
Bacteriophage
Dentin infection
Enterococcus faecalis phage therapy

ABSTRACT

Objective: *Enterococcus faecalis* is a gram-positive facultative anaerobic bacterium, which is present in 30–89% of teeth with postendodontic treatment failures. *E. faecalis* is capable of penetrating dentinal tubules and surviving as a monoculture after conventional endodontic therapy, indicating that it is resistant to commonly used endodontic disinfection protocols. Different *E. faecalis* strains have shown resistance to several antibiotics, and have been associated with both dental pathology and systemic infections. The aim of this study was to evaluate the efficacy of a genetically engineered bacteriophage to disinfect dentin infected with antibiotic resistant strains of *E. faecalis*.

Methods: Extracted human dentin root segments were cemented into sealable two-chamber devices, fabricated from syringe needle caps to form *in vitro* infected-dentin models. The models were inoculated with an overnight suspension of either *E. faecalis* V583 (vancomycin resistant strain) or *E. faecalis* JH2-2 (fusidic acid and rifampin resistant, vancomycin sensitive strain). After 7 days of incubation at 37 °C, a suspension of a genetically engineered phage, ϕEf11/ϕFL1C(Δ36)P^{nisA}, was added to the root canal of each infected dentin segment, and the incubation was continued for an additional 72-h. Dentin was harvested from the walls of each root canal and assayed for the residual titer of *E. faecalis* cells.

Results: The recovered *E. faecalis* titer was reduced by 18% for the JH2-2 infected models, and by 99% for the V583 infected models.

Conclusion: Treatment: of *E. faecalis*-infected dentin with bacteriophage ϕEf11/ϕFL1C(Δ36)P^{nisA} consistently resulted in a decrease in the residual bacterial population of both vancomycin-sensitive and resistant strains.

1. Introduction

One of the most important objectives of a root canal treatment is disinfection. However, complete elimination of microorganisms from the root canal system still remains as a target to be reached. Irrespective of improvements in instrumentation techniques, the use of thorough irrigation and intracanal medicaments, microorganisms may persist in dentinal tubules, apical ramifications and periapical areas (Peters & Wesselink, 2002). Furthermore, these microorganisms have the ability to form intra radicular (Nair, Sjogren, Kahnberg, & Krey, 1990; Sassone, Fidel, Fidel, & Dias, 2003) or less frequently, extra radicular (Tronstad, Barnett, Riso, & Slots, 1987; Siqueira & Lopes, 2001; Nori, Ehara, Kawahara, Takemura, & Ebisu, 2002), biofilms which may prevent resolution of apical periodontitis and lead to the failure of endodontic treatment (Lin, Skribner, & Gaengler, 1992; Nair, 2004; Siqueira, 2001).

Enterococcus faecalis is a gram-positive, facultatively anaerobic bacterium, which has been frequently isolated from root canals and associated with endodontic cases refractory to treatment (Sundqvist, Figdor, Persson, & Sjögren, 1998; Gomes et al., 2008; Siqueira and Lopes, 2001). Studies have shown the presence of *E. faecalis* infection in 30–89% of teeth with postendodontic treatment failure (Gomes et al.,

* Corresponding author at: Universidade do Estado do Rio de Janeiro, Boulevard 28 de Setembro, 157 −2º andar − sala 10 − Pavilhão Mario Franco Barrozo, CEP 20551-030 Vila Isabel, Rio de Janeiro, RJ, Brazil.
E-mail addresses: justinemonnerat@yahoo.com.br, justinetinoco@gmail.com (J.M. Tinoco).

http://dx.doi.org/10.1016/j.archoralbio.2017.06.005
Received 3 September 2016; Received in revised form 26 May 2017; Accepted 10 June 2017

2008; Haapasalo & Orstavik 1987; Sundqvist et al., 1998). *E. faecalis* is capable of penetrating dentinal tubules up to 800 micrometers from the root canal wall (Haapasalo & Orstavik, 1987), and can survive in the root canal as a monoinfection (Sedgley, Lennan, & Appelbe, 2005). Its persistence after conventional endodontic treatment indicates that it is resistant to commonly used endodontic disinfection protocols such as conventional therapy with calcium hydroxide (Orstavik & Haapasalo, 1990; Stevens & Grossman, 1983). Although sodium hypochlorite and chlorhexidine were found to be effective against *E. faecalis in vitro*, they require direct contact with the bacteria (D'Arcangelo, Varvara, & De Fazio, 1999). Consequently, novel approaches for managing endodontic infections caused by these organisms would be of considerable valuable in endodontic therapy.

Phage therapy is an important alternative to conventional treatments for infectious diseases caused by pathogenic bacteria (Chanishvili, Chanishvili, Tediashvili, & Barrow, 2001; Sulakvelidze, Alavidze, & Morris, 2001; Burrowes, Harper, Anderson, McConville, & Enrigh, 2011). It takes advantage of the natural lytic life cycle of bacterial viruses to infect and kill their host bacteria. On a previous static biofilm study, biomass of antibiotic-resistant *E. faecalis* strains were markedly reduced following infection by ϕEf11/ϕFL1C(Δ36)^{PnisA}, a genetically engineered bacteriophage (Tinoco et al., 2016).

In an era where antibiotic resistance is a major concern and the development of new drugs demands high investments of time and money, phage-based technologies may be valuable antimicrobial alternatives with widespread applications in medicine. This approach may also be useful in the treatment of root canal infections that are refractory to conventional endodontic therapy. Therefore, the purpose of this investigation was to conduct an *in vitro* study to evaluate the efficacy of ϕEf11/ϕFL1C(Δ36)^{PnisA} to disinfect dentin that is infected with an antibiotic resistant strain of *E. faecalis*.

2. Material & methods

2.1. Bacterial strains, and growth conditions

E. faecalis JH2-2 (fusidic acid and rifampin resistant, vancomycin sensitive) (Jacob & Hobbs, 1974) and *E. faecalis* V583 (vancomycin resistant) (Sahm, Kissinger, & Gilmore, 1989) were grown in brain heart infusion (BHI) broth, and transformed with plasmid pMSP3535, *cat*, *nis*R *nis*K, using procedures previously described by Zhang et al. (Zhang, Fouts, DePew, & Stevens, 2013).

2.2. Construction of phage

The wild type *E. faecalis* bacteriophage ϕEf11 was previously described (Stevens, Porras, & Delisle, 2009), as was its spontaneous recombinant, phage ϕEf11(Δ61-1,ϕFL1C40-44) (Zhang, H. et al., 2013). An allelic exchange plasmid was constructed to permit the deletion of the *CI* repressor gene (ORF36) and the replacement of the *CRO* promoter with a nisin-inducible promoter, from a ϕEf11(Δ61-1,ϕFL1C40-44) prophage within the *E. faecalis* lysogen JH2-2[ϕEf11(Δ61-1,ϕFL1C40-44)]. This recombinant pCR8/GW/TOPO vector contained a 1 kb DNA fragment upstream of ϕEF11 ORF36, a nisin promoter (P^{nisA}), an erythromycin selection marker (erm), and a 1 kb fragment downstream of phage ϕEf11 P^{cro} (the promoter controlling lytic cycle functions in phage ϕEf11), and was constructed essentially as described previously (Zhang, H. et al., 2013). This plasmid was electroporated into a competent strain of *E. faecalis* JH2-2[ϕEf11(Δ61-1, ϕFL1C40-44]. Following homologous recombination, transformant JH2-2[ϕEf11(Δ61-1, ϕFL1C40-44, Δ36, erm, ΔP^{cro}, P^{nisA})] clones were selected on erythromycin-containing BHI agar plates. The presence of ϕEf11 ORF31, erm, P^{nisA}, and the absence of ORF36 and P^{cro} in these cells, were confirmed by PCR analysis. The primers and predicted amplicon sizes have been published in a previous article (Tinoco et al., 2016).

These mutant clones, which will be referred to as *E. faecalis* JH2-2[ϕEf11/ϕFL1C(Δ36)P^{nisA}], were subsequently transformed with pMSP3535(*cat, nis*R/K), a plasmid that harbors the two-component, nisin-sensitive *nis*R/K system for regulating the P^{nisA} promotor in phage ϕEf11/ϕFL1C(Δ36)P^{nisA} (Kuipers, Beerthuyzen, de Ruyter, Luesink, & de Vos, 1995; Bryan, Bae, Kleerebezem, & Dunny, 2000), and a chloramphenicol selection marker (*cat*). The ϕEf11/ϕFL1C(Δ36)P^{nisA} prophages in these lysogens were induced with nisin (40 ng/ml), and the resulting phage was collected by centrifugation (see below).

2.3. Model preparation

The study was conducted using *in vitro* dentin infection models constructed from extracted human dentin segments which were cemented into sealable two-chamber devices, fabricated from syringe needle caps as previously described by Gordon et al. (Gordon, Atabakhsh, & Meza, 2007). 40 single-rooted teeth that previously had not been stored in sodium hypochlorite or any other disinfectant solution, were used. The teeth were sectioned at the cementoenamel junction and at a point 3 to 4 millimeters from the apex. The total root section had a length of 5 mm. Based on the findings of Haapassalo and Orstavik (Haapasalo & Orstavik, 1987) concerning factors affecting the growth of bacteria in dentin tubules, cementum and peripheral dentin of each root section was removed with a diamond bur, which resulted in a dentin cylinder approximately 5 mm in diameter that would fit within a hollowed-out plastic needle encasement.

The canal of each root section was enlarged with Gates Glidden sizes # 1 through # 4, resulting in a canal with an approximate volume of 8 microliters. The smear layer was removed with 17% ethylenediamine tetraacetic acid (EDTA) for 4 min, followed by 5.25% sodium hypochlorite (NaOCl) for another 4 min, and finally the dentin segments were rinsed in sterile water for 30 min. Each root section was placed and secured in a hollowed-out needle encasement. Kneadalite epoxy putty (Polymeric Systems, Phoenixville, Pa.) (certified at 300° F and 2000 pounds per square inch) was applied to the exterior surface and apical end of the root sections to create an airtight seal between the root sections and walls of the modified a 30-gauge blue syringe needle encasement. The needle encasement, containing the dentin cylinder, was cemented into a sealable two-chamber device, fabricated from a syringe needle cap. (Fig. 1A, B). The models were stored in a cold, damp location, to allow the putty to firmly set (in 12 h). 450 µl of buffered saline with gelatin/BSG (0.85% NaCl, 0.03% KH_2PO_4, 0.06% Na_2HPO_4, 0.01% gelatin) was added to the lower chamber of each model, and the models were autoclaved for 15 min at 120° C before infection.

2.4. Infection of models

Overnight broth cultures (grown at 37° C) of *E. faecalis* V583 [pMSP3535(cat, nisR nisK)] and JH2[pMSP3535(cat, nisR nisK)] were prepared in brain-heart infusion (BHI) broth (Difco Laboratories, Sparks, Md.). The broth cultures were used to infect the root dentin models.

Models were divided into 6 Groups, as follows:

Group 1 − (5 teeth) − JH2-2 control − bacterial load prior to phage infection, containing only JH2-2 *E. faecalis*, no virus

Group 2 − (10 teeth) − JH2-2 + 3 days phage therapy

Group 3 − (5 teeth) − JH2-2 control − bacterial load at conclusion of incubation without virus-only JH2-2 bacteria, no virus

Group 4 − (5 teeth) −V583 control − bacterial load prior to phage infection, containing only V583 *E. faecalis*, no virus

Group 5 − (10 teeth) −V583 + 3 days phage therapy

Group 6 − (5 teeth) − V583 control − bacterial load at conclusion of incubation without virus −only V583 bacteria, no virus

Groups G1, G2, and G3 were infected with overnight cultures of *E. faecalis* JH2-2[pMSP3535(cat, nisR nisK)], and Groups G4, G5 and G6 were infected with overnight cultures of *E. faecalis*