Endodontic and Salivary Isolates of *Enterococcus faecalis* Integrate into Biofilm from Human Salivary Bacteria Cultivated *In Vitro*

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Abstract

Introduction: The aim of this study was to examine whether *Enterococcus faecalis* isolates from endodontic patients (from saliva and from a root canal) are able to prevail against salivary bacteria when grown in coculture in a biofilm reactor. **Methods:** Saliva that was tested to be free of *E. faecalis* was used as the inoculum. The fate of *E. faecalis* was examined by using culture techniques and fluorescence in situ hybridization (FISH). **Results:** The root canal isolate accounted for 37.4% of the biofilm and 31.9% of the planktonic phase when examined by the culture technique, whereas the proportions examined by FISH showed 15.3% in the biofilm and 11.7% in the planktonic phase. The saliva isolate (as examined by the culture technique) accounted for 32.4% in the biofilm and 27.1% in the planktonic phase, respectively, compared with 14.1% in the biofilm and 9.5% in the planktonic phase when examined by FISH analysis. **Conclusions:** These results led to the suggestion that *E. faecalis* could persist in the biofilm of the human oral cavity. Because of the ubiquitous presence of *E. faecalis*, root canal infections may arise from different sources. *(J Endod 2009;35:986–991)*

Key Words

Duplex fluorescence *in situ* hybridization, endodontic infection, *Enterococcus faecalis*, oral biofilm

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Members of the species *Enterococcus faecalis* were found to be the predominant type of bacteria associated with periradicular lesions in root-filled teeth in which therapy had failed (1–5). Moreover, members of this species are ubiquitous and could be isolated from a wide range of habitats, including fermented food, water, plants, and the gastrointestinal tract of humans and many animal species (6).

Because persistent microorganisms after therapy or recontamination of the canal system caused by an inadequate seal are the main reason for root canal therapy failure, the origin and fate of persistent microorganisms in the oral cavity is of primary interest. *E. faecalis* has only been infrequently detected in the oral cavity. Cole et al (7) did not find any members of this species in the saliva probes from 10 infants. *E. faecalis* has rarely been isolated from the saliva of patients with endodontic treatment (5, 8, 9). However, the association of enterococci with subgingival periodontitis has been reported (10, 11).

Compared with standard culture techniques, fluorescence in situ hybridization (FISH) is a more useful method for the detection of bacteria because their natural environment is not disrupted (12). The combination of FISH and confocal laser scanning microscopy (CLSM) has already been used to obtain images of three-dimensional reconstructions of natural microbiological environments (13, 14).

The role of *E. faecalis* in supragingival plaque has not been investigated *in vitro* or *in situ* to date, even though this oral site is the origin of oral diseases such as caries and periodontitis and could be the origin of endodontic infections. Furthermore, it has been assumed that there is an association between caries lesions and *E. faecalis in vitro* (15). Because the source of oral *E. faecalis* is still unclear, the ability of this species to survive in supragingival plaque biofilms is a primary concern. Several virulence factors (ie, gelatinase and aggregation substance) were reported to have an important role in the pathogenicity of *E. faecalis* as an endodontic infectious agent (16, 17). The detection of these putative virulence factors in endodontic isolates by polymerase chain reaction (PCR) would suggest their possible putative functions in the role of *E. faecalis* infections in endodontic diseases.

In this study, we characterized two strains of *E. faecalis* from patients with periradicular lesions using multiplex PCR for different virulence factors. The fate of these isolates was investigated in coculture with human salivary bacteria in a biofilm reactor over a time period of 4 days using traditional culture methods and duplex FISH in combination with CLSM.

Material and Methods

Multiplex PCR

Multiplex PCR for the detection of the *asa1* (aggregation substance), *gelE* (gelatinase), *cylA* (cytolysin), *esp* (enterococcal surface protein), and *hyl* (hyaluronidase) genes was performed using five specific primer pairs as previously described (16). The PCR reactions consisted of 0.5 μL of each primer (0.1 μmol/L), 5 μL template DNA, 2 μL $MgCl_2$ (2 mmol/L), 1 μL nucleotides (0.2 mmol/L), 0.5 μL Taq polymerase (2.5 U), 5 μL PCR buffer (10×), and 31.5 μL distilled water and were performed on a Mastercycler Personal System (Eppendorf, Hamburg, Germany). All primers and PCR reagents were purchased from Invitrogen (Invitrogen Corporation, Carlsbad, CA). After

amplification, 9 μL of each reaction was mixed with 1 μL loading buffer (10×; Fermentas, St. Leon-Rot, Germany) analyzed electrophoretically on a 1.5% agarose (Invitrogen Corporation) gel using tris-borate-EDTA running buffer and stained with ethidium bromide (Sigma, St Louis, MO). PCR products were visualized with ultraviolet light in a Gel Doc 2000 (Bio-Rad, Life Science Group, Hercules, CA). All PCRs were conducted in duplicate. Positive controls consisting of the *E. faecalis* 12030 strain (positive for *gelE*, *asa1*, *cylA*, and *esp*) and *Enterococcus faecium* 137 (positive for *hyl*) were also included. The 1-kb Plus DNA ladder from Invitrogen was used as a molecular weight marker.

Preparation of Bovine Enamel Specimens

Bovine enamel slabs were used similarly to several earlier studies (18, 19) because this enabled the acquisition of standardized samples of homogenous quality and large surfaces. Furthermore, the chemical properties of human and bovine enamel are quite similar (20). Bovine incisors from bovine spongiform encephalopathy-free and freshly slaughtered cattle were taken to prepare 6 × 6-mm bovine enamel slabs. First, the teeth were separated from their roots and prepared using the grinding unit (Exakt-Mikroschleifsystem; Exakt-Apparatebau, Norderstedt, Germany). The final grinding of the bovine enamel was carried out with a grinding machine (Knuth-Rotor-3; Streuers, Willich, Germany) using sand paper of 1,200, 2,400, and 4,000 grits in decreasing order of grain sizes. The surface of the enamel specimen was monitored by using impinging light microscopy (Wild M3Z; Leica, Mannheim, Germany). Before their use for biofilm growth, the bovine enamel slabs were sterilized by ultrasonication for 2 minutes in 2% sodium hypochlorite and then 2 minutes in 70% ethanol. After this, the samples were washed twice in sterile distilled water. The sterility test was confirmed by aerobic and anaerobic cultivation on Columbia blood and yeast-cysteine blood agar, respectively.

Specification of the Biofilm Reactor and Biofilm Growth

The cultivation of biofilm using human salivary bacteria and *E. faecalis* isolates was conducted in a modified biofilm reactor (BioSurface Technologies, Corp, Bozeman, MT). The biofilm reactor was modified to be more robust and practicable and to prevent any contamination. It simulates the continuous microbiological culture of the oral cavity and allows studying the formation of biofilms on different substrata as well as testing of strategies to modulate or influence biofilm formation of single or mixed microorganisms. A basic mucin medium (BMM) described by Sissons et al. (21) was used to culture the multispecies biofilm in the bioreactor. The Biofilm reactor and composition of BMM are described in detail elsewhere (18).

The volume of the bioreactor in which the eight holders were incubated was 270 mL. After autoclaving the reactor at 120° for 20 minutes, two sterile bovine enamel specimens were fixed into each holder in a sterile hood. A Teflon stirrer was placed at the bottom of the reactor and was surrounded by the eight holders. We constructed a socket around the mean vessel to keep the temperature at 37°C using a water bath and pump. Each of the holders and the lid of the reactor were sealed with silicon rings. Air was diffused into the reactor through a 0.2-μm Teflon filter (Midisart 2000; Sartorius, Goettingen, Germany). Before inoculation with bacteria, the bovine enamel specimens were incubated in human sterile saliva for 2 hours in order to allow formation of a human pellicle. BMM was pumped into the reactor using peristaltic pumps (Ismatic, Munich, Germany) and sterile silicon tubing. The flow rate was adjusted to 0.35 mL/min, which corresponds to the average flow rate of saliva in healthy adults (22). A 1 mL logarithmic-phase culture of each *E. faecalis* isolate and 4 mL of unstimulated human saliva that was tested to be free of *E. faecalis* was used for the inoculation of the bioreactor. The absence of *E. faecalis* in the saliva was tested by the culture technique as well as by PCR as has been described elsewhere (5, 23). Additionally, the mixed population of this salivary inoculum was tested by FISH and shown to be free of *E. faecalis*. Before inoculation, the colony forming units (CFUs) of each *E. faecalis* culture and of human saliva were determined by plating on Columbia blood agar (CBA) as well as on bile esculin agar plates. The plates were incubated at 37°C in a 5% CO2 atmosphere for 3 days. The CFU of the planktonic phase in the reactor vessel were also determined immediately after inoculation. The proportion of *E. faecalis* was adjusted so that it made up 2% of all inoculated bacteria as determined aerobically on CBA. Biochemical tests were performed to confirm the isolates as *E. faecalis*. These biochemical tests were performed with API 20 Strep (Bio Merieux, Marcy-l'Etoile, France) according to the manufacturer's instructions (5). The biofilms were grown on bovine enamel slabs at 37°C for 4 days. The reactor experiments were conducted twice.

Determination of CFUs

To determine the CFU in the formed biofilm, eight slabs from each experimental period were washed with 0.9% sodium chloride. The dentine side as well as the margins were brushed off with sterile small sponge pieces (Voco GmbH, Cuxhaven, Germany) using sterile tweezers to exclude microorganisms that were not adherent to the enamel side. The enamel slabs overgrown with biofilm were then put into sterile tubes with 1 mL 0.9% sodium chloride, vortexed, and treated for 30 seconds in an ultrasonic bath on ice. To determine the CFU of the planktonic bacteria, 1 mL was removed from the biofilm reactor and then vortexed and treated for 30 seconds in an ultrasonic bath on ice. The solutions were serially diluted up to 1×10^5 in physiological saline, and 100 μL was plated on CBA and on bile esculin agar plates followed by incubation at 37°C for 3 days under aerobic conditions and 5% CO_2. Plating was conducted in triplicate. The CFU were counted using the Gel Doc EQ Universal Hood (Bio-Rad Life Science Group, Hercules, CA). The sampling of the planktonic state was carried out at the same time as the sampling of the biofim.

Duplex FISH

The sampling for FISH was performed at the same time as the sampling for the determination of CFUs. FISH was conducted according to Amann (24) with modifications as described elsewhere (14). In brief, biofilms grown on enamel chips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, 1.7 mmol/L KH_2PO_4-5 mM Na_2HPO_4 with 0.15 mol/L sodium chloride, pH = 7.2) for 12 hours at 4°C. After fixation, all specimens were washed with PBS and fixed again in an ethanol containing solution (50% in PBS, v/v) for 12 hours. The probes were washed twice with PBS and then incubated in a solution containing 7 mg of lysozyme per mL of 0.1 mol/L Tris-HCl-5 mmol/L EDTA, pH = 7.2, for 10 minutes at 37°C in order to permeabilize cells within the plaque biofilm. The biofilms were then dehydrated through a series of ethanol washes containing 50%, 80%, and 100% ethanol for 3 minutes each. Specimens were then incubated with the oligonucleotide probes at a concentration of 50 ng each per 20 μL of hybridization buffer (0.9 mol/L NaCl, 20 mmol/L Tris-HCl [pH = 7.2], 25% formamide [vol/vol], and 0.01% sodium dodecyl sulphate [w/vol]). Hybridization was conducted in 24-well plates (Greiner bio-one, Frickenhausen, Germany) at 46°C for 2 hours. After probe hybridization, specimens were incubated for 15 minutes at 48°C in wash buffer containing 20 mmol/L Tris-HCl (pH = 7.5), 5 mmol/L EDTA, 159 mmol/L NaCl, and 0.01% sodium dodecyl sulphate (w/vol).

All HPLC-purified oligonucleotide probes used in this study were synthesized commercially and 5'end labelled with different