Journal of Oral Biosciences 58 (2016) 62–65

Contents lists available at ScienceDirect

Journal of Oral Biosciences

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

Original Article

Profiling subgingival microbiota of plaque biofilms in the elderly

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ARTICLE INFO

Article history:
Received 24 July 2015
Received in revised form
11 November 2015
Accepted 30 November 2015
Available online 17 December 2015

Keywords:
Elderly
fimA
Genotype
Porphyromonas gingivalis

ABSTRACT

Objectives: The frequency of periodontitis among elderly is increasing in Japan. This study aimed to quantify the periodontitis-associated bacteria in subgingival plaques from elderly patients with periodontitis and from periodontally healthy subjects.
Methods: Subgingival plaque samples were collected from independent subjects (mean age=71.1 years, $n=518$). All samples were subjected to real-time PCR analysis to assess the presence of *Porphyromonas gingivalis*. In addition, we tested for *Tannerella forsythia*, *Eubacterium saphenum* and *Streptococcus oralis* in the earliest 95 samples; and performed *fimA* gene classification of *P. gingivalis* in the latest 49 samples.
Results: *P. gingivalis* and *T. forsythia* comprised a significantly higher proportion of total bacteria in subjects with periodontitis (1.1% and 5.1%) than periodontally healthy subjects (0.3% and 1.4%, respectively). The proportion of *E. saphenum* was low in both groups, whereas that of *S. oralis* was higher in periodontally healthy subjects. In 24 of 49 samples, *fimA* genotypes were detected and classified. Genotypes Ib ($n=5$) and II ($n=7$) were identified in those of subjects with periodontitis ($n=15$); while those of healthy subjects ($n=9$) were found to belong to genotypes I ($n=2$), II ($n=2$), III ($n=2$) and IV ($n=3$). In 4 out of the 5 subjects in whom *P. gingivalis* was detected at healthy sites, the *fimA* genotypes were identical between periodontitis and healthy sites, but the mean proportion of *P. gingivalis* was significantly higher at periodontitis sites (3.0%) than at healthy sites (0.5%) ($P<0.05$).
Conclusions: This study suggests that an increase in *P. gingivalis* and *T. forsythia* may be associated with periodontitis in the elderly, and we have identified characteristic pathogenic *fimA* genotypes that target this vulnerable group.

1. Introduction

Periodontitis and dental caries account for over 80% of the cases of tooth loss in Japan, and so they are considered to be medically important bacterial diseases of the human oral cavity. The microbiota of subgingival biofilms associated with periodontitis have been studied extensively. For example, *Porphyromonas gingivalis* [1–6], *Tannerella forsythia* [1–6] and *Eubacterium saphenum* [1–3,6,7] have been shown to be commonly found within periodontal pockets. However, these studies were performed with middle-aged patients with an elevated occurrence of periodontitis, and few studies have quantitatively analyzed biofilms in elderly subjects. Furthermore, previous studies on oral biofilms in elderly subjects have focused on those needing oral care; targeting opportunistic microorganisms such as *Candida* spp. on the tongue or in the saliva [8,9].

Therefore, we performed quantitative analysis using real-time polymerase chain reaction (PCR) in this study to characterize the microbiota present in subgingival biofilms from elderly subjects with periodontitis, and in periodontally healthy subjects. The incidence of *P. gingivalis*, *T. forsythia* and *E. saphenum* was quantified based on the 16S ribosomal RNA genes. The incidence of *Streptococcus oralis* was also quantified, since this organism is known to account for a high proportion of the microbiota in the gingival sulcus of healthy young subjects [6]. Additionally, we determined the *fimA* genotypes of *P. gingivalis* and compared these between sites of periodontitis and periodontally healthy sites in the same subjects.

2. Materials and methods

2.1. Subjects

A total of 518 elderly subjects (mean age ± SD=71.1 ± 7.0 years) participating in the program (for one-day) for the elderly at the

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http://dx.doi.org/10.1016/j.job.2015.12.002
1349-0079/

University of Hyogo, were included in the present study. Subjects had not received periodontal treatment or antimicrobial therapy for at least 6 months prior to sampling, and systemic diseases (hypertension, cardiovascular disease, diabetes mellitus, cerebrovascular disease, gout, dyslipidemia, arthralgia, benign prostatic hypertrophy, cataracts/glaucoma and cancer) were under control, either by hospital visits or medication. Probing depths were measured on all teeth, at six sites per tooth for each subject. We used this probe to assign subjects a score on the Community Periodontal Index, and the tooth with the deepest probing depth was selected as the target site for sampling. The deepest probing depths were ≥ 4 mm in subjects with periodontitis, and < 4 mm in periodontally healthy subjects. We also recorded whether bleeding occurred in the subjects during probing.

2.2. Sampling of the subgingival plaque biofilm and DNA extraction

Subgingival plaque biofilm samples were collected from 518 subjects using sterile toothpicks. All samples were immediately suspended in 200 μL of sterile distilled water and stored at −20 °C before extraction of genomic DNA. Genomic DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions.

2.3. Bacterial strains

Three species of anaerobic bacteria; *P. gingivalis*, *T. forsythia* and *E. saphenum* were selected for the present study, together with the facultative anaerobic bacterium; *S. oralis*. *P. gingivalis* W83, *T. forsythia* JCM 10827, *E. saphenum* ATCC 49989 and *S. oralis* JCM 12997 were cultured at 37 °C for 7 days on CDC Anaerobe 5% Sheep Blood Agar (BD, Franklin Lakes, NJ, USA) plates in an anaerobic glove box (Model AZ-Hard; Hirasawa, Tokyo, Japan) containing 80% N_2, 10% H_2 and 10% CO_2. One loopful of a colony from each strain was then suspended in 1 mL of sterile distilled water. Genomic DNA was extracted as described above. Concentrations of extracted DNA were measured using a microvolume UV–vis spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific Inc., Wilmington, DE, USA), and serial dilutions were utilized as standards for real-time PCR.

2.4. Quantitative analysis utilizing real-time PCR

In order to quantify total bacterial amounts and specific bacterial proportions in the samples, quantitative real-time PCR was undertaken using universal bacterial primers [6,10], specific primers [6] and iQ SYBR Green Supermix (Bio-Rad Laboratories, Richmond, CA, USA), in accordance with the manufacturer's instructions, although some criticisms have been reported on the universality of such primers [11]. Real-time PCR analysis for *P. gingivalis* was performed on all of the samples; while tests for *T. forsythia*, *E. saphenum* and *S. oralis* were only performed on the earliest 95 samples, due to limitations in sample availability. The sequences of the universal primers (directed against the 16S ribosomal RNA gene) used, 357F and 907R, were described elsewhere [6,10]. Sequences of specific primers against *P. gingivalis* (124F and 643R), *T. forsythia* (Tf-F and Tf-R), *E. saphenum* (551F and 981R), and *S. oralis* (MKR-F and MKR-R) were also described elsewhere [6]. Quantitative real-time PCR was performed using an iCycler (Bio-Rad Laboratories), as described previously [6,10]. Briefly, PCR cycling conditions were programmed for 3 min at 95 °C for initial heat activation, followed by 40 cycles of 15 s at 95 °C for denaturation, 30 s at 55 °C for primer annealing and 30 s at 72 °C for extension. During the extension step, fluorescence emissions were monitored and data were analyzed using iCycler iQ Software (Bio-Rad Laboratories). Standard curves (for total bacterial levels) were analyzed by comparing the universal primer set against a serial dilution of *P. gingivalis* W83 genomic DNA.

2.5. Genotyping of *fimA* by DNA sequence analysis

The genotypes of *fimA* were identified by sequence analysis of amplified DNA. Gene sequences for *fimA* were amplified by PCR using universal primers M1 and M2 [12] and *Taq* DNA polymerase (HotStar *Taq* Plus Master Mix plus; Qiagen GmbH), in accordance with the manufacturer's instructions. Primer sequences were as follows: M1, 5′- GCG CAG CAA GGC CAG CCC GGA GCA CAA CAC G -3′, and M2, 5′- GAG CGA ACC CCG TGA GG- 3′. Amplification proceeded using an iCycler (Bio-Rad Laboratories) programmed as follows: 5 min at 95 °C for initial heat activation; 30 cycles of 1 min at 94 °C for denaturation, 1 min at 65 °C for annealing, and 1.5 min at 72 °C for extension. The extension time was increased to 10 min for the final cycle. PCR products were separated on 1% agarose gels (High Strength Analytical Grade Agarose; BioRad Laboratories) in Tris-borate EDTA buffer (100 mM Tris, 90 mM borate, 1 mM EDTA; pH 8.4), stained with ethidium bromide and photographed under UV light. The sizes of the PCR products (ca. 1349 bp) were compared with molecular weight markers (100-bp DNA Ladder; Invitrogen Corp., Carlsbad, CA, USA). PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare UK Ltd., Buckinghamshire, UK), followed by sequencing at Fasmac (Atsugi, Kanagawa, Japan) using a BigDye Terminator Cycle Sequencing kit and an automated DNA sequencer (PRISM-3100; Applied Biosystem Japan, Tokyo, Japan). Primers M1 and M2 were used for sequencing, and the *fimA* gene sequences obtained were then compared with the sequences in the GenBank database using the BLAST search program through the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Bethesda, MD, USA). The *fimA* genes of *P. gingivalis* were classified into 6 genotypes (types I to V, and Ib) by DNA sequence analysis using BLAST search. We were only able to classify the *fimA* genotypes in the latest 49 samples due to limitations in sample availability.

2.6. Statistical analysis

Equality of variances were tested for the samples in Fig. 1 using Levene's test. Chi squared tests were performed for the samples in Fig. 2, and *t*-tests for samples in Table 1. *P*-values of < 0.05 were considered to be statistically significant, utilizing a statistical software (StatFlex, Ver. 6, Artech Co., Ltd., Osaka, Japan).

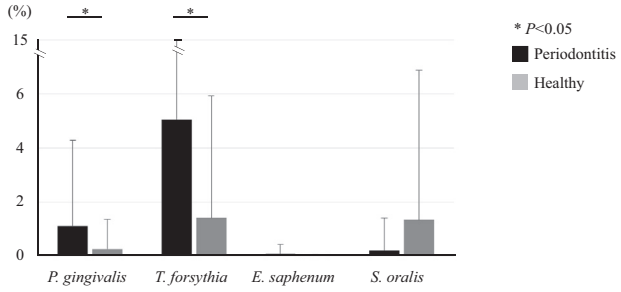

Fig. 1. Quantitative analysis of subgingival plaque biofilms in elderly subjects. Real-time PCR detection analysis was performed for *P. gingivalis* in all samples ($n = 518$). Similar analysis was also performed for *T. forsythia*, *E. saphenum* and *S. oralis* in the earliest 95 samples. Bar charts indicate the mean (±SEM) proportion (%) of each bacterium.