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Analysis of the Diversity of Oral Bacteria in Young Adults with Chronic Periodontitis

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Young adults are susceptible to chronic periodontal disease. Chronic periodontitis is thought to arise from the complex microflora in the oral cavity, which includes some putative periodontopathic bacteria. To unveil the diversity of periodontopathic bacteria, we conducted amplified ribosomal DNA restriction analysis (ARDRA) to investigate and compare the bacterial diversity in the subgingival plaque (SP), gingival crevicular fluid (GCF), and saliva samples of young adults with chronic periodontitis. Diversity indices were calculated by ARDRA results, which revealed that the bacterial communities in SP were more diverse than those in the GCF and saliva. In total, 65 operational taxonomic units from the SP library were identified and sequenced for phylogenetic analysis. These sequences could be assigned to 18 known bacterial genera, of which 15 are known to exist in the human oral cavity. The results of the phylogenetic study show that the predominant species in the SP of patients with chronic periodontitis are obligate anaerobes (*Selenomonas*, *Leptotrichia*, *Lachnospiraceae*, *Eubacterium*, and *Fusobacterium* species), and that Gram-negative bacteria are associated with chronic periodontitis. These findings demonstrate the diversity of bacteria that exist in chronic periodontitis, which should be considered in the treatment strategy for these patients.

Key words: ARDRA, Bacterial diversity, Chronic periodontitis, Subgingival plaque.

Periodontal disease refers to the inflammatory processes that occur in the tissues surrounding teeth in response to bacterial accumulations (dental plaque). It is also generally recognized that chronic periodontitis may be subsequently superimposed on both localized and generalized forms of aggressive periodontitis[1]. Chronic periodontitis is a major cause of tooth loss in adults and is an endemic inflammatory disease caused by subgingival microbiota. Several bacteria, mainly Gram-negative, have been shown to be strongly associated with periodontal infections[2]. Nevertheless, investigations thus far have been limited to species that could be cultivated on an artificial medium. Of the roughly 700 species detected in the oral cavity based on culture and molecular analysis, more than half have not yet been cultivated [3-5]. Therefore, molecular techniques such as amplified ribosomal DNA restriction analysis (ARDRA) and 16S rRNA gene sequencing are required to study the genetic diversity of the oral bacteria in chronic periodontitis.

The 16S rRNA gene sequencing method has been widely used as a reliable molecular marker for phylogeny identification. The 16S rRNA gene contains a conserved region, a unique sequence that is relatively unchanged both within and between species[6,7]. Restriction fragment length polymorphism (RFLP) is a method that is used to

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determine the differences in DNA fragment length (polymorphism) resulting from digestion by restriction enzymes. ARDRA is the name assigned to the RFLP analysis of the 16S rRNA gene; it is a useful method for phylogenetic analysis, and can be used to infer the degree of genetic variability and similarity between microorganisms[8]. This method has also been used to analyze the genetics and diversity of many bacteria such as *Streptococcus*[9], *Lactobacillus*[10], and *Mycobacterium*[11] species. Furthermore, one study has revealed that ARDRA can be used to identify the genetic diversity of the methylotrophic bacteria present in the human mouth[12].

The aims of the present study were to determine the oral bacterial diversity of the organisms associated with chronic periodontitis, and to compare the biodiversity of those found in subgingival plaque (SP), the gingival crevicular fluid (GCF), and saliva using ARDRA and 16S rRNA sequence analysis, with particular emphasis on the bacterial community associated with SP.

MATERIALS AND METHODS

Case Description, Sampling, and DNA Extraction

The study protocol was approved by the Ethics Committee of Fujian Normal University, and written informed consent to participate was obtained from the patients. Samples were taken from ten patients who had been referred to the Department of Stomatology, Fujian Provincial Hospital for root-canal or emergency treatment. Patients with chronic periodontitis were selected according to the following criteria: (i) aged 22–25 years; (ii) absence of present or past severe or chronic medical illness; (iii) presence of affected sites with a probable depth of 5–7 mm, concomitant loss of periodontal attachment, and visible gingival inflammation in all four quadrants of the dentition; and (iv) no antibiotic medication taken during at least the previous 2 months.

SP sampling

Samples of SP were removed with individual sterile Gracey curettes from the four deepest or most diseased sites.

GCF sampling

After removal of the SP, the crevicular site was gently dried with an air syringe. GCF was collected by placing filter paper strips into the sulcus/pocket until mild resistance was sensed and left in place for 30 seconds. Strips contaminated with saliva or blood were excluded from the analysis.

Saliva sampling

Samples of saliva (2 ml) were collected from each individual into a tube containing an equal volume of lysis buffer[13].

The SP and GCF samples were immediately injected into cryotubes containing Tris-EDTA buffer and frozen at "20°C. DNA was extracted from the clinical samples using the QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer's instructions. To maximize the DNA extraction from Gram-positive bacteria, a 30-minute preincubation step with lysozyme was added.

ARDRA

The 16S rRNA gene of the *Bacillus* species strains were amplified by polymerase chain reaction (PCR) using universal primers, with the forward primer 27f (5'- AGAGTT TGATCCTGGCTCAG -3') and the reverse primer 1492r (5'- ACGGTTACCTTGTTA CGACTT -3') [14]. The initial denaturation at 94°C for 4 minutes was followed by 30 cycles of denaturation at 94°C for 2 minutes, annealing at 55°C for 1 minute, and polymerization at 72°C for 1 minute; the final extension was carried out at 72°C for 10 minutes. The PCR products were further purified with the aid of a DNA purification kit according to the manufacturer's instructions (Promega, USA).

Purified 16S rRNA amplicons were cloned into the pEASY vector using the pEASY-T1 Cloning Kit (Beijing TransGen Biotech, Beijing, China). Recombinant plasmids were randomly chosen for colony PCR with M13F/M13R vector-derived primers. ARDRA was performed on selected PCR products[15, 16]. The amplicons were digested with the restriction enzyme AluI and analyzed by agarose gel electrophoresis. The single ARDRA pattern was identified and sequenced. The sequences (NCBI numbers: KC936892–KC936940) were aligned in BioEdit software (version 7.0.0). Phylogenetic analyses were conducted using Molecular Evolutionary Genetics Analysis software (version 5.2).

Calculation of community diversity

The various diversity indices were calculated from the AluI restriction types of three

clone libraries. Bacterial diversity indices of the 16S rRNA clone libraries were determined using the following formula:

C = [(total no. of clones examined per sample – no. of different AluI restriction patterns detected in sample) / total no. of clones examined per sample] × 100%.

Bacterial diversity was calculated on the basis of AluI restriction types by using the Shannon-Wiener index (H), Simpson's dominance index (D), Margalef's richness index (R), and Pielou's evenness index (E), as described by Tom[17].

RESULTS

Isolation of DNA from SP, GCF, and Saliva Samples, and Amplification of the 16S rRNA Gene

Total DNA was successfully isolated from the samples of SP, GCF, and saliva harvested from patients with chronic periodontitis (Fig. 1A). All of the DNA fragments were larger than 23 kb. PCR amplification of the 16S rRNA gene yielded DNA fragments with a single band at 1,500 bp for each sample (Fig. 1B). As many as possible of the 16S rRNA isolated from three samples was subjected

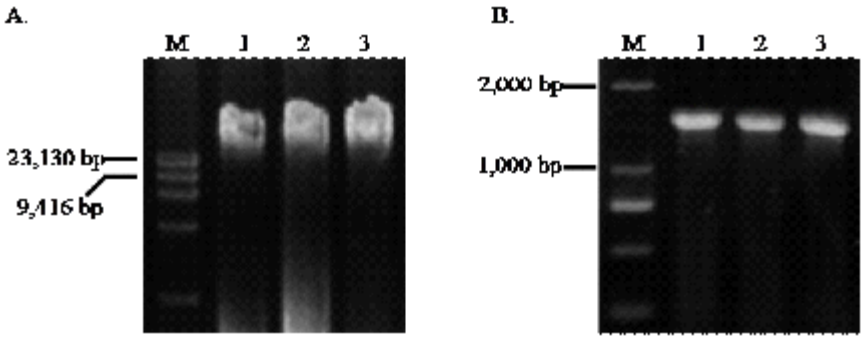

Fig. 1. A. Isolation of high-quality genomic DNA and amplification of the 16S rRNA gene from SP, GCF, and Saliva samples. A: The size of all total DNA >23 kb. B: The three 16s rRNA bands (Δ 1500 bp) are clearly visible. 1, SP; 2, GCF; 3, saliva

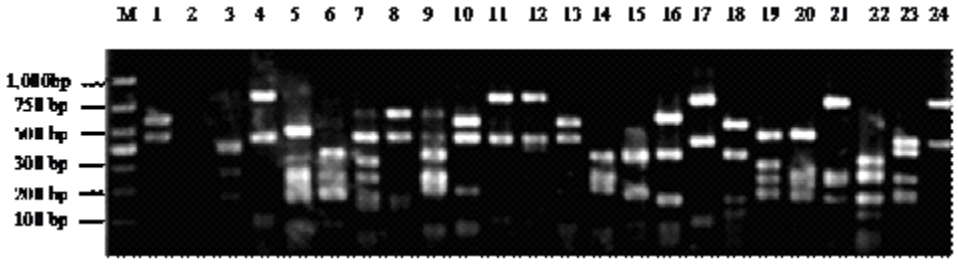

Fig. 2. The restriction patterns of amplified 16S rRNA genes from 24 colonies using the restriction enzyme AluI

to ARDRA by digesting the amplified 16S rRNA gene with the AluI restriction enzyme. The diversity of DNA fragments resulting from that digestion is shown in Fig. 2 (partial clones).

In total, 282 clones containing the full-length inserts (Δ 1500 bp) were digested with the AluI enzyme, which has been shown to be particularly effective at defining operational taxonomic units (OTUs). AluI digestion of full-length inserts resulted in two to six easily resolved bands, which were used for ARDRA. ARDRA patterns generated from AluI digestion of the cloned 282 inserts from 3 libraries were grouped into 65 different OTUs. An OTU was defined as a group of clones that had identical banding patterns obtained from independent digestions. Statistical analysis revealed the coverage of three libraries, as indicated in Table 1.

Diversity indices in 16S rRNA clone libraries from the SP, GCF, and Saliva libraries

The number of OTUs (richness) and the frequency distribution of the OTUs (evenness) in each of the clone libraries were evaluated using various standard diversity indices; the results are

Table 1. Diversity indices calculated from ARDRA profiles of the SP, GCF, and saliva libraries

16S rRNA library	OTUs	*C*	*H*	*D*	*R*	*E*
SP	65 (92)	33.7%	3.92	0.938	38.23	0.96
GCF	42 (96)	56.25%	3.37	0.867	17.29	0.92
Saliva	35 (94)	63.54%	3.21	0.863	12.26	0.91

Coverage(C). Shannon-Wiener index (H), Simpson's dominance index (D), Margalef's richness index (R), Pielou's evenness index (E).

summarized in Table 1. Table 1 indicates that the values of *H* and *D* were higher in the SP clone library than in the GCF and Saliva libraries, while the saliva clone library had the lowest values of *R* and *E*. According to values obtained from the 16S rRNA clone libraries, the SP library appears to be substantially more diverse than the GCF and Saliva libraries. The distribution of SP library clones in each sequence type tended to be more even than the other two libraries, and the SP library covers all OTUs types. Therefore, all of the OTUs in the SP library were sequenced to investigate the oral bacterial diversity in chronic periodontitis.

Fig. 3. Phylogenetic tree based on 16s rRNA sequences from SP library using neighbor-joining (NJ)

Sequence Analysis of the 16S rRNA Gene and the Bacterial Composition of the SP Library

Representative clones of all of the OTUs derived from the SP library were selected for 16S rRNA gene sequence analysis. A set comprising two or three clones from each of five randomly selected OTUs were sequenced to confirm the uniformity of the representative clones in the same OTU. Finally, one randomly selected clone from each OTU and unique representative(s) of different OTUs were sequenced. Altogether, 65 clones representative of the different groups were sequenced (nearly full-length sequencing of 16S rRNA gene, ~1500 nucleotides). The percentage similarities of the sequences were 85–99% with the 16S rRNA gene sequences in the database. All the 65 16s rRNA sequences from SP library were conducted to phylogenetic analyses (Fig.3). Of the total 65 cloned sequences of the SP library, four sequences (6.15%) clustered with sequences derived from uncultured bacterium clones. The sequenced clones fell into three lineages of the domain bacteria: obligate anaerobes [*Selenomonas* (12.31%), *Fusobacterium* (3.08%), *Eubacterium* (3.08%), *Firmicutes* (1.54%), *Lachnospiraceae* (6.15%), *Leptotrichia* (9.23%), *Treponema* (1.54%), *Prevotella* (3.08%), and *Bacteroidetes* (3.08%) species], facultative anaerobic [*Abiotrophiai* (1.54%), *Flavobacteriaceae* (1.54%), and *Capnocytophaga* (16.92%) species], and aerobic and microaerophilic [*Pseudomonas* (6.15%), *Neisseria* (7.69%), and *Campylobacter* (9.23%) species].

Fig.3

DISCUSSION

The present findings indicate that there is a significantly higher diversity of bacteria in subgingival plaque when compared to gingival crevicular fluid and saliva. Subgingival plaque located at depths of more than 4 mm in periodontal pockets provides more facilities for microorganisms to survive. These results indicate that the environment of SP in chronic periodontitis is anaerobic and favors the growth of anaerobes. Obligate anaerobes are more commonly found in patients with chronic periodontitis than in healthy subjects, including carriers of *Selenomonas*, *Fusobacterium*, *Eubacterium*, and *Lachnospiraceae* species. Most of these species have previously been associated with periodontitis. *Selenomonas* is a motile anaerobic Gram-negative bacterium associated with the etiology of generalized aggressive periodontitis[18]. *Fusobacterium* comprises several pathogenic strains, such as *F. nucleatum*, which is known for causing typical dental plaque on human teeth as well as for its involvement in periodontal diseases; it has the potential to be a periodontal pathogen[19]. *Asaccharolytic Eubacterium* species, which are obligate anaerobic, pleomorphic non-spore-forming, Gram-positive rods, have been isolated from human oral specimens, including those taken from periodontal pockets, infected pulp, and carious dentine. Some *Eubacterium* strains have also been isolated from subgingival areas associated with moderate or severe adult periodontitis. *Prevotella* is a genus of Gram-negative bacteria that is generally found in human dental plaque. *Leptotrichia* is a small genus that is closely related to *Fusobacterium*, and typically colonizes in the oral cavity. All *Leptotrichia* species are extremely fastidious and cannot be grown easily on conventional microbiologic media or by conventional methods[20]. The results of the present study show that six OTUs (with 65 clones) were clustered with *Leptotrichia*, indicating that this species may play a role in periodontal disease. *P. gingivalis* belongs to the phylum *Bacteroidetes*, and is found in the oral cavity, where it is implicated in certain forms of periodontal disease[21].

The facultative anaerobic bacteria often dominate in the supragingival plaque, as opposed to SP; therefore, few of these bacteria were detected in the SP library. In the present study, *Capnocytophaga* represented the most dominant subclass of facultative anaerobic, comprising 12 OTUs. *Capnocytophaga* is a prominent bacterium in subgingival samples from patients with chronic periodontitis[22]. *Abiotrophia* has been described as occurring in the oral cavity of healthy individuals[23].

Campylobacter, which was the most significant of the isolated aerobic and microaerophilic species in this study, is a microaerophilic and Gram-negative bacterium that is a potential pathogen in human periodontitis[24]. *Neisseria* species have been reported to comprise part of the microbial populations found on teeth,

in saliva, and on the tongue[25]. *Pseudomonas* species were the predominant species identified by culture-independent methods in the normal, gingivitis, and periodontitis samples[26].

Healthy and disease-causing bacteria can generally be grouped into two categories: (i) the harmless or helpful bacteria, called Gram-positive aerobic bacteria (which need oxygen to survive), and (ii) Gram-negative anaerobic bacteria (which do not need oxygen). In the present study, Gram-negative anaerobic bacteria (86.65%) were more predominantly isolated than the Gram-positive type (3.08%) in the cases with periodontitis. *Capnocytophaga* (16.92%), *Selenomonas* (12.31%), *Campylobacter* (9.23%), *Leptotrichia* (9.23%), and *Eubacterium* (3.08%) species were the most common organisms isolated. Gram-negative bacteria such as *Eubacterium*, *Selenomonas*, and *Leptotrichia* species are found in significantly higher levels in patients with chronic and aggressive periodontitis, and are therefore often called the red complex bacteria [27]. These Gram-negative bacteria colonize the gingival pockets; they are strong inducers of inflammation and tissue destruction, possess a highly developed capacity for evading host immune responses, and are resistant to antimicrobial treatment.

CONCLUSION

ARDRA is a useful approach for investigating bacterial diversity in samples of SP, GCF, and saliva. The diversity indices calculated from the ARDRA results reveal that the bacterial communities were more diverse in SP than in both GCF and saliva. Sixty-five OTUs from the SP library were identified and sequenced for phylogenetic analysis, revealing that obligate anaerobes and Gram-negative bacteria predominate in the SP of patients with chronic periodontitis. These findings demonstrate the diversity of bacteria in chronic periodontitis, which should be taken into account when deciding upon the treatment strategy for such patients.

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