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ORIGINAL ARTICLE

Association of uncultivated oral phylotypes AU126 and X112 with periodontitis

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OBJECTIVE: To discuss the relationship between uncultivated pathogenic bacteria and periodontitis.
SUBJECTS AND METHODS: Specific polymerase chain reaction (PCR) primers were designed for phylotypes AU126 and X112; PCRs were applied to determine the prevalence of these phylotypes in 35 patients with chronic periodontitis, 26 patients with plaque-induced gingivitis and 20 healthy control subjects.
RESULTS: The specificity of each primer is validated on the basis of the results from sequence analysis of PCR products. AU126 and X112 were detected in the subgingival plaque samples in all the three groups. The prevalence of AU126 in subgingival plaque in chronic periodontitis (77.1%) and plaque-induced gingivitis (61.5%) is relatively higher than that in the healthy subjects (10.0%), and the difference is statistically significant ($P < 0.01$). The prevalence of X112 in subgingival plaque in periodontitis patients (85.7%) is higher than that in healthy subjects (30.0%), the difference ($P < 0.01$) being equally statistically significant. The difference between the chronic periodontitis group and the plaque-induced gingivitis group (50.0%) is statistically significant ($P < 0.05$).
CONCLUSIONS: It might be assumed that the novel uncultivated AU126 phylotype could possibly be related to chronic periodontitis and plaque-induced gingivitis, and that X112 might play a role in the progress of lesion from gingivitis to periodontitis.
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Introduction

Most of the microorganisms on earth are yet to be known. Microbiologists estimate that $< 1\%$ of environmental bacteria can be cultured in the laboratory in accordance with the current standards and technologies. As estimated earlier, over 600 species of bacteria inhabit the human oral cavity (Kazor *et al*, 2003). Some reports have indicated that in subgingival microflora, about 50% of the species found remain uncultured, which are microscopically visualized and which have yet to form visible colonies on plates (Nishihara and Koseki, 2004).

Metagenomics is the culture-independent genomic analysis of microbial communities (Handelsman *et al*, 2002). This approach builds on recent advancements in oral microbial genomics and shares 16S rRNA gene sequence directly from human subgingival plaque samples (Kroes *et al*, 1999). Some putative pathogens have been detected from periodontal structures by the metagenomic approach as they are often identified from diseased sites, but only rarely from the healthy subjects (Kumar *et al*, 2003). Periodontitis, the inflammatory disease leading to destruction of the supporting tissues of teeth, is one of the major oral diseases resulting from the activity of a mixed bacterial biofilm growing under anaerobic conditions. Most analyses of the human oral putative pathogens such as *Actinobacillus actinomycetemcomitans*, *Campylobacter rectus*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Bacteroides forsythus*, and *Treponema denticola* prove to be limited by the conventional culture-dependent methods and no single species could be identified as the decisive pathogen in periodontitis (Mombelli *et al*, 2002; Socransky and Haffajee, 2005). It is important to find out which uncultured microorganisms present in the oral cavity are related to periodontitis. The novel approaches based on 16S rRNA gene may help find the uncultivated ones that are playing some roles in the periodontitis (Sakamoto *et al*, 2002).

The identification of novel organisms that are associated with the periodontitis does not, in itself, promote our understanding of the disease process. However, sequence

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Table 1 Primer sequences for the novel phylotypes

Phylotypes	*Primers*	*Sequences 5′ → 3′*	*Products size, bp*	*Annealing temperature, °C*
AU126	209F	TTA TTG ATT GGA GAT GGG TAT G	1228	60
	1428R	TCC TTG CGG TCA CGA AC		
X112	114F	CCC GAA GAT GGG TAT AAC TG	705	61
	818R	ACG ACA CTC CTG CCA ATA A		

data from the uncultivated organisms can be employed to design specific polymerase chain reaction (PCR) primers for a rapid detection of the organisms in clinical specimens. These can be used to determine the prevalence of the uncultured organisms in the diseased tissues of patients and healthy subjects. Any specific association found might constitute a useful sign of disease activity.

In this study, specific primers were designed for the uncultivated phylotype bacteroides-like sp. oral clone AU126 and the uncultured bacterium X112, which were selected from the many phylotypes that had been supposedly connected with periodontitis, but not with the healthy subjects (Paster *et al*, 2001). PCR was then applied to determine the prevalence of these phylotypes in patients with periodontitis and in healthy subjects.

Subjects and methods

Design of specific PCR oligonucleotide primer

The 16S rRNA gene sequences of AU126 and X112 were obtained from EMBL/GenBank/DDBJ databases. The primers for the specific amplification of the two novel phylotypes were designed by aligning the 16S rRNA gene sequences of their nearest neighbors in the phylogenetic tree by using the CLUSTAL W (Higgins *et al*, 1996) and Primer3 (Rozen and Skaletsky, 2000) programs, which are freely distributed at the web sites http://www2.ebi.ac.uk/clustalw/ and http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. The oligonucleotide primers were screened for specificity using the Ribosomal Database Project II (Maidak *et al*, 2001). The sequences of primers specific for each phylotype are shown in Table 1.

Multi-subgingival plaque samples were pooled and then centrifuged for 5 min at 13 000 × *g*, with the supernatant discarded and the pellet resuspended in 1 ml DNAzol Reagent (Invitrogen, Carlsbad, CA). The DNA of plaque samples were extracted according to the manufacturer's protocol. a PCR amplifications were performed with a PCR buffer containing 1.5 mM $MgCl_2$, 20 pmol of each primer, 40 nmol of deoxynucleoside triphosphate, 1 U of *Ex Taq* DNA polymerase (Takara, Dalian, China), and 10 μl template DNA in a total volume of 50 μl.

The best amplified protocol was determined after several tests using a thermocycler (Biometra Tgradient, Göttingen, Germany). The program for AU126 and X112 was performed for 36 cycles consisting of 95°C for 40 s, annealing temperature (listed in Table 1) for 50 s, and 72°C for 70 s, followed by a final extension step of 5 min at 72°C. PCR products were separated by electrophoresis in 1.5% agarose gels in 1x TAE buffer and visualized under UV light, following an ethidium bromide staining, before being sequenced with an ABI PRISM 3730 Sequencer (Invitrogen).

The specificity of primers was checked by sequencing the PCR products. RDP-II and BLAST were employed to check the PCR product sequences in comparison with the 16S rRNA gene sequences of the two uncultivated phylotypes (Altschul *et al*, 1997).

Clinical sample collection and detection procedure

Plaque samples were collected in sterile plastic tubes from 35 patients with untreated chronic periodontitis (Lindhe *et al*, 1999) (20 males and 15 females; mean age, 51.0 years), 26 patients with plaque-induced gingivitis (Caton *et al*, 1999) (nine males and 17 females; mean age, 26.2 years), and 20 periodontal healthy subjects (eight males and 12 females; mean age, 22.3 years). The sampling sites were isolated with sterile cotton rolls. Supragingival plaques and calculus were removed carefully with a scaler. Subgingival plaque samples from patients with chronic periodontitis were collected from the deepest pockets with a sterile Gracey curette and transferred into 0.1 mM PBS, and then stored at a temperature of −20°C immediately. Subgingival plaque samples from the other two groups were treated in the same manner.

In view of the fact that some clinical samples had shown the ability to inhibit *Taq* polymerase, the likelihood of false-negative results could be ruled out using an internal positive control (Garcia *et al*, 1998). PCR products from the first part of the experiment were diluted 10^4 times, and 1 μl was used as an internal positive control, amplified with another 9 μl DNA template from clinical plaque samples. The negative control did not react with any of the DNA templates. The extracted DNA from all clinical plaque samples was amplified in a manner similar to the protocol described above.

Results

To confirm the specificity of the PCR primers, PCR products obtained from pooled plaque samples that were positive for each of the phylotypes, were sequenced and compared with the original sequences from EMBL/GenBank/DDBJ and RDP-II. AU126, a product obtained by PCR, was 99% similar to the original sequences over 1228 bases by Blast, and Sequence Match (RDP Analysis Tool) arrived at a similar score: (S_ab) between the PCR product and the 16 S rRNA gene sequence of AU126 was 0.990. Similarly, a product from X112 was 99% similar to the sequence from the database over 705 bases, and the S_ab was 0.958. No

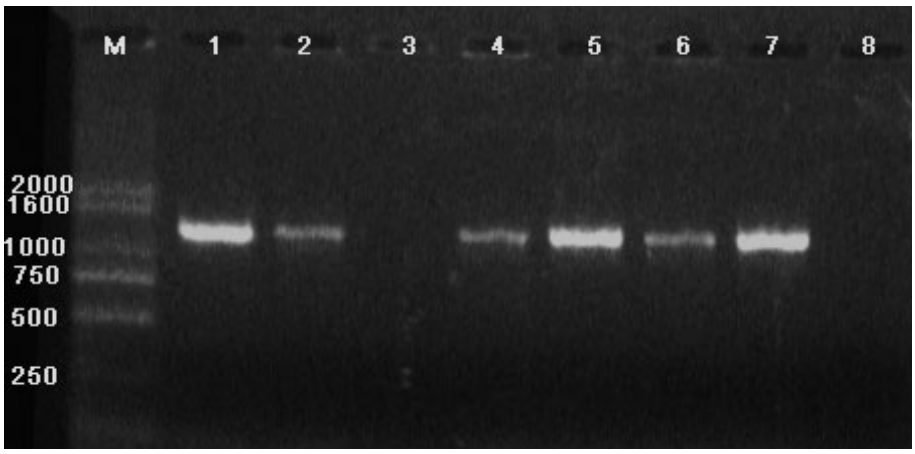

Figure 1 DNA amplified from 3 plaque samples (A,B,C) with chronic periodontitis for AU126. Lanes: M, molecular size marker; 1, positive control; 2, internal positive control for sample A; 3, sample A (negative); 4, internal positive control for sample B; 5, sample B (positive); 6, internal positive control for sample C; 7, sample C (positive); 8, negative control.

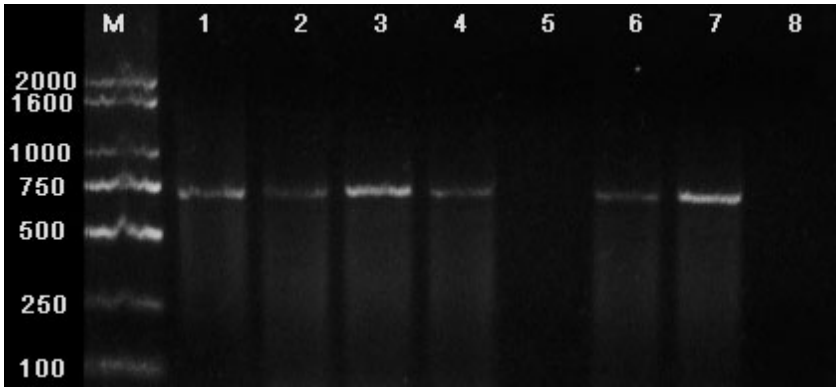

Figure 2 DNA amplified from 3 plaque samples (A,B,C) with chronic periodontitis for X112. Lanes: M, molecular size marker; 1, positive control; 2, internal positive control for sample A; 3, sample A (positive negative); 4, internal positive control for sample B; 5, sample B (negative); 6, internal positive control for sample C; 7, sample C (positive); 8, negative control.

other sequences in any database resemble so closely to those of the PCR products.

Samples from the three groups of subjects were checked for the presence of each uncultivated phylotype by PCR with the selected primers. Examples of PCR results are shown in Figures 1 and 2. In the identification of associations between phylotypes and diseases of various natures, the presence of the organisms was recorded as positive when the positive reaction with two controls displayed their corresponding signals. The prevalence of phylotypes in subgingival plaque samples from patients with chronic periodontitis, patients with plaque-induced gingivitis and the healthy subjects is shown in Table 2.

Table 2 Prevalence of phylotypes in patients with periodontitis, gingivitis and healthy subjects

Group	*AU126*	*X112*
Healthy ($n = 20$)	2 (10)	6 (30)
Gingivitis ($n = 26$)	16 (61.5)[a]	13 (50.0)
Periodontitis ($n = 35$)	21 (77.1)[a]	30 (85.7)[b]

Data are number and (percentage) of subjects.
[a]AU126 was significantly associated with subgingival plaque samples from periodontal sufferer and healthy ($P < 0.01$).
[b]X112 was significantly associated with subgingival plaque samples from patients with periodontitis and other two groups ($P < 0.01$).

Discussion

It is estimated that approximately half of the human oral flora has yet to be cultured. It might be assumed that currently unknown bacterial species may play roles in the etiology of periodontitis. Recent advancements in molecular biology have made it possible to study microbial communities, including uncultivated species (Wilson *et al*, 1997). The large databases of 16S rRNA sequences have been built, such as that made available by the RDP-II, which have supplied enough information for research into uncultivated microorganisms. The genes encoding the small-subunit rRNA from oral plaque are analyzed by purification and sequencing. A number of novel sequences, which do not correspond to the known cultured organisms, have been identified (Paster *et al*, 2001). These novel uncharacterized taxa identified by phylogenetic analysis in this way were designated as 'phylotypes'. They were more frequently detected from sensitive locations of the periodontum than from the healthy parts (Kumar *et al*, 2003).

For detection of the uncultivated pathogenic bacteria, specific PCR primers for the two phylotypes were designed in this study. The specificity of each primer was validated on the basis of the results of the sequence analysis of the PCR products, which are compared with

the known 16 S rRNA sequences with the BLAST 2.2.11 released on May 8, 2005 and RDP-II Release 9.27 containing 136 355 aligned 16 S rRNA sequences released on March 3, 2005. The PCR products of AU126 and X112 sequenced were 99% identical to sequences already in the EMBL/GenBank/DDBJ database. In relating a new sequence to known ones, most researches took related clusters with 97% or higher sequence identity to correspond to a species level relationship (Stackebrandt and Goebel, 1994). In this study, no other sequences in the EMBL/GenBank/DDBJ database are so close to the sequences acquired in this study. The results checked by Sequence Match of RDP-II have proved to be the same.

The mean age of the group with chronic periodontitis (51 years) is almost twice that of the group with plaque-induced gingivitis (26.2 years) and the healthy subjects (22.3 years). This age gap has resulted from a random selection of subjects from the clinical patients. The relationship between oral microbial composition and age gap is still a mystery and demands further discussion and investigation.

Phylotypes AU126 and X112 were detected in subgingival plaque samples in all the three groups. The prevalence of AU126 in the subgingival plaque is relatively higher in patients with chronic periodontitis and plaque-induced gingivitis than that in the healthy subjects, and the difference has turned out to be statistically significant ($P < 0.01$). But the difference is not statistically significant between the periodontitis group and the gingivitis group. The prevalence of X112 is higher in the subgingival plaque of periodontitis patients than in gingivitis patients and the healthy subjects, with the difference being equally significant ($P < 0.01$). There was no statistically significant difference between the gingivitis group and the healthy group. These results suggest that the novel phylotypes AU126 and X112 are associated with periodontitis. The novel uncultivated AU126 phylotype could be related to chronic periodontitis and plaque-induced gingivitis, while phylotype X112 may play a role in the progress of lesions from gingivitis to periodontitis.

Novel phylotypes AU126 and X112 will have to be cultured for research into the pathogenesis of periodontitis. The culture of these phylotypes has not yet been accomplished. The approach of this research is useful in detecting uncultivated organisms. The results demonstrate the wide distribution of the targeted phylotypes, which represent as-yet-uncultivated organisms in the human oral cavity. However, much remains to be known about the uncultivated phylotypes, and further studies of these putative pathogens are needed.

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