Prevalence of Putative Periodontopahogen TM7 and Dialister in Dental Plaque of Koreans

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TM7 is an uncultivated organism which is present in extremely diverse environments. Members of the Dialister genus are difficult to culture as a result of which many of these strains remain uncultivated. It has been suggested that TM7 and Dialister bacteria may belong to a group of suspected periodontal pathogens. In our current study, the presence of the sebacteria in Korean dental plaque samples was assessed using PCR detection methods with specific primers for 16S ribosomal RNA genes. The experimental group included 84 volunteers (35 males and 49 females). Plaque samples were collected from 4 non-adjacent proximal sites of the molar areas of the mandible in each subject and pooled. TM7 was detectable in 56% and the Dialister genus in 27.5% of the volunteers. Both TM7 and Dialister were present in 20.3% of volunteers. We found that 36.9% of the volunteers were negative for both bacteria. Further studies to evaluate the prevalence of these putative pathogenic bacteria in the Korean population are warranted.

Key words: dental plaque, periodontitis, prevalence

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Introduction

Anaerobic bacteria are known to play important roles in the pathogenesis of human periodontitis[1,2]. The presence of elevated levels of a specific complex of anaerobic bacteria in subgingival plaque is associated with periodontal disease severity [3]. Even though there is a consensus that bacteria play a causative role in the development of periodontal disease, any specific bacterial species have not been invariably detected at the lesion of periodontal diseases. In most previous studies, the search for pathogens has been limited to cultivable species. However, it is known that the in vitro cultivable bacterial species are estimated to comprise less than half of all oral floras [4]. It was reported that approximately 35% of the species present in subgingival plaque are as yet uncultivated, so their roles in inducing periodontal diseases are still unclear [5].

TM7 is an uncultivated organism which is present in extremely diverse environments, including soil, freshwater, seawater, hot springs, mouse feces and termite guts [6]. Through a survey of bacterial diversity in human subgingival plaque samples by broad-range PCR, TM7 bacteria were detected in human subgingival plaque[7]. Brinig *et al*. reported that the abundance of TM7 ribosomal RNA gene (rDNA) relative to total bacterial rDNA was higher in sites with mild periodontitis than in either healthy sites or sites with severe periodontitis[4]. Dialister cells are minute, obligately anaerobic, nonmotile, nonsporing, nonfermentative, gram-

negative bacilli[8]. Members of the Dialister genus are culture-difficult or remain uncultivated[9]. The presence of this genus was consistently confirmed in samples from gingival crevices [10-12] and endodontic infections [13]. *Dialister pneumosintes* was detected in 83% of patients having severe periodontitis and in 19% of patients having slight periodontitis [9]. It has been suggested that *D. pneumosintes* might be included in the group of suspected periodontal pathogens.

While the presences of the uncultivable TM7 and culture-difficult Dialister in dental plaque have been reported by several research groups, the prevalence of TM7 and Dialister in dental plaque of Korean population is not disclosed yet. In this study, the presence of TM7 and Dialister in dental plaque samples of Korean was examined by PCR detection methods with specific primers for ribosomal RNA genes of these bacteria. This study was carried out as a preliminary study before starting a research for revealing the relationship of these two species of bacteria with the periodontal diseases in Koreans.

Materials and Methods

Plaque samples

The experimental group consisted of 84 volunteers (35 males and 49 females; aged 22 ±4 yr).Mixed dental plaque samples of supragingival and subgingival areas were collected and pooled with anexplorer from four non-adjacent proximal sites of the molar areas of mandible, following saliva isolation with cotton rolls. Samples were placed in 1.5-ml microcentrifuge tubes and frozen until further analysis. Approximately 3-5 µl volume of plaque samples were collected from each person. All volunteers who wanted to donate their plaque for this study were thoroughly informed about the procedure and gave written consent for inclusion in the study. This study was approved by the Institutional Review Board of Gangneung-Wonju National University Dental Hospital (IRB2011-2).

Genomic DNA preparation and PCR

Genomic DNAs from each plaque sample were prepared with *AccuPrep*® Genomic DNA Extraction kit (Bioneer, Daejeon, Korea). After purification, the presence of bacterial genomic DNA in samples was examined by PCR using a pair of ubiquitous bacterial primers (Primer1 5'-AGA GTT TGA TCM TGG CTC-3' and Primer2 5'-GGY TAC CTT GTT ACG ACT T-3'). The following reaction conditions were used: annealing, 55°C for 30 sec; extension, 72°C for 1 min; and denaturation, 94°C for 30 sec. The cycles were repeated 34 times. To determine the presence of TM7 and Dialister in the plaque sample, PCR primers for specific ribosomal RNA genes were used. PCR primers specific for TM7 was prepared as reported in the previous study [6].

TM7580F 5'-AYT GGG CGT AAA GAG TTG C-3'
1492R 5'-TAC GGY TAC CTT GTT ACG ACT T-3'

PCR primers specific for Dialister was prepared as reported in the previous study [14].

D. pneumosintes 1 5'-TTC TAA GCA TCG CAT GGT GC-3'
D. pneumosintes 2 5'-GAT TTC GCT TCT CTT TGT TG-3'

PCR was performed in a reaction mixture that contained 2 µl of bacterial genomic DNA, 0.5 µM of primer 1, 0.5 µM of primer 2, 1× PCR buffer with 1.5 mM MgCl$_2$, 250 µM of dNTP, 1 unit of Taq polymerase (HotStart PCR Premix, Bioneer, Daejeon, Korea), and 16 µl of distilled water for a final volume of 20 µl. PCR amplification was performed in a DNA thermal cycler (GeneAMP PCR System 9700; Perkin Elmer, Waltham, MA, USA). PCR temperature profiles included initial denaturation at 96°C for 10 min, followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and an extension step at 72°C for 2 min for TM7.PCR temperature profiles included initial denaturation at 95°C for 2 min, followed by 36 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, and an extension step at 72°C for 2 min for Dialister. After completion of the final cycle, the PCR products finally were extended at 72°C for 5 min for TM7 and 72°C for 10 min for Dialister, respectively. PCR amplification products were electrophoresed on 1% agarose gel and were stained with ethidium bromide for 30 minutes; they then were visualized and photographed by ultraviolet (UV) transillumination.

Sequence analysis for confirming species

One of PCR products for each bacterial species was checked by DNA sequences analysis for confirming the identification of TM7 and Dialister. The PCR products were purified using an *AccuPrep*® PCRpurification kit (Bioneer, Daejeon, Korea). The purified PCR products were sent to Macrogen (Seoul, Korea) for nucleotide sequencing of the

16S rRNA genes. Sequences were compared with sequences from the reference organisms provided by BLAST (a genome database of the National Center for Biotechnology Information).

Results

Figure 1 shows PCR amplification products with specific primers for TM7 and Dialister in dental plaque samples of 84 Koreans. Among 84 persons, 17 persons (20.3%) possessed both TM7 and Diaslister and 31 persons (36.9%) had neither TM7 nor Dialister (Table 1). Thirty persons (35.7%) from 84 persons had only TM7 and 6 persons (7.1%) had only Dialister. Forty seven persons (56%) possessed TM7 with or without Dialister and 23 persons (27.4%) showed the presence of Dialister with or withour TM7.

The sequences of PCR amplified DNAs with specific primers for TM7 and Dialister in this study were matched with sequences from the reference organisms provided by BLAST.
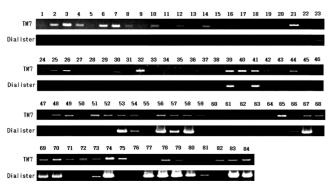

Fig. 1. The PCR detection of TM7 and Dialister in Korean dental plaque. Bacterial genomic DNAs were purified from plaque samples and the presence of TM7 and Dialister was determined by PCR with specific primers for 16S ribosomal RNA genes. PCR amplification products were electrophoresed on 1% agarose gel and stained with ethidium bromide.

Table 1. The prevalence of TM7 and Dialister in dental plaque of Koreans

	TM7+ Dialister+	TM7+ Dialister-	TM7- Dialister+	TM7- Dialister-
Male (n=35)	8(22.9)	14(40)	3(8.6)	10(28.5)
Female (n=49)	9(18.3)	16(32.7)	3(6.1)	21(42.9)
(n=84)	17(20.3)	30(35.7)	6(7.1)	31(36.9)

Discussion

Periodontal diseases are polymicrobial infections that can be induced by some of Gram negative anaerobic bacterial strains. A previous study reported that cultivated species including *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia* were determined to be periodontal pathogens[1,2,15].The subgingival bacterial species are largely uncultivated, and therefore, cultivation-based approaches should have limitations in searching the etiological bacterial species for periodontal diseases.

TM7 bacteria were recently detected in the subgingival plaque of periodontal disease patients through a survey of bacterial diversity in human subgingival plaque samples by broad-range PCR [7,16]. Five TM7 phylotypes were identified and clone I025 was suggested as a putative pathogen because it was found only in patients with various oral diseases [7]. It has been reported that the I025 phylotype was detected in 1 of 18 healthy samples and 38 of 58 disease samples among several division subgroups of TM7 [4]. In our study, phylotype of TM7 was not identified because general PCR primers for TM7 genus instead of phylotype specific primers were used. The further identification of phylotype of TM7 from Korean dental plaque will be necessary in future study.

In culture studies, an anaerobic, nonmotile, nonsporing, nonfermentative, gram-negative rod occurred in high proportions in several severe periodontitis lesions [9]. This organism was identified as *D. pneumosintes* [14]. One recent study reported that *D. pneumosintes* was detected in 47.8% of the biofilm samples, but only in 3% of saliva samples [17]. It also showed that significant associations between the prevalence of *D. pneumosintes* and pocket depth, attachment loss and bleeding on probing were observed. These findings corroborate the association of *D. pneumosintes* with periodontitis. It was also reported that subgingival *D. pneumosintes* occurred with significantly higher prevalence in older individuals and was closely associated with subgingival *T. forsythia* [18]. In that study, it was suggested that *D. pneumosintes* may play an important role in the microbial complex responsible for destructive periodontal disease [18]. It has been reported recently that *D. pneumosintes* may also have been implicated as a candidate

endodontic pathogen usually in a mixed infection [19,20]. They examined the prevalence of seven putative endodontic pathogens in samples of primary endodontic infections taken from patients of Brazil and Korea [21]. They showed that *D. pneumosintes* was one of the most prevalent species which were significantly more detected in Brazilian samples than in South Korean samples.

Kumar *et al*. carried out the investigation to identify potential periodontal pathogens among newly identified speciesor phylotypes by using species-specific ribosomal 16S primers for PCR amplification [22]. It was identified that associations with chronic periodontitis were observed for several new species or phylotypes, including uncultivated clones D084 and BH017 from the *Deferribacteres* phylum, AU126 from the *Bacteroidetes* phylum, *Megasphaera* clone BB166, clone X112 from the *OP11* phylum, and clone I025 from the TM7 phylum [22].

In this study, we used mixed plaque sample insupragingival and subgingival area and did not checkedsubgingival plaque and supragingival plaque separately. Because of this limitation, it is not clear at this point whether there is difference of prevalence of TM7 and Dialister between subgingival and supragingval plaque. It is still unclear whether TM7 and Dialister are detected with higher rates in subgingival plaque of periodontal patients than of healthy persons in Koreans. Researches to compare the prevalence of these putative pathogenic bacteria between disease patients and healthy persons in Korean should be performed to explain this question. In addition, it should be also investigated whether TM7 and *D. pneumosintes* are the cause or the consequence of the periodontal diseases. Through our present preliminary study,it has been shown that TM7 was present in about 56% of Korean and Dialister are present in approximately 27% of dental plaque of Koreans.

Acknowledgements

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