International Journal of Oral Biology, Vol. 38, No. 4 December 30 2013, p. 181-188
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http://dx.doi.org/10.11620/IJOB.2013.38.4.181

International Journal of Oral Biology

Oral Microbiota Comparison between Healthy volunteers, Periodontitis patients and Oral cancer patients

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(received December 3, 2013; revised December 18, 2013; accepted December 18, 2013)

The presence of distinct bacterial species is found to be dependent on age, diet, and disease. We compared the detection rate of several oral bacterial strains in a cohort of 36 subjects including healthy volunteers, periodontal patients, and oral cancer patients. Gargling samples were obtained from these subjects from which DNA was then extracted. Specific primers for 29 bacterial species were used for PCR detection. In the oral cancer patients, *Capnocytophaga ochracea*, *Gemella morbillorum*, and *Streptococcus salivarius* were detected more frequently compared with the healthy volunteers and periodontitis patients. *Fusobacterium nucleatum/ polymorphym* and *Prevotella nigrescens* were significantly less prevalent in oral cancer patients than the other groups. In periodontitis patients, *Porphyromonas gingivalis* and *Treponema denticola* were more frequently found compared with the healthy volunteers. In the healthy volunteer group, *Peptostreptococcus anaerobius* was more frequently found than the other groups. The detection rate of several oral bacterial species was thus found to differ between healthy volunteers, periodontitis patients and oral cancer patients.

Key words: Oral Microbiota, Periodontitis, Oral cancer

Introduction

The oral cavity harbors over 700 species of bacteria that contribute to the health and physiological status of oral cavity [1]. To understand the role of oral microbiota in the oral cavity, it is important to analyze its fundamental characteristics and dynamics. The oral microbiota in a healthy oral cavity versus a diseased one is distinctly different, which indicate that there may be a profile for oral microbiota [1]. Understanding the microbial differences between health and disease may give clinicians to recognize and diagnose diseases at an earlier and reversible stage [2].

In health, microbes may prevent disease progression in several ways. They can prevent the adherence of pathogens onto specific surfaces by occupying the niche preferred by pathogens. They can hinder pathogens' abilities to multiply and degrade pathogens' virulence factors [3]. In disease, the relationship between microbes are altered from mutualistic to

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parasitic and from commensal to opportunistic [4, 5]. As pathogenic bacteria flourish, host becomes infected or prones to infection [6].

Oral diseases such as periodontal disease and oral cancer are diseases worldwide affecting old ages [7, 8]. Periodontal disease results from subgingival plaque accumulation that causes shifts in the microbiota from healthy state to diseased state [9]. Microbes within biofilms begin to form pathogenic characteristics that aggravate and inflame the gingiva [10]. The predominant pathogens involved in periodontitis are *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Tannerella forsythia*, *Eikenella corredens*, and *Treponema denticola* [9].

Another oral disease that frequently affect the old ages is oral cancer. Oral cancer is the sixth most prevalent cancer, affecting over 300,000 people each year around the world [11]. Correlation between the structure and function of oral microbiota and oral cancer has been suggested [12]. Microbes can impact signal pathways that initiate and progress oral cancer [13]. Inflammation is usually the first symptom of compromised oral health and it gets worse as health regresses. Approximately 15–20% of human tumors contain pathogenic agents derived from inflammatory infections [12].

In this study, we compared the detection rate of oral bacteria species between healthy volunteers and patients with periodontitis or oral cancer. The purpose of present study was to determine whether the salivary microbiota in healthy subjects would differ from those of the patients with periodontitis or oral cancer in Korean.

Materials and Methods

Patient and healthy donor selection

Gargling samples used in this study were obtained from healthy volunteers and the patients with periodontitis or oral cancer. Since the definition of healthy state is quite controversial, samples from young volunteers were collected. Samples from healthy donor were obtained from School of Dentistry, Pusan National University. Samples from periodontitis patients were obtained from Pusan National University Dental Hospital. Samples from oral cancer patients were obtained from Dongnam Institution of Radiological and medical cancer. Criteria for the selection of periodontitis patient and healthy volunteers were: (1) absence of systematic diseases, (2) no antibiotics taken for at least 6 months before sampling, and (3) non-smokers. Sites with no overt signs of gingival inflammation and with a probing depth of < 3mm were defined as clinically healthy; sites with obvious alveolar bone loss detected radiographically and a probing depth > 3were defined as exhibiting signs of periodontitis. Informed consent was obtained from all donors and their rights were protected according to the protocol reviewed and approved by the institutional review board of Pusan National University Dental Hospital and Dongnam Institution of Radiological and medical cancer.

Sample collection

To collect the samples, participants gargled 15 ml of distilled water for 30 sec and the water was carefully collected in 50 ml tube. The samples were centrifuged at 3000 rpm for 10 min and the pellets were immediately transferred and stored at - 20°C before extraction of genomic DNA.

DNA isolation and PCR detection

DNA isolation was performed by using genomic DNA isolation kit (Qiagen, Valencia, CA, USA). The DNA concentrations in clinical samples and the concentrations of the reference DNA were determined by spectrophotometer measurement (Nanodrop, Thermo, Wilmington, DE, USA) of the absorbance at 260 nm. The PCR reaction used to assess the occurrence of all target taxa was performed with 20 μL of reaction mixture containing 1 pM of each specific primer and 2x master mix. PCR amplification was performed in a PCR Thermal Cycler (Eppendrof, Hambrug, Germany) programmed for 10 min at 95 °C for initial heat activation, followed by 35 cycles of 30 sec at 94 °C for denaturation, 30 sec at 60 °C for annealing, and 30 sec at 72 °C for extension, and 10 min at 72 °C for final extension. The predicted sizes of PCR products with species-specific primers are listed in Table 1. PCR products were separated on 1.5 % agarose gels, stained with ethidium bromide, and photographed under ultraviolet light. The sizes of PCR products were compared with a molecular size marker and confirmed to correspond to those listed in Table 1.

Table 1. Species-specific and ubiquitous polymerase chain reaction primers for 28 oral bacteria

Primer pairs (5'-3')	Amplicon length (bp)	reference
Aggregatibacter actinomycetemcomitans	557	[28]
AAA CCC ATC TCT GAG TTC TTC TTC		
ATG CCA ACT TGA CGT TAA AT		
Porphyromonas gingivalis	404	[28]
AGG CGA CTT GCC ATA CTG CG		
ACT GTT AGC AAC TAC CGA TGT		
Prevotella intermedia	575	[28]
TTT GTT GGG GAG TAA AGC GGG		
TCA ACA TCT CTG TGG GCT GCG T		
Prevotella nigrescens	804	[28]
ATG AAA CAA AGG TTT TCC GGT AAG		
CCC ACG TCT CTG TGG GCT GCG A		
Treponema denticola	316	[28]
TAA TAC CGA AGC TCA TTT ACA T TCA AAG TCT CTG		
TGG GCT GCG A		
Tannerella forsythensis	641	[28]
GCG TAT GTA ACC TGC CCG CA		
TGC TTC AGT GTG AGT TAT ACC T		
Capnocytophaga sputigena	185	[28]
AGA GTT TGA TCC TGG CTC AG		
GAT GCC GCT CCT ATA TAC CAT TAG G		
Capnocytophaga ochracea	185	[28]
AGA GTT TGA TCC TGG CTC AG		
GAT GCC GCT CCT ATA TAC TAT GGG G		
Capnocytophaga gingivalis	185	[28]
AGA GTT TGA TCC TGG CTC AG		
GGA CGC ATG CCC ATC TTT CAC CAC CGC		
Fusobacterium nucleatum/periodonticum	142	[28]
CTG AAC ATT GGA AAC TAT ATA GTA GAA CAA ACA AG		
GTC CTT CAT CGG CTC TTA CTA CCT AGG C		
Actinomyces israeli	230	[29]
AGA GTT TGA TCC TGG CTC AG		
CCA AAA CAC CAC AAA AGT GA		
Porphyromonas endodontalis	672	[29]
GCT GCA GCT CAA CTG TAG TC		
CCG CTT CAT GTC ACC ATG TC		
Prevotella melaniogenica	389	[29]
CGT CAT GAA GGA GAT TGG		
ATA GAA CCG TCA ACG CTC		
Streptoccus intermedius	500	[29]
AGA GTT TGA TCC TGG CTC AG		
GTA CCG TCA CAG TAT GAA CTT TCC		
Candida albicans	250	[30]
GCA TCG ATG AAG AAC GCA GC		
TCC TCC GCT TAT TGA TAT GC		
Gemella morbillorum		[31]
CGAGAGTCAGCCAACCTCATA		
GGTACTTAGATGTTTCAGTTC		
Neisseria mucosa	224	New
AAGCAACGACAGCGTGAAAC		
AGAACGCGCCTTGGTTTTTC		
Peptostreptococcus anaerobius	188	[32]
GCTCGGTGCCTTCACTAACG		
AGCCCCGAAGGGAAGGTGTG		
Streptococcus anginosus	445	[33]

Primer pairs (5’-3’)	Amplicon length (bp)	reference
ATG CAA TTG CAT CGC TAG T		
GCA GGC TTT GGA AAC TGT TTA ACT		
Streptococcus constellatus	445	[33]
GTG CAA GAG CAT CAC TAC C		
GCA GGC TTT GGA AAC TGT TTA ACT		
Streptococcus gordonii	440	[34]
CTATGCGGATGATGCTAATCAAGTG		
GGAGTCGCTATAATCTTGTCAGAAA		
Streptococcus oralis	374	[34]
TCCCGGTCAGCAAACTCCAGCC		
GCAACCTTTGGATTTGCAAC		
Streptococcus sanguinis	313	[34]
GGATAGTGGCTCAGGGCAGCCAGTT		
GAACAGTTGCTGGACTTGCTTGTC		
Streptococcus salivarius	544	[34]
GTGTTGCCACATCTTCACTCGCTTCGG		
CGTTGATGTGCTTGAAAGGGCACCATT		
Streptococcus mutans	415	[35]
AGCCATGCGCAATCAACAGGTT		
CGCAACGCGAACATCTTGATCAG		
Streptococcus sobrinus	329	[35]
GAAACCAACCCAACTTTAGCTTGGAT		
ATGGAGTGATTTTCCATCGGTACTTG		
Veillonella parvula	623	[36]
GAAGCATTGGAAGCGAAAGTTTCG		
GTGTAACAAGGGAGTACGGACC		
Eikenella corrodens	688	[37]
CTA ATA CCG CAT ACG TCC TAA G		
CTA CTA AGC AAT CAA GTT GCC C		
16S rRNA *	602	[37]
GAT TAG ATA CCC TGG TAG TCC AC		
CCC GGG AAC GTA TTC ACC G		

* Universal primers are from *Escherichia coli.*

Data analysis

Student t-test and Fisher’s exact probability test were used to analyze significance. P-values of < 0.05 were considered statistically significant.

Results

Patients and healthy volunteers

Bacteria in the saliva isolated from 36 subjects were determined in this study. Healthy volunteers were free from any oral disease (n=16). Periodontitis patients were diagnosed at the department of periodontology, Pusan National University (n=11). Oral cancer patients include hypopharynx (n=2), tongue (n=2), tonsil (n=4) and maxillary sinus cancer (n=1). The mean ± SD age of the healthy volunteers, periodontitis patients and oral cancer patients group was 30 ± 2.70, 62.13 ± 12.52, 55.22 ± 7.60 years old, respectively. The age of healthy volunteers was significantly lower than other groups ($p< 0.001$). The age difference between the periodontitis patients and oral cancer patients was insignificant.

Detection rate of oral bacteria

To characterize the oral bacteria frequently found in each study group, frequently isolated bacterial species were grouped together. Bacterial species more frequently detected in oral cancer patients are shown in Fig. 2A. *Capnocytophaga ochracea, Gemella morbillorum*, and *Streptococcus salivarius* were detected significantly more frequently in oral cancer patients. *Streptococcus constellatus* and *Streptococcus gordonii* were detected significantly more frequently in oral cancer patients compared to the healthy volunteers and periodontitis patients, respectively.

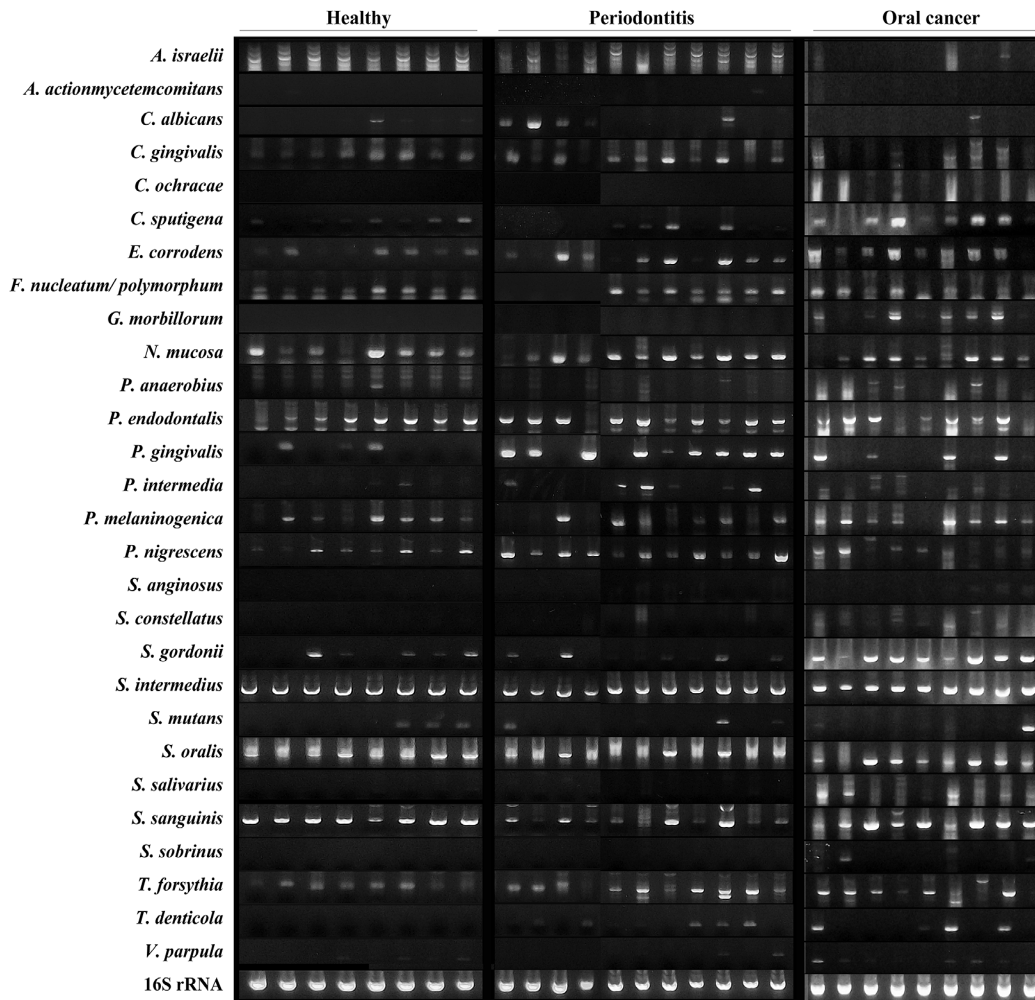

Fig. 1. Representative results of electrophoresis of PCR products from saliva samples. Saliva samples were collected from healthy volunteers, periodontitis patients and oral cancer patients. Bacterial DNA was extracted from saliva samples and PCR was performed as described in Materials and Methods. The PCR products were separated by electrophoresis on 1.5% agarose gels.

In Fig. 2B, bacterial species more frequently found in healthy volunteers and periodontitis patients are shown. *F. nucleatum/polymorphym* and *Prevotella nigrescens* were significantly lower in oral cancer patients compared to both the healthy volunteers and periodontitis patients. *Actinomyces israelii* was less frequently detected in oral cancer patients compared to the healthy volunteers.

In Fig. 2C, bacterial species that were more frequently found in periodontitis patient are shown. *P. gingivalis* and *T. denticola* were more frequently found in periodontitis patients. Among fungus, *C. albicans*, frequently found in oral cavity, was more frequently found in periodontitis patients.

In Fig. 2D, bacterial species more frequently detected in healthy volunteers are shown. *Peptostreptococcus anaerobius* was more frequently found in healthy volunteers compared to the periodontitis patients and oral cancer patients.

Discussion

Oral diseases such as dental caries and periodontal disease are the most prevalent diseases worldwide [14]. Most of periodontitis more frequently occur in the aged population and another oral disease which threatens the aged population is oral cancer. There are several studies which report oral bacterial species often found in periodontitis patients or oral cancer patients compared to healthy subjects. Since most of the studies were conducted in western nations, we surmised that different ethnic

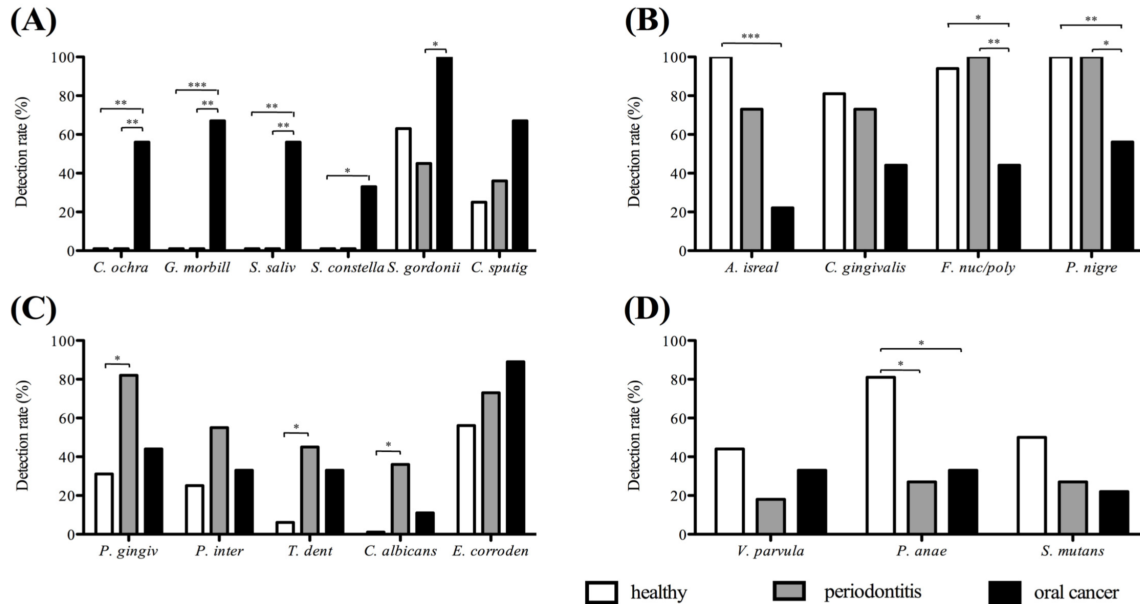

Fig. 2. Detection rates of oral bacteria in the saliva samples from healthy volunteers, periodontitis patients and oral cancer patients. (A) Bacterial species more frequently found in oral cancer patients. (B) Bacterial species less frequently found in oral cancer patients. (C) Bacterial species more frequently found in periodontitis patients. (D) Bacterial species more frequently found in healthy volunteers. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$.

background and lifestyle may influence the microbiota. In this study, we compared the detection rate of several oral bacterial species between young healthy volunteers and periodontitis patients and oral cancer patients in Korean.

Studies have reported that certain common oral bacteria are elevated in periodontitis patients. Most commonly accepted periodontopathogens are *T. denticola, P. gingivalis*, *T. forsythus, P. intermedia, and E. corrodens* [15, 16]. Consistent with other previous reports, high detection rate of *P. gingivalis* and *T. denticola* was observed. Also, *P. intermedia* and *E. corrodens* showed high rate of detection. Interestingly, *C. albicans* detection rate was also high in periodontitis patients. Our results showed similar pattern of bacteria species detection rate compared to other pervious reports studied in the western world [15, 16].

Certain common oral bacteria have been reported to be elevated in oral and esophageal cancer lesions [17-19]. Facultative oral streptococci, *Prevotella*, *Veillonella*, *Porphyromonas* and *Capnocytophaga* species were reported to be elevated [20-22]. Among *Streptococcus*, the detection rate of *S. constellatus* was significantly higher in oral cancer patient compared to periodontitis patients. *S. constellatus* belongs to the anginosus group of streptococci. The anginosus groups are facultative anaerobic gram-positive cocci and can cause serious infections in humans [23]. They possess pathogenicity based on tolerance to polymorphonuclear leukocytes, cellular components such as capsules, and extracellular enzymes [24, 25]. *S. constellatus* preferentially colonize the oral soft tissues and saliva compared to the teeth [26]. Since we used gargling wash to collect bacterial DNA, bacteria preferentially colonized to oral soft tissue could have been detected more frequently. Mager *et al*. reported that *Capnocytophaga gingivalis, Prevotella melaninogenica*, and *Streptococcus mitis* counts were significantly increased among the oral cancer patients [27]. In our study, only *C. ochracea* was significantly increased among the oral cancer patients while *C. gingivalis* showed rather decreased incidence.

Periodontitis and oral cancer develop mostly in aged population. Comparing the detection rate of oral bacterial species, several species were distinctively detected in periodontitis and oral cancer patients. Several similar results were observed between this study and other previous reports, suggesting that further study should improve our understanding on oral microbiota. However, small number of samples in this study limits its significance. Increasing the total number of clinical samples could strengthen the overall

result and its significance.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Ministry of Education, Science and Technology (MEST) (NRF-2011-0013215).

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