Prevalence of Oral Microbes in the Saliva of Oncological Patients

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This study examined the prevalence of oral microbes in the saliva of oncological patients and healthy subjects. PCR was used to assess the frequency of oral microbes including 3 cariogenic bacteria, 5 periodontopathic bacteria and 4 Candida species in the saliva of 104 oncological patients and 52 healthy subjects. Among these microorganisms, Streptococcus mutans, Fusobacterium nucleatum and Candida albicans were most frequently detected in both groups. There were no significant differences in the prevalence of cariogenic bacteria between the patient and healthy groups, whereas significant differences in the frequency of Porphyromonas gingivalis and Tannerella forsythia were observed between the two groups (p < 0.05). The prevalence of all five periodontopathogens was higher in the healthy group than in the patient group. The prevalence of C. albicans in patients was significantly higher than that of healthy group (p < 0.05). In conclusion, there were significant differences in the prevalence of P. gingivalis, T. forsythia and C. albicans between the oncological patient group and healthy group.

Key Words: Prevalence; Cariogenic bacteria; Periodontopathic bacteria; Candida; oncological; PCR

INTRODUCTION

A multitude of microorganisms thrive in the oral cavity interacting with each other at times causing clinical diseases. Mutans streptococci have been considered as major cariogenic bacteria (1–3), which are comprised of Streptococcus mutans, S. sobrinus, S. downei, S. rattus, S. cricetus, S. ferus, and S. macacae (4). Of these, S. mutans and S. sobrinus are strongly associated with human dental caries (2). In addition, lactobacilli which are part of the normal flora in the mouth, intestine and vagina (5, 6) are believed to play an opportunistic role in the development of dental caries due to their production of both lactic acid and extracellular polysaccharides (2).

Besides caries, another common dental disease is periodontitis. It has been shown that a group of microbes, such as Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter actinomycetemcomitans, Treponema denticola, Fusobacterium nucleatum, exist in complexes in subgingival plaque (7–9), and they are suspected to be the putative agents of periodontal diseases.

In addition, oral mucosal infections can be caused by potentially pathogenic microorganisms of extraoral origin or by a shift within the normal commensal microflora. For
example, oral mucosal lesions are often developed by an opportunistic microflora in immunocompromised patients. Oral candidiasis is an opportunistic disease due to some promoting factors that alter the equilibrium in the oral microflora, which assist in the transformation of yeasts from commensal organisms to pathogens (10). Oncologic therapy, such as irradiation or chemotherapy, further impairs the defense mechanism of the oral mucosa with the resultant proliferation of mucosal biofilms with subsequent overgrowth, particularly of yeast and anaerobic bacteria (11).

Various methods, such as biochemical tests, immunological tests, species-specific DNA probes, 16S rDNA sequencing comparison methods, and polymerase chain reaction (PCR) methods, have been used to both detect and identify oral microbes (4, 12–16). Among them, PCR provides a rapid, sensitive and specific modality. Therefore, in this study, the PCR technique was performed for the rapid identification of human oral microbes in the salivary samplings.

Prevalence of oral microbes in cancer patients may be affected by patient's health status, existence of any oral pathology, the timing of chemo-radiation, surgery, the stage of disease, the type of malignancy, pediatric vs. adult patients, or previous or current antibiotic treatments, etc. In addition, a study for the prevalence of oral microbes in cancer patients is important for providing the basic data to further control the oral complications of cancer therapy.

There is little data on the prevalence of oral microbes including caries-associated bacteria, major periodontal pathogens, and Candida species in saliva of oncological patients. This study examined and compared the frequency of cariogenic, periodontopathic bacteria and Candida spp. in the saliva of oncological patients and healthy subjects by PCR.

**MATERIALS AND METHODS**

**Bacterial strains**

The following bacterial strains were used: *S. mutans* Ingbritt, *S. sobrinus* B-13 and *Lactobacillus salivarius* subsp. *salicinius* ATCC 11742 were used as the cariogenic bacteria; *A. actinomycetemcomitans* ATCC 33384, *P. gingivalis* ATCC 33277, *F. nucleatum* ATCC 10953, *T. forsythia* ATCC 43037 and *T. denticola* ATCC 35405 were used as the periodontopathic bacteria; and *Candida albicans* KCTC 7965, *C. glabrata* KCTC 7219, *C. parapsilosis* KCTC 7214, and *C. tropicalis* KCTC 7212 were used as the *Candida* species. *S. mutans* and *S. sobrinus* were grown in brain heart infusion broth (BHI broth; Difco, Detroit, MI, USA). *L. salivarius* subsp. *salicinius* was grown in De Man, Rogosa, Sharpe broth (MRS broth; Difco). The bacteria were incubated at 37°C for 16 h under aerobic conditions. *A. actinomycetemcomitans* was grown in tryptic soy broth (Difco) supplemented with yeast extract (1 mg/ml) and horse serum (10%). *P. gingivalis* and *F. nucleatum* were grown in Brucella broth supplemented with yeast extract (1 mg/ml), hemin (10 μg/ml), and menadione (5 μg/ml). *T. forsythia* was grown in Brucella broth supplemented with a yeast extract (0.5 mg/ml), hemin (5 μg/ml), menadione (0.5 μg/ml), N-acetylmuramic acid (10 μg/ml) and fetal bovine serum (10%) (17). *T. denticola* was grown in TYGVS medium [tryptone (10 mg/ml), brain heart infusion broth (5 mg/ml), yeast extract (10 mg/ml), gelatin (10 mg/ml), (NH₄)₂SO₄ (0.5 mg/ml), MgSO₄ (0.1 mg/ml), K₂HPO₄ (1.13 mg/ml), KH₂PO₄ (0.9 mg/ml), NaCl (1 mg/ml), glucose (1 mg/ml), cysteine hydrochloride (1 mg/ml), thiamine pyrophosphate (12.5 μg/ml), sodium pyruvate (0.25 mg/ml), 0.027% acetic acid, 0.01% propionic acid, 0.0064% n-butyric acid, 0.0016% n-valeric acid, 0.0016% isobutyric acid, 0.0016% isovaleric acid, 0.0016% DL-methylbutyric acid, 10% heat-inactivated rabbit serum] (18). The bacteria were incubated anaerobically (85% N₂, 10% H₂, and 5% CO₂) at 37°C for 48 h. *Candida* species were grown in Sabouraud liquid medium (Difco) at 30°C for 24 h under aerobic conditions. For analysis, each culture was harvested by centrifugation at 12000 × g for 2 min and washed twice with PBS (pH 7.4).

**Subjects**

A total of 104 cancer patients of Chonnam National University Hwasun Hospital and 52 healthy subjects as control [90 males (M) and 66 females (F); age, 48.5±18.7
years (mean ± SD) were examined. Briefly, 52 healthy subjects (23 M and 29 F; age, 42.0±19.3 years) as well as 28 patients with head and neck tumors (22 M and 6 F; age, 58.0±9.7 years), 39 patients with hematological neoplasia (24 M and 15 F; age, 42.0±20.4 years) and 37 patients with other solid tumors (21 M and 16 F; age, 55.6±16.4 years) were enrolled. All subjects signed an informed consent form approved by the Ethics Committee of Chonnam

<table>
<thead>
<tr>
<th>Oligonucleotide sequence (5’→3’)</th>
<th>Amplicon size (bp)</th>
<th>References</th>
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</thead>
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<td><strong>S. mutans</strong></td>
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<td></td>
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aF, forward primer; bR, reverse primer
Saliva collection
The subjects were asked to refrain from eating, drinking, and dental hygiene for a minimum of 1 h before sialometry. The subjects were asked to hold their head slightly forward and to expectorate all accumulated saliva into the collection tubes, being careful not to swallow during the 5 min collection period. The saliva was expectorated into the tube at 1 min intervals. The amount of saliva was measured in milliliters to gauge the salivary flow. The saliva samples were stored immediately at -20°C before extracting the genomic DNA.

Genomic DNA preparation
Cultivated reference organisms and saliva samples were harvested by centrifugation at 12,000 × g for 5 min. The supernatants were discarded, and individual cell pellets were stored at -20°C until DNA isolation. The genomic DNA was prepared using an i-genomic BYF DNA Extraction Mini kit (iNtRON Biotech, Sungnam, Korea) according to the manufacturer’s instructions. The DNA concentrations in the samples from the culture and saliva were calculated by measuring the A260, and the quality was estimated from the A260/A280 ratio. The genomic DNA samples were stored at -20°C until use.

Polymerase chain reaction (PCR)
PCR amplifications for the detection of cariogenic and periodontopathic bacteria were performed in a reaction mixture (20 μl) containing Taq polymerase (Solgent, Daejon, Korea), 250 μM dNTP, 2 mM MgCl2, 30 mM KCl, and 10 mM Tris-HCl (pH 9.0), along with 200 nM primer and 40 ng of the genomic DNA (15, 19~21). Seminested PCR (snPCR) described by Ahmad et al. (22) was performed to detect the Candida species. Briefly, the first PCR was carried out in a total volume of 20 μl containing Taq polymerase, 200 nM each universal fungal primer (forward primer, 5’-TGCATCGATGAAGAACGCAGC-3’; reverse primer, 5’-TCCTTTCTCCCCGTTATGTGATGC-3’) and 40 ng of the genomic DNA. The second PCR was performed in a 20 μl reaction mixture containing 1 μl of the first PCR product and 200 nM of universal fungal reverse primer, together with 200 nM of the specific forward primer of each Candida species. Table 1 lists the sequences of the species-specific primers used in this study. The primers were synthesized commercially (Bioneer, Daejon, Korea). PCR cycling was carried out in a PCR Thermal Cycler Dice (Takara, Kyoto, Japan). The following PCR programs were used. For the cariogenic and periodontopathic bacteria: initial denaturation at 94°C for 3 min; followed by 40 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 30 s. For the Candida species (22): initial denaturation at 94°C for 3 min; followed by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. Each final cycle included an additional extension time of 5 min at 72°C. The PCR products were analyzed by 1.5% agarose gel electrophoresis, and visualized by staining with ethidium bromide. The sizes of the amplified DNA fragments were identified by comparison with a molecular size marker DNA (Invitrogen, Carlsbad, CA, USA).

Statistics
Each experiment was carried out in triplicate, and the mean value was analyzed further. Statistical analysis was performed using SPSS version 13.0 (Statistical packages for Social Science version 13.0; SPSS Inc., Chicago, IL, USA). A chi-square test was used to determine the statistically significant differences in the experiments. A p value < 0.05 was considered significant.

RESULTS
Salivary flow
Table 2 shows the salivary flow of the study group. The salivary flow of the oncological patients was lower than that of the healthy group. However, there were no significant differences between the patient and healthy groups.

Prevalence of cariogenic bacteria in saliva samples
PCR amplification was performed using reference strains to detect the presence of the three cariogenic bacteria in
Lactobacillus species were detected in all subjects. The frequency of S. mutans in the subjects was higher than that of S. sobrinus in the healthy group was significantly lower than that in the patients with head and neck tumors ($p < 0.05$). However, there were no significant differences in the frequency of cariogenic bacteria between the total cancer patient group and healthy group (Fig. 1).

**Prevalence of periodontopathic bacteria in saliva samples**

Five periodontopathogens were detected in the saliva of the study group, as shown in Fig. 2. Of the five periodontopathogens, F. nucleatum was detected most frequently in patients with head and neck tumors (74.2%), followed by P. gingivalis, T. forsythia, T. denticola, and A. actinomycetemcomitans, at frequencies of 71.0%, 67.7%, 64.5%, and 0.0%, respectively. F. nucleatum was most prevalent in the patients with hematological neoplasia at a frequency of 84.8%, followed by P. gingivalis and T. forsythia (67.4%), T. denticola (41.3%), and A. actinomycetemcomitans (0.0%). On the other hand, T. forsythia showed the highest frequency in patients with solid tumors at 86.8%, followed by F. nucleatum (84.2%), T. denticola (71.1%), P. gingivalis (28.9%), and A. actinomycetemcomitans (2.6%). In general, periodontopathic bacteria were more prevalent in the healthy group than in the patient group. There were significant differences in the frequency of P. gingivalis, T. forsythia and T. denticola between the patients with hematological neoplasia and healthy group ($p < 0.05$). In addition, the prevalence of T. forsythia in the healthy group was significantly higher than that in the patients with head and neck tumors.

**Table 2. Salivary flow of the study group**

<table>
<thead>
<tr>
<th></th>
<th>Patients with head and neck tumors (n = 28)</th>
<th>Patients with hematological neoplasia (n = 39)</th>
<th>Patients with solid tumors (n = 37)</th>
<th>Healthy group (n = 52)</th>
</tr>
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<tbody>
<tr>
<td>Salivary flow (ml)</td>
<td>2.45±1.22</td>
<td>2.75±1.41</td>
<td>2.16±1.81</td>
<td>2.94±1.69</td>
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</table>

**Figure 1.** The prevalence of cariogenic bacteria in the saliva of the study groups by PCR. HN, patients with head and neck tumors; HE, patients with hematological neoplasia; SO, patients with solid tumors; TO, total oncological patients; H, healthy subjects; Sm, S. mutans; Ss, S. sobrinus; Lb, Lactobacillus spp. *$p < 0.05$, vs. healthy subjects.

**Figure 2.** The prevalence of periodontopathic bacteria in the saliva of the study groups by PCR. HN, patients with head and neck tumors; HE, patients with hematological neoplasia; SO, patients with solid tumors; TO, total oncological patients; H, healthy subjects; Aa, A. actinomycetemcomitans; Pg, P. gingivalis; Tf, T. forsythia; Fn, F. nucleatum; Td, T. denticola. *$p < 0.05$, vs. healthy subjects.
Prevalence of *Candida* species in saliva samples

Of the four *Candida* species, *C. albicans* was also most prevalent in the patients with head and neck tumors at a frequency of 71.0%, followed by *C. glabrata* (16.1%), *C. parapsilosis* and *C. tropicalis* (12.9%). *C. albicans* was detected most frequently in patients with hematological neoplasia (63.0%), followed by *C. glabrata*, *C. tropicalis* and *C. parapsilosis*, at frequencies of 13.0%, 13.0%, and 8.7%, respectively. *C. albicans* with the highest frequency (73.7%) and *C. glabrata* with the lowest frequency (10.5%) were detected in the patients with solid tumors. *C. albicans* was detected most frequently in the healthy group (51.9%), followed by *C. parapsilosis*, *C. tropicalis* and *C. glabrata*, at frequencies of 25.0%, 21.2%, and 11.5%, respectively. The prevalence of *C. parapsilosis* in the healthy group was higher than that of the oncological patients. However, there were no significant differences in the prevalence of *Candida* species between patient group and healthy group, except in the case of *C. albicans*. In addition, the prevalence of *C. albicans* in total oncological patients was significantly higher than that of the healthy group (*p* < 0.05) (Fig. 3).

**DISCUSSION**

Radiotherapy or chemical therapy for cancer patients causes a pronounced decrease in saliva flow rate and the onset of clinical xerostomia (23–25). Xerostomia is always accompanied by pronounced shifts in the specific microbial components of the oral microflora (26, 27). An overgrowth of fungus can occur when the delicate balance of normal and abnormal bacteria is disturbed. In this study, with the regard to salivary flow, the results showed similar salivary flow in the cancer patients and healthy subjects.

PCR is used widely to identify various bacterial species. 16S rDNA can be used effectively for PCR assays because 16S rDNA is found universally in prokaryotic organisms, and a comparative analysis of 16S rDNA has shown that the variable sequence regions are interspersed with highly conserved regions (28). Therefore, in this study, PCR was performed for the detection of mutans streptococci, lactobacilli and periodontal pathogens from saliva samples using the species-specific PCR primers based on the 16S rDNA. Yoo et al. (20) reported that the PCR primers for the *S. mutans* and *S. sobrinus* based on the 16S rDNA gene were more specific for the clinical isolates of mutans streptococci in Koreans than those based on the dextranase gene. The major caries organisms are *S. mutans*, *S. sobrinus*, and lactobacilli. In particular, *S. mutans* and *S. sobrinus* are generally considered to be the primary etiological bacteria of human dental caries (2). Lactobacilli are part of the normal flora of the human body (5, 6). In this study, there were no significant differences in the prevalence of cariogenic bacteria between patient group and healthy group. *Lactobacillus* species were detected by PCR in all groups at a frequency of 100.0%, suggesting that *Lactobacillus* is not particularly pathogenic.

Periodontal diseases result from the complex actions of a group of periodontopathic bacteria, mostly gram-negative anaerobes. *A. actinomycetemcomitans* is an etiologic agent of juvenile periodontitis (29, 30), whereas red complex
species are strongly correlated with the severity of adult periodontitis (31). The red complex consists of a tightly related group containing species, such as *P. gingivalis*, *T. forsythia* and *T. denticola*, whereas the *F. nucleatum* subspecies are members of the orange complex (32). *F. nucleatum* is the most numerous gram-negative bacterium in the healthy oral cavity, but the mass of *F. nucleatum* increases significantly during active periodontal disease (33).

In this study, red complex species were significantly more prevalent in the healthy group than in the patients with hematological neoplasia. *F. nucleatum* was detected most frequently in the patient groups, even though it is probably not a major periodontal pathogen. A comparison of the bacterial diversity between the cancer groups showed that *P. gingivalis*, which is a minor bacteria in the solid tumor group, was detected preferentially in the other cancer groups. According to the results, the saliva of healthy individuals harbored larger amounts of periodontal pathogens than that of oncological patients. The lower prevalence of periodontopathogens in the cancer patient group than that in the healthy group might be affected by a range of factors, such as patient's health status or anticancer therapy. *A. actinomyctecomitis* is responsible for aggressive periodontitis and was detected at the lowest frequency in all groups tested. The low detection frequency of *A. actinomyctecomitis* in this study group might reflect the elderly aged group. This result is supported by the report of Müller et al (34) that *A. actinomyctecomitis* can be isolated occasionally in young adults.

The predominant fungi isolated from the human mouth belong to the genus *Candida*, and while there are more than 350 *Candida* species, approximately 10 of these colonize the oral cavity. In this study, the prevalence of four *Candida* species was examined by performing snPCR described previously (22). Consistent with the published data (36), oral *Candida* was common in oncological patients. However, compared with *Candida* species other than *C. albicans* in the healthy group, the values determined by PCR were considerably higher, as reported previously (35, 36). *C. albicans* is well known as an important opportunistic fungal pathogen. The data obtained in this study demonstrated that the cancer patients harbor more *C. albicans* than healthy subjects. However, all four *Candida* spp. were detected in the saliva of all groups tested. Furthermore, there was a higher prevalence of *C. parapsilosis* and *C. tropicalis* in the healthy group than in cancer patients. This suggests that these *Candida* species may be not pathogenic. This result is inconsistent with another report (37). There were differences in the prevalence of *Candida* species between cancer patients, which may be due to subject-based differences or a multitude of predisposing factors prevailing in cancer patients.

The presence of microorganisms including *Candida* or viruses in the body lesions of cancer patients has been well studied (38, 39). However, the prevalence of oral microflora in the saliva of oncological patients by PCR has not been investigated. In conclusion, PCR was used to compare the frequency of oral microbes in the saliva of oncological patients and healthy group. Among the oral microorganisms, *S. mutans*, *F. nucleatum* and *C. albicans* were detected most frequently in both groups. *P. gingivalis* and *T. forsythia* were significantly more prevalent in the healthy group than in the oncological patients, whereas *C. albicans* was significantly more prevalent in the oncological patients than in the healthy group. However, further studies on the quantitative analysis of these microorganisms will be needed.

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