

Analysis of Cariogenic Bacteria in Saliva of Cancer Patients

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This study examined salivary flow and salivary pH and the prevalence and levels of cariogenic bacteria in the saliva of oncological patients and healthy controls. Quantitative real-time polymerase chain reaction was used to assess the levels of microbes including *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus salivarius*, and *Lactobacillus acidophilus* in the saliva of 41 patients with a solid tumor (SO), 30 patients with a hematologic malignancy (HE), and 40 healthy controls. Salivary flow and pH were lower in oncological patients than in controls. The frequencies of all four cariogenic bacteria were highest in the SO group. *S. mutans* and *L. salivarius* were the most commonly detected in all three study groups. Mean numbers of *S. sobrinus* and *L. salivarius* in the SO group were significantly higher than in controls ($p < 0.05$). There were no significant differences between patients and controls with respect to mean numbers of *S. mutans* and *L. acidophilus* in saliva. However, the proportions of *S. mutans*, *S. sobrinus*, and *L. salivarius* versus total bacteria in the SO group were significantly higher than in controls. Within patients, both mean numbers and the proportions of *S. mutans* and *S. sobrinus* were significantly different ($p < 0.05$). In summary, significant differences were found in salivary pH values and the levels of *S. mutans*, *S. sobrinus*, and *L. salivarius* between SO patients and healthy controls.

Key Words: *Lactobacillus*; Real-time polymerase chain reaction; Saliva; *Streptococcus*

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INTRODUCTION

Radiotherapy or chemical therapy in cancer patients causes xerostomia and reduces saliva flow rates¹ and may induce highly specific changes in microbiota.² In addition, low saliva flow rates may be associated with markedly increased susceptibility to dental caries.³ The major organisms responsible for caries are mutans streptococci and lactobacilli⁴; of these, *Streptococcus mutans* and *Streptococcus sobrinus* are generally considered to be the primary etiological bacteria of human dental caries.^{5,6} Furthermore, *Lactobacillus* species have been reported to occur in high numbers in caries sites.^{7,8} In particular, *Lactobacillus salivarius* and *Lactobacillus acidophilus* are predominant species in the mouth.⁹

Investigations of bacterial profiles in saliva for the identification of caries risk groups are popular and produce reli-

able results.¹⁰ A variety of methods, such as conventional culture methods, direct enzyme tests, enzyme-linked immunosorbent assays, and conventional end-point polymerase chain reaction (PCR) have been used to detect and identify oral microbes.¹¹⁻¹³ However, these methods do not allow for accurate quantification; thus, reliance on them means that important diagnostic aspects are overlooked. However, quantitative real-time PCR (qRT-PCR) is a highly specific, relatively fast, and sensitive means of detecting and quantifying bacteria as compared with conventional culture methods and end-point PCR.¹⁴

Study of the prevalences of oral microbes in cancer patients is important because it provides basic data that aid in control of the oral complications of cancer therapies. Furthermore, the availability of an accurate quantitative assay for the detection of cariogenic bacteria could facilitate the monitoring of therapies and enable more accurate

epidemiological studies on the progression of caries. Recently, our research group showed that PCR can be used to compare the frequencies of oral microbes in the saliva of oncological patients and healthy controls.¹⁵ However, relatively few data are available on the quantification of salivary caries-associated bacteria in oncological patients by qRT-PCR.

The aim of this study was to determine and compare the physiologic values of salivary flow, pH, and the levels of *S. mutans*, *S. sobrinus*, *L. salivarius*, and *L. acidophilus* in saliva samples from oncological patients and healthy controls by use of qRT-PCR.

MATERIALS AND METHODS

1. Subjects and saliva collection

The study population consisted of 40 systemically healthy control subjects and 71 cancer patients, which included 30 patients with a hematologic malignancy (HE) and 41 patients with a solid tumor (SO) who visited Chonnam National University Hwasun Hospital for an oral examination. The 111 study subjects comprised 55 men and 56 women ranging in age from 13 to 78 years (mean, 53.6±14.4 years); their characteristics are shown in Table 1. None of the patients had received antibiotics or irradiation therapy during the preceding 3 months. Etiological factors including smoking and alcohol were not analyzed. All subjects signed an informed consent form approved by the Ethics Committee of Chonnam National University Hwasun Hospital (HCRI 09 032-3).

The subjects were asked to refrain from eating, drinking, and dental hygiene control for a minimum of 1 h before

sialometry. Subjects were asked to hold their head slightly forward and to expectorate accumulated saliva into a collection tube (SPL, Pocheon, South Korea) and to take care not to swallow during the 5-min collection period. Saliva was expectorated into the tube at 1-min intervals. Amounts of saliva collected were measured in milliliters to gauge salivary flow, and salivary pH was measured with a pH meter (pH-200L; iStek, Seoul, South Korea). Saliva samples were stored immediately at -20°C before genomic DNA extraction.

2. Bacterial strains

S. mutans Ingbritt, *S. sobrinus* KCTC 3308, *L. salivarius* KCTC 3157, and *L. acidophilus* KCTC 3164 were used as reference strains. *S. mutans* and *S. sobrinus* were grown in brain heart infusion broth (BHI broth; Difco, Detroit, MI, USA), and *L. salivarius* and *L. acidophilus* were grown in De Man, Rogosa, Sharpe broth (MRS broth; Difco). Bacteria were incubated at 37°C for 16 h under aerobic conditions.

3. Quantitative real-time PCR amplification

Bacterial DNAs were extracted from bacterial pellets obtained by centrifuging (12,000×g at 4°C for 2 min) 1-ml aliquots of overnight culture and clinical saliva samples by using a G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology Inc., Sungnam, South Korea).

Real-time PCR was performed by using the Rotor-Gene Q system (QIAGEN, Strasse, Hilden, Germany). Each reaction tube contained 10 µl of a mixture containing 2 µl of DNA template, 5 µl of 2×Rotor Gene™ Probe PCR Kit (QIAGEN), 2 µl of each primer (10 pmol), and 1 µl of probe

TABLE 1. Characteristics of the study group

	N (%)	Gender, N (%)		Age (years)	
		Male	Female	Mean±SD	Range
Hematologic malignancy	30	16 (53.3)	14 (46.7)	49.3±15.7	20-75
Acute leukemia	9 (30)				
Multiple myeloma	7 (23.3)				
Myelodysplastic syndrome	7 (23.3)				
Lymphoma	3 (10)				
Aplastic anemia	2 (6.7)				
Others	2 (6.7)				
Solid tumor	41	19 (46.3)	22 (53.7)	57.2±5.7	13-78
Breast cancer	10 (24.4)				
Colorectal cancer	10 (24.4)				
Lung cancer	8 (19.5)				
Pancreatic cancer	5 (12.2)				
Prostate cancer	3 (7.3)				
Thyroid cancer	2 (4.9)				
Others	3 (7.3)				
Healthy controls	40	20 (50)	20 (50)	53.2±11.1	30-76
p-value		0.842*		0.053 [†]	
Total	111	55 (49.5)	56 (50.5)	53.6±14.4	

*p value measured by Chi-square test. [†]p-value measured by Kruskal-Wallis test. N: number of subjects, SD: standard deviation.

(10 pmol). The primers and probes used for qRT-PCR targeted the *gtfB*, *gtfT*, and 16S-23S intergenic spacer regions of *S. mutans*, *S. sobrinus*, and *Lactobacillus* species, respectively. Another universal primer targeting the 16S rRNA gene was used to quantify the total bacterial load in samples. Primer and probe sequences are listed in Table 2. Probes were labeled with 6-carboxyfluorescein (FAM) at their 5'-ends and with Black Hole Quencher (BHQ) at their 3'-ends. Primers and probes were synthesized by Applied Biosystems (Foster City, CA, USA).

The PCR cycling conditions used were as follows: 1 cycle of denaturation at 95°C for 3 min, followed by 50 amplification cycles of 95°C for 3 s and 58°C for 10 s. Fluorescence was automatically measured during PCR and all amplifications were performed in duplicate. The data were analyzed by using complementary software from QIAGEN. Bacterial cell numbers were determined by using DNA from known amounts of the four different bacterial strains. Proportions of target bacteria were expressed as percentages of total bacteria.

4. Statistics

Statistical analysis was performed by using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). The chi-square and Kruskal-Wallis tests were used to determine statistical significance, which was accepted for p values of <0.05. Fisher's least significant difference test was used for non-parametric post-hoc multiple comparisons.

RESULTS

1. Salivary flows and pH values

Salivary flow and pH values are presented in Table 3. Salivary flow was nonsignificantly lower in patients than in controls. Salivary pH, however, was significantly lower in patients ($p < 0.05$).

2. Detection range of the real-time PCR system for cariogenic bacteria

A standard curve was plotted by using 10-fold serial dilutions of positive controls for all four target bacteria. Standard curves were generated from the amplification plots in the insets (correlation coefficients were 0.9955 for

TABLE 2. Oligonucleotide primers and probes used in qRT-PCR

Primer and probe	Sequence (5'→3')	Amplicon size (bp)	Reference
<i>S. mutans</i>			
Forward	GCCTACAGCTCAGAGATGCTATTCT	114	26
Reverse	GCCATACACCACTCATGAATTGA		
Probe	TGAAAATGACGGTTCGCCGTTATGAA		
<i>S. sobrinus</i>			
Forward	TTCAAAGCCAAGACCAAGCTAGT	88	26
Reverse	CCAGCCTGAGATTCAGCTTGT		
Probe	CCTGCTCCAGCGACAAAGGCAGC		
<i>L. salivarius</i>			
Forward	GTCGTAACAAGGTAGCCGTAGGA	97	27
Reverse	TAAACAAAGTATTCGATAAATGTACAGGTT		
Probe	CGGCTGGATCACC		
<i>L. acidophilus</i>			
Forward	GAAAGAGCCCAAACCAAGTGATT	85	28
Reverse	CTTCCCAGATAATTCAACTATCGCTTA		
Probe	TACCACTTTGCAGTCCTACA		
Universal			
Forward	CGCTAGTAATCGTGGATCAGAATG	69	14
Reverse	TGTGACGGGCGGTGTGTA		
Probe	CACGGTGAATACGTTCCCGGGC		

qRT-PCR: quantitative real-time polymerase chain reaction.

TABLE 3. Salivary flows and pH values in the study group

	Hematologic malignancy	Solid tumor	Healthy controls	p value
Salivary flow (ml/min)	2.34±1.17	2.15±1.44	2.50±1.14	0.275
Salivary pH	6.51±0.40 ^a	6.35±0.53 ^a	6.75±0.37 ^b	0.001*

Values are expressed as the mean±standard deviation. Values with the same superscript letter are not significantly different by Fisher's least significant difference test at $\alpha=0.05$. * $p < 0.05$, by Kruskal-Wallis test.

TABLE 4. Quantitative analysis of cariogenic bacteria in the saliva samples in the study groups

	Hematologic malignancy	Solid tumor	Healthy controls	p value
No. of total bacteria/ml	1.09±2.39×10 ⁹	1.66±5.58×10 ⁹	1.90±2.31×10 ⁹	0.065
No. of <i>S. mutans</i> /ml	0.56±2.86×10 ^{5a}	1.31±4.67×10 ^{5b}	0.49±2.17×10 ^{5ab}	0.032*
<i>S. mutans</i> levels (%)	2.19±5.20×10 ^{-3a}	1.26±7.03×10 ^{-1b}	0.33±1.35×10 ^{-2a}	0.033*
No. of <i>S. sobrinus</i> /ml	0.27±1.46×10 ^{4a}	0.23±1.36×10 ^{5b}	0.17±1.05×10 ^{4a}	0.036*
<i>S. sobrinus</i> levels (%)	0.33±1.82×10 ^{-4a}	1.05±6.13×10 ^{-2b}	0.63±3.94×10 ^{-4a}	0.031*
No. of <i>L. salivarius</i> /ml	1.93±6.46×10 ^{3ab}	2.89±8.13×10 ^{3a}	1.16±4.56×10 ^{3b}	0.035*
<i>L. salivarius</i> levels (%)	0.43±1.34×10 ^{-3ab}	0.97±6.15×10 ^{-1a}	1.48±5.39×10 ^{-4b}	0.031*
No. of <i>L. acidophilus</i> /ml	1.14±4.02×10 ¹	0.33±1.59×10 ¹	0.42±2.09×10 ²	0.892
<i>L. acidophilus</i> levels (%)	2.32±7.84×10 ⁻⁶	0.24±1.01×10 ⁻⁵	0.46±1.57×10 ⁻⁵	0.680

Bacterial levels (%) were expressed as a percentage of the total bacteria. Values are expressed as the mean±standard deviation. Values with the same superscript letter are not significantly different by Fisher's least significant difference test at $\alpha=0.05$. * $p<0.05$, by Kruskal-Wallis test.

S. mutans, 0.9968 for *S. sobrinus*, 0.9912 for *L. salivarius*, and 0.9899 for *L. acidophilus*). The real-time PCR assay was found to have a detection range of 10¹ to 10⁸ bacterial cells. To generate a standard curve, C_T values of standard dilutions were plotted against number of bacteria. Bacterial species used in this study had C_T values in the range of 12 to 37, which corresponded to bacterial counts of 10⁸ to 10¹, respectively.

3. Quantitative analysis of cariogenic bacteria levels in saliva samples

Saliva samples were evaluated by real-time PCR for the four cariogenic bacteria (Table 4). *S. mutans* was detected in 40.0% of HE samples, in 78.0% of SO samples, and in 60% of control samples. Mean numbers of *S. mutans* in cancer patients were not significantly higher than in healthy controls. However, mean numbers of *S. mutans* in the SO group were significantly higher than in the HE group ($p<0.05$). *S. sobrinus* was detected less frequently than *S. mutans*; *S. sobrinus* was detected in 3.3%, 22.0%, and 7.5% of subjects in the HE, SO, and control groups, respectively. The mean *S. sobrinus* number in the SO group was significantly higher than in both the HE group and the controls ($p<0.05$). *L. salivarius* was detected in 70% of the HE samples, in 85.4% of the SO samples, and in 70.0% of the control samples. The mean number of *L. salivarius* was significantly higher in the SO group than in the control group ($p<0.05$). *L. acidophilus* was detected less frequently than *L. salivarius*; *L. acidophilus* was detected in 40.0%, 48.8%, and 37.5% of the HE, SO, and control samples, respectively. However, the mean number of *L. acidophilus* in saliva was nonsignificantly lower in patients than in controls.

The proportions of the four cariogenic bacteria versus total bacteria by real-time PCR were also determined (Table 4). Proportions of *S. mutans*, *S. sobrinus*, and *L. salivarius* were significantly higher in the SO group than in the control group. Proportions of *S. mutans* and *S. sobrinus* were also significantly higher in the SO group than in the HE group. However, no significant intergroup differences were found in proportions of *L. acidophilus* among total bacteria.

DISCUSSION

The determination of accurate salivary flow rates is important in managing the altered oral environment of patients who have undergone radiotherapy or chemotherapy. Because saliva has a protective effect, clinically significant changes in salivary functions can contribute to the development or prevention of dental caries. In addition, it has been established that radiotherapy and chemical therapy in cancer patients causes marked reductions in the saliva flow rate and may cause clinical xerostomia.¹ In the present study, no significant intergroup differences were found for salivary flow rates, but salivary pH was significantly lower in cancer patients. This finding is consistent with published data.^{15,16}

Many researchers have reported that *S. mutans* levels in the oral cavity are correlated with the presence of dental caries.^{17,18} Blay et al.¹⁹ reported that the *S. mutans* levels in the oral cavity are correlated with a history of caries and caries susceptibility. In another study, the prevalence of *S. sobrinus* was found to be closely associated with high caries activity.²⁰ In the present study, consistent with that previously reported,¹⁵ the frequency of *S. mutans* among all study subjects was higher than that of *S. sobrinus*. Furthermore, the prevalences of *S. mutans* and *S. sobrinus* and the levels and proportions of *S. mutans* and *S. sobrinus*, as determined by qRT-PCR, were higher in patients than in controls. This result suggests that high proportions and levels of cariogenic bacteria in the saliva of cancer patients are sufficient to predict the occurrence of dental caries. On the other hand, the frequencies of *S. sobrinus* in the saliva were lowest in patients and controls. That finding concurs with a report issued by Homer et al.,²¹ in which the presence of N-acetylglucosamine in the oral cavity was found to inhibit the growth of *S. sobrinus* and to reduce the frequency of its isolation.

Lactobacilli constitute approximately 1% of cultivable oral microflora, and they are well known to play opportunistic roles during the development of dental caries by producing lactic acid and extracellular polysaccharides.

Several research groups have reported that lactobacilli including *L. salivarius* and *L. acidophilus* are numerically dominant in caries-susceptible sites.^{8,22,23} The results of the present study show that *L. salivarius* is detected most frequently in patients with a solid tumor, although it is probably not a major cariogenic pathogen. In addition, the levels and proportions of *L. salivarius* in saliva were higher in patients than in controls. In contrast, higher levels of *L. acidophilus* were present in controls, although its levels were lower than any other bacteria tested, which suggests that *L. acidophilus* levels are not correlated with dental caries. This result is not consistent with a study conducted by Martin et al.,⁸ in which *L. acidophilus* was found to be numerically dominant in caries samples. This discrepancy suggests that bacterial compositions are dependent on the carious sites included.

In the present study, saliva samples were collected and not plaque samples, because the latter is known to be unreliable for predicting the prevalence of caries-associated bacteria because bacterial counts on different tooth surfaces show large variations.²⁴ Emilson²⁵ reported that saliva better reflects the colonization of cariogenic bacteria on all dentition because it is in continuous contact with all oral surfaces. Thus, the use of saliva samples in the present study is believed to provide an accurate overall view of microbiological loadings.

The detection and quantification of mutans streptococci and lactobacilli in the oral cavity are important because these bacteria are relevant in the context of diagnosis and treatment planning for dental caries. In this study, we chose qRT-PCR for bacterial quantification because it is the most accurate and reliable tool for determining bacterial levels in clinical samples. By use of this technique, the prevalences and the levels of four cariogenic bacteria in the saliva samples of oncological patients and healthy controls were determined. High levels of three cariogenic bacteria were found in cancer patients. Nonetheless, the prevalences of mutans streptococci and lactobacilli were similar in patients and healthy controls. These results agree with our earlier PCR study,¹⁵ except that *Lactobacillus* species were not detected in all subjects. This finding indicates that real-time PCR is more specific and accurate than conventional end-point PCR.

In the present study, we focused on comparing the levels of cariogenic bacteria between cancer patients and a control group. Although the levels of cariogenic bacteria in cancer patients were higher than in controls, the caries status of cancer patients was not determined in this study. We did not find a significant correlation between salivary flow and salivary pH. High levels of cariogens are found to be closely associated with high caries activity. Nevertheless, we can suggest that the quantification of cariogenic bacteria is a valid method for evaluating caries susceptibility.

In conclusion, this study demonstrated that the saliva of oncological patients has significantly lower pH levels and harbors larger amounts of *S. mutans*, *S. sobrinus*, and *L. salivarius* than does the saliva of healthy individuals.

Therefore, periodic follow-up is required for dental caries detection and plaque removal in patients with cancer. Further studies on salivary microbial levels in relation to caries development in cancer patients are needed.

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