Measurement of the Total Number of Bacteria in Saliva Using Quantitative Real-Time PCR During Treatment for Head and Neck Malignancy: A Series of Cases

Ikuko Goto, Shungo Furudoi, Mayasa Akashi, Takahide Komori

Department of Oral and Maxillofacial Surgery, Graduate School of Medicine, Kobe University, Kusunoki-cho, Chuo-ku, Kobe, Japan.

Abstract

Purpose: Management of oral hygiene can reduce the negative side effects on the oral cavity caused by Chemoradiotherapy (CRT) for head and neck malignancy. Reduction of oral bacteria contributes to the prevention of these side effects. In this study, we assessed the total number of oral bacteria during treatment for head and neck malignancy, and we discuss the relationship between the change in the number of oral bacteria and the exacerbation of oral mucositis.

Methods: In 12 patients undergoing CRT or surgery for head and neck malignancies, the total number of oral bacteria in a mouthwash sample was measured using quantitative real-time PCR.

Results*:* All surgery cases showed a decrease in total number of bacteria after surgery, while in CRT cases the change of the total number of bacteria during treatment was variable. Although there was not a significant correlation between the number of bacteria in the oral cavity before irradiation and the onset of Grade 2 mucositis (P=0.11), the onset of oral mucositis Grade2 tended to be relatively later in patients who had less oral bacteria before CRT.

Conclusion: Quantitative real-time PCR has potential for assessment of oral hygiene in patients with head and neck malignancy.

Key Words: Head and neck malignancy, Chemoradiotherapy, Quantitative real-time PCR, Oral bacteria, Oral mucositis

Introduction

Chemotherapy and radiotherapy in head and neck malignancy have important roles such as the preservation of function. Despite their usefulness, these treatments cause damage to normal tissue. Oral mucositis is a common side effect caused by Chemoradiotherapy (CRT) [1-4]. Oral mucositis reduces the Quality of Life (QOL) of patients and often prevents completion of the CRT [1,3]. Oral mucositis can also lead to severe bacterial infections in the oral cavity [2,4], therefore management of oral hygiene to reduce the negative side effects of CRT is recommended [1,5]. Reduction of the number of bacteria in the oral cavity is thought to contribute to prevention of the exacerbation of oral mucositis, but there have been few reports regarding the sequential changes in the total number of oral bacteria during head and neck cancer treatment.

The total number of bacteria in saliva measured by quantitative real-time PCR (qPCR) has been effective in the risk assessment of periodontal disease and dental caries [6,7]. Evaluation of the total number of oral bacteria may also contribute to the risk assessment of complications during head and neck cancer treatment. In this study, we used qPCR to assess changes in the total number of oral bacteria in patients who underwent treatment for head and neck malignancy.

Patients and Methods

Patients

We enrolled 12 patients receiving treatment for head and neck cancer at the Department of Otolaryngology-Head and Neck Surgery and Oral and Maxillofacial Surgery in the Kobe University Hospital from September 2012 through June 2014. Six patients received CRT to oral areas for treatment head and neck cancer, and six patients underwent oral cancer surgery (i.e., radical cancer resection with primary free flap reconstruction). Both groups of patients received oral hygiene management with the same duration of hospitalization. The management protocol of this study was approved by the ethics committee of the Kobe University Graduate School of Medicine (No.1310), and informed consent was obtained from each subject.

Before medical treatment, all patients received oral health examinations. After primary dental treatments such as tooth extraction for serious caries or periodontosis, or removal of incongruous prosthetics, scaling and professional mechanical teeth cleaning were performed, and patients were given instructions in oral hygiene aiming to keep the O'Leary plaque control record under 20% [8]. Professional oral cleaning was continued in accordance with the patient's condition once per week during hospitalization. In CRT cases, the severity of oral mucositis was evaluated based on the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 3.0. Epidemiological data were retrospectively gathered from the medical charts as follows: age, sex, primary tumor, TNM classification, method of nutrition, and (in CRT cases) dose of radiation and chemotherapy regimen.

Bacterial strain and growth media

Porphyromonas gingivalis ATCC 33277 was used for sample of generating standard calibration curve [9], and grown in Torypticase Soy Broth (BD, Franklin Lakes, NJ, USA) with 1μg/ml (final conc.) menadione (Sigma, St. Louis, MO, USA), 500μg/ml (final conc.) hemin (Sigma, St. Louis, MO, USA) and 0.1% (w/v) yeast extract (BD, Franklin Lakes, NJ, USA), at 37°C and under anaerobic conditions.

Sampling and DNA extraction from mouthwash sample

Sampling by mouthwash sample (five seconds' washing with 10 ml sterile saline) was carried out approximately 2 hours after lunch at three time points as described below. The amount of saliva produced over 5 minutes in response to chewing gum was also calculated.

Corresponding author: Ikuko Goto, Department of Oral and Maxillofacial Surgery, Kobe University Graduate School of Medicine, Kusunoki-cho 7-5-1, Chuo-ku, Kobe 650-0017, Japan; Tel: +81-78-382-6213; Fax: +81-78-382-6229; e-mail: i.goto@people.kobe-u.ac.jp

In CRT cases, samples were collected at three time points as follows: point 1, on the date that oral primary care had been completed before the start of CRT; point 2, at the end of Radiotherapy (RT); point 3, on the date that oral mucositis had healed. In surgery cases, samples were collected at three time points as follows: point 1, on the date that oral primary care had been completed before surgery; point 2, on the date that oral intake was started after surgery; point 3, before discharge from hospital.

The mouthwash sample was processed using the method described by Yokoyama et al. [9] with a slight modification. A 1000 µl sub-sample of each mouthwash sample was transferred to a micro tube and centrifuged at 10,000×g for 10 minutes at 4°C, and the precipitate was washed twice with PBS.

Genomic DNA from the mouthwash sample was isolated using an Easy DNA kit (InstaGene Matrix: Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. After adding 200 µl InstaGene Matrix to the precipitate and incubating at 56°C for 30 minutes, the sample was stirred and incubated at 100°C for 8 min. All samples were stored at –20°C after the above processing. Samples were thawed immediately prior to qPCR and centrifuged at 10,000×g for 10 minutes at 4°C. The supernatant was used as a sample for measuring number of bacteria and generating standard calibration curve with qPCR.

Preparation of the standard calibration curve

P. gingivalis ATCC 33277 was cultured to OD=0.6, and seeded adjusted bacterial suspension into CDC blood agar (BD, Franklin Lakes, NJ, USA), and confirmed the number of bacteria by counting the colonies after 1 week. DNA from *P. gingivalis* (OD=0.6, 10^8cells/200µl) was added in serial dilutions from 10 to 10^8/200µl cells to a series of PCRs. Two hundred µl of each was then transferred to a micro tube, centrifuged at 10,000×g for 10 minutes at 4°C, and the precipitant was washed twice with PBS. In the same way as the mouthwash sample, genomic DNA was isolated using the InstaGene Matrix.

Total bacterial counts determined by qPCR

Referring to the method described by Yokoyama et al. [9], and using a Fast SYBR®Green Master Mix (Applied Biosystems, Foster City, CA, USA), amplification and detection were performed using the StepOne™ Real-Time PCR System (Applied Biosystems). The primer sequences used for determining the total number of bacteria are shown in *Table 1* [10]. Each reaction mixture contained 10 µl of Fast SYBR®Green Master Mix, 0.4 µl of each forward and reverse primer, 2.0 µl of the relevant DNA sample (either of the mouthwash sample, or *P. gingivalis* cell suspension for standard calibration curve), and sufficient nuclease-free H_2O to bring the final volume to 25 µl. The PCR cycle used was as follows: an initial denaturation step of 20 seconds at 95°C, followed by 30 cycles of denaturation (95°C for 3 seconds), and annealing and extending (60°C for 30 seconds). The total number of bacteria in the mouthwash sample was based on a copy number determined based on the standard calibration curve and the amplification curve.

Statistical analysis

Mann–Whitney U tests were used to evaluate the change between the measured values of saliva at each sampling point. The Pearson product-moment correlation coefficient was used to evaluate the relationship between total bacterial count before CRT, the amount of saliva before CRT and irradiation amount when patients were diagnosed with Grade 2 mucositis. Statistical significance was accepted for p values <0.05.

Results

Subject characteristics are summarized in *Table 2*.

In CRT cases, the mean age was 61.8 years (range, 42–73 years). All patients were male. The primary sites of tumors were the epipharynx in one patient, the oropharynx

Table 1. *Oligonucleotide primers and probes used for quantitative real-time PCR analysis.*

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Universal	TCCTACGGGAGGCAGCAGT	GGACTACCAGGGTATCTAATCCTGTT

Table 2. *Patient characteristics.*

CRT cases						
Case No.	**Gender**	**Age (years)**	**Primary tumor**	**TNM classification**	**Dose of RT (Gy)**	**CR regimen**
CRT case 1	Male	57	Oropharynx	cT2N0M0	70	cisplatin 80 mg/m^2 2 cycles
CRT case 2	Male	72	Oropharynx	cT2N2bM0	70	cisplatin 80 mg/m^2 1 cycle cisplatin 64 mg/m^2 2 cycles
CRT case 3	Male	42	Epipharynx	cT3N2bM0	70	cisplatin 80 mg/m^2 1 cycle cisplatin 64 mg/m^2 2 cycles
CRT case 4	Male	73	Hypopharynx	cT1N2bM0	70	cisplatin 80 mg/m^2 3 cycles
CRT case 5	Male	58	Hypopharynx	cT3N2bM0	70	cisplatin 100 mg/m^2 3 cycles
CRT case 6	Male	69	Oropharynx	cT2N2bM0	70	cisplatin 80 mg/m^2 2 cycles cisplatin 60 mg/m^2 1 cycle
Surgery cases						
Case No.	**Gender**	**Age (years)**	**Primary tumor**			**TNM classification**
Surgery 1	Male	63	Mandibular gingiva			cT2N2bM0
Surgery 2	Male	79	Tongue			cT2N0M0
Surgery 3	Female	62	Buccal mucosa			cT2N1M0
Surgery 4	Female	71	Mandibular gingiva			cT2N0M0
Surgery 5	Female	75	Oral floor			cT2N0M0
Surgery 6	Male	57	Mandibular gingiva			cT4aN2bM0

Percutaneous endoscopic gastrostomy (PEG) was used for nutritional support in CRT case 1, CRT case 2 and CRT case 5.

in three, and the hypopharynx in two. Clinical T-stages were as follows: T1 (n=1), T2 (n=3), and T3 (n=2); N-stages: N0 (n=1), and N2b (n=5); M-stage: M0 (n=6). The dose of radiation was 70 Gy in all patients. Percutaneous Endoscopic Gastrostomy (PEG) was used for nutritional support in three patients (50.0%), while three patients (50.0%) completed radiotherapy with oral intake alone. The worst NCI-CTCAE v3.0 grade was grade 2 in all patients.

In surgery cases, the mean age was 67.8 years (range, 57–79 years). Three patients were male and three female. The primary sites of tumors were the tongue in one patient, mandibular gingiva in three, buccal mucosa in one, and oral floor in one. Clinical T-stages were as follows: T2 (n=5), and T4a (n=1); N-stages: N0 (n=3), N1 (n=1), and N2b (n=2); M-stage: M0 (n=6). All patients could have oral intake, which was resumed 13.5 days (range, 7–18 days) after surgery.

Change in total bacterial count as determined by qPCR

In CRT cases, the number of bacteria in the mouthwash samples before CRT (point 1) was highly variable, ranging from 1.56×10^6 to 1.08×10^8 cells. When assessing changes in the number of bacteria at the end of CRT (point 2), three cases (case 3, case 4, and case 6) had increased levels, and three cases (case 1, case 2, and case 5) had reduced levels. There was thus not a clear tendency in the changes of bacterial numbers during treatment in CRT cases (*Figure 1A*).

In surgery cases, the number of bacteria in the mouthwash samples before surgery (point 1) ranged from 7.55×10^6 to 5.73×10^7 cells, so the variation was small compared to the CRT cases. The number of bacteria after surgery (point 2) was reduced in all cases (*Figure 1B*). There was a tendency for the number of bacteria to decrease initially after surgery and increase again by the day before discharge.

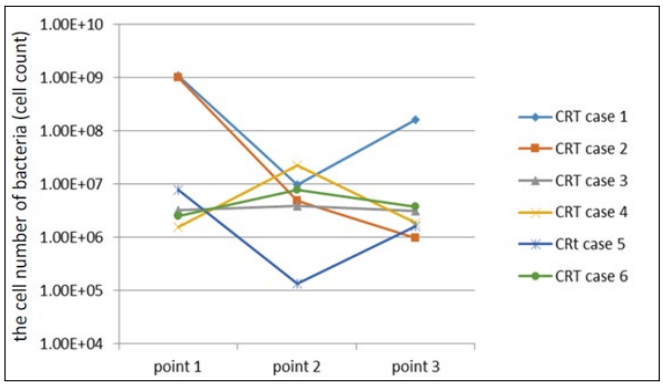

Figure 1A. *Change in total bacterial count as determined by Qpcr shows CRT case.*

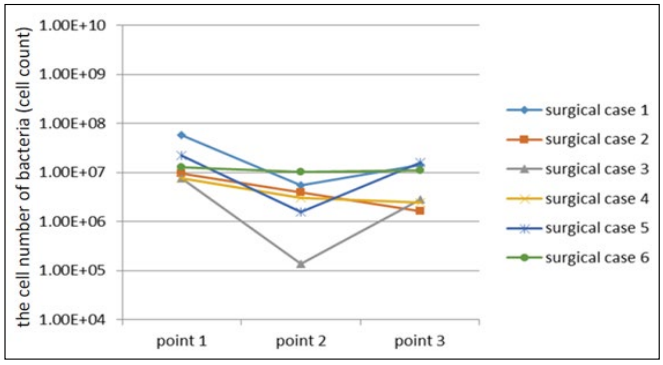

Figure 1B. *Change in total bacterial count as determined by Qpcr shows surgical case.*

Changes in the measured values of saliva secretion

In CRT cases, the mean values of the secreted saliva were as follows: before RT (point 1), 7.50 ± 2.08 ml; at the end of RT (at point 2), 2.00 ± 0.66 ml; and on the day after oral mucositis had healed (at point 3), 0.77 ± 0.38 ml (*Figure 2A*). A significant decrease in the measured values of secretion of saliva was found when comparing the values before CRT and at the end of CRT (P=0.02), and when comparing values before CRT and on the day after oral mucositis had healed (P=0.004). In surgery cases, the mean values of secreted saliva were as follows: before surgery (point 1), 6.75 ± 1.16 ml; after surgery and at the resumption of oral intake, 5.90 ± 1.42 ml; and on the day before discharge, 6.60 ± 1.14 ml (*Figure 2B*). There were no significant differences between these points.

Relationship between total bacterial count, the amount of saliva and oral mucositis in CRT cases

In CRT cases, all patients were diagnosed as Grade 2 in the NCI-CTCAE v3.0 at each irradiation dose. As shown in *Figure 3A*, there was not a significant correlation between total bacterial count before CRT and irradiation amount when patients were diagnosed with Grade 2 mucositis (P=0.11), but the onset of oral mucositis tended to be relatively later in patients who had less oral bacteria before CRT. There was no significant correlation between the amount of saliva before CRT and irradiation amount when patients were diagnosed with Grade 2 mucositis (P=0.61) (*Figure 3B*).

Discussion

Measurement of the number of bacteria in saliva using qPCR is useful for mass screening, because sample collection, storage and transport is easy compared with culture-based methods, and multiple specimens can be processed at once [9]. Yoshida

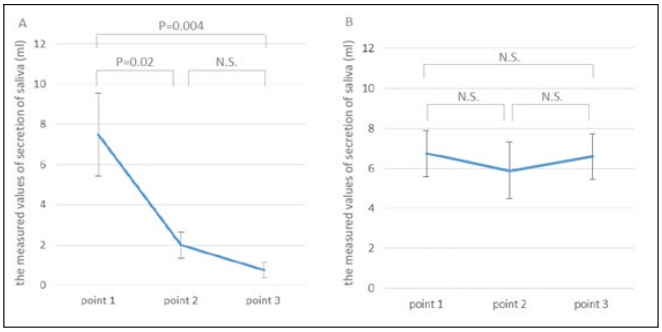

Figure 2. *Changes in the measured values of saliva secretion.* ***A*** *shows CRT case.* ***B*** *shows Surgical case.*

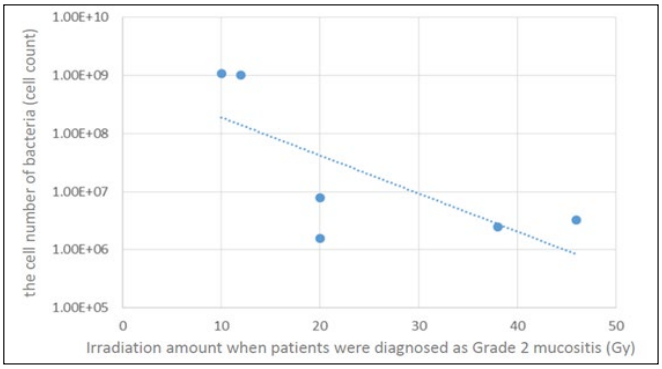

Figure 3A. *In CRT cases: shows relationship between total bacterial count before CRT and irradiation amount when patients were diagnosed as Grade 2 mucositis.*

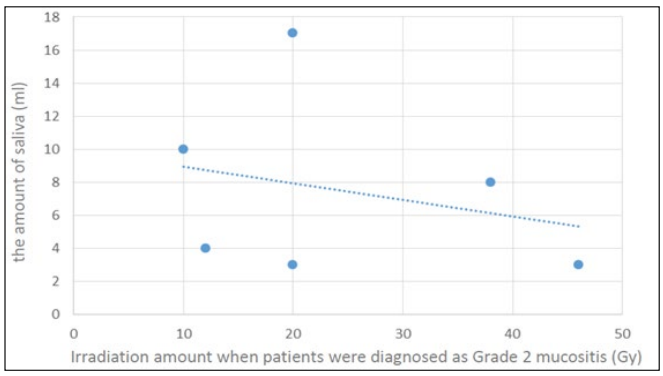

Figure 3B: *In CRT cases: shows between the amount of saliva before CRT and irradiation amount when patients were diagnosed as Grade 2 mucositis.*

et al. reported that cariogenic dental pathogens in saliva can be detected using qPCR [6], and Kurata et al. reported that periodontopathogenic bacteria in saliva investigated with qPCR might be associated with bad breath and periodontal factors [11]. These previous reports indicate that measurement of the number of bacteria in saliva is effective in screening for periodontal disease and dental caries.

Oral hygiene management has been recommended for the prevention of complications in head and neck cancer treatment [1,5], but there have been few studies of the relationship between the number of oral bacteria and the severity of complications, such as CRT-induced oral mucositis. We therefore aimed to evaluate sequential changes in the number of bacteria in the oral cavity during the treatment of head and neck cancer.

Our study revealed some technical problems in sample collection for the evaluation of oral bacterial number in CRT cases. As shown in *Figure 2*, the amount of saliva significantly decreased after irradiation, and it became difficult to collect saliva even in response to stimulation. A mouthwash was therefore used for sampling in this study. The numbers of bacteria detected were comparable to those previously reported [9] in which the number of bacteria in a stimulated saliva sample was measured. Sampling of saliva with gargling is simple and painless for patients. Our sampling method may be useful, but further studies using this method should be performed to confirm its suitability.

Various universal primer sequences have been used for measuring the total number of bacteria by qPCR, as previously reported [6,7,9-11,12-15]. The DNA sequences applied in this study can detect more than 80% of the species within the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacterioidetes* [14]. Lyons et al. have reported that there were no differences in the number of copies of the 16S rRNA gene between *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Escherichia coli*, and the group G *streptococcus*. *P. gingivalis* as a sample of the standard calibration line [15].

Postoperative reduction of the number of bacteria in all surgery cases was possibly caused by resection of the tumor, because the bacteria adhering to the surface of tumor (which forms a three-dimensional complex structure like the granular and ulcerative mucosa) had been eliminated by surgery. Prophylactic antibiotics were used perioperatively in all surgery cases, and samples were collected 1 week after the course of antibiotics had finished. While the effect of antibiotics on the number of oral bacteria in this study is unknown, we consider it likely that the number of bacteria in the oral cavity after surgery was primarily affected by complete removal of the tumor by surgery, rather than the effects of antibiotics. Also, there was little variation in the number of bacteria before surgery, possibly because all patients were admitted following referrals from their family dentist. This may have affected their oral management, with all patients having good oral care on a daily basis, such as through regular dental visits.

In CRT cases, variation in the number of bacteria before CRT was greater, and there was no consistent trend in changes in the number of bacteria after CRT. In case 1 and case 2, the number of bacteria before CRT was extremely high, which may have been affected by the following factors. In case 1, periodontal pockets of all of the teeth were greater than 5 mm so this patient was suffering from periodontal disease, but periodontal treatment had not been carried out before admission. In case 2, a number of required tooth extractions for severe dental caries were identified, and oral hygiene was poor. At the end of CRT, three cases (case 3, case 4, and case 6) had increased numbers of bacteria, and in case 4 and case 6, mucositis was evident at a number of locations, including the side of the tongue, soft palate, and pharynx. In three cases the number of bacteria was reduced at the end of CRT; in case 1 and case 2, mucositis was evident in only one location, while in case 5 mucositis had healed by this time. Although the number of bacteria tended to increase in cases where mucositis was present at several locations at the time of admission, this may be due to bacteria growing on the mucosal surface formed by ulcerative mucositis and self-care cannot be adequately performed because of the mucositis.

In CRT cases, although there was not a significant correlation between the number of bacteria in the oral cavity before irradiation and the onset of Grade 2 mucositis, the mucositis tended to deteriorate later in patients who had less oral bacteria before irradiation. Pathogenesis of primary oral mucositis in CRT is due to active oxygen generated by anticancer agents, which causes oxidative stress in the oral mucosa, and radiation is a physical stimulating factor of this process [5]. Suppression of mucositis onset is therefore difficult, even though the patient may be undergoing professional oral cleaning. However, if the number of bacteria remains relatively low, this may prevent the exacerbation of secondary oral mucositis development as a result of local oral bacterial infection [5]. Onset of Grade 2 mucositis tended to occur relatively later in patients who had less oral bacteria before CRT, which is not inconsistent with the above suggestion. Oral bacteria relates to the exacerbation of mucositis, as do a variety of other factors such as irradiation range, mucosal defense capacity of the host, and sensitivity to chemotherapy.

For an ideal risk assessment of the side effects caused by CRT in head and neck patients, evaluation of the number of bacteria in the oral cavity is an important component of the assessment of oral mucositis, but a comprehensive evaluation of various other factors should be completed at the same time. In this study, the irradiation range was not constant because the primary tumor was different.

Further studies in which a greater number of samples were available from cases with the same primary tumor would enable more in-depth investigation of the relationship between the number of bacteria and mucositis.

Conclusion

By measuring the number of bacteria contained in a mouthwash sample, we investigated changes in the number of bacteria in the oral cavity in response to treatment of head and neck cancer.

Disclaimer

The small sample size used in the project, limits the statistical power. Furthermore the results cannot be generalized to the general population.

Acknowledgements

The authors thank Jumpei Murakami, Osaka Universal Dental Hospital, for support in preparating the standard calibration curve.

References

1. Campos MI, Campos CN, Aarestrup FM, Aarestrup BJ. Oral mucositis in cancer treatment: Natural history, prevention and treatment. *Molecular Oncology - Journal*. 2014; **2**: 337-340.

2. Stokman MA, Spijkervet FK, Burlage FR, Dijkstra PU, Manson WL, de Vries EG, Roodenburg JL. Oral mucositis and selective elimination of oral flora in head and neck cancer patients receiving radiotherapy: A double-blind randomised clinical trial. *British Journal of Cancer*. 2003; **88**: 1012-1016.

3. Trotti A, Bellm LA, Epstein JB, Frame D, Fuchs HJ, Gwede CK, Komaroff E, Nalysnyk L, Zilberberg MD. Mucositis incidence, severity and associated outcomes in patients with head and neck cancer receiving radiotherapy with or without chemotherapy: A systematic literature review. *Radiotherapy & Oncology*. 2003; **66**: 253-262.

4. Köstler WJ, Hejna M, Wenzel C, Zielinski CC. Oral mucositis complicating chemotherapy and/or radiotherapy: Options for prevention and treatment. *CA: A Cancer Journal for Clinicians*. 2001; **51**: 290-315.

5. Keefe DM, Schubert MM, Elting LS. Updated clinical practice guidelines for the prevention and treatment of mucositis. Cancer. 2007; **109**: 820–831.

6. Yoshida A, Suzuki N, Nakano Y, Kawada M, Oho T, Koga T. Development of a 5' nuclease-based real-time PCR assay for quantitative detection of cariogenic dental pathogens Streptococcus mutans and Streptococcus sobrinus. *Journal of Clinical Microbiology*. 2003; **41**: 4438-4441.

7. Yoshida A, Suzuki N, Nakano Y, Oho T, Kawada M, Koga T. Development of a 5' fluorogenic nuclease-based real-time PCR assay for quantitative detection of Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis. *Journal of Clinical Microbiology*. 2003; **41**: 863-866.

8. O'Leary TJ, Drake RB, Naylor JE. The plaque control record. *Journal of Periodontology*. 1972; **43**: 38.

9. Yokoyama M, Fukui M, Masuda K, Takamatsu N, Okada J, Takebe H, Kataoka K, Ito H. Measurment of the total number of bacteria in saliva using quantitative real-time PCR (qPCR): evalution of the oral hygiene status. *Journal of Dental Health*. 2009; **59**: 183-189.

10. Nadkarini MA, Martin FE, Jacques NA. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology*. 2002; **148**: 257-266.

11. Kurata H, Awano S, Yoshida A, Ansai T, Takehara T. The prevalence of periodontopathogenic bacteria in saliva is linked to periodontal health status and oral malodour. *Journal of Medical Microbiology*. 2008; **57**: 636-642.

12. Okada T, Hashizume T, Atsuta W. Effect of one-time PMTC treatment on Mutans Streptococcal levels in brushing-plaque and stimulated-saliva samples. *Japanese Journal of Conservative Dentistry*. 2005; **48**: 34-39.

13. Price RR, Viscount HB, Stanley MC, Leung KP. Targeted profiling of oral bacteria in human saliva and in vitro biofilms with quantitative real-time PCR. *Biofouling*. 2007; **23**: 203-213.

14. Horz HP, Vianna ME, Gomes BP, Conrads G. Evaluation of universal probes and primer sets for assessing total bacterial load in clinical samples: general implications and practical use in endodontic antimicrobial therapy. *Journal of Clinical Microbiology*. 2005; **43**: 5332-5337.

15. Lyons SR, Griffen AL, Leys EJ. Quantitative real-time PCR for Porphyromonas gingivalis and total bacteria. *Journal of Clinical Microbiology*. 2000; **38**: 2362-2365.