Original Article

Quantitative Polymerase Chain Reaction Analysis of Cariogenic *Streptococcus mutans* in Saliva of Oral and Laryngeal Cancer Patients Undergoing Radiotherapy: A Clinical Study

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Abstract

Context: Radiotherapy leads to radiation-induced caries. There is limited knowledge about the quantification of cariogenic bacteria in the saliva of irradiated cancer patients. **Objective:** The aim of this study is to check salivary pH, flow rate, and the assessment of *Streptococcus mutans* in the saliva of irradiated oral and laryngeal cancer patients using quantitative real-time polymerase chain reaction (qRT-PCR). **Settings and Design:** This was time-bound study which consisted of 26 cancer patients undergoing radiotherapy (13-oral cancer 13-laryngeal cancer). **Subjects and Methods:** Resting saliva samples were gathered from oral (Group-I) and laryngeal (Group-II) cancer patients immediately before radiotherapy and after completion of radiotherapy (dose-60 Gy). pH of saliva and the salivary flow rate was measured. *S. mutans* were analyzed using qRT PCR. **Statistical Analysis Used:** Data were analyzed using SPSS software 20. Paired *t*-test was used to evaluate salivary pH, flow rate, and amount of *S. mutans* pre- and post-radiotherapy for Group I and II. Independent *t*-test was used to compare salivary pH, flow rate, and *S. mutans* pre- and post-radiotherapy between Group I and II. **Results:** Salivary pH and flow rate significantly reduced postradiotherapy in oral and laryngeal cancer patients ($P < 0.001$). The amount of *S. mutans* statistically increased postradiotherapy in oral cancer patients ($P = 0.001$). While *S. mutans* count was statistically insignificant in laryngeal cancer patients ($P = 0.091$). There was a significant increase in the amount of *S. mutans* in Group I when compared with Group II ($P = 0.002$). **Conclusion:** Amount of *S. mutans* increased postradiotherapy in oral cancer patients. While the salivary pH and salivary flow rate reduced postradiotherapy.

Keywords: *Radiation therapy, salivary flow rate, salivary pH, Streptococcus mutans*

Introduction

Cancer of the head-and-neck region includes tumors of pharynx, larynx, paranasal sinuses, and the oral cavity.[1] Oral cancer is considered the 11th most common cancer over the world.[2] There has been a significant advancement in treatment modalities of cancer from surgical procedures to radiotherapy and chemotherapy. Radiotherapy is a widely used treatment modality nowadays. Radiotherapy is considered to be a conservative treatment that attempts to preserve vital tissues situated nearby.[3] However, drawback attributed to radiotherapy for oral and laryngeal cases is, it leads to ill effects on salivary gland structures, saliva and teeth-producing inflammatory changes of the oral mucosa, hyposalivation and dental caries due to irradiation.[4] The buffering mechanism of saliva is also disrupted leading to changes in the growth of oral microflora in the oral environment.

Lactobacillus spp. and *Streptococcus* spp. are the main causative organisms leading to dental caries. *Streptococcus mutans* are the main cariogenic microbes among *Streptococcus* spp.[5]

The field of oral microbiology has revolutionized from traditional cultural techniques to advanced molecular biology. In molecular genetics, polymerase chain reaction (PCR) is newly introduced.[6] PCR is a molecular diagnostic tool that helps in accurate determination of bacteria, viruses, and fungi by amplification of small DNA fragments.[7] DNA is mainly available from blood. However now, saliva is a noninvasive medium available for DNA. Saliva is a source for a variety of microorganisms and toxins that they produce; thus, it helps in

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detecting diseases such as dental caries and periodontal disease.[8]

Thus, the purpose of the study was to evaluate *S. mutans* in the saliva of irradiated oral and laryngeal cancer patients using quantitative real-time PCR (qRT-PCR).

Subjects and Methods

The study population consisted of 26 oral and laryngeal cancer patients (thirteen oral cancer and thirteen laryngeal cancer). The present study was a time-bound study. Depending on the patient availability and to satisfy inclusion and exclusion criteria, 26 sample size was considered. Approval was obtained from the institutional ethical Board (BUETHICS/MPDC_082/ENDO-16/16). Patients were treated according to the Helsinki declaration. Patients were explained about the study and prior informed consent was obtained. Saliva samples of all the 26 patients were collected immediately before radiotherapy and after 60 Gy of radiotherapy. Patients were treated with two-dimensional conventional radiation (cobalt radiotherapy machine-Theratronics Int. Limited, Canada). Patients on antibiotic therapy during or 3 months before the treatment, patients who have received irradiation previously, patient having diagnosed Sjogren's syndrome and patients with acute radiation syndrome were excluded from the study.

These 26 patients belonged to different groups as – Group I: 13 patients of oral cancer, Group IA: Saliva samples taken preradiotherapy, Group IB: Saliva samples were taken postradiotherapy, Group II: 13 patients of laryngeal cancer, Group IIA: Saliva samples were taken preradiotherapy, Group IIB: Saliva samples were taken postradiotherapy.

Whole resting saliva samples of oral and laryngeal cancer patients were collected before the commencement of radiotherapy and after 6 weeks of radiotherapy for all patients (dose 60 Gy). Patients were instructed to avoid taking food or beverages or carrying out oral hygiene activity 1 h before saliva collection. Patients were explained not to swallow during the 5-min collection period and then to spit accumulated saliva into a graduated cylindrical container (J Sil Scientific Industries, Uttar Pradesh, India) through funnel. The collected saliva in the graduated container was measured to check the salivary flow. pH of the saliva was measured using the pH strips (Merck company, Mumbai, India). This collected saliva was then transferred to Eppendorf tube containing ethylenediaminetetraacetic acid (EDTA) buffer (TE buffer) (Thermofisher Scientific Inc., Massachusetts, United States) and was sent for real-time PCR analysis (thermal cycler PCR machine-Master cycler, Eppendorf, Germany) [Figure 1].

For PCR analysis, Eppendorf tubes containing saliva and TE buffer were centrifuged at 5000 rpm for 5 min and the process was recapitulated for four times. The

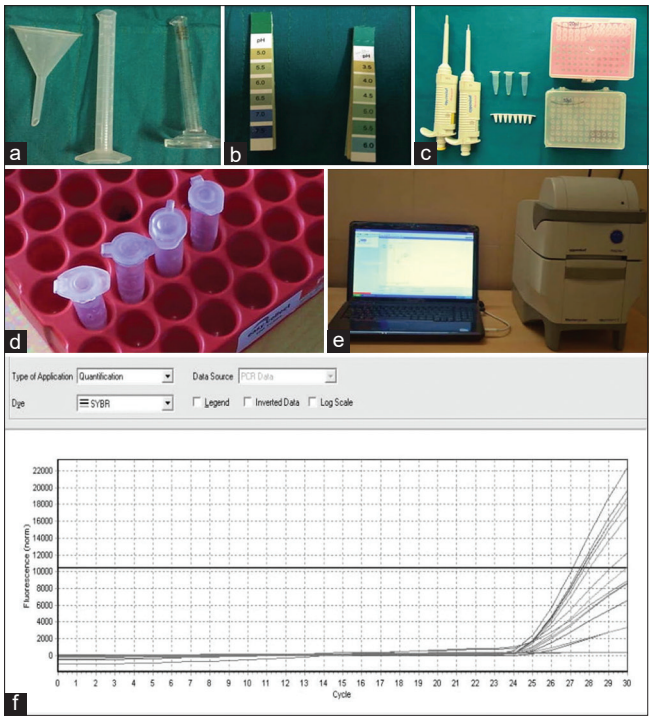


Figure 1: The equipment required for the study. (a) Cylindrical graduated tubes and funnel. (b) pH strips. (c) Micro tubes, micropipettes and Eppendorf tubes. (d) Eppendorf tubes containing saliva and tris ethylenediaminetetraacetic acid buffer. (e) Realplex software integrated with polymerase chain reaction machine. (f) SYBR green fluorescence chart produced in real-time polymerase chain reaction for amount of *Streptococcus mutans*

sediment formed was separated and lysis buffer I and II (Thermofisher Scientific Inc., Massachusetts, United States) were used for protein degradation and DNA extraction.

A mixture containing extracted DNA, SYBR green master mix, primers of *S. mutans* (Bioserve India Pvt Ltd., Hyderabad, India) and water were mixed and rotated at a slow speed. Qiagen quantitect SYBR green master mix (Qiagen, Hilden, Germany) was utilized for the study which comprises 2.5 mM $MgCl_2$, Taq polymerase enzyme, dNTP mix, and SYBR Green dye. The tube containing the mixture was placed inside the real-time PCR machine.

The sequence of primer used specific to *S. mutans* were: Forward primer: GTFB-Forward 5'-ACTACACTTTCGGGTGGCTTGG-3'; Reverse primer: GTFB-Reverse 5'-CAGTATAAGCGCCAGTTTCATC-3'.

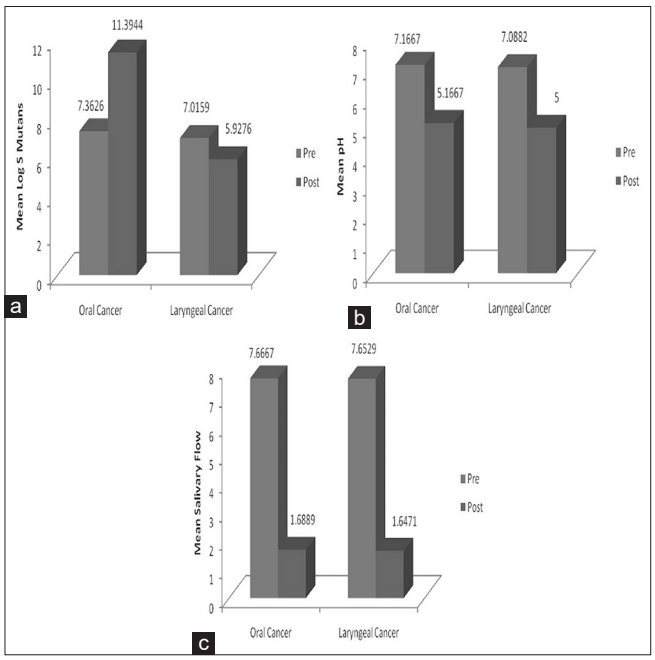
PCR procedure is a repetitive sequence consisting of three elemental stages: Denaturation: In this stage, at 95°C double-stranded helix of DNA was converted into single-stranded template DNA. The time duration to complete the procedure was 30 min; Annealing: In this phase, specific primers to *S. mutans* were annealed to the template DNA. The temperature was 56°C for 1 min; Extension: In this last stage, primer against the template was extended and multiple replica were produced. The

replica obtained again undergoes denaturation and serves as single template DNA and further, the cycle was repeated. The temperature was 72°C for 1 min. The cycle was repeated 35 times.

In SYBR Green dye procedure, dye united with double-stranded DNAs of extended copies of *S. mutans*. Fluorescence was emitted by the SYBR Green dye which was recorded in the form of graph. A graph was prepared on the basis of the quantity of fluorescence emitted by the extended copies in repetitive cycles. The graph was plotted as the amount of fluorescence against the number of the cycle [Figure 1f].

Results

Statistical analysis was performed using SPSS software 20 (SPSS Inc., Chicago, IL, USA). A total of 26 samples showed positive readings in PCR for *S. mutans*. Graph 1 shows the average value of quantification of *S. mutans*, salivary pH, and salivary flow rate in Group I and Group II. Table 1 shows the mean, standard deviation, standard error, and *P* value of Group I (oral cancer). Table 2 shows the mean, standard deviation, standard error, and *P* value of Group II (laryngeal cancer). Table 3 shows the mean, standard deviation, standard error, and *P* value comparing the amount of *S. mutans*, salivary flow rate, and salivary pH before and after radiotherapy between two groups.



Graph 1: (a) Average value of quantification of Streptococcus mutans before and after radiotherapy in Group I and II. (b) Mean value of salivary pH before and after radiotherapy in Group I and II. (c) Mean value of salivary flow rate before and after radiotherapy in Group I and II

Paired *t*-test was used to evaluate salivary pH, salivary flow rate, and amount of *S. mutans* pre- and post-radiotherapy for Group I and II individually.

In Group I, the mean value of *S. mutans* count was 7.3626 preradiotherapy, whereas 11.3944 postradiotherapy. The average pH was 7.1667 preradiotherapy and 5.1667 postradiotherapy, while the mean salivary flow rate was 7.6667 preradiotherapy and 1.6889 postradiotherapy. *S. mutans* increased significantly postradiotherapy compared to preradiotherapy (*P* value-0.001). Salivary pH and salivary flow rate reduced significantly postradiotherapy as compared to preradiotherapy ($P < 0.001$).

In Group II, the mean value of the amount of *S. mutans* was 7.0159 preradiotherapy, while 5.9276 postradiotherapy. The mean pH of saliva was 7.0882 preradiotherapy and 5.000 postradiotherapy, while the mean salivary flow rate was 7.6529 preradiotherapy and 1.6471 postradiotherapy. There was no significant difference in the amount of *S. mutans* postradiotherapy as compared to preradiotherapy ($P = 0.091$). Salivary pH and salivary flow rate reduced significantly postradiotherapy ($P < 0.001$).

Independent *t*-test was used to compare salivary pH, salivary flow rate, and *S. mutans* pre- and post-radiotherapy between Group I and II. *S. mutans* showed significant rise postradiotherapy in Group I as compared to Group II ($P = 0.002$). However, the difference in salivary pH and salivary flow rate between Group I and II were not statistically significant ($P > 0.05$).

Discussion

The mouth is a portal that leads to various diseases in the body from external sources. The oral microflora mainly resides in the saliva. The oral microbes live in harmony with the host. However, many of the microbes have capability to spread disease and are known as opportunistic pathogens. These organisms increase in the oral cavity when the oral equilibrium is disturbed.[9]

Saliva is an exocrine transparent seromucous fluid secreted by major and minor salivary glands.[10] It is mainly

Table 1: Comparison of *streptococcus mutans*, salivary pH and salivary flow rate pre- and post-radiotherapy in patients of oral cancer (Group-I)

	Mean	SD	SE mean	95% CI of the difference		*t*	Df	*P*
				Lower	Upper			
log_Smutans_Pre - log_Smutans_Post	−4.29438	2.35936	0.83416	−6.26685	−2.32191	−5.148	7	0.001
pH_Pre - pH_Post	2.00000	0.51450	0.12127	1.74415	2.25585	16.492	17	<0.001
SalivaryFlow_Pre - SalivaryFlow_Post	5.97778	0.39935	0.09413	5.77919	6.17637	63.508	17	<0.001

SD: Standard deviation; SE: Standard error; CI: Confidence interval

Table 2: Comparison of *streptococcus mutans*, salivary pH and salivary flow rate pre- and post-radiotherapy in patients of laryngeal cancer (Group-II)

	Mean	SD	SE mean	95% CI of the difference		*t*	Df	*P*
				Lower	Upper			
log_Smutans_Pre - log_Smutans_Post	−3.09228	3.12684	1.39837	−6.97477	0.79021	−2.211	4	0.091
pH_Pre - pH_Post	2.08824	0.56556	0.13717	1.79745	2.37902	15.224	16	<0.001
SalivaryFlow_Pre - SalivaryFlow_Post	6.00588	0.26094	0.06329	5.87172	6.14004	94.900	16	<0.001

SD: Standard deviation; SE: Standard error; CI: Confidence interval

Table 3: Comparison of *streptococcus mutans*, salivary pH and salivary flow rate pre- and post-radiotherapy between Group I and Group II

	t	Df	*P*	Mean difference	SE difference	95% CI of the difference	
						Lower	Upper
log_Smutans_Pre	0.185	20	0.855	0.34663	1.87307	−3.56053	4.25379
log_Smutans_Post	3.522	21	0.002	5.46679	1.55240	2.23839	8.69519
pH_Pre	0.755	33	0.456	0.07843	0.10395	−0.13306	0.28992
pH_Post	1.001	33	0.324	0.16667	0.16652	−0.17212	0.50545
SalivaryFlow_Pre	0.152	33	0.880	0.01373	0.09049	−0.17037	0.19782
SalivaryFlow_Post	0.432	33	0.668	0.04183	0.09674	−0.15499	0.23865
pH_Diff	−0.483	33	0.632	−0.08824	0.18258	−0.45970	0.28322
SalivaryFlow_Diff	−0.245	33	0.808	−0.02810	0.11477	−0.26161	0.20540

SE: Standard error; CI: Confidence interval

composed of proteins, mucins, enzymes, immunoglobulins, various electrolytes, etc.

Radiation to the oral and laryngeal regions in addition to cancer tissue also targets normal tissue like salivary gland. This results in altered composition of saliva. As a result, an increase in viscosity, decrease in immune response, altered buffering capacity, and reduction in pH are the changes that are noted. Thus, it provides an acidic medium for the growth of the cariogenic organisms.[11] *S. mutans* is a cariogenic organism that has a unique virulence property. It can survive under acidic environment and is detected as a dominant pathogen in culture.[12] It is also considered to be one main organism for the decay of teeth. Thus, in the present study, quantification of *S. mutans* was evaluated in irradiated patients.

Usually, the total radiation dose of 60–70 Gy is advised for 6–7 weeks for the treatment of oral and laryngeal cancers.[13] This dose of radiation produces xerostomia which is irreversible in nature.[14] Hence, in the present study dosage of 60 Gy was considered for taking postradiotherapy samples.

In the present study, saliva was used as a medium for sample collection and not plaque as the plaque sample shows the high discrepancy.[15] Saliva constantly stays in association with all the tooth surfaces, thereby providing a better source for the detection of pathogens.[16]

Whole resting saliva was collected since it prevents the modification of proteomic components in saliva. Bacteria constitute the salivary proteomics.[17] To prevent any dilution or contamination of saliva that may hamper the molecular testing, the patients were explained to avoid taking any food or drinks or carry out oral cleanliness activity before 1 h.

There are various molecular diagnostic testing procedures available like terminal restriction fragment length polymorphism, denaturing gradient gel electrophoresis, fluorescence *in situ* hybridization, DNA microarrays, DNA macro arrays, PCR, etc.[18] Mostly, conventional PCR techniques can only measure the presence of the pathogen and therefore are qualitative in nature. However, real-time PCR has a peculiar characteristic of measuring constant quantification of amplified products.[19] As the present study was based on the quantitative measurement of *S. mutans* pre- and post-radiotherapy, qRT-PCR was chosen for the detection of the bacterium. One advantage of choosing PCR was that all the samples could be investigated at the same time.

In the present study, TE buffer was used as transport media that helped in maintaining the microbial viability for over 48 h.

Many researchers have noted a fall in salivary pH from 7 to 5 along with altered buffering mechanism instantly postradiotherapy. In the present study, also average fall of pH from 7 to 5. This shows that the oral environment becomes acidic after radiotherapy.[20]

In the present study, the average fall in the salivary flow rate was from 7.6 ml/5 min to 1.6 ml/5 min after radiotherapy. Resting saliva flow <0.30 ml/min is considered as threat to develop caries.[21] Several researchers have found that the salivary flow of patients undergoing radiotherapy does not improve to normal even after a longer period of treatment.[22]

In the present study, there was a significant rise in *S. mutans* count after radiotherapy in oral cancer patients. However, the increase in *S. mutans* count after radiotherapy in patients of laryngeal cancer was not statistically significant. This was probably because the radiation given in laryngeal cancer patients spared the parotid glands.

In the present study, *S. mutans* count was significantly increased in saliva samples postradiotherapy as compared to preradiotherapy in oral cancer patients. The finding of the present study was in agreement with a previous study stating an increase in cariogenic microbe *Streptococcus sobrinus* in postradiotherapy samples.[23] Zhang *et al*. studied pathogens in the oral cavity of nasopharyngeal carcinoma in postradiotherapy patients. In this study, *Streptococcus* spp. was significantly higher in postirradiation saliva.[24] Hu *et al*. also noted a significant increase in *Streptococcus* spp. after radiotherapy in head-and-neck cancer patients.[25]

Based on the results, several measures can be taken for oral healthcare during radiation hyposalivation to reduce the risk of dental caries. The patient should be motivated for oral healthcare. Salivary substitutes like xylitol can be used to provide moisture and lubrication to oral mucosa that increases the flushing activity and helps in maintaining the pH. The patient should be advised to reduce the frequency of carbohydrate-rich diet. Mucositis, one of the consequences of radiation therapy, is a painful condition. It can affect oral hygiene badly and hinders the mechanical removal of plaque. Therefore, fluoride mouth rinse can be used to reduce the risk of dental caries.

The clinical relevance of this study is that radiation therapy reduces salivation and its pH and increases the cariogenicity leading to radiation-induced rampant caries. Thus, the patient should be encouraged for a regular dental check-up and should be explained about the risk of caries. In the era of conservative dentistry and to avoid osteoradionecrosis, restorative, and endodontic management should be planned for such cases.

Conclusion

Thus within the limitations of the study, we conclude from the results that the pH and flow of saliva reduce postradiotherapy in oral and laryngeal cancer patients. While the amount of *S. mutans* increases postradiotherapy in oral cancer patients.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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