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Oral Colonization of Aerobic Gram Positive and Gram Negative Bacteria in Individuals Irradiated For Head and Neck Malignancies

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Original Research Article

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Abstract: The oropharyngeal aerobic gram-negative bacillary carriage, uncommon in healthy people, is high in patients with head and neck cancer. A marked increase in oral Gram-negative enterobacteria and pseudomonads has particularly been shown during the period of radiation therapy. The aim of the study was to determine whether these bacteria could play a role in the causation of irradiation mucositis. The current study demonstrated that aerobic and facultatively anaerobic gram-negative rods and cocci and Gram-positive bacteria showed a marked increase in head and neck–irradiated subjects. There was a weak but non-significant correlation between the bacterial flora and oral mucositis.
Keywords: Oropharyngeal microflora; radiotherapy; mucositis.

INTRODUCTION

The presence of nutrients, epithelial debris, and secretions make the mouth an ecological niche for a great variety of bacteria. The composition of the normal oral flora changes with age and disease that establishes itself in varying ecological situations. At birth the oral cavity is sterile but rapidly becomes colonized from the environment, particularly from the mother in the first feeding. Oral bacteria include streptococci, lactobacilli, staphylococci and corynebacteria, with a great number of anaerobes, especially bacteroides and aerobes.

The role of human oral micro-flora in the healthy individuals needs to be defined before understanding the role of bacteria in oral disease. It is estimated that more than 700 species or phylotypes of bacteria, have been detected in the oral cavity, of which over 50% have not been cultivated [1].

Normal oropharyngeal flora consists of a variety of anaerobes (eg, Peptostreptococcus, Veillonella spp) at concentrations of approximately 1×10^8 cfu /mL of saliva. Aerobes are represented most prominently by viridans streptococci at approximately 1×10^6 cfu/mL of saliva. A variety of potentially pathogenic microorganisms are also found in the normal oropharyngeal flora, such as the ―community microorganisms, Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus and Candida spp [2].

"Abnormal" oropharyngeal flora are opportunistic aerobic Gram negative bacilli (AGNB), also termed ―hospital microorganisms (eg, Klebsiella, Enterobacter, and Pseudomonas spp). In patients with head and neck cancer, radiotherapy often leads to severe mucositis, promoting the adherence of AGNB. All AGNB are considered potential pathogens. The accurate estimation of mixed flora as in the human mouth is complicated by a number of technical difficulties, such as the impossibility of obtaining a homogeneous representative sample. Rods were predominant in the gingival crevice (60.5%) and cocci in saliva (69.1%) [3].

Mucositis induced by irradiation is defined as the reactive inflammatory-like process of the oropharyngeal mucosa following irradiation in head and neck cancer patients [4]. It is a transient side effect of therapeutic doses. Mucositis is basically a tissue reaction. Bacteria colonizing the oral tissues are thought to contribute to this inflammatory process.

Etiopathogenesis

The oral soreness and clinical inflammation of the oral mucosa, akin to the mucositis immediately following radiotherapy or chemotherapy, are common in terminally ill cancer patients. It has been suggested that release of endotoxin by gram-negative bacilli may be responsible for such symptoms. In a prospective study, mucositis in irradiated head and neck cancer patients was prevented by selective eradication of gram-negative bacilli from the oral flora. The microbiologic effects of the many factors may be responsible for the reduction in colonization resistance, more likely being

the microbial factors such as adhesion and interbacterial interference [5].

Mucositis develops as of a sequence of related and interacting biologic events, culminating in injury and apoptosis of basal epithelial cells, which results in the loss of epithelial renewal, atrophy, and ulceration [6]. The ulcerations of mucositis are generally deep, broad, and painful. A pseudo-membrane composed of dead cells and fibrin, a desirable environment for secondary bacterial colonization, usually covers them.

Both gram-positive and gram-negative organisms thrive within the psuedomembrane and may penetrate and invade the vessels of the submucosa to produce bacteremias. Bacterial cell wall products that find their way into the submucosa are excellent stimulators of macrophage pro- inflammatory cytokine production and the release of additional destructive MMPs. It is during the ulceration phase that the inflammatory infiltrate is most robust.

Donnelly JP *et al*., [7] found that Gram negative bacilli are believed to contribute to the cascade of events that aggravate oral lesions by releasing endotoxins (Lipopolysaccharide) that binds to the epithelial cells and activates one of the three pathways involved in initiating the inflammatory response. According to the authors, if this hypothesis is correct, eradication of Gram negative bacilli would lead to less mucositis [7].

The marked increase in the oral Gram negative Enterobacteria and Pseudomonads have particularly been shown as a possible aggravating factor for development of oral mucositis [8].

MATERIALS AND METHODS

The study was a non-interventional clinical trial done on forty-one patients with continuous sequential analysis. The saliva sample was also collected from twenty-one patients randomly in pre-radiotherapy phase to serve as control for the bacterial counts. The study design was based on the fact that Gram-negative bacteria or the Gram-positive bacteria could play a major role in the development of advanced stages of radiation mucositis, while the initial signs are basically related to irradiation only. The objective of the study was to determine the Grade of radiation mucositis (WHO Scale) [9] during the third week of radiotherapy and simultaneously estimate the colony count of Gram negative and Gram-positive bacteria in the saliva culture of the cancer patients. The study was done to correlate the severity of mucositis and count of Gram negative and Gram-positive bacteria in the given saliva sample.

Inclusion criteria for the study were:

- External bilateral irradiation via parallel-opposed portals. (4–6 MeV)
- Fractionation of 2 Gy daily, five times a week, with a prescribed dose of at least 50 Gy and at least 50% of the oral mucosa in the field of radiation. The dose specification was in line with ICRU 50 recommendations 1993

Criteria for exclusion were:

- An oral mucosa defect other than related to tumour surgery.
- Need for an obturator or resection prosthesis.
- Treatment with antibiotics for an oral infection the last 2 weeks before the start of irradiation.

The saliva samples were collected from the patients after the patients gargled and rinsed their mouth with 5 ml sterile saline for 30 seconds and spitted into a sterile vial. The saliva samples were plated on Nutrient agar and McConkey's agar media incubated overnight at 37^0 C. The colonies were counted on the plates and the viable number of the microorganisms per milliliter was estimated. The estimation and identification was performed by standard microbiological techniques. Typically numbers between 30 and 300 were considered to be in the range where one's data is statistically accurate.

The statistical analysis was done using Student's t-test and Spearman's rank order correlation coefficient. Student's t-test is used to test the significance of difference between means of small samples as small samples or their Z values do not follow normal distribution as the large values does. Spearman's rank order correlation coefficient used when two variables are correlated, but they do not follow normal distribution. Spearman's rank order correlation (*or rho*) determines the relationship between two sets of ordinal data (usually paired) that initially appear in rank order or have been converted to rank order. The software IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp. was used for analysis.

RESULTS

The pre Radiotherapy and post Radiotherapy Gram positive and Gram-negative bacterial colonies were counted and the mean colony count of the individual patients were recorded.

The following graph represents the distribution of oral cancer among patients selected for the study (Graph-1).

It was seen that the mean percentage increase in the post- radiotherapy Gram negative bacterial colony counts was greater than as compared to rise in the post radiotherapy Gram positive bacterial colony (Graph 2 & 3).

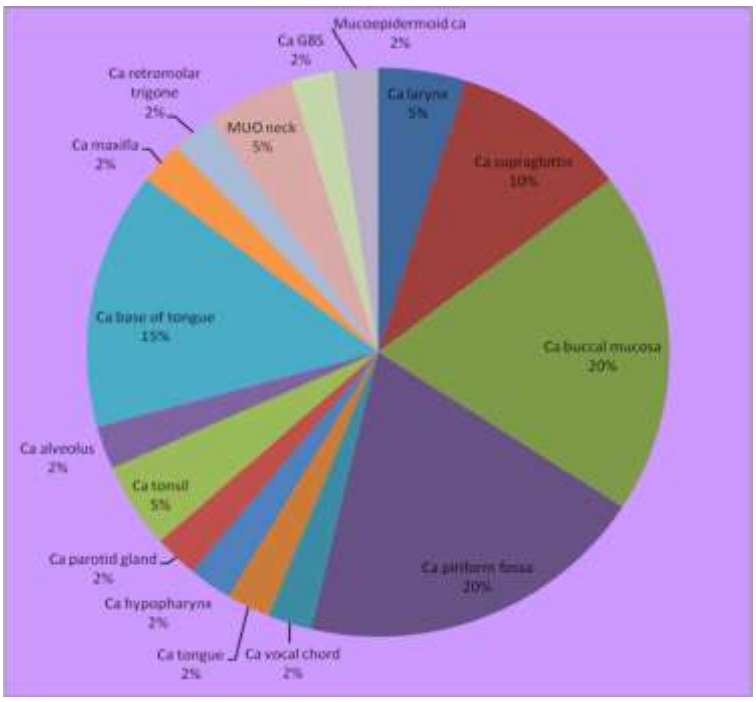
Comparison between mean baseline score and post RT bacterial colony counts (Graph-4, 5 & 6).

- Series 1- pre-RT mean Gram-positive Baseline score
- Series 2- pre-RT mean Gram-negative Baseline score
- Series 3- post-RT mean Gram-positive colony counts
- Series 4- post-RT mean Gram-negative colony counts

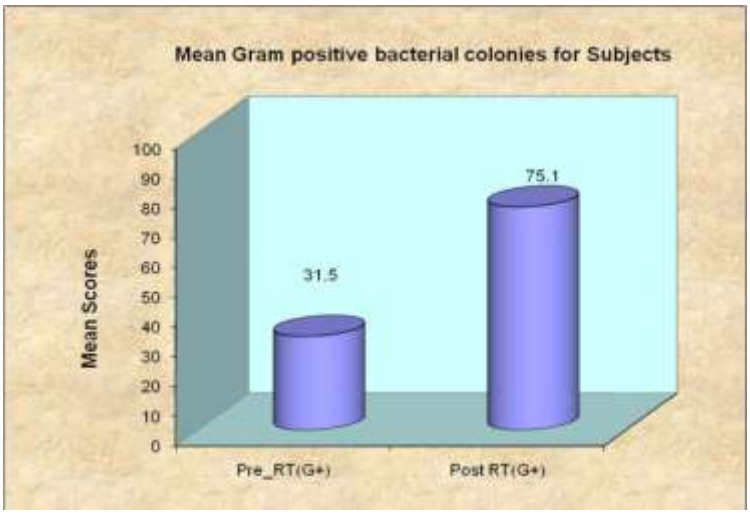
Comparison of Mucositis with Post Radiotherapy Bacterial Colony Counts. Table showing the correlation coefficient (ρ) and *P* value (Table-1).

As per the statistical data obtained, there was a definitive increase in the post radiotherapy counts of Gram positive and negative bacteria and there was weak and non- significant correlation between the grades of mucositis with either post radiotherapy Gram positive or Gram-negative organisms (Graph-4).

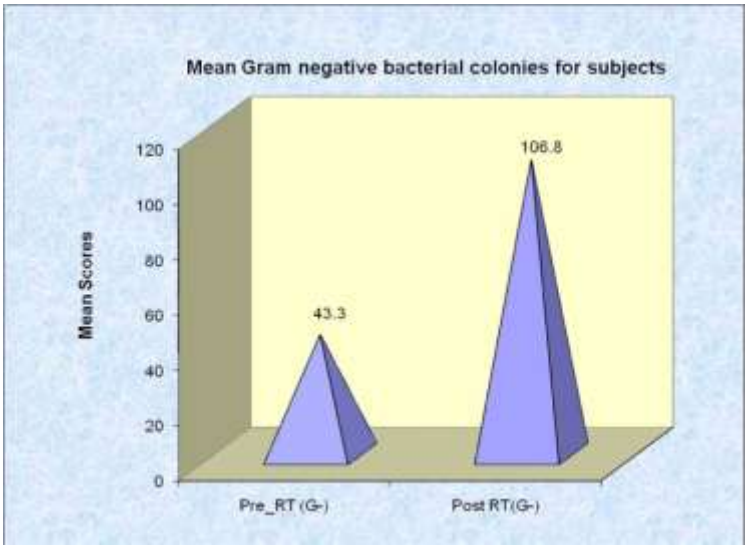
TABLES AND GRAPHS



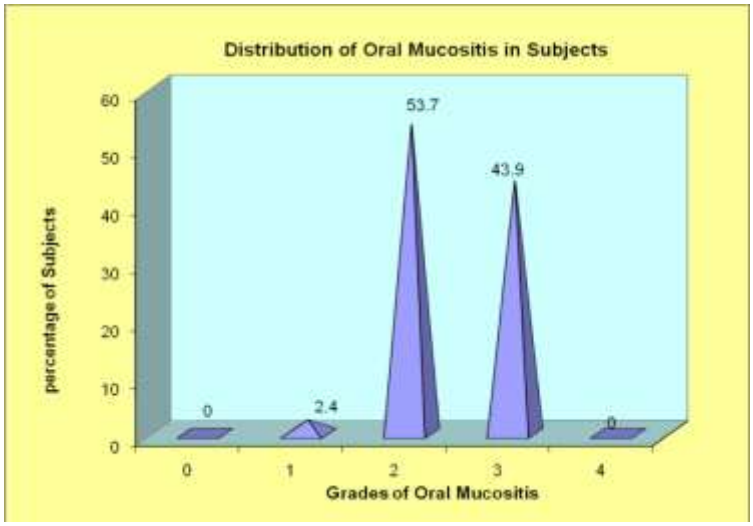
Graph-1: Distribution of head and neck cancers among patients



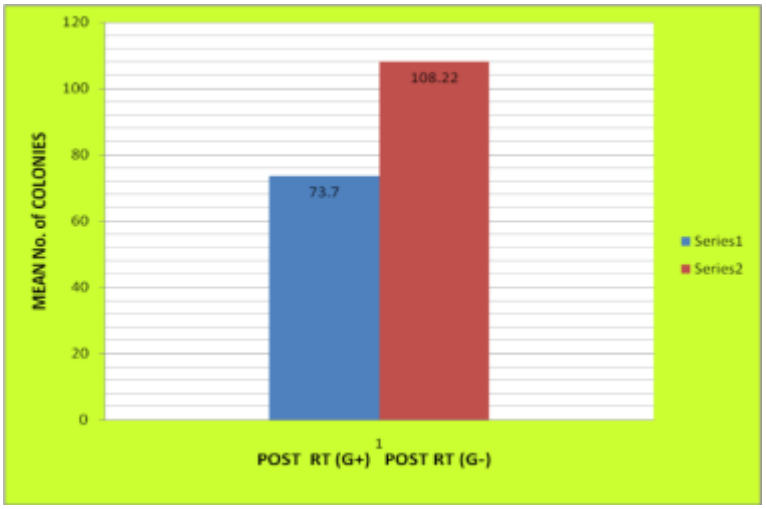
Graph-2: Graph depicting mean pre RT and post RT Gram positive bacterial colonies



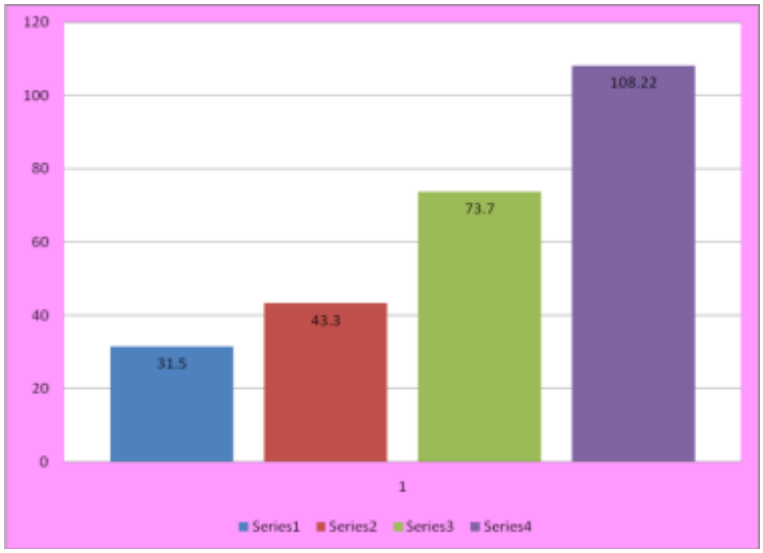
Graph-3: Graph depicting mean pre and post Gram negative bacterial colonies



Graph-4: Graph depicting percentage distribution of mucositis.



Graph-5: Graph depicting the post-RT Gram positive and Gram-negative bacterial colonies



Graph-6: Comparison between mean baseline score and post RT bacterial colony counts

Table-1: Comparison of Mucositis with Post Radiotherapy Bacterial Colony Counts. Table showing the correlation coefficient (ρ) and *P* value

	Correlation Coefficient (ρ)	*P* value
Pre-RT (G+) with Mucositis	0.103	*P* 0.658
Pre-RT (G-) with Mucositis	-0.05	*P* 0.84
Post-RT (G+) with Mucositis	0.221	*P* 0.165
Post -RT (G-) with Mucositis	0.003	*P* 0.97

DISCUSSION

Oral squamous cell carcinoma is the most common malignancy of the head and neck, with a worldwide incidence of over 300,000 new cases annually and is estimated by W.H.O to be the eighth most common cancer worldwide. The disease is characterized by a high rate of morbidity and mortality (about 50%) [10].

Chemotherapy, radiation therapy and their combination (chemo-radiotherapy) are the most widely used interventions for the treatment of cancer. The majority of new cases of invasive head and neck cancer are treated by radiotherapy as a primary treatment. The combination with chemotherapy is required in cases of metastasis and surgical management may be required as an adjunct or as palliation [16].

Oral complications that arise with chemotherapy and/or radiation therapy include oral ulcerations as in mucositis, xerostomia (dry mouth); bacterial, fungal, or viral infection (particularly in neutropenic patients), dental caries, loss of taste and osteoradionecrosis etc. Oral mucositis also represents a major non-hematologic complication of cytotoxic chemotherapy and radiotherapy associated with significant morbidity, pain, odynodysphagia, dysgeusia, and subsequent dehydration and malnutrition.

The oropharyngeal gram-negative bacillary carriage, uncommon in healthy people, is high in patients with head and neck cancer [11].

It is postulated that eradication of these bacteria almost completely prevents the severe signs of mucositis. Gram-negative bacilli are thought to exacerbate severe reactions of damaged oropharyngeal membranes by releasing endotoxins that are obnoxious to normal physiological state. Endotoxins are inflammatory mediators, which bring about changes onto mucosal membrane leading to symptoms of irradiation mucositis. Thus, eradication of the oropharyngeal gram-negative bacillary carrier state changes the 'abnormal' state into stable ‘physiological’ conditions.

Aerobic gram-negative bacillary carriage (AGNB) is likely to increase the LPS (lipopolysaccharide) concentrations of high endotoxicity, and after the absorption through the mucosal lining, enhances the generalized inflammation state triggered by the underlying conditions [12]. Gram-negative Enterobacteria and Pseudomonads (*P.aerugenosa and K.pneumoniae*) have particularly been shown as a possible aggravating factor for development of oral mucositis. The colonization of the oral cavity with these non-indigenous Gram-negative bacilli is shown to be in less than 10% of healthy individuals [12].

The atrophic changes in the epithelium of the oral membrane usually occur at a total dose level of 1600 to 2200 cGy when radiation is administered at a rate of 200 cGy per day [9]. The oral clearance of

microbial flora is based on normal shedding of surface cells, which has a role in controlling microorganisms. The normal surface cells are replaced by ulceration instead of newer epithelial cells and the pH balance is altered by changes in saliva, it is theorized that microbes and in particular gram-negative bacteria exacerbate the inflammatory response with localized infections and release of endotoxins, further enhancing the ulceration and pain that develops [13]**.**

The carriage and colonization of aerobic Gram-negative bacilli are thought to play a role in the pathogenesis of irradiation mucositis. A hypothesis has been proposed on the development of the mucositis in four consecutive phases, in which Ulcerative/ Bacterial phase is thought to play a role in the development of fibrous pseudomembranes of oral mucosa [14].

According to a study done by Spijkervet *et al*., [8] the presence of aerobic Gram negative bacilli has no influence on the development of radiation induced mucositis as no effect of selective oral flora elimination on mucositis was observed [14]**.** As per our study, there was weak and non-significant correlation between the grades of mucositis with post radiotherapy count of Gram negative organisms (ρ=0.003).

The irradiation-induced xerostomia seems, to favor frequent, repeated, transient intraoral colonization of aerobic and facultatively anaerobic gram-negative rods and cocci. In our study, there was a significant rise in the Gram-positive and Gram-negative bacterial colonies and the results were highly significant ($P<$ 0.001).

The selective elimination of AGNB of the oral flora did not result in a reduction of radiation-induced mucositis and therefore does not support the hypothesis that these bacteria play a crucial role in the pathogenesis of mucositis [15]. The results of our study were consistent as no significant correlation was established between grades of mucositis and Gram positive and Gram negative bacterial colonies.

The healthy individuals rarely carry oropharyngeal AGNB, suggesting effective oropharyngeal clearance in a healthy population predisposed to acquisition. Apparently, the oropharyngeal mucosa in healthy individuals is not receptive to adhesins of AGNB, resulting in rapid elimination of these bacteria. Thus, it was seen that in patients with head and neck cancer, radiotherapy often led to severe mucositis, promoting the adherence of AGNB. Oral AGNB carriage rates returned to control levels after the completion of radiotherapy. Thus, as per our study there was an increased AGNB carriage rate but its correlation with the mucositis was weak and non-significant.

The head and neck irradiation has a lasting effect on the oral aerobic and facultatively anaerobic gram-negative rods and cocci carriage, possibly due to disturbances in the normal physiological attributes of the oral cavity. Changes in the oral flora, colonising the oral mucosa, may aggravate the mucosal reaction because of radiation as the carriage and colonisation of aerobic Gram-negative bacilli are thought to play a role in the pathogenesis of irradiation mucositis. These early and late sequelae of head and neck radiotherapy have a large impact on the quality of life.

The current study demonstrates that aerobic and facultatively anaerobic gram-negative rods and cocci are highly prevalent in head and neck–irradiated subjects. The irradiation-induced xerostomia and related oral changes favour the initial oral colonization of aerobic and facultative anaerobic gram-negative rods and cocci, but most such microbes, unlike the dental plaque flora; fail to establish themselves intraorally in the longer term.

A better understanding of the role of the oral micro flora in the development of oral mucositis is therefore needed and further investigations in the future are necessary, if agents with selective properties to prevent or mitigate oral mucositis are to be developed.

CONCLUSION

The current study demonstrated that aerobic and facultatively anaerobic gram-negative rods and cocci and Gram-positive bacteria showed a marked increase in head and neck–irradiated subjects. There was a weak but non-significant correlation between the bacterial flora and oral mucositis.

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