Characterization of Salivary, Mucosal and Denture Surfaces Role in Denture Stomatitis: an Exploratory Study

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A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the school of Dentistry, (Prosthodontics).

Chapel Hill
2011

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ABSTRACT

SANDRA ALTARAWNEH: Characterization of Salivary, Mucosal, and Denture Surfaces
Role in Denture Stomatitis: an Exploratory Study
(Under the direction of Sompop Bencharit D.D.S., M.S., Ph.D.)

Objectives: To explore associations between clinical signs of stomatitis and tissue/denture
infection, as well as salivary and serum biomarkers**. Methods**: An IRB approved, case-
control study enrolled 32 edentate subjects, 17 with healthy palatal mucosa as controls and
15 with denture stomatitis (DS) (Newton's classification type II and III). Rate of salivary
flow, exfoliative cytology, culture, salivary cytokines levels, serum C-reactive protein levels,
and DNA-DNA checkerboard analyses were performed. **Results:** denture levels of yeast
were higher and more strongly predictive of clinical DS than salivary or mucosal levels.
Certain pro-inflammatory cytokines levels were increased in DS and associated with counts
of *C. albicans* in saliva. **Conclusions**: the denture, rather than the inflamed palatal mucosa, is
the primary *C. albicans* habitat. At this point, data suggest no evidence that DS can induce
systemic inflammation.

ACKNOWLEDGEMENTS

In recognition of their time and effort in making this project possible, I would like to thank

the members of my committee: Drs. Bencharit, Offenbacher, and Cooper, particularly Dr.

Bencharit for his enthusiasm and support. I would also like to thank my parents, my brothers

Tarek and Tamer, my Niece Zaina, and my best friend Abeer for always being there.

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Introduction

Candida-associated denture stomatitis (CADS) is the most common form of oral candidal infection in denture wearers. CADS has a multi-factorial etiology with both systemic and local factors being implied among the possible contributing factors such as nutritional deficiencies, malignancies, immune defects, dentures, commensal flora, and salivary factors. This multi-factorial etiology stems from the fact that no direct cause-effect relationship could be established with a specific factor/s, and this is clearly reflected in the various treatment regimens used for treatment from the use of antifungals, to antimicrobials, antiseptics, and relining or remaking the dentures.

Candida species, especially the hyphal-forming *C.albicans*, Gram negative anaerobes, impaired salivary flow, and ill-fitting dentures have been investigated as possible causative factors in previous studies. The role of *C.albicans* has been controversial as for whether its presence in saliva, or denture (within the denture and denture biofilm), or in the mucosal tissue underneath the denture is the main reservoir for this microorganism as related to denture stomatitis (DS). Xerostomia has been implicated as a contributing factor in DS. One theory suggests that the absence of cleansing effect in case of xerostomia makes the host more susceptible for such opportunistic infections. However, different studies yielded different results in this regard and most of the studies were more concerned with the quantity rather than the quality of saliva (i.e saliva composition especially the salivary proteins and

their antimicrobial characteristics). DS is often associated with trauma from ill-fitting dentures that are associated with atrophic osseous ridge anatomy. It is unclear whether the inflammatory state of DS is inducing ridge resorption that results in a loose and easily displaced denture, or whether the trauma associated with the denture (e.g. through poor tissue adaption, clenching or inadequate inter-ridge space) can provide a mechanical stress that induces mucosal inflammation and bone resorption via poor tissue perfusion, necrosis or trauma.

Although some progress has been made in revealing the mechanisms of epithelial and immunoregulatory interactions with Candida, there are major gaps in our knowledge regarding processes that lead to the establishment of candidiasis as an infection and the relationship between mucosal and systemic inflammatory responses.

Considering the number of potential factors that are related to DS, an exploratory case-control investigation was conducted aiming to examine the association between clinical signs of DS (measuring the severity of stomatitis with the Newton Score and the Denture fit using the Kapur index) and 1) the exfoliative cytology, 2) the microbial levels (bacteria and yeasts) present in saliva, on mucosal tissues and on denture surfaces, 3) the salivary flow rate and xerostomia symptoms, 4) and the level of inflammatory cytokines in saliva and systemic markers of inflammation that discriminate between DS and health.

The goal was to elucidate the molecular mechanisms associated with denture stomatitis that could possibly lead to identification of potential biomarkers of the disease for monitoring therapy as well as gain insight in to pathogenesis to improve prevention and treatment.

Chapter 1

Role of C. albicans in Saliva and on Mucosal and Denture Surfaces in Denture Stomatitis

Abstract

Purpose: To explore the association between the levels of Candida in saliva, and on denture and mucosal surfaces of subjects with and without denture stomatitis (DS), as diagnosed clinically and characterized by exfoliative cytology. Salivary flow rates were also examined as a possible disease modifier.

Methods: An IRB approved, case-control study enrolled 32 edentate subjects. Seventeen subjects with no signs or symptoms of DS as controls and 15 with (DS) [Newton's classification type II (moderate) and III (severe)] were selected. Subjects completed a xerostomia questionnaire and salivary flow rates were measured. Samples of unstimulated whole saliva (UWS) and stimulated whole saliva (SWS) were collected. UWS was used for fungal culturing, cytokine and proteomic analyses. Mucosal wetness was measured using sialopapers and periotron. Periodic acid Schiff (PAS) stain and quantitative exfoliative cytology was performed on samples from affected and unaffected mucosa from each subject. Levels of Candida species (*albicans* and non-*albicans*) were determined, expressed as colony-forming units, using salivary samples as well as swab samples obtained from denture's fitting surfaces, the affected and the unaffected mucosa. Biopsy samples were taken from the palate for whole transcriptome analyses by microarray. Cytokine, proteomic and transcriptomic analyses results are presented elsewhere.

Results: There were no significant differences in salivary flow rates, mucosal wetness or frequency of reported dry mouth comparing subjects with and without DS. Exfoliative cytology of mucosal smears demonstrated significantly higher (P=0.02) Inflammatory cells counts in DS subjects, as compared with healthy denture-wearers. *C. albicans* counts were

significantly higher in saliva (P=0.03) and on denture surfaces (P=0.002) of DS subjects, whereas mucosal candidal counts and the presence of cytological hyphae did not show significant difference comparing DS to health.

Conclusions: xerostomia does not appear to be associated with the presence of DS. Denture levels of yeast were higher and more strongly predictive of clinical DS than salivary or mucosal levels, suggesting that the denture, rather than the inflamed palatal mucosa, was the dominant *C. albicans* habitat.

Introduction

There are conflicting data regarding the etiology and pathogenesis of denture stomatitis (J. Barbeau, et al. ,2003, E. Budtz-Jorgensen. ,1990, C. G. Cumming, et al. ,1990, R. T. Glass, et al. ,1990, B. C. Webb, et al. ,1998a, B. C. Webb, et al. ,1998b). Several hypotheses have been offered as plausible etiologic and contributing factors in denture stomatitis depending on the fact that these factors cause an overgrowth of commensal microbes, especially yeasts such as candida, and of Gram negative anaerobes. The overgrowth of such oral pathogens has been suggested to be potentially due to impaired salivary flow and function, tissue trauma as a result of ill-fitting dentures, poor denture and oral hygiene and impaired immune response secondary to systemic conditions. Indeed, the higher prevalence of DS among immune-compromised individuals who are more susceptible to opportunistic yeast infections (e.g. diabetic subjects), coupled with the clinical evidence that antifungal agents can reduce musosal inflammation associated with DS provides strong evidence of the key role of fungal infections as potentially etiologic factor. However, after a course of antifungal therapy, the condition often returns even among immunocompetent individuals, and not all DS patients respond to antifungal therapy, In those refractory individuals, antibiotics have been reported to provide resolution, suggesting that anaerobic pathogens may potentially play a role under some circumstances (E. Budtz-Jorgensen, et al. ,1983, A. S. Koopmans, et al. ,1988, C. Pesci-Bardon, et al. ,2004, S. Redding, et al. ,2009). Thus, Gram negative anaerobes may also be important in the pathogenesis of DS. Although mucosal surfaces can harbor low levels of fungal microbes as well as anaerobic pathogens similar to those associated with periodontal diseases (A. Sachdeo, et al. ,2008), the denture can serve as a habitat for a high-density biofilm which can harbor high levels of bacteria and yeasts (A. Sachdeo, et al. ,2008,

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B. J. Coco, et al. ,2008, G. Ramage, et al. ,2004). Thus, the denture itself is believed to serve as a reservoir for triggering a local microbial infection-mediated inflammatory response. It should be noted, however, that these statements regarding etiology and effects of treatment are based upon data from relatively few case series and small single-centered trials.

The role of Candida is also controversial. Certain strains of Candida, specifically hyphal-forming clonal types, are more commonly found in mucosal infections and appear to be virulent because these strains are capable of epithelial binding, disruption of epithelial integrity and invasion (C. Salerno, et al. ,2010, G. Dahlen, et al. ,2009, H. Bilhan, et al. ,2009, J. E. Leigh, et al. ,2002, H. Nikawa, et al. ,2002, A. Rodriguez-Archilla, et al. ,1996). Overgrowth of Candida in DS is a common finding, and can be a precursor to oropharengeal candidiasis which can become a life-threatening disseminating infection among HIV/AIDS subjects and other immunocompromised conditions, especially those conditions that are associated with T cell functional deficits (J. E. Leigh, et al. ,2002, H. Nikawa, et al. ,2002, A. Rodriguez-Archilla, et al. ,1996). But the ecological pressures that cause the emergence of oral Candida and its role in pathogenesis in a normally healthy individual are less well understood. Antibiotic usage, especially prolonged suppression, can cause a shift in the oral flora and a fungal overgrowth. Impaired salivary flow or altered salivary composition, which may or may not be associated xerostomia syptomology, has been suggested to lead to a shift in the oral microbiome composition that favors fungal overgrowth. The association between the symptom of "dry mouth" and the amount of saliva flow are not necessarily concordant. Furthermore, salivary glycosaminoglycan content, as reflected in viscosity measures, does not agree well with xerostomia symptoms {{1541 Higuchi,Y. 2009; 1542 Wolff,M. 1998}}. Salivary secretory IgA level is a critical modulatory of microbial aggregation, microbial (Y.

Higuchi, et al. ,2009, M. Wolff, et al. ,1998) clearance and surface adherence. Thus, impaired sIgA has been attributed to microbial overgrowth (C. Fukushima, et al. ,2005, S. Hagewald, et al. ,2002, T. Tanida, et al. ,2003). The relation between salivary flow and viscosity has been suggested to also potentially play a role in DS by altering the epithelial resistance to Candidal binding and invasion.

In this study, we aimed to examine the association between clinical signs of DS (measuring the severity of stomatitis with the Newton Score and the Denture fit using the Kapur index) and 1) the exfoliative cytology, 2) the microbial levels (bacteria and yeasts) present in saliva, on mucosal tissues and on denture surfaces, and 3) the salivary flow rate and xerostomia symptoms, that discriminate between DS and health.

Materials and methods

Study design, Subjects and Sample Size Estimates

This was a case control study design intended to collect biological samples from participants without and with denture stomatitis, specifically with mucosal lesions (Newtons Classification Type II or Type III).

A total of 32 edentulous subjects were enrolled according to the inclusion and exclusion criteria listed in Table 1-1. The control group (n=17) had no signs or symptoms of denture stomatitis (DS), and the diseased group (n=15) presented with type II (n=8) or III (n=7) DS (Newton's classification) (A.V. Newton 1962). This level of DS includes the moderate (Type II) and severe (Type III) forms. Representative clinical appearance of cases and controls appear in Figure 1-1. All subjects that were enrolled completed the two visit study. For all parameters of interest there was no apparent gradient progressing from health to type II to

Type III, therefore the Type II and Type II remained grouped as a single DS diseased group, as initially intended.

This was an exploratory study and a targeted sample size of 30 (15 subjects per group) was determined to provide 80% power with two-sided alpha = 0.05 significance tests to detect changes in means from continuous variables between diseased and non-diseased subjects that are 1.06 times the standard deviation of the variable. The sample size was considered sufficient as effect sizes such as this, or larger, are common for levels of dental biofilm organisms and pro-inflammatory cytokines. To allow for an 8-10% possible drop-out rate, 32 subjects were enrolled to ensure that 30 (15 subjects per group) completed the study. In this report we limit the analyses to the clinical findings, the cytology and the cultivable data.

Table 1-1. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
1. At least 45 years of age 2. Male or females without menses for 12 consecutive months or who have had a complete hysterectomy 3. Wear complete maxillary denture (overdentures, implant or tooth retained dentures acceptable) without daily use of denture adhesive. 4. Must have read, understood and signed an informed consent form. 5. Must understand and be willing to comply with all study procedures and restrictions 6. Must be in good general health; diabetics included 7. Must have Type II or Type III denture stomatitis for denture stomatitis group 8. Must have no signs of denture stomatitis for control group	1. Less than 45 years of age. 2. Have chronic disease with oral manifestations other than denture/mucosal stomatitis. 3. Have gross oral pathology. 4. Have overt denture abrasion associated with symptoms. 5. Participants with clinically significant organic diseases, including impaired renal function, bleeding disorder, or any condition requiring antibiotic pre-medication for dental visits 6. Participants with active infectious diseases such as hepatitis, HIV or tuberculosis. 7. Participants who are immunosuppressed because of medications or condition. 8. Participants who have used antibiotics or antifungals for any medical or dental condition within 1 month prior to screening. 9. Participants using ongoing medications initiated less than 3 months prior to enrollment. 10. Participants with a known or suspected intolerance to local oral anesthesia. 11. Participants who have participated in another clinical study or have taken an investigational drug within 30 days of screening. 12. Participants who have used tobacco products within 6 months of screening. 13. Employees of the sponsor or the investigator or members of their immediate family. 14. Participants who have previously participated in this study. 15. Post-menopausalwomen on hormone replacement therapy.

Figure 1-1. Clinical photographs of (A) a control subject, (B) Newton classification
II stomatitis, and (C) Newton classification III stomatitis.

Clinical evaluation of dentures (Kapur Index)

A qualified examiner (prosthodontist) assessed the fit of the maxillary and mandibular
dentures (if present) using the Kapur Index (K. K. Kapur. ,1967)as following:

Retention:

3:      Good – maximum resistance to vertical pull and sufficient lateral force.

2:      Moderate – moderate resistance to vertical pull and little or no resistance to
        lateral force.

1:      Minimum – slight resistance to vertical pull and little or no resistance to
        lateral force.

0:      No retention – denture displaces itself.

Stability:

2:      Sufficient –demonstrates slight or no rocking on its supporting structures
        under pressure.

1:      Some –demonstrates moderate rocking on its supporting structures under
        pressure.

0:      No stability – demonstrates extreme rocking on its supporting structures under
        pressure.

Since not all of the subjects had a mandibular denture, mean values for the total scores of

retention and stability for the maxillary denture were calculated.

Clinically poor denture = sum score of<3

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Clinically fair denture = sum score of 3-4.

Clinically good denture = sum score of >4.

Unpaired T-test was performed to assess statistical difference between means of scores of the maxillary dentures for DS and healthy groups.

Unstimulated Saliva Collection

Subjects were instructed to remove their dentures and refrain from eating, drinking, smoking, brushing their teeth or chewing gum 15 minutes prior to salivary collections. All collections were performed between 9:00 and 11:00 AM. Subjects were instructed to swallow to clear the mouth of any accumulated saliva, and whole unstimulated saliva was allowed to pool in a sterile polypropylene graduated collection vial for 5 minutes. A fraction of the sample was sent immediately to the microbiology lab for rapid processing to prevent overgrowth of the Candida species. Samples were aliquoted into Eppendorf tubes, centrifuged for 10 minutes at 3000g, and the supernatant stored at $-80^{\circ}C$ for proteomic and cytokine analysis.

Stimulated Saliva Collection

Subjects were instructed to place their dentures back before the collection begins. Stimulated whole saliva was quantified by using a modification of the Saxon test. (Kohler PF, Winter ME, 1985) where each participant was asked to chew on a folded strip of paraffin for 2 minutes after swallowing to clear the mouth of the accumulated saliva. The collected saliva was then quantified as milliliters of saliva generated per minute (ml/min) and recorded on the CRF. Participants found to have a resting (unstimulated) saliva rate of less than 0.01 ml/min and stimulated salivary rate of less than 0.10 ml/min were characterized as having hyposalivation (M. Bergdahl, et al. ,2000, M. Bergdahl. ,2000)

Xerostomia questionnaire

Subjects were asked to complete the xerostomia questionnaire (Table 2) after the salivary collection. This questionnaire is derived from the validated questionare from the Dental ARIC study as described by Beck etal (J. D. Beck, et al. ,2001). Analysis of variance (items # 3, 13 and 14) and Spearman correlations (items #5-12) by regression analysis were used to analyze relationships between salivary flow rates and subject-reported symptoms of dry mouth.

Mucosal Wetness

Subjects were instructed to swallow and sialopaper was placed on the midline of the anterior third of the dorsum of the tongue for 5 seconds. Sialopaper was then transferred to Periotron (Model 6000) for reading and after reading the sialopaper was discarded. Measurement was done 2 times. T-test was used to determine differences between the means of readings comparing groups.

Table 1-2. Xerostomia questionnaire.

1) Do you have any difficulties in swallowing any foods? (yes/no)
2) Does your mouth feel dry when eating a meal? (yes/no)
3) Do you sip liquids to aid in swallowing food? (yes/no)
4) Does the amount of saliva in your mouth seem to be too little, too much, or you don't notice it?
5) Rate the difficulty you experience in speaking because of dryness in your mouth. (1-10 scale).
6) Rate the difficulty you experience in swallowing because of dryness in your mouth. (1-10 scale).
7) Rate how much saliva is in your mouth. (1-10 scale).
8) Rate the dryness of your mouth. (1-10 scale).
9) Rate the dryness of your throat. (1-10 scale).
10) Rate the dryness of your lips. (1-10 scale).
11) Rate the dryness of your tongue. (1-10 scale).
12) Rate the level of your thirst. (1-10 scale).
13) Dryness of lips (present or absent).
14) Dryness of buccal mucosa/ cheek areas (present or absent).

Exfoliative Examination

Smears were taken from the affected palatal mucosa and unaffected palatal mucosa for the

stomatitis group, and only one sample from the palatal mucosa for the control group.

Samples were taken from the buccal vestibular area and dorsum of the tongue as well.

Subjects with dry mouth (xerostomia) were instructed to rinse with a small amount of water

prior to collection of the sample. Selected areas were wiped firmly, using a wooden tongue

blade, until there was a visible accumulation of oral fluids present. Accumulated samples

were transferred to a clean glass slide until there is a thin coat visible when the slide is held

against light. Slides were then sprayed with Cytofix/Cytoperm™ with one or two swipes from a distance of about 1 foot and allowed to dry for 10 minutes.

When adequate samples could not be collected using this procedure, the sampling steps outlined above were repeated in a different area of the palatal mucosa, until an adequate sample was collected.

These Samples were used for cytology and PAS identification of fungal forms. The following scoring system was used for the PAS-cytology assessments:

0= INADEQUATE CELL SAMPLE, 1=BENIGN SMEAR, 2=BACTERIA ONLY, 3=BENIGN INFLAMMATORY SMEAR, 4=BACTERIA PLUS INFLAMMATORY CELLS, 5 = FUNGAL SPORES, 6= FUNGAL ORGANISMS.

Culture

BBL™CultureSwab™ was used to swab the denture and the mucosal surface, with total of 3 swabs for the diseased group (from the denture, mucosal surface of affected area and unaffected area), and two swabs for the control group (from the denture and the mucosal surface).

Samples were cultured on Sabouraud Dextrose agar containing quemicetine succinate. Samples were spiral plated to Sabaroud's dextrose plates to obtain a quantitative value of CFU/ml. Samples were also plated on a total aerobic plate to compute the percentage of total aerobic recoverable CFU on a non-selective medium to allow for identification of both *albicans* and non-*albicans* Candida. Candida colonies were counted after 48 h and the

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patients classified according to the number of CFU (Colony-forming units) as follows: negative (CFU/ml = 0), carrier (CFU/ml < 400), and positive (CFU/ml > 400). A fraction of the UWS was sampled in Sabouraud Dextrose agar containing quemicetine succinate, and Candida colonies were counted after 48 h and the patients classified in the same way as for the BBL culture swabs.

Results

Demographics

The mean age of the 32 participants was 64.8. The control group was comprised of 14 females, 3 males, 11 Caucasians, and 6 African Anericans. The stomatitis group was comprised of 9 females, 6 males, 9 Caucasians, 3 African Anericans, and 3 Asians. There were no significant differences in age, race or gender comparing the DS vs control groups. Baseline characteristics and Kapur index results are included in Table 1-3 and show no significant differences between groups.

Table 1-3. Demographics and baseline characteristics of the subjects.

	Control (n=17)	DS (n=15)
Age (mean ± SD)	66.2±9.7	63.2±8.83
Gender		
Male	3 (17.6%)	6 (40%)
Female	14 (82.4%)	9 (60%)
Race		
Caucasian	11 (64.7%)	9 (60%)
African American	6 (35.3%)	3 (20%)
Asian		3 (20%)
Kapur index of max. Denture (mean ± SD)	3.56 ± 1.46	3.00 ± 1.57

Salivary Flow Rates

There was no significant difference in the rate of stimulated or unstimulated salivary flow comparing Healthy individuals to DS patients. Table 1-4.

Table 1-4. Flow rate of unstimulated and stimulated whole saliva in contro, and DS subjects.

Subjects	Salivary flow (ml/min)	
	UWS (mean±SD)	SWS (mean±SD)
Control (n=17)	0.5 ± 0.23	1.43 ± 0.57
DS type II (n=8)	0.5 ± 0.15	1.14 ± 0.43
DS type III (n=7)	0.55 ± 0.23	1.34 ± 0.57
P value (ANOVA)	0.84	0.46
DS combined (n=15)	0.53 ± 0.19	1.23 ± 0.5
P value (unpaired T-test)	0.71	0.31

- UWS: unstimulated whole saliva collected for five minutes.
- SWS: stimulated whole saliva collected by chewing folded strip of paraffin for two minutes.
- Hyposalivation: unstimulated saliva rate of less than 0.01 ml/min, and stimulated saliva rate of less than 0.10 ml/min.

Xerostomia Symptoms

There was no significant difference in the frequency of reported dry mouth comparing DS to control subjects. However, among all subjects there were significant associations between symptomology and flow and wetness measures. A significant correlation was found between unstimulated salivary flow rate and perceived "Rate the dryness of your tongue", ($r^2 = 0.36$, p=0.0405). There was also a significant association between stimulated salivary flow rate and "Rate the level of your thirst", ($r^2=0.51$, p =0.0028). All other correlations were not

significant and were in the range of (-0.23 to 0.10). For questions #3, 13, and 14 there were no significant associations between the reported symptoms and the flow rate of both unstimulated and stimulated saliva.

Cytology

According to the cytology results of the palatal mucosal swabs (figure 1-2, Table 1-5), it was noticed that Type II and Type III DS are associated with higher scores reflecting inflammatory cells, bacterial and fungal morphotypes. Inflammatory cells in the palatal mucosa showed significant difference (P=0.02) between DS and control subjects. It is noteworthy that fungal forms were often found in control subjects. There was no statistically significant difference in the cytology of the vestibular and tongue swabs comparing DS Type 2 or DS Type 3 to Health. However, the vestibular area and the tongue as well as the palate had a trend for more prevalent fungal forms in DS subjects.

Figure 1-2. PAS exfoliative cytology representative slides.(A) a healthy control benign smear, (B) DS fungal hyphae.
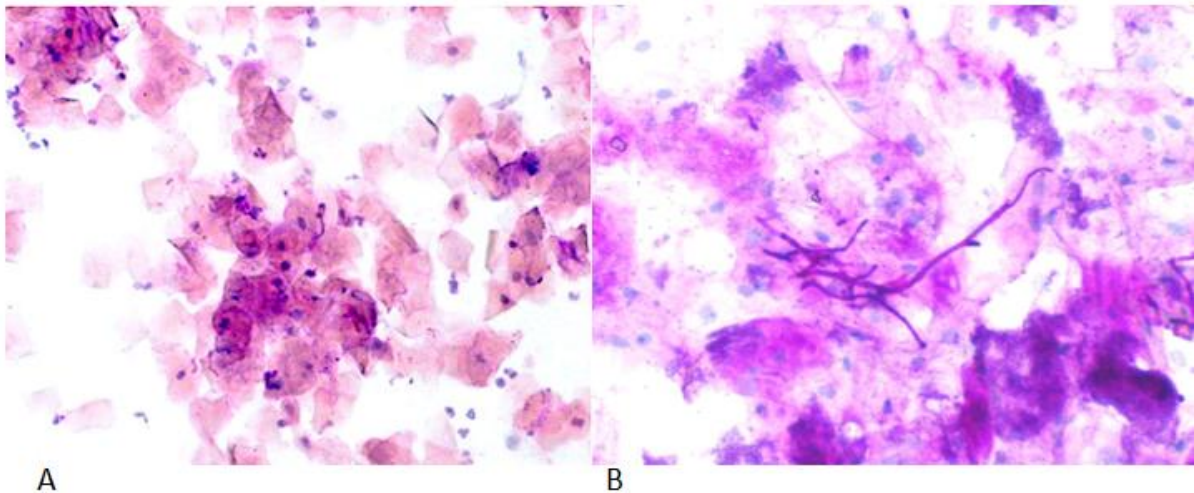
A) Oral cytologic smear of palatal mucosa showing typical squamous cells and scattered chronic inflammatory cells. (10 X)

B) Oral cytologic smear from palatal mucosa showing candidal hyphae. (40X)

Table 1-5. PAS exfoliative cytology results for palatal, and vestibular area and tongue.

	Palatal Area			Vestibular Area and Tongue		
	Healthy	Diseased	p-value	Healthy	Diseased	p-value
Adequate (Yes)	12 (44.4%)	15 (55.6%)		17 (53.1%)	15 (46.9%)	
No	5 (100.0%)	0 (0.0%)	0.02	..	..	..
Bacteria (Yes)	6 (40.0%)	9 (60.0%)		12 (46.2%)	14 (53.9%)	
No	11 (64.7%)	6 (35.3%)	0.16	5 (83.3%)	1 (16.7%)	0.10
Inflammatory Cells (Yes)	0 (0.0%)	4 (100.0%)		3 (37.5%)	5 (62.5%)	
No	17 (60.7%)	11 (39.3%)	0.02 *	14 (58.3%)	10 (41.7%)	0.31
Fungal Spores (Yes)	5 (35.7%)	9 (64.3%)		12 (46.2%)	14 (53.9%)	
No	12 (66.7%)	6 (33.3%)	0.08	5 (83.3%)	1 (16.7%)	0.10
Fungal Hyphae (Yes)	4 (40.0%)	6 (60.0%)		7 (43.8%)	9 (56.3%)	
No	13 (59.1%)	9 (40.9%)	0.32	10 (62.5%)	6 (37.5%)	0.29
Atypical Cells (Yes)	0 (0.0%)	1 (100.0%)		..	..	
No	17 (54.8%)	14 (45.2%)	0.28	17 (53.1%)	15 (46.9%)	..
Score						
Inadequate Cell Sample	6 (100.0%)	0 (0.0%)		..	..	
Benign Smear	4 (57.1%)	3 (42.9%)		4 (100.0%)	0 (0.0%)	
Bacteria Only	2 (50.0%)	2 (50.0%)		1 (50.0%)	1 (50.0%)	
Benign Inflammatory Cells	0 (0.0%)	1 (100.0%)		..	..	
Bacteria plus Inflammatory Cells	..	..		..	..	
Fungal Spores	1 (25.0%)	3 (75.0%)		5 (50.0%)	5 (50.0%)	
Fungal Organisms	4 (40.0%)	6 (60.0%)	0.13	7 (46.6%)	8 (53.3%)	0.28
Score Dichotomized (Fungal)	5 (35.7%)	9 (64.3%)		12 (48.0%)	13 (52.0%)	
No Fungal	12 (66.7%)	6 (33.3%)	0.08	5 (83.3%)	1 (16.7%)	0.12

- Statistically significant (P< 0.05)

Cultivable *C. albicans* levels in saliva and on Denture and Mucosal Surfaces

In the saliva, *C. albicans* was detected in 80% (12/15) of the DS subjects, but only 41.2% (7/17) of the control subjects (p=0.03, Chi Square). There was no greater detection rate of C. *albicans* in severe DS as compared to mild DS. In the dentures, *C. albicans* was present in 73.2% of dentures sampled from DS subjects and only in 11.8% of dentures sampled from healthy individuals (p=0.002), Chi Square. In the mucosa there was no significant difference in *C. albicans* counts between diseased (P=0.27) and healthy mucosa (p=0.2). There was a

high degree of concordance between the presence of *C. albicans* in the saliva vs that detectible on the denture (r^2=0.31, p=0.0007). *C. albicans* prevalence by location comparing saliva vs healthy mucosa was not significant (p=0.059), nor was it significant when compared to diseased mucosa (p=0.79).

Non-*albicans* Candida was also significantly detected especially in DS subjects. Table 1-6 shows the prevalence of both *C. albicans* and Non-*albicans* in all subjects.

Table 1-6. Prevalence of C.*Albicans* and Non-*Albicans* in Saliva, denture, and mucosal surfaces for control and DS subjects.

	Saliva (N=32)		Denture (N=32)		Unaffected Mucosa (N=32)		Affected Mucosa (N=15)	
	C.*Albicans*	Non-*Albicans*	C.*Albicans*	Non-*Albicans*	C.*Albicans*	Non-*Albicans*	C.*Albicans*	Non-*Albicans*
Control (N=17)	7 (41.2%)	7 (41.2%)	2 (11.8%)	8 (47.1%)	1 (5.9%)	7 (41.18%)		
DS type II (N=8)	7 (87.5%)	0 (0.00)	6 (75.0%)	0 (0.00%)	2 (25.0%)	2 (25.0%)	2 (25.0%)	3(37.5%)
DS type III (N=7)	5 (71.4%)	0 (0.00)	5 (71.4%)	2 (28.6%)	2 (28.6%)	4 (57.1%)	4 (57.1%)	3(42.9%)
Total (N,%)	19(59.4%)	7 (21.9%)	13 (40.6%)	10(31.3%)	5 (15.6%)	13 (40.6%)	6 (40.0%)	6(40.0%)
P values	0.03*	0.02*	0.002*	0.06*	0.27	0.45	0.20	0.83

- Statistically significant (P< 0.05)

Discussion

The role of xerostomia as an etiological factor in denture stomatitis has been a subject of controversy for many years. In this study, there was no difference in the frequency of reported dry mouth, or in the rate of stimulated or unstimulated salivary flow comparing control individuals to DS patients. In fact, most of the subject-reported symptoms of dry mouth did not correlate with saliva flow. Torres et al (S. R. Torres, et al. ,2003, T. Pereira-Cenci, et al. ,2008) found that 67.9% of the individuals with xerostomia were colonized by

Candida spp.; however, the difference between these patients and those without xerostomia was not statistically significant. Närhi et al (T. O. Narhi, et al. ,1993) found significantly higher counts of yeasts in individuals with salivary flow rates below normal. According to Tatiana et al (T. Pereira-Cenci, et al. ,2008) patients with low or impaired salivary flow and/or composition presented higher Candida species counts when compared with saliva from patients with normal salivary flow. Hibino et al (K. Hibino, et al. ,2009)reported that both stimulated and unstimulated salivary flow rates of the non-carriers were higher than the carriers although the difference was not statistically significant.

In their study to determine risk factors associated with oral candidiasis onset and chronic maintenance, Campisi et al (G. Campisi, et al. ,2008) found both denture wearing and xerostomia to be local risk factors according to their analysis. The sample size in our study is too small to refute or resolve any of these conflicting findings. One key issue is that the typical assessments of salivary function from a physiological perspective (e.g. salivary flow rate, mucosal wetness, xerostomia symptoms) may not be associated with the antifungal capacity of the saliva. Clearly, future studies which intend to examine the role of salivary immunoglobulins (e.g. sIgA) and specific anti-fungal components, such as lactoferrin, histatin-5, lysozyme, and histidine-rich polypeptides in recoverable yeast counts, in addition to physiological assessments would be informative.

Role of Candida

In this study *C. albicans* was approximately twice as likely to be present in saliva of DS subjects (80%) as compared to control subjects (41.2%). However, there was no clear trend for the CFU levels of salivary *C. albicans* to show a gradient of higher numbers associated with the transition from Type II to Type III DS.

A strong relationship between DS and the presence of Candida in saliva has been reported previously (I. Berdicevsky, et al. ,1980, B. C. Webb, et al. ,1998). On the other hand, patients with Candida in saliva may not develop DS (J. Wilson. ,1998) and this is in agreement with our findings. Since the frequency of detection of *C. albicans* is lower on the denture (11.8%) than present in the saliva (41.2%) of control subjects, it would appear that saliva and/or mucosa represents the reservoir of infectious agent in these healthy individuals and that the denture is less frequently colonized with *C. albicans*. According to Pires (F. R. Pires, et al. ,2002), the clinical resolution of Denture stomatitis was not related to the levels of Candida in saliva and, furthermore, a decrease of Candida counts in saliva usually is not followed by clinical improvement of DS.

In our study, there was a significant difference in the C. *albicans* prevalence in denture surfaces between DS and control subjects (p=0.03). C. *albicans* was 20.6 fold more likely to be cultured from the denture in DS patients compared to healthy. The notion that greater numbers of candida spp. were recovered from smears prepared from the fitting surfaces of the dentures than from those on the palatal mucosa has been known since the 1970's (J. C. Davenport.,1970, H. Lamfon, et al. ,2005). It has been shown that *C. albicans* colonies were recovered more frequently from the tissue fitting surface of the acrylic resin denture than from the corresponding palatal mucosa in DS subjects (B. C. Webb, et al. ,1998). However, according to Radford (D. R. Radford, et al. ,1999)it is difficult to attribute the etiology of the condition entirely to the presence of *C. albicans* in denture plaque as they found in their review that methods of sample collection accounted for individual variation, since palatal imprints yielded 55% of subjects with yeast present, denture plaque sampling 80%, and saliva sampling 95%. Interestingly, in our study we found a high degree of concordance

between the presence of C. *albicans* in the saliva vs. that detectible on the denture (r^2=0.31, p=0.0007). It was also noticed that non-*albicans* species are more prevalent in control subjects especially upon comparing prevalence in the unaffected mucosa in these subjects. However, whether non-*albicans* presence in patient's saliva or mucosa is associated with any protective effect or not, can't be withdrawn as a conclusion from this small cohort.

Exfoliative Cytology Findings in DS

Reports have been variable on whether the inflammation associated with denture stomatitis is secondary to denture trauma or it's just a result of candidal infection. According to Barbeau (J. Barbeau, et al. ,2003), the presence of yeasts on the denture in denture-related stomatitis is probably linked to extensive inflammation. Considering the hypothesis that inflammation could be present before Candida colonization, this could explain the variable results in the treatment of denture-related stomatitis with antifungal treatment alone (J. Barbeau, et al. ,2003). In a randomized clinical trial looking at frequency of DS in patients with mandibular two-implant overdentures as opposed to conventional dentures opposing conventional maxillary denture, Emami et al reported that inflammation due to trauma may create an environment favorable to micro-organisms found in denture stomatitis.

According to Edgerton (M. Edgerton, et al. ,1992), the pathogenesis of stomatitis may be through two separate mechanisms. Alterations in the composition of pellicle formed in stomatitis conditions, such as degradation of pellicle components, may directly promote colonization of *C. albicans* on "stomatitis" pellicle. Alternatively, *C. albicans* may be a secondary colonizer or may require bacterial cell products to stimulate adhesion as has been shown within vitro studies of *Streptococcus mutans* and C. *albicans* (C. Branting, et al.

,1989) . In this case, altered pellicle deposition in the disease process may initially enhance adhesion of other bacteria, which would subsequently promote adhesion of Candida. (M. Edgerton, et al. ,1992)

In a study by Ritchie et al. it was found that bacteria, leukocytes and yeast hyphae could be detected in all patients even when cultures were negative (G. M. Ritchie, et al. ,1969).

Examination of Periodic acid Schiff (PAS) stained smears prepared from denture scrapings showed higher number of yeast cells in DS patients by (E. Budtz-Jorgensen, et al. ,1983) as well. However, in our study the presence of hyphae, as determined from the PAS smear from tissue was not pathognomic for disease.

Conclusions:

hyposalivation, impaired mucosal wetness or xerostomia symptomology does not appear to be associated with the presence of DS. *C. albicans* counts in saliva and on denture surfaces were significantly elevated in DS as compared to health. Denture levels of yeast were higher and more strongly predictive of clinical DS than salivary or mucosal levels, suggesting that the denture, rather than the inflamed palatal mucosa, was the dominant *C. albicans* habitat.

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Chapter 2

Levels of Salivary Cytokines and Candida albicans and Serum C-reactive protein in Denture Stomatitis

Abstract:

Purpose: The aim of this study was to explore the microbial and inflammatory characteristics associated with Denture Stomatitis by characterizing 1) the levels of salivary cytokines and salivary cultivable C. *albicans*, 2) the composition of the biofilm attached to the tissue-bearing denture surfaces as determined by DNA-DNA checkerboard analyses and 3) the serum C-reactive protein levels as a marker of systemic inflammation.

Methods: Thirty-two subjects were enrolled in a case-control study with a control group (n=17), and a diseased group (n=15) with type II and III denture stomatitis (DS) (Newton's classification). Samples of unstimulated whole saliva (UWS), serum and swabs from denture surfaces were collected. Salivary levels of certain inflammatory mediators and serum levels of C-reactive protein were measured by flow multiplex analyses. Salivary cultivable levels of C. *albicans* were expressed as colony-forming units. Samples from denture and mucosal surfaces were analyzed with DNA-DNA checkerboard.

Results: In salivary samples from DS subjects there was a two fold increase in levels of IL-8 (p=0.04) and a 1.8 fold increase in IL-1β (p=0.04) with non-significant trends for increases in IL-1α, TNFα and IL-6. In DS C. *albicans* was found in significantly higher counts in saliva (p=0.03) and there was a significant association between salivary C. *albicans* CFU and levels of both IL-8 (r^2=0.16, P=0.03), and IL-1α (r^2=0.20, P=0.01) There were no significant differences in the serum CRP levels (p=0.74). Although previous studies using cultivable

methods had demonstrated the presence of periodontal pathogens on denture surfaces, using curette samples and DNA-DNA checkerboard analyses we found that typical periodontal pathogens were generally below the detection threshold of 10^4 organisms on both denture and inflamed mucosal surfaces.

Conclusions:

The data suggest that denture stomatitis is associated with significant salivary elevation of the levels of the chemokine IL-8, as well as increased levels of cultivable C. *albicans*. In denture stomatitis there was no evidence of systemic inflammation as measured by serum C-reactive protein levels. The findings from this relatively small exploratory study should be interpreted with caution but provide observations for future investigation.

Introduction

Candida-associated denture stomatitis (CADS) is the most common form of oral candidal infection in denture wearers, (B. C. Webb, et al. ,1998) with Candida *albicans* being the principal etiological agent (E. Budtz-Jorgensen. ,1990),(J. Chandra, et al. ,2001). CADS has been reported in 11%-67% of the otherwise healthy denture wearers (G. Ramage, et al. ,2004), and has been classified according to Newton's work in 1962 into 3 clinical types: Type I - a localized simple inflammation or a pinpoint hyperemia; Type II (mild) : an erythematous or generalized simple type presenting as more diffuse erythema, and Type III(severe): a granular or papillary type (A.V. Newton 1962). This condition has been considered to have a multifactorial etiology, which can include factors related to 1) trauma such as denture wearing, 2) local trauma resulting from salivary hypofunction (J. P. Lyon, et al. ,2006),(C. M. Abraham, et al. ,1998), 3) infection with candida species, or 4) factors related to impaired host defense as in systemic diseases such as HIV infection, leukemia, lymphoma, radiation therapy for head and neck malignancies, chemotherapy, diabetes, hormonal imbalance, anemia, malnutrition, or long-term use of corticosteroid or antibiotics. The multi-factorial etiology for denture stomatitis is reflected in the current treatment regimens which varies from antifungals(B. C. Webb, et al. ,1998), to denture hygiene measures, antiseptics, and use of denture reline materials containing antifungals (A. N. Ellepola, et al. ,2000).

Although some progress has been made in revealing the mechanisms of epithelial and immunoregulatory interactions with Candida, there are major gaps in our knowledge regarding processes that lead to the establishment of candidiasis as an infection and the relationship between mucosal and systemic inflammatory responses. Primary innate defense

mechanisms are known to play key roles in preventing yeast colonization of the oral cavity (R. D. Cannon, et al. ,1995). During oral infection with Candida, a large number of pro-inflammatory and immunoregulatory cytokines such as IL1α, IL1β, IL8, and TNFα, are generated by the oral mucosa (C. C. Villar, et al. ,2008, A. Dongari-Bagtzoglou, et al. ,2005, M. Schaller, et al. ,2004). The main sources of these cytokines have been reported to be of oral epithelial cells. Although infiltrating neutrophils are likely important contributors. Cytokines in saliva are also derived from salivary gland cells (secretory,epithelial cells, fibroblasts, endothelial cells), and leukocyte aggregates within the salivary gland tissues, tonsils, and the oral mucosa , including the periodontium. Therefore, cytokine profiles in whole saliva can depict the collective state of cytokine responses to infectious agents by all tissues in the oral cavity, including accessory salivary glands which are major contributors of secretory IgA(A. Dongari-Bagtzoglou, et al. ,2005). However, the influence of whole saliva composition on candida adherence and invasion is yet to be fully understood.

Biofilms are structured microbial communities in which the cells bind tightly to a surface and become embedded in a matrix of extracellular polymeric substances produced by these cells. Indeed components of saliva or serum proteins, such as mucin, fibrinogen and complementary factors specifically bind to Candida blastospores and germ tubes, possibly modifying the biofilm formation(H. Nikawa, et al. ,2000). Biofilms of differing compositions colonize oral surfaces including the teeth, the mucosa, the tongue and acrylic denture surfaces. These self-replenishing biofilms gain notoriety from their ability to resist antimicrobials and immune cell challenge (J. Nett, et al. ,2006, G. Ramage, et al. ,2001) .

The purpose of this study was to identify the candidate inflammatory mediators present within the saliva of DS subjects. We sought to confirm previous reports establishing the

relationship between DS and increased salivary levels C. *albicans* and to determine if the level of C. *albicans* was associated with the levels of salivary cytokine biomarkers. Finally, since DS is such a common infection among edentate people we accessed whether there was any evidence of systemic inflammatory response as indexed by serum levels of C-reactive protein. Although this was a relatively small exploratory pilot investigation, the data suggest that increased levels of certain salivary cytokines are associated with the presence of DS and related to salivary levels of C. *albicans*.

Materials and methods

An IRB approved case-control study was performed. Thirty-two subjects were enrolled and grouped into two groups: a control group (n=17) with no signs or symptoms of denture stomatitis (DS), and a diseased group (n=15) with type II or III DS (Newton's classification). Inclusion and exclusion criteria, and demographics of this cohort of patients are described in the previous chapter.

Unstimulated Saliva collection and cytokines analysis:

Subjects were instructed to remove their dentures and refrain from eating, drinking, smoking, brushing their teeth or chewing gum for 15 minutes prior to salivary collections. All collections were performed between 9:00 and 11:00 AM. Subjects were instructed to swallow to clear the mouth of any accumulated saliva, and whole unstimulated saliva was allowed to pool in a sterile polypropylene graduated collection vial for 5 minutes. A fraction of the sample was sent immediately to the microbiology lab to prevent overgrowth of the Candida species. Samples were aliquoted into Eppendorf tubes, centrifuged for 10 minutes at 3000g, and the supernatant stored at $-80^{\circ}C$ until analysis. Before cytokines analysis, the

samples were thawed and centrifuged for 10 minutes at 3000g to remove any solids. Specific proteins (IL-1α, IL-1$_β$, IL-6, IL-8, and TNFα) levels in Saliva samples were quantified by ELISA and/or using the Fluorokine[®] MAP cytokine multiplex kits and Luminex[®] analyzer system. Protein levels were log-transformed prior to application of multivariate tests comparing subjects with denture stomatitis to those without denture stomatitis, and comparing diseased and non-diseased sites within subjects with clinical stomatitis.

A fraction of the unstimulated saliva was sampled in Sabouraud Dextrose agar containing quemicetine succinate. Candida colonies were counted after 48 h and the patients were classified according to the number of CFU (Colony-forming units) as follows: negative (CFU/ml = 0), carrier (CFU/ml < 400), and positive (CFU/ml > 400).

Microbiology

Four bacterial biofilm samples were collected from the tissue and the corresponding surface area of the denture using a plastic curette. The palate was divided into four zones (according to the method described by (J. Barbeau, et al. ,2003) figure 2-1, and one biofilm sample was collected from each zone and recorded on a diagnostic diagram. The samples were pooled (4 mucosal samples and 4 denture samples) and placed into a separate Eppendorf tube containing 150 μL of TE buffer. 150 μL of 0.5 M NaOH were added to each sample and frozen at -80°C prior to analyses. The pooled biofilm samples were analyzed quantitatively and qualitatively to measure the presence, absence and levels of 18 dental biofilm organisms by DNA-DNA checkerboard tissue, where median cell count levels of 16 organisms was produced for comparison of subjects with clinical stomatitis to those without clinical

stomatitis using a nonparametric multivariate test. Mean Candida CFU counts were produced

for comparison of the diseased and non-diseased groups.

Figure 2-1. a diagram showing the division of the palate area into quadrants for bacterial
biofilm collection from each of the 4 zones .(J. Barbeau, et al. ,2003)
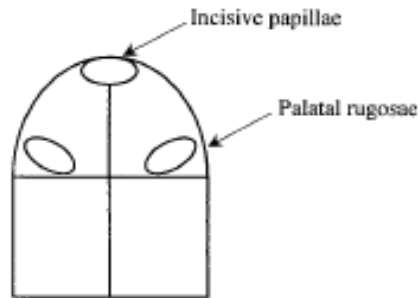
DNA-DNA Checkerboard

Sample deposition on nylon membranes

The samples were boiled in a water bath for 5 minutes and neutralized using 0.8 ml 5 M

ammonium acetate. The released DNA was placed into the slots of a Minislot device

(Immunetics, Cambridge MA) and thereby deposited as "lanes" onto a (formerly Boehringer

Mannheim, now Roche Diagnostics nylon membrane). The DNA was then permanently fixed

to the membrane by exposure to ultraviolet light, followed by baking at 120°C, for 20 min.

The Minislot device permits the deposition of up to 30 different bacterial samples in

individual "lanes" on a single 15 x 15 cm nylon membrane. In order to create reference

signals on each membrane, two of the 'lanes' were used to deposit pooled DNA of known

bacterial numbers (10^5 and 10^6) for each one of the microorganisms included in the analysis.

DNA-DNA hybridization

The membranes were prehybridized in "prehybridization" buffer [50% formamide, 5 x SSC (1 x SSC = 150 mM NaCl, 15 mM Na citrate, pH 7.0), 1% casein (Sigma), 5 x Denhardt's reagent, 25 mM sodium phosphate (pH 6.5) and 0.5 mg/ml yeast RNA (Boehringer-Mannheim)], at 42°C, for 1 hour and then placed back into a "Miniblotter 45", turned 90° to the original orientation. A 30 x 45 "checkerboard" pattern was produced. Digoxigenin-labeled DNA probes dissolved in "hybridization" buffer [45% formamide, 5 x SSC, 1 x Denhardt's reagent, 20 mM Na phosphate (pH 6.5),0.2 mg/ml yeast RNA, 20 ng/ml of labeled probe, 10% dextran sulfate and 1 % casein], were laid in individual lanes of the Miniblotter and the whole apparatus was placed in a sealed plastic bag. Hybridization was allowed to occur overnight, in a shaking water bath, at 42°C. Membranes were then washed once at low stringency to remove loosely bound probe and then twice at high stringency (68°C, 0.1 x SSC, 0.1% SDS, 20 min), in a Disk Wisk apparatus (Schleicher and Schuell).

Blood collection (serum CRP)

Venous blood (5-7 ml) was collected under a sterile technique in a purple top vaccutainer. Blood was processed into serum within 2 hours after collection: the whole blood was kept at room temperature for 30-45 minutes to allow a clot to form, and then centrifuged for 12 minutes to separate the serum from the clot. Serum was then aliquoted into barcode labeled microfuge tubes and quickly frozen at -80°C and stored until analysis using ELISA method. Mean serum CRP levels (mg/ml) were compared in diseased and non-diseased groups.

Statistical analyses

Salivary cytokine levels were not normally distributed and between group comparisons were made using the non-parametric, non-paired Wilcoxon test. Associations between salivary C. *albicans* CFU/ml and cytokine levels were compared by one-way ANOVA and regression analyses. The association between the presence or absence of C. *albicans* and the presence (pooling Type II and Type III) or absence of DS was determined by Chi Square test. A p value <0.05 was considered significant, however no adjustments were made for multiple comparisons and the results should be interpreted with caution.

Results

Salivary Markers comparing Control and Denture Stomatitis

We measured salivary levels of IL-1α, IL-1$_\beta$, IL-6, IL-8 and TNFα. The data from the DS and control subjects appear in Table 2-1.There was a statistically significant increase in salivary levels of IL-8 (p=0.04) and IL-1$_\beta$ (p=0.04) in DS as compared to health. In DS the apparent increase in salivary levels of IL-6, TNFα and IL-1$_\beta$ were not statistically significant. These increases in protein expression were consistent with the increased tissue mRNA expression (data not shown).

A statistically significant association between salivary counts of C. *albicans* and salivary levels of IL-8 (r^2=0.16, P=0.03), and IL-1α (r^2=0.20, P=0.01) was seen as illustrated in Figure 2-2.

Figure 2-3 shows that C. *albicans* was detected in saliva of 80% (12/15) of the DS subjects, and in 35.3% (6/17) of the Healthy subjects, (p=0.03), using the positive cut-off value of (CFU/ml > 400).

**Table 2-1**. Salivary Cytokines levels in control and DS groups

Cytokine	Control group Median (IQR) [ng/mL]	DS group Median (IQR) [ng/mL]	Wilcoxon P value
IL-1α	342 (202-752)	508 (140-1170)	0.47
IL-1ᵦ	**146 (77-328)**	**293 (119-1100)**	**0.04**
IL-6	4.2 (0.0-12.6)	5.2 (0.0-8.4)	0.50
IL-8	**446 (198-847)**	**792 (447-1970)**	**0.04**
TNFα	0.0 (0.0-6.2)	5.8 (0.0-14.2)	0.12

Numbers in bold show statistically significant differences

Figure 2-2: Scatter plots showing the relationship between *C.albicans* counts in saliva and salivary levels of both A) IL-8, and B) IL-1α.
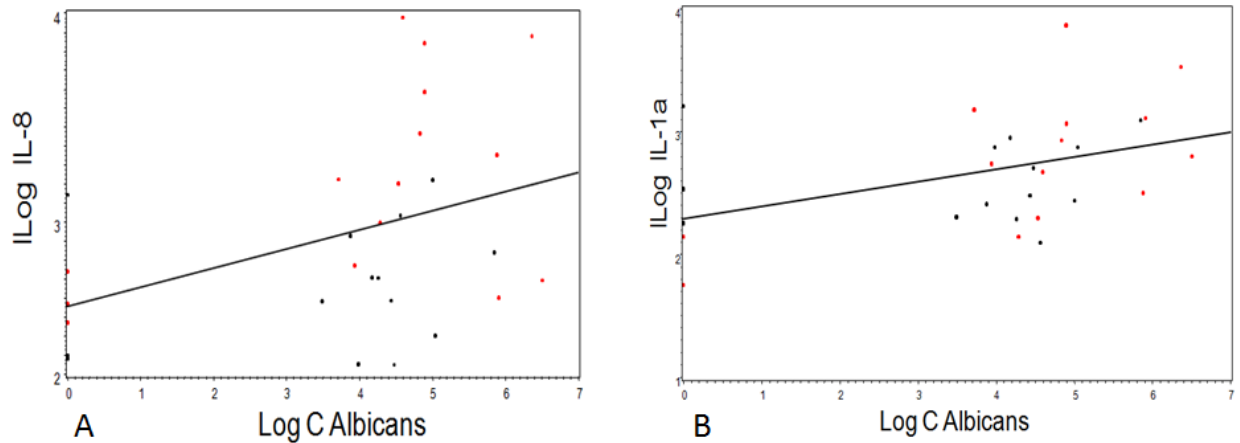
Figure 2-3. A bar graph showing the *C.albicans* prevalence in saliva of DS and control subjects.
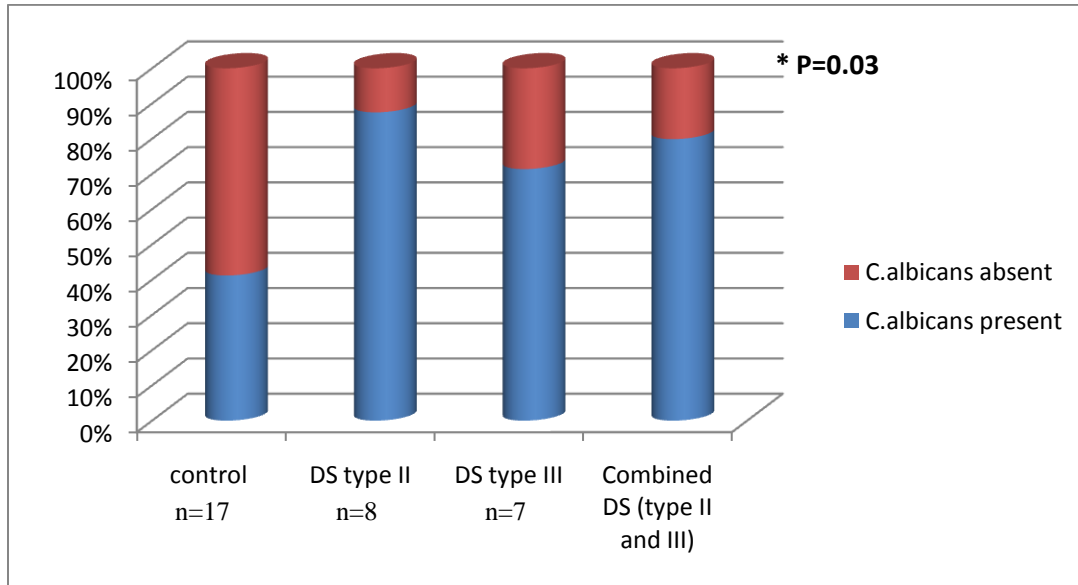
Bacterial Composition of Mucosal and Denture surface biofilm determined by DNA-DNA Checkerboard

Tissue and denture surfaces were sampled using a plastic curette to measure the presence, absence and levels of 16 dental biofilm organisms by DNA-DNA checkerboard. Biofilm samples from the palatal tissue surfaces did not show any detectible organisms, suggesting a level below 10^4 counts. Organisms were detected in the biofilm samples collected from dentures with (3/17) control subjects, (1/ 8) DS Type II, and (1/7) DS Type III. The data for the 3 healthy are compared to the 2 DS in Table 2-2. There were no significant differences in the counts comparing Health to Diseased samples.

Table 2-2. Organisms detected with DNA-DNA checkerboard of biofilm from denture surfaces. Numbers reflect counts x 10^6 CFU

Microorganism	Control (Mean ±SD)	DS (Mean±SD)	P value
P. gingivalis	0.8±1.6	0.9±2.2	0.93
P. intermedia	0.8±1.6	2. 2±4.9	0.42
P. nigrescens	1.8±3.9	2.4±6.0	0.80
B. forsythia	3.4±6.5	3.3±7.4	0.97
T. denticola	2.0±4.0	2.4±5.1	0.84
A. a	2.5±5.0	3.7±7.9	0.64
C. rectus	5.3±10.2	9.1±21.9	0.62
E. corrodens	1.8±3.5	2.2±5.00	0.82
F. nucleatum	15.1±29.1	17.9±38.3	0.85
S. noxa	2.5±5.0	4.0±9.4	0.65
C. ochracea	0.8±1.6	1.4±3.2	0.61
V. parvula	1.3±3.1	1.9±4.9	0.76
S. sanguis	2.2±4.3	2.5±5.4	0.86
S. intermedius	3.1±6.6	3.1±6.5	0.98
S. Oralis	1.6±3.1	3.0±6.4	0.53
A. viscosus	6.3±12.6	8.3±17.8	0.75

Serum CRP

There was no significant difference in the serum CRP level comparing health to DS individuals (p=0.74). We also examined levels of clinically elevated CRP values (CRP<3 mg/ml) as a threshold and again found no difference. Table 2-3.

Table 2-3. CRP levels in control and DS groups

Serum CRP (μg/ml)		
Groups	Mean ± SD	P value
Control (n=17)	4980.1 ± 2601.1	
DS (n=15)	4687.7 ± 2271.8	0.74

Discussion

Cytokines

Increased levels of GM-CSF, IFN-γ, TNF-α IL-8, IL-18 and IL-1α, IL-1β, IL-6, have been identified in the oral mucosa epithelial cell infection models and saliva of subjects with oral candidiasis and in experimental models of oral candidiasis (C. C. Villar, et al. ,2008, A. Dongari-Bagtzoglou, et al. ,2005, A. Dongari-Bagtzoglou, et al. ,2003)

We investigated levels of IL-1α, IL-1β, IL-6, IL-8, and TNFα in saliva and found a statistically significant two fold increase in median salivary levels of IL-8 (p=0.04) and a 1.8-fold increase in IL-1β. The increase in the chemokine IL-8 is consistent with neutrophilic infiltration and IL-1 β is a ubiquitous mediator of inflammation. These increases are in accordance with other studies that looked at these pro-inflammatory cytokines in oral epithelial cells infected with C. *albicans* (C. C. Villar, et al. ,2008, A. Dongari-Bagtzoglou, et al. ,2003, Y. Mostefaoui, et al. ,2004). A study that utilized epithelial cell lines (SCC4, SCC15, and OKF6/TERT-2), showed that all cell lines and primary cultures responded to C.*albicans* with an increase in IL-8 secretion. IL-8 responses were contact-dependent, strain-specific, required yeast viability and germination into hyphae, and were in part, autoregulated

by IL-1α (A. Dongari-Bagtzoglou, et al. ,2003). In an in vitro study on engineered human oral mucosa it was shown that IL-1β mRNA expression significantly increased during the early stages of infection and decreased during later stages (Y. Mostefaoui, et al. ,2004)

TNF-α is known to induce secondary production of other cytokines, such as IFN-γ, IL-1, IL-6, and IL-12 (A. D. Kennedy, et al. ,2007) which further amplifies the protective immune response to C.*albicans* infection (C. C. Villar, et al. ,2008). Reduced levels of C.*albicans* were found on the tongues of infected mice treated by oral administration of TNF-α during oral candidiasis consistent with the protective activity of TNFa which is a potent activator of monocyte-mediated microbial killing. Several studies have also provided a link between lower levels of TNF-α in the oral tissues or saliva of humans and animals with oral C. *albicans* infection (C. C. Villar, et al. ,2008). Thus, TNFα may be an important innate marker of inflammation that is protective by promoting C. *albicans* killing. However, both IL-1β and TNFα are important mediators of bone resorption and may contribute to the alveolar ridge resorption often associated with DS.

We found a significant association between the salivary counts of C. *albicans* and the levels of IL-8 and IL-1α (figure 2). This is suggestive of a dose response relationship where the increasing level of C. *albicans*, triggers increases in IL-8. The synthesis of IL-8 likely arises from mucosal epithelium secretion which is challenged by the presence of C. *albicans*. This increase in IL-8 would lead to neutrophil recruitment within the inflamed mucosa and passage into the saliva via disrupted epithelial integrity. An increase in neutrophils within the saliva has previously been reported in mucositis and in DS subjects (A. Dongari-Bagtzoglou, et al. ,2005, A. Dongari-Bagtzoglou, et al. ,2003, A. Ali, et al. ,2006). IL-8 is a potent

chemokine for neutrophils at nanomolar concentrations and the level in saliva ranged from 447-1970 ng/ml. Neutrophils role as a part of innate immunity also includes contribution towards initiation of cell-mediated immunity, and the importance of cell-mediated immunity against candidal infections through T-cells is well illustrated in AIDS patients that are prone to severe mucosal candidal infections.

C. *albicans* was detected in saliva of 80% (12/15) of the DS subjects, but only 41.2% (7/17) of the Healthy subjects, (p=0.03). Thus, C. *albicans* was twice as likely to be present in saliva if mild or severe DS was present. Although the presence of C. *albicans* was strongly associated with the presence of DS, numerically there was no clear trend for the salivary CFU of C. *albicans* relating to the clinical severity of DS.

Microbiology

It is well understood that adequate attachment of candidal organisms to denture surface is a crucial step in the pathogenesis of denture stomatitis. The formation of biofilms on the surface of the denture protects these organisms from detachment and removal by salivary flow and physical forces (G. Ramage, et al. ,2004, G. Ramage, et al. ,2001). It's also worth noticing that quorum sensing (QS) occurs both in single species bacterial communities and in complex mixed bacterial-yeast communities (J. R. Blankenship, et al. ,2006, T. Pereira-Cenci, et al. ,2008). However, the role played by saliva or serum pellicles during the colonization process and subsequent multilayer biofilm formation is poorly understood (H. Nikawa, et al. ,2000, T. Pereira-Cenci, et al. ,2008, H. Nikawa, et al. ,2006, H. Nikawa, et al. ,2005). Several investigators reported that saliva coating reduces the adherence of C. *albicans* in acrylic resin based materials (T. Pereira-Cenci, et al. ,2008, T. Pereira-Cenci, et al. ,2007, L. P. Samaranayake, et al. ,1980). On the other hand, other studies showed

increased adherence rates with saliva coating {{1427 Edgerton,M. 1992; 1509 Edgerton,M. 1993; 1453 Nikawa,H. 2000; 1510 Nikawa,H. 1993; (T. Pereira-Cenci, et al. ,2008) and other research groups found no effect at all of a(H. Nikawa, et al. ,2000, M. Edgerton, et al. ,1992, M. Edgerton, et al. ,1993, H. Nikawa, et al. ,1993)(H. Nikawa, et al. ,2000, M. Edgerton, et al. ,1992, M. Edgerton, et al. ,1993, H. Nikawa, et al. ,1993) saliva coating (H. Nikawa, et al. ,1992)(T. Pereira-Cenci, et al. ,2008, Y. Jin, et al. ,2004).

In this study, tissue and denture surfaces were sampled to measure the presence, absence and levels of 16 dental biofilm organisms by DNA-DNA checkerboard. Biofilm samples from the palatal tissue surfaces did not show any detectible organism suggesting a level below 10^4 counts. Thus, the test organisms were likely below our detection limit. On the other hand, organisms were detected in the biofilm samples collected from dentures in 3 control, 1 DS Type 2, and 1- DS Type 3. There were no significant differences in the counts comparing Health to Diseased samples. However, since all of the mucosal samples were negative as well as the majority of the denture samples, it appears that the DNA-DNA checkerboard which has a lower detection limit of 10^4 CFU, lacks adequate sensitivity for further analyses. This would suggest that in future studies different approaches can be utilized – such as cultivable, HOMIM (Human Oral Microbe Identification Microarray) or nested PCR (B. Riep, et al. ,2009, V. Lazarevic, et al. ,2010, M. R. Milward, et al. ,2007, S. S. Kirakodu, et al. ,2008, S. C. Gomes, et al. ,2008, R. C. Casarin, et al. ,2010, A. P. Colombo, et al. ,2009). Previous cultivable studies of denture surfaces have shown qualitative and/or quantitative differences between the microorganisms found on the surfaces and depths of the maxillary denture as compared to the respective surfaces and depths of the mandibular denture from different patients and between different geographical regions as well as within the same subject (R. T.

Glass, et al. ,1990, R. T. Glass, et al. ,2010). According to these studies by Glass et al. they found a wide array of oral and systemic disease-producing aerobic and anaerobic microorganisms including gram positive and negative cocci and rods and yeasts from the cultures that were taken from denture surfaces.

Sachdeo et al.(A. Sachdeo, et al. ,2008) have used a similar methodology to the one performed in our study to characterize the microbiota of edentulous oral cavities using checkerboard DNA-DNA hybridization, analyzing samples from mucosal and denture surfaces, and saliva from edentulous patients for 41 bacterial species where they found that A. actinomycetemcomitans and P. gingivalis, which were thought to be eliminated with the extraction of all natural teeth, were seen in significant numbers in the edentulous subjects. These organisms were detected in our study as well. Also, when they compared the mean total DNA probe counts among the dorsal surfaces of the tongue, denture palates, and the subjects' palates, the highest mean counts were found on the tongue dorsum, followed by the polished (exterior) surface of the denture palate, and were lowest on the hard palate. This finding is also consistent with our study where no organisms were detected from the mucosal samples. However, no samples were taken from the polished surfaces of the denture in our case, as we sampled the tissue-bearing surface.

Other studies (E. Theilade, et al. ,1988, E. Theilade, et al. ,1983), have shown that the predominant cultivable flora of denture plaque was Gram-positive cocci (*Streptococci*) and rod-shaped bacteria (D. R. Radford, et al. ,1999). Results from such studies suggest similarity between denture plaque bacterial composition and the periodontal niche in dentate patients. Therefore, consistent with the association of periodontal disease and specific periodontal pathogens on conditions such as cardiovascular disease and poor glycemic control among

diatbetics it has been suggested that denture cleaning is important to oral and systemic health and that the denture-wearing population requires care and follow-up similar to their non-denture-wearing counterparts (A. Sachdeo, et al. ,2008).

C-reactive Protein in Denture Stomatitis

We found no significant difference in levels of CRP between the two groups of patients. Same observation was found by Kostial el. al.(I. Kostiala. ,1984) where CRP values in patients with acute fungal stomatitis in non-immunocompromised patients were within normal ranges (I. Kostiala. ,1984). On the other hand, Ajwani et al. found that edentulous individuals with signs of oral candidosis or denture stomatitis showed elevated levels of serum CRP and salivary microbial counts (S. Ajwani, et al. ,2003). It has been reported that CRP levels are higher in patients with periodontitis (J. D. Beck, et al. ,2002, R. G. Craig, et al. ,2003, F. D'Aiuto, et al. ,2005, J. L. Ebersole, et al. ,2002, T. N. Salzberg, et al. ,2006, G. D. Slade, et al. ,2000), and in our study and elsewhere in the literature it has been demonstrated that there is similarity between the microbial content of denture plaque and that associated with periodontitis. However, CRP levels association with edentulism and the clues it may provide for DS diagnosis is still not understood. In addition, CRP is only marker of the hepatic acute phase response. It is possible that fungal infections, which activate a different microbial pathogen recognition system than bacteria, may specifically trigger the release of different hepatic acute phase reactants, such as alpha anti-trypsin, transferrin or orosomucoid synthesis.

Conclusions

Elevated levels of salivary IL-8 and IL-1$_\beta$ are associated with DS, and IL-8 is positively associated with increased levels of C. *albicans* in saliva. This finding suggests that topical therapeutics to reduce these inflammatory and infectious agents may be logical therapeutic strategies. Denture plaque has similar composition to periodontitis associated microbiota where C. *albicans* acts as an early colonizer facilitating the attachment of anaerobic microorganisms. At this point the data do not support the concept that DS induces systemic inflammation as measured by CRP levels.

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Conclusions

In this report we confirm the association of Candidal overgrowth and identify the denture as the primary infectious reservoir. Data suggests that denture plaque has similar composition to periodontitis associated microbiota which suggests that complete denture wearers should probably have regular follow ups similar to their dentate counter parts. Candida-associated denture stomatitis, even if asymptomatic, should be treated as it may act as reservoir for infections, and could encourage the resorption of the alveolar bone. No significant role was found for both hyposalivation and xerostomia symptoms as possible disease modifiers. Elevated levels of salivary IL-8 and IL-1$_\beta$ are associated with DS, and IL-8 is positively associated with increased levels of C. *albicans* in saliva. However, on the systemic level, data did not support the concept that DS induces systemic inflammation as measured by CRP levels. These findings provide new opportunities for the development of both new diagnostic and therapeutic tools to manage candidal infection including the possible utilization of the chemokines such as IL-8 in the topical therapeutics field. Finally, the findings from this relatively small exploratory study should be interpreted with caution but provide observations for future investigation.