Oral Microbiology Immunology 2008: 23: 377–383
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Mixed *Candida albicans* and *Candida glabrata* populations associated with the pathogenesis of denture stomatitis

B. J. Coco[1], J. Bagg[2], L. J. Cross[2], A. Jose[2], J. Cross[2], G. Ramage[2]

[1]Department of Biological and Biomedical Sciences, Glasgow Caledonian University, [2]Glasgow Dental School, Faculty of Medicine, University of Glasgow, Glasgow, UK

Coco BJ, Bagg J, Cross LJ, Jose A, Cross J, Ramage G. Mixed Candida albicans *and* Candida glabrata *populations associated with the pathogenesis of denture stomatitis. Oral Microbiol Immunol 2008: 23: 377–383.* © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard.

Introduction: Oral yeasts are an important component of the resident microbial ecology of the oral cavity, but they are also associated with various forms of oral candidosis, such as denture stomatitis. Although *Candida albicans* is the predominant oral fungal pathogen, other species may also play an integral role in pathogenesis. The aim of this study was to examine the mycological ecology in patients with denture stomatitis, using an improved sampling technique, to determine whether species diversity and species quantity were related to oral pathology.

Methods: Thirty-seven patients attending the Glasgow Dental Hospital were enrolled in this study following informed consent. A full clinical history was obtained, including details of their oral hygiene practices and the levels of erythema based on Newton's classification scale. Oral rinse, denture sonicate, and swab samples were taken, which were processed for quantitative and qualitative analysis of oral yeasts.

Results: The proportion of patients with no inflammation or Newton's Types I, II, and III were 31, 33, 25, and 14%, respectively. Denture sonication was a superior sampling procedure, with statistically greater quantities of yeasts isolated using this methodology ($P < 0.01$). The predominant oral yeasts isolated were *C. albicans* (75%) and *Candida glabrata* (30%), which were isolated in higher proportions in patients with the highest grades of inflammation (100 and 80%), and in combination from 80% of these patients.

Conclusions: This study has demonstrated that mixed *C. albicans* and *C. glabrata* biofilms may play an important role in the pathogenesis associated with severe inflammation in denture wearers.

Key Words: *Candida albicans*; *Candida glabrata*; clinical sampling; denture stomatitis

Gordon Ramage, Section of Infection and Immunity, University of Glasgow Dental School, 378 Sauchiehall Street, Glasgow, G2 3JZ, UK
Tel.: +44 141 211 9752;
fax: +44 141 353 1593;
e-mail: g.ramage@dental.gla.ac.uk
Accepted for publication January 7, 2008

Oropharyngeal candidosis is a common infection among the immunocompromised and elderly, resulting in significant morbidity including oral pain and burning, altered taste sensation, and nutritional compromise (13, 41, 48). Immunosuppression as a result of various diseases and their treatments, xerostomia secondary to autoimmune diseases, radiation therapy for head and neck cancer, and antibiotic therapies all predispose to the development of these infections as a result of changes in the local immune defenses and oral ecology (12, 14, 24, 31, 37, 39, 40). There are a variety of clinical presentations of oropharyngeal candidosis, including pseudomembranous candidosis (thrush) and erythematous forms such as denture-induced stomatitis, which are often recurrent (6, 28).

The wearing of prosthetic appliances is commonly associated with denture stomatitis, which is characterized by an inflamed mucosa, particularly under the upper

denture (47). Patients may complain of a burning sensation, discomfort, or bad taste, but in the majority of cases they are unaware of the problem. There are many factors that influence the onset and severity of denture stomatitis, including salivary flow, denture cleanliness, denture base material, age of denture, denture trauma, continuous denture wearing, smoking, dietary factors, and pH of denture plaque (29, 43–45). Denture stomatitis is a disease of fungal and bacterial origin, with *Candida albicans* the most frequently isolated yeast from the oral cavities of these patients (2, 26). In recent years, various studies have isolated other *Candida* species alongside *C. albicans*, which may be involved in the pathogenesis of patients with oropharyngeal candidosis (3, 8, 21, 39). A wide variety of organisms have been shown to cause infection, with *Candida glabrata*, *Candida dubliniensis*, *Candida parapsilosis*, *Candida krusei*, and *Candida tropicalis* being the most commonly described (23). The emergence of other *Candida* species is significant because they are frequently resistant to the commonly used antifungal agents (3, 21, 38). In a recent study by Bagg et al. it was reported that *C. glabrata* was isolated from the mouths of 22.7% of patients with advanced cancer, and that 72% of these strains were resistant to both fluconazole and itraconazole (3).

Nonetheless, *C. albicans* remains the most important and predominant oral fungal pathogen. It has the ability to adhere and proliferate upon both soft and hard tissue surfaces within the oral cavity, a growth modality referred to as biofilms (32, 33). *C. albicans* biofilms are heterogeneous, complex communities of cells that grow as a consortium on both biological and inanimate surfaces. Hyphae and extrapolymeric material are fundamental to the biofilm growth modality because of their maintenance of structural integrity, adhesive capacity, and contribution to antifungal resistance (1, 7, 35). The adhesive capacity of *Candida* spp. biofilms plays a key role in our ability to sample the oral cavity accurately in terms of qualitative and quantitative data. Conventional sampling techniques include oral rinses, imprint cultures, and swabbing of specific oral tissue sites (3). These techniques may provide qualitative information but they may not provide accurate quantitative data. Samaranayake et al. reported that concentrated oral rinse samples were more sensitive than neat rinse samples and imprint cultures (42). However, it is not clear whether adherent biofilm cells are removed during these procedures, nor whether they are simply loosely adherent cells residing at the outer peripheries of the biofilm, which has important implications for subsequent *in vitro* studies because of the phenotypic variations exhibited within complex monospecies biofilms (15). Furthermore, the denture remains the most prominent source and protective environment for fungal biofilm, but sampling of the denture is generally confined to swab cultures (33). This raises the question of whether these current sampling techniques provide a sufficient reflection of the complex oral mycological ecology developing in both immunocompetent and immunocompromised patient cohorts, and whether the data generated enable differentiation between colonization and infection. These issues are critical because of the increasing incidence of resistant species of *Candida*, such as *C. glabrata*, which will direct subsequent patient management through antifungal treatment.

The aim of this study was to examine the mycological ecology from a cohort of patients with denture stomatitis, using novel sampling procedures to determine if species diversity and species quantity are related to oral pathology.

Materials and methods

Patients

The protocol for the study was approved by the local ethical review committee (West Glasgow Ethics Committee; NHS Greater Glasgow), and all patients gave informed consent to their participation in the study. The study group comprised 37 wearers of complete dentures who were assessed for clinical evidence of denture stomatitis. They were recruited from the Oral Medicine and Prosthodontic Departments of Glasgow Dental Hospital and School. Each of the patients received standard dental hygiene advice during their assessment and was later provided with replacement dentures.

Clinical assessment

All patients were examined by one experienced investigator (L.J.C.), who collected a full medical and dental history and undertook a comprehensive oral examination. The age and fit of dentures, together with existing denture hygiene practices, were recorded. Denture stomatitis, if present, was classified as Newton's Type I (localized erythematous), Newton's Type II (diffuse erythematous), or Newton's Type III (hyperplastic granular) (27). Those patients with no evidence of denture stomatitis, exhibiting pink, healthy palatal mucosa (9), comprised the control group. Asymptomatic carriage was recorded for our uninfected control group (score of 0), represented by pink, healthy tissue (9).

Denture sonication

During clinical assessment the upper denture was removed from the mouth of the patient. The denture was subsequently placed inside a sterile polythene bag and immersed in 50 ml phosphate-buffered saline solution (PBS; Sigma, Gillingham, UK). Adherent microorganisms were removed from the denture by mild sonication at 35 kHz for 5 min (Fisherbrand®; Thermo Fisher Scientific Inc., Loughborough, UK). This has been shown previously to have no detrimental effect on microbial viability (46). The sonicate solution was then aseptically transferred into a sterile polypropylene centrifuge tube (Corning Incorporated - Life Sciences, Oneonta, NY) and retained for mycological analysis.

Conventional sampling

An oral rinse sample was collected while the dentures were being processed. Patients were asked to swish 9 ml sterile PBS solution in their mouths for 1 min, which was expectorated back into the bottle for collection and subsequent mycological processing. Oral swabs were also taken from the entire fitting surface of the denture before sonication, and from the palate, avoiding any relief areas if present. These samples were then processed as described below.

Mycological processing and identification

Oral rinse, swab, and sonicate samples were transported to the laboratory from the clinic and processed within 30 min of collection. Serial decimal dilutions from 10^0 to 10^{-2} of both the oral rinse and denture sonicate samples were prepared in PBS. These were vortexed vigorously for 30 s between dilutions to maximize the homogeneity of the suspensions. Each dilution (100 µl) was spread plated on both CHROMAgar ™ medium and Sabouraud agar (Life Technologies, Paisley, UK). These were incubated at 37°C for 48 h. Quantitative and qualitative microbiological assessments were performed, recording the total numbers of colonies, their color and their morphological characteristics. A tentative identification of specific *Candida* species was determined by colony color on the CHROMAgar™

medium, but all identifications were confirmed by biochemical tests. Distinct colonies were chosen for each species: three from the oral rinse sample, three from the denture sonicate sample, and one colony from the swab where appropriate. These were subcultured onto Sabouraud agar and incubated for 24 h at 37°C. Single morphotypes were identified using the API 32 C biochemical testing panel, following the manufacturer's instructions (bioMérieux UK Ltd, Basingstoke, UK). Each isolate was also subjected to identification validation using the Vitek® 2 automated identification system (bioMérieux UK Ltd). Subsequently, the colonies were subcultured and transferred to MicroBead storage tubes (ProLab Diagnostics, Neston, Cheshire, UK) and stored at −80°C for future analysis.

Statistical analysis

The quantities of *Candida* spp. isolated from each denture were compared by one-way analysis of variance and using Bartlett's test for homogeneity of variances and Bonferroni's multiple comparison post-test. A value of $P < 0.05$ was considered significant. The analyses were performed using SPSS 13.0 for Windows (SPSS Incorporated, Chicago, IL).

Results

Clinical assessment of denture stomatitis

Full patient histories, oral swabs, and oral rinse and denture sonicate samples were obtained for 37 patients at the Glasgow Dental Hospital and School. Eleven (31%) of the study participants comprised an uninfected control group (Type 0, healthy tissue). Twelve (33%) presented with Newton's Type I localized erythema (slightly red or swollen tissue), nine (25%) presented with Newton's Type II diffuse erythema (moderately red or swollen), and five (14%) presented with Newton's Type III severe erythema (severely red, swollen upper palate tissue). Table 1 provides a comprehensive description of the various species of oral yeast and the relative quantities isolated from specific oral sample sites.

Specimen collection

A swab from the denture, an oral rinse, and a denture sonicate were taken from each patient in the study. Of the samples processed, 14 swabs (39%), 29 oral rinses (81%), and 27 denture sonicates (75%) produced visible yeast growth on CHROMAgar™. Growth of yeasts from the same species was observed on both the swab and oral rinse in 83% of the study participants, from the swab and denture sonicate in 81% of the study participants, and for the oral rinse and denture sonicate in 72% of the study participants. When yeast growth upon the swabs, oral rinses, and denture sonicates was compared, the same species grew from all samples in 69% of the study participants examined. The sampling efficiency in the Newton's Type III patient group was superior, as 80% of the study participants had the same species identified from each clinical sample, whereas this figure in the Newton's Type II, I, and 0 groups was 78, 50, and 63%, respectively. In addition, there were more individual yeast species isolated from the oral rinsing procedure (43) than by denture sonication (37).

Identification of oral yeasts

C. albicans was the predominant oral yeast; it was isolated from 75% (28 patients) of the 37 study participants (Table 1). *C. glabrata* was the next most common yeast (29.73%; 11 patients), followed by *Saccharomyces cerevisiae* (10.81%, four patients), *C. krusei* (5.40%, two patients), *C. parapsilosis* (2.70%, one patient), *C. tropicalis* (2.70%, one patient), and *C. famata* (2.70%, one patient). When the distribution of the oral yeasts was analyzed in relation to Newton's classification, the proportion of patients from whom *C. albicans* was isolated increased relative to the highest grades of inflammation, i.e. Newton's Type 0, 54.45% (five patients); Newton's Type I, 66.67% (eight patients); Newton's Type II 100% (all nine patients); and Newton's Type III 100% (all five patients). Figure 1 illustrates the relative proportion of oral yeasts isolated from the study participants per Newton's type. The proportion of patients from whom *C. glabrata* was isolated was as follows: Newton's Type 0, 27.27% (three patients); Newton's Type I, 25% (three patients); Newton's Type II, 11.11% (one patient); and Newton's Type III, 80% (four patients); and for the other oral yeasts the proportions were: Newton's Type 0, 36.36% (four patients); Newton's Type I, 16.66% (three patients); Newton's Type II, 11.11% (one patient); and Newton's Type III, 20% (one patient).

Several study participants had more than one species of yeast within the oral cavity (35.10%, 13 patients). Mixed species populations were observed in each group of study participants: in 27.27% (three patients) of the control group, 33.33% (four patients) for Newton's Type I, 22.22% (two patients) for Newton's Type II, and 80% (four patients) for Newton's type III erythema. The combination of *C. albicans* and *C. glabrata* was the most prevalent of all yeast mixtures, isolated from 25% of all study participants. When placed into groups by Newton type this combination was greatest in patients with Type III disease (80%, four patients). In the other groups this was much less pronounced, i.e. Type 0, 9.09% (one patient); Type I, 25% (three patients); and Type II, 11.11% (one patient).

Quantitative analysis

Table 2 shows the mean total colony numbers grouped into the three Newton's Types, including the asymptomatic control. When the denture sonicates were compared to the oral rinses from each corresponding Newton Type there were at least 10-fold more total colonies isolated form the sonicates. Interestingly, comparison of the oral rinse samples for all groups showed no significant differences in the quantities of yeasts grown, whereas when each group was compared to the asymptomatic control samples for the denture sonicates, only Type II and Type III were significantly greater than the control ($P < 0.01$). There was no statistical difference between Type I and any of the other groups.

Oral hygiene

A detailed patient history was taken during sample collection, including details of each patient's age, smoking habits, age of denture, and denture fit, as well as the patient's brushing and oral hygiene habits, which are listed in Table 3. The average participant age in this study was 73 years (range 49–89 years), with dentures of an average age of 8.6 years (range 1–44 years). Seventy-two per cent of the asymptomatic control group removed their dentures overnight and 91% exercised at least a minimal level of oral hygiene. At least 63% of these participants soaked their denture for 8 h in denture cleansing products on at least 2 days of the week, and an equal proportion cleansed their denture by brushing. Overall, this group displayed good oral hygiene, with only 15% defined as smokers. Study participants with low level inflammation (Type I) exhibited generally poor oral hygiene, with 17% of these being smokers. Only 33% removed their denture overnight and 17% soaked

Table 1. Mycological ecology for Newton's Type 0 to Type III

		Oral rinse		Denture sonicate	
Patient	Swab	Species	Total CFU	Species	Total CFU
Newton's Type 0					
GDS 4	*C. albicans*	*C. albicans*	9.00×10^4	*C. albicans*	8.00×10^5
GDS 8	*C. albicans*	*C. albicans*	1.44×10^5	No growth	0
	C. glabrata	*C. glabrata*	4.32×10^5	No growth	0
GDS 10	*C. krusei*	*C. krusei*	1.89×10^5	*C. krusei*	5.75×10^3
GDS 12	No growth	*C. parapsilosis*	5.54×10^3	No growth	0
GDS 14	No growth	*C. albicans*	3.78×10^5	No growth	0
GDS 16	No growth	No growth	0	*C. albicans*	2.10×10^6
GDS 22	*C. glabrata*	*C. glabrata*	1.31×10^4	*C. glabrata*	2.15×10^6
	C. famata	*C. famata*	3.06×10^4	*C. famata*	1.70×10^7
GDS 23	*C. glabrata*	*S. cerevisiae*	7.20×10^4	*C. glabrata*	7.00×10^5
	S. cerevisiae				
GDS 25	*C. albicans*	*C. albicans*	5.85×10^3	*C. albicans*	2.70×10^6
GDS 34	No growth	No growth	0	No growth	0
GDS 35	No growth	No growth	0	No growth	0
Newton's Type I					
GDS 5	No growth	No growth	0	*C. albicans*	1.50×10^5
GDS 6	No growth	*C. albicans*	9.90×10^3	No growth	0
GDS 9	*C. albicans*	*C. albicans*	2.7×10^3	No growth	0
GDS 11	*C. krusei*	*C. krusei*	2.88×10^4	*C. krusei*	3.00×10^4
	S. cerevisiae	*S. cerevisiae*	1.26×10^5	*S. cerevisiae*	1.00×10^4
GDS 13	No growth	*C. albicans*	6.75×10^5	No growth	0
	No growth	*C. glabrata*	7.56×10^4	No growth	0
GDS 15	No growth	*C. albicans*	5.40×10^2	*C. albicans*	3.50×10^5
GDS 18	*C. albicans*	*C. albicans*	2.61×10^3	*C. albicans*	1.90×10^6
GDS 24	No growth	*S. cerevisiae*	2.34×10^3	No growth	0
GDS 26	*C. albicans*	*C. albicans*	2.79×10^5	*C. albicans*	2.70×10^6
	C. glabrata	*C. glabrata*	2.34×10^6	*C. glabrata*	2.50×10^8
GDS 29	*C. albicans*	*C. albicans*	5.76×10^5	*C. albicans*	1.04×10^8
	C. glabrata	*C. glabrata*	6.3×10^3	*C. glabrata*	2.5×10^8
GDS 36	No growth	No growth	0	No growth	0
GDS 37	No growth	No growth	0	No growth	0
Newton's Type II					
GDS 2	No growth	*C. albicans*	1.23×10^4	*C. albicans*	3.10×10^6
GDS 3	No growth	No growth	0	*C. albicans*	1.96×10^8
GDS 17	*C. albicans*	*C. albicans*	5.21×10^5	*C. albicans*	2.06×10^8
GDS 19	*C. albicans*	*C. albicans*	4.72×10^4	*C. albicans*	5.80×10^6
	C. tropicalis	*C. tropicalis*	9.45×10^2	*C. tropicalis*	5.00×10^4
GDS 20	*C. albicans*	*C. albicans*	2.43×10^4	*C. albicans*	3.41×10^6
GDS 27	*C. albicans*	*C. albicans*	9.36×10^4	*C. albicans*	1.80×10^7
GDS 28	*C. albicans*	*C. albicans*	1.05×10^6	*C. albicans*	3.00×10^8
	C. glabrata	*C. glabrata*	4.28×10^5	*C. glabrata*	3.00×10^8
GDS 30	*C. albicans*	*C. albicans*	1.07×10^5	*C. albicans*	1.00×10^4
GDS 33	*C. albicans*	*C. albicans*	7.25×10^5	*C. albicans*	7.00×10^7
Newton's Type III					
GDS 1	No growth	*C. albicans*	1.13×10^4	*C. albicans*	7.00×10^6
		C. glabrata	1.22×10^4	*C. glabrata*	1.18×10^6
GDS 7	*C. albicans*	*C. albicans*	5.77×10^5	*C. albicans*	1.75×10^6
	C. glabrata	*C. glabrata*	8.99×10^5	*C. glabrata*	4.05×10^7
GDS 21	*C. albicans*	*C. albicans*	6.35×10^4	*C. albicans*	3.50×10^7
GDS 31	*C. albicans*	*C. albicans*	9.00×10^3	*C. albicans*	4.50×10^5
	C. glabrata	*C. glabrata*	1.71×10^4	*C. glabrata*	1.05×10^8
GDS 32	*C. albicans*	*C. albicans*	3.65×10^5	*C. albicans*	1.91×10^8
		C. glabrata	1.39×10^5	*C. glabrata*	1.13×10^7
		S. cerevisiae	6.30×10^4	*S. cerevisiae*	7.45×10^6

CFU, colony-forming units; *C.*, *Candida*; *S.*, *Saccharomyces*.

their denture for up to 8 h in recognized cleansing products. Nevertheless, a total of 58% did soak their denture infrequently, and 83% chose to brush their denture and occasionally their palate. Patients with Newton's Type II denture stomatitis also exhibited poor oral hygiene, with 33% of these being smokers. Dentures were removed overnight from 45% of the patients in this group, and 33% did not undertake any oral hygiene. Although soaking the denture in a cleansing product was reported, only 22% of the patients did so on a regular basis. Those with the highest grade of oral inflammation showed the poorest oral hygiene, with 60% being smokers. None of these patients removed their dentures at night, and only one patient regularly cleansed his/her dentures. Sixty per cent reported that they soaked their dentures in cleansing agents for short periods of time, or brushed their dentures. The remaining 40% did not undertake any oral hygiene.

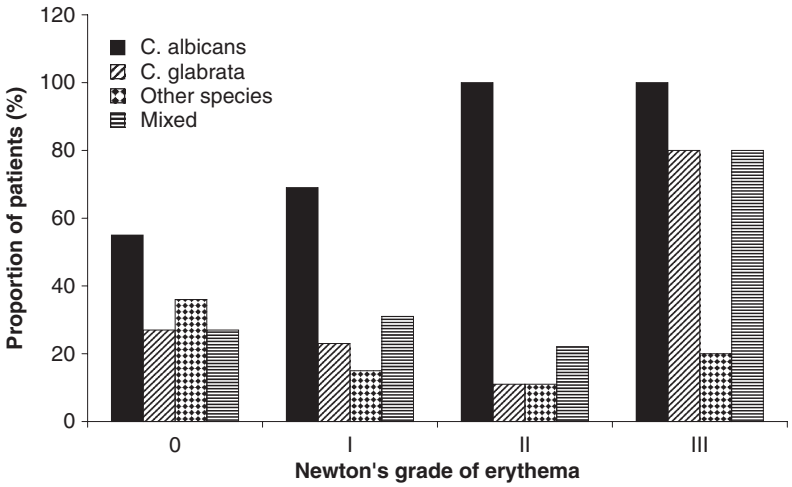

Fig. 1. Distribution of yeast species from the oral cavities of patients. Graph illustrating the relationship between yeast species isolated and Newton's type of erythema. Each group (Type 0, 11 patients; Type I, 12 patients; Type II, 9 patients; and Type III, 5 patients) represents the proportion of patients from whom *Candida albicans*, *Candida glabrata*, *Candida* sp., and mixed *Candida* sp. were isolated.

Table 2. Collective quantitative analysis of patients with denture stomatitis

	Total CFU/type	
Newton type	Rinse	Sonicate
0	1.24×10^5	2.31×10^6
I	4.12×10^5	5.08×10^7
II	3.35×10^5	6.59×10^7
III	4.31×10^5	8.01×10^7

CFU, colony-forming units.

Table 3. Summary of the oral hygiene of patients with denture stomatitis

Newton type	Mean age of denture (years)	Poor fitting denture (%)	Smoker (%)	Basic oral hygiene (%)	Overnight removal and cleansing (%)
0	10	63	9	91	72
I	7	58	17	83	33
II	4	67	33	67	44
III	21	60	60	60	0

Discussion

Denture stomatitis is a debilitating oral disease that can drastically reduce the quality of life for some individuals. Many denture wearers are also unaware that they have oral candidosis, and this could potentially lead to long-term complications if their immune status were to alter. In this study we have shown that *C. albicans* is the predominant oral yeast, isolated from 75% of the participants in this study, which was elevated to 81% in those with clinically defined denture stomatitis. This rate is similar to that reported by a study by Dar-Odeh and Shehabi who showed that *C. albicans* was isolated from 73% of patients with denture stomatitis (11). In another study, the rate of *C. albicans* colonization of denture wearers was 67% (10). Although *C. albicans* remains the predominant yeast associated with denture stomatitis, the levels of inflammation, especially on the palate, cannot be accounted for simply by the presence of this opportunistic yeast. Other factors must account for the severity of disease, such as the mycological ecology (i.e. species diversity), the quantities of yeast cells present, and the overall growth characteristics. In addition, oral hygiene practices play an important role.

One of the most significant findings from this study is the continued emergence of the yeast pathogen, *C. glabrata*, which is of growing cause for concern (21). In this study *C. glabrata* was isolated from 31% of those with clinically defined denture stomatitis, and from 31% of all study participants. These rates of colonization are similar to other studies of oral candidosis. For example, Bagg et al. isolated *C. glabrata* from 22.7% of the oral flora of patients with advanced cancer, and similarly Lockhart et al. demonstrated an isolation rate of 29% from elderly denture wearers (3, 22). However, Masia Cunuto et al. reported a lower isolation rate of 10% from the oropharyngeal flora of patients who were positive for human immunodeficiency virus (25). Nevertheless, there is an emerging trend developing that suggests that *C. glabrata* is much more than the innocent bystander that was once suspected. Studies by Pfaller et al. have shown that the mycological ecology and presence of *C. glabrata* is a function of age rather than of drug selection, which may account for the elevated rates we report herein (30). However, in this study *C. glabrata* was isolated from 80% of patients with the highest grade of inflammation, compared to 27% from the asymptomatic control groups.

Whether *C. glabrata* is able to contribute fully towards disease is not clear. Like *C. albicans*, it has the ability to adhere and colonize, produce extracellular enzymes, form filaments, and phenotypically switch, which is the subject of a recent review (21). Li and Dongari-Bagtzoglou recently demonstrated that *C. glabrata* exhibited the ability to induce proinflammatory cytokines within an oral epithelial cell line, but was overall less cytotoxic than *C. albicans* (19). Interestingly, *C. glabrata* was never isolated as a single species from any sample, and was instead found in combination with *C. albicans* on 83% of occasions. The complex interactions between these two species are not well defined, but these findings suggest that a synergistic relationship may be involved in the enhanced pathogenic potential of this combination. However, this has not been shown to be the case in limited *in vitro* investigations (20). Nevertheless, it has been shown that *C. albicans* and *C. glabrata* are able to form biofilms on inanimate substrates (7, 17, 36), and this may form the foundation for their coaggregation, colonization, and potential pathogenic relationship.

The formation of biofilms on the surface of the denture protects these organisms from detachment and removal by salivary flow and physical forces (33). As part of our rigorous study criteria we followed the assumption that the organisms associated with inflammation of the palate were attached to the denture as biofilm communities. To our knowledge, we are the first group to adopt an enhanced sampling technique to obtain an accurate quantitative and qualitative assessment of the fitted

denture, i.e. mild sonication of the denture, as opposed to crude swabbing techniques. Qualitatively there is an association between the species isolated during swabbing, oral rinsing, and denture sonication (72%), which was strongest in the Type III group (80%). Not surprisingly, the quantitative data obtained through this additional procedure produced a dramatic minimum 10-fold increase in the numbers of yeasts isolated. Oral rinse samples from each group showed no significant quantitative differences from one another, and could therefore not be used in isolation to diagnose denture stomatitis based on colony counts. Interestingly, denture sonicates from the two most inflamed cohorts had statistically greater quantities of yeast cells upon their dentures than those with asymptomatic carriage or low-level inflammation ($P < 0.01$), suggesting that this quantitative methodology could be used to differentiate colonization from infection.

Other factors may account for the dramatic quantitative differences reported here. For example, oral hygiene plays a strong role in the overall oral health of denture stomatitis patients, such as denture cleansing frequency and the age of the denture. Kanli et al. reported that only 16.7% of dentures worn by the elderly were regularly cleaned (16). It is clear from our analysis that those in the asymptomatic control group exhibited good general oral hygiene procedures (Table 2), whereas there was a gradual regression of oral hygiene in the patients with denture stomatitis, particularly those with Newton's Type III erythema. None of this group removed their denture overnight, which may reveal why greater quantities of yeasts were isolated. In addition, it is likely that those with better oral hygiene would clean their denture before arriving at the dental clinic. This may also explain why in some instances more individual yeast strains were isolated from oral rinses compared to denture sonicates. These factors cannot be controlled for during the study design. However, previous studies by Ramage et al. have shown that cells within the biofilm tend to be found in cracks and crevices upon the denture acrylic, which mechanical brushing and denture disinfectants are unable to remove (33). Therefore, although possible, it is unlikely that the higher yeast counts relate purely to oral hygiene. Microscopic examination of the denture sonicates revealed that large clumps of yeast and hyphal cells were present, in masses greater than 100 cells, whereas the oral rinse samples contained a homogeneous suspension of ovoid yeast cells and debris (results not presented). This indicates that the total colony counts reported for the denture sonicates are an underestimate. This is in agreement with other studies that have shown that hyphal formation is a requisite for biofilm formation (35). It is likely that these hyphal forms help to maintain the structural integrity of the denture-associated biofilm, and act as a sanctuary for the smaller *C. glabrata* cells. Interestingly, there were no statistically significant quantitative differences between the denture sonicates from Type II and Type III erythema, which suggests that the pathogenic process is not a sole function of yeast numbers, but of the specific organisms present. Although the number in the cohort of patients with Newton's Type III erythema examined was low (five), *C. glabrata* was isolated in significant numbers from all but one of these patients. Several studies have shown that *C. glabrata* is able to form pseudohyphae under certain environmental conditions, such as during nitrogen starvation (4, 5, 18). This morphology has been shown previously to support adhesion and biofilm formation by non-*C. albicans* species, which may enhance the pathogenicity of *C. glabrata* in the oral cavity (34).

Overall, we have shown that *C. glabrata* may be associated with denture stomatitis, particularly when in combination with *C. albicans*. Whether this relationship contributes to enhanced pathogenicity remains to be elucidated. An association with higher levels of inflammation suggests that this is indeed worth pursuing. This study is the first to use enhanced sampling techniques of the denture, which provided detailed quantitative and qualitative data that were invaluable for interpreting the mycology associated with denture stomatitis. Li et al. recently demonstrated no synergy between *C. albicans* and *C. glabrata* in terms of cytokine induction using an oral epithelial cell model (20). However, these observations were strain-specific, and whether the organisms isolated within this study from the higher grades of erythema exhibited similar effects remains to be elucidated. Further studies are currently ongoing to examine *C. albicans* and *C. glabrata* mixed biofilms in terms of antifungal therapy and pathogenicity.

Acknowledgments

We would like to acknowledge the Chief Scientist Office (CZG/1/152) for financial support. B.C. was funded by a Glasgow Caledonian University Department of Biological Sciences PhD studentship.

References

1. Al-Fattani MA, Douglas LJ. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. J Med Microbiol 2006: **55**: 999–1008.
2. Baena-Monroy T, Moreno-Maldonado V, Franco-Martinez F, Aldape-Barrios B, Quindos G, Sanchez-Vargas LO. *Candida albicans*, *Staphylococcus aureus* and *Streptococcus mutans* colonization in patients wearing dental prosthesis. Med Oral Patol Oral Cir Bucal 2005: **10**(suppl 1): E27–E39.
3. Bagg J, Sweeney MP, Lewis MA et al. High prevalence of non-albicans yeasts and detection of anti-fungal resistance in the oral flora of patients with advanced cancer. Palliat Med 2003: **17**: 477–481.
4. Brockert PJ, Lachke SA, Srikantha T, Pujol C, Galask R, Soll DR. Phenotypic switching and mating type switching of *Candida glabrata* at sites of colonization. Infect Immun 2003: **71**: 7109–7118.
5. Calcagno AM, Bignell E, Warn P et al. *Candida glabrata* STE12 is required for wild-type levels of virulence and nitrogen starvation induced filamentation. Mol Microbiol 2003: **50**: 1309–1318.
6. Cannon RD, Holmes AR, Mason AB, Monk BC. Oral *Candida*: clearance, colonization, or candidiasis? J Dent Res 1995: **74**: 1152–1161.
7. Chandra J, Mukherjee PK, Leidich SD et al. Antifungal resistance of candidal biofilms formed on denture acrylic *in vitro*. J Dent Res 2001: **80**: 903–908.
8. Coleman D, Sullivan D, Harrington B et al. Molecular and phenotypic analysis of *Candida dubliniensis*: a recently identified species linked with oral candidosis in HIV-infected and AIDS patients. Oral Dis 1997: **3**(suppl 1): S96–S101.
9. Cross LJ, Bagg J, Wray D, Aitchison T. A comparison of fluconazole and itraconazole in the management of denture stomatitis: a pilot study. J Dent 1998: **26**: 657–664.
10. Daniluk T, Tokajuk G, Stokowska W et al. Occurrence rate of oral *Candida albicans* in denture wearer patients. Adv Med Sci 2006: **51**(suppl 1): 77–80.
11. Dar-Odeh NS, Shehabi AA. Oral candidosis in patients with removable dentures. Mycoses 2003: **46**: 187–191.
12. Davies AN, Brailsford SR, Beighton D. Oral candidosis in patients with advanced cancer. Oral Oncol 2006: **42**: 698–702.
13. Finlay I, Davies A. Fungal infections. In: Davies A, Finlay I ed. Oral care in advanced disease, 1st edn. Oxford: Oxford University Press, 2005: 55–71.
14. Guggenheimer J, Moore PA, Rossie K et al. Insulin-dependent diabetes mellitus and oral soft tissue pathologies: II. Prevalence and characteristics of Candida and Candidal lesions. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2000: **89**: 570–576.
15. Jin Y, Samaranayake YH, Yip HK, Samaranayake LP. Characterization of switch

phenotypes in *Candida albicans* biofilms. Mycopathologia 2005: **160**: 191–200.
16. Kanli A, Demirel F, Sezgin Y. Oral candidosis, denture cleanliness and hygiene habits in an elderly population. Aging Clin Exp Res 2005: **17**: 502–507.
17. Kumar CP, Menon T. Biofilm production by clinical isolates of *Candida* species. Med Mycol 2006: **44**: 99–101.
18. Lachke SA, Joly S, Daniels K, Soll DR. Phenotypic switching and filamentation in *Candida glabrata*. Microbiology 2002: **148**: 2661–2674.
19. Li L, Dongari-Bagtzoglou A. Oral epithelium – *Candida glabrata* interactions in vitro. Oral Microbiol Immunol 2007: **22**: 182–187.
20. Li L, Kashleva H, Dongari-Bagtzoglou A. Cytotoxic and cytokine-inducing properties of *Candida glabrata* in single and mixed oral infection models. Microb Pathog 2007: **42**: 138–147.
21. Li L, Redding S, Dongari-Bagtzoglou A. *Candida glabrata:* an emerging oral opportunistic pathogen. J Dent Res 2007: **86**: 204–215.
22. Lockhart SR, Joly S, Vargas K, Swails-Wenger J, Enger L, Soll DR. Natural defenses against *Candida* colonization breakdown in the oral cavities of the elderly. J Dent Res 1999: **78**: 857–868.
23. Lyon JP, da Costa SC, Totti VM, Munhoz MF, de Resende MA. Predisposing conditions for *Candida* spp. carriage in the oral cavity of denture wearers and individuals with natural teeth. Can J Microbiol 2006: **52**: 462–467.
24. MacFarlane TW, Helnarska SJ. The microbiology of angular cheilitis. Br Dent J 1976: **140**: 403–406.
25. Masia Canuto M, Gutierrez Rodero F, Ortiz de la Tabla Ducasse V et al. Determinants for the development of oropharyngeal colonization or infection by fluconazole-resistant *Candida* strains in HIV-infected patients. Eur J Clin Microbiol Infect Dis 2000: **19**: 593–601.
26. McIntyre GT. Oral candidosis. Dent Update 2001: **28**: 132–139.
27. Newton A. Denture sore mouth: a possible aetiology. Br Dent J 1962: **112**: 357–360.
28. Odds FC. Candida and candidosis. A review and bibliography, 2nd edn. London: Baillière Tindall, 1988.
29. Oksala E. Factors predisposing to oral yeast infections. Acta Odontol Scand 1990: **48**: 71–74.
30. Pfaller MA, Messer SA, Boyken L, Tendolkar S, Hollis RJ, Diekema DJ. Variation in susceptibility of bloodstream isolates of *Candida glabrata* to fluconazole according to patient age and geographic location. J Clin Microbiol 2003: **41**: 2176–2179.
31. Radford DR, Challacombe SJ, Walter JD. Denture plaque and adherence of *Candida albicans* to denture-base materials *in vivo* and *in vitro*. Crit Rev Oral Biol Med 1999: **10**: 99–116.
32. Ramage G, Saville SP, Thomas DP, Lopez-Ribot JL. *Candida* biofilms: an update. Eukaryot Cell 2005: **4**: 633–638.
33. Ramage G, Tomsett K, Wickes BL, Lopez-Ribot JL, Redding SW. Denture stomatitis: a role for *Candida* biofilms. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004: **98**: 53–59.
34. Ramage G, Vande Walle K, Wickes BL, Lopez-Ribot JL. Biofilm formation by *Candida dubliniensis*. J Clin Microbiol 2001: **39**: 3234–3240.
35. Ramage G, VandeWalle K, Lopez-Ribot JL, Wickes BL. The filamentation pathway controlled by the *Efg1* regulator protein is required for normal biofilm formation and development in *Candida albicans*. FEMS Microbiol Lett 2002: **214**: 95–100.
36. Ramage G, Wickes BL, Lopez-Ribot JL. Biofilms of *Candida albicans* and their associated resistance to antifungal agents. Am Clin Lab 2001: **20**: 42–44.
37. Redding SW, Bailey CW, Lopez-Ribot JL et al. *Candida dubliniensis* in radiation-induced oropharyngeal candidiasis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001: **91**: 659–662.
38. Redding SW, Kirkpatrick WR, Saville S et al. Multiple patterns of resistance to fluconazole in *Candida glabrata* isolates from a patient with oropharyngeal candidiasis receiving head and neck radiation. J Clin Microbiol 2003: **41**: 619–622.
39. Redding SW, Marr KA, Kirkpatrick WR, Coco BJ, Patterson TF. *Candida glabrata* sepsis secondary to oral colonization in bone marrow transplantation. Med Mycol 2004: **42**: 479–481.
40. Reichart PA. Oral manifestations in HIV infection: fungal and bacterial infections, Kaposi's sarcoma. Med Microbiol Immunol (Berl) 2003: **192**: 165–169.
41. Rossie K, Guggenheimer J. Oral candidiasis: clinical manifestations, diagnosis, and treatment. Pract Periodontics Aesthet Dent 1997: **9**: 635–641.
42. Samaranayake LP, MacFarlane TW, Lamey PJ, Ferguson MM. A comparison of oral rinse and imprint sampling techniques for the detection of yeast, coliform and *Staphylococcus aureus* carriage in the oral cavity. J Oral Pathol 1986: **15**: 386–388.
43. Soysa NS, Ellepola AN. The impact of cigarette/tobacco smoking on oral candidosis: an overview. Oral Dis 2005: **11**: 268–273.
44. Soysa NS, Samaranayake LP, Ellepola AN. Cytotoxic drugs, radiotherapy and oral candidiasis. Oral Oncol 2004: **40**: 971–978.
45. Soysa NS, Samaranayake LP, Ellepola AN. Diabetes mellitus as a contributory factor in oral candidosis. Diabet Med 2006: **23**: 455–459.
46. Tunney MM, Patrick S, Gorman SP et al. Improved detection of infection in hip replacements. A currently underestimated problem. J Bone Joint Surg Br 1998: **80**: 568–572.
47. Wilson J. The aetiology, diagnosis and management of denture stomatitis. Br Dent J 1998: **185**: 380–384.
48. Wray D, Bagg J. Pocket reference to oral candidosis. London, UK: Science Press, 1997.