



Title	Sjögren's syndrome sufferers have increased oral yeast levels despite regular dental care
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4 **Sjögren's syndrome sufferers have increased oral yeast levels despite**
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6 **regular dental care**
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36 syndrome; xerostomia
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3 Abstract
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8 **AIM:** To investigate the prevalence and quantity of oral yeasts and their association with oral
9 candidiasis in Sjögren's Syndrome (SS) patients receiving regular dental care.
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11 **MATERIALS AND METHODS:** Yeasts in oral rinse and full mouth supra-gingival plaque
12 samples from 25 primary SS, 27 secondary SS and 29 control subjects were selectively
13 cultured. All yeasts except single-species isolates were genotyped using pulsed field gel
14 electrophoresis (PFGE).
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22 **RESULTS:** Ten (19%) SS sufferers had symptomless candidiasis. SS subjects had a higher
23 prevalence (73% vs 7%) and quantity of yeasts than controls in both oral rinse and plaque
24 samples ($P < 0.05$). Prevalence of yeasts in plaque was associated with candidiasis regardless
25 of denture wearing ($P \leq 0.04$). *C. albicans* was the predominant yeast isolated. PFGE showed
26 20 (66% of total) *C. albicans* isolate pairs, i.e. *C. albicans* species isolated from plaque and
27 oral rinse samples of the same individual, were of closely related genetic clonal types ($P <$
28 0.01).
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38 **CONCLUSIONS:** Despite effective oral hygiene, more SS subjects than controls had
39 detectable levels of oral yeasts and their presence in supra-gingival plaque was associated
40 with candidiasis. *C. albicans* colonised supra-gingival biofilm even in well-maintained SS
41 individuals, posing a challenge to the control of oral candidiasis.
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Sjögren's syndrome (SS) is a chronic, autoimmune disease characterized by lymphocytic infiltration of the exocrine glands, especially the lacrimal and salivary glands, leading to keratoconjunctivitis sicca and xerostomia. This syndrome is subclassified into primary SS (pSS), consisting of keratoconjunctivitis sicca and xerostomia, and secondary SS (sSS) when other connective tissue diseases such as rheumatoid arthritis are also associated (Fox, 2003).

Reduction in salivary output is the oral hallmark of SS. The oral manifestations of SS include dry mouth, oral soreness and ulceration, dysphagia, dysgeusia, an increased rate of dental caries, a higher prevalence of missing teeth, salivary gland enlargement, and oral candidiasis (Baudet-Pommel *et al*, 1994; Leung *et al*, 2004; McMillan *et al*, 2004).

Previous studies have indicated a high rate of yeast colonization in individuals with SS detected using oral rinse (Abraham *et al*, 1998; Radfar *et al*, 2003) and tongue or palate swabs (Rhodus *et al*, 1997). The prevalence of clinical oral candidiasis in these subjects appears to range widely (0% to more than 80%) (Tapper-Jones *et al*, 1980; Baudet-Pommel *et al*, 1994; Lundström & Lindström, 1995; Rhodus *et al*, 1997; Soto-Rojas *et al*, 1998; Almståhl *et al*, 1999; Pedersen *et al*, 1999), most likely due to those surveyed having differing oral hygiene status and/or different criteria used to diagnose oral candidiasis. The candidiasis state is generally symptomless. A variety of yeast species have been isolated from SS using different culture techniques (Kindelan *et al*, 1998; Radfar *et al*, 2003). Most reports indicate that *Candida albicans* is the predominant yeast isolated, although *Candida glabrata* and *Candida tropicalis* have also been found albeit infrequently (Kindelan *et al*, 1998; Torres *et al*, 2002). A Swedish study demonstrated increased prevalence (60% vs 25% in controls) but not proportion (%) of *C. albicans* from pooled supragingival plaque samples taken from the posterior teeth of non-denture wearing pSS subjects (Almståhl *et al*, 1999). No report is available describing the corresponding microbiology in sSS subjects.

An earlier study by this research group reported that southern Chinese SS sufferers in

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3 Hong Kong had a higher DMFT than controls despite good oral hygiene (Leung *et al*, 2004).
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5 In addition, SS subjects had significantly more clinical signs of candidiasis-related mucosal
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7 conditions than controls (35% vs 17%).
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10 The present study aimed to investigate oral yeast colonization in pSS and sSS patients
11 who were dentally well-maintained and had good oral hygiene. Putative associations between
12 oral yeasts isolated from oral rinse or full mouth supra-gingival plaque and oral candidiasis
13 were also investigated. The hypothesis tested was that for pSS, sSS and control subjects
14 under regular dental care, a similar proportion and minimal amount of yeast would be
15 detectable from their oral rinse samples and supra-gingival plaque; and oral yeast detection
16 would be independent of the clinical candidiasis state.
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32 **Material and methods**

33 *Subjects*

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35 SS sufferers were recruited from the Rheumatology Clinic, Faculty of Medicine, The
36 University of Hong Kong, Queen Mary Hospital, Hong Kong SAR, China. The subjects were
37 further classified as pSS or sSS group depending on the clinical diagnosis, based on the
38 revised version of the European Community Diagnostic Criteria for the Classification of
39 Sjögren's syndrome (Vitali *et al*, 2002). All control and SS subjects had attended regular
40 dental review at 6 months intervals for at least 12 months before study commencement. The
41 control group comprised medically clear patients attending the Prince Philip Dental Hospital.
42 No subjects were under active dental treatment at the time of the study. Exclusion criteria
43 included subjects with a history of systemic autoimmune connective tissue disease, prior
44 radiotherapy in the head and neck region, or any concurrent condition other than SS in test
45 subjects which might cause altered saliva flow. Participants in the control group were selected
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3 for similar age and gender using the age/gender characteristics of both pSS and sSS groups.
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5 All subjects in the study were dentate southern Chinese. The Faculty of Dentistry Ethics
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7 Committee, the University of Hong Kong, approved the study. Written informed consent was
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9 obtained from each participant prior to admission to the study.
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12 13 14 15 *Oral examination*

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17 All clinical examinations and sampling were performed by one calibrated examiner (KCML).
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19 The presence of clinical signs of oral candidiasis, i.e. angular cheilitis, erythematous lesions,
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21 ~~and~~ or white removable plaque, was recorded (Soto-Rojas *et al*, 1998). Oral hygiene was
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23 assessed using the Plaque (PI) (Löe and Silness, 1964) and Calculus Indices (CI) (Silness and
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25 Löe, 1964). Denture stomatitis in denture wearers, if any, was classified as reported by
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27 Newton (1962). In brief, the condition at the denture bearing area was classified as follows.
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29 Stage 1: pin point hyperaemia; Stage 2: diffuse hyperaemia; Stage 3: granular inflammation,
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31 i.e. the mucosa has a nodular appearance with a hyperaemic surface normally present over the
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33 entire denture-bearing area but is more commonly restricted to the central areas and, in
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35 particular, under relief areas. Denture hygiene of denture wearers was accessed by scoring the
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37 cleanliness of the fitting surface as described by Blair *et al*, (1995): 0 = no visible plaque
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39 detected by scraping fitting surface with flat plastic; 1 = no visible plaque but plaque
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41 detectable by scraping of fitting surface; 2 = plaque visible upon careful examination; 3 =
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43 plaque deposits clearly visible; 4 = gross plaque deposits.
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52 53 *Sialometry*

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55 All subjects were asked to rinse their mouths for 60s with 10 ml of sterile 0.01 M
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57 phosphate-buffered saline, pH 7.2 (oral rinse microbial sampling; as described below) before
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59 stimulated whole saliva (SWS) sampling. SWS was then obtained as described in an earlier
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3 report (Leung *et al*, 2004). In brief, each subject was instructed to chew on a piece of
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5 sterilized silicone rubber tubing for 5 minutes and to expectorate all SWS into a sterile,
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7 pre-weighed plastic cup. All samples were collected between 9 a.m. to 12 noon. The flow rate
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9 was determined and expressed as ml/min, as described previously (Leung *et al*, 2004).
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12 13 14 15 *Microbial sampling*

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17 An oral rinse sampling protocol was used as described previously (Leung *et al*, 2000).
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19 Dentures, if present, were not removed during rinsing. Supra-gingival plaque samples were
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21 collected after the oral rinse. Plaque was removed with a sterile sickle scaler passing along
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23 the gingival margins of all standing teeth after air dry and cotton wool isolation. The sample
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25 was dispersed into a pre-weighed vial containing 1 ml reduced transport fluid. Care was taken
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27 to avoid sampling plaque sub-gingivally and at sites covered by removable denture
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29 components or with open carious lesions. Full mouth supra-gingival plaque was removed and
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31 the net wet weight in grams was calculated.
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36 37 38 *Culture and identification of yeast isolates*

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40 All oral rinse and plaque samples were used neat, and vortexed for 30 s at maximum setting
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42 (Autovortex Mixer SA2, Stuart Scientific, London, UK). Fifty microlitre of each of the oral
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44 rinse and plaque samples were serially diluted and spiral plated (Model DU, Spiral Systems,
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46 Cincinnati, OH) on to CHROMagar *Candida* plates (Becton Dickinson Europe, Le Pont de
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48 Claix, France), then incubated aerobically for 48 h at 37°C. After incubation, plates with
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50 appropriate number of colonies that were well-separated and evenly dispersed with up to
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52 approximately 100 colony-forming units (c.f.u.) were chosen for counting. The number of
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54 yeast c.f.u. per ml of oral rinse or per gram wet weight of plaque was calculated. Then a
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56 stainless steel template was used to subdivide the agar into sectors with a defined area (Leung
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3 *et al*, 2005). A sector with 6-10 colonies including all different colony morphotypes observed
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5 was selected and all colonies within the sector were subcultured using Sabouraud's dextrose
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7 agar (SDA; Oxoid, Unipath Ltd, Basingstoke, Hampshire, UK) to obtain pure isolates. For
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9 samples that gave rise to ≤ 10 yeast colonies, all colonies were subcultured. The estimated
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11 lowest detection level for yeast using the current protocol is 20 c.f.u. per ml oral rinse or 20
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13 c.f.u. per full mouth plaque sample.
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17 The yeasts were identified and speciated based on the following: colony morphology in
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19 CHROMagar and SDA, cell morphology, gram staining reaction, germ tube test; and API
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21 20C AUX test (Leung *et al*, 2000). To differentiate *C. albicans* from *C. dubliniensis*, all
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23 isolates with the appropriate presumptive identity were incubated aerobically on SDA, 48 h at
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25 45°C, and on phenol red agar for 48h at 26°C and 37°C, respectively (Sancak *et al*, 2005). All
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27 plates were examined macroscopically for colony morphology and then microscopically for
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29 chlamydospore formation. Isolates from the same specimen with identical characteristics/test
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31 results based on the above identification protocol were considered as one isolate in the
32
33 following experiments. Isolates that could not be identified in this manner were further
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35 characterized by MicroSeq D2 LSU rDNA fungal identification system (Applied Biosystems,
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37 Foster City, CA, USA). Yeast strains, of the same species, isolated from both oral rinse and
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39 plaque samples of an individual with identical biochemical or API 20C AUX characteristics,
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41 or identical MicroSeq D2 LSU rDNA fungal identification result, i.e. of same presumptive
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43 biotype or genetic identification, were considered to be an isolate pair. Their genetic
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45 relatedness would then be tested by PFGE.
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55 *Total anaerobic plaque bacteria count*

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57 All plaque samples were vortexed for 20 s at maximum setting (Autovortex Mixer) and then
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59 serially diluted in 10-fold increments up to a ten thousand fold in trypticase soy broth (TSB).
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The diluted samples were plated onto enriched Columbia blood agar (CBABS, Columbia agar base with 5% defibrinated horse blood, 0.0005% hemin and 0.00005% Vitamin K) (Leung *et al*, 2005) using a Spiroplater. CBABS plates were incubated in an anaerobic chamber (Forma Scientific Inc, Marietta, OH) under an atmosphere of 10% CO₂, 10% H₂, 80% N₂ for 5 - 7 days at 37°C.

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CBABS plates with appropriate number of colonies that were well separated and evenly dispersed were selected, i.e. plates with 30 – 300 c.f.u., were chosen for counting. With reference to the counts, inoculum size and the dilution factor, the total anaerobic viable counts (TVC) per gram wet weight supra-gingival plaque were measured. The percentage proportion of yeast over the corresponding total anaerobic viable count was then calculated.

29 30 31 32 *Chromosomal analysis of yeast isolates by PFGE*

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All yeast isolates were subjected to PFGE analysis. Preparation of the yeast DNA was performed according to the instruction manual of CHEF Genomic DNA Plug kits (Bio-Rad Laboratories, Hercules, CA, USA).

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The chromosomes of the yeast isolates were separated employing the contour-clamped homogeneous electric field (CHEF) technique using the CHEF-DR III Variable angle system (Bio-Rad Laboratories, Hercules, Calif.) in 0.5 × TBE buffer at 10°C as described earlier with modifications (Leung *et al*, 2000). The electrophoretic conditions used were as follows: two state mode with linear ramping factors; run time, 72 h; initial and final switching times, 3 min and 17 min, respectively; constant voltage of 3 V/cm, switching angle, 106 degrees. *Hansenula wingei* and *Saccharomyces cerevisiae* chromosomes (Bio-Rad) were used as the molecular size standards. The resulting gel was stained with 0.5 µg/ml ethidium bromide (Sigma) in distilled water for 15 min and then destained in distilled water for 3 h. DNA bands were visualized and photographed under UV transillumination. The PFGE profiles of the

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3 yeast isolates were confirmed by repeating the assay two more times on separate occasions.
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7 8 *Dendrogram analysis of yeast isolates*

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10 PFGE pattern of all yeast strains group under the same species, except those of a single
11 isolate was analysed using the Dendron® 3.0 Program (Soll-tech, Oakdale, CA, USA). The
12 unweighed pair group method with arithmetic means (UPGMA) was used for clustering of
13 the isolates on the dendrogram. The method was applied to 61, 5, 4, 3 and 2 strains each of *C.*
14 *albicans*, *C. parapsilosis*, *C. tropicalis*, *S. cerevisiae*, and *C. dubliniensis*, *C. glabrata*, or
15 *Trichosporon mucoides*, respectively. One strain each of *C. guilliermondii*, *C. krusei*, and *C.*
16 *lusitaniae*, *C. pelliculosa*, *C. pseudointermedia* isolate from control or sSS oral rinse samples,
17 respectively was not analyzed. Closely related genetic clusters of individual yeast species
18 were identified and the genetic similarities were measured. Isolate pairs were considered to
19 be of the related genetic clonal type when $S_{AB} \geq 0.85$ (Clemons *et al*, 1997).
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36 37 *Statistical analysis*

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39 The demographic and microbiological parameters were analyzed and compared among the
40 pSS, sSS and control groups. Differences in categorical variables among the three groups
41 were tested by χ^2 test or Fisher's exact test whichever appropriate. One-way ANOVA,
42 unpaired t, or Kruskal-Wallis tests, whichever appropriate, was performed for evaluating
43 differences in continuous variables among the three groups. Multiple comparisons were
44 carried out with Bonferroni correction of *P*-values. Spearman's rank correlation coefficient
45 was used to explore associations between parameters of interest. A 5% level of significance
46 was used for the statistical tests. All data were analyzed using SPSS for Windows 12.0.1.
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Results

Participants

The study population consisted of 25 pSS (1 male; mean age 50.7, range 33-74) and 27 sSS (1 male; mean age 42.7, range 26-66) patients. The mean time since diagnosis was for 7.2 and 3.9 years respectively for pSS and sSS. The control group had 29 participants (2 men; mean age 43.9, range 27-75). All except one woman in the control group were non-smokers. There was no age-related difference among the three groups ($P > 0.05$). Duration of last dental recall visit was from within 5 months-2 weeks to 6 months-2 weeks for all participants. All subjects were partially or fully dentate. Other than fluoridated tooth-paste, no control or SS subject used homecare fluoride products. None of the SS patients were taking medication for dry mouth symptoms. Seven (28%) pSS and eight (30%) sSS patients were taking hydroxychloroquine for management of extra-glandular manifestation of SS. None of the patients were receiving anticholinergic therapeutic agents. Eighteen sSS patients had SLE, four had RA, two had sicca symptoms, two had SLE-like disease one had autoimmune hepatitis.

Sialometric parameters

SS sufferers had significantly lower stimulated whole saliva flow than the controls (pSS/sSS/control: $0.3 \pm 0.4/0.4 \pm 0.4/0.9 \pm 0.5$ ml/min, ANOVA $P < 0.001$; pSS/sSS < control, Bonferroni multiple comparison).

Oral hygiene and denture status

Among the pSS/sSS/control subjects, 88/89/100% tooth-sites respectively had plaque score of ≤ 1 and 84/78/79% respectively had a calculus score of 0 (calculus free) ($P = 0.111$ and 0.884 respectively, χ^2 test), indicating on average a satisfactory level of oral cleanliness.

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There was a statistical significant difference in number of removable denture wearers between groups ($P = 0.001$, Fisher's exact test, control vs total SS). Eight pSS subjects (32%) wore at least one removable denture, no sSS subjects wore removable dentures whereas six (22%) of them presented with fixed prostheses in at least one jaw. Two controls subjects (7%) wore a single removable partial denture and one had a mandibular fixed prosthesis. Regarding denture hygiene (Blair *et al*, 1995), 4 pSS subjects had score 0, 3 had score 1, 1 had score 2 while the 2 control denture wearers had score 0. None of the subjects surveyed had denture stomatitis.

Soft tissue

Prevalence of oral candidiasis based on clinical signs among the 3 groups was as shown (Table 1). None of those affected experienced symptoms. There was a statistical significant difference in the prevalence of oral candidiasis between the control and SS subjects (Table 1).

Isolation of oral yeasts

The mean wet weight of full-mouth supra-gingival plaque sampled was 10.4 ± 32.0 mg (range: 0.3 - 295.2 mg). The prevalence and quantity of yeast colonization are summarized in Table 1. More pSS (84%) and sSS (63%) sufferers than control subjects (7%) had oral yeast detectable from either oral rinse or full mouth plaque samples ($P < 0.0001$, χ^2 test). For SS cases as a whole, there was a yeast prevalence of 56% for both oral rinse and plaque and 73% for yeast in either oral rinse or plaque in SS subjects. Fifteen SS subjects had no detectable level of yeast in plaque or oral rinse samples. Primary SS subjects also had a significantly higher mean yeast count in both samples compared with sSS subjects and controls ($P < 0.001$). All SS patients who wore dentures had oral yeast detectable in both samples. A positive correlation was detected between oral colonization by yeast and removable denture

wearing ($r_s = 0.334$, $P = 0.004$). When non-denture wearers were considered, a similar pattern of more yeasts detectable from oral rinse or full mouth supra-gingival plaque of SS than control subjects was observed (pSS/sSS/control; $n = 17/27/27$; mean oral rinse yeast count: $242.4 \pm 351.3/155.0 \pm 327.2/0.7 \pm 3.9$ c.f.u./ml; mean full mouth supra-gingival plaque yeast count [$\times 10^6$]: $0.0 \pm 0.0/2.3 \pm 5.0/0.4 \pm 0.8$ c.f.u./g wet weight; mean percentage proportion yeast per total viable anaerobic count [$\times 10^{-3}$]: $0.0 \pm 0.0/0.3 \pm 0.7/1.2 \pm 5.2$ %, $P < 0.001$, Kruskal-Wallis test). Four pSS, 10 sSS and 27 control subjects did not carry yeasts above detectable level in their oral rinse or full-mouth plaque samples.

A total of 84 yeast strains were isolated. Six pSS and six sSS sufferers harboured more than 1 species of yeast, and 3 species of yeast were recovered from two sSS sufferers. The most predominant yeast isolated was *C. albicans*, with 28 and 33 strains isolated from oral rinse and full mouth supra-gingival plaque samples, respectively. Growth characteristics, API 20C AUX test, and chlamydospores production on phenol red agar confirmed the identity for all *C. dubliniensis* and all except 5 *C. albicans* isolates which had API 20C AUX profile as *C. albicans* but did not grow under 45°C and formed chlamydospores on phenol red agar at 26°C. The final *C. albicans* identity of these isolates was confirmed by MicroSeq D2 LSU rDNA fungal identification. The various types of yeasts cultivable from both samples are shown in Table 2. No statistically significant difference was detected between the mean c.f.u. of the various yeast species among the three groups apart from *C. albicans* ($P < 0.001$).

Oral yeast isolation and candidiasis

Seven out of 10 SS subjects with candidiasis had yeast detectable from both oral rinse and plaque samples. One sSS subject with candidiasis had yeast detectable from supra-gingival plaque while the remaining two affected SS subjects did not have any detectable yeast from plaque or oral rinse samples. They were all non-denture wearers. Among the two with no

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yeasts detectable, one subject (pSS) had no intra-oral mucosal lesions except mild angular cheilitis. The other sSS subjects had clinical oral mucosa manifestations of candidiasis. Sjögren's syndrome subjects with oral candidiasis tended to have higher mean yeast and *C. albicans* counts in both oral rinse and plaque samples than those who did not, although the difference was not statistically significant (SS subjects with candidiasis vs without candidiasis: mean yeast/*C. albicans* in oral rinse, 426/418 vs 361/355 cfu/ml; mean yeast/*C. albicans* in plaque, $2.7 \times 10^6/2.7 \times 10^6$ vs $4.3 \times 10^5/4.2 \times 10^5$ cfu/gm). However, a positive correlation was observed between the prevalence of oral candidiasis and the prevalence of yeast isolated in plaque but not in the oral rinse ($r_s = 0.269$, $P = 0.02$ and $r_s = 0.223$, $P = 0.082$, respectively). Despite the high isolation rate of yeast associated with removable denture wearing (Table 1), there was no correlation between oral candidiasis prevalence and the appliance usage among subjects in all three groups ($P = 0.604$). When denture wearers were excluded from the data analysis, subjects with oral candidiasis had higher but not statistically significant mean yeast or *C. albicans* counts in both oral rinse and plaque samples (mean yeast/*C. albicans* in oral rinse, 467/460 vs 73/68 cfu/ml; mean yeast/*C. albicans* in plaque, $3.3 \times 10^6/3.2 \times 10^6$ vs $3.8 \times 10^5/3.8 \times 10^5$ cfu/gm). Nonetheless, positive correlations were observed between the prevalence of oral candidiasis and the prevalence of yeast isolated from oral rinse or plaque in non-denture wearers ($r_s \geq 0.257$, $P \leq 0.044$).

PFGE analysis of yeast isolates from SS subjects

All yeast isolates except one strain each of *C. guilliermondii*, *C. krusei*, *C. lusitaniae*, *C. pelliculosa*, and *C. pseudointermedia* isolate were subject to PFGE karyotyping as described above. The genetic relatedness of the total 61 *C. albicans* strains are shown in Fig. 1, a dendrogram constructed based on UPGMA clustering analysis of the PFGE results. The S_{AB} values of the *C. albicans* isolates ranged from 0.71 to 1.0. There were six clusters (i-vi)

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showing fairly even distribution of the isolates. Subjects harbouring *C. albicans* belonging to cluster vi were significantly older than the corresponding subjects of cluster i (58.8 vs. 37.0 years, $P = 0.032$, Bonferroni multiple comparison). Subjects harbouring *C. albicans* belonging to cluster v had a) lower quantity of the yeast in oral rinse samples than subjects with cluster vi (mean *Candida*: 38 vs 4060 c.f.u./ml, $P = 0.01$, Kruskal-Wallis test), or b) larger quantity of the yeast in full mouth supra-gingival plaque than subjects with cluster iv (mean *Candida*: 4.2×10^6 vs. 5.9×10^4 c.f.u./g, $P = 0.047$, Bonferroni multiple comparison).

Using $S_{AB} \geq 0.85$ to identify subgroups with closely related genetic clonotypes or karyotypes, fourteen (78%), and eight (80%) of the identified *Candida* isolate pairs from pSS and sSS groups respectively were found to be closely related ($P < 0.001$, Kruskal-Wallis test). One pSS sufferer had one isolate pair of PFGE identical *C. parapsilosis* while another pSS sufferer had an identical pair of *C. tropicalis* (Figure 2). Twenty *C. albicans* isolate pairs out of a total of 61 isolates showed close karyotypic or genetic identity ($S_{AB} \geq 0.85$, Figure 1). Out of these 20 pairs, seven and three isolate pairs from the pSS and sSS groups respectively were karyotypically identical, i.e. $S_{AB} = 1.0$ ($P = 0.008$, Kruskal-Wallis test). One isolate pair each of *C. parapsilosis*, *C. tropicalis*, *S. cerevisiae* and *T. mucoides* isolates showed close or identical karyotypes ($S_{AB} \geq 0.85$). One pSS subject had two karyotypically identical isolate pairs, i.e. one pair each of *C. albicans* and *T. mucoides*. The rest of the yeast isolates did not appear to be karyotypically related to each other. In summary, 22 of 52 SS subjects (42%) had genetically related *Candida* or yeasts strains isolatable from oral rinse sample and supra-gingival plaque at the same time, 5 more subjects had positive *Candida* isolation from both oral rinse and supra-gingival plaque but were apparently not genetically related; another SS subject had unrelated *C. albicans* isolate-pair which the yeasts were genetically related to isolates from 2 other SS individuals; 5 or 7 subjects had single *Candida* species isolated only from oral rinse or plaque, respectively.

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3 Among SS subjects with a *C. albicans* isolate pair, their oral mucosa (as detected by oral
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Among SS subjects with a *C. albicans* isolate pair, their oral mucosa (as detected by oral
rinse) and full mouth supra-gingival plaque were apparently colonized by a genetically
related, if not identical, strain of the same yeast (*C. albicans* isolates form isolate pairs or not
vs $S_{AB} < 0.85$ or $S_{AB} \geq 0.85$; $P < 0.001$, Fisher exact test). They also showed a higher but not
statistically significant mean *Candida* count than individuals with no *C. albicans* isolate pair
detectable (1124 cfu vs 44 cfu).

One representative isolate pair from each of the 5 *C. albicans* karyotypic clusters (except
cluster v which lacks isolate pair), and one each of *C. parapsilosis*, *C. tropicalis*, *S. cerevisiae*
and *T. mucoides* isolate pair are shown (Fig. 2).

Discussion

The prevalence of clinically detectable oral candidiasis among SS sufferers in the present
study was around 20% ($n = 10$) which is at the lower end of the reported range. The low rate
of oral candidiasis observed in the current study is possibly due to good oral and/or denture
hygiene and the stringent diagnostic criteria for oral candidiasis used (Soto-Rojas *et al*, 1998).
The current study revealed that oral candidiasis in the present cohort of regularly dentally
cared for SS sufferers seemed to be associated with the presence of yeast in full mouth
supra-gingival plaque. The current understanding that *Candida* residing in oral biofilm as one
determinant of the agent in oral mycotic infection (Coogan *et al*. 2006) provided theoretical
support for this observation. The fact that no swab or imprint sample was taken at candidiasis
sites apparently prevented the direct comparison between the yeast causing the infection and
those residing in supra-gingival plaque. Hence one must consider this limitation when
interpreting the current data. It is known however, that the turnover rate for the intact oral
epithelium layer is 4 – 14 days (Squier *et al*, 1976) implying the quick exfoliation of the

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3 superficial cell layers. Therefore, for successful colonization by yeasts, a recurring source of
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5 “pioneering micro-colonies of yeasts” from an already established biofilm reservoir would be
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7 pivotal.
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11 Although many sampling methods for oral mucosal flora have been devised it is now
12
13 generally recognised that the oral rinse sampling method designed by Samaranayake *et al*,
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15 (1986) two decades ago is the simplest and practical method for the purpose, especially when
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17 focal erythematous or pseudo-membranous lesions are unapparent. Furthermore, the
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19 employment of this standard methodology provides a comparator useful for relative
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21 assessment between different studies conducted in the past for normal, non-SS (Leung *et al*,
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23 2003), SS (Kindelan *et al*, 1998; Radfar *et al*, 2003), or other xerostomic subjects (Leung *et*
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25 *al*, 2000).
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31 No report, however, is available reporting how often and how many “pioneering
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33 micro-colonies” would be released from supra-gingival plaque per unit time (60s in the
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35 current case) after oral rinse. On the other hand, considering the subjects in this study were of
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37 generally good oral hygiene (up to 90% tooth sites without visible plaque) and hence with
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39 small amount of supra-gingival plaque and thus the total surface area of oral mucosa exposed
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41 to oral rinse sampling is substantially greater than the supra-gingival plaque, we postulated
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43 that our oral rinse sampling “predominantly detected the mucosal colonization of oral yeast”
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45 among the subjects surveyed in this particular situation.
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50 It was in only one sSS subjects that negative oral rinse and supra-gingival plaque culture
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52 was associated with clinical candidiasis. Perhaps in such subject the infection was associated
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54 with yeasts at below the detection level. Another SS subject was classified as suffering from
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56 candidiasis because of angular cheilitis at the left corner of mouth. The same subject had
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58 otherwise normal intact oral mucosa. Failure of yeast detection in that individual was perhaps
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60 due to inability of the oral rinse sample to reach corners of mouth, or again the offending

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3 yeast was at a level below our detection limit, or the possibility that non-mycotic infectious
4 agent might be involved (Dias & Samaranayake, 1995). There is no guideline available
5 regarding the critical yeast count above which clinical signs of oral candidiasis will be
6 evident, although it is generally agreed that oral candidiasis is observed in individuals with
7 high yeast counts, e.g. reported median c.f.u. of 1349 as measured by oral rinse or swab
8 (Abraham *et al*, 1998). Comparing the reported data to our current low median level of 280
9 c.f.u./ml oral rinse in regularly dentally cared SS subjects with candidiasis, one would readily
10 appreciate why in a small proportion of the affected individual we failed to detect the
11 presence of yeast. The corresponding yeast levels from oral rinse in candidiasis or
12 non-candidiasis SS subjects were not significantly different might also help explain such
13 observation. In a cohort of elderly institutionalized people with poor oral hygiene and 19% of
14 them xerostomic, Grimoud *et al* (2005) showed that after a 3-month oral hygiene programme,
15 the prevalence of oral candidiasis and total quantity of yeasts reduced measurably reflecting
16 that oral yeast colonization in susceptible subjects could be reduced by attention to oral
17 cleanliness. The current SS sufferers and controls, however, demonstrated reasonable oral
18 hygiene with up to 90% tooth sites without visible plaque. Despite the similar levels of oral
19 hygiene, the prevalence of yeast colonization in controls was only detectable from oral rinse
20 samples at a low level (7%) while the corresponding prevalence were $\geq 50\%$ for SS sufferers.
21 In order to establish a clearer linkage between clinical candidiasis, oral hygiene and oral yeast
22 colonization, a longitudinal investigation, involving a larger sample size, correlating the
23 microbiology, presence or absence of oral candidiasis vs plaque control as well as
24 supra-gingival plaque yeast prevalence or counts would be needed.

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Only two out of eight pSS denture wearers (25%) surveyed had oral candidiasis, one of
whom had a denture hygiene score of 2. In contrast to common belief, candidiasis did not
appear to be associated with removable denture wearing in the present SS cohort who

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3 incidentally had reasonable denture hygiene and were free of denture-related stomatitis. Such
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5 negative findings between candidiasis and denture wearing are supported by Soto-Rojas *et al*
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7 (1998).
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11 In the current study, prevalence of yeast colonization was 84 % and 63% respectively for
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13 pSS and sSS groups which is significantly higher than controls (7%). This finding compares
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15 favourably with a number of similar studies which reported 59% to almost 100% yeast
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17 colonization in SS sufferers (Tapper-Jones *et al*, 1980; Lundström & Lindström, 1995;
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19 Abraham *et al*, 1998, Radfar *et al*, 2003). The increased rate of yeast colonization is
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21 considered to be the effect of hyposalivation as significantly lower salivary flow rates were
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23 observed.
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27 Two previous reports (Kindelan *et al*, 1998; Pedersen *et al*, 1999) have demonstrated a
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29 higher rate of yeast colonization in primary compared with secondary SS sufferers which was
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31 also observed in the current study. However, previous studies did not explain the observation.
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33 A lower, but not statistically significant, salivary flow rate has been uniformly detected in
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35 pSS sufferers compared to sSS subjects (Daniels *et al*, 1975; van der Reijden *et al*, 1996;
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37 Almståhl *et al*, 1999). It is postulated that two factors may account for the higher rate of yeast
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39 colonization in the pSS group, i.e. reduced saliva production and/or denture wearing. The fact
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41 that none of our sSS subjects were denture wearers limited our ability to genuinely
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43 substantiate the impact of reduced saliva production and yeast colonization among pSS or
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45 sSS subjects.
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51 All SS denture wearers in this study had positive yeast cultures for both oral rinse and
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53 plaque samples. A similar observation was also reported by Radfar *et al* (2003), although
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55 Tapper-Jones *et al* (1980) reported a slightly lower figure. In the present study, a strong
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57 correlation between removable denture wearing and yeast colonization was detected which
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59 had not been reported previously in SS patients. Yet, this finding should be interpreted with
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3 caution as there were only eight SS denture wearers involved. Nonetheless, removable
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6 prostheses wearing has been long recognized as a risk factor for yeast carriage as the effect of
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8 diminished salivary flow in SS subjects on yeast growth is further augmented by removable
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10 denture wearing. Oral prostheses are believed to cause persistent trauma to the oral mucosa
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12 and promote proliferation of yeast (Nyquist, 1992). They also provide microscopically rough
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14 surface for the yeast to inhabit (Taylor *et al*, 1998).

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17 Using the oral rinse sampling technique, *Candida* counts of pSS sufferers have been
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19 reported to be around 1000 c.f.u./ml by a number of investigators and is significantly
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21 different to healthy controls (Abraham *et al*, 1998; Kindelan *et al*, 1998; Almståhl *et al*,
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23 1999). Our results compare favourably with previous work. A lower mean yeast count was
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25 obtained from sSS sufferers, which supports the findings of some researchers (Kindelan *et al*,
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27 1998; Rhodus *et al*, 1998) but not others (Rhodus *et al*, 1997; Almståhl *et al*, 1999). To our
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29 knowledge, there is only one study which has reported the amount of *C. albicans* in the
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31 supra-gingival plaque of pSS subjects (Almståhl *et al*, 2001). They reported 60% and 25% of
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33 pSS and controls respectively had *C. albicans* in supra-gingival plaque samples, and a
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35 significantly higher mean *C. albicans* count in pSS than controls. However, a significant
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37 proportion of their cohort frequently used saliva-stimulant which has been shown to lead to
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39 an increased salivary flow rate and a decreased amount of *C. albicans* colonization (Rhodus
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41 *et al*, 1998), making direct comparison with the current report difficult. Nonetheless, it was
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43 noteworthy that the mean *C. albicans* counts (c.f.u./g wet weight plaque) of the present pSS
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45 cohort was deduced to be almost 2000-fold that obtained by the Swedish group while the
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47 mean proportion viable count of *C. albicans* in pSS non-denture wearers was 100-fold less
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49 than the Swedish report (0.0003% vs 0.04%) (Almståhl *et al*, 2001).

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52 Among the yeast species identified, the majority were *C. albicans* which is in agreement
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54 with many other investigations (Hernandez & Daniels, 1989; Kindelan *et al*, 1998;
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Soto-Rojas *et al*, 1998; Torres *et al*, 2002; Radfar *et al*, 2003). This can be explained by the fact that *C. albicans* are more robust and can survive and adapt to diverse environments by phenotypic and/or genotypic switching (Cannon *et al*, 1995; Leung *et al*, 2000). Moreover, six SS subjects from each group (24% pSS and 22% sSS) showed mixed colonization which compares favourably with the 28% noted by Torres *et al* (2002). Mixed colonization has also been observed, though less frequently, in patients with HIV (Tsang & Samaranayake, 2000) and advanced malignancy (Jobbins *et al*, 1992). It is mostly due to alteration in host defence which promotes colonization by other non-*C. albicans* opportunistic pathogens such as *C. dubliniensis*, *C. krusei* and *C. parapsilosis* (Weems, 1992; Samaranayake & Samaranayake, 1994; Gutierrez *et al*, 2002). Furthermore, the use of CHROMagar *Candida* as the isolation medium has been shown to have high sensitivity and specificity in detection of mixed cultures (Kindelan *et al*, 1998; Willinger & Manafi, 1999).

To our knowledge, this is the first time that oral *Candida* colonization has been characterized and studied from oral rinse and full mouth supra-gingival plaque samples of SS sufferers. A variety of molecular based techniques are available including electrophoretic karyotyping, restriction fragment length polymorphisms (RFLP) (Merz 1990, review), randomly amplified polymorphic DNA (RAPD) (Leung *et al*, 2000; Dassanayake and Samaranayake 2003, review), and other PCR based approaches such as repetitive sequence-based PCR (rep-PCR) (Chen *et al*, 2005). High variability or low reproducibility are frequently encountered using RFLP or RAPD particularly under low-stringency conditions (Leung *et al*, 2000; Dassanayake and Samaranayake 2003) while the rep-PCR technique appears to rectify, to some extent, the short fall of PCR based techniques (Chen *et al*, 2005). The PCR or the RFLP based methods gave high discriminatory indices and were effective in discriminating particular pathogenic strain of the yeast from regular isolates (Chen *et al*, 2005). On the other hand, to identify clonal relatedness of isolates from the same

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3 host, electrophoretic karyotyping appeared to be among the best approaches (Leung *et al*,
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6 2000; Chen *et al*, 2005) and was thus used in the current study.

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8 Using electrophoretic karyotyping, we were able to show that out of the 52 SS sufferers,
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10 22 (42%) had genetically related *Candida* or yeast strains colonizing oral mucosa and
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12 supra-gingival plaque at the same time. Comparing genetically related *C. albicans* isolation
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14 pairs to the other *C. albicans* strains obtained from the same cohort, interesting findings were
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16 observed. No two subjects were colonized by *C. albicans* with same PFGE genetic identity,
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18 although 10 subjects colonized by *C. albicans* strains showed PFGE genetic relatedness to a
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20 *C. albicans* strain from one other individual among their group of which the genetic
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22 relatedness detected by PFGE (mean $S_{AB} \pm SD = 0.87 \pm 0.02$) was smaller than that between
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24 the *C. albicans* isolate pair from the same individual ($S_{AB} = 0.94 \pm 0.07$, $P = 0.0003$, unpaired
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26 t test with Welch correction). We postulated that the *C. albicans* recovered from the SS
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28 subjects most likely had diverse origins.
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34 In the majority of the subjects the oral mucosa and supra-gingival plaque were colonized
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36 by closely genetically related or identical yeast strains which lead us to postulate that
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38 persistent oral mucosal yeast infections might be aggravated by supra-gingival plaque
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40 colonization of the offending yeast or its genetically related form. The fact that sessile rather
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42 than planktonic *C. albicans* is more resistant to antifungal in vitro (Ramage *et al*, 2001)
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44 favours the notion that the yeast would be in a protected, stable environment within the
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46 plaque biofilm. The yeast might, from time to time be released by the biofilm and cause
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48 mucosal infection in those who were affected. The observation that *Candida* colonization of
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50 the gastrointestinal tract originated from carious teeth which constituted an ecologic niche
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52 (Hossain *et al*, 2003), tends to support this hypothesis albeit indirectly. Further investigations,
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54 however, are needed to establish the above hypothesis. We postulate that SS sufferers who
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56 had supra-gingival plaque colonized with yeast or *Candida* species might be more readily
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3 affected by mucosal colonization of the same or genetically related yeast species.
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6 No *Candida* was recovered in the full mouth supra-gingival plaque samples of the age-
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8 and gender-matched controls but was detectable at high prevalence among all SS sufferers,
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10 despite more pSS subjects being denture wearers. This implies that *Candida* is able to
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12 establish itself in the supra-gingival plaque, a natural biofilm, of SS sufferers even if they
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14 practise good oral hygiene as was the case in our current cohort. Whether this establishment
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16 is due to colonization by biofilm-forming *C. albicans* strains in SS, or due to diminished
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18 salivary flow rate and defence mechanisms leading to reduced selection pressure against *C.*
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20 *albicans*, or both, needs to be further explored. However, a recent in-vitro study found no
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22 quantitative differences in biofilm formation ability between *C. albicans* from HIV-infected
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24 versus HIV-free individuals on a micro-titre plate surface (Jin *et al*, 2003) possibly favouring
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26 the latter contention. Nonetheless, the clinical implication of such a finding is that once
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28 *Candida* is established in plaque, clinical and subclinical oral candidiasis might become
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30 persistent and difficult to manage, as treatment might involve not only topical application of
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32 antifungal agents to the oral mucosa but also an exceptionally high standard of plaque control.
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34 The reason being as in the case of the current SS subjects, they had *C. albicans* established in
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36 supra-gingival plaque despite good oral hygiene and high dental awareness (Leung *et al*,
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38 2004). Another concern is that *Candida* colonization of supra-gingival plaque can also serve
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40 as a reservoir for disseminated infections such as aspiration pneumonia and gastrointestinal
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42 infections in compromised individuals (Cannon *et al*, 1995; Hossain *et al*, 2003).
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51 Within the limitations of the present study, Chinese SS sufferers with good oral hygiene
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53 appeared to be prone to oral *Candida* colonization. Clinical oral candidiasis was only
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55 relatively commonly detected. *C. albicans* was the predominant oral yeast isolated. *Candida*
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57 species were also found to be established in the supra-gingival plaque of many of the SS
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59 sufferers.
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Table 1 Quantity and prevalence of yeast isolated from oral rinse and supra-gingival plaque samples

	<i>pSS</i> (<i>n</i> = 25)	<i>sSS</i> (<i>n</i> = 27)	<i>Control</i> (<i>n</i> = 29)
% oral candidiasis ^a	20	18.5	0
% yeast colonization			
oral rinse ^b	72.0	48.1	6.9
plaque ^b	84.0	55.6	0.0
oral rinse and plaque ^b	72.0	40.7	0.0
% denture wearers with yeast colonization	<i>n</i> = 8	<i>n</i> = 0	<i>n</i> = 2
oral rinse	100.0	NA ^c	50.0
plaque ^d	100.0	NA	0.0
oral rinse and plaque ^d	100.0	NA	0.0
yeast count (mean ± SD)			
oral rinse (c.f.u./ml) ^e	1025.5 ± 3882.1	155.0 ± 327.2	2.1 ± 8.2
denture wearers	2689.8 ± 6836.3	NA	20.0 ± 28.3
non-denture wearers ^e	242.4 ± 351.3	155.0 ± 327.2	0.7 ± 3.9
plaque (×10 ⁶ c.f.u./g) ^e	1.8 ± 4.1	0.4 ± 0.8	0.0
denture wearers	0.9 ± 1.0	NA	0.0
non-denture wearers ^e	2.3 ± 5.0	0.4 ± 0.8	0.0
<i>Candida</i> count (mean ± SD)			
oral rinse (c.f.u./ml) ^e	1017.5 ± 3883.7	149.1 ± 322.7	2.1 ± 8.2
plaque (×10 ⁶ c.f.u./g) ^e	1.8 ± 4.1	0.4 ± 0.8	0.0
% per total anaerobic viable count in supra-gingival plaque			
yeast (×10 ⁻³) ^e	0.4 ± 0.7	1.2 ± 5.2	0.0
denture wearers	0.5 ± 0.5	NA	0.0
non-denture wearers ^e	0.3 ± 0.7	1.2 ± 5.2	0.0
<i>Candida</i> (×10 ⁻³) ^e	0.4 ± 0.7	1.1 ± 4.8	0.0

^a*P* = 0.012, Fisher's exact test, *pSS* and *sSS* subjects grouped together.

^bSignificant difference among the groups, *P* < 0.0001, χ^2 test.

^cNA = not applicable.

^d*P* = 0.022, Fisher's exact test; no removable denture wearer in *sSS* group.

^eSignificant difference among the groups, *P* < 0.001, Kruskal-Wallis test.

Table 2 Identity, frequency and quantity of cultivable yeast isolated from oral rinse samples and full mouth supra-gingival plaque^a.

	Oral rinse						Supra-gingival plaque ^b			
	pSS (n = 25)		sSS (n = 27)		Control (n = 29)		pSS (n = 25)		sSS (n = 27)	
	Prevalence	Mean viable count (c.f.u./ml)	Prevalence	Mean viable count (c.f.u./ml)	Mean viable count (c.f.u./ml)	Mean viable count (c.f.u./ml)	Prevalence	Mean viable count (c.f.u./g)	Prevalence	Mean viable count (c.f.u./g)
Candida										
<i>Candida albicans</i> ^c	16 (64%)	1015.1	12 (44%)	149.1	0	0	18 (72%)	18 × 10 ⁵	15 (56%)	4 × 10 ⁵
<i>Candida dubliniensis</i>	0	0	0	0	0	0	1 (4%)	5846	1 (4%)	898
<i>Candida glabrata</i>	2 (8%)	1.6	0	0	0	0	0	0	0	0
<i>Candida guilliermondii</i>	0	0	0	0	1 (3%)	1.4	0	0	0	0
<i>Candida krusei</i>	0	0	0	0	1 (3%)	0.7	0	0	0	0
<i>Candida lusitaniae</i>	0	0	1 (4%)	0.7	0	0	0	0	0	0
<i>Candida parapsilosis</i>	2 (8%)	2.4	2 (7%)	1.5	0	0	1 (4%)	143	0	0
<i>Candida pelliculosa</i>	0	0	1 (4%)	1.5	0	0	0	0	0	0
<i>Candida pseudointermedia</i>	0	0	1 (4%)	0.7	0	0	0	0	0	0
<i>Candida tropicalis</i>	1 (4%)	2.4	1 (4%)	0.7	0	0	2 (8%)	1.7×10 ⁴	0	0
Non-Candida										
<i>Saccharomyces cerevisiae</i>	1 (4%)	1.6	1 (4%)	0.7	0	0	0	0	1 (4%)	6173
<i>Trichosporon mucoides</i>	1 (4%)	2.4	0	0	0	0	1 (4%)	1739	0	0

^aSome individuals carried more than a single yeast species.

^bNo yeast was detected from full mouth supra-gingival plaque of control subjects (n=29).

^cSignificant difference regarding c.f.u. counts (per ml oral rinse or per gram wet weight supra-gingival plaque) among the groups, $P < 0.001$, Kruskal-Wallis test.

LEGEND

Fig. 1. Dendrogram showing the genetic divergence of 61 strains of *C. albicans* isolated from oral rinse (R) and full mouth supra-gingival plaque (P) of pSS (p) and sSS (s) subjects analyzed by pulse-field gel electrophoresis (PFGE). The dendrogram was constructed by unweighed pair group method with arithmetic means (UPGMA clustering analysis). A dashed line is drawn at $S_{AB} = 0.85$ to denote the threshold selected to demarcate genetic relatedness of isolates (Clemons *et al*, 1997). Note that the *C. albicans* isolates formed 6 PFGE karyotype clusters ($S_{AB} \geq 0.73$, clusters i-vi). Ten isolate pairs of *C. albicans* (7 and 3 pairs from pSS and sSS respectively) were karyotypically/genetically identical, i.e. $S_{AB} = 1.0$; another 10 isolate pairs appeared to be genetically related. Representative *C. albicans* PFGE patterns are shown in Fig. 2.

Fig. 2. Representative PFGE separation of chromosome-sized DNA of yeast isolate pairs of different karyotypic clonal types from oral rinse samples and full mouth supra-gingival plaque from subjects with SS. Lane M1: *Saccharomyces cerevisiae* chromosome molecular size standard (Bio-Rad); lanes 1, 3, 5, 7, 9: *C. albicans* strains isolated from oral rinse samples; lanes 2, 4, 6, 8, 10: *C. albicans* strains isolated from full mouth supra-gingival plaque sample of the corresponding subjects; lanes 11 and 12, 13 and 14, 15 and 16, 17 and 18: *C. tropicalis*, *C. parapsilosis*, *S. cerevisiae*, or *T. mucoides* isolate pairs from oral rinse sample and full mouth supra-gingival plaque from the same subject, respectively; lane M2: *Hansenula wingei* chromosome molecular size standard (Bio-Rad). All isolate pairs except lanes 5 and 6, 15 and 16 ($S_{AB} \geq 0.86$) were found to be karyotypically identical i.e. $S_{AB} = 1.0$.

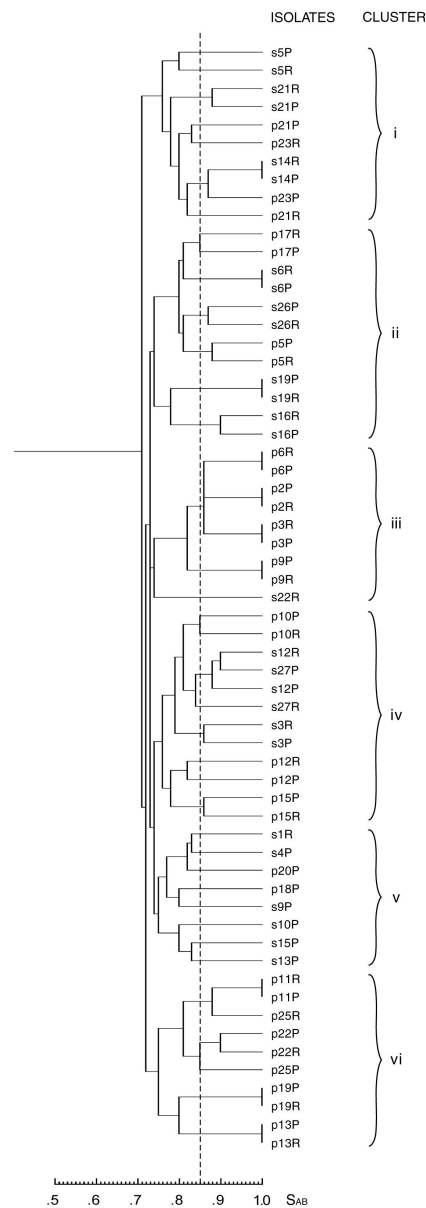


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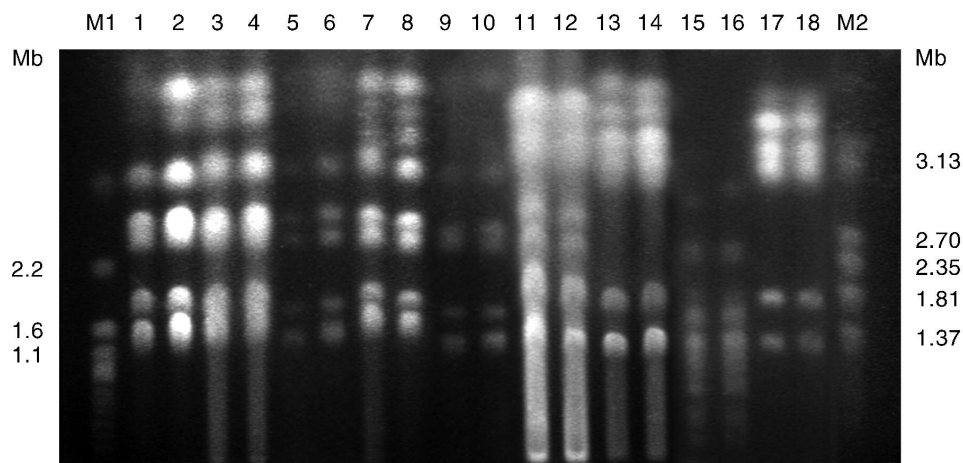


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