Clonal distribution of Candida in the oral cavity and its differentiation based on the internally transcribed spacer (ITS) regions of rDNA

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Abstract

This study aimed to determine the distribution of Candida species in the oral cavity and differentiate the species based on PCR amplification, including HindIII and MspI digestion, in order to assess the effectiveness of using the rDNA region for species identification. Samples from saliva as well as palate, tongue and cheek mucosa surfaces were collected from 45 individuals, consisting of three groups: periodontal disease patients; denture-wearers; and the control group. The samples were serially diluted, spread on BHI and YPD agar plates and scored for colony-forming units (CFUs). Fifteen random candidal colonies were isolated and subjected to genomic DNA extraction, based on glass beads disruption. Four primers were used to amplify regions in the rDNA, and the ITS1-5.8S-ITS2 PCR product was digested by HindIII and MspI restriction enzymes. The microbial loads on all sites of the denture-wearers were found to be significantly higher than control, while in the periodontal disease group only the microbial loads on the tongue were significantly higher than control. Meanwhile, there was no significant difference at other sites. The restriction fragment lengths of the clinical samples were compared to those of seven control species, allowing the differentiation of all seven species and the identification of 14 species from the clinical samples. The MspI restriction digest was not able to distinguish between C. albicans and C. dubliniensis, whereas the HindIII digest could not distinguish between C. tropicalis and C. parapsilosis. It was concluded that PCR-RFLP of the candidal rDNA region has potential for species identification. This study demonstrates the potential use of candidal rDNA as a means for identifying Candida species, based on genotype. The results also indicate the possibility of constructing genetic probes that target specific restriction fragments in the ITS1-5.8S-ITS2 region, enabling swift and precise identification of Candida species. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: Candida albicans; C. tropicalis; C. krusei; C. parapsilosis; C. dubliniensis; C. glabrata; C. lusitaniae

Introduction

Candida is a genus of opportunistic yeasts, unicellular fungi that can cause oral, vaginal, lung and sometimes systemic infections. Typical colonization sites in the oral cavity include the mucosal surfaces of the cheek and palate, which are sparsely populated (Theilade, 1990), although certain specialized surfaces, such as the keratinized stratified squamous epithelium of the palate, can influence the microbial distribution of the oral cavity, as well as the surface of the tongue, which consists of saliva-coated desquamated epithelium for microbial adhesion (Takahashi, 2005) and has a comparatively higher microbial density (Theilade, 1990; Takahashi, 2005). In addition, the average concentration of oral yeasts in saliva has been reported to be about 300–500 cells/ml (Cannon and Chaffin, 1999). Oral Candida species
are typically harmless, but they can become pathogenic under conditions that allow them to increase their relative proportion to other members of the local flora, such as during drug therapy (Akpan and Morgan, 2002), the presence of dentures (Shulman et al., 2005) and impaired salivary flow rate (Peterson, 1992). Pathogenic oral Candida usually manifests as oropharyngeal candidiasis (OPC), with symptoms including a redness of the palate and tongue, the formation of thick white plaque, mouth soreness, burning tongue sensation and taste changes (Samaranayake and Yaacob, 1990). Several candidal species of clinical interest include C. albicans, C. tropicalis, C. krusei, C. parapsilosis, C. dubliniensis, C. glabrata and C. lusitaniae. Correct and accurate identification of the candidal species infecting an oral candidiasis patient is highly important, as different antifungal agents are effective against different candidal species (Ellepola et al., 2003). Furthermore, candidal infections are typically treated withazole antifungal drugs, mainly fluconazole; however, many infections are caused by non-albicans Candida (NAC) species that may have fluconazole resistance (Niimi et al., 1999). As the most conserved region in the fungal genome, the rDNA region is suitable for analysis of phylogenetic differences (Iwen et al., 2002), and thus is potentially useful for candidal species identification. Several studies have already investigated the use of the rDNA region for candidal species differentiation, including PCR amplification of the ITSII region (Turenne et al., 1999), single-strand confirmation polymorphism analysis (Kumar and Shukla, 2005), multiplex PCR (Fujita et al., 2001) and PCR–RFLP (Shokohi et al., 2010). This study aims to determine the distribution of candidal species at different sites in the oral cavity in addition to differentiating between candidal species based on PCR amplification and digestion by the restriction enzymes HinflI and MspI, in order to assess the effectiveness of using the rDNA region for candidal species identification (Figures 1 and 2).

Materials and methods

Sample collection

Samples were collected from the oral cavities of 45 individuals of three groups (15 individuals from each group): the control group consisting of non-denture-wearers with a healthy oral cavity, mean age 35.8 (median 32) years; the denture wearer group, mean age 59.44 (median 59) years, and the periodontal disease group, mean age 46.65 (median 48) years. Consent was obtained from volunteers prior to sampling, in accordance with ethical code DF 0B0702/2002(L). Individuals from the periodontal disease group were confirmed by a clinician to have adult-stage periodontitis of 5 mm pocket depth. All individuals were aged 35–65 years and were also non-smokers, non-diabetic and had not taken any antimicrobial treatment for the past 6 months prior to sampling. Samples were taken from the saliva as well as from the surfaces of the cheek mucosa, tongue and palate. Salivary samples were obtained by soaking a pea-sized cotton ball on the floor of the mouth. Samples from the tongue, palate and buccal mucosa surfaces were taken by brushing against the surface 10 times consecutively with a cytobrush. All of the samples were then transferred into 1.5 ml phosphate-buffered saline (PBS) transport medium and brought to the laboratory on ice.

Microbial load determination

The samples were serially diluted to $10^5$ in preparation for agar plating. Then, 100 μl was pipetted from each of the dilution tubes, as well as from the undiluted sample, and plated on brain–heart infusion (BHI) and yeast peptone dextrose agar (YPD) plates. The plates were incubated at 37°C for 48 h under aerobic conditions, after which each plate was scored for colony-forming units (CFUs). This enabled the calculation of microbial loads of candidal colonies based on the CFUs scored on the YPD plates, the total microbial loads of both candidal and bacterial colonies based on the CFUs scored on the BHI agar plates, and the microbial loads of bacterial colonies based on taking the difference between the CFUs on the BHI agar plates and the CFUs on the YPD plates.

Statistical analysis

Statistical analysis was carried out using SPSS software v 17.0 in order to compare the microbial loads for each site between the three different groups. Results were computed from three determinations performed in triplicate ($n = 9$). The Shapiro–Wilk test for normality was used to determine the
distribution of the obtained data, with analysis of variance (ANOVA) using the Kruskal–Wallis test. Furthermore, the Mann–Whitney test was used to determine the significance of the microbial loads at each of the oral sites of the periodontal disease and denture-wearers groups, in comparison to the control group as the baseline. For all tests, $p < 0.05$ was considered significant.

DNA extraction

Fifteen random candidal colonies were isolated for DNA extraction. Colonies were cultured on Chromagar for tentative identification of \textit{C. albicans}, \textit{C. krusei} and \textit{C. tropicalis}. In addition, seven ATCC Candida species were obtained as controls: \textit{C. albicans} (ATCC 14053); \textit{C. tropicalis} (ATCC 13803); \textit{C. krusei} (ATCC 14243); \textit{C. parapsilosis} (ATCC 22019); \textit{C. dubliniensis} (ATCC MYA 2975); \textit{C. glabrata} (ATCC 90030); and \textit{C. lusitaniae} (ATCC 64125).

Genomic DNA extraction was carried out with a slightly modified protocol employed by Mirhendi \textit{et al.} (2006), in which yeast cells were lysed through mechanical disruption using glass beads. In brief, yeast cultures were pelleted and then suspended in a lysis buffer containing Triton X-100, mixed together with phenol–chloroform–isopropanol (PCI) and glass beads. The mixture was vortexed before centrifugation to separate the cell lysate, and treated with chloroform to remove traces of phenol. DNA precipitation was then carried out, using sodium acetate and isopropanol, before washing with ethanol.

PCR amplification

PCR amplifications of the ITS1, 5.8S-ITSII and ITS1-5.8S-ITSII regions were carried out on the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Gel pictures showing the band patterns for the ATCC control species of: (A) PCR products of ITS1-5.8S-ITSII; (B) PCR products of ITS1; (C) PCR products of 5.8S-ITSII; (D) \textit{Hinf}I restriction fragments; and (E) \textit{Msp}I restriction fragments for the seven ATCC control species}
\end{figure}
candidal genomic DNA extracts, based on slightly modified versions of the protocol employed by Kumar and Shukla (2005). Three PCR reactions were carried out using different pairs of the following four primers: ITS1, TCCGTAGGTGAACCTGCGG; ITS2, GCTGCGTTCTTCATCGATGC; ITS3, GCATCGATGAAGAACGCAGC; and ITS4, TCCTCCGCTTATTGATATGC. Each PCR reaction tube contained a mixture of 4 μl DNA template, 5 μl 10× PCR buffer, 0.1 μM of each of the primers used (ITS1 and ITS2 for amplification of the ITS1 region, ITS3 and ITS4 for the 5.8S-ITSII region, and ITS1 and ITS4 for the ITS1-5.8S-ITSII region), 100 μM dNTP mixture and 1 U Taq polymerase. In general, the amplification process consisted of an initial denaturation step at 96°C (95°C for the ITS-5.8S-ITSII amplification) for 10 min, followed by 30 cycles of denaturation at 95°C for 1 min, and then the annealing step (60°C for ITS1 amplification, 56°C for 5.8S-ITSII amplification and 55°C for ITS1-5.8S-ITSII amplification) for 1 min, extension at 72°C for 1 min (90 s for the 5.8S-ITSII amplification) and a final extension step of 72°C for 10 min.

Restriction enzyme digestion

Following PCR amplification, the ITS1-5.8S-ITSII PCR products were digested with HindIII and MspI restriction enzymes, following the manufacturer’s specifications. All PCR products and restriction fragments were visualized by running gel electrophoresis at 90 V for 100 min, using 1.5% agarose gels, and viewed under UV light.

Figure 2. Gel pictures showing the band patterns for the clinical samples of: (A) PCR products of ITS1-5.8S-ITSII; (B) PCR products of ITS1; (C) PCR products of 5.8S-ITSII; (D) HindIII restriction fragments; and (E) MspI restriction fragments for the 15 random clinical samples (Note: samples that were found to have identical banding patterns were grouped together and labelled accordingly)
Results

Microbial loads

The Shapiro–Wilk test for normality determined that the collected data was not Normally distributed. The Kruskal–Wallis non-parametric independent variable test was employed to compare between the microbial loads at each oral site between the three groups, in which the Kruskal–Wallis H is used to represent the χ² test value. No significant differences were found between the three groups for bacterial colonies in the saliva and candidal and bacterial colonies on the buccal mucosa surface. Meanwhile, microbial loads were significantly different between the three groups for candidal colonies in the saliva (H = 12.84, p = 0.006), candidal (H = 12.601, p = 0.006) and bacterial (H = 14.28, p = 0.006) colonies on the tongue surface, as well as candidal (H = 15.973, p = 0.003) and bacterial (H = 14.605, p = 0.003) colonies of the palate.

The Mann–Whitney two independent-variables test was carried out to assess the significance of the microbial loads at the oral sites of the denture-wearers and periodontal disease groups, in comparison to the control group. The results of this test, as well as the average microbial loads at the oral sites of each of the three groups, represented in medians, are summarized in Table 1. As can be seen, the average bacterial loads of the saliva and the buccal mucosa were found to have no significant difference with the control group, while all the other average microbial loads were significantly higher in comparison to the control group. Meanwhile, in the periodontal disease group, the average microbial loads of the tongue were significantly higher than in the control group, while the average microbial loads of the other three sites had no significant differences from the control group.

It should be noted that the enumeration of oral bacteria used in this study is limited to cultivable, aerobic bacterial species and does not take into account anaerobic and uncultivable bacterial species.

Mean percentages of candidal loads

The percentage of candidal load out of total cultivable microbial load was calculated for each sample. Data for mean percentage and standard deviation (SD) of candidal load out of total cultivable microbial load for each sampled oral site of each of the three groups are summarized in Table 2.

For the control group, bacteria clearly dominated at each of the oral sites, as the candidal load at each oral site constitutes only 0.6–17% of the total microbial load. Of the five sampled oral sites in the control group, the surface of the tongue had

### Table 1. Median microbial loads of the saliva, tongue, palate and buccal mucosa sites of the healthy control, periodontal disease and denture-wearers groups

<table>
<thead>
<tr>
<th>Site</th>
<th>Healthy control group</th>
<th>Denture-wearers group</th>
<th>Periodontal disease group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median candidal CFU/ml</td>
<td>Median bacterial CFU/ml</td>
<td>Median candidal CFU/ml</td>
</tr>
<tr>
<td>Saliva</td>
<td>0.02 × 10³</td>
<td>54.93 × 10³</td>
<td>0.80 × 10³</td>
</tr>
<tr>
<td>Tongue</td>
<td>2.61 × 10³</td>
<td>281.57 × 10³</td>
<td>244.77 × 10³</td>
</tr>
<tr>
<td>Palate</td>
<td>2.52 × 10³</td>
<td>44.06 × 10³</td>
<td>40.07 × 10³</td>
</tr>
<tr>
<td>Buccal</td>
<td>2.68 × 10³</td>
<td>296.53 × 10³</td>
<td>12.04 × 10³</td>
</tr>
<tr>
<td>mucosa</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*No significant difference from the control group.

*Significantly higher (U = 34, p = 0.001) than the control group.

*Significantly higher (U = 47, p = 0.002) than the control group.

*Significantly higher (U = 37, p = 0.003) than the control group.

*Significantly higher (U = 29, p = 0.001) than the control group.

*Significantly higher (U = 39, p = 0.012) than the control group.

*Significantly higher (U = 36, p = 0.005) than the control group.

*Significantly higher (U = 46, p = 0.006) than the control group.

*Significantly higher (U = 45, p = 0.009) than the control group.
the highest percentage of candidal load, while saliva contained the lowest.

In the denture-wearers group, the palate surface was the oral site with the highest percentage of candidal load, followed by the surface of the tongue. Meanwhile, saliva once again had the lowest percentage. The saliva, tongue and palate surfaces had higher percentages of candidal load compared to the control group, while the cheek mucosal surface and the gingival sulcus had lower percentages.

Meanwhile, for the periodontal disease group, the candidal load was in the range 1.6–14%. Saliva once again contained the lowest percentage of candidal load, while the palate now had the highest. The percentages of candidal load at each site were higher than in the control group, with the exception of the tongue surface, which had a comparatively lower percentage. Apart from the saliva, the candidal load at all of the sampled oral sites was in the range 11–14%.

### PCR amplification of the ITS1 region

As shown in Table 3, PCR amplification produced bands of variable length in the range 120–480 base pairs (bp). Three of the ATCC control species, *C. albicans*, *C. tropicalis* and *C. parapsilosis*, had identical band sizes of 220 bp, and *C. dubliniensis* had a band size (210 bp) that was very close to the aforementioned three, indicating difficulties if ITS1 were to be used for species identification. Meanwhile, *C. krusei* and *C. lusitaniae* were found to have distinctively smaller band sizes compared to the other samples (170 and 120 bp, respectively), while *C. glabrata* had a distinctively larger band size of 480 bp. Of the clinical samples, nine had band sizes matching *C. albicans*, *C. tropicalis* and *C. parapsilosis*, while four matched *C. glabrata*, one matched *C. dubliniensis* and one did not match any of the control species.

### PCR amplification of the 5.8S-ITSII region

As can be seen in Table 3, the band sizes of the 5.8S-ITSII region were in the range 250–420 bp. Once again, *C. glabrata* had the largest band size of 420 bp, while *C. lusitaniae* had the smallest at 120 bp, making both easily distinguishible. Unlike the ITS1 region, *C. albicans*, *C. krusei* and *C. dubliniensis* were found to have the same 5.8S-ITSII region size of 340 bp, whereas *C. tropicalis* had a band size that was close to them (320 bp) and thus difficult to distinguish visually. Of the clinical samples, five matched *C. albicans*, *C. krusei* and *C. dubliniensis*, four matched *C. glabrata*, one matched *C. tropicalis* and, once again, one did not match any of the control species.

### Table 2. Mean percentages and SDs of candidal loads out of total microbial loads

<table>
<thead>
<tr>
<th>Group</th>
<th>Oral site</th>
<th>Mean (%)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control</td>
<td>Saliva</td>
<td>0.61</td>
<td>1.09</td>
</tr>
<tr>
<td></td>
<td>Tongue</td>
<td>16.82</td>
<td>30.78</td>
</tr>
<tr>
<td></td>
<td>Palate</td>
<td>7.75</td>
<td>9.65</td>
</tr>
<tr>
<td></td>
<td>Buccal mucosa</td>
<td>11.81</td>
<td>18.84</td>
</tr>
<tr>
<td>Denture-wearers</td>
<td>Saliva</td>
<td>1.41</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>Tongue</td>
<td>19.11</td>
<td>24.95</td>
</tr>
<tr>
<td></td>
<td>Palate</td>
<td>22.27</td>
<td>25.85</td>
</tr>
<tr>
<td></td>
<td>Buccal mucosa</td>
<td>8.71</td>
<td>12.61</td>
</tr>
<tr>
<td>Periodontal disease</td>
<td>Saliva</td>
<td>1.67</td>
<td>3.77</td>
</tr>
<tr>
<td></td>
<td>Tongue</td>
<td>11.42</td>
<td>14.31</td>
</tr>
<tr>
<td></td>
<td>Palate</td>
<td>13.41</td>
<td>17.54</td>
</tr>
<tr>
<td></td>
<td>Buccal mucosa</td>
<td>12.44</td>
<td>23.87</td>
</tr>
</tbody>
</table>

### Table 3. PCR product and restriction fragment sizes (bp) of the control species

<table>
<thead>
<tr>
<th>Control species</th>
<th>ITS1</th>
<th>5.8S-ITSII</th>
<th>ITSI-5.8S-ITSII</th>
<th>MspI restriction fragment</th>
<th>Hinfl restriction fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>220</td>
<td>340</td>
<td>540</td>
<td>300, 240</td>
<td>(270, 270)</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>220</td>
<td>320</td>
<td>520</td>
<td>340, 180</td>
<td>(260, 260)</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>170</td>
<td>340</td>
<td>510</td>
<td>260, 250</td>
<td>240 (150, 150)</td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>220</td>
<td>300</td>
<td>520</td>
<td>520</td>
<td>(260, 260)</td>
</tr>
<tr>
<td><em>C. dubliniensis</em></td>
<td>210</td>
<td>340</td>
<td>540</td>
<td>300, 240</td>
<td>290, 260</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>480</td>
<td>420</td>
<td>900</td>
<td>570, 330</td>
<td>360 (270, 270)</td>
</tr>
<tr>
<td><em>C. lusitaniae</em></td>
<td>120</td>
<td>250</td>
<td>380</td>
<td>260, 120</td>
<td>(190, 190)</td>
</tr>
</tbody>
</table>
PCR amplification of the ITS1-5.8S-ITSII region

As summarized in Table 3, PCR amplification of the ITS1-5.8S-ITSII region generated band sizes of around 500 bp for almost all of the tested samples, with C. glabrata and C. lusitaniae once again distinctively having the largest (900 bp) and smallest band sizes (380 bp), respectively. Two pairs of control species, C. albicans and C. dubliniensis, as well as C. tropicalis and C. parapsilosis, had identical band sizes, 540 and 520 bp, respectively. Of the clinical samples, five matched C. albicans and C. dubliniensis, five matched C. tropicalis and C. parapsilosis, four matched C. glabrata and one did not have any match.

Restriction enzyme digestion by MspI and HinfI

As seen in Table 3, MspI digestion of the ITS1-5.8S-ITSII region produced unique restriction fragment patterns for all of the control species, with the exception of C. albicans and C. dubliniensis, which had identical patterns of 300 and 240 bp fragments. The PCR product of C. parapsilosis was not cut at all, retaining its original size of 520 bp, indicating that it lacks the MspI recognition site. The other patterns were very distinctive and easy to distinguish. For the clinical samples, digestion generated five different restriction fragment patterns, four of which could be matched to the restriction fragment pattern of at least one control species. Only one clinical sample could not be matched to any of the control species.

The HinfI digestion produced many restriction fragments that were too close in size to the point that the fragments did not separate from each other within the gel and appeared as a single band; in these cases the presence of multiple fragments represented by one band was extrapolated based on the total size of the ITS1-5.8S-ITSII PCR product. As summarized in Table 3, HinfI digestion generated unique patterns for all the control species with the exception of C. tropicalis and C. parapsilosis, which had identical patterns of two 260 bp fragments. Furthermore the fragment sizes of these two species were very similar to that of C. albicans (two 270 bp fragments), making distinguishing between them prone to error. Apart from that, HinfI digestion generated easily distinguishable restriction fragment patterns for the other species. One clinical sample could not be matched to any of the control species.

Species identification based on band patterns

Of the 15 clinical samples that were tested, four had band patterns that matched C. albicans, another four matched C. parapsilosis and yet another four matched C. glabrata. There was also one match for C. tropicalis and one match for C. dubliniensis. Lastly, there was one sample that could not be matched to any of the control species. DNA sequencing of the ITS1-5.8S-ITSII PCR product and a subsequent basic local alignment search tool (BLAST) query of the Candida Genome Database revealed that it was a match for C. guilliermondii, with an E value of 0, bit score of 1008 and 98.5% identical residues.

Discussion

Candidal loads in the control group

As microorganisms in the saliva are derived from microbes dislodged from other oral surfaces, salivary microbiota can be considered to reflect changes in the microbiota of the rest of the oral cavity (Li et al., 2005). Hence, the very low percentage of Candida in the saliva of the control group could be a reflection of the low yeast carriage in comparison to bacterial carriage in the oral cavity. Furthermore, the presence of antimicrobial factors such as histatin, secretory IgA and lysozyme in the saliva (Tenovuo et al., 1992) could be another reason why candidal loads in the saliva are so low.

Meanwhile, the surface of the tongue is considered to be an ideal environment for candidal colonization because of the humidity, temperature and existence of hidden niches between the papillae of the tongue (Zadik et al., 2010). This is also supported by a study which concluded that the tongue is the primary oral reservoir for candidal species (Arendorf and Walker, 1980). As the most common oral candidiasis typically affects the surface of the tongue (Samaranayake and Yaacob, 1990), it can be surmised that the tongue may harbour many of the potentially pathogenic candidal species responsible for oral candidal infection.

The squamous stratified epithelium of the buccal mucosa was found to be less ideal for candidal colonization compared to the papillary surface of the tongue. A possible reason for this is the
continuous shedding by exfoliation of the buccal mucosa, as buccal epithelial cells have an estimated turnover rate of 5–6 days (Harris and Robinson, 1992).

The unique qualities of the squamous epithelium of the palate, which is keratinized and is also less permeable compared to buccal mucosa (Gibbons, 1989), may be the reason why the palate was found to have a lower percentage of candidal occurrences compared to the tongue and buccal mucosa. Furthermore, as different oral sites have different cell surface receptors (Gibbons, 1989), it is also a possibility that the cell surface receptors of the palate favour the cell surface adhesins of bacterial species over candidal species.

Candidal loads in the denture-wearers group

One of the most important roles of saliva in the oral microenvironment is the removal and clearance of microorganisms from the oral cavity. As the presence of dentures in the oral cavity has the effect of slowing down salivary flow rate (Akpan and Morgan, 2002), this of course encourages the growth of oral microorganisms. Furthermore, denture use encourages the colonization of oral Candida (Shulman et al., 2002) in addition to predisposing towards candidal infection (Pires et al., 2002). Thus, candidal species in the oral cavity become increasingly competitive in the presence of dentures, which are reflected in the saliva.

The slowed salivary flow rate in the oral cavity also encourages microbial growth on the tongue surface, hence the significantly higher candidal load; however, unlike at the other sites, this does not appear to allow candidal species to compete any better than bacteria, as candidal load percentage is not much different from the control. This is perhaps unsurprising, as the tongue surface is known to have dense (Theilade, 1990) and diverse (Takahashi, 2005) bacterial populations, and thus it would be very tough for candidal populations to compete with them.

Dentures protect the palate from the washing action of saliva by trapping saliva in the palatal–denture space, and furthermore it has been found that immobilized salivary mucins can promote candidal adhesion to surfaces (Edgerton et al., 1993); thus, the arrested salivary flow in the palatal–denture space is responsible for greatly enhancing candidal growth, leading to a higher proportion of candidal species on the palate surface.

The presence of dentures was also found to encourage candidal colonization on the cheek mucosal surface, which is in agreement with previous studies (Shulman et al., 2005), but had little to no effect on bacterial colonization of the buccal mucosa.

Candidal loads in the periodontal disease group

With the onset of periodontal disease, among the responses of the host immune system is the release of antibacterial factors, the production of antibodies stimulated by bacterial antigens and the elevation of non-specific antimicrobial factors in the saliva (Lamster et al., 2003). The elevation of salivary antimicrobial factors may inhibit any increased growth of oral microorganisms that could occur as a result of periodontal disease, thus maintaining microbial growth at the same levels as in the control.

The tongue of periodontal patients may provide a better environment for bacterial species to compete against oral Candida species, which is consistent with the suggestion that the tongue is a reservoir for bacterial species that cause periodontal disease (Van der Velden et al., 1986). It can thus be surmised that, as periodontal disease-linked bacterial species become more competitive on the tongue surface during the onset of periodontal disease, the candidal colonization of the tongue is adversely affected, leading to lower percentages of candidal loads.

As saliva continuously bathes the oral cavity, the microbial growth inhibitory effect would also extend to the palate and cheek mucosal surfaces. Furthermore, the oral epithelial cells of both the palate and cheek mucosal surfaces can also be stimulated by bacterial infection to produce interleukins, such as interleukin-6, that inhibit microbial growth on the mucosal surface (Hedges et al., 1992), in addition to being actively involved with neutrophils in the immune response to bacterial infection (Dale et al., 2000). It is possible that both of these oral epithelium-mediated immune responses are better at suppressing bacterial species rather than candidal species, allowing candidal populations on oral mucosal surfaces to compete better against oral bacterial populations. This is supported by studies that show that disrupting bacterial populations in the oral cavity promote the growth of oral Candida (Scully et al., 1994).
Distribution and differentiation of oral candidal species

In periodontal patients, the palate may be the best environment for candidal species to compete against bacterial species, as it has the highest mean percentage of candidal load, overtaking both the tongue and cheek mucosal surfaces. This is perhaps because the flow of gingival crevicular fluid, containing antibodies, neutrophils, leukocytes and other antimicrobial factors that have been stimulated by periodontitis (Lamster, 1992) on the cheek mucosal surface is acting to suppress candidal colonization on the buccal mucosa, whereas this has no effect on the palate surface.

Differentiation of oral Candida based on ITS regions of rDNA

The PCR product sizes of the ITS1 region for C. albicans, C. tropicalis and C. krusei are approximately in agreement with those reported by Kumar and Shukla (2005). The 5.8S-ITSII amplification PCR product sizes for C. albicans, C. krusei and C. lusitaniae are consistent with the reports of Fujita et al. (2001), but the sizes for C. tropicalis and C. parapsilosis differ by about 10 bp. Meanwhile, the band sizes of the ITS1-5.8S-ITSII region for C. albicans, C. tropicalis, C. krusei and C. parapsilosis are in agreement with Mirhendi et al. (2006), but the reported product size for C. glabrata differs by 20 bp. Elsewhere, Fujita et al. (2001) reported similar product sizes for the species C. tropicalis, C. parapsilosis and C. lusitaniae, but the reported product sizes for C. albicans and C. krusei both differ with those observed in this study by about 10 bp. Lastly, the MspI restriction fragment sizes of the ITS1-5.8S-ITSII amplicons for C. albicans, C. tropicalis, C. krusei and C. parapsilosis are consistent with those reported by Mirhendi et al. (2006). However, the fragment sizes for C. glabrata differ by 13–16 bp.

Comparing the ITS1, 5.8S-ITSII and the ITS1-5.8S-ITSII band sizes, there is very little difference between C. albicans, C. dubliniensis, C. tropicalis, C. krusei and C. parapsilosis. Meanwhile, the sizes of C. glabrata and C. lusitaniae are markedly different, indicating that they are, by comparison, genetically more dissimilar from the other four species.

The sizes of the C. albicans and C. dubliniensis 5.8S-ITSII as well as ITS1-5.8S-ITSII bands are identical, which is unsurprising as these two species have always been closely linked and difficult to distinguish morphologically (Pincus et al., 1999). Meanwhile, C. tropicalis and C. parapsilosis have identical ITS1 and ITS1-5.8S-ITSII bands, despite their differences in morphology. On the other hand, their 5.8S-ITSII bands differ by as much as 20 bp. Interestingly, both C. tropicalis and C. parapsilosis have shorter 5.8S-ITSII regions compared to C. albicans, C. dubliniensis and C. krusei.

Each of the control species has one MspI recognition site in the amplified region, with the exception of C. parapsilosis, which has none. Meanwhile, they each have two or three Hinfl recognition sites, and for some species, they produced bands of similar sizes that overlapped each other when visualized in the agarose gel.

As no single PCR amplification was able to distinguish between all of the control species, clinical species identification would be problematic unless multiple PCR reactions were employed. Likewise, neither Hinfl nor MspI digestion by themselves could distinguish between all seven of the control species. Similar to Shokohi et al. (2010), who employed the restriction enzymes MspI and BlnI, combining the results of the two digestions enables the differentiation of candidal species, potentially providing a quick, easy and reliable method of candidal species identification.

This study is limited by its small sample size (n = 15) as well as the limited number of ATCC colonies used as control species.

Conclusion

The candidal loads of the saliva, tongue, palate and buccal mucosa of denture-wearers were found to be significantly higher than the control group, while only the candidal loads of the tongue of periodontal patients were found to be significantly higher, whereas at all the other sites there were no significant differences. Meanwhile, the mean percentages of candidal load were lowest in the saliva for all three groups, while they were highest on the tongue surface of the control group, and highest on the palate surface of denture-wearers and periodontal patients.

Fifteen clinical samples were randomly isolated and were then differentiated into six different genotypes, based on the sizes of their ITS regions and restriction fragments. Of these six different genotypes, five were successfully matched to the
genotype profile of a control species, enabling identification of their species.

Of the seven control species, C. albicans, C. tropicalis, C. krusei, C. parapsilosis, C. dubliniensis, C. glabrata and C. lusitaniae, MspI restriction digestion of the amplified ITS-5.8S–ITSII region was successful in differentiating between all those species, with the exception of not being able to distinguish between C. albicans and C. dubliniensis, whereas the HinfI digest could differentiate all of the aforementioned species with the exception of not being able to distinguish between C. tropicalis and C. parapsilosis. However, combining the results of both MspI and HinfI digestions would enable quick identification based on the resulting band patterns.

Further investigation of these and other restriction fragments has potential for the development of a system of candidal species identification that is faster and also more accurate than traditional methods of identification. It would also be less expensive than DNA sequencing, making it a viable alternative for diagnostic purposes. Furthermore, there is the possibility of the development of hybridization probes for rapid genotype-based species identification.

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