Quantitative detection of *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* in human oral epithelial cells from subjects with periodontitis and periodontal health

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Epithelial cells in oral cavities can be considered reservoirs for a variety of bacterial species. A polymicrobial intracellular flora associated with periodontal disease has been demonstrated in buccal cells. Important aetiological agents of systemic and nosocomial infections have been detected in the microbiota of subgingival biofilm, especially in individuals with periodontal disease. However, non-oral pathogens internalized in oral epithelial cells and their relationship with periodontal status are poorly understood. The purpose of this study was to detect opportunistic species within buccal and gingival crevice epithelial cells collected from subjects with periodontitis or individuals with good periodontal health, and to associate their prevalence with periodontal clinical status. Quantitative detection of total bacteria and *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* in oral epithelial cells was determined by quantitative real-time PCR using universal and species-specific primer sets. Intracellular bacteria were visualized by confocal microscopy and fluorescence *in situ* hybridization. Overall, 33 % of cell samples from patients with periodontitis contained at least one opportunistic species, compared with 15 % of samples from healthy individuals. *E. faecalis* was the most prevalent species found in oral epithelial cells (detected in 20.6 % of patients with periodontitis, *P* = 0.03 versus healthy individuals) and was detected only in cells from patients with periodontitis. Quantitative real-time PCR showed that high levels of *P. aeruginosa* and *S. aureus* were present in both the periodontitis and healthy groups. However, the proportion of these species was significantly higher in epithelial cells of subjects with periodontitis compared with healthy individuals (*P* = 0.016 for *P. aeruginosa* and *P* = 0.047 for *S. aureus*). Although *E. faecalis* and *P. aeruginosa* were detected in 57 % and 50 % of patients, respectively, with probing depth and clinical attachment level ≥ 6 mm, no correlation was found with age, sex, bleeding on probing or the presence of supragingival biofilm. The prevalence of these pathogens in epithelial cells is correlated with the state of periodontal disease.

INTRODUCTION

The oral cavity harbours a complex microbiota that is commonly related to oral health. Nonetheless, it is common knowledge that only some microbial consortia are associated with carious lesions and periodontal disease (Aas et al., 2005; Socransky & Haffajee, 2005). On the other hand, classically pathogenic micro-organisms can find favourable environmental conditions within and occupy specific niches of the oral cavity (Fourrier et al., 1998). Bacterial species commonly associated with nosocomial infections and multi-resistance to antimicrobials, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Enterococcus faecalis* and the *Enterobacteriaceae* family, have been detected in high proportions and levels in the subgingival biofilm of individuals with periodontitis (Slots et al., 1990; Colombo et al., 2002) and in the endodontic canal (Brito et al., 2007). Furthermore, there seems to be a strong association between the presence of these pathogens in dental plaque and respiratory infections in hospitalized patients (El-Solh et al., 2004; Didilescu et al., 2005; Bahrani-Mougeot et al., 2007).
It remains unclear whether these micro-organisms of medical importance are involved in the process and progression of periodontal disease or if, conversely, the presence of deep pockets in periodontal disease might act as an excellent reservoir for pathogens and, consequently, a risk factor for systemic infection (Raghavendran et al., 2007; Paju & Scannapieco, 2007). Several studies have evaluated the participation of S. aureus, one of the most common causes of nosocomial and community-acquired infections, in chronic and aggressive periodontitis and peri-implantitis (Leonhardt et al., 2003; Murdoch et al., 2004; Souto et al., 2006; Fürst et al., 2007; Fritschi et al., 2008; Persson et al., 2008). Although isolated from dental plaque, carious lesions and gingival pockets (Dahlén & Wikström, 1995; Colombo et al., 2002; Didilescu et al., 2005; Kouidhi et al., 2010), S. aureus is not considered a resident oral bacterium. E. faecalis is an opportunistic pathogen found in human gastrointestinal microbiota. This species is also frequently isolated from failed root canals undergoing re-treatment and represents the third most common cause of nosocomial infections (Sedgley et al., 2006). The occurrence of E. faecalis in periodontal pockets seems to be higher in individuals with periodontitis, including patients with chronic refractory periodontitis (Rams et al., 1992; Souto & Colombo, 2008; Balaei-Gajan et al., 2010), compared with those without periodontitis, but the correlation between the prevalence of this pathogen and periodontal disease remains unclear. Strong associations have been found between colonization of the oral cavity by P. aeruginosa and respiratory infections, with some results even suggesting that poor oral hygiene and periodontal disease may contribute to this condition, especially in hospitalized and immunosuppressed patients (Raghavendran et al., 2007; Persson et al., 2008). In addition, interactions between periodontal pathogens and P. aeruginosa might contribute to the ability of P. aeruginosa to invade epithelial cells and disseminate systemically (Pan et al., 2009).

Recent studies have demonstrated the presence of periodontopathic bacteria inside buccal epithelial cells. The polymicrobial intracellular nature of these cells, similar to that of dental biofilm, suggests that these cells may provide a protective environment in vivo and a reservoir for bacterial re-colonization of the gingival crevice (Rudney et al., 2005a; Colombo et al., 2007). However, few studies have been performed to determine whether opportunistic species that are frequently isolated from the oral cavity can also be found within buccal and gingival epithelial cells. The aims of the present study were therefore to determine the presence of S. aureus, P. aeruginosa and E. faecalis within oral epithelial cells collected from subjects with and without periodontitis by quantitative real-time PCR (qPCR) and fluorescence in situ hybridization (FISH), and to investigate the prevalence of these species and their association with periodontal status.

METHODS

Subjects. Forty-six adults with periodontal disease and 12 with periodontal health (PH) underwent a complete periodontal clinical examination by a calibrated examiner at the Dental School of the University of São Paulo, Brazil. This study was approved by the research ethics committee of the University of São Paulo. All participants were informed about the nature of the study and signed a consent form. Periodontal clinical measurements were performed at six sites per tooth for each subject, and included probing depth (PD), clinical attachment level (CAL), presence of supragingival biofilm and bleeding on probing (BOP). The deepest PDs were ≤ 3 mm (range 2.0–3.0 mm; mean ± SD 2.0 ± 0.60 mm) in PH subjects and ≥ 4 mm (range 4.0–16.0 mm; mean ± SD 6.17 ± 2.30 mm) in subjects with periodontitis. In each subject, the three teeth with the deepest PDs were chosen as the target sites for sampling. Exclusion criteria included pregnancy, use of local or systemic antimicrobial agents within 6 months prior to entry into the study, diabetes and other systemic conditions that could affect the periodontal status (Armitage, 2004).

Collection of oral epithelial cells. After removal of supragingival and subgingival biofilms, gingival crevice epithelial cell and buccal epithelial cell samples were collected with sterile cytological brushes from a pool of three sites in each subject. Cell samples were placed in individual tubes containing 1 ml of Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10 % fetal bovine serum and 50 µg gentamicin ml⁻¹ (Amersham Pharmacia Biotech). A portion of each sample was stored in liquid nitrogen for FISH and another was frozen at −20 °C for extraction of genomic DNA (Dibart et al., 1998).

DNA extraction. Epithelial cells were harvested, washed gently with PBS to remove dead or non-attached bacteria and submitted to DNA extraction. P. aeruginosa PA14, S. aureus ATCC 33591 and E. faecalis ATCC 29212 strains were used as standard DNA for qPCR. Genomic DNA was extracted using the WIZARD® Genomic DNA Purification kit (Promega), according to the manufacturer’s instructions.

Quantitative real-time PCR. In order to quantify the total amount of eubacteria and opportunistic species present in the cell samples, qPCR was undertaken using universal primers for 16S rRNA gene and species-specific primer sets (Table 1). Standard curves containing 10²–10⁵ DNA copies µl⁻¹ of E. faecalis ATCC 29212, S. aureus ATCC 33591 and P. aeruginosa PA14 PCR products were used. For amplification reactions, triplicate samples were routinely used and assays were performed in a total volume of 20 µl containing 10.0 µl SYBR Green Quantomix (Biotools), 1.0 µl each forward and reverse primer (final concentration 200 nM each), 2 µl template DNA solution (10 ng µl⁻¹) and 8 µl sterilized nuclease-free water (Ambion, Life Technologies). Amplification reactions were performed in a thermocycler iCycler (Bio-Rad Laboratories) using optical-grade 96-well plates programmed for initial denaturation at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s, as described previously (Abiko et al., 2010). During the annealing and extension step, fluorescence emissions were monitored and data were analysed using iCycler iQ Software (Bio-Rad Laboratories). Non-template controls were included for all reactions. A melting curve analysis was performed to assess reaction specificity.

Fluorescence in situ hybridization. The three species-specific oligonucleotide probes and the universal probe EUB 338, which hybridizes with a conserved region of all eubacteria, were obtained as conjugates to fluorescent dyes (Table 1). Each cell sample positive for opportunistic species by qPCR was hybridized separately with each species-specific and the universal probe. The FISH protocol used has been previously described by Rudney et al. (2001) with modifications. Briefly, oral epithelial cell samples and bacterial suspensions were washed in PBS and fixed in cold acetone 100 % for 5 min. A 100 µl aliquot was then spotted onto poly-L-lysine-coated glass slides (Sigma-Aldrich) and permeabilized with 0.1 % Triton X-100 (Bio Basic) for 10 min at 37 °C. In order to detect Gram-positive species,
cells were also treated with a permeabilization buffer (PBS) containing 1 mg lysosome ml⁻¹ (Sigma-Aldrich) and 1 mg lysostaphin ml⁻¹ (Sigma-Aldrich) for 5 min at 37 °C. Pre-warmed hybridization buffer [100 µl; 20 mM Tris/HCl (pH 7.6), 0.9 M NaCl, 0.01 % SDS and 30 % formamide] containing each FITC or Cy5-labelled probe (50 ng µl⁻¹) was carefully added onto the smears. Slides were incubated for 2 h in a dark, humid chamber at 50 °C and washed twice for 15 min at 60 °C in 50 ml wash solution (20 mM Tris, 180 mM NaCl and 0.01 % SDS). Finally, slides were rinsed with distilled water and air-dried in the dark. ProLong Gold anti-fade reagent (Molecular Probes) was added to the glass slides, which were mounted and sealed with coverslips.

Confocal microscopy. A laser-scanning confocal microscope (model LSM 510, Carl Zeiss) was employed in epithelial cell samples to determine whether bacteria detected by FISH were inside the epithelial cells. A series of 7 to 32 confocal sections were scanned with a series of z increments of stacks of 20 µm. A laser-scanning confocal microscope (Sigma-Aldrich) for 5 min at 37 °C. Pre-warmed hybridization buffer [100 µl; 20 mM Tris/HCl (pH 7.6), 0.9 M NaCl, 0.01 % SDS and 30 % formamide] containing each FITC or Cy5-labelled probe (50 ng µl⁻¹) was carefully added onto the smears. Slides were incubated for 2 h in a dark, humid chamber at 50 °C and washed twice for 15 min at 60 °C in 50 ml wash solution (20 mM Tris, 180 mM NaCl and 0.01 % SDS). Finally, slides were rinsed with distilled water and air-dried in the dark. ProLong Gold anti-fade reagent (Molecular Probes) was added to the glass slides, which were mounted and sealed with coverslips.

Statistical analysis. Mean values ± SD were calculated for each bacterial species were examined by Spearman’s correlation coefficient. A significance level of P<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Several studies have attempted to elucidate the role of periodontal disease as a source of opportunistic non-oral pathogens and a risk factor for systemic infections. Studies have focused on the gingival pocket as reservoir for these bacterial species (El-Soh et al., 2004; Gebara et al., 2006; Raghavendran et al., 2007; Paju & Scannapieco, 2007). The present study examined oral epithelial cells from individuals with periodontitis and PH for the presence and quantification of E. faecalis, S. aureus and P. aeruginosa using qPCR.

Demographic and periodontal clinical features

Table 2 shows the distribution of age, sex and smoking status according to clinical groups. Mean PD, CAL, BOP and supragingival biofilm differed significantly (P<0.05) between the periodontitis and PH groups. As expected, subjects with periodontitis showed greater mean PD and CAL than PH subjects. BOP and the percentage of sites with supragingival biofilm accumulation were significantly higher in patients with periodontitis (57 % and 78 %, respectively) compared with PH subjects. The mean age of the PH individuals was significantly lower (37.3 ± 12.1 years) than that of subjects with periodontal disease (47.2 ± 13.7 years). This fact has been observed in several demographic studies. Moreover, it has been reported that the subgingival microbiota of PH subjects does not change markedly with age and may not influence our results (Haffajee et al., 1998; Abiko et al., 2010).

Detection frequency of opportunistic species in oral epithelial cells by qPCR

Our microbiological findings revealed that epithelial cell samples from periodontitis patients presented a higher prevalence of total opportunistic species (33.3 %, P<0.05) than samples from individuals with PH (15 %), regardless of epithelial cell type (Table 3). Among species detected by qPCR, E. faecalis had the highest prevalence among subjects with periodontitis. Data correlating periodontal infection with E. faecalis colonization are limited. Previous

Table 1. Sequences of oligonucleotide primers used for quantitative real-time PCR and probes used for FISH

<table>
<thead>
<tr>
<th>Species</th>
<th>Primer/ Probe sequence (5-3)</th>
<th>Product size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterococcus faecalis</td>
<td>forward: GTT TAT GCC GCA TGG CAT AAG AG</td>
<td>310</td>
<td>Rocos et al., 2004</td>
</tr>
<tr>
<td>Target gene: 16S rRNA</td>
<td>reverse: CCG TCA GGA GAC GGT CAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENF191*</td>
<td>probe: GAA AGC GCC TTT CAC TCT TAT GC</td>
<td>93</td>
<td>Wellinghausen et al., 2007</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>forward: AAT TAA CGA AAT GGG CAG AAA CA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Target gene: fem B</td>
<td>reverse: TGC GCA ACA CCC TGA ACT T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sau*</td>
<td>probe: GAA GCA AGC TTC TCG TCC G</td>
<td></td>
<td>Kempf et al., 2000</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>forward: ATG AAC AAC GTT CTG AAA TTC TCT GCT</td>
<td>249</td>
<td>De Vos et al., 1997</td>
</tr>
<tr>
<td>Target gene: oprl</td>
<td>reverse: CTT GCG GCT GGG TTT TTC CAB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSEUDOAOER*</td>
<td>probe: GGA CGT TAT CCC CCA CTA T</td>
<td></td>
<td>Jansen et al., 2000</td>
</tr>
<tr>
<td>Universal</td>
<td>357F: CTC CTA CGG GAG GCA GCA G</td>
<td>~620</td>
<td>Yamaura et al., 2005</td>
</tr>
<tr>
<td>Target gene: 16S rRNA</td>
<td>907R: CCG TCA ATT CMT TTR AGT TT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EUBIS38†</td>
<td>probe: GCT GCC TCC GCT AGG AGT</td>
<td></td>
<td>Amann et al., 1990</td>
</tr>
</tbody>
</table>

*Probes 5’ labeled with sulfoindocyanine dye Cy 5 (Bioneer®, Alameda, CA, USA).
†Probes 5’ labeled with fluorescein isothiocyanate (FITC) (Molecular Probes®, Life Technologies, USA).
studies have detected *E. faecalis* in different sites of the oral cavity by PCR and qPCR, but the data are divergent not only in the frequency of detection, but also in their determination of the role of this micro-organism in the pathogenesis of periodontitis (Roças et al., 2004; Sedgley et al., 2004, 2006; Souto & Colombo, 2008). In general, the prevalence of *E. faecalis* in the oral cavity seems to increase in individuals with periodontitis (Slots et al., 1990; Souto et al., 2006; Balaei-Gajan et al., 2010).

Our results showed a significantly higher frequency of *E. faecalis* associated with epithelial cell samples (20.6%) from periodontitis patients compared with PH subjects (P=0.03; Fig. 1). Interestingly, *E. faecalis* was not detected in oral epithelial cells from subjects with PH. In addition, cell samples containing *E. faecalis* were detected in 57% of individuals with PD and CAL ≥6 mm, but no significant correlations were found (data not shown). The frequency of detection of *S. aureus* and *P. aeruginosa* was similar among the periodontitis and PH groups, ranging between 6.0% and 12.5%, regardless of epithelial cell type (Table 3). No difference was observed in the prevalence of *P. aeruginosa* between periodontitis and PH subjects (Fig. 1), or between the types of epithelial cells. This species has previously been associated with periodontal disease (da Silva-Boghossian et al., 2011) and has been detected at a higher prevalence in the subgingival biofilm of HIV-infected subjects with chronic periodontitis (Gonçalves et al., 2009).

### Quantification and proportions of opportunistic pathogens associated with oral epithelial cells

The qPCR analysis results demonstrated that *P. aeruginosa* and *S. aureus* were found in higher counts (DNA copies numbers) than *E. faecalis* in both groups of individuals.

#### Table 2. Demographic and clinical parameters from the individuals with periodontitis and periodontally healthy subjects

Values are n (%) or mean ± sd.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Periodontitis (n=46)</th>
<th>Periodontal health (n=12)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>27 (59)</td>
<td>9 (75)</td>
<td>0.342†</td>
</tr>
<tr>
<td>Male</td>
<td>19 (41)</td>
<td>3 (25)</td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>10 (22)</td>
<td>2 (17)</td>
<td>1.000†</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>47.2 ± 13.7</td>
<td>37.3 ± 12.1</td>
<td>0.025‡</td>
</tr>
<tr>
<td>PD (mm)*</td>
<td>6.17 ± 2.3</td>
<td>2.0 ± 0.6</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td>CAL (mm)*</td>
<td>6.6 ± 2.1</td>
<td>1.4 ± 0.5</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td>BOP (no. sites)*</td>
<td>26 (57)</td>
<td>8 (1)</td>
<td>0.003†</td>
</tr>
<tr>
<td>Supragingival biofilm (no. sites)*</td>
<td>36 (78)</td>
<td>2 (17)</td>
<td>&lt;0.001†</td>
</tr>
</tbody>
</table>

*Significantly different between groups with periodontitis and PH (P<0.05).
†Fisher’s exact test.
‡Student’s t-test.

#### Table 3. Prevalence of opportunistic species associated with oral epithelial cells

<table>
<thead>
<tr>
<th>Species</th>
<th>Subjects with periodontitis (%)</th>
<th>Subjects with PH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buccal epithelial cells</td>
<td>Gingival crevice epithelial cells</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>24.2</td>
<td>17.1</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>6.1</td>
<td>0</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>9.2</td>
<td>5.7</td>
</tr>
<tr>
<td><em>E. faecalis</em> + <em>P. aeruginosa</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. aureus</em> + <em>E. faecalis</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. aureus</em> + <em>P. aeruginosa</em></td>
<td>3.0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. aureus</em> + <em>E. faecalis</em> + <em>P. aeruginosa</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>39.5</td>
<td>22.8</td>
</tr>
</tbody>
</table>

Periodontitis = 33.3†

PH = 15.0†

*Significant difference between periodontitis and PH groups (P<0.05, Student’s t-test).
†Prevalence of total non-oral bacteria (percent) detected in each group.
when compared with the total amount of bacterial DNA (Fig. 2). Although no difference was observed in the prevalence and quantities of *P. aeruginosa* among periodontitis and PH subjects, or in the types of epithelial cells, the proportion of this species was higher in cells from individuals with periodontitis (9.2 % ± 2.94) than in cells from PH subjects (*P* 0.016; Fig. 3). Moreover, 50 % of individuals with PD and CAL 6 mm harboured *P. aeruginosa* in their epithelial cells; however, there was no statistically significant correlation (data not shown).

Interactions between periodontopathic bacteria and *P. aeruginosa* may facilitate the adhesion and internalization of this respiratory pathogen to epithelial cells lines (Scannapieco *et al.*, 2001; Pan *et al.*, 2009). Studies by da Silva-Boghossian *et al.* (2011) showed that red complex associated with *P. aeruginosa* present in the subgingival microbiota significantly increased the likelihood of a subject having aggressive periodontitis. *P. aeruginosa* also seemed to have synergy with *Aggregatibacter actinomycetemcomitans*, increasing the risk for periodontal disease. Multivariate logistic regression analysis demonstrated significant associations between periodontal disease and combinations of *P. aeruginosa* with *Tannerella forsythia* and with *Treponema denticola*. Furthermore, a study using the microarray technique reported that *P. aeruginosa* was significantly more predominant in refractory periodontitis than in successfully treated or PH subjects (Colombo *et al.*, 2009). Likewise, Persson *et al.* (2008) showed that *P. aeruginosa* might be of potential use as a diagnostic tool for periodontitis. We speculate that a possible interaction between *P. aeruginosa* and periodontal pathogens, associated with the disease profile, may contribute to the high proportion of internalized *P. aeruginosa* observed in our study.

*E. faecalis* presented the lowest proportion (0.235 ± 0.12 %) and quantities when compared with total bacterial DNA present in cell samples (Figs 2 and 3). A higher frequency in periodontitis patients and low amounts of *E. faecalis* agree with previous studies that reported on oral rinse, saliva, dental plaque and subgingival biofilm samples (Rams *et al.*, 1992; Sedgley *et al.*, 2004; Souto & Colombo, 2008). Although studies have shown adhesion of *E. faecalis* to epithelial cells such as in the intestine, very little is known about their invasive behaviour. *In vitro*, enterococci are able to invade HeLa, HT-29 and CaCo-2 cells, and have been shown to survive within phagocytes when an m.o.i. of 1000 was used (Bertuccini *et al.*, 2002; Baldassarri *et al.*, 2005). To our knowledge, this is the first study to detect *E. faecalis* associated with oral epithelial cells *ex vivo*. One reason for these findings may be the possibility that other
bacteria of the oral microflora – such as streptococcal species, as demonstrated by Rudney et al. (2005b) – have a greater ability to adapt to epithelial cells than *E. faecalis*.

In the present study, *S. aureus* showed the lowest prevalence in both groups (2.9% in periodontitis subjects and 10% in PH subjects; Fig. 1), but the proportion of this species among the total bacteria associated with oral epithelial cells was significantly higher in patients with periodontal disease than in healthy subjects (*P*<0.05; Fig. 3). Our report also found relatively lower amounts of *S. aureus* than previous studies (Fig. 2) (Murdoch et al., 2004; Souto et al., 2006). Fritschi et al. (2008) observed high levels of *S. aureus* (>10^5 cells) in subgingival samples of individuals with chronic periodontitis and aggressive periodontitis. This discrepancy may be due to different techniques used in previous studies, which did not include epithelial cell samples. Studies have documented that *S. aureus* can adhere to and invade different cells and that it persists intracellularly for various periods of time (Garzoni & Kelley, 2009). Furthermore, some studies have shown interactions between periodontopathic pathogens and *S. aureus* strains, which may contribute to colonization of *S. aureus* in the oral cavity and enhance its invasion (Kamaguchi et al., 2003; Tada & Hanada, 2010).

**Correlation between intracellular detection of opportunistic species within oral epithelial cells and periodontal status**

Until now, there has been no evidence of a relationship between the prevalence of opportunistic pathogens within buccal and gingival epithelial cells and periodontitis. In this study, we found that the prevalence of *E. faecalis, S. aureus* and *P. aeruginosa* within oral epithelial cells was related to disease periodontal status (Table 3; Figs 1 and 2). No significant correlations were found between the frequency of opportunistic species detected in epithelial cells and smoking, age, sex, CAL, BOP and PD >6 mm (Spearman’s rank correlation; data not shown).

**Intracellular localization visualized by FISH and confocal microscopy**

Intracellular localization of *S. aureus, E. faecalis* and *P. aeruginosa* was confirmed by FISH and confocal microscopy, occurring in the range of 1–100 bacteria (Fig. 4c, d). Various epithelial cells without internalized bacteria were visualized and no signs of apoptosis or cellular damage were observed in the cells (Fig. 4a). Similar results have been shown in other studies, supporting the suggestion that several species, including extra-oral bacteria, have the ability to invade and probably survive as facultative intracellular micro-organisms (Rudney et al., 2001, 2005a, b). Low amounts and proportions of intracellular enterococci were also determined when we examined gingival crevice epithelial cells by FISH and confocal microscopy (Fig. 4d).

Likewise, there is a polymicrobial flora associated with buccal cells; our findings suggest that this also occurs in gingival sulcus cells. When the universal probe EU388 was used with FISH, confocal microscopy allowed the visualization of aggregates with different morphotypes (Fig. 4b) indicating a pattern of co-association, as occurs on biofilm. Our qPCR results detected two opportunistic species associated with epithelial cells in both groups (Table 3). In addition, recent work has suggested that coaggregation between periodontopathic bacteria and other oral pathogens may promote attachment and invasion of normally non-invasive bacteria in epithelial cells (Lafontaine et al., 2004; Edwards et al., 2006).

Although the intracellular localization of a broad range of oral bacteria in different types of oral epithelial cells seems a common phenomenon, intracellular periodontopathic species have been found to be more prevalent in cells from periodontitis patients (Colombo et al., 2007; Edwards et al., 2006; Rudney et al., 2001, 2005a). One can speculate that, in the periodontal pocket site, the persistence of an inflammatory process associated with the establishment of higher numbers of pathogenic subgingival microbiota may induce epithelial cells to express specific molecules that favour the adherence and internalization of certain bacterial species (Vitkov et al., 2005). Similarly, the presence of non-oral opportunistic pathogens internalized in epithelial cells may reflect a risk factor for systemic infections, since this ulcerated site from the gingival pocket could favour haematogenous spread (Murdoch et al., 2004).

This report demonstrates the ability of opportunistic species present in the oral cavity to invade buccal and gingival epithelial cells. In this study, we found that the prevalence of *E. faecalis, S. aureus* and *P. aeruginosa* within oral epithelial cells was related to disease periodontal status (Table 3; Figs 1 and 2). No significant correlations were found between the frequency of opportunistic species detected in epithelial cells and smoking, age, sex, CAL, BOP and PD >6 mm (Spearman’s rank correlation; data not shown).

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Intracellular localization of *S. aureus, E. faecalis* and *P. aeruginosa* was confirmed by FISH and confocal microscopy, occurring in the range of 1–100 bacteria (Fig. 4c, d). Various epithelial cells without internalized bacteria were visualized and no signs of apoptosis or cellular damage were observed in the cells (Fig. 4a). Similar results have been shown in other studies, supporting the suggestion that several species, including extra-oral bacteria, have the ability to invade and probably survive as facultative intracellular micro-organisms (Rudney et al., 2001, 2005a, b). Low amounts and proportions of intracellular enterococci were also determined when we examined gingival crevice epithelial cells by FISH and confocal microscopy (Fig. 4d).

Likewise, there is a polymicrobial flora associated with buccal cells; our findings suggest that this also occurs in gingival sulcus cells. When the universal probe EU388 was used with FISH, confocal microscopy allowed the visualization of aggregates with different morphotypes (Fig. 4b) indicating a pattern of co-association, as occurs on biofilm. Our qPCR results detected two opportunistic species associated with epithelial cells in both groups (Table 3). In addition, recent work has suggested that coaggregation between periodontopathic bacteria and other oral pathogens may promote attachment and invasion of normally non-invasive bacteria in epithelial cells (Lafontaine et al., 2004; Edwards et al., 2006).

Although the intracellular localization of a broad range of oral bacteria in different types of oral epithelial cells seems a common phenomenon, intracellular periodontopathic species have been found to be more prevalent in cells from periodontitis patients (Colombo et al., 2007; Edwards et al., 2006; Rudney et al., 2001, 2005a). One can speculate that, in the periodontal pocket site, the persistence of an inflammatory process associated with the establishment of higher numbers of pathogenic subgingival microbiota may induce epithelial cells to express specific molecules that favour the adherence and internalization of certain bacterial species (Vitkov et al., 2005). Similarly, the presence of non-oral opportunistic pathogens internalized in epithelial cells may reflect a risk factor for systemic infections, since this ulcerated site from the gingival pocket could favour haematogenous spread (Murdoch et al., 2004).
gingival crevice epithelial cells. In the present study, these pathogens were most frequently detected within oral epithelial cells in the periodontitis group, suggesting that this condition may contribute to their establishment and re-colonization in oral sites. Attention should be given to these groups regarding the risk for the development of infections by these species in other sites of the body.

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