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Subgingival plaque microbiota in HIV positive patients

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Abstract

Aim: To describe and compare the predominant bacterial and fungal species associated with gingivitis, periodontitis, and linear gingival erythema (LGE), in HIV positive subjects with different immune status.

Methods: Viral loads and CD4 levels determined HIV disease status. From pooled subgingival plaque, 16S and 18S rDNA were cloned and sequenced to determine species identity.

Results: One hundred and nine bacterial species were identified from 14 subjects. Nearly half of the species were not cultivable. Notably, the classical putative periodontal pathogens, *Treponema denticola, Porphyromonas gingivalis* and *Tannerella forsythia* were below the limit of detection and were not detected. Species of *Gemella, Dialister, Streptococcus* and *Veillonella* were predominant. In one HIV positive subject with periodontitis and low viral load, *Gemella morbillorum*, a known opportunistic pathogen, constituted 84% of the clones. *Saccharomyces cerevisiae* was the only fungal species detected in an LGE subject and in periodontitis subjects with high viral loads. In periodontitis patients with low viral loads, *Candida albicans* was predominant, while *S. cerevisiae* was only a minor component.

Conclusion: These case studies suggest that other bacterial species, rather than the classical periodontal pathogens, may be involved in periodontal diseases of subjects with HIV. These data are indicative of opportunistic infections in a highly susceptible immunocompromised host.

Key words: 16S and 18S rDNA; HIV positive; periodontitis; subgingival plaque

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In the past 20 years, 20 million people have died from AIDS. The number of people living with human immunodeficiency virus (HIV) still continues to grow, from 35 million people in 2001 to almost 39.4 million in 2004 (http://www.unaids.org/bangkok2004/GAR2004_html/GAR2004_00_en.htm http://www.cdc.gov/hiv/stats.htm). Oral disease manifestations are often the first clinical expression of HIV infection (Piluso et al. 1993).

A multitude of oral lesions have been described in individuals infected with HIV (Reichart et al. 1987, Makimura et al. 1994, Horning & Cohen 1995, Robinson et al. 1996, Stanford & Rivera-Hidalgo, 1999), such as oral candidiasis, hairy leucoplakia (Kappe et al. 1996), recurrent herpes infections, papilloma virus infection, and mucosal Kaposi's sarcoma (Chapple et al. 1992).

Infections such as necrotizing ulcerative periodontitis (NUP) and linear gingival erythema (LGE) are more commonly seen in the HIV positive (HIV^+) population (Kinane et al. 1989, Murray et al. 1989). LGE, characterized by a marginal band with apical, focal and/or diffuse areas of erythema that may extend beyond the mucogingival line, is associated with the early stage of HIV infection and CD4 T lymphocyte suppression (Robinson et al. 1997). HIV^+ patients are predisposed to periodontal infections. It has been documented that conventional gingivitis is more prevalent, extensive and severe in HIV^+ individuals while chronic periodontitis progresses more rapidly in HIV^+ individuals than in the HIV^- population (Robinson et al. 1997).

Based on a limited number of species tested, recent studies have indicated that the microbial components of HIV-associated periodontal diseases are not significantly different from those in

Conflict of interest and source of funding statement

The authors declare that they have no conflict of interest.

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Table 1. The mean clinical measurements of the four sampled sites

Age	Pack years.	CD4	Viral load	PI.	GI	BOP	PD	AL
Low HIV, high CD4 gingivitis								
41	17.25	442	0	1	0	0	2.5	0.5
41	0.25	498	0	1.5	1	0	2.25	0.25
59	29	401	0	1	1	0.5	2	0
49	3	1101	0	1	1	0	2	0.75
40	0	772	0	1.5	1	0	2.25	1.5
High HIV, low CD4 periodontitis								
35	30	143	26166	3	1.5	0.75	6	6.75
51	0.75	170	21758	2	1	0.25	4.5	3.75
56	3	161	20067	2	1	0.5	4	4.75
39	5	66	44929	1	1	0.5	4	3.75
Low HIV, high CD4 periodontitis								
52	45	531	0	2.5	1	0	5	5.75
36	15	296	0	1	1.5	0.75	4.5	2.5
41	2	548	0	1	1	0.5	4.5	4.25
38	12.5	356	0	2.5	1.25	0.25	6.25	8
Low HIV, high CD4 LGE								
37	0.45	640	0	3	2.6	0.83	3.5	1.83

All patients in the study were white male. BOP, bleeding on probing; pack years, number of packs of cigarettes smoked/day multiplied by number of years smoked; PD, probing depth; AL; attachment level; PI, plaque index; GI, gingival index.

HIV^- individuals (Rams et al. 1991, Moore et al. 1993, Alpagot et al. 2004). Studies based on culture methods, selective media, and microscopy did not register substantial differences in the microbiota of HIV-infected subjects with or without periodontal disease from that of seronegative subjects (Brady et al. 1996). Similarly, an association between LGE and *Candida* infections has been described (Kappe et al. 1996), but it is not known whether additional fungi are also involved. Consequently, the microbial associations of periodontal HIV diseases are not clearly understood.

The aim of the present study was to use molecular methods to describe and compare the predominant bacterial and fungal species associated with common periodontal infections in HIV^+ subjects, including gingivitis, periodontitis, and LGE in HIV^+ patients with different immune status.

Material and Methods

Subject population

A total of 14 HIV infected male subjects were recruited from the CARE clinic at the University of Pacific, School of Dentistry. Subjects were classified into three periodontal disease categories groups as defined: gingivitis, periodontitis, and LGE (with no sign of attachment loss). Five gingivitis and four periodontitis subjects with low viral load (<2,000 copies/ml) and high CD4 levels (>300 cells/mm^3) were analysed. Four periodontitis subjects with high viral load values (>20,000 copies/ml) and low CD4 levels (<200 cells/mm^3) were also analysed. Furthermore, one LGE subject with high CD4 and low viral loads values was analysed. Subjects were >18 years of age, had >20 teeth, did not require premedication with antibiotics for a periodontal examination, did not have other systemic health problems, did not have periodontal therapy within the last 6 months, or had not taken antibiotics, steroids, or non-steroidal anti-inflammatory drugs within the last 3 months. The demographic, immunologic, and clinical data are shown in Table 1. The protocol for all procedures was approved by the Institutional Review Board of the Pacific Medical Center and The Forsyth Institute. All study subjects signed the committee-approved informed consent.

Disease category definitions

LGE

HIV^+ subject had at least four gingivitis sites with evidence of LGE, i.e., a marginal band of intense erythema with apical, focal and/or diffuse areas of erythema that occasionally extended beyond the mucogingival borderline.

Gingivitis

Subjects with red to bluish red edematous gingival tissue usually had swollen interdental papillae and increased tendency to bleeding. Each subject had at least four gingivitis sites based on the following criteria: gingival index >0, probing depth <3 mm, and attachment loss = 0.

Periodontitis

Subjects with periodontitis had at least 20 teeth, and at least four sites with the following criteria: gingival index >0, probing depth ⩾5 mm, and attachment loss ⩾5 mm. Clinical measurements and vertical bite wing X-rays that were taken within the last 6 months were used to characterize the periodontal condition in each of the study groups.

Sampling

Supragingival plaque from the LGE subject was collected using a Gracey curette. Before sampling the subgingival plaque, supragingival plaque was removed, and all teeth were professionally cleaned. All sample sites were isolated with cotton roles preventing saliva contamination. The subgingival plaque samples were then collected by placing a Gracey curette at the apical extent of the pocket and drawing it coronally with slight pressure against the root. A minimum of three passes were made for each site to ensure that most subgingival plaque was removed. Samples from a total of four sites, including two molars and two anterior teeth, from each participant were collected and pooled. All samples from each participant met the criteria of the actual disease category. The subgingival plaque samples were collected and transferred into a microfuge tube containing 200 μl of transport

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fluid, then stored at -80°C until the day of analysis.

Bacterial and fungal lysis

For bacterial lysis, subgingival plaque samples were directly suspended in 50 μl of 50 mM Tris buffer (pH 7.6), 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8, and 0.5% Tween 20. Proteinase K (200 μg/ml) (Roche Applied Science, Indianapolis, IN, USA) was added to the mixture. The samples were then heated at 55°C for 2 h. Proteinase K was inactivated by heating at 95°C for 5 min. For fungal lysis, pooled subgingival plaque samples from four actual sites (2 μl from each) were suspended in 10 μl Zymolase solution (2.5 mg/ml Zymolase 20T, 1.2 M sorbitol, 0.1 M sodium phosphate, pH 7.4) (Roche Applied Science) and incubated for 15–60 min. at 37°C.

PCR amplification of 16S rDNA

The 16S rDNA was amplified under standardized conditions using a universal primer set (forward primer-5;-GAG AGT TTG ATY MTG GCT CAG-3; reverse primer 5;-GAA GGA GGT GWT CCA RCC GCA-3;) (Paster et al. 2001). Primers were synthesized commercially (Operon Technologies, Alameda, CA, USA). PCR conditions were as previously described (Aas et al. 2005).

PCR amplification of 18S rDNA

For cloning of 18S rDNA, universally conserved fungal primers (Makimura et al. 1994), forward primer B2F (5′-ACT TTC GAT GGT AGG ATA G-3) and reverse primer B4R (5′-TGA TCG TCT TCG ATC CCC TA-3) were used to amplify $\sim$ 690-base 18S rDNA from clinical samples. These primers were purported to amplify *Candida* spp. and eight divergent genera, including *Hansenula* spp., *S. cerevisiae*, *Cryptococcus neoformans*, *Trichosporon beigelii*, *Malassezia furfur*, *P. carinii*, *Aspergillus* spp., and *Penicillium* spp. PCR conditions were as previously described (Aas et al. 2005).

Cloning procedures

Cloning of PCR-amplified DNA was performed using the TOPO TA cloning kit (Invitrogen, San Diego, CA, USA) according to the instructions of the manufacturer. Sizes of the inserts were determined in a PCR using an M13 (−20) forward primer and an M13 reverse primer (Invitrogen). Before sequencing of the fragments, the PCR-amplified 16S rDNA fragments were purified and concentrated according to Paster et al. (Paster et al. 2001).

rDNA sequencing

Purified PCR-amplified 16S rDNA inserts were sequenced using an ABI Prism cycle sequencing kit (BigDye Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase FS) applying a Gene amp PCR system 9700 (ABI, Foster City, CA, USA). Sequencing reactions and the primers used for bacterial DNA sequencing have been described previously (Paster et al. 2001). Fungal DNA sequencing were performed with the 3.2 μM M13 (−20) forward and reverse primer (Invitrogen).

16S and 18S rDNA sequencing and data analysis of unrecognized inserts

A total of 875 clones with a 16S rDNA insert of the correct size of approximately 1500 bases were analysed. The number of 16S rDNA clones per subject that were sequenced ranged from 53 to 70 with an average of 59.5 and a standard deviation of 4.5. A total of 306 clones with an 18S rDNA insert of the correct size of approximately 700 bases were also analyzed from six subjects; one LGE subject, two periodontitis subjects with low viral counts and three periodontitis subjects with high viral loads. The number of 16S rDNA clones per subject that were sequenced ranged from 41 to 67 with an average of 51 and a standard deviation of 9.5. For bacterial identification, a sequence of approximately 500 bases was obtained first to determine identity or approximate phylogenetic position. Full sequences of about 1500 bases were obtained by using five to six additional sequencing primers (Kazor et al. 2003) for those species deemed novel. For fungal identification, a sequence of approximately 600 bases, assembled with the forward primer B2F, was obtained first to determine identity or approximate phylogenetic position. For identification of the closest relatives, the sequences of the unrecognized inserts were compared with the 16S rDNA and 18S rDNA sequences of over 10,000 microorganisms in our database and over 100,000 sequences in the Ribosomal Database Project (Cole et al. 2005) and GenBank. The similarity matrices were corrected for multiple base changes at single positions by the method of Jukes & Cantor (1969). Similarity matrices were constructed from the aligned sequences by using only those sequence positions for which data were available for 90% of the strains tested. Phylogenetic trees were constructed by the neighbor-joining method of Saitou & Nei (1987). TREECON, a software package for the Microsoft Windows environment, was used for the construction and drawing of evolutionary trees (Van de Peer & De Wachter 1994). We are aware of the potential creation of 16S rDNA and 18S rDNA chimera molecules assembled during the PCR (Liesack et al. 1991). The percentage of chimeric inserts in 16S rDNA and 18S rDNA libraries ranged from 0% to 4%/clonal library. Chimeric sequences were identified by using the Chimera Check program in RDP, by treeing analysis, or by base signature analysis. Species identification of chimeras was obtained, but the sequences were not examined for phylogenetic analysis.

Nucleotide sequence accession number

The complete 16S rDNA sequences of clones representing novel phylotypes defined in this study, sequences of known species not previously reported, and published sequences are available for electronic retrieval from the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession numbers shown in Fig. 1.

Results

As shown in Fig. 1, the subgingival plaque from all the HIV^{+} subjects harbored a diverse bacterial community with 109 bacterial species detected. Nearly half (42%) of these species have not yet been cultivated and are designated as ''clones'' in Fig. 1. Species detected fell into seven bacterial phyla, namely, the *Firmicutes* (including *Streptococcus*, *Eubacterium*, *Peptostreptococcus* and related genera), the *Actinobacteria* (including *Actinomyces*, *Corynebacterium* and related genera), the *Cyanobacteria* (including *Synechococcus*), the *Fusobacteria* (including the *Fusobacterium* and *Leptotrichia* genera),

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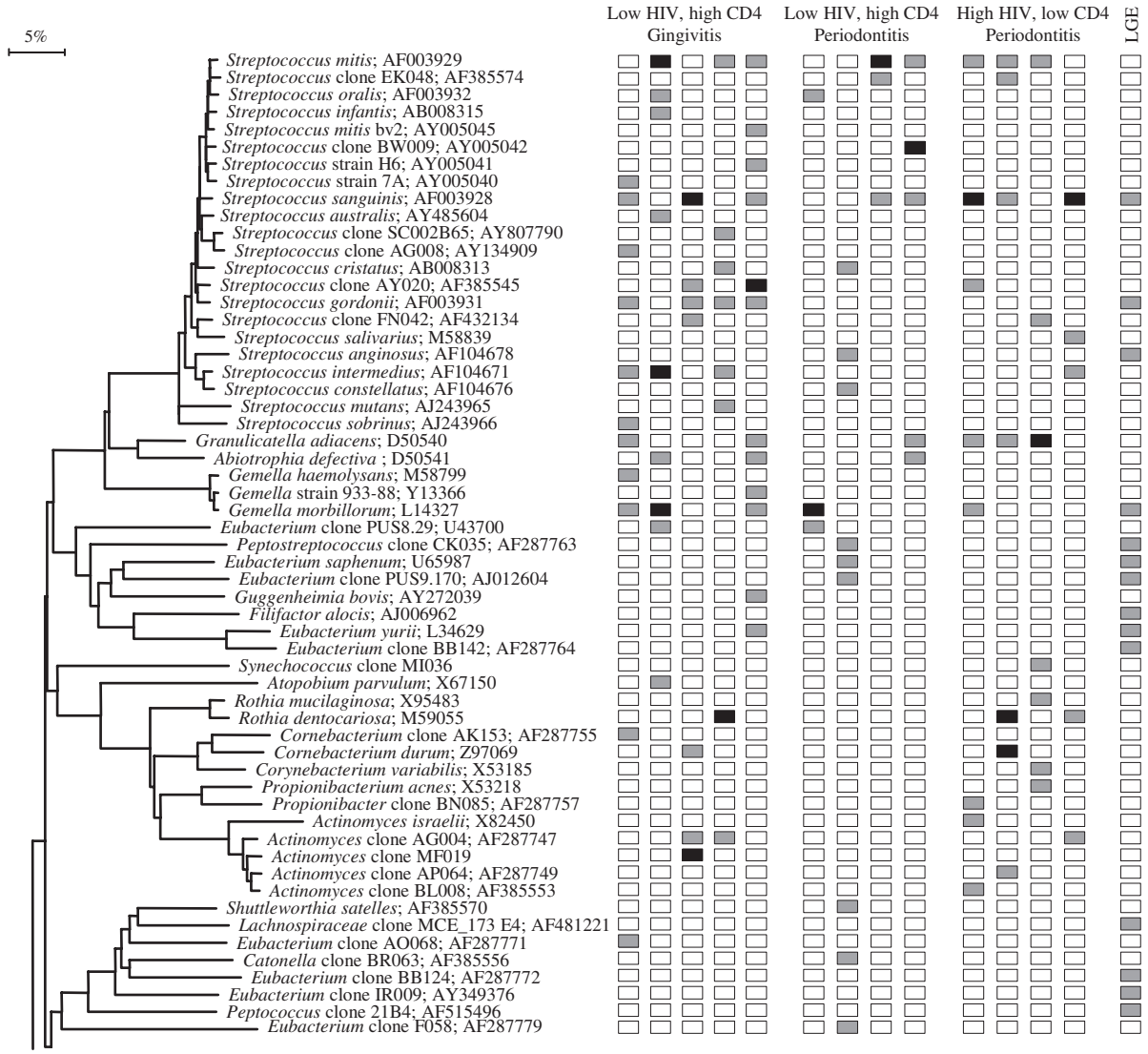

Fig. 1. Phylogenetic tree of bacterial phylotypes detected in HIV$^+$ subjects relative to immune status. Each column of boxes represents the bacterial profile/subject. Grey-shaded boxes indicate presence of species detected at $<15\%$ of the total number of clones analysed. Black-shaded boxes indicate presence of species detected at $>15\%$ of the total number of clones analysed. Clear boxes indicate that species were not detected (below the limit of detection). The marker bar represents a 5% difference in nucleotide sequences.

Synergistes, the *Proteobacteria* (including *Haemophilus*, *Neisseria*, *Campylobacter*, and related genera), and the *Bacteroidetes* (including genera *Porphyromonas* and *Capnocytophaga*).

Differences in bacterial profiles for subjects in each disease category are also presented in Fig. 1. For example, species of *Streptococcus* were more commonly detected in gingivitis HIV$^+$ subjects with low viral loads. Furthermore, species of *Corynebacterium*, *Actinomyces*, and related genera, and species of *Fusobacterium* and *Leptotrichia*, were notably absent from periodontitis HIV$^+$ subjects with low viral loads and the LGE subject, but were detected in gingivitis HIV$^+$ subjects with low viral loads and periodontitis subjects with high viral loads (Fig. 1). Species of *Proteobacteria*, such as *Pseudomonas*, *Neisseria,* and related genera, predominated in samples of subjects who were severely immunocompromised, i.e., in subjects with high viral load and low CD4 counts. Overall, the bacterial profile for the LGE subject was quite different from that of all other subjects (although clearly more subjects have to be analysed).

In many samples, some specific species predominated among the species recovered. For example, in a low viral load subject with periodontitis, the opportunistic pathogen *Gemella morbillorum* constituted 84% of the clones identified, and was detected overall in five of the subjects. Other predominant bacterial species detected were *Dialister* spp., *Streptococcus* spp., and *Veillonella* spp., *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, species often associated with periodontal disease were below the limit of detection and were not detected.

Analysis of the 18S rDNA amplified from an LGE subject revealed that *S. cerevisiae* was the only fungal species detected. In three severely immunocompromised HIV$^+$ subjects with periodontitis, again only *S. cerevisiae* was detected. Conversely, in two periodontitis subjects with low viral loads and high

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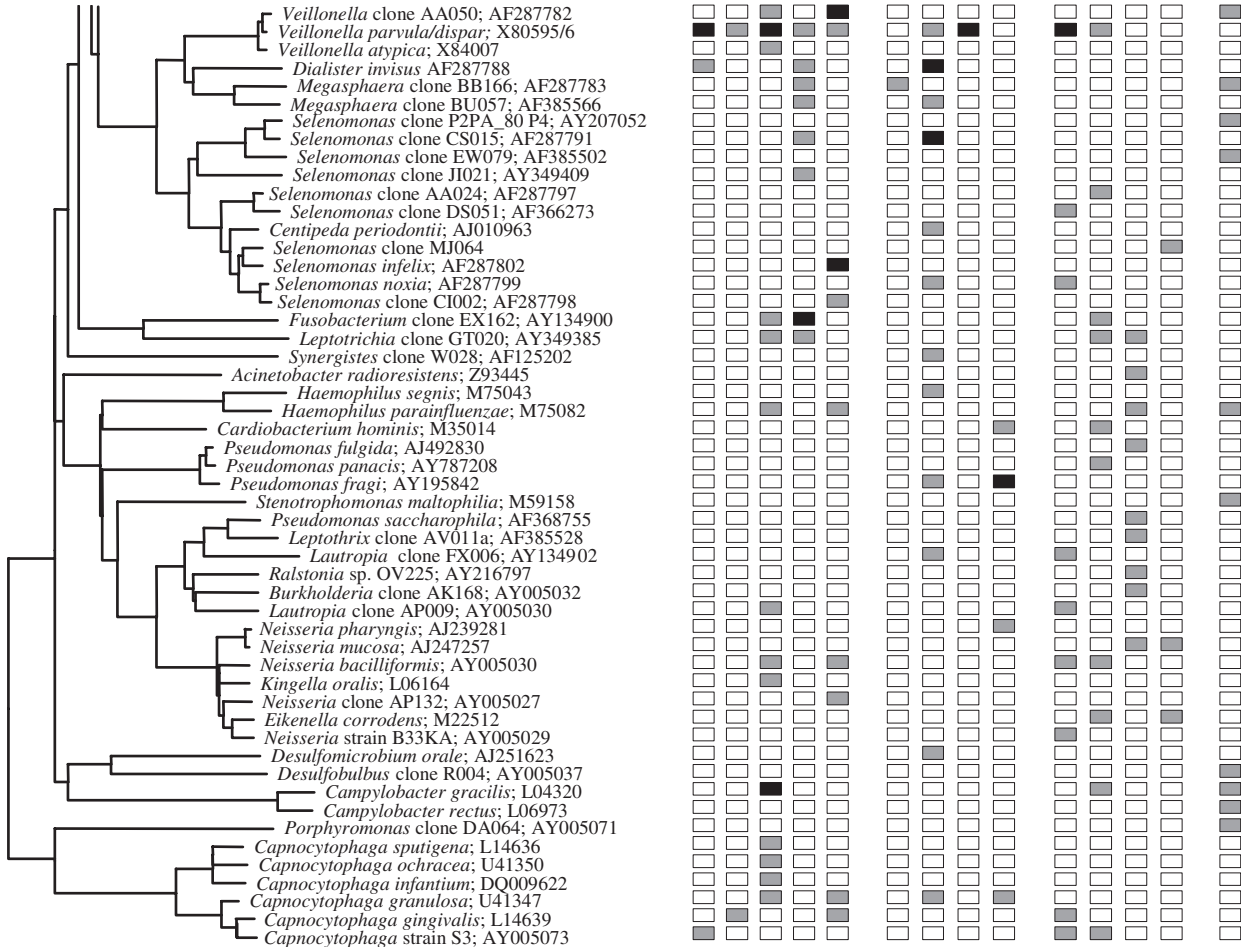

Fig. 1. Continued

CD4 levels, C. *albicans* was the predominant fungal species and *S. cerevisiae* was only a minor component. No other fungal species were detected in analysis of 306 18S rDNA clones.

Discussion

Our study represents one of the relatively few studies available describing the bacterial and fungal associations of periodontal infections in HIV^+ subjects. In previous studies, Tenenbaum, et al. (1997) found that *Campylobacter rectus* was only slightly more prevalent in HIV^+ periodontitis compared with HIV seronegative subjects with periodontitis. However in that study, only six periodontal pathogens were assayed, namely *Actinobacillus actinomycetemcomitans*, *P. gingivalis, Prevotella intermedia, T. forsythia, Eikenella corrodens* and C. *rectus*. In other studies, the bacterial microflora in HIV periodontitis was considered similar to that found in adult periodontitis (Rams et al. 1991, Moore et al. 1993). For example, species of *A. actinomycetemcomitans, C. rectus, Peptostreptococcus micros*, and *P. intermedia* were the predominant species recovered from 14 subjects with HIV periodontitis (Rams et al. 1991). However, these and other authors suggested that HIV periodontitis lesions might also contain organisms that are rarely found in common types of periodontitis, such as enterics and *Mycoplasma salivarium* (Zambon et al. 1990, Moore et al. 1993, Lamster et al. 1997). Indeed, in the present study, enterics and related species (i.e. *Proteobacteria*) were commonly detected in severely immunocompromised subjects with periodontitis.

Socransky et al. (1998) described five bacterial complexes in periodontal disease and health, with the red complex (*P. gingivalis, T. forsythia,* and *T. denticola*) most associated with periodontitis. As noted above, members of the red complex were below the limit of detection in the present study which supports the results of the previous study by Moore et al. (1993). Of course, it is possible that the red complex was present in lower amounts, i.e., $<2\%$ of the total population (approximately 50 clones were analyzed for each library, thus 1/50 is 2%). It should be noted that Paster et al. (2001) using identical cloning and sequencing methods, found all red complex members in HIV^- subjects with periodontal diseases. In another study using the same protocol, Paster et al. (2002) demonstrated that HIV^+ subjects with NUP did not have detectable levels of *P. gingivalis* and *T. forsythia*, although *T. denticola* and other spirochetes were detected. In this study, it is clear that species other than the red complex were the most prevalent species, which we feel may likely play an important role in HIV-associated periodontitis. For example in one subject, the opportunistic pathogen *G. morbillorum* constituted 84% of the clones identified, and was detected in five of the subjects. This bacterial species has been associated with endocarditis, bacteremia, noma, central nervous system infection, purulent meningitis, skeletal infections,

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and cerebral and lung abscesses (La Scola & Raoult 1998, Brouqui & Raoult 2001). Among the other predominant bacteria detected were *Dialister* spp., *Streptococcus* spp., and *Veillonella* spp. *Dialister pneumosintes* is an obligate anaerobic, non-motile, nonsporing rod that is normally present in the oral, nasopharyngeal, intestinal, and vaginal microflora, and has been considered a potential opportunistic pathogen (Ghayoumi et al. 2002, Rousee et al. 2002). Limited clinical and microbiological information has been available on human diseases caused by *D. pneumosintes* due to the difficulties associated with culturing and the lack of an accurate biochemical identification method (Rousee et al. 2002). In recent years, with the help of molecular techniques, there has been a growing interest in *D. pneumosintes* as a clinically important pathogen, often associated with a mixed microflora. Contreras et al. (2001) detected *D. pneumosintes* in 83% of severe periodontitis lesions but only in 19% of mild periodontitis lesions in HIV^- subjects. Slots et al. (2002) showed that *D. pneumosintes* and subgingival human cytomegalovirus (HCMV) were closely associated with disease-active periodontitis. These data are suggestive of opportunistic infections in HIV^+ subjects, namely, immunocompromised hosts are prone to microbial infection.

It has long been suggested that subgingival yeasts also play an important role in periodontal diseases in HIV-infected individuals. A study by Grbic et al. (1995) and Robinson et al. (1996) suggested a close association of intraoral candidiasis and occurrence of LGE. *C. albicans* was the species most often isolated from HIV-infected subjects, although other *Candida* species such as *Candida tropicalis*, *Candida krusei* and *Candida glabrata* were also recovered, but at lower levels (Gugnani et al. 2003). Even though oral candidiasis is more likely to develop in individuals with low CD4 T lymphocyte counts, the lesions have been observed in subjects with a wide range of CD4 counts (Brady et al. 1996, Gugnani et al. 2003). Surprisingly, *S. cerevisiae*, and not *C. albicans*, was the only fungal species detected from the LGE subject and in two severely immunocompromised HIV^+ subjects with periodontitis. However, *C. albicans* was predominant in two periodontitis subjects with low viral loads and high CD4 levels. *S. cerevisiae* was only a minor component in these latter subjects. Other studies (Jabra-Rizk et al. 2001) isolated S. cerevisiae from periodontal lesions of HIV-infected patients. *S. cerevisiae* is closely related to *Candida* species, which is considered as the most commonly observed pathogenic yeasts. It is noteworthy that *Saccharomyces* spp. are now among the emerging causative agents of opportunistic mycoses in patients who are compromised due to severe immunosuppression, prolonged hospitalization, prosthetic cardiac valves, and prior antibiotic therapy (Morrison et al. 1993, Salonen et al. 2000). *S. cerevisiae* has been implicated as a potentially important opportunistic pathogen, and it has so far been associated with pneumonia, endocarditis, liver abscess, fungemia, and sepsis (Eschete & West 1980, Aucott et al. 1990).

This descriptive study illustrates the breadth of bacterial and fungal diversity of HIV^+ subjects relative to different immune status. Although only a limited number of subjects were analysed, it was notable that the classical putative periodontal pathogens were below the limit of detection in periodontal disease subjects with HIV. These data suggest that species other than the red complex members are associated in these subjects. The bacterial species that were associated with periodontal infections of subjects with HIV may be more opportunistic in nature, such as species of *Gemella*. As this study represents a case series, it is premature to make definitive conclusions about the specific bacterial species or complexes of species that are associated with periodontal disease in HIV^+ subjects. However, our results do begin to determine the microbial associations with these oral diseases. In other studies, we have utilized species-specific oligonucleotide 16S rDNA-based probes in checkerboard hybridization assays to better assess bacterial associations with health and disease (Becker et al. 2002, Paster et al. 2002). We are currently developing DNA probes for approximately 200 known species and novel phylotypes for use in DNA microarray formats (Paster et al. 2006). Consequently, microbial profiles of a statistically significant number of samples from HIV^+ subjects can be readily determined to identify and compare those species or complexes of species that are associated with health and disease.

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Clinical relevance

Scientific rationale for the study: HIV^+ subjects are prone to periodontal diseases. However, only limited information is available describing the microbial composition of subgingival plaque of HIV^+ subjects with periodontal diseases. Our goal was to identify and compare the microbial diversity of the gingival crevice of HIV^+ patients with different immune status.

Principal findings: Bacterial species other than typical periodontal pathogens were predominant in subgingival plaque of HIV^+ subjects with periodontal diseases. These bacterial profiles differed with severity of HIV infection. *S. cerevisiae*, and not *C. albicans*, was predominant in periodontal subjects with high viral loads.

Practical implications: The results suggest that periodontal disease in HIV^+ subjects is opportunistic and alternative therapies may be required. The clinical significance of not cultivable bacteria is so far unclear.

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