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Microbiological evaluation of primary endodontic infections in teeth with and without sinus tract

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Abstract

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Aim To examine the microbiological status of primary endodontic infections in teeth with and without a sinus tract.

Methodology Samples were collected by means of a size 15 H-type file and two sterile paper points from 30 cases of primary endodontic infections with ($n = 15$) or without ($n = 15$) a sinus tract. The presence of 40 bacterial species was determined by the checkerboard DNA-DNA hybridization method.

Results The species found at the highest levels and prevalence were *Fusobacterium nucleatum sp. vincentii*, *Porphyromonas gingivalis*, *Veillonella parvula*, *Enterococcus faecalis*, *Campylobacter gracilis* and *Neisseria mucosa*. Total bacterial counts were similar between teeth with (44×10^5) and without (50×10^5) a sinus tract (*t*-test: $P > 0.05$). *E. faecalis*, *Streptococcus anginosus*, *Capnocytophaga sputigena* and *Capnocytophaga gingivalis* had significantly higher counts in the absence of sinus tract (Mann–Whitney test, $P < 0.05$). Higher levels of *P. gingivalis* and *Fusobacterium nucleatum sp. nucleatum* were observed in cases with a sinus tract. *Leptotrichia buccalis* (OR = 1.83; CI 95%) and *Porphyromonas endodontalis* (OR = 2.15; CI 95%) were associated with an increased chance of subjects having a sinus tract.

Conclusions Primary endodontic infections were associated with a large variety of bacterial species. Specific differences between the composition of the microbiota of primary root canal infections were observed in cases with or without a sinus tract.

Keywords: bacteria, clinical, DNA probes, endodontics, symptoms.

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Introduction

Over the years, several studies have demonstrated that pulpal and periapical pathosis are closely related to the presence of microorganisms (Miller 1894, Kakehashi *et al.* 1965, Sundqvist 1976). Many culture studies have showed that these infections are associated with a mixed microbiota, mainly composed of obligate anaerobes such as *Fusobacterium*, *Porphyromonas* and *Prevotella* (Sundqvist *et al.* 1989, Baumgartner & Falkler 1991, Gomes *et al.* 1996). The introduction of microbiological molecular diagnostic methods represented an improvement on the identification of the microbiota associated with endodontic infections. These techniques do not require bacterial viability for their identification, allowing the detection of some fastidious species difficult to cultivate in solid media, such as *Treponema* spp. (Siqueira *et al.* 2000, 2001a,b, Jung *et al.* 2001). One of these methods, called the checkerboard DNA–DNA hybridization is able to hybridize large numbers of DNA samples against large numbers of DNA probes on a single support membrane (Socransky *et al.* 1994).

Primary endodontic infections are associated with clinical signs and symptoms such as pain, presence of a periapical pathosis, swelling and sinus tract. The presence of a sinus tract close to a tooth with pulp necrosis characterizes this lesion as a chronic apical abscess. This pathological process is a result of the exit

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of irritant substances from the infected root canal to the periapical tissues or may result from an acute apical abscess that became chronic (Walton & Torabinejad 1996).

Some bacterial species, for example, black-pigmented bacteria (van Winkelhoff *et al.* 1988), or *Prevotella* spp. and *Peptostreptococcus* spp. (Gomes *et al.* 1994, 1996) have been related to specific signs and symptoms of endodontic infections such as pain. However, few studies have determined the composition of the microbiota of endodontic infections associated with a sinus tract.

Therefore, the purpose of this study was to evaluate, by the checkerboard DNA–DNA hybridization technique, the composition of the microbiota of primary endodontic infections associated with and without the presence of a sinus tract.

Materials and methods

Subject population

The subject population was composed of 30 subjects selected from the Endodontics Clinic of three different Brazilian Dental Schools: Rio de Janeiro State University, Gama Filho University and UNIGRANRIO University. All subjects were informed about the nature of the study and following the signing of an Ethics Committee approved informed consent, they were entered into the study. This work was approved by The Research Ethics Committee of Rio de Janeiro State University (#745-CEP/HUPE).

Inclusion and exclusion criteria

To be included in the study subjects had to have good systemic health, with at least one single-rooted tooth with necrotic pulp and radiographic evidence of apical pathosis, with (n = 9) or without the presence of pain (n = 21) and with (n = 15) or without (n = 15) the presence of a sinus tract. Exclusion criteria were pregnancy, nursing mothers and any systemic condition that could affect the progression of infectious disease or required antibiotic cover for routine dental therapy. In addition, subjects who received antibiotic or anti-inflammatory therapy in the previous 6 months were excluded.

Sample collection

Samples were collected from each of the 30 teeth under strict aseptic conditions. Initially, the tooth was cleaned with pumice and isolated with rubber dam. The tooth and the rubber dam were cleaned with a solution of 1 mL of 3% hydrogen peroxide and then disinfected with 1 mL of 2.5% NaOCl solution. The coronal access into the pulp chamber was made with the use of sterile round burs without water spray. The pulp chamber and the operatory field were disinfected again using a swab soaked in 1 mL of 2.5% NaOCl. This solution was inactivated with 1 mL of sterile 5% sodium thiosulfate. Samples were collected from the root canal by means of a size 15 H-type file (Dentsply Maillefer®, Ballaigues, Switzerland) with the handle removed using a discrete filing motion, introduced 1 mm short of apical foramen. This length was determined by means of a periapical radiograph and a metallic ruler. Subsequently, two sterile paper points were introduced in the root canal to the same level of the file and each left for 1 min, in order to soak up the tissue fluid. Both file and paper points were then transferred to single rigid Eppendorf tubes containing 150 μL of TE (10 mmol L^{-1} Tris–HCl, 1 mmol L^{-1} EDTA, pH 7.6). 0.1 mL of 0.5 mol L^{-1} NaOH was added to each tube and the samples were frozen at −20 °C until being processed.

Microbiologic assessment

The presence and levels of 40 bacterial species (Table 1) were determined in each sample using the checkerboard DNA–DNA hybridization method described by Socransky *et al.* (1994).

Briefly, the samples were boiled for 10 min and neutralized using 0.8 mL of 5 mol L^{-1} ammonium acetate. The released DNA was then fixed in individual lanes of a positively charged nylon membrane (Amersham Biosciences, Chicago, IL, USA) using a Minislot 30™ apparatus (Immunetics, Cambridge, MA, USA). A Miniblotter 45™ (Immunetics) device was used to hybridize 40 digoxigenin-labelled whole genomic DNA probes (Table 1) perpendicular to the lines of the clinical samples. The DNA probes were prepared using the *random primer digoxigenin labeling Kit* (Boehringer Mannheim, Indianapolis, IN, USA) (Feinberg & Vogelstein 1983). Bound probes were detected by using phosphatase-conjugated antibody to digoxigenin and chemiluminescence (*CDP-Star Detection Reagent*™, Amershan Biosciences). Signals were evaluated visually by comparison with the standards at 10^5 and 10^6 for the test species on the same membrane. They were recorded as follows: 0, not detected; 1, $<10^5$ cells; 2, approximately 10^5 cells; 3, 10^5 to 10^6 cells; 4, approximately 10^6 cells; 5, $>10^6$

Table 1 Strains employed for the development of DNA probes

Species strains		*Species strains*	
Actinobacillus actinomycetemcomitans	*	*Leptotrichia buccalis*	14201
Actinomyces gerencseriae[a]	23860	*Micromonas micros*[a]	33270
Actinomyces israelii[a]	12102	*Neisseria mucosa*	19696
Actinomyces naeslundii genospecies 1[a]	12104	*Porphyromonas endodontalis*[a]	35460
Actinomyces odontolyticus[a]	17929	*Porphyromonas gingivalis*[a]	33277
Capnocytophaga gingivalis[a]	33624	*Prevotella intermedia*[a]	25611
Capnocytophaga ochracea[a]	33596	*Prevotella melaninogenica*[a]	25845
Capnocytophaga sputigena[a]	33612	*Prevotella nigrescens*[a]	33563
Campylobacter gracilis[a]	33236	*Propionibacterium acnes*	**
Campylobacter rectus[a]	33238	*Selenomonas noxia*[a]	43541
Campylobacter showae[a]	51146	*Streptococcus anginosus*[a]	33397
Eikenella corrodens[a]	23834	*Streptococcus constellatus*[a]	27823
Enterococcus faecalis[a]	29212	*Streptococcus gordonii*[a]	10558
Eubacterium nodatum[a]	33099	*Streptococcus intermedius*[a]	27335
Eubacterium saburreum[a]	33271	*Streptococcus mitis*[a]	49456
Fusobacterium nucleatum ss. polymorphum[a]	10953	*Streptococcus oralis*[a]	35037
Fusobacterium nucleatum ss. nucleatum[a]	25586	*Tanaerela forsythensis*[a]	43037
Fusobacterium nucleatum ss. vicentii[a]	49256	*Treponema denticola*[b]	B1
Fusobacterium periodonticum[a]	33693	*Treponema socranskii*[b]	S1
Gemella morbillorum[a]	27284	*Veillonella parvula*[a]	10790

[a]ATCC (American Type Culture Collection, Rockville, MD).
[b]FDC (Forsyth Dental Center).
*ATCC strains 43718 e 29523; **ATCC strains11827 e 11828.

cells. The sensitivity of this assay was adjusted to permit detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe.

Statistical analysis

The data were expressed in prevalence (presence or absence) and mean counts ($\times 10^5$ cells) of each species. Differences between cases with and without an associated sinus tract were sought. Odds radio and confidence interval of the prevalence data was used to measure the association of bacterial species and the presence of sinus tract associated. The data were recorded in six levels but they were dichotomized in under or above 10^4 cells for this analysis. The statistical associations were tested by Fisher's exact test. The association extent was calculated by chances in logistic regression. The mean counts data were evaluated by means of the Mann–Whitney test for individual bacterial species or group of species. The comparison of the mean total counts of the 40 species evaluated between the two groups was assessed using a *t*-test. The level of significance was established at 5%.

Results

The population age ranged from 18 to 70 years (mean age = 34 years) and 65% of the subjects were women. Five cases with an associated sinus tract and four cases without an associated sinus tract presented with pain. Each of the 40 bacterial species evaluated was present in at least four samples. The mean number of species found in the 30 samples was 23.73, ranging from 7 to 36 species per sample. In samples from teeth with an associated sinus tract, the mean number of species detected was 20.40, ranging from 7 to 36, and 27.07 ranging from 11 to 35 species in cases without an associated sinus tract.

Figure 1 presents the prevalence of detection of the 40 species evaluated in the 30 samples. The most prevalent species, found in more than 75% of the samples were *Prevotella melaninogenica, Leptotrichia buccalis, Enterococcus faecalis, Campylobacter gracilis, Neisseria mucosa, Fusobacterium nucleatum sp. vincentii, Eubacterium saburreum, Streptococcus anginosus, Veillonella parvula* and *Porphyromonas gingivalis*. The less prevalent species, found in less than 25% of the samples, were *Streptococcus intermedius, Actinomyces naeslundii, Capnocytophaga ochracea* and *Actinomyces gerencseriae*.

The mean counts of each species in all samples evaluated are presented in Fig. 2. *F. nucleatum sp. vincentii* (9.8×10^5), *P. gingivalis* (4.88×10^5), *V. parvula* (4.88×10^5), *F. nucleatum sp. nucleatum* (4.20×10^5), *E. faecalis* (3.0×10^5), *C. gracilis* (2.10×10^5), *N. mucosa* (3.0×10^5) and *E. saburreum*

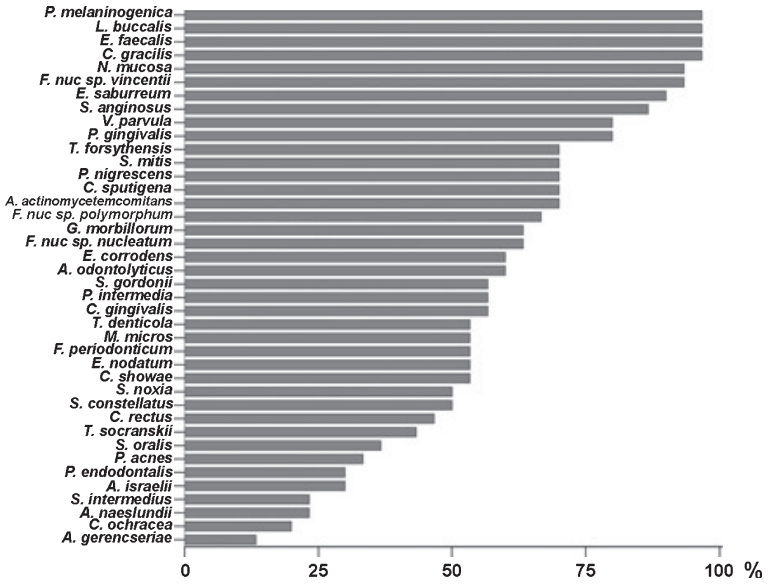

Figure 1 Bar chart of the prevalence of the 40 test bacterial species in all 30 root canal.

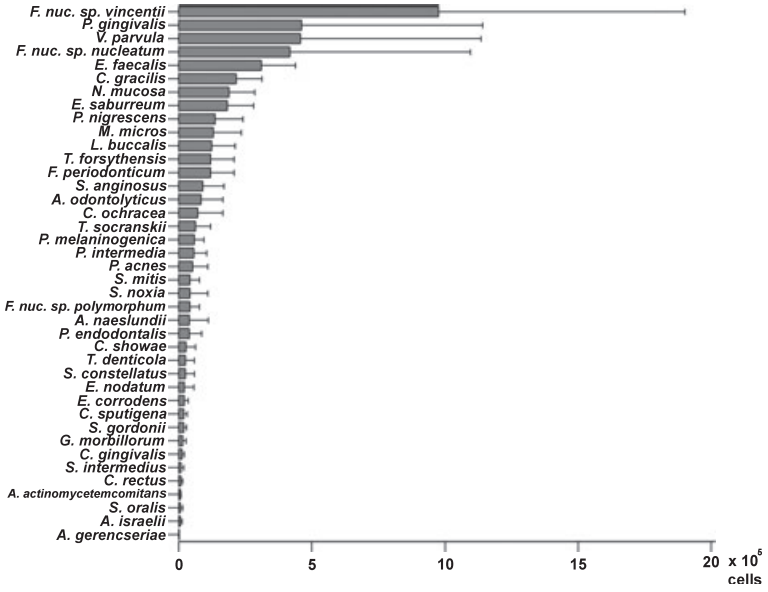

Figure 2 Bar chart of mean counts ($\times 10^5$) of the test bacterial species in all 30 root canal samples.

(2.92×10^5), were the species found in highest levels; whilst *S. intermedius* (0.26×10^5), *Campylobacter rectus* (0.18×10^5), *Actinobacillus actinomycetemcomitans* (0.12×10^5), *Streptococcus oralis* (0.09×10^5), *Actinomyces israelii* (0.08×10^5) and *A. gerencseriae* (0.05×10^5), were found at the lowest levels. When the bacterial species were divided within genera, *Fusobacterium* was found in highest levels (19.12×10^5) followed by *Prevotella* (3.91×10^5), *Campylobacter* (3.87×10^5), *Streptococcus* (2.16×10^5), *Capnocytophaga* (1.78×10^5) and *Actinomyces* (1.48×10^5).

The mean total bacterial counts in cases associated with a sinus tract was 44×10^5 (SEM = 21×10^5) and 50×10^5 (SEM = 13×10^5) in cases not associated with a sinus tract. The difference observed between the two groups was not statistically significant ($P > 0.05$) (Fig. 3).

Figure 4 presents the mean counts ($\times 10^5$) of each species evaluated in the two groups.

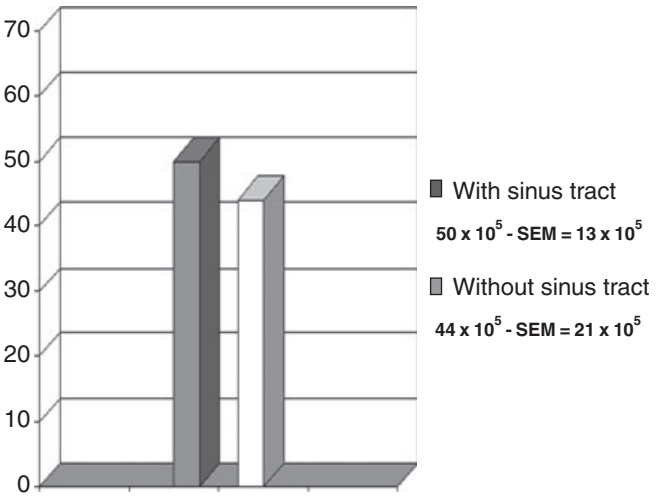

Figure 3 Total mean counts ($\times 10^5$ cells) of the 40 test bacterial species in cases with and without associated sinus tract ($P < 0.05$; *t*-test).

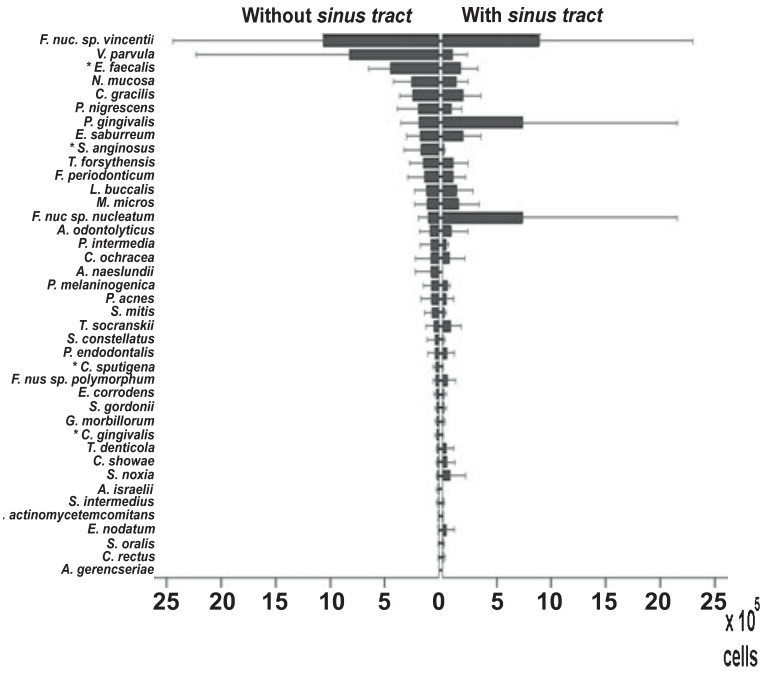

Figure 4 Bar charts of the mean counts ($\times 10^5$ cells) of the 40 test bacterial species in cases with and without sinus tract. The species were ordered according the mean counts of the samples without sinus tract (*$P < 0.05$; Mann–Whitney test).

In cases with a sinus tract, the bacterial species detected in highest levels were: *F. nucleatum* sp. *vincentii* (8.90×10^5), *P. gingivalis* (7.38×10^5), *F. nucleatum* sp. *nucleatum* (7.36×10^5), *C. gracilis* (1.92×10^5), *E. saburreum* (1.90×10^5) and *E. faecalis* (1.70×10^5). The mean count levels of G+ were 7.48×10^5 (SD 8.17) and of G− were 36.90×10^5 (SD 11.28) whilst rods were detected in 37.18×10^5 (SD 8.15), cocci in 6.08×10^5 (SD 1.06) and spirochaete in 1.12×10^5 (SD 2.04). In cases without a sinus tract, the species detected at the highest levels were: *F. nucleatum* sp. *vincentii* (10.61×10^5), *V. parvula* (8.23×10^5), *E. faecalis* (4.48×10^5), *N. mucosa* (2.50×10^5), *C. gracilis* (2.41×10^5) and *Prevotella nigrescens* (1.91×10^5). *E. faecalis*, *S. anginosus*, *Capnocytophaga sputigena* and *Capnocytophaga gingivalis* were found in significantly higher levels ($P < 0.05$) in samples from teeth without a sinus tract. The species *F. nucleatum sp. vincentii* was present in all cases with a sinus tract (data not shown). The mean count levels of G+ were 12.89×10^5 (SD 1.03) and of G− were 37.15×10^5 (SD 4.52), whilst rods were detected in 30.07×10^5

(SD 3.64), cocci in 19.35×10^5 (SD 3.34) and spirochaete in 0.62×10^5 (SD 0.12).

Possible associations between bacterial taxa and sinus tract were determinated by calculating the odds ratio amongst bacterial species and groups of species. The Fisher's exact test showed that there was no statistical significance association amongst any single bacteria species with teeth with and without a sinus tract ($P > 0.05$). Similarly, none of the bacterial groups evaluated (genera; cocci, rods or spirochete; G+ or G−), had a significant association with the presence or absence of a sinus tract. The association extent was calculated by logistic regression. *L. buccalis* (OR = 1.83; CI 95%) and *P. endodontalis* (OR = 2.15; CI 95%) were associated with an increased chance of subjects having a sinus tract.

Discussion

It is well established in the literature that primary root canal infections are caused by microorganisms that colonize necrotic pulp tissue. However, difficulties in cultivating some of these species have delayed a better comprehension of the composition of the endodontic microbiota, as well as possible associations between specific species and clinical signs and symptoms. The development of molecular biology techniques represented an improvement in this scenario, because these tests are not dependent on microbial viability for their detection. The aim of the present investigation was to evaluate the microbiota of primary endodontic infections with or without the presence of sinus tract, using a molecular biology test called checkerboard DNA–DNA hybridization (Socransky *et al.* 1994).

Previous studies using different diagnostic tests have showed that the microbiota associated with primary endodontic infections is mixed and predominated by anaerobic bacteria such as *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Treponema*, *Peptostreptococcus* and *Campylobacter* species, and by some facultative microorganisms such as *Streptococcus* species (Gomes *et al.* 1996, Siqueira *et al.* 2001a, Siqueira *et al.* 2001b, Siqueira *et al.* 2002, Gomes *et al.* 2004, Soriano de Souza *et al.* 2005). The present findings are, to some extent, in agreement with these results, showing high levels and prevalence of *F. nucleatum sp. vincentii*, *P. gingivalis*, *V. parvula*, *E. faecalis*, *C. gracilis* and *N. mucosa*. However, the *Streptococcus* species evaluated in the present study were found in low counts. Even *S. anginosus* and *Streptococcus mitis* that were detected in relatively high prevalence (87 and 70%, respectively) were not present in high levels (0.8 and 0.46×10^5, respectively).

Using the checkerboard DNA–DNA hybridization method, Soriano de Souza *et al.* (2005) also found high counts and prevalence of *P. gingivalis*, *F. nucleatum sp. vincentii* and *V. parvula* in teeth with primary endodontic infections.

It is interesting to note that overall, the studies that used microbial culture have found lower bacterial counts compared to the present study (Sundqvist 1976, Haapasalo *et al.* 1986, van Winkelhoff *et al.* 1988, Haapasalo 1989, Gomes *et al.* 2004).

E. faecalis was found in almost 90% of the samples, data that differ substantially from previous studies that have detected a much lower prevalence of this species using PCR and DNA probes (Siqueira *et al.* 2002, Rôças *et al.* 2004, Soriano de Souza *et al.* 2005). Usually this enteric species is associated more with failing endodontic cases, with a low prevalence in primary infections (Rôças *et al.* 2004). This could be explained by differences in the methodology of those studies such as the sampling method, the medium in which the sample was stored, the *ATCC* strain used, the number of samples and a different clinical scenario. However, recent investigations using PCR have also detected high levels of this microorganism in primary endodontic infections (Foschi *et al.* 2005, Gomes *et al.* 2006). Also, the significant association of *E. faecalis* with the absence of a sinus is understandable in view of its involvement with asymptomatic periradicular disease (Siqueira *et al.* 2002, Rôças *et al.* 2004).

Some well-known periodontal pathogens (Socransky *et al.* 1998) such as *T. denticola*, *T. socranskii* and *T. forsythensis* were found in relatively high prevalence and mean levels. This data are in agreement with some recent studies that used molecular methods (Siqueira *et al.* 2000, 2001a, Siqueira & Rôças 2003a,b, Soriano de Souza *et al.* 2005) but is in contrast with some culture studies where these species were not detected (Haapasalo 1989, Sundqvist *et al.* 1989, Baumgartner & Falkler 1991). The discrepancies amongst those studies could be explained, to some extent, by the advantage of molecular methods of detecting nonviable bacterial cells, allowing the identification of species that are difficult to grow on agar plates.

Many ecological factors such as O_2 tension, pH, nutritional factors and association of bacterial species may interfere in the pulpal space environment and consequently with the species that colonize the root

canal system (Sundqvist 1992a,b, 1994, Siqueira 2002). Time period of the infection also interferes with colonization and bacterial succession (Fabricius *et al.* 1982); but this aspect can not be measured in *in vivo* studies.

In addition, it has been suggested that the endodontic microbiota can differ according to the type of perirradicular disease present (Siqueira 2002). Some authors correlate cases of acute periapical lesions (Griffee *et al.* 1980, Haapasalo *et al.* 1986, Sundqvist *et al.* 1989, Gomes *et al.* 1996, 2004) with the presence of Gram-negative species such as *Prevotella*, *Porphyromonas* and *Fusobacterium*. However, a small number of reports have attempted to find some relationship between the presence of sinus tract and specific bacterial species. In the present study, the total bacterial counts were similar between the two groups (Fig. 3). The endodontic microbial composition of teeth with or without a sinus tract did not present striking discrepancies; however, a few important differences could be observed between the colonization profiles of the two groups (Fig. 4).

Even though not statistically significant, a higher mean level of *P. gingivalis* and *F. nucleatum sp. nucleatum* was detected in cases with a sinus tract compared to cases without a sinus. These two species are Gram-negative, strict anaerobes. *P. gingivalis* has been described as the most proteolytic and pathogenic species amongst black-pigmented microorganisms (van Winkelhoff *et al.* 1988). The high levels of this species detected in root canals associated with sinus tract lesions might help to maintain the persistent exudate observed in these infections. Besides, *P. endodontalis* was statistically associated with increasing chances of a presenting sinus tract. This species could release outer membrane blebs, probably containing lipopolysaccharide, which may be an important virulence factor in the pathogenesis of perirradicular lesions (Haapasalo 1989). In contrast, four species not considered to have an aggressive profile were found in significantly higher levels in cases without a sinus tract: *E. faecalis, S anginosus, C. sputigena and C. gingivalis. V. parvula* was also found in much higher levels in cases without a sinus tract.

Conclusion

Primary endodontic infections presented a large variety of bacterial species. Specific differences between the composition of the microbiota of root canal infections with or without a sinus tract were observed.

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