NOTE / NOTE

Occurrence of herpes simplex virus 1 and three periodontal bacteria in patients with chronic periodontitis and necrotic pulp

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Abstract: Viral and bacterial associations appear to be implicated in the development of periodontal infections. Little information is available describing the periodontopathic agents in root canals with necrotic pulp. In this study, the occurrence and the combinations among herpes simplex virus type 1 (HSV-1) and *Dialister pneumosintes*, *Tannerella forsythia*, and *Treponema denticola* in patients with chronic periodontitis and necrotic pulp were evaluated. Clinical samples from healthy subjects and patients with periodontal or pulp infections were analyzed using a nested polymerase chain reaction PCR to detect HSV and PCR to detect the 3 periodontal bacteria. The presence of *Tannerella forsythia* and *Treponema denticola* was observed in healthy, periodontitis, and necrotic pulp patients. HSV was observed in periodontitis and necrotic pulp patients, and no healthy subject harbored *D. pneumosintes* or HSV. The occurrence of *Tannerella forsythia* was not statistically significant in patients with necrotic pulp ($P = 0.704$). Periodontal bacteria were observed varying from 10.3% to 20.7% in periodontitis and necrotic pulp patients. The presence of *Treponema denticola* – HSV association was predominant in patients showing necrotic pulp (24.1%); however, HSV alone was observed in one patient with periodontitis and in another patient with necrotic pulp. The presence of double association among bacteria or bacteria – HSV could indicate a role in both periodontitis and necrotic pulp, and *Tannerella forsythia* – *Treponema denticola* – HSV and *Tannerella forsythia* – *D. pneumosintes* – *Treponema denticola* – HSV associations might be important in periodontitis.

Key words: herpes simples virus, *Dialister pneumosintes*, *Tannerella forsythia*, *Treponema denticola*, chronic periodontitis, necrotic pulp.

Résumé : Les associations de virus et de bactéries semblent impliquées dans le développement d'infections périotondales. Il existe peu d'informations qui décrivent les agents périodontopathogènes dans les canaux des racines avec pulpe nécrotique. Dans cette étude, l'occurrence du virus herpes simplex type 1 (HSV-1), de *Dialister pneumosintes*, de *Tannerella forsythia* et de *Treponema denticola* et leur combinaison a été évaluée chez des patients souffrant de périodontites chroniques avec pulpe nécrotique. Des échantillons cliniques de sujets sains et de patients affectés d'une infection périodontale ou pulpaire ont été analysés par PCR nichée pour détecter le HSV et par PCR pour détecter les 3 bactéries périodontales. La présence de *Tannerella forsythia* et de *Treponema denticola* a été observée chez les sujets sains et chez les patients affectés de périodontite et de pulpe nécrotique. Le HSV a été observé chez les patients affectés de périodontite et de pulpe nécrotique, et aucun sujet sain n'était porteur de *D. pneumosintes* ou du HSV. L'occurrence de *Tannerella forsythia* n'était pas statistiquement significative chez les patients avec pulpe nécrotique ($P = 0{,}704$). Les bactéries périodontales ont été observées, variant de 10,3 % à 20,7 %, chez les patients affectés de périodontite et de pulpe nécrotique. La présence d'une association *Treponema denticola* – HSV était prédominante chez les patients présentant une pulpe nécrotique (24,1 %), cependant le HSV seul était observé chez un patient souffrant de périodontite et chez un autre affecté de pulpe nécrotique. La présence d'une double association de bactéries ou de bactéries – HSV pourrait être indicative d'un rôle de telles associations dans la périodontite et la pulpe nécrotique, et les associations *Tannerella forsythia* – *Treponema denticola* – HSV et *Tannerella forsythia* – *D. pneumosintes* – *Treponema denticola* – HSV pourraient être importantes dans la périodontite.

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Periodontal disease represents a variety of clinical manifestations of infectious disorders affecting tooth-supporting tissues. Periodontitis patients often have sporadic advancing disease with stability that may last for several years (Slots 2007).

Studies have shown the identification at least 700 bacterial species in the human oral cavity and over 400 bacterial species in the periodontal pocket, and any particular individual may harbor approximately 100–200 oral bacterial species (Paster et al. 2006).

Important periodontal bacteria include *Porphyromonas gingivalis*, *Tannerella forsythia*, *Dialister pneumosintes*, *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*, and *Treponema denticola* (Holt and Ebersole 2005). During the last years, herpesviruses have emerged as periodontal pathogens, and the herpes simplex virus (HSV), Epstein-Barr virus, and type 1 human cytomegalovirus seem to play a major pathogenic role in aggressive periodontitis (Slots 2005).

Moreover, the etiology of periodontitis and necrotic pulp has been focused on the presence of anaerobic periodontal bacteria; however, little or no consideration has been given to the possible involvement of human herpesvirus in the etiology and pathogenesis of the root canal infection (Kamma and Slots 2003).

The interaction among different bacterial species or genera that colonize the root canal can activate the host's immunologic defense, producing the chronic or acute inflammatory stages of periradicular disease (Slots 2005). The most common bacterial genera isolated from necrotic pulp are *Peptostreptococcus*, *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Eubacterium*, *Actinomyces*, and *Treponema*. However, the participation of these microorganisms in endodontic infections remains unclear (Slots 2005, 2007).

Studies have shown the association between periodontal bacterial and herpesvirus and an increase in the severity of the periodontal disease (Parra and Slots 1996; Sabeti et al. 2003). As herpesvirus and anaerobic bacteria are both closely associated with periodontitis, it may be that these 2 types of infectious agents act cooperatively in the breakdown of periodontal tissues (Slots 2007).

The occurrence of herpesvirus and selected periodontopathic bacteria in various types of periodontal disease has been studied by means of qualitative and quantitative polymerase chain reaction (PCR) identification techniques (Slots 2005). Moreover, studies have shown that cytomegalovirus and Epstein-Barr virus are more common in both periodontitis patients and healthy individuals than is herpesvirus (Slots 2004). In addition, the relationships between HSV and periodontal or periapical processes are still not fully understood. Therefore, in this study the occurrence and the combinations of herpesvirus, *D. pneumosintes*, *Tannerella forsythia*, and *Treponema denticola* in patients with chronic periodontitis and necrotic pulp were determined.

One hundred and fifty patients attending the Clinics of Periodontics and Endodontic of the University of São Paulo Dental School were selected. All the patients were from 18 to 59 years old (mean age: 33 years). None of the patients received antibiotic therapy 3 months prior to sample collection nor showed a history of HSV infection in the past, as determined by anamnesis. The Research Ethics Committee of the Institute of Biomedical Sciences, USP, approved this study (process No. 259/CEP). Patients were grouped according to their clinical features, as follows:

- Group 1: 50 patients (23 male and 27 female) with chronic periodontitis showing periodontal pockets with ≥5 mm of depth and radiographic and clinical signs of progressive attachment and alveolar bone loss, as well as the absence of periapical lesion.
- Group 2: 50 patients (26 male and 24 female) with carious lesions showing periapical lesion and radiographic and clinical signs of necrotic pulp. No patient displayed periodontitis.
- Group 3: 50 healthy individuals (14 male and 36 female) without clinical signs of periodontal disease or necrotic pulp were considered as control. Samples were collected from the gingival crevice. In this study, only patients with chronic periodontitis, periapical lesion with necrotic pulp, and HSV-negative were included. Patients with systemic diseases or severe acute apical periodontitis were excluded. Clinical samples were collected from March 2004 to March 2006.

In all the patients, the supragingival biofilm was removed by using a gauze and (or) curette type — Gracey. The subgingival samples were collected using 2 sterilized paper points (No. 30, Endopoints Ind. Ltd., Rio de Janeiro, RJ, Brazil) that were introduced in the apical region of the gingival crevice or periodontal pocket. All the sampled sites were isolated with cotton rolls, preventing saliva contamination. The necrotic pulp sample was collected after coronary opening using 2 sterilized paper points. Initially, the premolar tooth was isolated by a rubber dam, and the tooth and the surrounding field were cleansed with 30% (*v*/*v*) hydrogen peroxide and decontaminated with a solution of 5% (*v*/*v*) iodine. The iodine was inactivated with 5% (*v*/*v*) sodium thiosulfate. The sterility of the tooth surface after cleaning and disinfection was evaluated by samples taken from the surface and was processed by PCR for each periodontopathic agent. After coronal access, the root canals were lightly irrigated with 0.9% (*v*/*v*) saline solution, avoiding flooding. Cones remained for 60 s in each sample site and were then transferred to microcentrifuge tubes containing 300 μL of sterilized ultra-pure water (Avila-Campos and Velasquez-Melendez 2002). Collected samples were homogenized by vigorous vortexing, boiled for 15 min, and centrifuged (14 000*g*, 10 min), and supernatants (DNA) were stored at –20 °C until they were used.

A nested PCR method was used to detect herpesvirus

Table 1. Primer pairs and polymerase chain reaction (PCR) conditions used for detection of 4 periodontopathic agents in chronic periodontitis and necrotic pulp samples.

Periodontopathic agent	Oligonucleotide 5′→3′	Amplicon (bp)	Amplification cycles	Reference
Herpes simplex virus type 1	Forward-1—GGGCCAGGCGCTTGTTGGTGTA, Reverse-1—TACATCGGCGTCATCTGCGGGG; Forward-2—CAGTTCGGCGGTGAGGACAAA, Reverse-2—GCGTTTATCAACCGCACCTCC	222	Denaturation: 94 °C × 1 min; 30 cycles of 94 °C × 1 min, 60 °C × 1 min (first PCR) or 55 °C × 1 min (second PCR), 72 °C × 1 min; final extension: 72 °C × 1 min	Parra and Slots 1996
Dialister pneumosintes	Forward—TTCTAAGCATCGCATGGTGC, Reverse—GATTTCGCTTCTCTTTGTTG	1100	Denaturation: 94 °C × 5 min; 36 cycles of 94 °C × 30 s, 55 °C × 1 min, 72 °C × 2 min; final extension: 72 °C × 5 min	Doan et al. 2000
Tannerella forsythia	Forward—GCGTATGTAACCTGCCCGCA, Reverse—TGCTTCAGTGTCAGTTATACCT	641	Denaturation: 94 °C × 5 min; 30 cycles of 94 °C × 30 s, 60 °C × 1 min, 72 °C × 2 min; final extension: 72 °C × 5 min	Ashimoto et al. 1996
Treponema denticola	Forward—TAATACCGAATGTGCTCATTTACA, Reverse—TCAAAGAAGCATTCCCTCTTCTTCTTA	316	Denaturation: 94 °C × 5 min; 30 cycles of 94 °C × 30 s, 60 °C × 1 min, 72 °C × 2 min; final extension: 72 °C × 5 min	Ashimoto et al. 1996

DNA, using specific primers (Table 1). Amplifications were carried out in final volumes of 25 μL, containing 10× PCR buffer, 50 mmol/L $MgCl_2$, 200 mmol/L dNTP mix, 0.4 μmol/L (each) primer (Forward-1/Reverse-1), 0.5 U Platinum *Taq* DNA polymerase (Invitrogen do Brasil, São Paulo, SP, Brazil), and 5 ng of DNA. PCR amplification was used to detect bacterial DNA, using the same master mix used for herpesvirus, but with 10 ng of DNA template. Amplification reactions were performed in a thermal cycler (Perkin Elmer, Gene Amp PCR System 9700), programmed in accordance with Table 1. Positive controls included purified DNA of HSV (Perkin Elmer Cetus, Norwalk, Connecticut), *D. pneumosintes* ATCC 33048, *Tannerella forsythia* ATCC 43037, and *Treponema denticola* ATCC 33521, which were kindly supplied by Dr. Casey Chen (Department of Periodontology, School of Dentistry, University of Southern California, Los Angeles, California, USA). Negative controls without respective DNA were used. PCR products were analyzed by electrophoresis in 2% agarose gel, at 60 V, for 2.5 h, stained with ethidium bromide (0.5 μg/mL), and photographed under an UV transilluminator, using a Digital Kodak Science 120 system. One kilobase DNA ladder digest (Invitrogen, São Paulo, SP) was used as the molecular size marker. Chi-square (χ^2), significance level (P), Odds Ratio (OR), and Confidence Bounds (IC 95%), with Excel and SPSS (Version 10.0) programs were employed in statistical comparisons of clinical features and microbial data.

Of the 50 periodontitis and 50 necrotic pulp patients examined 13 (46.4%) and 15 (53.6%) were HSV-positive, respectively. None of the healthy individuals harbored HSV. In both periodontitis and necrotic pulp patients, the presence of HSV was statistically significant ($P = 0.000$) (Table 2). In Table 3 the results for the presence of the 4 periodontopathic agents and their combination in healthy (18%), periodontitis (58%), and necrotic pulp (58%) samples can be observed. Moreover, the association of HSV with *Treponema denticola* (OR 11.12), *Tannerella forsythia* (OR 6.68), and *D. pneumosintes* (OR 4.28) showed a relationship with periodontitis and necrotic pulp. A *Treponema denticola* – HSV association was observed in 24.1% of the patients with necrotic pulp. In addition, the presence of the 4 periodontopathic agents in patients with periodontitis and necrotic pulp was observed, respectively, in 20.7% and 17.2% for *D. pneumosintes*, 13.8% and 10.3% for both *Tannerella forsythia* and *Treponema denticola*, and 3.4% and 3.4% for HSV. Healthy individuals harbored *Tannerella forsythia* (33.3%) and *Treponema denticola* (56.0%).

The occurrence of herpesvirus and selected periodontopathic bacteria in various types of periodontal disease has been shown (Slots 2007). The HSV-1 primers showed high specificity in accordance with Parra and Slots (1996). This study detected a significantly high prevalence of HSV in necrotic pulp (30%) and periodontal pocket (26%) specimens. This finding might suggest that HSV can infect periodontal and pulp tissues and can also serve as a factor in destructive oral process.

Michalowicz et al. (2000) observed a strong relationship between cytomegalovirus and *P. gingivalis* in Jamaican adolescents with localized juvenile periodontitis. Ting et al. (2000) affirmed that during the root formation of the perma-

Table 2. Presence of 4 periodontopathic agents in clinical samples of healthy (n = 50), periodontitis (n = 50), and necrotic pulp patients (n = 50).

		Presence		Absence		
Periodontopathic agent	Clinical sample	No.	%	No.	%	*P*
Dialister pneumosintes	Healthy	0	0.0	50	39.7	—
	Periodontitis	11	45.8	39	30.1	0.000
	Necrotic pulp	13	54.2	37	29.4	0.000
Tannerella forsythia	Healthy	4	14.8	46	37.4	—
	Periodontitis	16	59.3	34	27.6	0.000
	Necrotic pulp	7	25.9	43	34.9	0.704
Treponema denticola	Healthy	6	17.1	44	38.3	—
	Periodontitis	15	55.6	35	30.4	0.000
	Necrotic pulp	14	40.0	36	31.3	0.006
Herpes simplex virus	Healthy	0	0.0	50	40.9	—
	Periodontitis	13	46.4	37	30.3	0.000
	Necrotic pulp	15	53.6	35	28.7	0.000

Table 3. Presence of periodontopathic agents and their combinations in healthy (n = 50), periodontitis (n = 50), and necrotic pulp patients (n = 50).

	Healthy		Periodontitis		Necrotic pulp	
Periodontopathic agent	No.	%	No.	%	No.	%
Dialister pneumosintes	0	0.0	6	20.7	5	17.2
Tannerella forsythia	3	33.3	4	13.8	3	10.3
Treponema denticola	5	56.0	4	13.8	3	10.3
Herpes simplex virus (HSV)	0	0.0	1	3.4	1	3.4
Combinations						
T. forsythia – *D. pneumosintes*	0	0.0	0	0.0	1	3.4
T. forsythia – *T. denticola*	1	11.1	2	6.9	0	0.0
D. pneumosintes – *T. denticola*	0	0.0	0	0.0	2	6.9
T. forsythia – HSV	0	0.0	2	6.9	1	3.4
D. pneumosintes – HSV	0	0.0	0	0.0	3	10.3
T. denticola – HSV	0	0.0	2	6.9	7	24.1
T. forsythia – *D. pneumosintes* – HSV	0	0.0	1	3.4	1	3.4
T. forsythia – *T. denticola* – HSV	0	0.0	3	10.3	1	3.4
D. pneumosintes – *T. denticola* – HSV	0	0.0	0	0.0	1	3.4
T. forsythia – *D. pneumosintes* – *T. denticola* – HSV	0	0.0	4	14.8	0	0.0

nent incisors and first molars at 3–5 years of age, a cytomegalovirus active infection in tissue surrounding the tooth germ alters the root surface structure, increasing the susceptibility to future periodontal breakdown.

Dialister pneumosintes (Contreras et al. 1999), *Tannerella forsythia* (Tanner and Izard 2006), and *Treponema denticola* (Holt and Ebersole 2005) are considered periodontal pathogens. These microorganisms may produce potent virulence factors, including a capability of expressing adhesins (fimbriae, capsular polysaccharide. and hemagglutinin) and antiphagocytic and immunosuppressive factors (capsule, immunoglobulin proteases, and leukotoxin) (Slots et al. 2002). These bacterial factors protect against antibacterial defenses of periodontium and active herpesviral infection and permit an overgrowth of microorganisms that contribute to the occurrence of the periodontal disease and periapical processes (Contreras et al. 1999).

Since the mid-1990s, herpeviruses have emerged as putative pathogens in various types of periodontal disease (Contreras and Slots 2000; Slots et al. 2006), and it is known that periodontal sites with herpesviruses infections harbor high levels of periodontal bacteria, such as *D. pneumosintes*, *Tannerella forsythia*, and *Treponema denticola. Tannerella forsythia* and *Treponema denticola* belong to the red complex described by Socransky et al. (1998), and they are considered classic periodontal pathogens. However, the presence of *Tannerella forsythia* was not statistically significant in necrotic pulp (P = 0.704) because in necrotic pulp this microorganism does not find ecological and nutritional conditions for its establishment. The presence of herpes simplex virus observed in gingival tissue has suggested that other common types of human viruses may be involved in pulp disease and in the development of periapical lesions (Heling et al. 2001). Moreover, the presence of virus inside the root canal may increase the risk for iatrogenic exacerbations (flare ups) when dentinal debris is transported into the apical region (Siqueira and Rôças 2005).

This study aimed to delineate whether there was an association of HSV with the presence of *D. pneumosintes*, *Tannerella forsythia*, and *Treponema denticola* in clinical features of periodontal disease and necrotic pulp process. It was possible to detect a significant relationship between the

presence of HSV and the clinical features of periodontitis (26%) and pulp infections (30%). Moreover, our results support the notion that the presence of HSV associated with *D. pneumosintes*, *Tannerella forsythia*, and (or) *Treponema denticola* might influence some types of periodontal and periapical processes.

In conclusion, many questions remain unanswered on herpesvirus–bacteria associations in etiopathogenesis of these oral diseases. However, the presence of HSV and others virus and virus–bacteria associations can lead to differences in the composition of the periodontal and endodontic microbiota.

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