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Note

The use of a rapid assay to detect the neuraminidase production in oral *Porphyromonas* spp. isolated from dogs and humans

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ABSTRACT

Neuraminidase was produced by 32.1% and 28.5% of *Porphyromonas* from dogs with and without periodontitis, respectively; and by 31.8% of bacteria from humans. The presence of neuraminidase in *Porphyromonas* spp. suggests that this enzyme can be involved with the pathogenesis of the periodontal disease, and the use of this assay to detect the neuraminidase production in oral *Porphyromonas* species is suggested.

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Species of *Porphyromonas* are important etiological agents in human oral infections, such as chronic periodontitis. Species of *Porphyromonas* display an ability to colonize the periodontal pocket and form coaggregations with *Tannerella forsythia*, *Fusobacterium nucleatum*, *Treponema denticola*, and *Prevotella* spp., as a requisite for developing of periodontal diseases (Pihlstrom et al., 2005).

Gram-negative anaerobic bacteria especially black-pigmented bacteria, such as *Porphyromonas* spp. and Prevotela spp., have been isolated from periodontal pockets of small animals, such as dogs and cats. Studies have shown the presence of human periodontitis-related bacteria, such *as P. gingivalis*, *Prevotella intermedia*, *T. denticola*, *T. forsythia* and *Aggregatibacter actinomycetemcomitans* from subgingival samples of dogs with and without periodontitis; however, the oral microbiota of animals has been poorly characterized (Senhorinho et al., 2012). In addition, a high incidence of periodontal disease in dogs has been caused by others species of *Porphyromonas*, such as *P. gulae*, *P. macacae*, *P. cangingivalis*, *P. gingivicanis*, and *P. circumdentaria*, and the etiologic factors of gingivitis and periodontitis seem to be similar to humans (Isogai et al., 1999).

Considering dogs as domestic animals and keep a close contact with man, the presence of periodontal bacteria in oral cavity may represent a source of cross transmission of virulent oral bacteria (Forsblom et al., 2002).

Porphyromonas gingivalis produce different virulence factors, such as fimbriae, haemagglutinin, gingipains and capsule affecting the periodontium (Hajishengallis, 2009). Neuraminidases and sialopeptidases are produced by *P. gingivalis* and play a role in bacterial coaggregation and in the breakdown of carbohydrates and glycoproteins (Sharma et al., 2005).

The sialic acids are widely distributed on cell surface of eukaryotes and the presence or absence of these substances on cells or tissues appears to be an important determinant of their physiological status (Schauer, 1985). The neuraminidase production by anaerobic bacteria is associated with its pathogenicity, and the exposure of neuraminidase on tissues causes in certain bacteria an increased adhesion and invasion (Briselden et al., 1992). In this study, the prevalence of oral species of *Porphyromonas* producing neuraminidase by using a rapid assay was determined.

Ninety nine *Porphyromonas* spp. isolated from dogs with and without periodontitis (77 strains) and from humans with periodontitis (22 strains) were evaluated. All clinical samples were collected from a private dental veterinary clinic (Odontovet, Sao Paulo, SP, Brazil) of dogs and from clinic of periodontology (Dentistry school, Univeristy of Sao Paulo). Clinical samples were collected from March 2010 to January 2011. Strains were grown on Brucella agar (Difco Laboratories, USA) and incubated in anaerobiosis (90% N_2/10% CO_2), 37 °C for 48 h (Jousimies-Somer et al., 2002). After identification were stored at −80 °C until use.

Neuraminidase production was determined in microtitre plates by using normal human erythrocytes drawn from healthy volunteers according to Nakano et al. (2006). Briefly, the erythrocytes were collected in Alsever's solution (10%) and bacterial cells resuspended (1.5×10^8 bacteria/mL) in brain heart infusion (BHI) broth. Then, 1 mL of bacteria was added to tubes with 10 μL of the erythrocytes and incubated in anaerobiosis (37 °C, 4 h). Serial dilutions of the lectin from peanut (2.5 mg/mL) (*Arachis hypogaea*; PNA, Sigma Chemical Co.) in phosphate buffered saline (PBS) were performed, and 20 μL of each dilution was

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added to the mixture bacteria-erythrocytes (20 μL) and incubated at 4 °C, overnight. The presence of the neuraminidase was noticed when an agglutination (bacteria-erythrocytes-lectin) was observed. A mixture of bacteria-erythrocytes without lectin was used as negative control. The agglutination titer was defined as the reciprocal of the end point dilution. The strains *Actinomyces viscosus* ATCC 91014, *Porphyromonas gingivalis* ATCC 33277 and *Prevotella intermedia* ATCC 33563, were used as positive controls, and all of them showed titers > 512. All the assays were performed in duplicate.

The inhibition of the neuraminidase production was verified in neuraminidase-positive strains. Briefly, bacteria were treated with 1 mL of 1 mM galactose at room temperature for 30 min. Then, 2 μL of diluted lectin were mixed with 18 μL of bacteria treated with 1 mM galactose, and added to 20 μL of erythrocytes (1% final concentration). Plates were incubated (37 °C, 1 h), and the enzyme inhibition was noticed by a precipitation of erythrocytes. A mixture of bacteria-erythrocytes-lectin was used as negative control.

Hemagglutination (HA) was tested qualitatively by mixing 50 μL of bacterial suspension with 50 μL of erythrocyte suspension. Then, 50 μL erythrocytes were added to each dilution and incubated (4 °C, overnight). HA titers were expressed as the reciprocal of the highest bacterial dilution showing agglutination activity.

The ability of D-arabinose, D-galactose, D-mannose or D-xylose to inhibit hemagglutination was evaluated. Each carbohydrate was dissolved in 80 mM of PBS. The hemagglutination inhibition (HAI) was qualitatively performed by mixing 50 μL of bacterial suspension with 50 μL of erythrocytes treated with each carbohydrate suspension. Two-fold serial dilutions of the bacterial suspension were performed. Then, 50 μL of erythrocytes treated with carbohydrate were added and incubated (4 °C, overnight). Erythrocytes treated with sugar and mixed with PBS were used as negative control. In all the assays *Fusobacterium nucleatum* ATCC 10953 was also used as negative control.

The production of neuraminidase and haemagglutination of oral *Porphyromonas* spp. isolated from dogs (77 strains) and humans (22) are showed in Table 1. The neuraminidase production was observed in 32.1% of the strains isolated from periodontal dogs and in 28.5% from healthy dogs. In addition, 31.8% of the strains isolated from humans produced this enzyme.

Maltose was able to inhibit the neuraminidase production in most of strains isolated from dogs with and without periodontitis. Thirty one out of 99 strains belonging to *P. gulae* (from dogs) and *P. gingivalis* (from humans) were able to hemagglutinate erythrocytes, showing HA titers from 2 to 64 (data not showed). Strains isolated from dogs (11.7%) and humans (31.8%) produced neuraminidase and haemagglutination; and of them 54.5% and 50%, respectively, no enzyme activity was observed.

Table 1
Strains neuraminidase and haemagglutination producer isolated from dogs with and without periodontitis and humans with periodontitis.

Source/Strains (n°)	Neuraminidase Production			Haemagglutination Production		
	n°	(%)	Titers	n°	(%)	Titers
Dogs with periodontitis (56)						
P. creviocanis (5)	1	20	4	0	0	0
P. gingivicanis (4)	4	100	2–4	0	0	0
P. gulae (36)	12	33.3	2 - >512	14	38.8	2–64
P. macacae (8)	1	12.5	2	0	0	0
P. cangingivalis (3)	0	0	0	0	0	0
Total	18	32.1		14	25	
Dogs without periodontitis (21)						
P. catoniae (2)	0	0	0	0	0	0
P. circumdentaria (6)	2	33.3	2–8	0	0	0
P. gulae (13)	4	30.8	2–16	6	46.1	2–16
Total	6	28.5		6	28.5	
Humans with periodontitis (22)						
P. gingivalis (22)	7	31.8	2–8	11	50	2–32
Total	7	31.8		11	50	

In companion animals, clinically both gingivitis and periodontitis are similar to humans (Hardham et al., 2005). In animals different *Porphyromonas* spp. appears to be associated with periodontal disease, but their pathogenicity is still unclear (Conrads et al., 2004). The presence of neuraminidase in *Porphyromonas* spp. suggests that this enzyme is involved with the pathogenesis or that it may be important to colonize mucosal as observed in *P. gingivalis* (Nakano et al., 2006).

Li et al. (2012) demonstrated that neuraminidase is an important virulence factor in *P. gingivalis* contributing to the biofilm formation, capsule biosynthesis and pathogenicity, and it can collaborate as a new target for developing of therapeutic agents against *P. gingivalis* infection. Moncla et al. (1990) showed neuraminidase activity in 25 *P. gingivalis* strains suggesting that this enzyme plays an important role in the pathogenesis of this microorganism.

In this study, 20 (26%) *P. gulae* and 11 (50%) *P. gingivalis* were able to agglutinate erythrocytes with titers from 2 to 64. The adherence ability of *P. gulae* appears to be lower than *P. gingivalis* and it might be explained because of absence of fimbrillin (Hamada et al., 2008). On the other hand, the lack of the neuraminidase activity in *F. nucleatum* ATCC 10953 was observed.

To assure the specificity of this assay its inhibition was noticed when galactose was used as peanuts lectin is highly specific for the GalNac (Pereira et al., 1976). In conclusion, this neuraminidase assay based in PNA hemagglutination is quick, simple and highly sensitive, and neuraminidase-producing *Porphyromonas* spp. present in dogs with periodontitis suggests a possible role in the developing of periodontal disease.

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