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Research Article

Pathogenicity and genetic profile of oral *Porphyromonas* species from canine periodontitis

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ABSTRACT

Objective: In this study, the presence of the *prtC* and *fimA* genes involved in the pathogenicity of oral *Porphyromonas* spp. isolated from dogs with periodontitis and healthy, as well as their genetic diversity was investigated.

Design: Thirty-two Beagle dogs, 24 with periodontitis and 8 healthy were evaluated. Subgingival samples from only one gingival site of both groups were collected. Bacteria grown in anaerobiosis were identified by RAPID ID 32A kits. From each strain the respective DNA was obtained and used to genotyping by conventional PCR and AP-PCR.

Results: Dogs with periodontitis harbored 28 *P. gulae*, 2 *P. creviocaricanis*, 1 *P. cangingivalis* and 7 *P. macacae*; and from healthy dogs, 11 *P. gulae* and 5 *P. circumdentaria*. In *P. gulae* isolated from periodontal dogs the gene *prtC* was observed in 19 (67.85%) and in 7 (63.63%) from healthy dogs. *P. gulae* strains from periodontal dogs harbored either the gene *fimA* I or *fimA* II; while strains from healthy dogs harbored the gene *fimA* I, *fimA* II, *fimA* III or *fimA* IV, as well as 1 *P. circumdentaria* the gene *fimA* II. By AP-PCR strains were grouped in different clusters suggesting heterogeneity of these microorganisms.

Conclusions: The results presented herein inform that *Porphyromonas* spp. isolated from dogs with and without periodontitis harbored the *prtC* and *fimA* genes and it could be a role in the establishment of the infectious process.

1. Introduction

It is well known that periodontitis is a complex infection, in which bacteria are responsible to initiate the immune inflammatory process and the result is a loss of support of the affected teeth (Meyle & Chapple, 2015). This process is characterized by destruction of the periodontal attachment apparatus, increased bone resorption with loss of crestal alveolar bone, apical migration of the epithelial attachment, and formation of periodontal pockets (Graves, Li, & Cochran, 2011). Among all oral pathogenic and non-pathogenic microorganisms, species of the genus *Porphyromonas* are able to invade and to damage the epithelial layer as well as to induce inflammatory responses leading to attachment loss and periodontal destruction (Paster et al., 2001). *Porphyromonas gingivalis* has been described as a key pathogen in periodontal infections due to the production of different virulence factors, such as gingipain, amino peptidases, invasin, capsule, collagenases, fimbriae, and lipopolysaccharide (Amano, 2003; Jotwani & Cutler, 2004; Preshaw, Schifferle, & Walters, 1999).

Fimbriae-producing *P. gingivalis* plays an important role in the attachment and invasion to periodontal tissues (Hamada et al., 1998) and it is critical for promotion of the bacterial infection (Nakagawa, Amano, & Ohara-Nemoto, 2002). The collagenolytic activity of *P. gingivalis* has been associated to the collagen destruction and progression of periodontitis (Kato, Takahashi, & Kuramitsu, 1992). This collagenase produced by *prtC* gene has been described as responsible for cleavage of type I collagen (Wittstock, Schmidt, Flemmig, & Karch, 2000).

Studies have shown that periodontitis is also common in dogs (Weinberg & Bral, 1999), leading to bone resorption and tooth loss. In animals, the anaerobic oral microbiota is represented by others important species of *Porphyromonas,* such as *P. gulae, P. circumdentaria, P. creviocaricanis, P. casulci, P. canoris, P. denticanis, P. salivosa, P. cangingivalis*, *P. canis*, *P. gingivicanis,* and *P. catoniae* (Allaker, de Rosayro,

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Table 1
Nomenclature, oligonucleotide and PCR conditions.

Primer	Oligonucleotide 5′ → 3′	Annealing Temperature (°C)	Amplicon (bp)	References
Universal	AGA GTT TGA TCC TGG CTC AG GGC TAC CTT GTT ACG ACT T	58	3480	Amano, Nakagawa, Okahashi, and Hamada, 2004
prtC	CGA GAT CGG AGT AGA AGT GCA TC CCA CGG TTT GCA GTT CGT ATC G	55	815	Odell et al. (1999)
fimA I	CTG TGT GTT TAT GGC AAA CTT C AC CCC GCT CCC TGT ATT CCG A	58	392	Amano et al. (2004)
fimA II	ACA ACT ATA CTT ATG ACA ATG G AAC CCC GCT CCC TGT ATT CCG A	58	257	Amano et al. (2004)
fimA III	ATT AC ACCT ACA CAG GTG AGG C AAC CCC GCT CCC TGT ATT CCG A	58	247	Amano et al. (2004)
fimA IV	CTA TTC AGG TGC TAT TCA CCA A AAC CCC GCT CCC TGT ATT CCG A	58	251	Amano et al. (2004)
fimA V	AAC ACC AGT CTC CTT GAC AGT G TAT TGG GGG TCG AAC GTT ACT GTC	58	462	Nakagawa et al. (2002)
OPA-03	AGT CAG CCA C	42	–	Chen and Slots (1994)
OPA-05	AGG GGT CTTG	42	–	Chen and Slots (1994)
OPA-13	CGG CAC CCA C	42	–	Chen and Slots (1994)
OPA-17	GAC CGT CTT GT	42	–	Chen and Slots (1994)

Young, & Hardie, 1997; Hardham, Dreier, Wong, Sfintescu, & Evans, 2005; Isogai, Kosako, Benno, & Isogai, 1999). *Porphyromonas gulae* is a gram-negative, black-pigmented producing, and is considered the commonly species detected in subgingival biofilm of dogs displaying periodontitis (Kato et al., 2011). Similarly to *P. gingivalis* the fimbriae is an important cell structure involved in the adherence and invasion of host's cells, and stimulates the production of inflammatory cytokines by macrophages and fibroblasts. This adhesive ability is considered to be a major pathogenic characteristic that causes periodontal tissue destruction (Amano, Nakagawa, Kataoka, Morisaki, & Hamada, 1999; Sasaki, Watanabe, Toyama, Koyata, & Hamada, 2015).

On the other hand, little is known about the presence of *Porphyromonas* spp. isolated from dogs carrying the *prtC* gene, as well as its relationship with the canine periodontitis. Since pathogenic bacteria from animal origin, particularly dogs, may be transferred to people through bites, for example; and considering their involvement in human and animal periodontal disease; this study, was performed to determine the presence of the *prtC* and *fimA* genes in oral *Porphyromonas* spp. isolated from dogs with and without periodontal disease, as well as, the genetic diversity of these species by using an AP-PCR technique.

2. Materials and methods

2.1. Animal study design

Subgingival biofilm samples were collected from thirty-two Beagle dogs of 7-months to 10-years-old at the School of Veterinary Medicine and Zootechny of the University of Sao Paulo (Sao Paulo, SP, Brazil). Dogs were grouped in: (1) Eight dogs without periodontitis, and (2) 24 dogs with periodontitis. As inclusion criteria, only the animals had received no antibiotic treatment within the previous three months of the sample collection. Prior to collection, the animals with periodontitis were examined in relation to clinical conditions: degree of gingival inflammation, supragingival plaque level, probing pocket depths (5 mm), bleeding on probing, tooth mobility, and alveolar bone loss.

The animals underwent anesthesia with a mixture of propofol (2 mg/kg) and diazepam (5.5 mg/kg) administered via intramuscular injection (Senhorinho et al., 2011). Briefly, supragingival biofilms were removed with sterile gauze to avoid any contamination of the paper points. Then, subgingival samples from only one gingival site of both groups were collected with two fine sterile paper points (N. 30, Tanariman Ind Ltd, AM, Brazil), introduced into the apical region for 60 s. Paper points were then placed into VMGA III transport medium and processed within 4 h of collection.

The protocols used in this study were approved by the Ethics Committee for Animal Experimentation at the Institute of Biomedical Science/USP (116/CEEA), and followed all recommendations of the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines for the execution and submission of studies in animals.

2.2. DNA extraction, PCR assay and AP-PCR genotyping

Bacteria were isolated on Brucella blood agar supplemented with hemine (0.5 μg/ml) and menadione (0.1 μg/ml), at 37 °C, under anaerobic conditions (10% CO_2/90% N_2), and identified in accordance with Jousimies-Somer et al. (2002) or by RAPID ID 32A kits (bioMérieux) according to the manufacturer's instructions. The broth bacterial cultures were centrifuged at 14.000*g* for 10 min to spin down the pellet and used to DNA extraction. The total genomic DNA was isolated by Easy-DNA commercial kit (Invitrogen do Brasil, Ltd, Sao Paulo, SP, Brazil) and the concentration of bacterial DNA determined with a spectrophotometer (OD_{260nm}).

PCR Amplifications were performed as described: each 25 μL-PCR contained 1 X PCR buffer, 2.5 mM $MgCl_2$, 20 μM dNTP (Invitrogen), 0.5 U Platinum *Taq* DNA polymerase (Invitrogen), 0.4 μM of each primer (Table 1) (Chen & Slots, 1994; Odell, Baumgartner, Xia, & David, 1999) and 1 μL of DNA. The cycle conditions were optimized according to the gene. For *prtC* gene, denaturation 1 cycle of 94 °C (5 min); amplification, for 33 cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (1 min); and 1 cycle of 72 °C (10 min). For *fimA* gene detection the same PCR conditions were used, but with 30 cycles of 94 °C (30 s), 58 °C (30 s), and 72 °C (30 s). Combinations were used in all PCR reactions and amplifications were performed in a thermal cycler (Perkin Elmer Amp PCR System 2400).

In order to investigate the AP-PCR genotyping, four arbitrary primers OPA-03, OPA-05, OPA-13 and OPA-17 (Table 1) and 10 μL of DNA were used, as mentioned above. Conditions were further optimized, as follow: denaturation 1 cycle of 94 °C (5 min); amplification, for 35 cycles of 94 °C (1 min), 42 °C (2 min), 72 °C (2 min); and 1 cycle of 72 °C (10 min). From all the resulting PCR products, twenty microlitres of each amplification product were evaluated by electrophoresis in 1.0% agarose gel. Reference strains *P. gingivalis* ATCC 33277 and *F. nucleatum* ATCC 10953 were used as control.

2.3. Statistic analysis

Pairwise similarities were computed by the package NTSYS program (Applied Biostatistics, Inc. version 1.7) program using the Dice coefficient of similarity and unweighted pair group method using arithmetic

Table 2
Presence of the *prtC* and *fimA* genes in *Porphyromonas* strains from dogs with and without periodontitis.

Origin Strains (no.)	Genes					
	prtC	*fimA* I	*fimA* II	*fimA* III	*fimA* IV	*fimA* V
Dogs with Periodontitis						
P. gulae (n = 28)						
D1, D4, D7, D20, D22	+	+	−	−	−	−
D3	+	−	+	−	−	−
D2, D8, D12, D21, D25, D26, D27, D28, D30, D32, D33, D34, D35	+	−	−	−	−	−
D9, D23, D29	−	+	−	−	−	−
D5, D6, D10, D11, D24, D36	−	−	−	−	−	−
P. creviocaricanis (n = 2)						
D37, D38	−	−	−	−	−	−
P. cangingivalis (n = 1)						
D31	+	−	−	−	−	−
P. macacae (n = 7)						
D13, D14, D15, D16, D17, D18, D19	−	−	−	−	−	−
Healthy Dogs						
P. gulae (n = 11)						
S2	+	+	−	+	+	−
S1	+	−	−	+	+	−
S3	+	+	+	−	+	−
S4	+	−	+	−	−	−
S5, S6	+	+	+	−	−	−
S7	+	−	−	−	−	−
S8, S10, S12, S13	−	−	−	−	−	−
P. circumdentaria (n = 5)						
S11	−	−	+	−	−	−
S16	+	−	−	−	−	−
S9, S14, S15	−	−	−	−	−	−
Reference strains						
P. gingivalis ATCC 33277	+	+	−	−	−	−
F. nucleatum ATCC 10953	−	−	−	−	−	−

Presence (+); absence (−).

averages (UPGMA) clustering.

3. Results

Fifty-four strains were isolated and identified from 24 dogs with periodontitis (38 strains) and from eight healthy dogs (16 strains) (Table 2). The *prtC* gene was detected in 19 (67.86%) of 28 *P. gulae* strains, and in 1 *P. cangingivalis* isolated from dogs with periodontitis. Neither two *P. creviocaricanis* nor seven *P. macacae* strains harbored that gene. Of the 16 strains from healthy dogs, 7 (63.64%) *P. gulae* and 1 *P. circumdentaria* harbored the *prtC* gene. The *fimA* I gene was observed only in eight *P. gulae* strains from periodontitis and in four strains from healthy dogs. The presence of *fimA* II gene was noted in 1 *P. gulae* from periodontitis, and in 4 *P. gulae* and 1 *P. circumdentaria* from healthy dogs. Interestingly, the *fimA* III and *fimA* IV were not observed in *P. gulae* strain from periodontal dogs, but both genes were noted in 2 *P. gulae,* and *fimA* IV in another strain isolated from dogs without periodontitis. Moreover, neither periodontal nor healthy dogs harbored strains with the *fimA* V gene (Table 2). Double or triple bacterial associations with fimbriae types were observed only in strains isolated from healthy dogs. Reference strains *P. gingivalis* ATCC 33277 harbored the *prtC* and *fimA* I genes, and *F. nucleatum* ATCC 10953 did not harbor both genes.

In Fig. 1 is observed the genetic analysis of the 38 *Porphyromonas* strains. A High degree of DNA polymorphism was detected with OPA-03 primer, which produced clear bands and a good bacterial distribution showing the presence of 15 clusters with 50% of similarity. In addition, the primers OPA-05, OPA-13 and OPA 17, revealed weak amplification bands. These bands were not constantly detected by visual inspection, and were not considerate in this analysis. The presence or absence of *prtC* and *fimA* genes did not show any relationship with the clusters. The genotyping of the strains by AP-PCR produced from 4000 to 400 bp DNA fragments, with 15 different genetic profiles in strains isolated from dogs with and without periodontitis. In addition, in Fig. 1 can also be observed three main groups: group A, harbored only strains isolated from periodontitis including *P. gingivalis* ATCC 33277 (*P.g* ATCC); group B, strains isolated from dogs with and without disease, and group C, containing only strains from healthy dogs. The cluster IX was formed for two sub-group containing *P. macacae* strains isolated from dogs with periodontitis. These strains showed 100% of similarity. Strains isolated from healthy dogs, were grouped from XI to XV. The cluster XIV grouped three *P. circumdentaria* (*P.c*) with 100% of similarity. Some strains of *Porphyromonas* and *F. nucleatum* ATCC 10953 did not amplify fragments.

4. Discussion

Dog periodontitis-related bacterial species have been found in human oral cavity and are involved with aggressiveness of the disease. Similarly to the human periodontal disease, species of *Porphyromonas* are also important etiological agents in dog oral infections (Pihlstrom, Michalowicz, & Johnson, 2005), and among them, *P. gulae* is considered the most prevalent (Kato et al., 2011). In order to investigate the presence of black-pigmented anaerobic bacteria from animal origin and their virulence factors involved in periodontal disease, we determined the presence of *Porphyromonas* species in dogs with and without periodontal disease. The genetic diversity of these species isolated from periodontal and healthy dogs were demonstrated by AP-PCR analysis.

Porphyromonas gulae from dogs with periodontitis were significantly present in higher number than those of the healthy group. Furthermore, we demonstrated that bacterial species isolated from periodontal dogs displayed both *prtC* and *fimA* genes, that promote the adherence and colonization to the host's tissues. Importantly, we also found a high level of polymorphism among the *Porphyromonas* spp., which might reflect the potential of virulence and its involvement with periodontal disease. This is an important finding since competition among clones might select more resistant clones able to survival in the human host.

Fimbriae are also considered to be critical factors that mediate bacterial interactions in the adherence and invasion to the host's cells. FimA is encoded by the *fimA* gene and its virulence is related to the genetic variation. The presence of *P. gingivalis fimA* genotypes II and IV have been shown to be significantly more prevalent than other genotypes in human chronic marginal periodontitis processes (Amano et al., 2000; Feng et al., 2014). The pathogenicity of the various *fimA* genotypes has also been evaluated in animal models. Strains *fimA* genotypes II, Ib or IV appear to cause severe infectious symptoms and inflammatory changes, when compared to strains *fimA* genotypes I and III (Amano et al., 1999; Nakano et al., 2004). Additionally, studies on the pathogenic potential of *Porphyromonas* spp. have shown that *fimA* genotypes II are prevalent in patients with aggressive periodontitis (Miura, Hamachi, Fujise, & Maeda, 2005). These results support the proposal that *P. gulae* could be considered as a possible risk factor for the periodontitis development (Senhorinho et al., 2011).

In line with previous studies, our findings show the presence of *Porphyromonas* spp. *fimA* genotype I in both groups of dogs with periodontitis and healthy, suggesting no association between that genotype and the disease evolution. On the other hand, *fimA* genotype II associated with human aggressive oral diseases was found in only one dog with periodontitis. These results suggest that *Porphyromonas* spp. harboring genes, such as *prtC* and *fimA* can be pathogenic to animals, but it might not be true to produce an oral disease in humans, in accordance with (Amano et al., 2000). Some studies affirm that it is difficult for oral bacteria from canine origin to survive and colonize the human oral

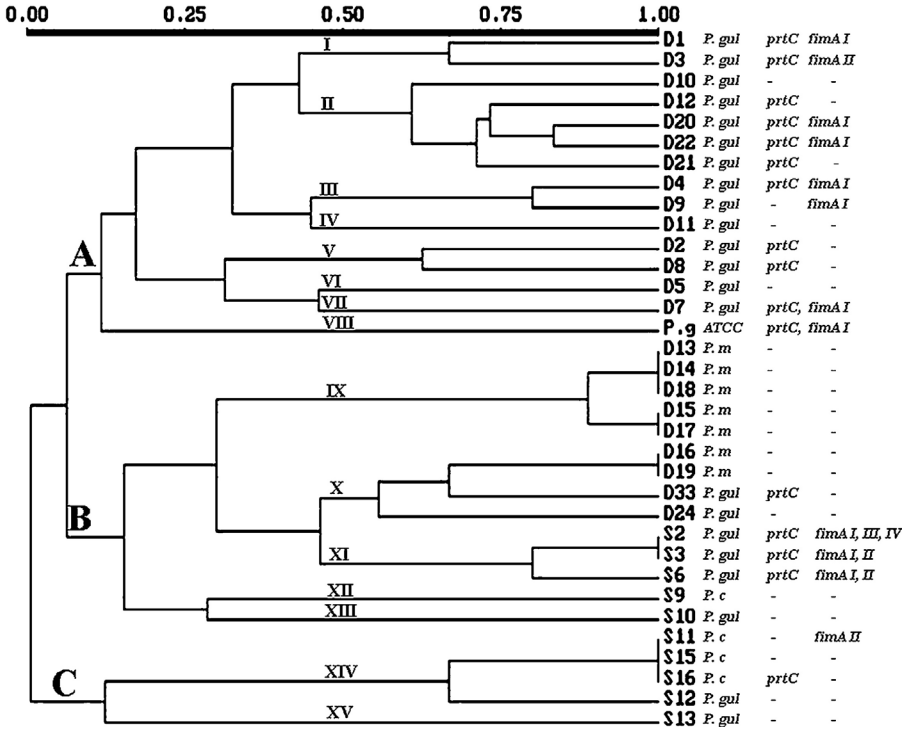

Fig. 1. Dendrogram showing the genetic profile of oral *Porphyromonas* strains isolated from dogs with and without periodontitis. (D) Strains from dogs with periodontitis; (S) strains from healthy dogs; (−/+) absence/presence of genes; (*prtC* and *fimA*) presence of the genes; (*P. gul*) *P. gulae;* (*P. m*) *P. macacae;* (*P. c*) *P. circumdentaria;* and (*ATCC*) *P. gingivalis* ATCC 33277.

cavity (Oh et al., 2015) due to the physiological differences between the two environments, such as: difference of pH (Li & Bowden, 1994) and by the fact that humans brush their teeth removing the supra- and subgingival bacterial biofilms. In addition, when canine oral bacteria are transmitted to the human oral cavity, these microorganisms begin an ecological competition with the human resident oral microbiota, and it can influence the expression of some virulence factor for the initiation of a disease (Senhorinho et al., 2012). The bacterial dynamic relationship in different microbial ecosystem needs more studies.

Our results show that periodontal dogs harbored strains *fimA* I and *fimA* II, and healthy dogs harbored *fimA* I, *fimA* II, *fimA* III, and *fimA* IV. This result suggests that species of *Porphyromonas* with genotypes *fimA* III and *fimA* IV are not implicated in periodontal disease of dogs. Since in humans, the presence of *P. gingivalis* harboring the *fimA* gene may be associated with the severity of the periodontitis; it is possible that *P. gulae* with the genotype *fimA* may also be associated with the pathogenesis of periodontitis in dogs. Studies have shown that the presence of long fimbriae observed in *P. gingivalis* serve to mediate the co-adhesion with other bacteria, such as *Actinomyces viscosus, Treponema denticola* and *Streptococcus oralis* (Goulbourne & Ellen, 1991; Hashimoto, Ogawa, Asai, Takai, & Ogawa, 2003; Maeda et al., 2004).

The FimA protein produced by *P. gulae* has been classified into genotypes A–C. The bacterial detection with different *fimA* genotypes is considered a risk factor to the periodontitis development (Maeda et al., 2004). In addition, differences in the fimbriae protein from *P. gulae* are related to the level of aggressiveness determined by their bone destruction capacity (Nomura et al., 2012). The 41-kDa fimbriae protein is able to induce the osteoclast differentiation as well as the inflammatory cytokine production by macrophages, in murine model, as well as it was similar to LPS in the osteoclast activation in periodontal diseases (Nomura et al., 2012). Thus, the presence of specific fimbriae proteins in *P. gulae* can explain the divergent results observed in different studies, and it might be useful to understand the association of the presence of *fimA* genotypes with either oral health or disease.

In order to examine the potential role of the bacterial collagenases in oral diseases, we investigated the presence of the *prtC* gene in oral *Porphyromonas* isolated from dogs with and without periodontal disease. Collagenase is a potential virulence factor, which is expressed by *P. gingivalis* associated with periodontal disease (Odell et al., 1999), and it is suggested that *prtC* gene-positive *Porphyromonas* spp. plays an important role in the progression of periodontitis; however, studies of this enzyme produced by *P. gulae* on host's cells need to be performed. A limitation of this study was the sample size and the differences on number of dogs in both with and without disease groups. Furthermore, we selected dogs with a large age range, and since the total number of bacterial species detected in these animals was positively correlated with their chronological age, this variation could be interfered our findings (Harvey, Shofer, & Laster, 1994).

Further studies focusing the understanding of the relationship among the bacterial transmission, bacterial genes and protein expression in oral environment, and the relationship with oral disease of pathogenic oral bacteria from dogs to humans might be important to help us to better understand the complex oral microbial ecosystem. In conclusion, the high level of *Porphyromonas* spp. from animal origin takes many paths to a reinfection of humans, due to colonized or infected pets might influence the pathogenesis of disease in humans. Our findings strongly suggest that the colonization and adaptation of *Porphyromonas* spp. from dogs in human oral cavity is a possible fact. Also, these results allow us to correlate the presence of the *prtC* and *fimA* genes, particularly *fimA* I and *fimA* II, with periodontal disease in dogs.

Conflict of interests

The authors declare that they have no competing interests.

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