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Note

Detection of *Porphyromonas gulae* from subgingival biofilms of dogs with and without periodontitis

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ABSTRACT

A rapid PCR approach was developed to detect *Porphyromonas gulae* strains from subgingival samples of dogs with and with periodontitis. The presence of *P. gulae* was observed in 92% and 56%, respectively, in dogs with and without periodontitis. The new primer pair was specific to detect this microorganism, and this technique could be used to evaluate a correlation between periodontitis and *P. gulae* in companion animals.

Periodontal disease is a chronic and multi-factorial disease that leads to inflammation of the gingival destruction of periodontal tissue and loss of alveolar bone in humans and animals [1]. Gingivitis and periodontitis are common in dogs; however, their oral microbiota has been poorly characterized [2].

In animals, particularly dogs, periodontitis occurs spontaneously increasing in prevalence and severity with age, and different species of *Porphyromonas* appear to be associated with periodontal disease [3], but their pathogenicity is still unclear [4]. Differences between human and companion animal *Porphyromonas* isolates have been reported. Strains of animal origin, closely resembling *P. gingivalis* of humans, and both can be distinguished phenotypic and genotypically. Catalase-producing *P. gingivalis* strains are referred as *Porphyromonas gulae*, and this microorganism has been recovered from subgingival samples of different companion animals displaying periodontitis [3–5]. Because the bacterial dispersion from dog bites to humans is observed, the study of its oral microbiota is important [6,7].

Molecular methods are currently available for detecting periodontopathogens and polymerase chain reaction has been used to identify these pathogens from subgingival microbiota [8,9]. In this study, a rapid detection of *P. gulae* from subgingival biofilm of dogs with and without periodontitis was performed.

Subgingival samples were taken from 50 dogs with and 50 without periodontitis undergoing routine dental treatment at the Private Dental Veterinary Clinic (Odontovet, São Paulo, SP, Brazil) and at the School of Veterinary Medicine and Zootechny of the University of São Paulo. Dogs (62) and bitches (38) with and without periodontitis from 7-months to 10-years of age were selected and represented 12 different breeds. Periodontitis was verified by different clinical indices (degree of gingival inflammation, amount of supragingival plaque, probing pocket depths (≥5 mm), bleeding on probing, tooth mobility and alveolar bone loss. None animal had received antibiotic treatment within the previous three months of the collection. The Ethics Committee for Animal Experimentation at the Institute of Biomedical Science/USP (116/CEEA) approved this study.

Animals were anesthetized with propofol (2 mg/kg) and diazepam (5.5 mg/kg) by an intramuscular injection, and received isoflurane and oxygen by an endotracheal way. Supragingival biofilm was removed by using a sterile gauze, and subgingival samples from only one gingival site (healthy dogs) or periodontal pocket (dogs with periodontitis) were collected by using two fine sterile paper points (N°. 30, Tanariman Ind Ltd, AM, Brazil), placed for 60 s.

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Points were then placed into VMGA III transport medium and processed within 4 h of collection. All samples 10-fold diluted were plated (0.1 ml) onto Brucella blood agar (Difco Laboratories) containing 5% defibrinated horse blood, 0.0005 mg/ml hemin and 0.0001 mg/ml menadione, and incubated in atmosphere of 10% CO_2 + 90% N_2, at 37 °C for 5 days.

Black-pigmented hemolytic colonies were selected and identified as *P. gulae* by using a Rapid ID 32A kit (bioMérieux), by a β-hemolitic activity and producing catalase and indole. *P. gulae* ATCC 51700 was used as control. Then, bacterial genomic DNA was extracted and purified from cells in the mid-logarithmic growth phase using a QIAamp DNA mini kit (Qiagen Inc, Chatsworth, CA). The 16S rRNA gene fragments were amplified [10]. Briefly, two subregions of 16S rRNA gene were amplified by using two primer pairs. Subregion A was defined as 899-bp sequences between primers 8UA and 907B, and subregion B was defined as 711-bp sequences between primers 774A and 1485B. PCR was performed for 35 cycles of 30 s at 95 °C, 30 s at 45 °C, and 1 min at 72 °C, with a final extension at 72 °C for 5 min. PCR products were excised from a 1% agarose gel after electrophoresis and purified using a QIA quick gel extraction kit (Qiagen). An ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA) was used for DNA sequencing.

Analysis of *P. gulae* isolates was performed by comparing the type strains sequences determined in this study, as well as related sequences retrieved from GenBank, as follow: *P. gulae* ATCC 51700 [AY546476]; *P. gulae* [AF285873]; *P. gulae* [AF285872]; *P. gulae* [AF285874] and *P. gulae* [AF285871] which were analyzed by multisequence alignment with CLUSTAL-W (http://genome.kribb.re.kr). Based on the multi-alignment analysis data, two primers were designed as follows: F-primer: 5′- TTG GTT GCA TGA TCG GG -3′ and R-primer: 5′- GCT TAT TCT TAC GGT ACA TTC AYA -3′. Sequences were analyzed for secondary structure formation, G + C content, and primer–dimer formation with the NetPrimer analysis software (http://www.premierbiosft.com/netprimer). The specificities of the primers were compared to all available sequences by using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST). Primers were tested by running PCR with DNA from nine different reference strains (*P. gingivalis* ATCC 33277, *P. macacae* ATCC 33141, *Prevotella intermedia* ATCC 25611, *P. nigrescens* ATCC 33563, *Fusobacterium nucleatum* ATCC 25586, *Treponema denticola* ATCC 35308, *Tannerella forsythia* ATCC 25540, *Bacteroides fragilis* ATCC 25285 and *Parabacteroides distasonis* ATCC 8503). A colony from Brucella blood agar was resuspended in distilled water (200 μl), boiled (20 min), and 2 μl of supernatant (DNA) was used for subsequent amplifications.

PCR amplification was carried out in a 30-μL reaction mix containing 1 × PCR buffer, 50 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer pair, 0.5 U *Taq* Platinum DNA polymerase (Invitrogen) and 1 ng of DNA. Amplification was carried out in a thermal cycle (GeneAmp PCR System 9700), programmed for 1 cycle of 94 °C (5 min) followed by 35 cycles at 94 °C (30 s), 58 °C (1 min) and 72 °C (30 s), and 1 cycle of 72 °C (5 min). A negative control without DNA was included in each PCR run, and PCR products were analyzed by electrophoresis in 1% agarose gel. A 300 bp amplicon was expected (Fig. 1).

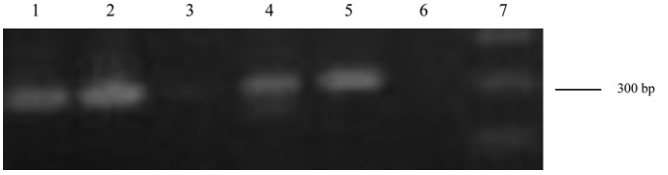

Fig. 1. DNA from *P. gulae* isolates and another non-related organism amplified with the specific primer pair. Lanes 1 to 4, *P. gulae* isolates; lane 5, *P. gulae* ATCC 51700; lane 6, negative control; lane 7, 100-bp DNA ladder.

Species of *Porphyromonas* were recovered from dogs with (50) and without (50) periodontitis, respectively, in 46 (92%) and 28 (56%) dogs. Of the 46 periodontal and of the 28 healthy dogs, 38 and 15 *P. gulae*, respectively, were isolated. The presence of *P. gulae* in dogs without periodontitis did not show any statistically significant difference; however, the presence of this microorganism in dogs with periodontitis showed statistically significant values ($P = 0.008$).

Because periodontal disease is considered as the most common disease in small animals, previous companion animal studies determining the oral microbiota using biochemical assays have been performed; however, these procedures are limited by the quality of the biochemical databases. In this study, we detected the presence of *P. gulae* in canine periodontal lesions. Paper point samples from the crevicular space that are diluted and plated on growth media have proven to be the most reliable method for isolating bacteria from crevicular fluid. The traditional culture and identification methods may limit the bacterial recovery due to the absence of viable cells and growth requirements [3]. The identification of canine periodontal pathogens, such as *P. gulae* allows for a more directed approach to companion animal periodontitis prevention and treatment [3,11].

Hardham et al. [7] using the 16S rRNA gene sequences identified 26 different species of black-pigmented anaerobic bacteria, including *P. gulae* in the most evaluated dogs. The new synthesized primers were specific to all isolated *P. gulae*. In addition, all the PCR products belonging to *P. gulae* were confirmed by sequencing. No amplification with the other bacterial DNA was observed (data not shown). These primers distinguished *P. gulae* from *P. gingivalis*-like organisms and they proved to be reliable and applicable for a rapid identification of *P. gulae*. In addition, these primers might be used to a rapid diagnosis of periodontitis in animals.

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