Clinical microbiology

Occurrence and antimicrobial susceptibility of Porphyromonas spp. and Fusobacterium spp. in dogs with and without periodontitis

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ABSTRACT

The occurrence of Porphyromonas gulae, Porphyromonas macacae, Fusobacterium nucleatum and Fusobacterium canifelinum in subgingival plaque from dogs with and without periodontitis as well as their antimicrobial susceptibility were evaluated. From 50 dogs with periodontitis were identified 38 P. gulae, 8 P. macacae, 26 F. nucleatum and 15 F. canifelinum, and from 50 dogs without periodontitis were identified 15 P. gulae, 12 F. nucleatum and 11 F. canifelinum. All strains were susceptible to most of the antibiotics tested, however, different resistance rates to clarithromycin, erythromycin and metronidazole among strains were observed. The role of P. gulae, P. macacae, F. nucleatum and F. canifelinum in periodontal disease of household pets needs to be defined to a better prevention and treatment of the canine periodontitis.

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1. Introduction

In man, periodontal diseases, such as gingivitis and periodontitis, are chronic and multi-factorial diseases affecting the tissues supporting the teeth [1]. Gingivitis and periodontitis lesions in dogs are more closely related to humans than other animals [2]; although, gingivitis did not necessarily progress to periodontitis. The etiologic factors of gingivitis and periodontitis seem to be identical in humans and dogs [2,3]; however, the oral microbiota in animals has been poorly characterized.

It is estimated that approximately 80% of dogs and cats display some degree of periodontal disease around 4 years of age [4]. In animals, different species of Porphyromonas and Fusobacterium appear to be associated with periodontal disease [5,6], but their pathogenicity is still unclear.

Black-pigmented anaerobic bacteria have been isolated from the periodontal pockets of dogs and cats; however, several differences between human and companion animal Porphyromonas strains have been reported [5,7].

Fusobacterium nucleatum is one of the most common bacterial species in gingival crevice of humans and animals and it is associated with periodontal disease. Fusobacterium canifelinum is closely related to F. nucleatum and it has been recovered from human wounds produced by animal bites, particularly, cat or dog [6,8].

Molecular methods are currently available for typing and subtyping of periodontopathogens, such as Porphyromonas gingivalis, but they can vary in efficiency and in amount of required labor [9,10]. Techniques such as bacterial culture are used to identify putative anaerobic pathogens from destructive periodontal disease but can be somewhat cumbersome and expensive [10]. Polymerase chain reaction (PCR) has been used for direct identification of periodontal pathogens from subgingival specimens [11], and for elucidating the role of specific bacteria in the periodontal disease because of ability to accurately detect bacterial species from mixed populations.
In anaerobic bacteria, the inadequate use of antibiotics has produced an alarming and graduate increase of the resistance to several drugs. Since antimicrobials can produce disequilibrium in oral or intestinal resident microbiota, the choice of a suitable antibiotic therapy is needed, and it must be taken in consideration to the treatment of infectious diseases. Gram-negative anaerobic bacteria, such as Porphyromonas spp. and Fusobacterium spp. isolated from humans has shown high resistance to several drugs [12]; however, data showing the susceptibility of oral bacteria from animal origin to different antimicrobials are scarce [13].

In this study, the occurrence of Porphyromonas spp. and Fusobacterium spp. from subgingival biofilm of dogs with and without periodontitis, as well as their antimicrobial susceptibility were evaluated.

### 2. Materials and methods

#### 2.1. Cohorts

Subgingival plaque samples were taken from 50 dogs with and 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 Zootecny of the University of São Paulo (São Paulo, SP, Brazil). Male and female dogs with (11 different breeds) and without (10 different breeds) periodontitis from 7-months to 10-years of age were selected. All animals with periodontitis were 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 of the animals had received antibiotic treatment within the previous three months of the sample collection. The Ethics Committee for Animal Experimentation at the Institute of Biomedical Science/USP (116/CEEA) approved this study.

#### 2.2. Sample collection and processing

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 sterile gauze and area was isolated by using cotton rolls. 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), introduced into the apical region of healthy or periodontal site and allowed to remain for 60 s. Paper points were then placed into VMGA III transport medium [14] and processed within 4 h of collection. All collected samples 10-fold diluted were plated (0.1 mL) onto Brucella blood agar (Difco Laboratories) containing 5% defibrinated horse blood, 0.0005 mg/mL hepin and 0.0001 mg/mL menadione to isolate Porphyromonas gulae and Porphyromonas macacae, and onto Omata and Disraely agar to isolate F. nucleatum and F. canifelimum. Plates were incubated in atmosphere of 90% N2 and 10% CO2, at 37 °C, for 7 days in anaerobiosis.

#### 2.3. Bacterial identification

Characteristic colonies of each bacterial species were presumptively identified by Gram staining; catalase, H2S and indol production; and esculin and starch hydrolysis. Further identification by biochemical tests [15] or by RAPID ID 32A kits (bioMérieux) according to the manufacturer’s instructions was performed. The resistance to levofloxacin was performed as an additional assay to distinguish F. nucleatum from F. canifelimum [6].

#### 2.4. Serum resistance and hemolytic activity

Bacterial resistance to human, dog or horse serum was determined [16]. Inactivated sera at 56 °C for 30 min were used as controls. 1 mL (1.5 × 10^8 cfu/mL) of each bacterial growth was mixed with 1 mL of dog, horse or human serum, and incubated in anaerobiosis at 37 °C for 4 h. 0.1 mL of each mixture was then inoculated onto Brucella blood agar and incubated in anaerobiosis at 37 °C for 72 h. A total inhibition was considered as being susceptible to serum. Hemolysin production was observed onto blood agar and each strain grown in BHI broth was inoculated by using a Steers replicator (ca. 10^6 cfu/spot). After incubation as described above, the hemolytic activity was defined as a clear zone around the bacterial growth.

#### 2.5. Hemagglutination (HA) and hemagglutination inhibition (HAI) assays

Each bacterial suspension, in PBS (0.01 M, pH 7.3), was adjusted to ca. 1.5 × 10^6 cfu/mL [17]. Human erythrocytes were resuspended in 1% Alsever’s solution. HA was qualitatively assessed by mixing 0.1 L of bacterial suspension to 0.1 mL of human, dog or horse serum, and incubated in anaerobiosis at 37 °C for 1 h, and after at 4 °C overnight. Hemagglutination titers were expressed as the reciprocal of the highest bacterial dilution showing agglutination.

The ability of α-glucose, α-galactose, α-mannose and α-lactose to inhibit the HA was evaluated. Carbohydrates were prepared in 80 mM in PBS and sterilized by filtration (0.45 micron pore size, Millipore). 0.1 mL of each strain was treated with 9.9 mL of each carbohydrate. The HAI was qualitatively performed by mixing 50 μL of bacteria with 50 μL of erythrocytes treated with each carbohydrate. Two-fold serial dilutions containing 50 μL/bottle were performed, and 50 μL of erythrocytes were then added to each dilution. Plate was gently shaken and incubated at 37 °C for 1 h, and after at 4 °C overnight. Hemagglutination titers were expressed as the reciprocal of the highest bacterial dilution showing agglutination.

#### 2.6. Antimicrobial susceptibility testing

An agar dilution method in Brucella blood agar was used according to CLSI [18]. The antibiotics used were: amoxicillin, ampicillin, clindamycin, erythromycin, and tetracycline (Luper Ind Ltd, SP, Brazil), amoxicillin/clavulanic acid (Smithkline Beecham Ltd, SP, Brazil), penicillin G (Prodoti Lab Farm Ltd, SP, Brazil), metronidazole (Aventis Farm Ltd, SP, Brazil), cefoxitin (Merck, Sharp and Dohme, SP, Brazil), and clarithromycin (Boehringer Ingelheim do Brasil Quim Farm Ltd, SP, Brazil). Breakpoints used for erythromycin and clarithromycin were according to NCCLS [19].

### 3. Results

#### 3.1. Bacterial isolation and identification

In ten out of eleven dogs with, and six out of ten without periodontitis of different breeds harbored at least one microorganism evaluated (Table 1). From forty-six (92%) out of 50 dogs with periodontitis, 38 Porphyromonas gulae, 9 Porphyromonas macacae, 26 F. nucleatum and 15 F. canifelimum strains were isolated, and 28 (56%) out of 50 dogs without periodontitis 15 P. gulae, 12 F. nucleatum and 11 F. canifelimum were isolated. No healthy dog harbored P. macacae. From each subgingival clinical sample, one to two colonies were picked.
Interestingly, in this study, poodle with periodontitis and yorkshire dogs with periodontitis was statistically significant without periodontitis.

The phenotypic characteristics of the four bacterial species are shown in Table 2. All 26 F. canifelinum strains were resistant to levofloxacin (MIC > 4). All P. gulae strains were β-hemolytic and P. macacae, F. nucleatum or F. canifelinum strains did not produce hemolysis. Most of P. gulae, P. macacae, F. nucleatum and F. canifelinum isolated from dogs with or without periodontitis were resistant to human, dog or horse serum.

### Table 1

<table>
<thead>
<tr>
<th>Presence of oral Porphyromonas spp. and Fusobacterium spp. in dogs with and without periodontitis.</th>
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<tbody>
<tr>
<td>Breed of dogs</td>
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<tr>
<td>With periodontitis</td>
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<td>Argentine mastiff</td>
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<td>Australian cattle dog</td>
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<td>Basset hound</td>
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<td>West highland white terrier</td>
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<td>Yorkshire terrier</td>
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<td>Yorkshire terrier</td>
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<td>Total</td>
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### 3.2. HA and HAI assays

Only 21 P. gulae and 2 F. nucleatum were able to agglutinate erythrocytes with titers from 2 to 64 and 4, respectively, d-glucose, d-galactose, d-mannose and d-lactose were able to inhibit the hemaggulination in most of P. gulae and F. nucleatum strains. Glucose was not able to inhibit HA in F. nucleatum.

### 3.3. Antimicrobial susceptibility

All strains tested were susceptible to amoxicillin, ampicillin, amoxillin/clavulanate, ceftoxitin, clindamyacin, penicillin G, and tetracycline. P. gulae and P. macacae strains isolated from dogs with periodontitis were respectively resistant to clarithromycin (68.4% and 50%) and erythromycin (34.2% and 12.5%). One (2.6%) metronidazole-resistant P. gulae strain was observed (MIC ≥ 512 μg/mL). In addition, F. canifelinum and F. nucleatum were respectively, 100% and 96% resistant to clarithromycin and erythromycin (Table 3). P. gulae strains from dogs without periodontitis were resistant to clarithromycin (86.7%), erythromycin (73.3%) and metronidazole (13.3%). F. canifelinum and F. nucleatum strains were resistant to clarithromycin (100%) and erythromycin (90.9% and 91.7%, respectively) (Table 4).

### 4. Discussion

Studies has shown the presence of human periodontitis-related bacterial species, such as P. gingivalis, Prevotella intermedia, Treponema denticola, T. forsythia, A. actinomycetemcomitans, C. rectus and E. corrodens from subgingival samples of dogs with and without periodontitis [20,21,24], suggesting that these microorganisms should be regarded as members of the oral microbiota in dogs.

Since the 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.

Studies have shown that black-pigmented anaerobic bacteria isolated from animal or human periodontal lesions are phenotypically similar, particularly in oral Porphyromonas species [7,22]; however there is little information about the occurrence of oral anaerobic bacteria such as Porphyromonas spp. and Fusobacterium spp. in animals.

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 [23]. Traditional methods of bacterial isolation and identiﬁcation may limit the bacterial recovery due to the lack or absence of viable cells and growth requirements [5].

In this study, only catalase-producing P. gulae and P. macacae were observed. This enzyme might allow the bacterial colonization in gingival crevice or periodontal pocket by reducing the superoxide effect [25,26].

The production of hemagglutinins and hemolysis by P. gingivalis collaborate with the colonization of diseased sites, and its attachment on erythrocytes might be an important role in the establishment and progression of the infection [27]. Fournier et al. [5] analyzing P. gulae and P. macacae strains showed that only P. gulae strains produced erythrocytes agglutination. On the other hand, the adherence ability of P. gulae appears to be lower than P. gingivalis and it can be explained because of absence of fimbrillin a protein that influence the adherence [28].

All strains were 100% resistant to dog’s serum. The serum bactericidal effect is an important host’s defense mechanism.
responsible for the bacterial resistance, and it has been a target of several studies [32]. All tested strains were susceptible to amoxicillin, ampicillin, amoxicillin/clavulanate, cefoxitin, clindamycin, penicillin G and tetracycline. Studies have shown the presence of bacterial strains isolated from small animals, such as cats and dogs, as highly susceptible to several antimicrobials [33].

In addition, high MIC values to clarithromycin and erythromycin of *P. gulae*, *F. caninum* and *F. nucleatum* were observed, as well as, *P. macacae* resistant to clarithromycin. The bacterial resistance to macrolides has increased, particularly in those considered as resident microbiota in humans and animals. Clarithromycin and erythromycin act on the bacterial protein synthesis, however, their MIC values for anaerobic bacteria have not been yet established [18]. The resistance to macrolides observed in *P. gulae*, *F. caninum* and *F. nucleatum* strains suggests the presence of resistance-encoding genes, but it was not determined. Moreover, further studies might be needed to determine the real role that *P. gulae*, *P. macacae*, *F. caninum* and *F. nucleatum* could play in dogs’ periodontal disease.

5. Conclusions

The presence of oral *P. gulae*, *P. macacae*, *F. caninum* and *F. nucleatum* was determined in oral cavity from dogs of different breeds, and it could play an important role in dogs’ periodontal disease. All strains were susceptible to most of the antibiotics tested.
Conflict of interest statement

Authors declare no conflict of interest.

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References


