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Black-pigmented anaerobic bacteria associated with ovine periodontitis

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ABSTRACT

Periodontitis is a polymicrobial infectious disease that causes occlusion change, tooth loss, difficulty in rumination, and premature culling of animals. This study aimed to detect species of the genera *Porphyromonas* and *Prevotella* present in the periodontal pocket of sheep with lesions deeper than 5 mm (n = 14) and in the gingival sulcus of animals considered periodontally healthy (n = 20). The presence of microorganisms was evaluated by polymerase chain reaction (PCR) using specific primers for *Porphyromonas asaccharolytica*, *Porphyromonas endodontalis*, *Porphyromonas gingivalis*, *Porphyromonas gulae, Prevotella buccae, Prevotella intermedia, Prevotella loescheii, Prevotella melaninogenica, Prevotella nigrescens, Prevotella oralis,* and *Prevotella tannerae.* Prevalence and risk analysis were performed using Student's *t*-test and Spearman's correlation. Among the *Prevotella* and *Porphyromonas* species detected in the periodontal lesions of sheep, *P. melaninogenica* (85.7%), *P. buccae* (64.3%), *P. gingivalis* (50%), and *P. endodontalis* (50%) were most prevalent. *P. gingivalis* (15%) and *P. oralis* (10%) prevailed in the gingival sulcus. *P. gulae* and *P. tannerae* were not detected in the 34 samples studied. Data evaluation by *t*-test verified that occurrence of *P. asaccharolytica, P. endodontalis, P. gingivalis, P. buccae, P. intermedia, P. melalinogenica,* and *P. nigrescens* correlated with sheep periodontitis. The findings of this study will be an important contribution to research on pathogenesis of sheep periodontitis and development of its control measures.

1. Introduction

Periodontitis is a multifactorial disease caused by a complex of bacterial species that interact with host tissues and cells, causing the release of inflammatory cytokines, chemokines, and mediators, some of which lead to destruction of the periodontal structures, namely the tooth supporting tissues, alveolar bone and periodontal ligament (Holt and Ebersole, 2005).

In many countries, sheep periodontitis is considered to be one of the major reasons for premature culling of animals in flocks (Ridler and West, 2007), because the disease causes premature loosening and loss of teeth in its natural course (Spence et al., 1988). With its own epidemiological characteristics and multifactorial aetiology associated with the environment, its subgingival microbiota (Frisken et al., 1989; McCourtie et al., 1990) is compatible with that found in periodontitis of humans (Haffajee and Socransky, 1994), bovine (Döbereiner et al., 1974; Dutra et al., 1993; Borsanelli et al., 2015a,b), and other animal species (Hardam et al., 2005; Riggio et al., 2011).

The polymicrobial subgingival composition associated with destructive periodontitis is predominantly gram-negative, and differs from that found in healthy sites, where it is predominantly gram-positive (Darout, 2014). Among the putative periodontal pathogens, there are species belonging to *Porphyromonas* and *Prevotella* genera that produce black pigment which are profoundly associated with other periodontopathogens, and during dysbiosis they can induce an inflammatory response and production of virulence factors that directly result in the destruction of periodontal tissues (Holt and Ebersole, 2005; Hajishengallis, 2015).

Members of the *Porphyromonas* and *Prevotella* genera express potent virulence factors such as collagenase, proteinase, endotoxin, hemolysins, cellular invasiveness and fibroblast inhibiting factor and are commonly associated with periodontitis in humans and in several other species (Haffajee and Socransky, 1994; Hardam et al., 2005). Thus, although some aspects of the disease such as pathology, bacteriology

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and epidemiology are known, the objective composition of the microbiota associated with periodontitis in sheep still needs to be elucidated. In order to expand the knowledge about the microbial flora involved in sheep periodontitis, the present study focused on identifying species from the *Porphyromonas* and *Prevotella* genera using polymerase chain reaction (PCR) in subgingival biofilms from sheep with or without periodontitis.

2. Materials and methods

2.1. Clinical characterization of periodontitis and sample collection

The clinical status of sheep was established after intra-oral and periodontal evaluation, and the criteria laid down by the Ethics Committee on Animal Experiment (Process FOA n° 2015-00280) were considered during all stages of the study. Samples were obtained from the injured ovine periodontal pocket (n = 14) and from the gingival sulcus of animals considered periodontally healthy (n = 20). Gingival sulcus sampling of healthy animals was performed between the palatal medial edge of the third premolar and the first molar jaw tooth, and in diseased animals only those whose pockets were deeper than 5 mm as measured by probing were sampled.

The gingival sulcus or periodontal pocket material was sampled according to the procedures described by Gaetti-Jardim et al. (2012). After removal of the supra-gingival bacterial biofilm with a sterile gauze pad, samples were collected using a paper cone, which was then left undisturbed for about 60 s. The cone was then transferred to a tube containing 1.0 ml of sterile ultrapure water and stored at −80 °C until DNA extraction.

2.2. Bacterial identification by polymerase chain reaction (PCR)

Detection of each bacterial DNA sample in sterile ultrapure water was performed firstly using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, St. Louis, USA) according to manufacturer's instructions. In addition, specific primers were used to identify *Porphyromonas asaccharolytica*, *Porphyromonas endodontalis*, *Porphyromonas gingivalis*, *Porphyromonas gulae, Prevotella buccae, Prevotella intermedia, Prevotella loescheii, Prevotella melaninogenica, Prevotella nigrescens, Prevotella oralis* and *Prevotella tannerae* (Borsanelli et al., 2015b) by PCR.

Amplifications were performed in 25.0 µl volumes containing 11.9 µl water, 5 µl PCR/Mg^{2+} buffer (Boehringer Mannheim, Indianapolis, IN, USA), 1.0 µl dNTP (Pharmacia Biotech, Piscataway, NJ, USA), 0.1 µl Taq DNA polymerase (Invitrogen do Brasil, São Paulo, SP, Brazil), 0.2 µl of each primer pair (Invitrogen do Brasil), and 5.0 µl sample. This amplification was performed in a PCR thermocycler (Perkin Elmer GeneAmp PCR System 9700, Norwalk, CT, USA) programmed for one cycle at 94 °C (5 min) and 30 to 36 cycles at 94 °C (1 min). The cycle at the annealing temperature of each primer was programmed for a time ranging from 30 s to 1 min followed by 2 min at 72 °C, and a final extension of 5 min at 72 °C. The PCR amplification products were subjected to electrophoresis on 1.0% agarose gel and stained with ethidium bromide (0.5 mg/ml). DNA samples of references strains were used as positive controls (Gaetti-Jardim et al., 2012).

2.3. Statistical analysis

Data were plotted and analyzed using SPSS software. Prevalence and risk analysis were performed using Cochran and Mantel-Haenszel statistics for dichotomous variables or Pearson's Chi-square test for analysis of proportions when variables had three or more categories. Interrelations between clinical and microbiological parameters were assessed by Student's *t*-test and Spearman's correlation test. Statistical tests were performed using Bonferroni's correction with the p-value adjusted from 0.05 to 0.00357, due to detection of 11 microbial species.

Table 1
Porphyromonas and *Prevotella* species detected by PCR in periodontal pocket (n = 14) of sheep with periodontitis and gingival sulcus of healthy animals (n = 20).

Species	Periodontal pocket n (%)	Gingival sulcus n (%)	*P* Student's *T*-test	Correlaction Index (CI) Spearman
Porphyromonas asaccharolytica	6 (42.9)	0 (0)	0.0006[a]	0.55[b]
Porphyromonas endodontalis	7 (50)	1 (5)	0.0015[a]	0.52[b]
Porphyromonas gingivalis	7 (50)	3 (15)	0.0274[a]	0.38[b]
Porphyromonas gulae	0 (0)	0 (0)		
Prevotella buccae	9 (64.3)	1 (5)	0.00004[a]	0.64[b]
Prevotella intermedia	3 (21.4)	0 (0)	0.0303[a]	0.37[b]
Prevotella loescheii	1 (7.1)	1 (5)	0.8012	0.04
Prevotella melaninogenica	12 (85.7)	1 (5)	0.00000[a]	0.82[b]
Prevotella nigrescens	6 (42.9)	0 (0)	0.0006[a]	0.55[b]
Prevotella oralis	0 (0)	2 (10)	0.2351	−0.21
Prevotella tannerae	0 (0)	0 (0)		

[a] Significant values of *p* by Student's *T*-test 2 tailed.
[b] Significant values of CI by Spearman's correlation test.

3. Results

Among the black-pigmented *Porphyromonas* and *Prevotella* species detected in samples of sheep with periodontitis (n = 14), *P. melaninogenica* (85.7%), *P. buccae* (64.3%), *P. gingivalis* (50%), and *P. endodontalis* (50%) were the most predominant. In healthy sheep (n = 20), *P. gingivalis* (15%) and *P. oralis* (10%) were most commonly found. *Porphyromonas gulae* and *Prevotella tannerae* were not detected in any of the 34 samples studied (Table 1, Fig. 1).

Data evaluated by Student's *t*-test (Table 1), verified that the occurrence of *P. asaccharolytica, P. endodontalis, P. gingivalis, P. buccae, P. intermedia, P. melaninogenica,* and *P. nigrescens* was associated with sheep periodontitis. Similar results were obtained by Spearman's correlation test (Table 1).

An analysis of detection frequency of different microorganisms using the Spearman's correlation test suggests associations between members of the subgingival biofilms. *P. asaccharolytica* seemed to have synergistic associations with *P. endodontalis* (Correlation Index [CI] = 0.47), *P. gingivalis* (CI = 0.55), *P. intermedia* (CI = 0.67), *P. melaninogenica* (CI = 59) and *P. nigrescens* (CI = 0.60), whereas *P. gingivalis* established synergistic associations with *P. intermedia* (CI = 0.48), *P. melaninogenica* (CI = 0.42), and *P. nigrescens* (CI = 0.38).

Strong interactions were found between *Porphyromonas endodontalis* and *P. loescheii* (CI = 0.45), *P. melaninogenica* (CI = 0.42) and *P. nigrescens* (CI = 0.47). In the same way, a clear association was observed between *P. intermedia* and *P. loescheii* (CI = 0.36), *P. melaninogenica* (CI = 0.40), and *P. nigrescens* (CI = 0.40). *P. nigrescens* showed an association with *P. buccae* (CI = 0.38) and *P. oralis* was associated with *P. loescheii* (CI = 0.47).

Prevotella oralis showed antagonistic associations with *P. asaccharolytica* (CI = −0.12), *P. gingivalis* (CI = −0.16), *P. buccae* (CI = −0.16), *P. intermedia* (CI = −0.08), *P. melaninogenica* (CI = −0.20), and *P. nigrescens* (CI = −0.12).

4. Discussion

Sheep periodontitis is an infectious disease that affects adult animals and is characterized by gingival bleeding, gingival edema, periodontal pocket formation, accumulation of food and loosening or

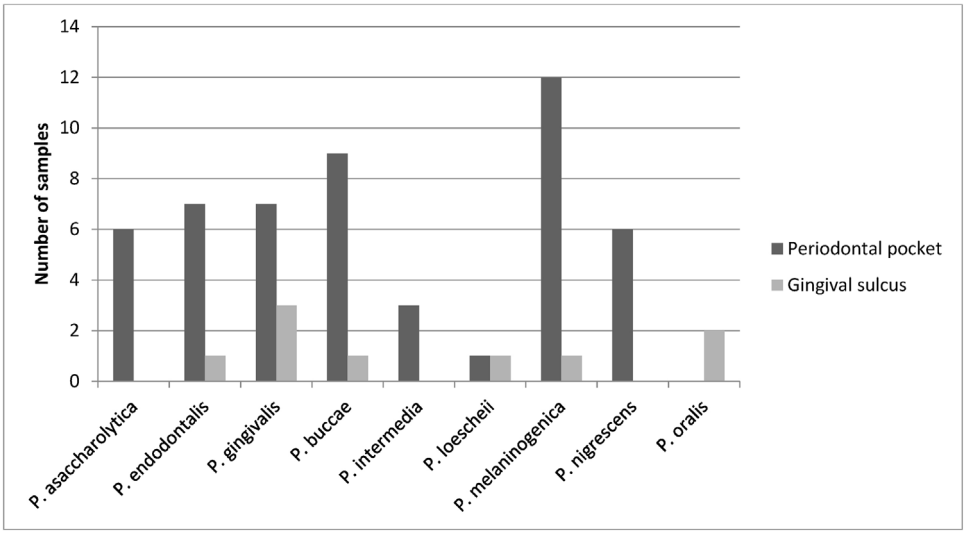

Fig. 1. Prevalence of *Porphyromonas* and *Prevotella* species identified by PCR in periodontal pocket of sheep with periodontitis and gingival sulcus of healthy animals.

loss of incisors, premolars, and molars (Spence et al., 1988). In addition to being a painful condition, it reduces the efficiency of grazing in sheep, which contributes to malnutrition, weight loss and systemic health problems (Anderson and Bulgin, 1984; Baker and Britt, 1990).

Gram-negative black-pigmented anaerobes belonging to the genera *Prevotella* and *Porphyromonas* have been commonly isolated from human patients with periodontitis, especially *P. gingivalis.* Other species such as *Porphyromonas gingivalis*, *Porphyromonas endodontalis, Prevotella melaninogenica, Prevotella intermedia, Prevotella loescheii,* and *Prevotella denticola* have been isolated from healthy sites albeit in smaller proportions (Darout, 2014). Several species of *Porphyromonas* and *Prevotella* have been identified in dogs with periodontitis (Hardam et al., 2005; Riggio et al., 2011) and in cats with periodontal disease (Booij-Vrieling et al., 2010). In other animal species, such as non-human primates, species of the two genera prevailed in individuals with periodontal inflammation and attachment loss (Gaetti-Jardim et al., 2012). *Porphyromonas* species were isolated from the oral cavity of kangaroos with different levels of periodontal disease (Mikkelsen et al., 2008) and several species of *Prevotella* were isolated from the oral cavity of donkeys (Takada et al., 2010) and some species of marsupials (Bird et al., 2002).

Botteon et al. (1993) and Dutra et al. (2000) showed the predominance of black-pigmented *Bacteroides*, that were both saccharolytic and non-saccharolytic in cultures of periodontal lesions (“cara-inchada”) from cattle. Borsanelli et al. (2015b) demonstrated that the occurrence of *P. asaccharolytica, P. endodontalis, Prevotella buccae, P. intermedia, P. melaninogenica,* and *P. oralis* was associated with bovine periodontitis using PCR.

In sheep with “broken mouth” periodontitis, Frisken et al. (1989) demonstrated the increasing incidence of species of the black-pigmented *Bacteroides*, in particular *P. gingivalis.* According to McCourtie et al. (1990) black-pigmented *Bacteroides* (*Prevotella* and *Porphyromonas*) and species of *Fusobacterim* accounted for 70% of isolates from sheep in New Zealand and *P. gingivalis* was isolated from animals with active disease. However, there are few studies involving culture-independent methods to evaluate the presence of these bacteria in the subgingival microbiota of sheep with and without periodontitis. Riggio et al. (2013) identified, by bacterial 16S rRNA gene sequencing, *Porphyromonas* sp., *P. asaccharolytica, Porphyromonas cangingivalis, Porphyromonas cansulci, P. endodontalis, Prevotella* sp. and *Prevotella salivae* in subgingival microflora of sheep with ‘broken mouth’ periodontitis but these bacteria represented a very small proportion of the total microflora.

Statistical analysis showed that the occurrence of *P. asaccharolytica, P. endodontalis, P. gingivalis, P. buccae, P. intermedia, P. melaninogenica,* and *P. nigrescens* was associated with sheep periodontitis. The results of this study reinforce the relationship between these anaerobes and the presence of periodontal inflammatory conditions, but also have some peculiarities that were not previously described, such as the presence of *P. endodontalis* and *P. asaccharolytica* in the evaluated samples.

The observed presence of *Porphyromonas gingivalis* is unusual or is observed in reduced numbers at healthy sites, being more often found in destructive forms of periodontitis (Haffajee and Socransky, 1994). Its levels increase with the depth of the periodontal pocket and are more abundant at sites with suppuration (Haffajee and Socransky, 1994). *P. gingivalis* has been considered an important pathogen in sheep with “broken mouth” periodontitis (Frisken et al., 1989; McCourtie et al., 1990) and our study also showed a strong association with periodontitis.

The levels of *Prevotella intermedia* are particularly high in certain types of periodontitis and at progressive sites of the chronic condition (Haffajee and Socransky, 1994). This is the black-pigmented species most frequently isolated from suppurative infections such as periodontal abscesses and apical periodontitis, in addition to extra-oral infections (Mättö et al., 1997; Jaramillo et al., 2005). *Prevotella intermedia* and *Prevotella nigrescens* are not distinguished by conventional methods of identification by cultivation (Ashimoto et al., 1996). In the present study, it was possible to verify that both species were associated with sheep periodontitis.

In dogs, *Prevotella buccae* is found significantly more frequently at sites of periodontitis than at healthy sites (Forsblom et al., 1997). *Prevotella melaninogenica* is commonly found in the oral cavity of healthy individuals (Könönen, 1993; Nadkarni et al., 2012) and is the most frequently identified black-pigmented species in the dental plaque of children between 7 and 9 years of age (Dahlén et al., 2012). In the current study, *P. buccae* and *P. melaninogenica* also showed association with periodontitis.

A few studies emphasize that *Porphyromonas asaccharolytica* is rarely found in the human oral microbiota and that it is not able to colonize periodontal pockets (Slots, 1979; Haffajee and Socransky, 1994; Moore and Moore, 1994). However, according to Syed et al. (1981) this microorganism predominates in the supragingival plaque of dogs with periodontitis and the subgingival and supragingival plaque of dogs with gingivitis. In our study, *P. asaccharolytica* showed strong association with sheep periodontitis and was often found in the subgingival biofilm of the evaluated animals.

The presence of *Porphyromonas endodontalis* in oral infections is rarely reported by studies based on cultivation since this microorganism rarely grows in culture (Lillo et al., 2004). Nevertheless, in our current

study, *P. endodontalis* demonstrated a strong association with periodontitis.

Species of *Porphyromonas* and *Prevotella* were selected as the focus of this study because of their notable species diversity and association with periodontal disease in several species. However, owing to the diversity of the two genera, there may be other species whose presence has not yet been identified. In the present study, *P. asaccharolytica*, *P. endondotalis*, *P. gingivalis, P. buccae*, *P. intermedia*, *P. melaninogenica,* and *P. nigrescens* showed strong association with the lesions of sheep periodontitis. Identification of species belonging to the genera *Prevotella* and *Porphyromonas* in the periodontal pockets of sheep is an original and important contribution for studies on the pathogenesis and control measures of sheep periodontitis.

These correlation data suggest the existence of ecological interactions between the microorganisms, particularly between *P. asaccharolytica* and *P. intermedia* (CI = 0.67), *P. endodontalis* and *P. nigrescens* (CI = 0.47), *P. gingivalis* and *P. asaccharolytica* (CI = 0.55), *P. buccae* and *P. melaninogenica* (CI = 0.55), and *P. melaninogenica* and *P. asaccharolytica* (CI = 0.59), although positive correlations were also observed between the other anaerobes.

Conflicts of interest statement

The authors have no conflict of interest.

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