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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