Presence of *Porphyromonas* and *Prevotella* species in the oral microflora of cattle with periodontitis[1]

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Bovine periodontitis is a progressive purulent infectious process associated with the presence of strictly and facultative anaerobic subgingival biofilm and epidemiologically related to soil management in large geographic areas of Brazil. This study aimed to detect species of the genera *Porphyromonas* and *Prevotella, which* occurr in periodontal pockets of cattle with lesions deeper than 5mm (n=26) and in gingival sulcus of animals considered periodontally healthy (n=25). Presence of the microorganisms was evaluated by independent-culture medium diagnostic method, using polymerase chain reaction (PCR) with specific primers of *Porphyromonas asaccharolytica*, *P. endodontalis, P. gingivalis, P. gulae, Prevotella buccae, P. intermedia, P. loescheii, P. melaninogenica, P. nigrescens, P. oralis* and *P. tannerae*. The species *P. endodontalis* (80.7%), *P. melaninogenica* (73.1%) and *P. intermedia* (61.5%) were the most predominant in samples of cattle with periodontitis. Regarding non-injured gingival sulcus of cattle, *P. endodontalis* (40%) and *P. loeschei* (40%) prevailed. *Porphyromonas gingivalis, P. gulae* and *Prevotella tannerae* were not detected in the 51 samples studied. Data evaluation by T test, enabled to verify that ocorrence of *Porphyromonas asaccharolytica* (p=0.000003), *P. endodontalis* (p=0.0023), *Prevotella buccae* (p=0.0017), *P. intermedia* (p=0.0020), *P. melaninogenica* (p=0.00006) and *P. oralis* (p=0.0028) is correlated with bovine periodontitis.

INDEX TERMS: *Porphyromonas* spp., *Prevotella* spp., periodontitis, cattle.

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RESUMO.- [**Presença de espécies de *Porphyromonas* e *Prevotella* na microbiota oral de bovinos com periodontite.**] A periodontite bovina é um processo infeccioso purulento e progressivo associado à presença de biofilme subgengival anaeróbio estrito e facultativo e de incidência em extensas áreas geográficas do Brasil. O trabalho teve por objetivo detectar espécies dos gêneros *Porphyromonas* e *Prevotella* presentes na bolsa periodontal de bovinos com lesões de profundidade maior que 5mm (n=26) e do sulco gengival de animais com idade de 6 a 24 meses e considerados periodontalmente sadios (n=25). A presença dos microrganismos foi avaliada pelo método independente de cultivo bacteriano, por meio da reação em cadeia da polimerase (PCR) com iniciadores específicos para *Porphyromonas asaccharolytica*, *P. endodontalis, P. gingivalis, P. gulae, Prevotella buccae, P. intermedia, P. loescheii, P. melalinogenica, P. nigrescens, P. oralis* e *P. tannerae*. *P. endodontalis*

(80,7%), *P. melalinogenica* (73,1%) e *P. intermedia* (61,5%) foram os mais prevalentes nas amostras de bovinos com periodontite. Já no sulco gengival de bovinos sem lesões prevaleceram *P. endodontalis* (40%) e *P. loeschei* (40%). *Porphyromonas gingivalis*, *P. gulae* e *Prevotella tannerae* não foram detectados nas 51 amostras pesquisadas. A partir da avaliação dos dados pelo teste T, verificou-se que a ocorrência de *Porphyromonas asaccharolytica* (p=0.000003), *P. endodontalis* (p=0.0023), *Prevotella buccae* (p=0.0017), *P. intermedia* (p=0.0020), *P. melalinogenica* (p=0.00006) e *P. oralis* (p=0.0028) está associada à periodontite bovina.

TERMOS DE INDEXAÇÃO: *Porphyromonas* spp., *Prevotella* spp., periodontite bovina.

INTRODUCTION

"Cara inchada" in cattle is a progressive, purulent periodontitis, with macroscopic and histological changes, which usually initiate in the papilla between the second and third deciduous maxillary premolars, with periodontal pocket formation. Next, food particles are accumulated, fact that worsen the process, determining injury increase, extension and depth; resulting in a chronic periostitis ossificans. With alveolar purulent process development, the tooth roots become exposed with loosening and loss of teeth (Döbereiner et al. 1974).

The occurrence of the periodontitis is associated with presence and prevalence of anaerobic Gram-negative non-sporulating bacteria forming black-pigmented colonies in culture medium with hemin and K vitamin (Blobel et al. 1984, Botteon et al. 1993, Dutra et al. 1986, 2000). The disease has great economic and sanitary impact on Brazilian livestock and shows peculiar epidemiological characteristics. Initially associated with pasture formation in extensive areas of south-eastern, central-western and northern regions of the country (Döbereiner et al. 2000), the disease reoccurs with great prevalence of its apparent clinical manifestation in herds after pasture reformation or when cattle in dentition stage were fed with forage cultivated in endemic areas (Dutra et al. 1993, Döbereiner et al. 2000).

Anaerobic bacteria are predominant in the oral microflora of humans and several animal species, highlighting the anaerobic Gram-negative producers of black pigment, which belong to the *Porphyromonas* and *Prevotella* genera, and were identified in cases of chronic periodontitis, biofilm associated gingivitis and osteomyelitis in humans (Ashimoto et al. 1996, Socransky et al. 1998, Mayanagi et al. 2004, Gaetti-Jardim Jr et al. 2010). In pet animals, these microorganisms were also identified in the oral microflora of cats with or without periodontal disease (Mallonee et al. 1988, Love et al. 1989, Love et al. 1990) as well as in periodontal pockets of dogs (Hardham et al. 2005, Nishiyama et al. 2007, Riggio et al. 2011, Senhorinho et al. 2011). The two genera are likely to predominate in lesions of bovine periodontitis (Blobel et al. 1987) and in sheep with "broken mouth" (McCourtie et al. 1989, Duncan et al. 2003).

Porhyromonas and *Prevotella* genera have a wide range of virulence factors, such as collagenase production, a series of proteases, superantigens, endotoxins, fatty acids, hydrogen sulfide, ammonium, NH_3, H_2S, cytolysins and hemolysins that collaborate to the destruction of periodontal tissues (Haffajee & Socransky 1994, Deshpande & Khan 1999, Holt & Ebersole 2005), that occurs in a significantly elevated pace in ungulate species when compared to pet animals and humans. Thus, although there is awareness of several aspects of pathology, bacteriology and epidemiology that corroborates the infectious etiology, the disease etiopathogenesis and detailed composition of the microbial flora associated with "cara inchada" are aspects further to be elucidated. Aiming to expand the knowledge about the microbial flora involved in bovine periodontitis, the present study focused on identifying species of *Porphyromonas* and *Prevotella* genera using polymerase chain reaction (PCR) in samples of bovine subgingival biofilm with or without periodontitis.

MATERIALS AND METHODS

Clinical characterization of periodontitis and sample collection. The clinical status of 6 to 24-month-old cattle was established after intra-oral and periodontal examination, considering during all stages the Ethics Committee on Animal Experiment criteria (Process FOA nº 2013-01402). Periodontal lesions were identified by the same indicators proposed by Döbereiner et al. (1974), which consist of some visible dental aspects. We performed oral examination after animal containment with the aid of a mouth opener and a probe to measure the depth of periodontal pockets.

Samples were obtained from periodontal pocket of injured cattle (n=26) and from gingival sulcus of cattle considered periodontally healthy (n=25). The first were collected from animals raised in farms considered endemic and the second were taken from cattle reared in areas harmless for the disease. The gingival sulcus collection was carried out between the palatal medial portion of the second and the third premolar. In animals with injuries, only pockets with probing depth deeper than 5 mm were collected. In both groups the samples were collected with sterilized paper point, according to the procedures described by Gaetti-Jardim Jr et al. (2012).

The examined cattle were categorized according to the following characteristics: (1) presence or absence of recession of gums, (2) destruction of supporting tissues characterized by the existence of periodontal pockets (measured by periodontal probe), and (3) the presence or absence of halitosis, as reported by Döbereiner et al. (1974). Twenty-six animals with periodontitis had gingival recession and consequently bone loss (periodontal pockets); therefore, it was used as an indicator of periodontitis in the statistical analysis.

When needed, samples of periodontal pocket were gathered after food removal, and the sampling procedures for collection of gingival sulcus or periodontal pocket material were performed as described by Gaetti-Jardim Jr et al. (2012).

Bacterial identification by polymerase chain reaction (PCR). Each sample for bacterial DNA detection in sterile ultrapure water was priory performed by commercial DNA extraction kit (GenElute Mammalian Genomic DNA Miniprep Kit, Sigma). The presence of *Porphyromonas asaccharolytica, P. endodontalis, P. gingivalis, P. gulae, Prevotella buccae, P. intermedia, P. loescheii, P. melaninogenica, P. nigrescens, P. oralis* and *P. tannerae* was evaluated with the use of specific primers and conditions of DNA amplification (Table 1).

Amplifications were performed in 25µl volumes containing 11.9µl water for PCR, 5µl PCR/Mg^{++}buffer (Boehringer Mannheim, Indianapolis, IN, USA), 1µl dNTP (Pharmacia Biotech, Piscataway, NJ, USA), 0.1µl Taq DNA polymerase (Invitrogen do Brasil, São Paulo, SP, Brazil), 0.2µl of each primer pair (Invitrogen do Brasil)

Table 1. Polymerase chain reaction (PCR) primers used to identify Porphyromonas and Prevotella species within subgingival microflora of cattle with periodontitis and healthy sites of animals without clinical evidence of the disease

Porphyromonas/Prevotella species	Primers (5´- 3´)	Annealing temperature	Primers references
Porphyromonas asaccharolytica	CTC-TAG-CTA-GAG-TGT-ACT-GG ATA-GGG-TTT-ATA-GAT-TAG-CTC-TCT	60°C	Tran et al. 1997
Porphyromonas endodontalis	GCT-GCA-GCT-CAA-CTG-TAG-TC CCG-CTT-CAT-GTC-ACC-ATG-TC	60°C	Fouad et al. 2002
Porphyromonas gingivalis	AGG-CAG-CTT-GCC-ATA-CTG-CG CTG-TTA-GCA-ACT-ACC-GAT-GT	60°C	Ashimoto et al. 1996
Porphyromonas gulae	TTG-CTT-GGT-TGC-ATG-ATC-GGG-CTT-ATT-CT TAC-GGT-ACA-TTC-ACA	60°C	Kato et al. 2011
Prevotella buccae	TCC-TCC-TTT-GAA-GGC-ATC-TGAGTT-GGG-CCG-CTG-CTT-TT	60°C	Nadkarni et al. 2012
Prevotella intermedia	CGT-GGA-CCA-AAG-ATT-CAT-CGG-TCTT-TAC-TCC-CCA-ACA-AAA-GCA	55°C	Ashimoto et al. 1996
Prevotella loescheii	TGC-CAA-CTC-CCG-ATT-TCTAC-ACC-AAG-GTT-TTC-CCC	58°C	Nadkarni et al. 2012
Prevotella melaninogenica	CGT-CAT-GAA-GGA-GAT-TGGATA-GAA-CCG-TCA-ACG-CTC	59°C	Nadkarni et al. 2012
Prevotella nigrescens	ATG-AAA-CAA-AGG-TTT-TCC-GGT-AAGCCC-ACG-TCT-CTG-TGG-GCT-GCG-A	55°C	Ashimoto et al. 1996
Prevotella oralis	TTC-CCA-TTA-CTA-CGG-CAT-ACC-CCCG-CCT-GCT-TAC-TGC-GTA-C	60°C	Nadkarni et al. 2012
Prevotella tannerae	CTT-AGC-TTG-CTA-AGT-ATG-CCGAGC-TGA-CTT-ATA-CTC-CCG	60°C	Mayanagi et al. 2004

and 5µl of the sample. This amplification was performed in a PCR apparatus (Perkin Elmer GeneAmp PCR System 9700, Norwalk, CT, USA) programmed for one cycle at 94°C (5min), and 30 to 36 cycles at 94°C (1min). The annealing temperature of each primer was programmed for a time ranging from 30 seconds to 1 minute, 2min at 72°C and a final extension of 5min at 72°C. PCR amplification products were subjected to electrophoresis on 1% agarose gel and staining with ethidium bromide (0.5mg/ml). As positive controls were used DNA samples of reference strains (Gaetti-Jardim Jr et al. 2012).

Statistical analysis. Data were plotted and analyzed using SPSS software. Prevalence and risk analysis was performed using Cochran and Mantel-Haenszel statistics for dichotomous variable or Pearson´s Chi-Square test for analysis of proportions when variables had 3 or more categories. Interrelations between clinical and microbiological parameters were assessed by Student´s T test and Spearman Correlation Test. Statistical tests were carried out using Bonferroni correction with p-value adjusted from 0.05 to 0.00357, due to detection of 8 microbial species.

RESULTS

Among black-pigmented *Porphyronomas* and *Prevotella* detected in samples of cattle with periodontitis, *P. endodontalis* (80.7%), *P. melaninogenica* (73.1%) and *P. intermedia* (61.5%) were the most predominant. Regarding non-injured cattle (n=25) *P. endodontalis* (40%) and *P. loeschei* (40%) prevailed. *Porphyromonas gingivalis*, *P. gulae* and *Prevotella tannerae* were not detected in the 51 samples studied (Table 2, Fig.1).

Table 2. Porphyromonas and Prevotella species detected by PCR in periodontal pocket (n=26) of cattle with periodontitis and gingival sulcus of healthy animals (n= 25)

Species	Periodontal pocket n (%)	Gingival sulcus n (%)	*P*
Porphyromonas asaccharolytica	14 (53,8)	0 (0.0)	0,000003*
Porphyromonas endodontalis	21(80,7)	10 (40,0)	0,0023*
Porphyromonas gingivalis	0 (0.0)	0 (0.0)	
Porphyromonas gulae	0 (0.0)	0 (0.0)	
Prevotella buccae	12 (46,1)	2 (8,0)	0,0017*
Prevotella intermedia	16 (61,5)	5 (20,0)	0,0020*
Prevotella loescheii	14 (53,8)	10 (40,0)	0,33
Prevotella melaninogenica	19 (73,1)	5 (20,0)	0,00006*
Prevotella nigrescens	11 (42,3)	2 (8,0)	0,0042
Prevotella oralis	13 (50,0)	3 (12,0)	0,0028*
Prevotella tannerae	0 (0.0)	0 (0.0)	

* Significant values of p by Student's T test.

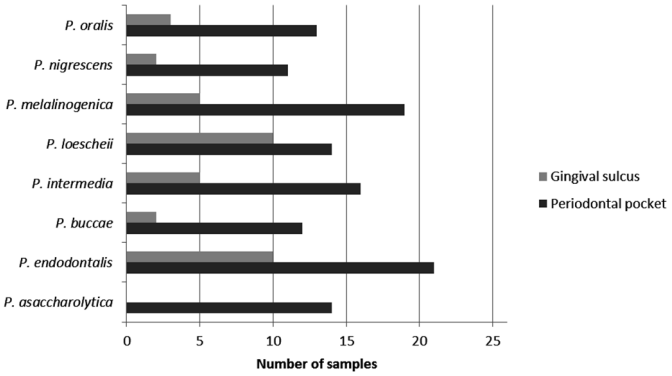

Fig.1. Prevalence of *Porphyromonas* and *Prevotella* species identified by PCR in periodontal pocket of cattle with periodontitis and gingival sulcus of periodontally healthy animals.

Table 2 displays that the presence of *P. asaccharolytica*, *P. endodontalis, Prevotella buccae*, *P. intermedia*, *P. melaninogenica* and *P. oralis* is linked to bone loss. Evaluating other characteristics of the animals, it was verified that *P. asaccharolytica* (p=0.0000001), *P. buccae* (p=0.0000001), *P. melaninogenica* (p=0.0018) and *P. oralis* (p=0.0038) were more prevailing in animals with halitosis.

By Spearman correlation test, a strong positive association between presence of *P. asaccharolytica* and *P. buccae* (Correlation Index - CI varying from 0.663 to 0.76) was observed, followed by *P. intermedia*, *P. melaninogenica*, *P. oralis* and *P. nigrescens* (CI of 0.313 to 0.426).

The correlation data suggest the existence of ecological interaction between tested microorganisms, particularity between *P. asaccharolytica* and *P. buccae* (CI=0.507), *P. endodontalis* and *P. melaninogenica* (CI=0.435), *P. intermedia* and *P. melaninogenica* (IC=0.408), *P. loescheii* and *P. nigrescens* (CI=0.440), *P. oralis* and *P. buccae* (IC=0.436), although positive correlation has been observed between other anaerobes studied.

DISCUSSION

Periodontal breakdown in humans is strongly associated with dysbiosis of the periodontal microbiota, producing relevant changes in the relative abundance of individual components of the dental biofilm and modification in host-microbe relationship sufficient to mediate destructive inflammation and bone loss, which are linked to presence

of black pigmented anaerobes, *P. gingivalis* in particular (Hajishengallis 2014, Hajishengallis 2015, Amaliya et al. 2015). Other species such as *P. intermedia*, *P. melaninogenica* and *P. loescheii* have been isolated from healthy periodontal sites and their populations and occurrence are significantly higher in patients and periodontal sites presenting conjunctive attachment loss (Darout 2014, Dahlén et al. 2014, Amaliya et al. 2015). Similar results have been described in other host species, as in cats (Pérez-Salcedo et al. 2015) and in dogs (Hardham et al. 2005, Nishiyama et al. 2007, Riggio et al. 2011, Senhorinho et al. 2011).

Bovine periodontitis occurs under specific epidemiological conditions and is predominantly associated with presence of anaerobic bacterial microflora in subgingival biofilm, especially by black-pigmented *Bacteroides*, *Fusobacterium* and other microorganisms (Döbereiner et al. 2000, Dutra et al. 2000). Despite of scarce data regarding microbiological aspects of this disease, it was observed through culture method, that the black-pigment species constitute 80% of subgingival microflora in diseased calves (Botteon et al. 1993, Dutra et al. 2000), although distribution of different species has not been widely evaluated yet.

Dutra et al. (2000) observed that while periodontal pockets in calves with periodontitis contained black-pigment average percentage of 71.3% of total microflora cultivated in anaerobic conditions and specific culture method, the same animals showed only 1.7% after the vanishing of inflammation clinical symptomatology, suggesting that these microorganisms can be more prevailing and abundant during the most active disease period, by the time conjunctive attachment loss occurs, as observed in humans.

Different species of *Porphyromonas* and *Prevotella* were identified in dogs with periodontitis; among them were *Porphyromonas gingivalis*, *P. endodontalis, P. gulae, P. cangingivalis*, *P. denticanis, P. salivosa, Prevotella intermedia* (Hardham et al. 2005, Nishiyama et al. 2007, Riggio et al. 2011, Senhorinho et al. 2011), while *Porphyromonas* spp., *P. gulae* and *Tannerella forsythia* (Booij-Vrieling et al. 2010) were identified in cats with periodontal disease. In other animal species, like non-human primates, the species of the two genera prevailed and in individuals with periodontal inflammation and attachment loss (Holt & Ebersole 2005). *Porphyromonas gingivalis* and *P. gulae* were also isolated from oral cavities of kangaroos suffering from periodontal disease (Mikkelsen et al. 2008). Moreover, various species of *Prevotella* were isolated from oral cavity of donkeys, such as *Prevotella dentasini*, *P. denticola*, *P. intermedia*, *P. loescheii*, *P. melaninogenica* and *P. nigrescens* (Takada et al. 2010). Additionally, *P. gingivalis* and *P. intermedia* were frequently observed in sheep with periodontitis (Duncan et al. 2003), as well as *P. asaccharolytica* and *P. buccae* (McCourtie et al. 1989).

Statistical analysis enabled to verify that the occurrence of *Porphyromonas asaccharolytica*, *P. endodontalis, Prevotella buccae*, *P. intermedia*, *P. melaninogenica* and *P. oralis* is associated with bone loss and as consequence to bovine periodontitis. Our results reinforce the relationship between these anaerobes and the inflammatory periodontal conditions, but also present few peculiarities not yet described, like absence of *P. gingivalis* in evaluated samples. However, other species of this genus and of the entire pigmented group were very frequent, suggesting, that the ecological niche which this species plays in humans is being occupied by other microorganisms in cattle, such as *P. asacharolytica* and *P. endodontalis* (Table 2).

The presence of *Porphyromonas endodontalis* in oral infections is unusually reported by studies based on cultivation, once this microorganism rarely develops in culture mediums (Lillo et al. 2004). Nevertheless, in this study, using PCR techniques, *P. endodontalis* demonstrated a substantial association with periodontitis.

Prevotella intermedia is the second pigmented to receive considerable interest in human periodontitis. The levels of this rod are particularly high in certain types of periodontitis and in progressive sites of the chronic type (Socransky & Haffajee 2010). *P. intermedia* is the black-pigmented most frequently isolated from suppurative infections such as periodontal abscesses and apical periodontitis, in addition to extra-oral infection (Mättö et al. 1997, Herrera et al. 2000, Jaramillo et al. 2005). *Prevotella intermedia* and *Prevotella nigrescens* are not distinguished by conventional methods of cultivation identification (Ashimoto et al. 1996, Nishiyama et al. 2007). On the other hand, PCR use enabled to evidence that *P. intermedia* is associated with bovine periodontitis, while *P. nigrescens* did not demonstrate significant values.

Porphyromonas asaccharolytica is a black-pigmented prevailing in intestinal and urogenital tract beyond its importance in several non-oral infections. A few studies affirmed that this microorganism is rarely found in human oral microbiota and it is not able to colonize periodontal pockets (Slots 1979, Haffajee & Socransky 1994, Moore & Moore 1994, Tran et al. 1997). Distinctly, our study showed a clear association of *P. asaccharolytica* with bovine periodontitis.

Nadkarni et al. (2012) evidenced the correlation of *P. oralis* with periodontal disease and deep periodontal pockets, while *P. melaninogenica* is associated to healthy sites. In this study, both microorganisms showed great associations with bovine periodontitis.

We had selected the genera *Porphyromonas* and *Prevotella* as focus of this study because of their notable diversity in species and association with periodontal disease of several species. Nonetheless, such diversity would increase likelihood of non-identified species. In present study, *P. asaccharolytica*, *P. endondotalis*, *P. buccae*, *P. oralis*, *P. intermedia* and *P. melaninogenica* showed strong association with lesions of bovine periodontitis. The identification of the species belonging to *Prevotella* and *Porphyromonas* genera in periodontal pockets of cattle is an original and important contribution for studies on pathogenesis and control measures of bovine periodontitis.

CONCLUSIONS

This investigation suggests the etiologic role of bacteria in bovine periodontitis, probably initiating the inflammatory response and leading to attachment bone loss derived from the action of mediated factors (IL-1, TNF, prostaglan-

dins, complement, RANKL), as observed in human periodontitis (Hajishengallis 2015, Hajishengallis et al. 2015, Pandit et al. 2015).

The present results also highlight the significance of black pigmented anaerobes in the etiology of periodontal inflammation in cattle.

The presence of periodontal pathogen *Porphyromonas* and *Prevotella* species and their association with periodontal lesions corroborate the evidence of the bacterial dysbiosis effect in the infectious multifactorial etiology of bovine periodontitis.

The use of PCR allowed identification of the black pigmented species that do not grow in standard anaerobic culture media.

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