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Short communication

Molecular identification of bacteria associated with canine periodontal disease

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ABSTRACT

Periodontal disease is one of the most common diseases of adult dogs, with up to 80% of animals affected. The aetiology of the disease is poorly studied, although bacteria are known to play a major role. The purpose of this study was to identify the bacteria associated with canine gingivitis and periodontitis and to compare this with the normal oral flora. Swabs were obtained from the gingival margin of three dogs with gingivitis and three orally healthy controls, and subgingival plaque was collected from three dogs with periodontitis. Samples were subjected to routine bacterial culture. The prevalent species identified in the normal, gingivitis and periodontitis groups were uncultured bacterium (12.5% of isolates), *Bacteroides heparinolyticus*/*Pasteurella dagmatis* (10.0%) and *Actinomyces canis* (19.4%), respectively. Bacteria were also identified using culture-independent methods (16S rRNA gene sequencing) and the predominant species identified were *Pseudomonas* sp. (30.9% of clones analysed), *Porphyromonas cangingivalis* (16.1%) and *Desulfomicrobium orale* (12.0%) in the normal, gingivitis and periodontitis groups, respectively. Uncultured species accounted for 13.2%, 2.0% and 10.5%, and potentially novel species for 38.2%, 38.3% and 35.3%, of clones in the normal, gingivitis and periodontitis groups, respectively. This is the first study to use utilise culture-independent methods for the identification of bacteria associated with this disease. It is concluded that the canine oral flora in health and disease is highly diverse and also contains a high proportion of uncultured and, in particular, potentially novel species.

1. Introduction

Periodontal disease (gingivitis and periodontitis) is one of the most common infectious diseases affecting adult dogs, with up to 80% of animals of all breeds affected (Golden et al., 1982; Harvey and Emily, 1993; Harvey, 1998). The incidence of the disease increases markedly with advancing years and causes significant oral pain and suffering. Periodontal disease has been described as a multi-factorial infection (Lindhe et al., 1973), and plaque bacteria are known to be an important causative factor. Gingivitis is completely reversible and is recognised by the classic signs of halitosis, bleeding, inflammation, redness and swelling of the gingivae. Periodontitis is irreversible and attacks the deeper structures that support the teeth, permanently damaging the surrounding bone and periodontal ligament and resulting in increased periodontal pocket depth and tooth loss. The aetiology of canine periodontal disease remains unknown, although gram-negative anaerobic bacteria have been implicated in the disease (Hennet and Harvey, 1991a,b; Boyce et al., 1995).

In recent years, the use of culture-independent (bacterial 16S rRNA gene sequencing) methods has supplemented traditional culture-dependent methods to detect bacteria in

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clinical specimens. 16S rRNA gene sequencing has permitted the identification of bacteria which are uncultivable, fastidious in their growth requirements and even novel, in addition to detecting known cultivable species (Clarridge, 2004; Spratt, 2004). In the current study, the bacteria associated with canine periodontal disease, and with the normal canine oral cavity, were identified using both culture-dependent and culture-independent methods.

2. Materials and methods

2.1. Sample collection and processing

Ethical approval for the study was obtained from the Local Research Ethics Committee. Samples were classified into normal and diseased groups as follows: no gingival inflammation, no periodontal pockets (normal); gingival inflammation and/or periodontal pockets less than 3 mm in depth (gingivitis); periodontal pockets at least 4 mm in depth (periodontitis). Dental plaque was collected using sterile swabs from the gingivae of periodontally healthy dogs (three samples) and animals with gingivitis (three samples). For the periodontitis cases (three samples), subgingival plaque was collected using a sterile curette from the periodontal pocket. Swabs were placed into sterile reduced transport medium and subgingival plaque was immersed into 1 mL of fastidious anaerobe broth (FAB) and immediately sent for laboratory analysis. Each swab was then immersed into 1 mL of FAB. All samples were mixed for 30 s to remove bacteria.

2.2. Bacterial culture

Ten-fold serial dilutions (to 10^{-6}) were prepared for each sample and spiral plated onto both Columbia agar containing 7.5% (v/v) defibrinated horse blood (for aerobic culture) and fastidious anaerobe agar (FAA) (BioConnections, Wetherby, UK) containing 7.5% (v/v) defibrinated horse blood (for anaerobic culture). Columbia blood agar plates were incubated in 5% CO_2 at 37 °C, and FAA plates were incubated at 37 °C in an anaerobic chamber with an atmosphere of 85% N_2/10% CO_2/5% H_2 at 37 °C. Plates were incubated for up to seven days, and up to eight morphologically distinct colonies (visually representing the most abundant colony types) were then subcultured in order to obtain pure cultures. Bacterial isolates were identified by 16S rRNA gene sequencing as described below.

2.3. Extraction of DNA from samples

A bacterial DNA extract was prepared from each sample by digestion with 1% SDS and proteinase K (100 μg/mL) at 60 °C for 1 h, followed by boiling for 10 min. Extraction of DNA from bacterial isolates was carried out using the same method.

2.4. PCR amplification of bacterial 16S rRNA genes

Bacterial 16S rRNA genes were amplified by PCR using the universal primers 5′-CAGGCCTAACACATGCAAGTC-3′ (63f) and 5′-GGGCGGWGTGTACAAGGC-3′ (1387r) (Marchesi et al., 1998). PCR reactions were carried out in a total volume of 50 μL containing 5 μL of the extracted DNA and 45 μL of reaction mixture comprising 1× GoTaq® PCR buffer (Promega, Southampton, UK), 1.25 units of G*oTaq*® polymerase (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (New England Biolabs, Hitchin, UK), and each primer at a concentration of 0.2 μM. The PCR cycling conditions comprised an initial denaturation phase of 5 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and primer extension at 72 °C for 1.5 min, and finally a primer extension step at 72 °C for 10 min.

2.5. PCR quality control

Stringent procedures were adhered to in order to prevent contamination during the PCR process (Riggio et al., 2000). Negative and positive control reactions were included with every batch of samples being analysed. The positive control comprised a standard PCR reaction mixture containing 10 ng of *Escherichia coli* genomic DNA instead of sample, whereas the negative control contained sterile water instead of sample. PCR products (10 μL) were electrophoresed on 2% (w/v) agarose gels, stained with ethidium bromide (0.5 μg/mL) and visualised under ultraviolet light.

2.6. Cloning of 16S rRNA PCR products

PCR products were cloned into the pSC-A-amp/kan plasmid vector using the StrataClone™ PCR Cloning Kit (Stratagene) in accordance with the manufacturer's instructions.

2.7. PCR amplification of 16S rRNA gene inserts

Following cloning of the 16S rRNA gene products amplified by PCR for each sample, approximately 50 clones from each library were selected at random. The 16S rRNA gene insert from each clone was amplified by PCR with the primer pair 5′-CCCTCGAGGTCGACGGTATC-3′ (M13SIF) and 5′-CTCTAGAACTAGTGGATCCC-3′ (M13SIR). The M13SIF binding site is located 61 base pairs downstream of the M13 reverse primer-binding site, and the M13SIR binding site is located 57 base pairs upstream of the M13–20 primer-binding site, in the pSC-A-amp/kan plasmid vector.

2.8. Restriction enzyme analysis

16S rRNA gene inserts amplified by PCR were subjected to restriction enzyme analysis. Approximately 0.5 μg of each PCR product was digested in a total volume of 20 μL with 2.0 units of each of the restriction enzymes *Rsa*I and *Mnl*I (Fermentas Life Sciences, York, UK) at 37 °C for 2 h and the generated restriction fragments visualised by agarose gel electrophoresis. For each library, clones were initially sorted into groups based upon the *Rsa*I restriction digestion profiles and further discrimination was achieved by digestion of clones with *Mnl*I. Clones with identical 16S rRNA gene restriction profiles for both enzymes were assigned to