The Prevalence of Canine Oral Protozoa and Their Association with Periodontal Disease

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ABSTRACT

Periodontal disease is one of the most important health concerns for companion animals. Research into canine forms of periodontitis has focused on the identification and characterization of the bacterial communities present. However, other microorganisms are known to inhabit the oral cavity and could also influence the disease process. A novel, broad spectrum 18S PCR was developed and used, in conjunction with next-generation sequencing analyses to target the identification of protists. Trichomonas sp. and Entamoeba sp. were identified from 92 samples of canine plaque. The overall prevalence of trichomonads was 56.52% (52/92) and entamoebae was 4.34% (4/92). Next-generation sequencing of pooled healthy, gingivitis, early-stage periodontitis, and severe periodontitis samples revealed the proportion of trichomonad sequences to be 3.51% (health), 2.84% (gingivitis), 6.07% (early periodontitis), and 35.04% (severe periodontitis), respectively, and entamoebae to be 0.01% (health), 0.01% (gingivitis), 0.80% (early-stage periodontitis), and 7.91% (severe periodontitis) respectively. Both genera of protists were statistically associated with plaque from dogs with periodontal disease. These findings provide the first conclusive evidence for the presence of oral protozoa in dog plaque and suggest a possible role for protozoa in the periodontal disease process.

ORAL disorders are an important causes of concern in dog health and of these, periodontal (gum) disease is the most common (Kyllar and Witter 2005). Periodontal disease results from localized inflammatory responses in the oral periodontium (soft tissues around the tooth root) upon exposure to plaque. Plaque build-up initially results in gingivitis characterized by mild inflammatory signs that are reversible. However, if left uncontrolled, gingivitis can progress to painful periodontal ligament destruction, alveolar bone loss, and eventual loss of teeth (severe periodontitis).

Plaque is composed primarily of bacterial communities; however, evidence from the human field suggests that protist organisms, such as Entamoeba gingivalis and Trichomonas tenax, are also present and correlate with periodontal disease (Athari et al. 2007; Dao et al. 1983; Decarnieri and Giannone 1964; Dudko and Kurnatowska 2007; Kurnatowska et al. 2004; Linke et al. 1989). At present it remains unclear if these protists play a key role in the aetiology of periodontal disease or if they opportunistically take advantage of the reduced health state of the mouth and consequent enhanced nutrient availability. Understanding periodontal disease at all stages of its progression still requires extensive research. In canine periodontal disease a considerable amount of research has been undertaken towards the discovery of the bacterial species interactions associated with oral health and disease (Davis et al. 2013; Dewhirst et al. 2012; Holcombe et al. 2014; Wallis et al. 2015), however, many other microorganisms may be involved in this complex pathogenesis. This study was undertaken to characterize the protozoan composition of canine oral plaque, and elucidate any associations resident protozoa may have with health and/or periodontal disease. We describe the development and use of a novel, targeted 18S PCR method in conjunction with next-generation sequencing to identify and screen for the presence of protozoa in canine gingival plaque.
MATERIAL AND METHODS

Sequence alignment and 18S primer design

Protist ribosomal small subunit DNA sequences (Table S1) were obtained from the National Institutes of Health (NIH) genetic sequence database, GenBank (Benson et al. 2014). Protist sequences for alignment were chosen based on literature analyses indicating pathogenic potential or ability to inhabit humans or animals. All sequences chosen for alignment were greater than 1,000 base pairs and represented the most complete rRNA small subunit gene for that particular organism at the time of alignment. Sequences were aligned using the VectorNTI software (Thermofisher Scientific, Life Technologies, Warrington, U.K.), using the default alignment settings. A consensus sequence was calculated and a conserved region for PCR primer design chosen manually, the goal being to encompass as wide a region of the gene as possible, and to cover as many organisms as possible within the alignment. The degenerate PCR primers identified through this process were np-SSU-570fwd-5’tgccacagcyggggttaacctc’ and np-SSU-1633-rev-5’tgtgtaNcaaggcagggacgt’ and were synthesized by Eurofins Genomics, Ebersberg, Germany. These primers allow the identification of protists present in the sample based on their PCR product size (Table S2).

Ribosomal small subunit gene PCR amplification and optimization

Amplification of the small subunit rRNA gene was performed via a touchdown PCR protocol using Platinum® Pfx DNA Polymerase kit (Thermofisher Scientific, Life Technologies), a high fidelity enzyme preparation with proofreading (3’ to 5’ exonuclease) activity. After optimization of the PCR conditions, the following PCR reaction mix was used: 5 μl Pfx amplification buffer, 5 μl PCRx Enhancer Solution, 1.5 μl deoxynucleotide triphosphates 10 mM (dNTPs—Promega Ltd, Southampton, U.K.), 1.5 μl of each primer (10 mM), 1.0 μl magnesium sulphate (50 mM), and 1 unit Platinum® Pfx DNA Polymerase. To the reaction mix, 10–50 ng of genomic DNA template was added. The final volume was brought to 50 μl using nuclease-free water (Qiagen Ltd, Manchester, U.K.). A GeneAmp PCR 9700 thermal cycler (Thermofisher Scientific, Applied Biosystems) was used to amplify the small subunit rRNA gene using the following touchdown cycle conditions: 94 °C for 2 min followed by 15 cycles of 94 °C for 45 s, 63 °C for 45 s (decreasing by 0.5 °C per cycle), and 68 °C for 2.5 min. This was followed by 20 cycles of 94 °C for 45 s, 53 °C for 45 s, and 68 °C for 2.5 min and a final extension step of 68 °C for 10 min. The reaction was then stopped by holding at 4 °C.

18S PCR screen of periodontal health state samples

Individual animal subgingival plaque samples were collected from client-owned dogs recruited in 2008/2009 from a veterinary dental referral practice (Wey Referrals, Surrey, U.K.) as part of other research projects undertaken at the WALTHAM® Centre for Pet Nutrition. The study was approved by the WALTHAM® Centre for Pet Nutrition ethical review committee and owner consent was obtained. Table S3 lists a total of 92 samples taken from periodontally healthy animals and from dogs presenting with various stages of periodontal disease; healthy gingiva (n = 20, average age 4.64 yrs), gingivitis (n = 28, average age 4.91), periodontal disease stage 1 (n = 26, average age 7.94 yrs), and severe periodontal disease stages 3–4 (n = 18, average age 10.00 yrs). Animals were sampled during treatment for other nonrelated veterinary procedures requiring anaesthesia, including extractions of fractured teeth and orthopaedic treatments or during the course of their normal treatment for early periodontal disease.

For inclusion into the healthy group (Health), clinically healthy gingiva were required with no or only low levels of localized gingivitis. Where such gingivitis was present, this was at locations away from the sampling sites. Gingivitis group samples (Gingivitis) were collected from animals displaying localized gingivitis but no attachment loss (disease stage 1). For inclusion into periodontal disease group 1 (PD1), samples were collected from animals displaying periodontal disease of at least stage 2; equivalent to less than 25% attachment loss. Severe disease group plaque samples (Severe Disease) were required to have a minimum of four sites displaying periodontal disease of at least stage 3; equivalent to between 25% and 50% or greater attachment loss. Animals below the age of two were not sampled to avoid exaggerating any age bias between healthy and diseased groups.

Samples were collected into 350 μl of 50 mM Tris (pH 7.6), 1 mM EDTA, and 0.5% v/v Tween 20 and stored immediately at –20 °C prior to DNA extraction. Genomic DNA from each sample was extracted using the Qiagen DNAeasy Blood and Tissue DNA extraction Kit (Qiagen Ltd) following the manufacturers standard protocol.

The 18S gene of organisms present in each sample was amplified using the 18S gene PCR protocol described above. The resultant amplicons produced from samples were separated through electrophoresis on standard 1.5% (w/v) agarose gels, stained with 3X GelRed (Biotium Inc., Fremont, CA) and visualized using a Kodak IS200R gel documentation system (Kodak Ltd, Watford, U.K.). Protozoan identities were determined on the basis of amplicon size according to Table S2.

Statistical analysis 18S PCR screen

For the 18S PCR screen results, the proportion of samples identified with each protist were compared between the “Health” and each subsequent group (“Gingivitis”, “PD1”, and “Severe disease”), using two-sample binomial tests with a normal approximation (GenStat v18.1, VSN International Ltd, Hemel Hempstead, U.K.). The percentage of positive samples in each health state and the difference from “Health” were calculated with 95% confidence.
intervals. A Bonferroni significance level of $p < 0.025$ was used to adjust for two protozoa being tested.

**Next-generation sequencing of pooled canine plaque samples**

Four DNA pools were created from subgingival plaque DNA, one pool representing each health state (“Health”, “Gingivitis”, “PD1”, and “Severe disease”), see Table S3. From each individual sample 1 μl of extracted genomic DNA was included in the pool. Amplification of the eukaryotic 18S genes present in each of the pools was performed using the 18S gene PCR protocol described above. Products from each reaction were purified using the QIAquick PCR Purification Kit (Qiagen Ltd), following the manufacturer’s standard protocol and 1,500 ng of DNA from each was used for sequencing using the Roche 454™ GS FLX+ platform. Library preparation and sequencing of the samples for 454™ sequencing was undertaken by Source BioScience, Nottingham, U.K. The amplicons were fragmented using Covaris Adaptive Focused Acoustics technology (Covaris Inc., Woburn, MA), a sonication based method to shear the PCR products to sizes compatible for 454™ sequencing. The sheared samples were analysed on a 2100 Bioanalyzer instrument (Agilent Technologies, Edinburgh, U.K.) to assess sample integrity and provide sample quantification. Sheared PCR products of 400–700 base pairs in size were purified for library preparation from each sample using a Pippin Prep Targeted Size Selection System (Sage Science Inc., Beverly, MA). For 454™ library preparation, each sample was processed using a NEBNext® DNA Library Prep Master Mix Set for 454™ (New England Biolabs, Hitchin, U.K.). Each library was then pooled and sequenced to target 250,000 total reads on a quarter of a plate on the Roche 454™ GS FLX+ platform.

**Analysis of Roche 454™ GS FLX+ sequencing data**

Sequences were processed for quality and analysed using QIIME (Quantitative Insights Into Microbial Ecology) software (Caporaso et al. 2010), a pipeline for performing microbial community analysis. The analysis was carried out according to the 18S sequence identification methodology outlined in QIIME tutorials and modified as follows.

To reduce the amount of erroneous OTUs and to increase the accuracy of the pipeline, the returned raw sequences were denoised in QIIME using the de-noise_wrapper.py script with default parameters. Chimeric sequences were removed from the analysis using the QIIME identify_chimeric_seqs.py script and employing Chimera Slayer as the method of chimeric sequence identification. Operational Taxonomic Units (OTUs) were picked by clustering the sequences with uclust using the pick_otus.py script and default settings (97% sequence similarity and no reverse strand matching). A representative sequence for each OTU, the most abundant sequence in each, was chosen using the pick_rep_set.py script. Each representative sequence was assigned taxonomy using the assign_taxonomy.py script using the RDP classifier (to genus level, 97%) employing the Silva 104 taxonomic map and reference sequences (Quast et al. 2013: http://www.arb-silva.de/download/archive/qiime/).

**Statistical analysis of 454™ GS FLX+ sequencing data**

For each protozoa, the proportion of sequences identified within the “Health” pool sample was compared to those found in “Gingivitis”, “PD1”, and “Severe Disease” pool samples using two-sample binomial tests with a normal approximation. The proportion of positive samples for each protist identified for each health state and the difference in proportions from “Health” to each disease group, along with 95% confidence intervals were calculated. A Bonferroni significance level of $p < 0.025$ was used to adjust for two protozoa being tested.

**RESULTS**

**Ribosomal small subunit gene PCR specificity and sensitivity**

The novel PCR protocol was tested using extracted genomic DNA samples and was found to successfully amplify small subunit PCR products from a wide range of protist type strains and environmental isolates (Table S2). As little as 3.2 pg of protist genomic DNA template per PCR and as few as 20 cells of a single type in individual and mixed cell conditions were detectable (N. Patel., unpubl. data). Table S2 lists the small sub unit amplicon sizes produced by this PCR from the species tested. DNA preparations from canine oral bacteria isolates were tested and found to not produce an amplicon using this PCR protocol (N. Patel., unpubl. data).

**Detection of protozoa in canine plaque—novel 18S PCR**

Based on 18S PCR amplicon size, two distinct protists were identified as being present in canine plaque. An amplicon of approximately 950 bp was identified as representing Trichomonas sp. and was observed in 20% of healthy dogs, 61% of dogs with gingivitis, 73% of dogs with periodontal disease stage 1, and 67% of dogs with severe periodontal disease (Fig. 1). Entamoeba sp. gave rise to an 18S PCR amplicon of approximately 1,250 bp and this was identified in 22% of severe disease samples but was not seen in any of the other sample types (Fig. 1). The overall prevalence of trichomonads and entamoebae detected in the total sample population was 56.52% (52/92) and 4.34% (4/92) respectively.

Two-sample binomial tests for Trichomonas sp. showed statistically significant differences ($p < 0.025$) between the “Health” and “Gingivitis” groups ($p = 0.005$), the “Health” and “PD1” groups ($p < 0.001$), and the “Health” and “Severe Disease” groups ($p = 0.004$) (Fig. 1).

Entamoebae comparisons (Fig. 1) were calculated for each “Health” versus disease state group and showed a
difference between the “Health” group and the “Severe Disease” group, but this was nonsignificant after Bonferroni adjustment (p = 0.026). No significant differences were found between any of the other groups and “Health”.

Detection of protozoa in canine plaque—next-generation sequencing

The four pooled plaque samples resulted in a total of 34,207 reads after denoising and chimeric sequence removal. The number of reads per sample ranged from 7,265 to 11,574, with a median read length of 7,941 bases across all four samples (Table 1). OTU sequence numbers identified by the QIIME pipeline as protozoan in origin, and their abundance within each sample type, are shown in Fig. 2. Unassignable, nonrelevant (i.e. canine, yeast or fungal) and ambiguous sequences were excluded.

Two distinct protist genera were identified in the canine pooled samples. 18S protozoan sequences belonging to the genus *Entamoeba* made up 0.01% of the “Health” pool sample, 0.01% of the “Gingivitis” pool sample, 0.80% of the “PD1” pool, and 7.91% of the “Severe Disease” pool sample (Fig. 2). 18S protozoan sequences belonging to the genus *Trichomonas* were 3.51% of the “Health” pool sample, 2.84% of the “Gingivitis” pool sample, 6.07% of the “PD1” pool, and 35.04% of the “Severe Disease” pool (Fig. 2).

Statistical analysis of the 454 sequences showed a significant difference between the proportion of sequences identified as *Trichomonas* sp. when comparing the “Health” pool to the “PD1” and “Severe Disease” pool samples (Table 2). No statistical difference was seen between the “Health” sample and the “Gingivitis” sample. A significant difference was also observed when comparing the proportion of sequences identified as *Entamoeba* sp. in the “Health” group to those in the “PD1” and “Severe Disease” groups (Table 2). No statistically significant difference in *Entamoeba* sp. sequences was seen between the “Health” sample and the “Gingivitis” sample.

**DISCUSSION**

The aim of this study was to investigate the presence of protists in canine dental plaque and to determine any
 associations with canine periodontal disease. A novel, broad spectrum 18S PCR was developed to target the identification of protists and was used in conjunction with next-generation sequencing to characterize the protist content of canine subgingival plaque. Both techniques reveal for the first time the presence of trichomonads and entamoebae in the dental plaque of dogs. The overall prevalence of each genus in canine plaque was 56.52% (52/92) and 4.34% (4/92) respectively. Binomial statistical analysis of the PCR detections and next-generation sequencing data concluded both genera of protozoa were more likely to be associated with periodontal disease samples.

There is a growing body of evidence indicating the association of human oral protists and their potential contribution to periodontal disease (Athari et al. 2007; Decarneri and Giannone 1964; Dudko and Kurnatowska 2007; Linke et al. 1989; Marty et al. 2015; Ribeiro et al. 2015). In this study, trichomonads were found to be widely distributed in canine dental plaque. Trichomonad PCR positive samples and small subunit rRNA sequences were detected in all sample health groups, their prevalence increasing as the disease severity increased. Further investigation is required to discover if they are important to the disease progression or simply proliferate in numbers due to a change to more favourable conditions. In the human mouth, trichomonads are commonly detected with prevalence rates varying between 12.7% and 37%, showing associations to periodontal disease (Athari et al. 2007; Cuevas et al. 2008; Dudko and Kurnatowska 2007). Several recent studies have also indicated the parasitic potential of oral trichomonads, and question their prior description as commensal organisms (El Sibaei et al. 2012; Ribeiro et al. 2015; Yamamoto et al. 2000).

In contrast to trichomonads, entamoebae PCR positive samples and small subunit rRNA sequences were found principally in the later stage disease canine plaque.

| Table 1. Total number of 454 reads and percentage Trichomonad (a) and Entamoebae (b) 454 sequence reads, with 95% confidence interval values, in each categorized pool sample |
|---------------------------------|-----------------|-----------------|-----------------|
| (a) Trichomonad 454 reads       | Total number of 454 reads (%) | Trichomonad reads (%) | 95% Confidence intervals |
| “Health” pool                   | 7,265            | 3.50            | 3.09             | 3.95             |
| “Gingivitis” pool               | 6,750            | 2.84            | 2.46             | 3.26             |
| “PD1” pool                      | 8,618            | 6.06            | 5.57             | 6.59             |
| “Severe Disease” pool           | 11,574           | 35.03           | 34.17            | 35.91            |
| (b) Entamoebae 454 reads        | Total number of 454 reads (%) | Entamoebae reads (%) | 95% Confidence intervals |
| “Health” pool                   | 7,265            | 0.013           | 0.000            | 0.077            |
| “Gingivitis” pool               | 6,750            | 0.014           | 0.000            | 0.083            |
| “PD1” pool                      | 8,618            | 0.800           | 0.624            | 1.012            |
| “Severe Disease” pool           | 11,574           | 7.914           | 7.429            | 8.421            |

| Table 2. Probability scores for Trichomonad (a) and Entamoebae (b) 454 sequence reads in each categorized pool sample |
|---------------------------------------------------------------|-----------------|-----------------|-----------------|
| Comparator Proportion of “Health” reads Proportion of comparator reads Approximate SE of difference Probability (p Value) |
| (a) Trichomonad reads                                         |                 |                 |                 |
| “Gingivitis”                                                  | 0.031           | 0.028           | 0.0029          | 0.379            |
| “PD1”                                                        | 0.031           | 0.061           | 0.0033          | < 0.001*         |
| “Severe disease”                                              | 0.031           | 0.350           | 0.0049          | < 0.001*         |
| (b) Entamoebae reads                                          |                 |                 |                 |
| “Gingivitis”                                                  | 0.00014         | 0.0001          | 0.00020         | 0.959            |
| “PD1”                                                        | 0.00014         | 0.0080          | 0.00097         | < 0.001*         |
| “Severe disease”                                              | 0.00014         | 0.0791          | 0.00251         | < 0.001*         |

Binomial statistical analysis was conducted to analyse the proportion of Trichomonads and Entamoebae “Health” sequences and compare against the proportion of sequences found in each of disease states. p values were calculated for each pair of groups using the test.

*Statistically significant differences.
samples—“PD1” and “Severe Disease”. These findings point to a stronger association between entamoebae and the progression of the mouth from a healthy to more diseased state, however, more investigation is required to separate cause and effect. Entamoebae prevalence in human plaque varies between 37.6% and 81% (Bonner et al. 2014; Decarneri and Giannone 1964; Trim et al. 2011), and their presence is reported to be confined to gingival pockets. In our study entamoebae were more commonly found in periodontally diseased animals, and as such may prove useful as a diagnostic target for the disease process.

The findings of this study provide the first conclusive evidence for the ubiquitous presence of canine oral protozoa in dog plaque and suggest a possible role for protists in the periodontal disease process. Over recent decades, researchers have predominantly investigated the presence and functions of bacterial communities in both human and other mammalian mouths along with their contribution to periodontal disease. It is thought that the disease results as a consequence of the complex interaction between bacterial species and the host immune system (Genco and Slots 1984), however, our finding indicate that protists may also be involved in this process.

Culture, species identification and investigation into the functional activity of the protists found in this study will be required to understand their biology and their relation to canine periodontal disease. These studies and the identification of other protist species may identify novel targets for therapies and contribute to intervention strategies that have the potential to help prevent or control the damage resulting from this common disease.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. NCBI GenBank (Benson et al., 2013) protozoan sequences chosen for sequence alignment during small subunit rRNA PCR primer design.

Table S2. Small subunit RNA gene amplon sizes produced from protozoa and other eukaryotic organisms using the PCR developed in this study.

Table S3. Metadata associated with canine subgingival plaque collections collected from animals presenting with severe periodontal disease (stages 3–4), periodontal disease stage 1, gingivitis, or from healthy animals.

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