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Characterization of the oral microbiota of healthy cats using next-generation sequencing

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ABSTRACT

The healthy feline oral cavity harbours a rich assemblage of microorganisms, which have not previously been well characterized using modern sequencing technology. The goal of this study was to accurately describe the oral microbiota of 11 healthy cats using next-generation sequencing.

Sequencing generated a total of 10,177 operational taxonomic units, representing 273 genera from 18 bacterial phyla. Eight bacterial phyla made up 97.6% of sequences: Proteobacteria (75.2%), Bacteroidetes (9.3%), Firmicutes (6.7%), SR1 (2.7%), Spirochaetes (1.8%), Fusobacteria (1.3%), and Actinobacteria (0.6%). The most prevalent genus-level phylotypes were: an unclassified Pasteurellaceae (18.7%), *Moraxella* (10.9%), *Thermomonas* (6.9%), an unclassified Comamonadaceae (5.6%), *Neisseria* (4.9%), an unclassified Moraxellaceae (4.4%), and *Pasteurella* (4.3%). Results suggest that the feline oral microbiota are largely conserved between cats at the phylum level, and that the population is highly diverse, rich and even. A strong core microbiome was evident among all cats, yet significant differences in oral bacterial populations were observed across cats in each household.

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Introduction

The oral cavity is home to complex, diverse and abundant microbial populations, which play a critical role in health and disease. The microbial populations (the microbiota) at this site and their genetic composition (the microbiome) have been poorly characterized because of limitations in the methods used to characterize the uncultivable and unknown species that are likely to be present in this environment. Recent advances in next generation sequencing and bioinformatics have revolutionized the description of polymicrobial environments and characterizing these members is now feasible. This technology has been applied to study the human oral microbiome, and revealed estimates of diversity to be over an order of magnitude higher than previously recorded, and species estimates to be >19,000 phylotypes (Keijser et al., 2008).

Oral bacterial infections (e.g. periodontal disease) can be an important cause of morbidity in cats; however, there has been limited study of the feline oral microbiota. This is especially concerning given that periodontal disease is the most prevalent disease in cats (Verhaert and Van Wetter, 2004), yet it is poorly understood. Additionally, knowledge of the feline oral microbiota is important when cat bites are investigated, as the bacteriology of cat bite wounds generally reflects species from the feline oral microbiome (Love et al., 1989, 1990). Given that cats are implicated in 8% of all yearly reported animal bites in humans (Palacio et al., 2007), with an incidence of infection of approximately 50% (Goldstein, 1992), the influence of the feline oral microbiota should not be underestimated. Understanding the role of the oral microbiota in these diseases and infections will depend first on characterizing the 'normal' bacterial populations at this site. This improved understanding could provide critical insight into the pathophysiology of disease, as well as new approaches to prevent or treat oral diseases.

Current knowledge of the feline oral microbiota has been principally discovered through the cultivation of samples taken directly from the oral cavity (Mallonee et al., 1988; Love et al., 1990; Dolieslager et al., 2011) or from cat bite abscesses (Love et al., 1979, 1989; Norris and Love, 1999; Talan et al., 1999; Westling et al., 2006; Hariharan et al., 2011). Although the most frequently recovered genera in these studies are *Bacteroides*, *Fusobacterium* and *Pasteurella* (Love et al., 1979, 1990), results are likely to be hindered by the tendency of culture to overestimate the importance of species that are easily cultivated, and underestimate the fastidious organisms that grow poorly on culture media.

To date, there has been only one study characterizing the healthy feline oral microbiota using cultivation-independent methods (Dolieslager et al., 2011). This study used cloning-based

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techniques for sequencing the 16S rRNA gene of bacteria and identified *Capnocytophaga canimorsus* as the most prevalent species in the feline oral cavity, followed by uncultured bacterium, *Bergeyella* spp., and a Xanthomonadaceae bacterium. Many fastidious organisms that were identified by cloning failed to be recovered using cultivation techniques in that study. Conversely, some species that were recovered through cultivation were not identified through cloning. This suggests that cultivation-, cloning- and/or primerbias might have affected the efficiency of community characterization in the study, and given the small sample size ($n = 3$) and relatively low number of sequences that were evaluated, the oral cavity remains to be effectively characterized in healthy cats.

The objective of the current study was to characterize the resident microbial populations of the healthy feline oral cavity using next-generation, 16S rRNA gene sequencing.

Materials and methods

Sample collection

Oral samples were collected from 11 cats living in households in Guelph, Ontario, Canada. Samples were collected from two cats in the same household for five of the six households studied. Cats were clinically healthy, had been spayed or neutered, were fed commercial diets free from raw or unpasteurized meat or milk products, and had no history of dental procedures, surgery, general anesthesia, or antimicrobial exposure within the preceding 6 months. Ethical approval was obtained from the University of Guelph Animal Care Committee (eAUP 1917).

A composite oral sample was collected using sterile cotton-tipped swabs by inserting the swab into the mouth and swabbing the gums, teeth and cheeks for 10–15 s. Swabs were broken off into sterile 2 mL microfuge tubes and transported at room temperature.

DNA extraction

Genomic DNA was extracted from each sample within 24 h of collection using InstaGene Matrix (Bio-Rad Laboratories), with slight modifications to the manufacturer's 'DNA preparation from bacteria' protocol. Briefly, 200 µL of homogenized InstaGene matrix was added directly to a 2 mL microfuge tube containing the cotton swab and the collected material. Samples were spun in a vortex at medium speed for 30 s, incubated for 30 min at 56 °C and 8 min at 100 °C. Excess solution was removed from the swabs using QIAshredder spin columns (Qiagen). The resulting supernatant was transferred back into the 2 mL microfuge tube, spun in a vortex at high speed for 10 s, and centrifuged for 3 min at 12,000 g. This supernatant was transferred to a sterile microfuge tube and stored at –20 °C until further treatment.

PCR amplification of bacterial 16S rRNA gene

The V4 region of the 16S rRNA gene was amplified in a two-step PCR reaction using forward (S-D-Bact-0564-a-S-15 (5′-AYTGGGYDTAAAGNG-3′)) and reverse (S-D-Bact-0785-b-A-18 (5′-TACNVGGGTATCTAATCC-3′)) 16S primers. These primers were designed with overhang adapters: (TCGTCGGCAGCGTCAGATGTGTATAA-GAGACAG forward and GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG reverse) for annealing to Illumina primers with 8 bp identifier indices and sequencing adaptors (AATGATACGGCGACCACCGAGATCTACAC-index-TCGTCGGCAGCGTC forward and CAAGCAGAAGACGGCATACGAGAT-index-GTCTCGTGGGCTCGG reverse).

The first PCR was performed in a 48 µL reaction mixture containing 25 µL of Kapa2G Fast HotStart ReadyMix 2X (KapaBiosystems), 1 µL of 50 mM $MgCl_2$ (Invitrogen), 1.3 µL of BSA (Bio-Rad), 17.9 µL of PCR-grade H_2O, 2 µL of DNA template, and 0.4 µL of both the forward and the reverse 16S primers (100 pmol/µL).

Samples were amplified on a Mastercycler pro S thermal cycler (Eppendorf) under the following conditions: initial denaturation at 94 °C for 3 min, followed by 28 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 1 min, and elongation at 72 °C for 1 min 30 s, followed by final elongation for 72 °C for 10 min. Samples were held at 4 °C. Subsequently, 1 µL of a forward Illumina primer and 1 µL of a reverse Illumina primer were added to the PCR products and run under the following conditions: 94 °C for 3 min, followed by seven cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 40 s, and elongation at 72 °C for 1 min, with a final elongation at 72 °C for 5 min. Genomic DNA from a mixed microbial community was used as a positive control, and water was used in the place of a DNA template as a negative control.

Purification and dilution

The amplicon library was purified with Agencourt AMPure XP beads (Beckman Coulter) with slight modification to the manufacturer's protocol. Briefly, 72 µL of AMPure beads were added to 20 µL of library and incubated for 10 min at room temperature. Samples were washed twice with 80% ethanol, and eluted with 20 µL of PCR-grade H_2O. Purified samples were quantified by spectrophotometry (NanoDrop, Thermo Fisher Scientific), evaluated by electrophoresis on a 1% agarose gel, and diluted to 5 ng/µL.

Sequencing

Samples were sequenced at the University of Guelph's Advanced Analysis Centre using an Illumina MiSeq (Illumina RTA v1.17.28; MCS v2.2) with the 2 × 250 kit.

Read trimming and taxonomic assignment

Sequences were downloaded from the BaseSpace website and processed using the open-source software package, mothur (version 1.32.0; Schloss et al., 2009). Sequences with ambiguous base calls, an inappropriate length (≠240 bp), runs of homopolymers of >8 bp, and sequences corresponding to chloroplasts, mitochondria, Archeae and Eukaryotes were removed. Chimeras were detected using uchime (Edgar et al., 2011) and removed. The remaining sequences were clustered into operational taxonomic units (OTUs) at a 3% dissimilarity level and aligned with the SILVA 16S rRNA gene reference database (Quast et al., 2013). The core microbiome, those phylotypes shared among all sampled cats, was identified at the genus level.

Alpha and beta diversity

Subsampling was performed to normalize sequence numbers. OTUs were used to calculate community diversity (Shannon and Simpson diversity indices), and evenness (Shannon equitability index). An estimate of species richness was also made using CatchAll (Bunge et al., 2012) to compute parametric and non-parametric estimators of richness and return the best estimate based on goodness-of-fit and standard error. Completeness of sampling effort was evaluated using Good's coverage and rarefaction curves.

A phylip-formated distance matrix was created to describe the dissimilarity amongst the cats using the Yue and Clayton measure of dissimilarity. The distance matrix was visualized using a non-metric multidimensional scaling (NMDS) plot. The statistical significance of the observed spatial separation was determined using the analysis of molecular variance (AMOVA) statistic. $P < 0.05$ was considered statistically significant. A phylogenetic tree was also generated using the Jaccard index of dissimilarity to compare the cats, and was visualized using Dendroscope (Huson and Scornavacca, 2012).

Results

Sample population

Eleven clinically healthy cats were enrolled from six households, with two cats enrolled from each of five households. The population characteristics of these cats are presented in Table 1. None of the cats were diagnosed with oral or dental disease in the 6-month period following sampling.

Sequencing

Sequencing generated a total of 2,439,936 reads for all 11 samples, with a mean of 221,812 sequences per sample. Removing

Table 1
Characteristics of enrolled cats.

Sample	Household	Age (years)	Sex	Indoor/outdoor	Diet type
C1	House 1	4	F	Both	Wet and dry
C2	House 1	4	M	Both	Wet and dry
C3	House 2	10	M	Indoor	Dry
C4	House 3	11	M	Indoor	Dry
C5	House 3	5	M	Indoor	Dry
C6	House 4	10	F	Indoor	Dry
C7	House 5	7	M	Indoor	Wet and dry
C8	House 5	17	M	Indoor	Wet and dry
C9	House 4	10	M	Indoor	Dry
C10	House 6	3.5	M	Indoor	Wet and dry
C11	House 6	2.5	M	Indoor	Wet and dry

F, female; M, male.

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