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Novel bacterial phylotypes associated with the healthy feline oral cavity and feline chronic gingivostomatitis

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ABSTRACT

Feline chronic gingivostomatitis (FCGS) is a painful inflammatory disease of the oral cavity. Treatment options for FCGS are very limited and little is known regarding its aetiology. The aim of this study was to investigate the presence of putative novel species in the oral cavity of cats with and without FCGS. Bacterial DNA was extracted from oral swabs and identified by 16S rRNA gene sequencing. The 16S rRNA genes of 54 clones representing distinct potentially novel species were sequenced (1202–1325 base pairs). Obtained sequences were compared to the BLAST database, aligned using the ClustalW2 alignment tool and a phylogenetic tree created. Twenty-two clones (18 from control and four from FCGS samples) had a similarity of less than 97% and were considered novel. The proportion of novel phylotypes in each group was 19.6% (control) and 2.3% (FCGS). In the derived phylogenetic tree, 15 novel phylotypes clustered together and branched away from known species and phyla. This suggests the presence of a group of novel, previously unidentified bacteria that are associated with the feline oral cavity in both health and disease.

Feline chronic gingivostomatitis (FCGS) is an inflammatory disease of the oral cavity that causes pain and distress in affected cats. Treatment of this disease is challenging and full dental extraction, which is commonly used in severe cases, has a recovery success rate of only 50–60% (Hennet, 1997; Bellei et al., 2008). The disease is assumed to have a multifactorial aetiology and both viruses and bacteria have been implicated (Thompson et al., 1984; Mallonee et al., 1988; Tenorio et al., 1991; Addie et al., 2003).

To further understand the role of bacteria in the disease, it is important to compare the bacteria associated with both feline oral health and FCGS. Previous studies on the feline oral bacterial flora have generally identified only cultivable bacteria (Mallonee et al., 1988; Love et al., 1989). On the basis that approximately 50% of the human oral microflora is uncultivable (Socransky et al., 1963), our previous study focused on the identification of the oral microflora in cats using culture-independent (16S rRNA gene sequencing) methods (Dolieslager et al., 2011). For the three control and five FCGS samples examined, 158 and 253 clones were analysed by partial 16S rRNA gene sequencing (500–800 base pairs), respectively, and their identities determined by comparison with bacterial 16S rRNA gene sequences deposited in public access sequence databases. The predominant species identified in the control and FCGS groups were *Capnocytophaga canimorsus* (10.8%) and *Pasteurella multocida* (51.8%), respectively (Dolieslager et al., 2011). However, 69 (43.7%) clones from the control group were tentatively identified as novel phylotypes (sequence identities of less than 97%), compared to only 12 (4.7%) clones in the FCGS group.

The aim of the current study was to confirm, by sequencing of almost the entire 16S rRNA gene (over 1300 base pairs), which of those clones previously tentatively classified as novel phylotypes (from five FCGS and three control feline oral samples) were novel. An additional FCGS sample was also included in the analysis. Phylogenetic analysis of clones from all samples was also carried out.

Ethical approval was obtained from the Local Research Ethics Committee. Nine oral swab samples (three control, six FCGS) from the palatoglossal folds were obtained from nine privately owned cats by two veterinary surgeons, using identical sampling conditions to ensure consistency, as previously described (Dolieslager et al., 2011). Control samples (no FCGS) were collected from cats euthanised due to feline immunodeficiency virus (FIV) infection 10–30 min after euthanasia. In cats with FCGS, oral lesions ranged from mild to severe, the time since onset of disease was 2–14 months and the time since antimicrobial treatment ranged from 13–265 days. None of the control cats had received antimicrobial treatment. The signalment and results of routine virological tests performed on each of the cats are shown in Table 1.

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Table 1
Signalment and viral status of six cats with FCGS and three control cats.

Cat ID	Viral status	Age (months)	Breed	Sex
F1	ND	102	Domestic shorthair	FN
F2	FCV	164	Siamese cross	FN
F3	Negative	104	Domestic shorthair	MN
F4	FCV	164	Siamese	MN
F5	FCV	90	Domestic shorthair	MN
F6	FCV	47	Norwegian Forest	MN
H1	FIV	61	Domestic shorthair	FN
H2	FIV/FCV	96	Domestic shorthair	M
H3	FIV	35	Domestic shorthair	MN

H1–H3, control group; F1–F6, FCGS group. ND, not determined. FCV, feline calicivirus; FIV, feline immunodeficiency virus. M, male; F, female; N, neutered. Viruses were tested for as follows: feline herpesvirus-1, virus isolation and real-time PCR; FCV, virus isolation; FIV, immunofluorescent antibody test; feline leukaemia virus, p27 antigen ELISA. All cats tested negative for the presence of feline herpesvirus-1 and feline leukaemia virus.

Bacterial DNA was extracted from each swab eluate by digestion with 1% SDS and proteinase K (100 μg/mL) at 60 °C for 60 min and boiling for 10 min. Bacterial 16S rRNA genes were amplified by PCR (using primer pair 63f/1387r) and cloned into the cloning vector pSC-A-amp/kan using the StrataClone™ PCR Cloning Kit (Stratagene) as previously described (Dolieslager et al., 2011).

Following cloning of the 16S rRNA gene products amplified by PCR for each sample, approximately 50 clones from each generated library were randomly selected. The 16S rRNA gene insert from each clone was initially 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 binding site for the M13 reverse primer, and the M13SIR binding site is located 57 base pairs upstream of the M13 −20 primer binding site, within the pSC-A-amp/kan cloning vector.

In total, 158 clones from the three control samples and 304 clones from the six FCGS samples were subjected to restriction enzyme analysis with each of the restriction enzymes *Rsa*I and *Mnl*I as described previously (Dolieslager et al., 2011), resulting in the generation of distinct restriction fragment length polymorphism (RFLP) groups (control, 85; FCGS, 119).

From each RFLP group one representative clone was initially partially sequenced (500–800 base pairs) using the SequiTherm EXCEL™ II DNA Sequencing Kit (Cambio, Cambridge, UK) and IRD800-labelled 357f sequencing primer (5′-CTCCTACGGGAGGCAGCAG-3′) with the following cycling parameters: (i) initial denaturation at 95 °C for 30 s, (ii) 10 s at 95 °C, 30 s at 57 °C and 30 s at 70 °C, for 20 cycles and (iii) 10 s at 95 °C and 30 s at 70 °C for 15 cycles. The products of DNA sequencing were visualised on a LI-COR Gene ReadIR 4200S automated DNA sequencing system.

Sequence data were compared with bacterial 16S rRNA gene sequences from the EMBL and GenBank sequence databases using the advanced gapped BLAST program, version 2.1 (Altschul et al., 1997) run through the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gow/BLAST). Sequences with less than 97% identity with any known sequence from the databases were tentatively classified as potentially novel phylotypes and subjected to near full-length sequencing of the 16S rRNA gene using forward (M13SIF) and reverse (M13SIR) sequencing primers (yielding 1202–1325 base pairs of contiguous sequence).

Near full-length 16S rRNA gene sequences were aligned using the ClustalW2 multiple sequencing alignment tool and phylogenetic analysis carried out using the Molecular Evolutionary Genetics Analysis (MEGA 5) program (Tamura et al., 2011). The maximum likelihood method was used to perform phylogenetic analysis based on the Tamura–Nei model (Tamura and Nei, 1993). Chimeric sequences were searched for using Greengenes (DeSantis et al., 2006), but none were identified.

Within the FCGS samples a total of 25 clones from 17 different RFLP groups were tentatively classified as potentially novel phylotypes by partial 16S rRNA gene sequencing (sequence identity of <97% with sequences in public access sequence databases), which compared with 69 clones from 37 RFLP groups for the control samples. Sequencing of almost the entire 16S rRNA gene for all 54 clones representing potentially novel phylotypes from both sets of samples (one from each RFLP group) demonstrated that only

Table 2
Novel bacterial phylotypes identified by sequencing of the 16S rRNA gene of clones from control and FCGS samples.

Accession no.	Most closely related bacterium (novel phylotypes)	Control		FCGS	
		No. of clones sequenced (% of total) $n = 85$	No. of clones within RLFP group (clones analysed) (% of total) $n = 158$	No. of clones sequenced (% of total) $n = 119$	No. of clones within RFLP group (clones analysed) (% of total) $n = 304$
EU535726.1	Uncultured bacterium clone nbt10b01 (H1cl11, H3cl55)	2 (2.4)	2 (1.3)		
AB243853.1	*Virgibacillus halophilus* (H1cl29)	1 (1.2)	1 (0.6)		
GQ111117.1	Uncultured bacterium (H1cl32)	1 (1.2)	1 (0.6)		
DQ232854.1	Uncultured *Enterococcus* sp. (H1cl40)	1 (1.2)	1 (0.6)		
HM336282.1	Uncultured bacterium (H1cl43)	1 (1.2)	1 (0.6)		
EU681996.1	Uncultured bacterium (H1cl63)	1 (1.2)	1 (0.6)		
FJ672502.1	Uncultured bacterium (H2cl4)	1 (1.2)	3 (1.9)		
AM420030.1	Uncultured *Capnocytophaga* sp. (H3cl8, H3cl9, H3cl25, H3cl27, H3cl40, H3cl57)	6 (7.1)	10 (6.3)		
FJ669153.1	*Capnocytophaga canimorsus* (H3cl23, H3cl29, H3cl41)	3 (3.5)	9 (5.7)		
FJ960029.1	Uncultured bacterium (H3cl37)	1 (1.2)	2 (1.3)		
EU409846.1	Uncultured gamma proteobacterium (F1cl64)			1 (0.8)	1 (0.3)
AB270004.1	Uncultured rumen bacterium (F2cl10)			1 (0.8)	1 (0.3)
AB195883.1	Uncultured bacterium (F2cl20)			1 (0.8)	3 (1.0)
AM420050.1	Uncultured *Eubacterium* sp. (F3cl37)			1 (0.8)	2 (0.7)

The 18 clones from the control samples (H) and the four clones from the FCGS samples (F), which were confirmed as representing novel phylotypes, are shown. The novel phylotypes (denoted in brackets) are shown adjacent to the most closely related sequence in the database as determined by BLAST analysis.