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Identification of bacteria associated with feline chronic gingivostomatitis using culture-dependent and culture-independent methods

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Abstract

Feline chronic gingivostomatitis (FCGS) is a chronic inflammatory disease of the oral cavity that causes severe pain and distress. There are currently no specific treatment methods available and little is known regarding its aetiology, although bacteria are thought to play a major role. The purpose of this study was to identify the oral bacterial flora in normal and diseased cats. Oral swabs were obtained from the palatoglossal folds of eight cats (three normal, five FCGS) and were subjected to microbiological culture. *Pasteurella pneumotropica* and *Pasteurella multocida* subsp. *multocida* were the most prevalent species identified by culture methods in the normal and FCGS samples, respectively. Bacteria were also identified using culture-independent methods (bacterial 16S rRNA gene sequencing). For the normal samples, 158 clones were analysed and 85 clones were sequenced. *Capnocytophaga canimorsus* (10.8% of clones analysed) was the predominant species. Uncultured species accounted for 8.2% of clones analysed, and 43.7% of clones analysed represented potentially novel species. For the FCGS samples, 253 clones were analysed and 91 clones were sequenced. The predominant species was *Pasteurella multocida* subsp. *multocida* (51.8% of clones analysed). Uncultured species accounted for 8.7% of clones analysed and 4.7% of clones analysed represented potentially novel species. It is concluded that oral flora in cats with FCGS appears to be less diverse than that found in normal cats. However, *Pasteurella multocida* subsp. *multocida* is found to be significantly more prevalent in FCGS than in normal cats and consequently may be of aetiological significance in this disease.

Keywords: feline gingivostomatitis / bacteria / microbiological culture / 16S rRNA / polymerase chain reaction

1. Introduction

Feline chronic gingivostomatitis (FCGS) is a severe inflammation of the feline oral cavity that causes much pain and distress that can lead to euthanasia of affected animals (White et al., 1992; Diehl and Rosychuk, 1993; Healey et al., 2007). The syndrome presents as a proliferative and ulcerative inflammation of the oral cavity, mostly on the palatoglossal folds (often referred to as the fauces) and the buccal gingiva. Other areas that can be affected are the pharynx, tongue and lips. The palate can become inflamed at the sites of the molar and premolar teeth. Clinical signs, generally caused by the inflammation which induces pain when opening the mouth, are dysphagia, weight loss, loss of grooming behaviour, excessive salivation, pawing at the mouth and halitosis (Bonello, 2007; Bellei et al., 2008).

FCGS is the most challenging of the oral inflammatory diseases to treat and its aetiology remains unknown. Many different bacterial species, including *Prevotella* and *Porphyromonas* species associated with human periodontal disease, have been implicated in FCGS (Mallonee et al., 1988; Love et al., 1989). However, no reliable treatments or preventative measures are available for the disease.

The purpose of this study was to identify the bacteria associated with FCGS, and with a normal feline oral cavity, using both culture-dependent and culture-independent (bacterial 16S rRNA gene sequencing) methods. The strength of culture-independent methods is that as well as detecting cultivable bacteria they can also be used to identify bacteria that are uncultivable or very fastidious in their growth requirements and, in addition, identify novel species.

2. Materials and methods

2.1. Sample collection and processing

Ethical approval was obtained from the Local Research Ethics Committee. Samples were collected, using sterile swabs, from the palatoglossal folds of cats with a normal oral cavity (three samples) which had been euthanatised for reasons unrelated to the oral cavity, and from cats with FCGS (five samples). All cats were older than 18 months of age. Swabs were placed into sterile reduced transport medium and immediately sent for laboratory analysis. Each swab was immersed into 1.0 mL fastidious anaerobe broth (FAB) and mixed for 30 s to remove bacteria.

2.2. Microbiological culture

Ten-fold serial dilutions (neat to 10^{-6}) of material eluted from each swab were prepared and spiral plated onto both Columbia agar containing 7.5% (v/v) defibrinated horse blood (aerobic culture) and FAA (BioConnections, Wetherby, UK) containing 7.5% (v/v) defibrinated horse blood (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, and 5% H_2 at 37°C. Plates were incubated for up to seven days, and up to eight morphologically distinct colonies were subcultured to obtain pure cultures. Isolates were identified by 16S rRNA gene sequencing as described below.

2.3. DNA extraction

A crude bacterial DNA extract was prepared from each swab eluate by digestion with 1% SDS and proteinase K (100 ug/mL) at 60ºC for 60 min, followed by boiling for 10 min. DNA was stored at –20ºC until required. DNA was also extracted from bacterial isolates using the same method.

2.4. PCR amplification of bacterial 16S rRNA genes

Bacterial 16S rRNA genes were amplified by PCR using universal primers. The primer sequences were 5′-CAGGCCTAACACATGCAAGTC-3′ (63f) and 5′-GGGCGGWGTGTACAAGGC-3′ (1387r) (Marchesi et al., 1998). Primers were synthesised commercially (Sigma Genosys, Cambridge, UK). The 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 x GoTaq® PCR buffer (Promega, Southampton, UK) 1.25 U Go*Taq*® 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 consisted of an initial denaturation phase of 5 min at 95ºC, followed by 35 cycles of denaturation at 95ºC for 1 min, annealing at 58ºC for 1 min and primer extension at 72ºC for 1.5 min, and finally a primer extension step at 72ºC for 10 min.

2.5. PCR quality control

When performing PCR, stringent procedures were employed to prevent contamination. Negative and positive controls were included with each batch of samples being analysed. The positive control comprised a standard PCR reaction mixture containing 10 ng of *E. coli* genomic DNA instead of sample; the negative control contained sterile water instead of sample. Each PCR product (10 μL) was subjected to electrophoresis on a 2% agarose gel, and amplified DNA was detected by staining with ethidium bromide (0.5 μg/mL) and examination under ultraviolet illumination.

2.6. Cloning of 16S rRNA PCR products

PCR products were cloned into the cloning vector pSC-A-amp/kan 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 generated library were randomly selected. 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 cloning vector.

2.8. Restriction enzyme analysis

Each re-amplified 16S rRNA gene insert was 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 U of each of the restriction enzymes *Rsa*I and *Mnl*I (Fermentas Life Sciences, York, UK) at 37ºC for 1 h. Restriction fragments were visualised by agarose gel electrophoresis. For each library, clones were initially sorted into groups based upon their *Rsa*I restriction digestion profiles. Further discrimination was achieved by digestion of clones with *Mnl*I, and clones with identical restriction profiles for both enzymes were finally grouped together in distinct restriction fragment length polymorphism (RFLP) groups.

2.9. DNA sequencing

The 16S rRNA gene insert of a single representative clone from each RFLP group was sequenced. Sequencing reactions were performed 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 95ºC for 30 s; (ii) 10 s at 95ºC, 30 s at 57ºC and 30 s at 70ºC, for 20 cycles and (iii) 10 s at 95ºC and 30 s at 70ºC for 15 cycles. Formamide loading dye (6 μL) was added to each reaction mixture after thermal cycling and 1.5 μL of each reaction mixture was run on a LI-

COR Gene ReadIR 4200S automated DNA sequencing system (MWG Biotech, Milton Keynes, UK).

2.10. DNA sequence analysis

Sequence data were compiled using LI-COR Base ImagIR 4.0 software, converted to FASTA format and 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). The program was run through the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gow/BLAST). Clone sequences that demonstrated at least 97% identity with a known sequence from the database were considered to be the same species as the matching sequence with the highest score. Sequences with less than 97% identity were classified as potentially novel phylotypes.

3. Results

3.1. Culture-dependent methods

The bacterial isolates obtained by culture-dependent methods were identified by 16S rRNA gene sequencing.

The bacteria identified from the three normal samples (N1 to N3) are shown in Table 1. A total of 29 isolates were obtained and the most frequently isolated bacteria were *Pasteurella pneumotropica* (3 isolates, 10.3%) and uncultured bacterium (3 isolates, 10.3%).

The bacteria identified from the five FCGS samples (F1 to F5) are shown in Table 1. A total of 59 isolates were obtained and the most frequently isolated bacteria were *Pasteurella multocida* subsp. *multocida* (11 isolates, 18.6%), uncultured bacterium (8 isolates, 13.6%) and *Pasteurella multocida* subsp. *septica* (8 isolates, 13.6%). A further 6 isolates (10.2%) were identified as either *Pasteurella multocida* subsp. *multocida* or *Pasteurella multocida* subsp. *septica*.

3.2. Culture-independent methods

All three normal and five FCGS samples were positive for the presence of bacteria as determined by 16S rRNA PCR analysis.

In total, 158 clones were analysed and 85 clones were sequenced across the three normal samples. The bacteria identified (23 phylotypes) are grouped according to species in Table 2. The predominant species was *Capnocytophaga canimorsus* (10.8% of clones analysed). Uncultured species accounted for 13 (8.2%) of clones analysed.

In total, 253 clones were analysed and 91 clones were sequenced across the five FCGS samples. The bacteria identified (19 phylotypes) are grouped according to species in Table 2. The predominant species was *Pasteurella multocida* subsp. *multocida* (51.8% of clones analysed). Uncultured species accounted for 22 (8.7%) of clones analysed.

In the normal samples, sixty-nine (43.7%) of clones analysed represented potentially novel species (Table 3). In the FCGS samples, twelve (4.7%) of clones analysed represented potentially novel species (Table 3).

4. Discussion

FCGS is a common and debilitating disease of unknown aetiology, although bacteria are thought to play an important role in the disease process. Bacterial species which have been implicated include *Bartonella* species and Gram-negative anaerobes. Initial small-scale serological studies suggested a link between *Bartonella henselae* and FCGS (Ueno et al., 1996; Glaus et al., 1997) but other larger-scale studies utilising a combination of ELISA, Western blot immunoassay and PCR failed to find any correlation (Quimby et al., 2008; Dowers et al., 2009). Serological responses to the Gram-negative anaerobes *Actinobacillus actinomycetemcomitans*, *Bacteroides intermedius* and *Bacteroides gingivalis* have been demonstrated in cats with FCGS (Sims et al., 1990) and several *Bacteroides* species have also been isolated from the oral cavity (Love et al., 1989).

In the current study, we used molecular cloning and sequencing of bacterial 16S rRNA genes (culture-independent methods), in tandem with conventional culture-dependent methods, to identify the bacteria associated with FCGS and health. This is the first study to use such an approach in an attempt to identify the microbial flora associated with FCGS and the healthy feline oral cavity. The key finding of our study was that the proportion of *Pasteurella multocida* subsp. *multocida* was greatly increased in FCGS compared with the healthy samples, representing over half the identified microbial flora as determined by the culture-independent approach. Therefore, this species may be considered to be of aetiological importance in FCGS. *P. multocida* subsp. *multocida* is commonly found in the healthy feline oral cavity (Love et al., 1990) and is associated with cat-bite infections (Love et al., 2000). The organism is also found in feline periodontal disease, although its numbers decrease with increasing severity of the disease

(Mallonee et al., 1988). The massive overgrowth of *P. multocida* subsp. *multocida* in the FCGS samples resulted in a dramatic reduction of some bacteria found at high levels in the normal samples, most notably *C. canimorsus* and *Desulfomicrobium orale*, and this is most likely due to increased competition for nutrients.

Overall, microbial diversity was less in the FCGS samples compared to the normal group. Culture-independent methods identified 23 different phylotypes in the normal samples, compared to 19 in the FCGS group. Uncultured bacteria were found at similar levels in the normal and FCGS groups (8.2% and 8.7%, respectively). Potentially novel species were present at significantly higher levels in the normal samples than in the FCGS group (43.7% and 4.7%, respectively). The very high prevalence of potentially novel species in the normal samples is perhaps unsurprising given that this is the first study to use culture-independent methods to identify bacteria in the feline oral cavity. However, confirmation of such species as being novel would require sequencing of the entire 16S rRNA gene.

The finding that *Pasteurella multocida* subsp. *multocida* was the predominant species identified by culture-independent methods in the FCGS samples is corroborated by the culture data obtained, with 18.6% of bacterial isolates from the FCGS group being identified as this species. However, there were also some differences in the bacterial species identified by the culture-dependent and culture-independent methods used in the current study. For example, *Capnocytophaga canimorsus* was the predominant species identified by culture-independent methods in the normal samples but was not isolated by the culture methods employed. One possible reason for this is the use of standard culture media and incubation conditions, which were used to ensure that as many types of bacteria as possible were cultured. However, this approach may not have been suitable for the culture of fastidious species. This lends credence to

the suggestion that culture-independent methods should be conducted in parallel with the conventional culture methods in order to identify as many bacterial species as possible in each sample. Conversely, some bacteria were isolated by culture methods yet were not identified by culture-independent methods. This could be attributed to the phenomenon of primer bias (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998), which leads to unequal amplification of PCR products and consequent inaccuracies in the true numbers of species present within the sample.

It is concluded that a wide range of bacteria are present in the healthy feline oral cavity. However, the microbial diversity significantly decreases in cats with FCGS, in which the predominant species is *Pasteurella multocida* subsp. *multocida*. This species is associated with the normal feline oral flora but a huge increase in its prevalence in FCGS suggests that it may be an important aetiological agent of this disease.

Conflicts of interest

The authors have no conflicts of interest.

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Table 1 Bacterial species identified by 16S rRNA sequencing of isolates obtained following microbiological culture from three normal samples (N1 to N3) and five FCGS samples (F1 to F5).

Species	Normal	FCGS
	No. of isolates (% of total) *n=29*	**No. of isolates (% of total) *n=59***
Actinomyces canis	2 (6.9)	
Anaerococcus sp. / *Peptostreptococcus* sp.*	1 (3.4)	
Bacillus sp.	1 (3.4)	
Bacteroides tectus		2 (3.4)
Bergeyella sp.	1 (3.4)	
Catonella sp.	1 (3.4)	
Chryseobacterium sp.		2 (3.4)
Clostridium perfringens	1 (3.4)	
Corynebacterium felinum	1 (3.4)	
Cupriavidus basilensis		1 (1.7)
Cytophaga sp.	1 (3.4)	
Enterobacter sp.	1 (3.4)	
Enterococcus casseliflavus	1 (3.4)	
Enterococcus faecalis		1 (1.7)
Enterococcus sp.	1 (3.4)	
Eubacteriaceae[1] bacterium	1 (3.4)	
Filifactor villosus	2 (6.9)	
Gemella palaticanis		1 (1.7)
Moraxella ovis		1 (1.7)
Mycoplasma arginini	1 (3.4)	
Neisseria sp.	2 (6.9)	
Pantoea agglomerans	1 (3.4)	
Pasteurella multocida subsp. *multocida*		11 (18.6)
Pasteurella multocida subsp. *septica*	2 (6.9)	8 (13.6)
Pasteurella pneumotropica	3 (10.3)	5 (8.5)
Pasteurella sp.		1 (1.7)
Pasteurella subsp. *multocida* / *septica**		6 (10.2)
Porphyromonas sp. (oral)		1 (1.7)
Pseudomonas reactans		1 (1.7)
Pseudomonas sp.		2 (3.4)
Staphylococcus aureus		1 (1.7)
Staphylococcus sp.		1 (1.7)
Streptococcus minor	1 (3.4)	
Streptococcus sobrinus		1 (1.7)
Uncultured bacterium	3 (10.3)	8 (13.6)
Uncultured *Haemophilus* sp.	1 (3.4)	
Uncultured *Micrococcus*		1 (1.7)
Virgibacillus halophilus		4 (6.8)

[1]Family; *Unable to distinguish between two or more species, therefore grouped generically.

Table 2 Bacterial species (at least 97% identity) identified by 16S rRNA sequencing of clones from three normal control samples (N1 to N3) and five FCGS samples (F1 to F5).

Species	Normal		FCGS	
	No of clones analysed (% of total) *n=158*	**No of clones sequenced (% of total)** *n=85*	**No of clones analysed (% of total)** *n=253*	**No of clones sequenced (% of total)** *n=91*
Abiotrophia defectiva	1 (0.6)	1 (1.2)		
Advenella sp. C12 / *Pelistega europaea* / *Tetrathiobacter kashmirensis**	1 (0.6)	1 (1.2)		
Bacterium cp04.13	3 (1.9)	3 (3.5)		
Bacteroides tectus			1 (0.4)	1 (1.1)
Bergeyella sp.	11 (7.0)	7 (8.2)		
Capnocytophaga canimorsus	17 (10.8)	2 (2.4)	1 (0.4)	1 (1.1)
Capnocytophaga cynodegmi			1 (0.4)	1 (1.1)
Capnocytophaga sp.	1 (0.6)	1 (1.2)		
Citrobacter amalonaticus / *Citrobacter* sp. R3*	1 (0.6)	1 (1.2)		
Clostridium botulinum / *Clostridium sporogenes**	1 (0.6)	1 (1.2)		
Comamonas sp.	2 (1.3)	1 (1.2)		
Desulfomicrobium orale	9 (5.7)	2 (2.4)		
Fusobacterium canifelinum			4 (1.6)	4 (4.4)
Lysobacter sp.	1 (0.6)	1 (1.2)		
Moraxella ovis			3 (1.2)	2 (2.2)
Pasteurella multocida subsp. *multocida*	4 (2.5)	4 (4.7)	131 (51.8)	24 (26.4)
Pasteurella multocida subsp. *septica*	5 (3.2)	1 (1.2)	1 (0.4)	1 (1.1)
Pasteurella pneumotropica	4 (2.5)	4 (4.7)	11 (4.3)	4 (4.4)
Pasteurella sp.			1 (0.4)	1 (1.1)
Pasteurella stomatis	1 (0.6)	1 (1.2)		
Pasteurella trehalosi	1 (0.6)	1 (1.2)		
Pasteurellaceae[1] bacterium R46	1 (0.6)	1 (1.2)		
Peptococcus sp. (oral)			24 (9.5)	7 (7.7)
Peptostreptococcus sp.	1 (0.6)	1 (1.2)		
Porphyromonas cangingivalis			1 (0.4)	1 (1.1)
Porphyromonas canoris			1 (0.4)	1 (1.1)
Porphyromonas circumdentaria			3 (1.2)	2 (2.2)
Pseudomonas reactans			13 (5.1)	6 (6.6)
Pseudomonas sp.			22 (8.7)	13 (14.3)
Pseudomonas synxantha			1 (0.4)	1 (1.1)
Simonsiella sp.	2 (1.3)	2 (2.4)		
Uncultured bacterium	12 (7.6)	7 (8.2)	14 (5.5)	8 (8.8)
Uncultured *Capnocytophaga* sp.			2 (0.8)	2 (2.2)
Uncultured Prevotellaceae[1]	1 (0.6)	1 (1.2)		
Uncultured *Pseudomonas* sp.			6 (2.4)	2 (2.2)
Virgibacillus sp. / *Salibacillus* sp.*	1 (0.6)	1 (1.2)		
Xanthomonadaceae[1] bacterium	8 (5.1)	3 (3.5)		

[1]Family; *Unable to distinguish between two or more species, therefore grouped generically.

Table 3 Potentially novel bacterial species (less than 97% identity) identified by 16S rRNA sequencing of clones from three normal samples (N1 to N3) and five FCGS samples (F1 to F5).

Most closely related species	**Normal**		**FCGS**	
	No of clones analysed (% of total) *n=158*	**No of clones sequenced (% of total)** *n=85*	**No of clones analysed (% of total)** *n=253*	**No of clones sequenced (% of total)** *n=91*
Actinomyces sp.	2 (1.3)	2 (2.4)		
Bacteroides sp. XB1A			2 (0.8)	1 (1.1)
Capnocytophaga canimorsus	9 (5.7)	3 (3.5)		
Catonella sp. (oral)			1 (0.4)	1 (1.1)
Chryseobacterium sp.	1 (0.6)	1 (1.2)		
Eubacterium brachy			2 (0.8)	1 (1.1)
Mannheimia sp.	1 (0.6)	1 (1.2)		
Micromonas micros			2 (0.8)	1 (1.1)
Neisseria sp. (oral)	1 (0.6)	1 (1.2)		
Pasteurella multocida subsp. *multocida*			1 (0.4)	1 (1.1)
Pasteurella pneumotropica / *Pasteurella stomatis**			1 (0.4)	1 (1.1)
Porphyromonas sp. (oral)	10 (6.3)	5 (5.9)	1 (0.4)	1 (1.1)
Prevotella sp. (oral)	4 (2.5)	1 (1.2)		
Uncultured bacterium	16 (10.1)	10 (11.8)	2 (0.8)	2 (2.2)
Uncultured Bacteroidetes[2] bacterium	3 (1.9)	1 (1.2)		
Uncultured *Capnocytophaga* sp.	10 (6.3)	6 (7.1)		
Uncultured *Catonella* sp.	2 (1.3)	1 (1.2)		
Uncultured Firmicutes[2] bacterium	1 (0.6)	1 (1.2)		
Uncultured *Fusibacter* sp.	2 (1.3)	1 (1.2)		
Uncultured *Peptococcus* sp.	1 (0.6)	1 (1.2)		
Virgibacillus marismortui	6 (3.8)	2 (2.4)		

[2]Phylum; *Unable to distinguish between two or more species, therefore grouped generically.