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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.

Keywords: canine periodontal disease / bacteria / microbiological culture / 16S rRNA / polymerase chain reaction

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 & 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 & 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 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 ug/mL) at 60ºC 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 x GoTaq® PCR buffer (Promega, Southampton, UK) 1.25 units of 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 comprised 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

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.

145 *2.8. Restriction enzyme analysis*

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147 16S rRNA gene inserts amplified by PCR were subjected to restriction enzyme analysis.
148 Approximately 0.5 μg of each PCR product was digested in a total volume of 20 μL with 2.0
149 units of each of the restriction enzymes *Rsa*I and *Mnl*I (Fermentas Life Sciences, York, UK) at
150 37ºC for 2 h and the generated restriction fragments visualised by agarose gel electrophoresis.
151 For each library, clones were initially sorted into groups based upon the *Rsa*I restriction digestion
152 profiles and further discrimination was achieved by digestion of clones with *Mnl*I. Clones with
153 identical 16S rRNA gene restriction profiles for both enzymes were assigned to distinct
154 restriction fragment length polymorphism (RFLP) groups.

155

156 *2.9. Sequencing of bacterial 16S rRNA genes*

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158 The 16S rRNA gene insert of a single representative clone from each RFLP group was
159 sequenced. Sequencing was performed with the SequiTherm EXCEL™ II DNA Sequencing Kit
160 (Cambio, Cambridge, UK) and IRD800-labelled 357f sequencing primer (5′-
161 CTCCTACGGGAGGCAGCAG-3′) using the following cycling conditions: (i) initial
162 denaturation at 95ºC for 30 s; (ii) 10 s at 95ºC, 30 s at 57ºC and 30 s at 70ºC, for 20 cycles and
163 (iii) 10 s at 95ºC and 30 s at 70ºC for 15 cycles. Formamide loading dye (6 μL) was added to
164 each reaction mixture after thermal cycling and 1.5 μL of each reaction mixture was run on a LI-
165 COR Gene ReadIR 4200S automated DNA sequencing system

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167

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2.10. 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 with at least 98% identity with a known sequence from the database were designated the same species as the matching sequence with the highest score. Clone sequences with less than 98% identity were tentatively classified as putative novel phylotypes.

2.11. Statistical analysis

To determine whether the observed differences in the microflora between each of the three groups were of statistical significance, a cross-tabulation using Fisher's exact test was performed. A level of statistical significance was indicated by $p<0.0167$ (Bonferroni correction).

3. Results

3.1. Culture-dependent identification of bacteria

Bacterial isolates obtained following microbiological culture of samples were identified by 16S rRNA gene sequencing, and the results are shown in Table 1. All isolates had identities of at least 98% with a known database sequence. Of the 32 isolates obtained from the normal samples, the predominant bacteria identified were uncultured bacterium (4 isolates, 12.5%) and *Neisseria*

193 *weaveri* (three isolates, 9.4%). Thirty isolates were obtained from the gingivitis samples, of
194 which three (10%) were identified as *Bacteroides heparinolyticus* and three (10%) as *Pasteurella*
195 *dagmatis*. For the periodontitis samples, 36 isolates were identified and the predominant species
196 was *Actinomyces canis* (seven isolates, 19.4%).
197
198 *3.2. Culture-independent identification of bacteria*
199
200 Following 16S rRNA PCR analysis, all nine samples were shown to be positive for the
201 presence of bacteria. For the three normal samples, 152 clones were analysed and 83 clones were
202 sequenced. Bacteria with identities of at least 98% with a known database sequence are grouped
203 according to species in Table 2, with a total of 19 phylotypes being identified. The predominant
204 species was *Pseudomonas* sp. (30.9% of clones analysed). Uncultured species (three phylotypes)
205 accounted for 20 (13.2%) of clones analysed. Fifty-eight (38.2%) of clones analysed (17
206 phylotypes) represented potentially novel species (Table 3).
207 In total, 149 clones were analysed and 96 clones were sequenced across the three gingivitis
208 samples. The bacteria identified (24 phylotypes) are grouped according to species in Table 2. The
209 predominant species was *Porphyromonas cangingivalis* (16.1% of clones analysed). Uncultured
210 species (two phylotypes) accounted for 3 (2.0%) of clones analysed. Fifty-seven (38.3%) of
211 clones analysed (15 phylotypes) represented potentially novel species (Table 3).
212 Analysis of the three periodontitis samples resulted in 133 clones being analysed and 109
213 clones being sequenced. The bacteria identified (20 phylotypes) are grouped according to species
214 in Table 2. The predominant species was *Desulfomicrobium orale* (12.0% of clones analysed).
215 Uncultured species (four phylotypes) accounted for 14 (10.5%) of clones analysed. Forty-seven
216 (35.3%) of clones analysed (17 phylotypes) represented potentially novel species (Table 3).

3.3. Statistical analysis

In order to determine if the differences in the microflora observed in each of the three groups was statistically significant, a three-way comparison between groups using cross-tabulation with a Fisher's exact test was performed for the data presented in Tables 1-3.

For bacteria identified by culture-dependent methods (Table 1) statistically significant differences were observed between the gingivitis and periodontitis groups ($p=0.0090$) and the normal and periodontitis groups ($p=0.0043$). However, no statistical difference was observed between the normal and gingivitis groups ($p=0.181$).

For known bacteria identified by culture-independent methods (Table 2) statistically significant differences were observed between all three groups ($p<0.000001$).

For potentially novel bacteria identified by culture-independent methods (Table 3), statistically significant differences were observed between all three groups ($p<0.00020$).

4. Discussion

Canine periodontal disease is one of the most common infectious diseases of companion animals and is characterised by gingival inflammation and tooth loss (Hennet & Harvey, 1992; Harvey, 1998). Black pigmenting anaerobic bacteria, in particular *Porphyromonas* and *Prevotella* species, have been isolated from the periodontal pockets of dogs with periodontal disease (Watson, 1994; Gorrel and Rawlings, 1996). Isogai et al. (1999) isolated several pigmented *Porphyromonas* species from cases of canine periodontal disease. Several new *Porphyromonas* species (*Porphyromonas cangingivalis, Porphyromonas cansulci, Porphyromonas gulae, Porphyromonas creviocanis, Porphyromonas gingivacanis, Porphyromonas canoris,*

Porphyromonas denticanis) associated with the disease have also been described (Collins et al., 1994; Hirasawa and Takada, 1994; Love et al., 1994; Fournier et al., 2001; Hardham et al., 2005).

Our current study is the first to use molecular cloning and sequencing of bacterial 16S rRNA genes, in addition to conventional microbiological culture methods, to identify the bacteria associated with canine gingivitis, periodontitis and oral health. Given the relatively small number of samples analysed, it was not possible to age- and sex- match the animals used in the study, although this would be desirable in future large-scale studies. Despite the relatively small number of samples analysed in each group in our current study, clear correlations were seen to emerge between disease status and the prevalence of specific bacterial species, and differences in the microflora between the three groups was statistically significant. The most prevalent species found in the normal, gingivitis and periodontitis groups were *Pseudomonas* sp. (30.9%), *Porphyromonas cangingivalis* (16.1%) and *Desulfomicrobium orale* (12.0%), respectively. *Porphyromonas cangingivalis* was first isolated from cases of canine periodontitis (Collins et al., 1994) and *Desulfomicrobium orale* has been isolated from cases of human periodontitis (Langendijk et al., 2001). Other prevalent species identified included *Porphyromonas canoris*, *Tannerella forsythensis* and *Capnocytophaga cynodegmi* (gingivitis), and *Actinomyces* sp. and *Capnocytophaga cynodegmi* (periodontitis). The association of *Tannerella forsythensis* with human periodontal disease is well documented, *Capnocytophaga cynodegmi* is found in the oral cavity of the vast majority of dogs (van Dam et al., 2009) and *Porphyromonas canoris* has been isolated from canine dental plaque samples (Allaker et al., 1997). Culture-independent methods showed that microbial diversity was similar in all three groups (19 to 24 phylotypes). Uncultured bacteria were found at a higher proportion in the normal samples (13.2%) compared to the gingivitis (2.0%) and periodontitis (10.5%) samples. Potentially novel species were found at high proportions in all three groups (35.3% to 38.3%), a finding that is unsurprising when one

considers that this is the first study to use culture-independent methods to identify bacteria in the canine oral cavity. However, sequencing of the entire 16S rRNA gene would be required to confirm these species as being novel.

The finding that *Pseudomonas* sp., *Porphyromonas cangingivalis* and *Desulfomicrobium orale* were the predominant species identified by culture-independent methods in the normal, gingivitis and periodontitis samples, respectively, is not corroborated by the culture data obtained. However, some concordance does exist between the data since some species were detected by both identification methods (three, nine and five species in the normal, gingivitis and periodontitis samples, respectively). In addition, previously uncultured bacteria were identified by both methods in all three groups. However, many bacteria were identified by culture-independent methods but not by culture methods. A possible explanation for this anomaly is the use of standard culture media and incubation conditions, which were adopted to ensure that as many different types of bacteria as possible were cultured. However, this approach may not have been suitable for the culture of many species, particularly those with fastidious growth requirements. Consequently, we advocate that culture-independent methods should be used in conjunction with conventional culture methods in order to identify the total microflora in clinical samples. Conversely, some bacteria isolated by culture methods were not identified by culture-independent methods. The most likely explanation for this additional anomaly is PCR primer bias, which is caused by self-annealing of the most abundant templates in the late stages of amplification (Suzuki and Giovannoni, 1996) or as a result of differences in the amplification efficiency of different templates (Polz and Cavanaugh, 1998). This results in the differential amplification of PCR products, leading to an inaccurate reflection of the true numbers of species present within the sample.

In conclusion, a wide range of bacteria is present in the oral cavity of healthy dogs and those with gingivitis and periodontitis and a distinct microbial flora appears to be associated with each of the three groups. Potentially novel bacterial species may play a significant role in gingivitis and periodontitis.

Conflict of interest statement

The authors have no conflicts of interest.

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380 **Table 1** Bacterial species identified by 16S rRNA gene sequencing of isolates obtained from
381 three normal, three gingivitis and three periodontitis samples (all at least 98% identity).
382

Species	Normal	Gingivitis	Periodontitis
	No. of isolates (% of total) *n=32*	**No. of isolates (% of total) *n=30***	**No. of isolates (% of total) *n=36***
Actinomyces bowdenii	1 (3.1)		
Actinomyces canis	1 (3.1)		7 (19.4)
Actinomyces coleocanis		1 (3.3)	
Actinomyces hordeovulneris	1 (3.1)		2 (5.6)
Bacteroides heparinolyticus		3 (10.0)	
Bergeyella sp.		1 (3.3)	
Brachybacterium zhongshanense		1 (3.3)	
Brevundimonas sp.	1 (3.1)		
Buttiaxella agrestis	2 (6.3)		
Capnocytophaga canimorsus		1 (3.3)	
Capnocytophaga cynodegmi			3 (8.3)
*Capnocytophaga cynodegmi / canimorsus**			2 (5.6)
Corynebacterium lipophiloflavum		1 (3.3)	
Corynebacterium sp.		1 (3.3)	2 (5.6)
Cytophaga sp.			2 (5.6)
Filifactor vilosus		1 (3.3)	1 (2.8)
Fusobacterium alosis			1 (2.8)
Fusobacterium canifelinum	1 (3.1)		
Fusobacterium russii		1 (3.3)	
Gemella palaticanis	1 (3.1)		
*Lactobacillus casei / lactis**	1 (3.1)		
*Leucobacter chromireducens / solipictus**	1 (3.1)		
Moraxella bovoculi			2 (5.6)
Moraxella canis		1 (3.3)	
Moraxella sp.	1 (3.1)		
Neisseria canis		2 (6.7)	2 (5.6)
Neisseria weaveri	3 (9.4)	1 (3.3)	1 (2.8)
Neisseria zoodegmatis	1 (3.1)		
Pasteurella canis		1 (3.3)	1 (2.8)
Pasteurella dagmatis	1 (3.1)	3 (10.0)	1 (2.8)
Pasteurella multocida subsp. *septica*	1 (3.1)		
Pasteurella multocida subsp. *septica / multocida**	2 (6.3)		

Pasteurella stomatis	1 (3.1)		
Pasteurella trehalosi	1 (3.1)		
Porphyromonas canoris	1 (3.1)	2 (6.7)	
Propionibacteriaceae bacterium[1]		1 (3.3)	
Pseudoclavibacter sp.		2 (6.7)	
Pseudomonas aeruginosa	1 (3.1)		
Pseudomonas brenneri			2 (5.6)
Pseudomonas sp.			3 (8.3)
Pseudomonas stutzeri	1 (3.1)		
Serratia grimesii	1 (3.1)		
Serratia sp.	1 (3.1)		
Streptococcus minor	1 (3.1)		
Uncultured bacterium	4 (12.5)	2 (6.7)	3 (8.3)
Uncultured Bacteroidetes bacterium[2]			1 (2.8)
Virgibacillus halophilus	1 (3.1)		
Xanthomonadaceae bacterium[1]		2 (6.7)	
Xanthomonas sp.		1 (3.3)	
Xenophilus sp.		1 (3.3)	

*Unable to distinguish between species: [1]Family; [2]Phylum.

397 **Table 2** Bacterial species identified by 16S rRNA sequencing of clones from three normal, three

398 gingivitis and three periodontitis samples: at least 98% identity.

Species	**Normal**	**Gingivitis**	**Periodontitis**
	No. of clones analysed (% of total) *n=152*	**No. of clones analysed (% of total)** *n=149*	**No. of clones analysed (% of total)** *n=133*
Acinetobacter junii			1 (0.8)
Acinetobacter sp.	1 (0.7)		
Actinomyces hordeovulneris		1 (0.7)	
Actinomyces sp.	1 (0.7)	1 (0.7)	11 (8.3)
Bergeyella sp.	5 (3.3)	6 (4.0)	
Capnocytophaga canimorsus		1 (0.7)	2 (1.5)
Capnocytophaga cynodegmi		6 (4.0)	10 (7.5)
CDC Group NO-1		1 (0.7)	
Clostridiales bacterium (oral)[3]			6 (4.5)
Clostridium sporogenes / *botulinum**	1 (0.7)		
Desulfomicrobium orale			16 (12.0)
Filifactor villosus		4 (2.7)	
Fusobacterium russii		2 (1.3)	
Gemella palaticanis	1 (0.7)		
Klebsiella pneumoniae	1 (0.7)		
Methylobacterium radiotolerans	1 (0.7)		
Moraxella bovoculi		3 (2.0)	3 (2.3)
Moraxella canis			1 (0.8)
Moraxella nonliquefaciens	1 (0.7)		
Neisseria canis		1 (0.7)	
Orodibacter denticanis		2 (1.3)	
Pasteurella canis		5 (3.4)	4 (3.0)
Pasteurella dagmatis			5 (3.8)
Peptococcus sp. (oral)		3 (2.0)	
Peptoniphilus sp. 'Oral Taxon 386'			2 (1.5)
Peptostreptococcus sp. (oral)			5 (3.8)
Porphyromonas cangingivalis		24 (16.1)	2 (1.5)
Porphyromonas canis		1 (0.7)	
Porphyromonas canoris		8 (5.4)	1 (0.8)
Pseudomonas sp.	47 (30.9)		
Psychrobacter pulmonis	2 (1.3)		
Salibacillus sp.	1 (0.7)		
Serratia grimesii	3 (2.0)		
Serratia proteomaculans	2 (1.3)		
Serratia proteomaculans quinovora	1 (0.7)		
Simonsiella steedae	2 (1.3)		

Tannerella forsythensis		7 (4.7)	
Treponema genomosp.		2 (1.3)	
Treponema sp.		4 (2.7)	1 (0.8)
Uncultured *Acinetobacter* sp.	5 (3.3)		
Uncultured bacterium	12 (7.9)	1 (0.7)	7 (5.3)
Uncultured *Capnocytophaga* sp.			1 (0.8)
Uncultured Peptostreptococcaceae bacterium[1]			4 (3.0)
Uncultured Prevotellaceae bacterium[1]		2 (1.3)	
Uncultured *Pseudomonas* sp.	3 (2.0)		
Uncultured rumen bacterium			2 (1.5)
Virgibacillus halophilus	4 (2.6)		
Wernerella denticanis		1 (0.7)	
Xanthomonadaceae bacterium[1]		3 (2.0)	2 (1.5)
Xenophilus sp.		3 (2.0)	

*Unable to distinguish between species; [1]Family; [3]Order.

417 **Table 3** Potentially novel bacterial species identified by 16S rRNA sequencing of clones from

418 three normal, three gingivitis and three periodontitis samples: less than 98% identity.

Species [% identity range]	Normal	Gingivitis	Periodontitis
	No. of clones analysed (% of total) *n=152*	No. of clones analysed (% of total) *n=149*	No. of clones analysed (% of total) *n=133*
Actinomyces sp. [95.5-97.4]	1 (0.7)		2 (1.5)
Actinomyces hordeovulneris [96.9]	1 (0.7)		
Arthrobacter sp. [93.8]	2 (1.3)		
Bacteroides sp. [89.9]			2 (1.5)
Brachymonas sp. [96.9]		2 (1.3)	
Capnocytophaga canimorsus [96.7-97.0]		3 (2.0)	
Capnocytophaga cynodegmi [97.1]			1 (0.8)
Clostridiales bacterium (oral)[3] [85.4-97.2]		13 (8.7)	6 (4.5)
Corynebacterium pseudotuberculosis [94.6]		1 (0.7)	
Desulfomicrobium orale [96.2-97.2]			3 (2.3)
Desulfovibrio sp. [89.9]			1 (0.8)
Flexistipe-like sp. (oral) [95.7]		1 (0.7)	
Haemophilus haemoglobinophilus [96.2]	1 (0.7)		
Klebsiella sp. [97.1]	1 (0.7)		
Marine bacterium [96.6]	1 (0.7)		
Moraxella bovoculi [96.2]			1 (0.8)
Moraxella canis [97.1]			1 (0.8)
Mycobacterium sp. [95.7]	1 (0.7)		
Mycoplasma canis [91.5]		1 (0.7)	
Pasteurella sp. [97.2]			1 (0.8)
Porphyromonas cangingivalis [85.5-97.4]		4 (2.7)	1 (0.8)
Porphyromonas canoris [95.6-97.1]		2 (1.3)	2 (1.5)
Prevotella genomosp. P9 (oral) [90.2]			2 (1.5)
Pseudomonas fluorescens [93.2]	1 (0.7)		
Pseudomonas sp. [96.4-97.3]	20 (13.2)		
Psychrobacter pulmonis [94.7]	1 (0.7)		
Salibacillus sp. [96.2-96.9]	2 (1.3)		
Serratia proteomaculans quinovora [97.2]	1 (0.7)		
Simonsiella steedae [92.3-97.1]	2 (1.3)		
Tannerella forsythensis [97.3]		1 (0.7)	
Uncultured bacterium [87.7-96.8]	5 (3.3)	16 (10.7)	18 (13.5)
Uncultured beta-proteobacterium [94.0]	2 (1.3)		
Uncultured Lachnospiraceae (oral)[1] [94.6]		1 (0.7)	
Uncultured *Lautropia* sp. (oral) [97.2-97.4]		5 (3.4)	
Uncultured Moraxellaceae bacterium (oral)[1] [96.4]			1 (0.8)

Uncultured *Peptococcus* sp. (oral) [96.9]		1 (0.7)	
Uncultured *Porphyromonas* sp. (oral) [93.2-93.6]			3 (2.3)
Uncultured *Prevotella* sp. (oral) [80.6]			1 (0.8)
Uncultured rumen bacterium [88.5]			1 (0.8)
Uncultured γ-proteobacterium [94.1]		1 (0.7)	
Virgibacillus marismortui [95.7-97.0]	13 (8.6)		
Virgibacillus sp. [96.0]	3 (2.0)		
Xanthomonadaceae bacterium1 [96.0-96.8]		5 (3.4)	

[1]Family; [3]Order.

The % identity range indicates the % identities of analysed clones from each phylotype with best matching sequences in the database.