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1 **Molecular identification of bacteria associated with canine periodontal disease**

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25 **Abstract**

26 Periodontal disease is one of the most common diseases of adult dogs, with up to 80% of  
27 animals affected. The aetiology of the disease is poorly studied, although bacteria are known to  
28 play a major role. The purpose of this study was to identify the bacteria associated with canine  
29 gingivitis and periodontitis and to compare this with the normal oral flora. Swabs were obtained  
30 from the gingival margin of three dogs with gingivitis and three orally healthy controls, and  
31 subgingival plaque was collected from three dogs with periodontitis. Samples were subjected to  
32 routine bacterial culture. The prevalent species identified in the normal, gingivitis and  
33 periodontitis groups were uncultured bacterium (12.5% of isolates), *Bacteroides heparinolyticus* /  
34 *Pasteurella dagmatis* (10.0%) and *Actinomyces canis* (19.4%), respectively. Bacteria were also  
35 identified using culture-independent methods (16S rRNA gene sequencing) and the predominant  
36 species identified were *Pseudomonas* sp. (30.9% of clones analysed), *Porphyromonas*  
37 *cangingivalis* (16.1%) and *Desulfomicrobium orale* (12.0%) in the normal, gingivitis and  
38 periodontitis groups, respectively. Uncultured species accounted for 13.2%, 2.0% and 10.5%, and  
39 potentially novel species for 38.2%, 38.3% and 35.3%, of clones in the normal, gingivitis and  
40 periodontitis groups, respectively. This is the first study to use utilise culture-independent  
41 methods for the identification of bacteria associated with this disease. It is concluded that the  
42 canine oral flora in health and disease is highly diverse and also contains a high proportion of  
43 uncultured and, in particular, potentially novel species.

44  
45 **Keywords:** canine periodontal disease / bacteria / microbiological culture / 16S rRNA /  
46 polymerase chain reaction

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## 49 1. Introduction

50

51 Periodontal disease (gingivitis and periodontitis) is one of the most common infectious  
52 diseases affecting adult dogs, with up to 80% of animals of all breeds affected (Golden et al.,  
53 1982; Harvey & Emily, 1993; Harvey, 1998). The incidence of the disease increases markedly  
54 with advancing years and causes significant oral pain and suffering. Periodontal disease has been  
55 described as a multi-factorial infection (Lindhe et al., 1973), and plaque bacteria are known to be  
56 an important causative factor. Gingivitis is completely reversible and is recognised by the classic  
57 signs of halitosis, bleeding, inflammation, redness and swelling of the gingivae. Periodontitis is  
58 irreversible and attacks the deeper structures that support the teeth, permanently damaging the  
59 surrounding bone and periodontal ligament and resulting in increased periodontal pocket depth  
60 and tooth loss. The aetiology of canine periodontal disease remains unknown, although gram-  
61 negative anaerobic bacteria have been implicated in the disease (Hennet & Harvey, 1991a, b;  
62 Boyce et al., 1995).

63 In recent years, the use of culture-independent (bacterial 16S rRNA gene sequencing)  
64 methods has supplemented traditional culture-dependent methods to detect bacteria in clinical  
65 specimens. 16S rRNA gene sequencing has permitted the identification of bacteria which are  
66 uncultivable, fastidious in their growth requirements and even novel, in addition to detecting  
67 known cultivable species (Clarridge, 2004; Spratt, 2004). In the current study, the bacteria  
68 associated with canine periodontal disease, and with the normal canine oral cavity, were  
69 identified using both culture-dependent and culture-independent methods.

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## 73 2. Materials and methods

74

### 75 2.1. Sample collection and processing

76

77 Ethical approval for the study was obtained from the Local Research Ethics Committee.  
78 Samples were classified into normal and diseased groups as follows: no gingival inflammation,  
79 no periodontal pockets (normal); gingival inflammation and/or periodontal pockets less than 3  
80 mm in depth (gingivitis); periodontal pockets at least 4 mm in depth (periodontitis). Dental  
81 plaque was collected using sterile swabs from the gingivae of periodontally healthy dogs (three  
82 samples) and animals with gingivitis (three samples). For the periodontitis cases (three samples),  
83 subgingival plaque was collected using a sterile curette from the periodontal pocket. Swabs were  
84 placed into sterile reduced transport medium and subgingival plaque was immersed into 1 mL of  
85 fastidious anaerobe broth (FAB) and immediately sent for laboratory analysis. Each swab was  
86 then immersed into 1 mL of FAB. All samples were mixed for 30 s to remove bacteria.

87

### 88 2.2. Bacterial culture

89

90 Ten-fold serial dilutions (to  $10^{-6}$ ) were prepared for each sample and spiral plated onto both  
91 Columbia agar containing 7.5% (v/v) defibrinated horse blood (for aerobic culture) and fastidious  
92 anaerobe agar (FAA) (BioConnections, Wetherby, UK) containing 7.5% (v/v) defibrinated horse  
93 blood (for anaerobic culture). Columbia blood agar plates were incubated in 5% CO<sub>2</sub> at 37°C,  
94 and FAA plates were incubated at 37°C in an anaerobic chamber with an atmosphere of 85% N<sub>2</sub> /  
95 10% CO<sub>2</sub> / 5% H<sub>2</sub> at 37°C. Plates were incubated for up to seven days, and up to eight  
96 morphologically distinct colonies (visually representing the most abundant colony types) were

97 then subcultured in order to obtain pure cultures. Bacterial isolates were identified by 16S rRNA  
98 gene sequencing as described below.

99

### 100 2.3. *Extraction of DNA from samples*

101

102 A bacterial DNA extract was prepared from each sample by digestion with 1% SDS and  
103 proteinase K (100 ug/mL) at 60°C for 1 h, followed by boiling for 10 min. Extraction of DNA  
104 from bacterial isolates was carried out using the same method.

105

### 106 2.4. *PCR amplification of bacterial 16S rRNA genes*

107

108 Bacterial 16S rRNA genes were amplified by PCR using the universal primers 5'-  
109 CAGGCCTAACACATGCAAGTC-3' (63f) and 5'-GGGCGGWGTGTACAAGGC-3' (1387r)  
110 (Marchesi et al., 1998). PCR reactions were carried out in a total volume of 50 µL containing 5  
111 µL of the extracted DNA and 45 µL of reaction mixture comprising 1 x GoTaq<sup>®</sup> PCR buffer  
112 (Promega, Southampton, UK) 1.25 units of GoTaq<sup>®</sup> polymerase (Promega), 1.5 mM MgCl<sub>2</sub>, 0.2  
113 mM dNTPs (New England Biolabs, Hitchin, UK), and each primer at a concentration of 0.2 µM.  
114 The PCR cycling conditions comprised an initial denaturation phase of 5 min at 95°C, followed  
115 by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and primer extension  
116 at 72°C for 1.5 min, and finally a primer extension step at 72°C for 10 min.

117

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121 2.5. PCR quality control

122  
123 Stringent procedures were adhered to in order to prevent contamination during the PCR  
124 process (Riggio et al., 2000). Negative and positive control reactions were included with every  
125 batch of samples being analysed. The positive control comprised a standard PCR reaction  
126 mixture containing 10 ng of *Escherichia coli* genomic DNA instead of sample, whereas the  
127 negative control contained sterile water instead of sample. PCR products (10 µL) were  
128 electrophoresed on 2% (w/v) agarose gels, stained with ethidium bromide (0.5 µg/mL) and  
129 visualised under ultraviolet light.

130

131 2.6. Cloning of 16S rRNA PCR products

132

133 PCR products were cloned into the pSC-A-amp/kan plasmid vector using the StrataClone™  
134 PCR Cloning Kit (Stratagene) in accordance with the manufacturer's instructions.

135

136 2.7. PCR amplification of 16S rRNA gene inserts

137

138 Following cloning of the 16S rRNA gene products amplified by PCR for each sample,  
139 approximately 50 clones from each library were selected at random. The 16S rRNA gene insert  
140 from each clone was amplified by PCR with the primer pair 5'-  
141 CCCTCGAGGTCGACGGTATC-3' (M13SIF) and 5'-CTCTAGAACTAGTGGATCCC- 3'  
142 (M13SIR). The M13SIF binding site is located 61 base pairs downstream of the M13 reverse  
143 primer-binding site, and the M13SIR binding site is located 57 base pairs upstream of the M13  
144 -20 primer-binding site, in the pSC-A-amp/kan plasmid vector.

145 2.8. Restriction enzyme analysis

146

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 *RsaI* and *MnII* (Fermentas Life Sciences, York, UK) at  
150 37°C 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 *RsaI* restriction digestion  
152 profiles and further discrimination was achieved by digestion of clones with *MnII*. 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

157

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 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  
163 (iii) 10 s at 95°C and 30 s at 70°C 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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168



169 *2.10. Sequence analysis*

170  
171 Sequence data were compiled using LI-COR Base ImagIR 4.0 software, converted to FASTA  
172 format and compared with bacterial 16S rRNA gene sequences from the EMBL and GenBank  
173 sequence databases using the advanced gapped BLAST program, version 2.1 (Altschul et al.,  
174 1997). The program was run through the National Centre for Biotechnology Information website  
175 (<http://www.ncbi.nlm.nih.gov/BLAST>). Clone sequences with at least 98% identity with a  
176 known sequence from the database were designated the same species as the matching sequence  
177 with the highest score. Clone sequences with less than 98% identity were tentatively classified as  
178 putative novel phylotypes.

179  
180 *2.11. Statistical analysis*

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

184  
185 **3. Results**

186  
187 *3.1. Culture-dependent identification of bacteria*

188  
189 Bacterial isolates obtained following microbiological culture of samples were identified by  
190 16S rRNA gene sequencing, and the results are shown in Table 1. All isolates had identities of at  
191 least 98% with a known database sequence. Of the 32 isolates obtained from the normal samples,  
192 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).

### 217 3.3. Statistical analysis

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

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

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

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

229

## 230 4. Discussion

231

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

240 *Porphyromonas denticanis*) associated with the disease have also been described (Collins et al.,  
241 1994; Hirasawa and Takada, 1994; Love et al., 1994; Fournier et al., 2001; Hardham et al., 2005).

242 Our current study is the first to use molecular cloning and sequencing of bacterial 16S rRNA  
243 genes, in addition to conventional microbiological culture methods, to identify the bacteria  
244 associated with canine gingivitis, periodontitis and oral health. Given the relatively small number  
245 of samples analysed, it was not possible to age- and sex- match the animals used in the study,  
246 although this would be desirable in future large-scale studies. Despite the relatively small number  
247 of samples analysed in each group in our current study, clear correlations were seen to emerge  
248 between disease status and the prevalence of specific bacterial species, and differences in the  
249 microflora between the three groups was statistically significant. The most prevalent species  
250 found in the normal, gingivitis and periodontitis groups were *Pseudomonas* sp. (30.9%),  
251 *Porphyromonas cangingivalis* (16.1%) and *Desulfomicrobium orale* (12.0%), respectively.  
252 *Porphyromonas cangingivalis* was first isolated from cases of canine periodontitis (Collins et al.,  
253 1994) and *Desulfomicrobium orale* has been isolated from cases of human periodontitis  
254 (Langendijk et al., 2001). Other prevalent species identified included *Porphyromonas canoris*,  
255 *Tannerella forsythensis* and *Capnocytophaga cynodegmi* (gingivitis), and *Actinomyces* sp. and  
256 *Capnocytophaga cynodegmi* (periodontitis). The association of *Tannerella forsythensis* with  
257 human periodontal disease is well documented, *Capnocytophaga cynodegmi* is found in the oral  
258 cavity of the vast majority of dogs (van Dam et al., 2009) and *Porphyromonas canoris* has been  
259 isolated from canine dental plaque samples (Allaker et al., 1997). Culture-independent methods  
260 showed that microbial diversity was similar in all three groups (19 to 24 phylotypes). Uncultured  
261 bacteria were found at a higher proportion in the normal samples (13.2%) compared to the  
262 gingivitis (2.0%) and periodontitis (10.5%) samples. Potentially novel species were found at high  
263 proportions in all three groups (35.3% to 38.3%), a finding that is unsurprising when one

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

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

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

291

#### 292 **Conflict of interest statement**

293

294 The authors have no conflicts of interest.

295

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297

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300

#### 301 **References**

302

303 Allaker, R.P., de Rosayro, R., Young, K.A., Hardie, J.M., 1997. Prevalence of *Porphyromonas*

304 and *Prevotella* species in the dental plaque of dogs. *Veterinary Record* 140, 147–148.

305 Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J.,

306 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search

307 programs. *Nucleic Acids Research* 25, 3389–3402.

308 Boyce, E.N., Ching, R.J.W., Logan, E.I., Hunt, J.H., Maseman, D.C., Gaeddert, K.L., King, C.T.,

309 Reid, E.E., Hefferren, J.J., 1995. Occurrence of gram-negative black-pigmented anaerobes in

- 310 subgingival plaque during the development of canine periodontal disease. *Clinical Infectious*  
311 *Diseases* 20 (Suppl 2), S317–S319.
- 312 Clarridge, J.E. III, 2004. Impact of 16S rRNA gene sequence analysis for identification of  
313 bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews* 17,  
314 840–862.
- 315 Collins, M.D., Love, D.N., Karjalainen, J., Kanervo, A., Forsblom, B., Willems, A., Stubbs, S.,  
316 Sarkiala, E., Bailey, G.D., Wigney, D.I., Jousimies-Somer, H., 1994. Phylogenetic analysis of  
317 members of the genus *Porphyromonas* and description of *Porphyromonas cangingivalis* sp.  
318 nov. and *Porphyromonas cansulci* sp. nov. *International Journal of Systematic Bacteriology*  
319 44, 674–679.
- 320 Fournier, D., Mouton, C., Lapierre, P., Kato, T., Okuda, K., Ménard, C., 2001. *Porphyromonas*  
321 *gulae* sp. nov., an anaerobic, Gram-negative coccobacillus from the gingival sulcus of various  
322 animal hosts. *International Journal of Systematic and Evolutionary Microbiology* 51, 1179–  
323 1189.
- 324 Golden, A.L., Stoller, N., Harvey, C.E., 1982. A survey of oral and dental diseases in dogs  
325 anesthetized at a veterinary hospital. *Journal of the American Animal Hospital Association*  
326 18, 891–899.
- 327 Gorrel, C., Rawlings, J.M., 1996. The role of a ‘dental hygiene chew’ in maintaining periodontal  
328 health in dogs. *Journal of Veterinary Dentistry* 13(1), 31–34.
- 329 Hardham, J., Dreier, K., Wong, J., Sfintescu, C., Evans, R.T., 2005. Pigmented-anaerobic  
330 bacteria associated with canine periodontitis. *Veterinary Microbiology* 106, 119–128.
- 331 Harvey, C. E., Emily, P.P., 1993. Periodontal disease. In: Harvey, C.E., Emily, P.P. (Eds.), *Small*  
332 *Animal Dentistry*. Mosby, St. Louis, USA, pp. 89–144.

- 333 Harvey, C.E., 1998. Periodontal disease in dogs. Etiopathogenesis, prevalence, and significance.  
334 Veterinary Clinics of North America: Small Animal Practice 28, 1111–1128.
- 335 Hennes, P.R., Harvey, C.E., 1991a. Anaerobes in periodontal disease in the dog: a review.  
336 Journal of Veterinary Dentistry 8(2), 18–21.
- 337 Hennes, P.R., Harvey, C.E., 1991b. Spirochetes in periodontal disease in the dog: a review.  
338 Journal of Veterinary Dentistry 8(3), 16–17.
- 339 Hennes, P.R., Harvey, C.E., 1992. Natural development of periodontal disease in the dog: a  
340 review of clinical, anatomical and histological features. Journal of Veterinary Dentistry 9(3),  
341 13–19.
- 342 Hirasawa, M., Takada, K., 1994. *Porphyromonas gingivicanis* sp. nov. and *Porphyromonas*  
343 *crevioricanis* sp. nov., isolated from beagles. International Journal of Systematic Bacteriology  
344 44, 637–640.
- 345 Isogai, H., Kosako, Y., Benno, Y., Isogai, E., 1999. Ecology of genus *Porphyromonas* in canine  
346 periodontal disease. Journal of Veterinary Medicine. Series B 46, 467–473.
- 347 Langendijk, P.S., Kulik, E.M., Sandmeier, H., Meyer, J., van der Hoeven, J.S., 2001. Isolation of  
348 *Desulfomicrobium orale* sp. nov. and *Desulfovibrio* strain NY682, oral sulfate-reducing  
349 bacteria involved in human periodontal disease. International Journal of Systematic and  
350 Evolutionary Microbiology 51, 1035–1044.
- 351 Lindhe, J., Hamp, S.-E., Löe, H., 1973. Experimental periodontitis in the Beagle dog. Journal of  
352 Periodontal Research 8, 1–10.
- 353 Love, D.N., Karjalainen, J., Kanervo, A., Forsblom, B., Sarkiala, E., Bailey, G.D., Wigney, D.I.,  
354 Jousimies-Somer, H., 1994. *Porphyromonas canoris* sp. nov., an asaccharolytic, black-  
355 pigmented species from the gingival sulcus of dogs. International Journal of Systematic  
356 Bacteriology 44, 204–208.



- 357 Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J., Wade, W.G., 1998.  
358 Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding  
359 for bacterial 16S rRNA. *Applied and Environmental Microbiology* 64, 795–799.
- 360 Polz, M.F., Cavanaugh, C.M., 1998. Bias in template-to-product ratios in multitemplate PCR.  
361 *Applied and Environmental Microbiology* 64, 3724–3730.
- 362 Riggio, M.P., Lennon, A., Wray, D., 2000. Detection of *Helicobacter pylori* DNA in recurrent  
363 aphthous stomatitis tissue by PCR. *Journal of Oral Pathology and Medicine* 29, 507–513.
- 364 Spratt, D.A., 2004. Significance of bacterial identification by molecular biology methods.  
365 *Endodontic Topics* 9, 5–14.
- 366 Suzuki, M.T., Giovannoni S.J., 1996. Bias caused by template annealing in the amplification of  
367 mixtures of 16S rRNA genes by PCR. *Applied and Environmental Microbiology* 62,  
368 625–630.
- 369 van Dam, A.P., van Weert, A., Harmanus, C., Hovius, K.E, Claas, E.C.J., Reubsaet, F.A.G.,  
370 2009. Molecular characterization of *Capnocytophaga canimorsus* and other canine  
371 *Capnocytophaga* spp. and assessment by PCR of their frequencies in dogs. *Journal of Clinical*  
372 *Microbiology* 47, 3218–3225.
- 373 Watson, A.D.J., 1994. Diet and periodontal disease in dogs and cats. *Australian Veterinary*  
374 *Journal* 71, 313–318.

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378

379

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

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

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384 \*Unable to distinguish between species: <sup>1</sup>Family; <sup>2</sup>Phylum.

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

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

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\*Unable to distinguish between species; <sup>1</sup>Family; <sup>3</sup>Order.

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

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

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420 <sup>1</sup>Family; <sup>3</sup>Order.  
421  
422 The % identity range indicates the % identities of analysed clones from each phylotype with best  
423 matching sequences in the database.  
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