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Predominant bacterial species in subgingival plaque in dogs

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Background and Objective: The dog has been used extensively for experimental and microbiological studies on periodontitis and peri-implantitis without detailed knowledge about the predominant flora of the subgingival plaque. This study was designed to evaluate the predominant cultivable bacterial species in dogs and compare them phenotypically and genotypically with corresponding human species.

Material and Methods: Four subgingival samples were taken from two upper premolars in each of six Labrador retrievers. The samples from each dog were processed for anaerobic culture. From the samples of each dog, the five or six predominating bacteria based on colony morphology were selected and pure cultured. Each of the strains was characterized by Gram stain, anaerobic/aerobic growth and API-ZYM test. Eighteen strains showing clear-cut phenotypic differences were further classified based on DNA sequencing technology. Cross-reactions of DNA probes from human and dog strains were also tested against a panel of both human and dog bacterial species.

Results: Thirty-one strains in the dogs were isolated and characterized. They represented 21 different species, of which six belonged to the genus *Porphyromonas*. No species was found consistently in the predominant flora of all six dogs. *Porphyromonas crevioricanis* and *Fusobacterium canifelinum* were the two most prevalent species in predominant flora in dogs. DNA probes from human and dog species cross-reacted to some extent with related strains from humans and dogs; however, distinct exceptions were found.

Conclusion: The predominant cultural subgingival flora in dogs shows great similarities with the subgingival bacteria from humans at the genus level, but distinct differences at the species level; however, a genetic relatedness could be disclosed for most strains investigated.

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Dogs have been used in experimental gingivitis and periodontitis studies for many years (1–5). Likewise, dogs have now also come into use for experimental peri-implantitis studies. Ligature-induced breakdown of periodontal and peri-implant tissues in animals is a well-described and commonly used experimental model to study mechanisms involved in tissue destruction (6–10). The dog model has several advantages compared with other animal models. Thus, the dog is susceptible to periodontal disease and the jaws are large enough for the use of standard types of dental implants in designated areas. A further development of the experimental peri-implantitis model in dogs, including the study of spontaneous progression of the lesion after ligature-induced breakdown, has recently been introduced (11,12). Thus, following ligature removal and ongoing plaque accumulation at implants, a chronic

inflammation persisted in the peri-implant tissues, and destruction of supporting bone and connective tissue continued. While it is clear from experimental studies in dogs that the bacterial plaque on the surface of teeth and implants (biofilm) and in the periodontal/peri-implant pocket plays a major role in the progression of the lesion, the nature and composition of this subgingival flora in dogs is less well known. It has been shown that its general characteristics are similar to that harboured in humans, with a gram-negative predominantly anaerobic flora (13–17). This picture has been found by using microscopic and culture analysis and evaluates the flora to genus level at best. Several studies have shown distinct differences in species between dog and human bacterial strains, even if they share commonalities phenotypically (18–21). By means of DNA-based methods, the microflora in the experimental dog model can be further investigated to species and genotype level in order to achieve more accurate identification of the bacteria.

The aim of this study was to identify the predominant bacterial flora in dogs used for experimental periodontitis and peri-implantitis and to compare bacterial species from dogs phenotypically and genotypically with corresponding human species.

Material and methods

Animals

The regional Ethics Committee for Animal Research, Gothenburg, Sweden, approved the study protocol. Six Labrador retriever dogs, about 1 year old, which were used in a study on experimental peri-implantitis (22), were included. Microbiological samples were obtained during the phase when all implants were submerged, i.e. before experimental peri-implantitis was induced. During the microbiological sampling procedure, the animals were sedated with an intramuscular injection of a combination of butarphanol (0.1 mg/kg; Dolorex vet.; Intervet International BV, Boxmeer, The Netherlands) and medetomidine (25 μg/kg; Dexdomitor®; Orion Corporation, Esbo, Finland). After sampling, the sedation was reversed with an intramuscular injection of atipamezole (125 μg/kg; Antisedan® vet.; Orion Corporation).

Sampling and culture

At the time of sampling, the dogs had accumulated plaque on their teeth and the gingival tissue was inflamed, with gingival pocket depth of about 3–4 mm; however, no periodontal breakdown was recorded. Subgingival plaque could easily be collected using the paper point technique. The fourth maxillary premolars, one on each side, were used as sampling sites. The paper points were kept in position for 10 s, and all four paper points were pooled in the same bottle with 3.3 mL of transport medium VMGA III (23). After reaching the laboratory (< 3 h), the sample was diluted into a series of 1:10 and 1:1000 and spread uniformly over the entire surface of a Brucella agar plate (BBL Microbiological Systems, Cockeysville, MD, USA) enriched with 5% defibrinated horse blood, 0.5% haemolysed horse blood and 5 mg/L of menadione. The plates were anaerobically incubated for 7 d in jars at 37°C, with hydrogen combustion in 95% H_2 and 5% CO_2. The bacteria were recorded based on colony morphology, selecting the five or six different morphotypes. All isolates were pure cultured, Gram stained and categorized as obligate or facultative anaerobic. All strains were also subjected for phenotypic enzyme production using the API-ZYM test (24), according to the manufacturer's instructions (API bioMérieux, Marcy l'Etoile, France). Catalase production and haemagglutination of horse erythrocytes were also tested.

Identification with the Culture Collection University of Gothenburg (CCUG) system

Eighteen strains with differing phenotypical characteristics isolated from the dogs were sent to the CCUG laboratory for a final classification. Gram staining was performed with a commercial kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. The strains were biochemically characterized by using one or more of the API rapid ID32AN, API rapid ID32Strep, API CAMPY and API ZYM systems (API bioMérieux). The 16S rRNA genes of the isolates were amplified by PCR using universal primers. The amplified products were purified, using a QIAquick PCR Purification Kit (Qiagen Ltd, Dorking, UK), and directly sequenced, using primers directed towards conserved positions of the 16S rRNA gene and the Big Dye terminator cycle sequencing kit (version 3.1; Applied Biosystems, CA, US), with an automatic DNA sequencer (model 3100 Avant; Applied Biosystems). The closest known relatives were determined by performing database searches, using the program FASTA [European Bioinformatics Institute (EBI), Cambridge, UK] (25). These sequences and those of other related strains were retrieved from GenBank and aligned with the newly determined sequences, using the program SEQtools (Søren W. Rasmussen, Denmark) (26). The resulting multiple sequence alignment was corrected manually, using the program GeneDoc (Pittsburgh Supercomputing Center, Pennsylvania, US) (27).

Identification with DNA–DNA hybridization technique

DNA probes were produced for main species identified by CCUG. Thus, probes were made for *Porphyromonas canoris*, *Porphyromonas gulae*, *Porphyromonas crevioricanis*, *Porphyromonas cangingivalis*, *Porphyromonas* sp., *Fusobacterium canifelinum*, *Fusobacterium russii*, *Filifactor alocis*, *Filifactor villosus*, *Tannerella forsythia*, *Campylobacter oricanis* (for designation in the OMGS (Oral Microbiology, Gothenburg, Sweden) and CCUG system see Table 1). In addition, DNA probes were made for the following human strains: *Porphyromonas gingivalis* (strain FDC381), *Porphyromonas endodontalis* (OMGS 1205), *Tannerella forsythia* (ATCC 43037), *Parvimonas micra* (ATCC 33270), *Fusobacterium nucleatum* (ATCC 10953), *Prevotella*

Table 1. Dog strains and their characteristics (OMGSno stands for Oral Microbiology, Gothenburg, Sweden number, CFU for colony forming units, TVC total viable count, CCUG Culture Collection University of Gothenburg, API-ZYM method for semi-quantification of enzymatic activities)

Dog:strain	OMGSno.	CFU (% TVC)	Identification CCUG (2008)	CCUG no.	API-ZYM	Catalase	Haem-agglutination	Checkerboard test	Final designation
1:1	3832	11	*Porphyromonas canoris*	57077	α-Chymotrypsin + + + Phosphatases + + + *N*-Acetyl-glucosaminidase + + +	+	+	n.t.	*P. canoris*
1:2	3846	5.9	*Fusobacterium canifelinum*	57078	Naphtol-hydrolase +	−	+	n.t.	*F. canifelinum*
1:3	3847	16.1	*Filifactor villosus*	57079	Leucine arylamidase + + +	+	+	n.t.	*F. villosus*
1:4	3856	19.4	*Bacteroides tectus*	57080	Phosphatases + + + β-Glucuronidase + + + α-Glucosidase + + + *N*-Acetyl-glucosaminidase + +	−	−	n.t.	*B. tectus*
1:5	3857	12.9	n.d.	n.d.	Phosphatases + + + *N*-Acetyl-glucosaminidase + + +	−	−	*Bacteroides* sp.	*Bacteroides* sp. (n.f.s.)
2:1	3831	15.2	n.d.	n.d.	α-Chymotrypsin + + + Phosphatases + + + *N*-Acetyl-glucosaminidase + + +	+	+	*P. canoris*	*P. canoris*
2:2	3854	5.7	n.d.	n.d.	Phosphatases + + +	−	+	*F. canifelinum*	*F. canifelinum*
2:3	3858	12.7	n.d.	n.d.	Phosphatases + + + Naphtol-hydrolase + + + β-Galactosidase + + + β-Glucosidase + + + *N*-Acetyl-glucosaminidase + + +	−	(+)	*Bacteroides* sp.	*Bacteroides* sp. (n.f.s.)
2:4	3859	28.6	*Tannerella forsythia*	57306	Trypsin + + + Phosphatases + + + Naphtol-hydrolase + + + α-Glucosidase + + + *N*-acetyl-glucosaminidase + + + α-Fucosidase + + +	+ +	+ +	n.t.	*T. forsythia* (dog)
2:5	3845	3.2	*Peptostreptococcus* sp.	57081	α-Glucosidase + +	−	+	n.t.	*Peptostreptococcus canis*
3:1	3860	34.2	*Porphyromonas gulae*	57312	Trypsin + + + Phosphatases + + + Naphtol-hydrolase + + + *N*-Acetyl- glucosaminidase+ + +	+	+	n.t.	*P. gulae*
3:2	3850	15.8	n.d.	n.d.	Phosphatases + + + Naphtol-hydrolase + + +	−	+	*Porphyromonas crevioricanis*	*P. crevioricanis*
3:3	3834	36.7	*Porphyromonas* sp.	57313	Trypsin + + + Phosphatases + + + Naphtolhydrolase + + + α-Galactosidase + + + *N*-Acetyl-glucosaminidase + + +	+	−	n.t.	*Porphyromonas* sp. (n.f.s.)

Table 1. (Continued)

Dog:strain	OMGSno.	CFU (% TVC)	Identification CCUG (2008)	CCUG no.	API-ZYM	Catalase	Haem-agglutination	Checkerboard test	Final designation
3:4	3861	2.5	n.d.	n.d.	Phosphatases + + + Naphtol-hydrolase + + + *N*-Acetyl-glucosaminidase + + +	–	+	*Bacteroides* sp.	*Bacteroides* sp. (n.f.s.)
3:5	3849	8.3	*Peptostreptococcus* sp.	57314	α-Glucosidase + + +	–	+	n.t.	*P. canis*
3:6	3848	2.5	*Pasteurella stomatis*	57315	Phosphatases + + +	+	+	n.t.	*P. stomatis*
4:1	3833	12.8	n.d.	n.d.	Trypsin + + + Phosphatases + + + *N*-Acetyl-glucosaminidase + + +	+	+	*P. gulae*	*P. gulae*
4:2	3843	8.1	*Campylobacter oricanis*	57305	Phosphatases + + Leucine arylamidase + +	(+)	+	n.t.	*C. oricanis*
4:3	3862	4.1	*Porphyromonas cangingivalis*	57082	Phosphatases + + + α-Chymotrypsin + + Naphtol-hydrolase + +	(+)	+	n.t.	*P. cangingivalis*
4:4		9.3	n.d.	Lost on subcultivation	Lost on subcultivation				
4:5	3844	2.3	n.d.	n.d.	Esterase + + +	–	+	*F. canifelinum*	*F. canifelinum*
5:1	3853	5.2	*P. crevioricanis*	57307	Phosphatases + + + Naphtol-hydrolase + +	–	+	n.t.	*P. crevioricanis*
5:2	3835	33.8	n.d.	n.d.	Trypsin + + + α-Chymotrypsin + + + α-Galactosidase + + + *N*-Acetyl-glucosaminidase + + +	+	–	*Porphyromonas* sp.	*Porphyromonas* sp. (n.f.s.)
5:3	3852	20.8	n.d.	n.d.	Esterase lipase + + Naphtol-hydrolase + +	+	–	*Pasteurella* sp.	*Pasteurella* sp. (n.f.s.)
5:4	3851	5.2	*P. cangingivalis*	57308	α-Chymotrypsin + + + Phosphatases + + +	+ +	+	n.t.	*P. cangingivalis*
5:5	3836	10.4	*Filifactor alocis*	57083	Leucine arylamidase + + +	–	–	n.t.	*F. alocis*
6:1	3837	18.3	n.d.	n.d.	Phosphatases + + + Naphtol-hydrolase + + +	–	+	*P. crevioricanis*	*P. crevioricanis*
6:2	3838	6.7	*Filifactor villosus*	57309	Leucine arylamidase + + +	+ +	+	n.t.	*F. villosus*
6:3	3839	24.4	n.d.	n.d.	Phosphatases + + + Naphtol-hydrolase + + +	+ +	+	*Pasteurella* sp.	*Pasteurella* sp. (n.f.s.)
6:4	3840	24.4	*C. oricanis*	57310	Phosphatases + + + Leucine arylamidase + +	(+)	+	n.t.	*C. oricanis*
6:5	3841	7.3	n.d.	n.d.	Esterase + + Valine arylamidase + + Naphtol-hydrolase + +	(+)	+	*Campylobacter* sp.	*Campylobacter* sp. (n.f.s.)
6:6	3842	6.1	*Fusobacterium russii*	57311	Phosphatases + + +	–	+	n.t.	*F. russii*

Abbreviations: (+), weak reaction; +, slight activity/positive reaction; + +, moderate activity/very positive reaction; + + +, strong activity; –, negative reaction; n.d., not deposited in CCUG, n.t., not tested; (n.f.s.), not further specified.

intermedia (ATCC 25611), *Filifactor alocis* (ATCC 35896), *Campylobacter rectus* (ATCC 33238) and *Treponema denticola* (OMGS 3271).

Whole genomic probes were made according to the methods described previously (28). In brief, digoxigenin-labelled, whole genomic DNA probes were prepared by random priming using the High-Prime labelling kit (Boehringer-Mannheim, Mannheim, Germany). The sensitivity and specificity of whole genomic probes constructed as above have been previously described (29,30).

The DNA–DNA hybridization method ('the checkerboard technique') was used for identification of the remaining 13 isolates that were not sent for identification by CCUG. They were tested against the available DNA probes. The checkerboard technique has been described thoroughly (30,31). Briefly, the microbial samples were placed in sterile Eppendorf tubes and then transferred to 100 μL TE buffer (10 mM Tris HCl and 1 mM EDTA, pH 7.6). One hundred microlitres of 0.5 M NaOH was added and the suspensions were boiled for 5 min. After boiling, 800 μL of 5 M ammonium acetate was added to each tube and the samples were processed according to standardized procedures. The hybrids formed between the bacterial DNA and the probes were detected after a series of stringency washes, by application of an antidigoxigenin antibody conjugated with alkaline phosphatase and incubation with a chemiluminiscent substitute (CSPD; Boehringer-Mannheim). Evaluation of the signal was performed at a LumiImager™ workstation (Boehringer-Mannheim) by comparing the obtained signals with those of pooled standard samples containing 10^6 (high-standard) or 10^5 (low standard) of each of the 12 bacterial species of the panel. The obtained chemiluminiscent signals were transformed into a scale of scores from 0 to 5 (28). The score 1 cut-off is selected to contrast colonized vs. noncolonized sites and the score 3 cut-off to contrast heavily colonized (score 3 or more) vs. noncolonized and less heavily colonized sites.

Identification of cross-reactions between strains from dogs and humans

All DNA probes that were prepared and used for identification were cross-tested against the bacterial species used in the study, using the checkerboard technique. Thus, probes from associated human strains were cross-tested against dog strains and, vice versa, probes from dog strains tested against closely related human bacteria. Digoxigenin-labelled whole genomic DNA probes were constructed using DNA from both human and dog oral isolates. Potential cross-reactions would enable us to reveal the degree of relatedness of 'human-like' dog species to human species.

Results

Five or six bacterial strains with different phenotypical characteristics, based on colony morphology after anaerobic culture, were isolated from the predominant flora of six dogs; altogether, 31 strains. One was lost on subculture. Phenotypic characteristics of all strains are shown in Table 1. Eighteen strains were further identified at CCUG by phenotypic characteristics and 16S rRNA sequencing and were identified as currently designated dog bacterial species (*P. canoris*, *P. gulae*, *P. cangingivalis*, *P. crevioricanis*, *F. canifelinum* and *C. oricanis*) or were classified as an animal variant of a closely related human variant [*F. russii*, *F. alocis*, *F. villosus*, *Bacteroides tectus*, *Pasteurella stomatis* and *T. forsythia* (dog)] or did not fit to known current species designations and were defined only at genus level (*Porphyromonas* sp., *Bacteroides* sp., *Peptostreptococcus* sp., *Pasteurella* sp., *Campylobacter* sp.; Table 1). Having performed the API-ZYM test, we could identify trypsin-like enzyme in *P. gulae*, *Porphyromonas* sp., *T. forsythia* (dog) and chymotrypsin activity in *P. canoris* and *P. cangingivalis*. Interestingly, one strain belonging to *Porphyromonas* sp. (5:2), showed strong activity for both trypsin and chymotrypsin. Leucine arylamidase could be identified in *F. villosus*, *F. alocis* and *C. oricanis*. In addition, *P. canoris*, *P. gulae*, *P. cangingivalis*, *Porphyromonas* sp., *T. forsythia* (dog), *Campylobacter* sp., *C. oricanis*, *F. villosus*, *Pasteurella* sp. and *P. stomatis* were catalase positive using a slide test with 3% hydrogen peroxide. *P. canoris*, *P. gulae*, *P. crevioricanis*, *P. cangingivalis*, *Bacteroides* sp., *T. forsythia* (dog), *Campylobacter* sp., *C. oricanis*, *F. villosus*, *F. canifelinum*, *F. russii*, *Peptostreptococcus canis*, *Pasteurella* sp. and *P. stomatis* produced haemagglutination of horse erythrocytes. Thirteen strains that were not identified by CCUG were further identified using probes from the CCUG-identified species. Thus, six strains were identified to species level and the remaining seven to genus level (Table 1). The two *Peptostreptococcus* strains were both catalase negative but positive to haemagglutination and were later suggested as a new species, *P. canis* (32).

The study identified 21 different species among the 31 isolated strains from dogs (Table 2). Most common were *P. crevioricanis* and *F. canifelinum* that were found in the predominant flora of three dogs each. No species were found in the predominant flora of all dogs. The strains constituted 71.7 ± 19.9% (mean ± SD) of total viable count (TVC) in the subgingival flora of the dogs. The predominance of strains as a percentage of TVC varied between 2.5 and 36.7%. *Porphyromonas* sp., *T. forsythia* (dog) and *Pasteurella* sp. were detected in the greatest magnitude as a percentage of TVC.

The relatedness between dog and human isolates was cross-tested using the checkerboard method and DNA probes from both dog and human strains (Tables 3 and 4). Cross-reactions were quite common between species with a suspected relatedness within genus *Porphyromonas*, *Fusobacterium* and *Campylobacter*. Generally, the probes from dog strains cross-reacted more strongly against the pooled standard of dog strains compared with the pooled standard of human strains (data not shown).

DNA probes from human strains cross-reacted with dog strains in the pattern shown in Table 3. *P. gingivalis* cross-reacted strongly with *P. gulae* as

Table 2. Frequency of predominating species in subgingival plaque of six dogs

Species	*n*[a] (%[b])	Mean value (% TVC)
Porphyromonas crevioricanis (3:2, 5:1, 6:1)	3 (50)	13.1
Porphyromonas gulae (3:1, 4:1)	2 (33.3)	23.5
Porphyromonas canoris (1:1, 2:1)	2 (33.3)	13.1
Porphyromonas cangingivalis (4:3, 5:4)	2 (33.3)	4.7
Porphyromonas sp. (3:3)	1 (16.7)	36.7
Porphyromonas sp. (5:2)	1 (16.7)	33.8
Fusobacterium canifelinum (1:2, 2:2, 4:5)	3 (50)	4.6
Fusobacterium russii (6:6)	1 (16.7)	6.1
Filifactor villosus (1:3, 6:2)	2 (33.3)	11.4
Filifactor alocis (5:5)	1 (16.7)	10.4
Bacteroides tectus (1:4)	1 (16.7)	19.4
Bacteroides sp. (1:5)	1 (16.7)	12.9
Bacteroides sp. (2:3)	1 (16.7)	12.7
Bacteroides sp. (3:4)	1 (16.7)	2.5
Peptostreptococcus canis (2:5, 3:5)	2 (33.3)	5.8
Pasteurella stomatis (3:6)	1 (16.7)	2.5
Pasteurella sp. (5:3)	1 (16.7)	20.8
Pasteurella sp. (6:3)	1 (16.7)	24.4
Campylobacter oricanis (4:2, 6:4)	2 (33.3)	16.25
Campylobacter sp. (6:5)	1 (16.7)	7.3
Tannerella forsythia (dog) (2:4)	1 (16.7)	28.6
Total number of strains	31	—
Total number of species	21	—

[a]Number of dogs as an absolute count.
[b]Percentage of dogs.

well as with strain 3:3, which belongs to the *Porphyromonas* sp. Human *T. forsythia* cross-reacted strongly with *Tannerella* sp. but not *T. forsythia* (dog). *F. nucleatum* cross-reacted strongly with *F. canifelinum*, whereas *F. alocis* cross-reacted strongly with *F. villosus*. Notably, *C. rectus* cross-reacted very strongly with *C. oricanis* but not at all with the dog strain, belonging to *Campylobacter* sp.

Cross-reactions between DNA probes from dog strains and bacteria from humans are shown in Table 4. Thus, *P. gulae* cross-reacted strongly with *P. gingivalis* but less with *P. endodontalis* and *T. forsythia*. *Porphyromonas canoris* and *P. crevioricanis* reacted to a similar extent with *P. gingivalis* and *P. endodontalis*, but *P. crevioricanis* cross-reacted more strongly with *T. forsythia* and additionally with *F. nucleatum*. Strain 5:2, which belongs to *Porphyromonas* sp., cross-reacted in a similar way to *P. cangingivalis*, i.e. strong reactions to *P. gingivalis*, *P. endodontalis* and *T. forsythia*, thus confirming it to be a *Porphyromonas* species, although its final species designation was not disclosed. In contrast, strain 3:3 followed a different pattern, implying that it belongs to the genus *Porphyromonas* but could be a different species. *F. canifelinum* and *F. russii* showed strong cross-reaction with human *F. nucleatum*, and the former a slight reaction also against human *F. alocis*. *F. alocis* and *F. villosus* from the dog reacted strongly against the human *F. alocis* and *F. villosus* also reacted weakly with *F. nucleatum*. *T. forsythia* (dog) reacted strongly with human *T. forsythia* and slightly with *P. gingivalis*, *P. endodontalis* and *F. nucleatum*. The *Bacteroides* strain (1:5) behaved differently in the cross-reactions and was not linked strongly to human *T. forsythia*, thus confirming our designation as a different species from *Tannerella*. *C. oricanis* and the dog strain *Campylobacter* sp. showed no cross-reaction with human *C. rectus*, and the dog *P. canis* showed no cross-reaction with human *P. micra* (formerly *Peptostreptococcus micros*). It was notable that none of the DNA probes from dog species reacted with human *T. denticola*, *P. intermedia*, *C. rectus* and *P. micra*.

In Table 4 it is noteworthy that the two *P. gulae* strains (3:1 and 4:1) may be different. Strain 3:1 had a markedly weaker reaction against other *Porphyromonas* species and the pooled standard. A similar difference was also noted for the two *P. canoris* strains (1:1 and 2:1).

Discussion

This study identified five or six predominating species in each of six Labrador retriever dogs used for experimental periodontitis and periimplantitis studies. Of the total of 31 isolated strains, 28 strains were obligate anaerobic and three (all *Pasteurella* species) facultative anaerobic. This finding confirms the results from previous studies that the subgingival flora in dogs resembles that in humans and is predominantly constituted of anaerobes (33–37). The isolated species constituted 71.7% of TVC, thus confirming their predominance in the subgingival oral microflora in dogs, although none was isolated in all six dogs. This may seem surprising in view of the fact that the dogs were of similar age and were bred and caged together with similar food and environmental factors. On the other hand, it is likely that all dogs in fact have all the identified species, although not detected in the predominant flora. The heterogeneity found in composition between the dogs should be kept in mind when analysing the microbiology in the experimental situation.

The general character (microbial ecology) of the subgingival flora in dogs and humans is quite similar. However, distinct differences were noticed. *Streptococcus* and *Actinomyces*, which are regularly found in humans, were not among the predominant species in dogs. In contrast, *Pasteurella* sp. was predominant in dogs. This is no surprise because Talan *et al.* (38) found *Pasteurella* species to be the most common species isolated from dog bites. In humans, *Pasteurella* spp. are seldom recorded either with culture or molecular biology methods. In the broad approach used by Aas *et al.* (39), no *Pasteurella* phylotype was found. It would, however, be of interest to test *Pasteurella* species in a checkerboard panel so as to confirm

Table 3. Cross-reactions between DNA probes from human strains and dog strains

	DNA probes from human strains								
Dog strains	*Porphyromonas gingivalis*	*Porphyromonas endodontalis*	*Tannerella forsythia*	*Parvimonas micra*	*Fusobacterium nucleatum*	*Prevotella intermedia*	*Filifactor alocis*	*Campylobacter rectus*	*Treponema denticola*
Porphyromonas canoris 1:1	–	–	–	–	–	–	–	–	–
P. canoris 2:1	+	–	–	–	–	–	–	–	–
Porphyromonas gulae 3:1	+ + +	–	–	–	–	–	–	–	–
P. gulae 4:1	+ + +	–	(+)	–	–	–	–	–	–
Porphyromonas crevioricanis 3:2	(+)	–	–	–	–	–	–	–	–
P. crevioricanis 5:1	(+)	–	–	–	–	–	–	–	–
P. crevioricanis 6:1	–	–	–	–	–	–	–	–	–
Porphyromonas cangingivalis 4:3	(+)	–	–	–	–	–	–	–	–
P. cangingivalis 5:4	–	–	–	–	–	–	–	–	–
Porphyromonas sp. 3:3	+ +	–	–	(+)	–	(+)	+ +	–	–
Porphyromonas sp. 5:2	(+)	–	–	–	–	–	–	–	–
T. forsythia 2:4	–	–	–	–	–	–	–	–	–
Fusobacterium canifelinum 1:2	–	–	–	(+)	+ +	–	(+)	–	–
F. canifelinum 2:2	(+)	–	–	(+)	+ +	–	+	+	–
F. canifelinum 4:5	(+)	–	–	(+)	+ +	–	(+)	+	–
Fusobacterium russii 6:6	–	–	–	(+)	–	–	(+)	–	–
F. alocis 5:5	–	–	–	–	–	–	(+)	–	–
Filifactor villosus 1:3	+	–	(+)	(+)	–	(+)	+ +	–	–
F. villosus 6:2	–	–	–	+	–	–	+ +	–	–
Bacteroides tectus 1:4	–	–	–	–	–	–	–	–	–
Bacteroides sp. 1:5	(+)	–	+ +	–	–	–	–	–	–
Bacteroides sp. 2:3	(+)	–	–	–	–	–	–	–	–
Bacteroides sp. 3:4	(+)	–	–	–	–	–	–	–	–
Campylobacter oricanis 4:2	(+)	–	–	–	–	(+)	–	+ + +	–
C. oricanis 6:4	–	–	–	(+)	–	–	+	+ + +	–
Campylobacter sp. 6:5	–	–	–	–	–	–	–	–	–
Pasteurella stomatis 3:6	–	–	–	–	–	–	–	–	–
Pasteurella sp. 5:3	–	–	–	–	–	–	–	–	–
Pasteurella sp. 6:3	–	–	–	–	–	–	–	–	–
Peptostreptococcus canis 2:5	(+)	–	–	(+)	–	–	–	–	–
P. canis 3:5	(+)	(+)	(+)	–	–	–	–	–	–

Abbreviations: –, no cross-reaction; (+), very weak cross-reaction; +, weak cross-reaction; + +, moderate cross-reaction; + + +, strong cross-reaction.

whether it belongs to the subgingival flora of humans or not. The three strains included in our study, all belonging to the genus *Pasteurella*, were catalase positive. Two of these strains (3:6 and 6:3) haemagglutinated horse erythrocytes. They also shared the same rapidly amplified polymorphic DNA (RAPD) banding patterns, implying that they belong to the same species, i.e. *P. stomatis* (data not shown). The third strain (5:3) was negative to haemagglutination.

This study also confirms previous studies showing that *Porphyromonas* species are the predominant genus in dogs, although it is split into several different species designations. Madianos *et al.* (40) found up to six different genotypes of '*P. gingivalis*-like' isolates in similar types of experimental dogs. They probably represented different species using the current taxonomy. Thus, seven different *Porphyromonas* species have been found in dogs/cats, and their relationship is nicely described by Mikkelsen *et al.* (41). They indicate that *P. gingivalis* and *P. gulae* are closely related genetically, and this is confirmed

Table 4. Cross-reactions between DNA probes from dog strains and human strains

	DNA probes from dog strains														
Human strains	*Porphyromonas canoris* 1:1 2:1	*Porphyromonas gulae* 3:1 4:1	*Porphyromonas crevioricanis* 3:2 5:1 6:1	*Porphyromonas cangingivalis* 4:3 5:4	*Porphyromonas* sp. 3:3	*Porphyromonas* sp. 5:2	*Fusobacterium canifelinum* 1:2 2:2 4:5	*Fusobacterium russii* (6:6)	*Filifactor alocis* (5:5)	*Filifactor villosus* 1:3 6:2	*Tannerella forsythia* (dog) 2:4	*Bacteroides* sp. 1:5	*Campylobacter oricanis* 4:2 6:4	*Campylobacter* sp. 6:5	*Peptostreptococcus canis* 2:5 3:5
Porphyromonas gingivalis	(+) +	++ +++	++ (+) ++	++	+	++	(+) (+) (+)	–	–	– –	(+)	+	– –	–	– –
Porphyromonas endodontalis	(+) +	– (+)	++ (+) ++	+ ++	(+)	++	– (+) (+)	–	–	– –	(+)	(+)	– –	–	– –
Tannerella forsythia	(+) +	(+) ++	++ + ++	(+) ++	(+)	++	(+) (+) (+)	–	–	– –	+++	+	– –	(+)	– –
Treponema denticola	– –	– –	– – –	– –	–	–	– – –	–	–	– –	–	–	– –	–	– –
Fusobacterium nucleatum	– +	– +	+ + +	+ +	(+)	(+)	++++ ++++ ++++	++	–	– (+)	(+)	–	– –	–	(+) –
F. alocis	– (+)	– (+)	(+) – (+)	(+) (+)	(+)	(+)	(+) + +	–	++	(+) ++	–	(+)	– (+)	(+)	++ (+)
Prevotella intermedia	–	– –	– – –	– –	–	–	– – –	–	–	– –	–	–	– –	–	– –
Campylobacter rectus	–	– –	– – –	– –	–	–	– – –	–	–	– –	–	–	(+) –	–	– –
Parvimonas micra	–	– –	– – –	– –	–	–	– – –	–	–	– –	–	–	– –	–	– –

Abbreviations: (+), very weak cross-reaction; +, weak cross-reaction; ++, moderate cross-reaction; +++, strong cross-reaction; ++++, very strong cross-reaction; –, no cross-reaction.

phenotypically by being positive for trypsin and hemagglutination and showing no fluorescence under ultraviolet light. However, *P. gulae* is catalase positive compared with *P. gingivalis* (42). All other *Porphyromonas* species isolated from dogs apart from *P. crevioricanis* are quite distant from human strains and are catalase postive. The corresponding *P. endodontalis* is catalase negative, although it is indicated by Mikkelsen *et al.* (41) to be related to *Porphyromonas gingivicanis*, *Porphyromonas circumdentaria* (cats) and *Porphyromonas canis*. None of these species was identified in this study, and *P. endodontalis* showed no cross-reactions, explaining why *P. endodontalis*-related species were not found in the dogs. The *Porphyromonas* spp. isolates in the study were tested for a potential cross-reaction with *P. endodontalis*, but it was negative. Neither of these isolates (3:3 or 5:2) could be verified to be *P. canis* or *P. gingivicanis*, and it is possible that we are dealing with yet another species. However, from data not shown, it was revealed that they share the same RAPD banding patterns, and we could conclude that they belong to the same novel species.

Fusobacterium and *Filifactor* species are apparently among the predominating bacteria in dogs. All three *F. canifelinum* strains from dogs cross-reacted strongly with *F. nucleatum*, indicating a close relation, as proposed by Citron (43) in an update of *Fusobacterium* taxonomy. Likewise, *F. alocis* (human) cross-reacted almost equally well with *F. alocis* and *F. villosus* (dogs). Human *F. alocis* probes could distinguish between dog *F. alocis* and *F. villosus*, indicating a genetic difference between the dog and human strains.

Bacteroides sp. was included in the predominant flora and was catalase negative. *B. tectus* (1:4) and one *Bacteroides* strain (1:5) were additionally negative to haemagglutination. They shared the same RAPD banding patterns, implying that they most probably belong to the same species, *B. tectus* (data not shown). The other two strains (2:3 and 3:4) belonging to *Bacteroides* sp. proved to be positive to haemagglutination. In humans, most oral *Bacteroides* species have been moved or reclassified to other species designations, e.g. genus *Prevotella* and *Campylobacter*. From dog bites, *Bacteroides* sp. can be isolated. While *B. tectus* was the most common, *B. ureolyticus* (now *Campylobacter ureolyticus*), *B. forsythia* (now *T. forsythia*) and *Bacteroides gracilis* (now *Campylobacter gracilis*) were isolated (38,44). No *Bacteroides* species is included in the orange complex (45). Dahlén and Leonhardt (31) included *B. ureolyticus* in their checkerboard panel; however, this species was not found to be in the predominant flora in periodontal pocket samples from chronic periodontitis. In contrast, no *Prevotella* sp. was found in dogs, which is suggested to be one of the predominant bacteria in human subgingival flora represented by black-pigmented *P. intermedia*, *P. nigrescens* and *P. tannerae* (46) as well as nonpigmented (e.g. *P. oris*, *P. buccae*; 47–49). No pigmented isolates apart from the *Pophyromonas* isolates were found in dogs (19). The human *P. intermedia* probe did not cross-react with any of the dog strains, whereas it gave a cross-reaction with plaque material from dogs, indicating that *Prevotella* species may be present in low numbers in dogs. The most common *Prevotella* isolate in dog bite wounds is *Prevotella heparinolytica* (38). It was also included in the panel of Dahlén and Leonhardt (31), but only giving weak reactions, and does not seem to belong to the subgingival flora in humans. In summary, we noticed some distinct differences between dogs and humans with respect to *Bacteroides* (*Prevotella*) species.

Campylobacter was found in the predominant flora in two dogs, representing either *C. oricanis* or a strain identified to genus level, *Campylobacter* sp. (6:5). *Campylobacter* sp. did not cross-react with the human *C. rectus*, in contrast to *C. oricanis*, which cross-reacted strongly. Moreover, *C. oricanis* produced leucine arylamidase, similar to the human *C. rectus*. The strain *Campylobacter* sp. (6:5) behaved differently, as it produced valine arylamidase and not leucine arylamidase and could be a new species.

Peptostreptococus was also found among the predominant flora in dogs. It was distinctly different from human *Peptostreptococcus*, including the former *Peptostreptococcus micros* (now *P. micra*). This was suggested to be a new species and is now proposed as *P. canis* (32). Both strains (2:5 and 3:5) shared the same RAPD banding patterns, confirming that they are the same novel species (data not shown). We also found one isolate belonging to the genus *Tannerella* among the predominant bacteria in dogs. Sequencing technology revealed that it was one more novel species, i.e. *T. forsythia* (dog). It was strongly positive for haemagglutination and reacted intensely with human *T. forsythia*, implying strong relatedness.

This study was performed on six dogs. It is clear that the 21 predominating species identified do not represent dog subgingival flora in general, but are limited to the dogs used in the present periodontitis and peri-implantitis experiment. From a microbiological standpoint, the strong relationship of human and dog strains seems to establish the periodontitis and peri-implantitis dog model as a valid experimental tool for the study of the pathogenesis of these diseases. However, more dogs used for the same purpose need to be recruited in future studies in order to verify that the observed findings are reproducible in a larger number of samples.

Although the general oral microbiology of dogs and humans may be fairly similar, there are distinct differences at genus and species level. Before the era of DNA sequencing technology, *Porphyromonas* dog strains were vaguely described as '*Porphyromonas*-like' strains or '*Porphyromonas* animal' strains, and any rough genomic taxonomy was based on human probes (40,50). This proves to be inaccurate and inadequate, because there are marked differences between human and dog strains at species level. DNA probes from human strains cannot indisputably be used for detection and quantification of bacteria in dogs, as done even after the start of the DNA sequencing era (51). Specific probes made from the relevant dog species, as done in this study, should be the principle, not only for taxonomic purposes, but also for

clarifying the properties and activity of the canine oral microbiology.

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