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Molecular microbiological characteristics of gingival pockets in the periodontal diseases of dogs

Izabela Polkowska[1], Barbara Tymczyna-Borowicz[2], Magdalena Gołyńska[1], Beata Nowicka[1]✉

[1]Department and Clinic of Animal Surgery, Faculty of Veterinary Medicine, University of Life Sciences, 20-612 Lublin, Poland
[2]Chair and Department of Conservative Dentistry with Endodontics , Medical University of Lublin, 20-093 Lublin, Poland
beatanowicka@aol.com

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Abstract

Introduction: Canine periodontitis results among other factors from a disturbed balance of dental plaque microflora and an inadequate host inflammatory response to a stimulus. This investigation sought to identify microorganisms associated with canine periodontitis. **Material and Methods:** Microbiological analysis was undertaken of gingival pockets in an experimental group of 36 dogs with periodontal diseases. Swabs were collected with the use of Pet Test (MIP Pharma, Berlin, Germany) from patients with gingival pockets deeper than 5 mm. Samples were aggregated and placed in separate shipping containers with the Pet Test kit. **Results:** Identification was made of the most common microorganisms, *e.g. Porphyromonas gingivalis*, *Treponema denticola* and *Prevotella intermedia*. The red complex constituted the largest proportion of all analysed organisms (84.26%). *Capnocytophaga gingivalis* was isolated from 33 dogs, *Peptostreptococcus micros* from 32 dogs, *Fusobacterium nucleatum* from 29 animals and *P. intermedia* from 20. **Conclusion:** The highest percentage of pathogens was supplied by *P. gingivalis* (61%). It is thought that dogs acquire them by means of cross-species transmission. The inter-study variability of results may depend not only on the method of periopathogen detection, but also on environmental factors, host immune status or genetic background. Depending on the state of periodontal disease, patients show varied microbiological profiles of the gingival pockets.

Keywords: periodontal diseases, gingival diseases, dogs, oral diseases, infectious diseases.

Introduction

The oral microbiota in dogs is composed of microorganisms belonging to several thousand species (19, 23). The composition of the bacteria forming dental plaque varies depending on the degree of plaque accumulation. In the balanced ecosystem of the gingival fissure, the microorganisms colonising the oral cavity do not have a pathogenic effect on the periodontium – they are a commensal microbiota. Disturbances in the living conditions of the bacteria lead to changes in the composition of the gingival pocket bacterial population (10, 18, 20). Microorganisms become pathogenic only if the host is susceptible to pocket colonisation. Host susceptibility enables the periopathogens to propagate and reach the spectrum of virulence. The normal bacterial flora is primarily conditioned by Gram-positive aerobic bacteria (4, 6, 18, 28). Under conducive conditions, a bacterial flora favoured by them adheres to the surfaces of the teeth, tongue, lips, cheeks and gums permanently and irremovably, breaks down the defence system of the host and penetrates its tissues. As inflammation of the periodontal tissues deepens and periodontal disease develops, the surface of the teeth becomes colonised with Gram-negative bacteria (19, 28, 29). Restricted oxygen diffusion and low oxidation reduction potential facilitates the development of obligate anaerobes, and the bacterial flora of the gingival pockets changes from a composition of aerobic Gram-positive species to one of anaerobic Gram-negative species (14, 16, 23). As a result of gingival swelling and deepening of the pocket, a subgingival biofilm is developed, where colonies of anaerobic Gram-negative (*Bacterioides* spp., *Capnocytophaga* spp., *Fusobacterium* spp., *Porphyromonas* spp., *Prevotella* spp., *Tannerella* spp. and *Treponema* spp.) and Gram-positive (*Actinomyces* spp., *Corynebacterium* spp., *Eubacterium* spp., *Peptostreptococcus* spp. and *Streptococcus* spp.) bacteria exist (10, 18, 22, 30). Microorganisms may affect periodontal tissues directly or indirectly. Direct tissue damage results from the activity of toxins, enzymes and the bacterial products of

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metabolism. An indirect effect involves the impairment of the defence response of the host by activation of the inflammatory cells, which produce and release mediators with high pro-inflammatory and catabolic activity (4). This activity plays a key role in the destruction of periodontal tissues. Moreover, certain bacteria may affect the host defence mechanism by inactivation of specific antibodies or inhibition of cellular phagocytosis (21). They can also penetrate periodontal tissues and consequently create specific subgingival reservoirs (16, 22).

Material and Methods

Group of animals qualified for the studies. The studies were conducted in dogs which were patients of the Department of Animal Surgery, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Poland. All procedures performed on the animals were accepted by the 2nd Local Ethics Committee for Animal Experiments in Lublin on 26 May 2015 (Resolution no. 29/2015). The owners of dogs qualified for the studies were informed about the purpose of the undertaken activities and gave their informed consent to the procedures explained to them. The studies were carried out on 105 dogs, comprising 65 males and 40 females of various breeds. The patients were aged 3–16 years and had body weights of 3–10 kg. The breeds qualified for the studies were mixed (n = 28), Yorkshire terrier (n = 28), poodle (n = 8), English cocker spaniel (n = 8), short-haired dachshund (n = 8), miniature schnauzer (n = 7), fox terrier (n = 3), pug (n = 3), Pekingese (n = 3), beagle (n = 2), papillon (n = 2), miniature pinscher (n = 2), Italian greyhound (n = 2) and Welsh terrier (n = 1). The animals were divided into two groups: experimental and control. The control group (C) comprised 18 clinically healthy dogs which came to the Department of Animal Surgery for periodic or prophylactic examinations. The dogs comprised 9 males and 9 females of various breeds. They were aged 5–13 years and had body weights of 5–10 kg. The breeds qualified for the studies in the control group were mixed (n = 8), Yorkshire terrier (n = 8) and poodle (n = 2). A physical examination and other examinations confirmed the good general condition of the animals.

Experimental group with periodontal diseases. The experimental group (E) comprised 87 dogs which were diagnosed with periodontal disease. They were examined for dental disorders because of the following symptoms: food intake disorders, foetor *ex ore*, gum redness, swelling and bleeding, purulent exudate, erosions and ulceration on the mucosa, and the presence of dental calculus.

Physical dental examination, periodontal examination and swabbing for microbiological tests were performed on the dogs under general anaesthesia before performing cavity cleaning, as part of the treatment of the periodontal disease. The anaesthesia regimen presented below was used for each patient. The premedication was xylazine (Sedazin; Biowet Puławy, Poland) at a dose of 2 mg/kg body weight (b.w.). After 15 min, the patient had an intravenous line inserted and received ketamine (Vetaketam; VetAgro, Lublin, Poland) at a dose of 8 mg/kg b.w., combined at a 1:1 ratio with diazepam (Relanium; Polfa, Warsaw, Poland) at a dose of 0.5 mg/kg b.w. (administered proportionately to the effect seen). Next, the patient was intubated, and a device monitoring vital signs (SurgiVet Advisor, Smiths Medical, Dublin, OH, USA) was connected. In order to maintain anaesthesia, inhalation (Matrx VMS Plus; Midmark Animal Health, Versailles, OH, USA) was used of a mixture of oxygen and isoflurane at a concentration of 2% (Aerrane; Baxter, Deerfield, IL, USA).

Bacteriological examination of the gingival pocket. Swabs were collected with the use of sterile paper points (Pet Test; MIP Pharma, Berlin, Germany) from patients with gingival pockets deeper than 5 mm. Five swabs were collected from each patient from several sites in the periodontium, which were used as a pooled sample according to the procedure recommended by the manufacturer. Prior to insertion of a paper point into the gingival pocket, supragingival plaque was gently removed with the use of sterile gauze pads, paying attention not to cause bleeding. Then, the sites were dried with the use of compressed air. Each sterile point was inserted with tweezers into the bottom of the gingival pocket and left there for 15 s. Next, the points were combined into a pooled sample, which was placed in the separate transport containers supplied with the Pet Test kit. The samples were collected from 36 dogs in total (Fig. 1).

The samples were sent to the MIP Pharma GmbH analytical laboratory, where the material was analysed. The Pet Test uses a molecular-biological polymerase chain reaction – a real-time RT-PCR with the use of the TaqMan probe. Reference strains were cultured in accordance with the recommendations of the American Type Culture Collection. Sets of primers and probes of specific species were designed from the 16S rRNA gene sequence variable regions and used for a qualitative determination of the total proportion of bacterial cells in the samples.

Free DNA fragments were obtained from bacterial cells by lysis, which were then amplified and hybridised with fluorescence-labelled primers specific to particular periopathogens. Quantification was performed with the use of a reader measuring the fluorescence intensity versus reference samples.

The occurrence of the following bacteria was analysed: *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Prevotella intermedia*, *Peptostreptococcus micros*, *Fusobacterium nucleatum*, *Eubacterium nodatum* and *Capnocytophaga gingivalis* (Fig. 1).

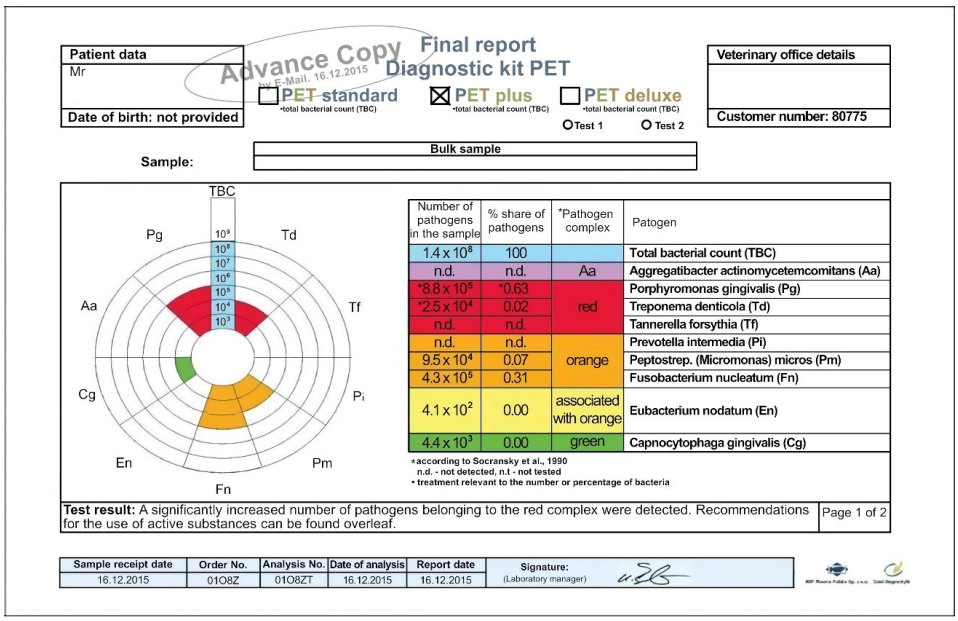


Fig. 1. Each patient from which a swab was collected received a card with the qualitative and quantitative assessment of bacterial microflora of the gingival pocket

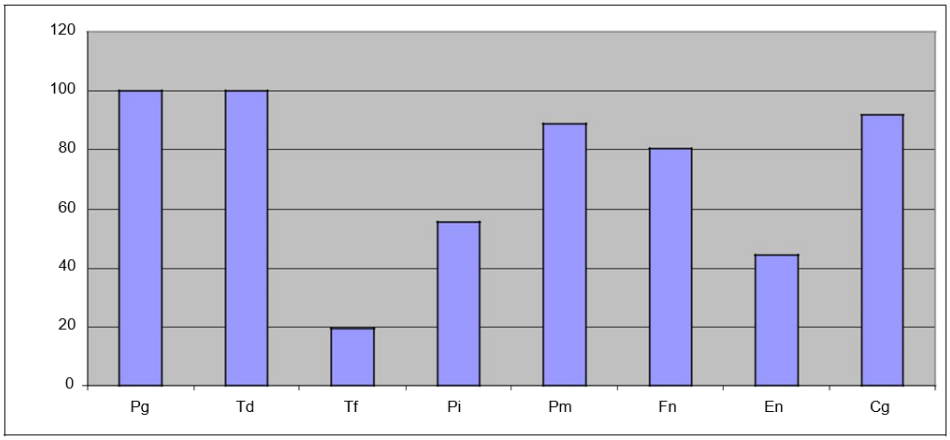


Fig. 2. Frequency of isolation of particular bacteria in the study dogs, expressed as percentages
Aa – *Aggregatibacter actinomycetemcomitans*; Pg – *Porphyromonas gingivalis*; Td – *Treponema denticola*; Tf – *Tannerella forsythia*; Pi – *Prevotella intermedia*; Pm – *Peptostreptococcus micros*; Fn – *Fusobacterium nucleatum*; En – *Eubacterium nodatum*; Cg – *Capnocytophaga gingivalis*

Porphyromonas gingivalis and *T. denticola* were isolated from the samples from all 36 patients. The bacterium isolated in most cases (from 33 dogs' samples) was *C. gingivalis*; *P. micros* was isolated from 32 dogs' samples, *F. nucleatum* from 29, and *P. intermedia* from 20. *Eubacterium nodatum* was present in 16 samples and *T. forsythia* in 7, and these were isolated from the lowest number of patients (Fig. 2). Only by comparing the subgroups in stage III of periodontal disease (E3) and stage IV of periodontal disease (E4) were statistically significant differences found in the number of *Eubacterium nodatum* isolated from gingival pockets (Fig. 3).

Statistical analysis of research results. All calculations were performed with computer methods using the Microsoft Excel 2010 spreadsheet and the Statistica 10.0 statistical package (StatSoft, now Tibco, Tulsa, OK, USA). Statistical significance was calculated using the Mann–Whitney U test. Relationships between the selected variables were calculated using Spearman's correlation.

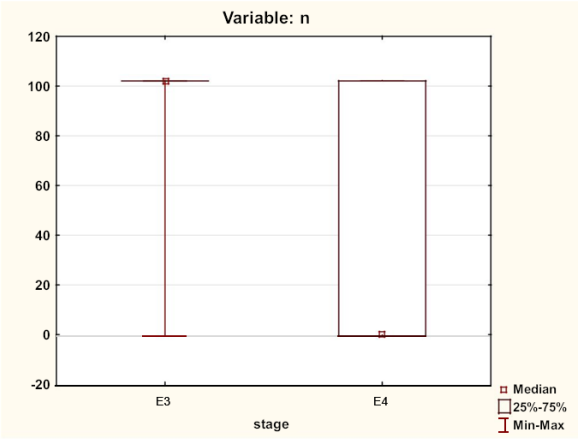


Fig. 3. Statistical significance at p = 0.027189 in the number of isolated *E. nodatum* in dogs at stage III (E3) and stage IV (E4) of the periodontal disease (moderate periodontitis and severe periodontitis, respectively)

Table 1. Mean number of bacteria isolated from gingival pockets presented in three subgroups

Subgroups		Aa	Pg	Td	Tf	Pi	Pm	Fn	En	Cg
E2	x	-	41.00	13.06	-	1.18	1.10	4.62	0.01	0.30
	SD	-	37.50	8.30	-	0.32	0.71	3.61	-	0.18
E3	x	-	47.56	26.72	0.15	5.35	1.32	10.13	0.18*	0.43
	SD	-	38.61	39.70	0.11	7.91	2.63	14.69	0.22	0.42
E4	x	-	89.35	27.42	0.25	4.15	4.66	9.69	0.10*	1.02
	SD	-	136.57	33.58	0.41	11.16	12.39	17.14	0.17	1.39

E2, E3, E4 – stage II, stage III and stage IV of the periodontal disease, respectively; x – mean bacterial count (× 10^4 CFU/mL); SD – standard deviation; * – statistically significant differences between particular bacteria in the analysed subgroups for $P \leq 0.05$; Aa – *Aggregatibacter actinomycetemcomitans*; Pg – *Porphyromonas gingivalis*; Td – *Treponema denticola*; Tf – *Tannerella forsythia*; Pi – *Prevotella intermedia*; Pm – *Peptostreptococcus micros*; Fn – *Fusobacterium nucleatum*; En – *Eubacterium nodatum*; Cg – *Capnocytophaga gingivalis*

Results

Inflammation assessment based on the parameters of periodontological diagnostics. Based on the analysis of the oral cavity clinical assessment results from patients of the experimental group (n = 87), four stages of periodontal disease advancement were identified and dogs were accordingly allocated to subgroups:

Stage I (E1) – 19 dogs
Stage II (E2) – 24 dogs
Stage III (E3) – 20 dogs
Stage IV (E4) – 24 dogs.

The criterion of inclusion in a particular stage group was the degree of periodontal tissue destruction.

In none of the patients was *Aggregatibacter actinomycetemcomitans* detected. Red complex bacteria were the most common among all microorganisms screened for and comprised 84.26% of the total isolates. The red complex pathogen constituting the highest proportion of those identified was *Porphyromonas gingivalis*, and 61% were this species. The second isolated bacterium with regard to quantity was *Treponema denticola* with 23%. *Tannerella forsythia*, the third bacterium of the red complex, represented only 0.26% of all the bacteria. Bacteria of the orange complex were isolated as *Prevotella intermedia* for 4% of species, *Peptostreptococcus micros* for 3% and *Fusobacterium nucleatum* for 8%. *Eubacterium nodatum* and *Capnocytophaga gingivalis* represented less than 1% of the isolated pathogens (0.09% and 0.64%, respectively). Despite the small presence of these last five bacteria, in every case their population increased as the inflammation advanced, which apparently is the result of increased reproduction of bacteria in moderate and severe periodontitis (Table 1).

Both *Porphyromonas gingivalis* and *Treponema denticola* were isolated from all the examined dogs. The mean count of *P. gingivalis* was higher with more severe tissue inflammation, and ranged from 41 × 10^4 CFU/mL (in stage II of the periodontal disease) to 89.35 × 10^4 CFU/mL (in stage IV). During stage II of the periodontal disease, with a lower degree of inflammation, the mean count of *T. denticola* was 13.06 × 10^4 CFU/mL, while at stages III and IV it was isolated in greater numbers: 26.72 × 10^4 CFU/mL and 27.42 × 10^4 CFU/mL, respectively. Finally, the last bacterium of the red complex – *Tannerella forsythia* – was isolated only in seven dogs' samples (19%) during stages III and IV of the periodontal disease and was not detected in any of the dogs in the course of early periodontitis. This bacterium was isolated in low amounts compared to the other bacteria of the red complex (0.15–0.25 × 10^4 CFU/mL).

Prevotella intermedia was isolated in 20 of the studied dogs (55.5%). Similarly to *P. gingivalis* and *T. denticola*, it was isolated in greater numbers in dogs with more advanced damage to periodontal tissues (subgroups E3 and E4), reaching 26.72–27.42 × 10^4 CFU/mL, than in dogs with less severe inflammation (subgroup E2), where it did not exceed 13.06 × 10^4 CFU/mL.

Discussion

In 1998, Socransky *et al.* (25) isolated bacterial complexes of 40 species by identifying the constituents of the subgingival biofilms with a molecular method and grouping them into five major microbial complexes (red, orange, yellow, green and purple). They assigned the highest importance to red complex bacteria, which in humans represent the marker bacteria for periodontitis. Bacteria belonging to red complexes are Gram-negative, non-spore-forming, anaerobic organisms carrying several virulence factors responsible for developing the inflammatory process, such as fimbriae, proteinases, exopolysaccharides and haemin-binding proteins (10). These include *Treponema denticola*, *Tannerella forsythia* and the main pathogen causing periodontitis in humans, *Porphyromonas gingivalis* (2). In dogs, various species of *Porphyromonas* are also considered important pathogens causing periodontitis, but their true significance in the pathogenesis is not fully known (19). The species of *Porphyromonas* isolated from the oral cavity of dogs include *Porphyromonas gulae*, *P. macacae*, *P. cangingivalis*, *P. gingivicanis* and *P. circumdentaria* (23). The second important complex in the course of periodontal diseases in humans is the green complex, which includes *Capnocytophaga gingivalis*, *Campylobacter concisus* and *Eikenella corrodens*. The orange complex, consisting of *Fusobacterium nucleatum*, *Prevotella intermedia*, *P. nigrescens*, *Peptostreptococcus micros*, *Campylobacter rectus*, *C. gracilis*, *C. showae* and *Streptococcus constellatus* is important because the

presence of bacteria belonging to this complex determines formation of the red complex (9). The colonisation of specific bacterial species in the biofilm is an aetiological factor of periodontitis, and knowledge of the occurring pathogens makes it easier to select an adequate local and systemic therapy (10). However, while the presence of bacterial microflora causing periodontal diseases is a necessary condition for the development of the disease, by itself it is not sufficient for this (11, 17, 21, 24).

In the authors' studies, clinical evaluation of the periodontal tissue condition was based on cross-referencing the depth of the gingival pockets to the Russell clinical index of periodontal diseases. An indicator and other indices used to assess the oral cavity in dogs used in studies include the presence of bleeding during probing, plaque index, calculus index modified by Logan and Boyce, gingivitis index, tooth mobility index or furcation index (17). However, authors have most frequently assessed the depth of the gingival pockets and the loss of connective tissue attachment. It should be noted that the results of the periodontal disease evaluation in dogs may be affected by the selection of dogs with regard to age and breed.

According to the literature data, most anaerobic bacteria isolated from gingival pockets in dogs belong to the *Fusobacterium*, *Porphyromonas* and *Prevotella* genera, members of which have for many years been considered key microorganisms that cause periodontal diseases, while *Porphyromonas gingivalis* in humans is considered a bacterium initiating periodontal disease and highly associated therewith (14, 15, 21). However, several differences have been documented between *Porphyromonas* in humans and their companion animals (23). *Porphyromonas gingivalis* isolated from dog and cat samples is catalase-positive and shows high phenotypic similarity to *P. gulae*, while the one isolated from human samples is catalase-negative (14). Moreover, both *Porphyromonas* species show similar virulence traits. The literature states that the high incidence of periodontal diseases in dogs may also be related to the occurrence of such *Porphyromonas* species as: *P. macacae*, *P. cangingivalis*, *P. gingivicanis* and *P. circumdentaria* (23). In the authors' studies, *Aggregatibacter actinomycetemcomitans* was not isolated in any of the dogs undergoing microbiological analysis. This is probably due to the fact that this bacterium is highly correlated with aggressive periodontitis when identified in human cases. This form of inflammation is characterised by rapid loss of soft tissue attachment and destruction of the alveolar bones, while in dogs the disease is of a chronic nature whereby the development of periodontitis may last years (11, 12, 14).

In the authors' studies, the presence of *P. gingivalis* was positively correlated with all the indices assessed in the dental examination, which may indicate the significant influence of this bacterium on periodontal tissue damage. *Porphyromonas gingivalis* was also strongly correlated with *T. denticola* as indicated by the calculated r coefficient. This may indicate a synergistic effect between bacteria and one between them and related occurrences. Analysing the rising trend of the counts of *P. gingivalis* and *T. denticola* with the stage of advancement of periodontal disease, one may suppose that they play an important role in progressive tissue destruction in the course of periodontitis. At the early stage of periodontitis in the analysed dogs, both bacteria were isolated in lower numbers than during more advanced stages, where the loss of periodontal tissues was much greater.

Bacteria of the *Porphyromonas* genus and some species of the *Prevotella* genus are called black-pigmented bacteria (12). Members of the latter genus were also frequently detected in the studied dogs. For many years, some bacteria of these genera have been identified as key factors involved in the pathogenesis of periodontitis in dogs. Black-pigmented bacteria have also been confirmed to be good markers of periodontal disease in dogs and cats. In the present studies, an upward tendency of the *P. gingivalis* and *P. intermedia* counts was observed with higher inflammation severity, which may classify them as indicator bacteria. Khazandi *et al.* (14) isolated Gram-negative bacteria producing black-pigmented colonies in 95% of the collected samples from canine and feline gingival pockets after four days of incubation, finding the majority of isolates to be *Porphyromonas gulae*. Charalampakis *et al.* (4) also used human strains of *P. intermedia* and *T. denticola* in their studies, recording an increased count of these bacteria in dogs with experimentally induced periodontitis.

Fusobacterium species show synergism with other bacteria. Existing in the gingival pocket, they have the ability to aggregate with other microorganisms. *Fusobacterium nucleatum* is one of the most frequently occurring bacterium in the gingival fissure of humans and dogs associated with periodontal diseases (26). The results of the analysis conducted by Conrads *et al.* (5) showed the existence of new strains of *Fusobacterium* belonging to species that are closely related to *F. nucleatum*. In the present studies, *F. nucleatum* was isolated in 80.5% of the experimental dogs. With regard to the total count of isolated pathogens, this bacterium made up 8%. A lower count of *F. nucleatum* was obtained in dogs with less severe inflammation of the periodontal tissues (4.62×10^4 CFU/mL) than in dogs with severe periodontitis (9.69×10^{bn} CFU/mL). Similar results were obtained by Senhorinho *et al.* (23), who showed the presence of *Fusobacterium nucleatum* in the subgingival region in dogs with periodontitis. These researchers suggest that *Fusobacterium* plays an important role in periodontal diseases in dogs. Numerous examples of isolates from samples taken in the present research resolved to *F. nucleatum*, which raises the question of this bacterium possibly supporting other bacteria (*e.g. Porphyromonas* spp.) and facilitating their binding (2).

Such bacteria as *Peptostreptococcus micros*, *Eubacterium nodatum* and *Capnocytophaga gingivalis* were detected in some of the examined dogs. Given the low number of these pathogens in the collected samples and their low value as compared to other pathogens, it may be concluded that these bacteria are not significant for the pathogenesis of periodontal diseases in dogs. However, it must be noted that in each case, low numbers of these bacteria in the analysed samples gave way to increased counts as the inflammatory process deepened, which is probably related to an increased propagation of bacteria during moderate and severe periodontitis. Dilegge *et al.* (8) isolated not only the *Capnocytophaga* species typical of dogs (*C. ochracea* and *C. haemolytica*) but also those typical of humans (*C. gingivalis* and *C. granulosa*) in the dental plaque of dogs.

Wallis *et al.* (27) did not identify red complex bacteria (*P. gingivalis*, *T. denticola* and *T. forsythia*) in the subgingival plaque. Allaker *et al.* (2) did not identify *Treponema* sp. in studied dogs, which is in contrast to the authors' own studies, since *T. denticola* was isolated from all the dogs, and as compared to other bacteria, it represented a major part of the pool of pathogens. The most frequently isolated bacteria in the studies of Riggio *et al.* (20) were *Pseudomonas* spp. (30.9%) and *P. cangingivalis* (16.1%). Other bacteria isolated in lower amounts included *Tannerella forsythia*. In a metagenomic analysis of the bacteria colonising the oral cavity of dogs, Sturgeon *et al.* (26) showed that the dominant genus among the isolates was *Porphyromonas* (39.2%). They isolated *Fusobacterium* spp. from 4.5%, *Capnocytophaga* spp. from 3.8%, *Tannerella* spp. from 2.2%, and *Treponema* spp. from 1.6% of samples. Other researchers identified *P. gingivalis* in 68% of the examined animals, while in 91% of animals they found the presence of black-pigmented bacteria (2). Nishiyama *et al.* (16) also showed the presence of *P. gingivalis* in 64% of individuals. Moreover, they showed the presence of *P. intermedia* and *T. forsythia* in 20% of the studied dogs, and *F. nucleatum* in 16% of the dogs. They did not isolate *T. denticola* from any of the dogs. In addition, they isolated *P. gingivalis* from one dog without periodontitis. Di Bello *et al.* (7) analysed in their studies the presence of red complex bacteria in dogs in the course of periodontal diseases. In 49.3% of the dogs, they observed a mixed *P. gingivalis* and *T. forsythia* infection, which was statistically significant. In other studies where various methods were used to treat experimentally induced periodontal disease, high levels of *Prevotella intermedia* and *T. forsythia* were observed. Where red complex bacteria were detected, Hall *et al.* (11) observed an increased mean count of these bacteria even after a four-week treatment period. These results are in contrast to the present results, since *T. forsythia* was only isolated in low amounts from 19% of canine samples. On the other hand, *T. forsythia* was isolated from humans from dog and cat bite wounds, which suggests the presence of these bacteria in the oral cavities of the animals (12). Senhorinho *et al.* (23) also reported the *Porphyromonas* genus as the one most often isolated from dogs with periodontal diseases (92% of the studied dogs), but also as a genus isolated from 56% of healthy dogs without periodontitis. Out of 46 dogs from which they isolated *Porphyromonas* spp., from 38 animals they also isolated *P. gulae*. Among isolates from gingival pockets in dogs studied by Radice *et al.* (19), the majority was represented by *P. gingivalis* (30.8%), *Peptostreptococcus* spp. (30.8%) and *Prevotella intermedia* (23.1%). Kato *et al.* (13) studied the occurrence of *P. gingivalis*, *T. denticola*, *T. forsythia*, *Prevotella intermedia* and *A. actinomycetemcomitans* in the saliva of dogs with gingivitis. The main bacteria isolated by them was *T. forsythia*, and in this the present investigation's outcome differs. Regarding *A. actinomycetemcomitans*, they did not detect it in any of the dogs, as was the case in the authors' own studies. They isolated *P. intermedia* from only three dogs and *Porphyromonas gingivalis* and *T. denticola* from only one dog. The authors also studied the occurrence of *P. gulae* and successfully isolated it from almost all the studied dogs. Yamasaki *et al.* (30) studied the occurrence of 10 bacteria associated with periodontitis in humans. In their study, the most frequently isolated pathogen was *T. forsythia* (77.3%), with which our results do not concur. However, they rarely isolated *P. gingivalis*, *T. denticola*, *Prevotella intermedia* or *A. actinomycetemcomitans*. In addition, in those authors' studies the occurrence of *Porphyromonas gulae* was observed, being isolated from 71.2 % of the studied dogs. *Porphyromonas gulae* seems to be highly associated with periodontal disease in dogs but not in humans.

In their studies conducted on dogs with periodontal disease, Di Bello *et al.* (7) showed the dominance of *Actinomyces*, *Peptostreptococcaceae* and *Porphyromonas* bacteria. The authors also demonstrated a correlation between the presence of *Treponema denticola* and mild periodontitis. This contrasts with the findings of this research, because in dogs showing stage II periodontal disease (early periodontitis), a negative correlation between *T. denticola* and the clinical indicator of periodontal disease was observed. In dogs in stage III (moderate periodontitis), this correlation was positive and high. The periodontal tissue damage was proportional to the number of isolated *T. denticola*. Perhaps together with the development of the periodontal disease, there is greater colonisation of the plaque with *T. denticola*, which in turn results in a further progressive loss of tissues.

Charalampakis *et al.* (4) showed an increased total bacteria count after removing ligatures causing inflammation of the periodontium when conducting a microbiological analysis of experimentally induced periodontitis in dogs, and demonstrated the formation of anaerobic Gram-negative bacterial flora. These data are in contrast with those of the authors' present studies, since no correlation was observed between the total

bacterial count and the age of the examined dogs. The analysis of the age of animals qualified to particular subgroups showed that the mean age of the dogs was directly proportional to the advancement of the inflammatory process.

The results of available studies confirm that periodontal diseases are caused by bacterial complexes. In recent years, traditional methods of bacterial culture have been replaced with methods of molecular analysis of the gene coding ribosomal RNA (16S rRNA). Sequencing of the 16S rRNA gene enabled the identification of bacteria which, for various reasons, cannot be cultured in media (1, 20). Test Pet uses a molecular and biological reverse transcriptase polymerase chain reaction in real time. This method enables quick and easy identification of specific microorganisms. Test Pet copies selected DNA/RNA fragments rapidly and makes it possible to analyse the number of copies in particular samples. It is characterised by high sensitivity – one fragment may give 10^6 copies. Moreover, it enables the analysis of material even at low baseline DNA levels. Real-time PCR enables the specific and sensitive identification and quantification of the pathogens causing periodontitis. Comparing the real-time PCR with cell culture of five periopathogens for the purpose of their identification and quantification, Boutaga *et al.* (3) showed the high level of conformity of these analyses. They defined the real-time PCR method as an effective alternative for the quantitative assessment of anaerobic bacteria isolated from the subgingival region. In a subsequent investigation, they demonstrated the possibility of using the real-time PCR method to detect very low amounts of periopathogens in collected samples and particularly amounts below the detection limit with the use of the cell culture technique.

Elliott *et al.* (9) compared the DNA sequences used in their studies with human microflora DNA sequences in GenBank and showed approximately 7% difference in the 16S rRNA gene. They showed that most isolates from dogs were not typical of those occurring in the human oral cavity, while the bacteria were similar to those isolated from humans overall. Other researchers (7, 22) also conducted an analysis of bacterial species isolated from the dental plaque of dogs. Using the method of 16S rRNA gene sequencing, they identified 353 taxons, wherein 80% were completely new and only 16.4% were common to the microflora of the human oral cavity. With reference to the studies by Dahlen *et al.* (6), there are controversies related to the detection of microorganisms in the gingival pocket of dogs using microbiological tests based on molecular probes of human strains. The authors suggested the occurrence of cross-reactions between the DNA probes of human strains and strains isolated from dogs. According to them, *P. gingivalis* showed a strong cross-reaction with *P. gulae* and other *Porphyromonas* species. "Human" *T. forsythia* cross-reacts with *Tannerella* spp., but not with "canine" *T. forsythia*. *Fusobacterium nucleatum* cross-reacts with *F. canifelinum*. However, *Prevotella intermedia* does not cross-react with other canine species. Patients with periodontitis, regardless of the varied microbiological profile of the oral cavity, show a good response to treatment with the use of cavity cleaning (30).

Periodontal pocket microbiological analysis shows that microorganisms, namely *Porphyromonas gingivalis*, *Treponema denticola* and *Prevotella intermedia*, are present in the oral cavity of dogs with periodontal disease. In therapeutic categories, it is crucial to assess the role of these microorganisms in the aetiology of periodontitis in domestic animals. As the inflammatory process progresses, the number of isolated bacteria increases, showing a greater colonisation of the subgingival area associated with deepening local inflammation and progressive damage to periodontal tissues.

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