Vol. 20, No. 3, Autumn, 2024, 20-34
DOI: 10.22055/ivj.2024.454864.2710
Journal Homepage: http://www.ivj.ir/

Original Article

Molecular identification of *Fusobacterium spp.* in dogs with or without gingivitis/ periodontitis in Ahvaz and Tehran districts

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Received: 29.04.2024 Accepted: 03.08.2024

Abstract

Periodontal disease is one of the most common disorders seen in small animal practices. Oral bacteria play an important role in periodontitis. *Fusobacterium spp.* is one of the important bacterial agents in the progression of periodontitis. The aim of the present study was to investigate the association between the presence of *fad*A and *leukotoxin* genes in *Fusobacterium spp.* isolated in dogs with or without gingivitis/ periodontitis in districts of Tehran and Ahvaz. One hundred and fifty samples (75 dogs from Tehran and 75 from Ahvaz district), between 2 to 11 years old, 78 males and 72 females, were studied during ten months. The studied major breeds included White Terrier, Poodle, Pomeranian, Shih Tzu, Yorkshire terrier, Pug, Spitz, Maltese and the rest were other breeds. They were fed with dry, homemade or mixed food. Twenty samples had healthy gums (13.33 %), 32 cases periodontitis grade 1 (21.33%), 47 other cases periodontitis grade 2 (31.33%) and 51 samples periodontitis grade 3 (34%). Twenty-seven out of 150 samples were infected with *Fusobacterium* (18.0%; 95% CI: 11.8%-24.1%). The percentages of the relative frequency of these bacteria were 21.3% (95% CI: 12.0%-30.6%) and 14.6% (95% CI: 6.6%-22.6%) in Tehran and Ahvaz, respectively. Survey of *leukotoxin* gene in 18 samples of *Fusobacterium necrophorum* showed that 11 samples (61.11%) (9 cases from Tehran and 2 other cases from Ahvaz) had this gene; the observed difference in the presence of this gene, was not statistically significant (p-value=0.43; df=1; X^2=0.62). Nine out of 26 samples (34.61%) had *fad*A virulence gene and the relationship between the presence of *fad*A gene and periodontitis grades was not statistically significant (p-value=0.41; df=1; X^2=0.68). Multivariable logistic regression showed that age, gender, breed, periodontitis, district, and type of food explained 97.6% of the infection and only gender and periodontitis had a significant effect on infection. The presence of *fadA* gene in *Fusobacterium nucleatum* isolated from dental plaques of dogs suffering from periodontitis and *leukotoxin* gene in *Fusobacterium necrophorum* subspecies *necrophorum* were not significant in periodontitis in related to two different districts of Tehran and Ahvaz. In conclusion, the prevalence of *Fusobacterium* was 16% and 2% in periodontitis grade 3 and healthy gums, respectively.

Key words: Periodontal diseases, *Fusobacterium*, *Leukotoxin*, *Fad*A, Gingivitis, Dog

Introduction

Periodontal disease (PD) is one of the most common diseases seen in small animal practices. It is reported that 80% or more of dogs over five years of age have PD. This disease occurs as a result of infection and chronic inflammation of the gums, bone,

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dental cement, and surrounding tissues that support the tooth (Jamshidi et al, ۲۰۰۵; Riggio et al, 2011; Robinson et al, 2016; Stella et al, 2018; Wallis et al, 2020). Periodontitis is a collective term used to describe two conditions: gingivitis and periodontitis. Gingivitis is where the gingiva becomes erythematous and inflamed as a result of dental plaque bacterial activity and is reversible. Periodontitis is where the supporting structures of the tooth are destroyed as a result of the host's inflammatory response to the dental plaque and can be resulted in odontogenic infection and, ultimately, tooth loss. Periodontitis is irreversible without surgical intervention, but is often controllable. The occurrence of PD depends on many factors, such as the breed and size of the dog, increasing of age, weight loss, changing behavior, hygiene and dental care (Niemiec, 2013; Lobprise et al, 2019; Wallis et al, 2020).

The pathogenesis of periodontitis, from early stages of gingivitis to advanced periodontitis, is that plaque formed first on the outer surface of the tooth. Salivary glycoproteins that have adhesive properties stick to the tooth surface, then the bacteria in the oral cavity are added to this composition and finally, plaque is formed. This plaque can be mineralized and become calculus (tartar). Undoubtedly, bacteria play a major role in the oral cavity in the process of periodontitis. The diagnosis of PD is based on taking history, clinical examination, and radiological evaluation (Niemiec, 2008; Gorrel, 2013). According to the American College of Veterinary Dentistry classification, periodontitis can be classified or staged into four stages based on clinical signs and severity of lesions. Non-surgical periodontal therapy is always the first line of treatment and involves scaling plaque and calculus removal from the tooth crown, gingival sulcus, and root surfaces is essential for the prevention and control of PD (Niemiec, 2008; Lobprise, 2019).

Fusobacteria is one of the most common bacteria found in the gums of humans and dogs (Conrads et al, 2004). This bacterium belongs to the *Bacteriodaceae* family and is a dominant microorganism in the periodontal tissue. *Fusobacteria* are obligate anaerobic bacteria, gram-negative, non-spore-forming, non-motile, pleomorphic rod-shaped bacilli (Signat et al, 2011). *F. necrophorum* is classified into four biotypes A, B, AB, and C according to biochemical characteristics. Based on the 16S ribosomal RNA sequence, it is closely related to both A and B biotypes (biotype A of *Fusobacterium necrophorum* subspecies *necrophorum*, and biotype B of *F. necrophorum* subspecies *funduliforme*) (Nagaraja et al, 2005; Wright, 2016). The main virulence factor in *F. necrophorum* is *leukotoxin*, and variation in this gene enables *Fusobacterium* cause different diseases in multiple hosts (Bennett et al, 2011). The *F. necrophorum* subspecies *necrophorum* has been isolated from infections more than the *F. necrophorum* subspecies *funduliforme* while the *F. necrophorum* subspecies *funduliforme* is usually isolated from infections as a secondary agent and in a non-specific manner (Nagaraja et al, 2005). These bacteria are a part of the normal flora of the oral cavity, but *F. nucleatum* is also considered as a pathogenic organism in periodontitis. These bacteria can accumulate with other pathogens and be on the enamel surface as a bridge to connect new bacteria to the older group of plaque pathogens. They are the main focal point in the physical interactions between gram-positive and gram-negative species (Machuca et al, 2010; Signat et al, 2011). Adhesion gene *fad*A has been identified, which makes *F. nucleatum* capable of high adhesion to host cells (Liu et al, 2014).

The prevalence of *Fusobacterium* was reported in healthy gingiva 7%, gingivitis 25% and periodontitis 10-40% (Hennet et al, 1991). It was announced that the frequency rates of gingivitis and

periodontitis were 24% and 12%, respectively in the referred dogs to the small animal hospital of Tehran University (Jamshidi et al, 2005). Also, it was sated that *F. canifelinum* was the most predominant flora in sub-gingival plaques of dogs (Dahlen et al., 2012). The prevalence of *F. nucleatum* was estimated up to 52% with periodontitis and 24% without periodontitis (Senhorinho et al, 2012). According to the previous studies and the investigation of virulence genes of different subspecies in different districts, determining the existing genetic factors is crucial at the molecular level. Considering the prominent role of *Fusobacterium* in the creating of periodontitis, the present study was conducted for the first time in Ahvaz district and also a comparative evaluation between Ahvaz and Tehran districts.

Materials and methods

Sample collection

In the present survey, after the determining of the periodontitis/gingivitis levels, dental plaque samples were taken from 75 small breed dogs referred to the Veterinary Faculty and clinics in Ahvaz district (Iranian Vet Clinic and Royal Vet Clinic), as well as 75 small breed dogs from the Veterinary clinics in Tehran (Oxygen Pet Hospital and Nella Pet Clinic) during ten months from January to November 2022. Characteristics of dogs such as age, gender, breed, and type of feed (dry, homemade, or mixed), dental hygiene and administration of immunosuppress drugs or antibiotics were recorded. All the studied dogs were in the age range of 2 to 11 years. Seventy-two out of 150 dogs were female and 78 male. Among the studied dogs in each district, at least 30 cases had periodontitis or gingivitis. Dogs that did not allow oral examination were sampled under sedation drugs with ketamine (10 mg/kg IM) and acepromazine (0.05mg/kg IM). Silness-Loe index was used to detect grading, diagnosis and severity of periodontitis. The normal depth of the gum pocket is 1 to 3 mm in dogs using the probe, and depths greater than these amounts were considered as periodontitis. The probe was entering the gum pocket vertically, and different parts of the tooth were examined around the tooth. The measurement had been done from the free edge of the gum to the end of the gum pocket based on references (Gorrel, 2013). In the following, sampling was done by scrubbing plaque with scaler, and calculus forceps. Calculus and plaque were collected from the surface of the tooth in cases of periodontitis and in gingivitis and healthy gums, from the border between the gum and the tooth by sterile cotton swab and placed in a sterile 1.5 ml and finally stored at -20 °C until the DNA extraction.

DNA extraction

Following the protocol indicated by the manufacturer (DENAzist-Iran), 10 mg of plaque tissue was combined with 450 µl AT1 solution in a 2 ml microtube. The sample was mixed and adjusted to the desired volume before adding the correct solution. Glass beads were added to plaque solutions and vortexed until tissue dissolved. 5 µL proteinase K added and incubated at 60°C for 20 minutes. 450 µl of AT2 buffer was mixed with the solution and incubated for 5 minutes at 60°C. The solution was then centrifuged and the supernatant was moved to a new tube. 10 µL RNAse A was added and incubated for 30 minutes at 37°C. 700 µl of lysate was transferred to a column, centrifuged, and discarded. The remaining solution was transferred into the column and the process was repeated. Finally, 700 µL AT3 solution was centrifuged at 10,000 rpm for 1 minute. 100 µl of AT4 solution was added to the column and incubated for 5 minutes. The column and 1.5 ml tube were centrifuged at 10,000 rpm for 1 minute. The collected liquid was returned to the column, centrifuged at 13000 rpm for 3 minutes, and the DNA was stored at -20 °C.

Detection and confirmation of 16S rRNA-based PCR for *Fusobacterium spp*. in the obtained samples and virulence genes

After DNA extraction, the presence of *Fusobacterium spp.* was investigated first in all 150 samples by PCR. The primers were designed to detect the *Fusobacterium* genus, including the $Fuso_1$ (F) and $Fuso_2$ (R) primers. DNA extracts from samples using *F. nucleatum* primers of $F.N_1$ (F) and $F.N_2$ (R) to amplify a 360-bp region of the 16S rRNA gene (Figures 1-5). They were also used to identify the *fad*A virulence gene and differentiate *F. necrophorum* subspecies. Specific primers were utilized for the *lekotoxin* virulence gene. Detailed gene sequences, primers, and fragment lengths are provided in Tables 1 and 2.

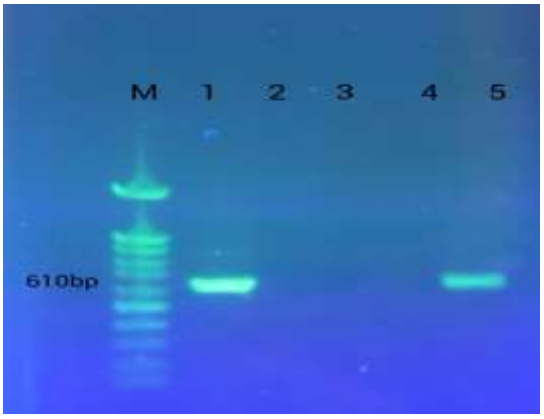

Figure 1: PCR detection of *Fusobacterium* genus in 610bp. M: Molecular ladder, No. 1: Positive control, No. 5: Positive sample, No. 2, 3, 4: Negative samples.

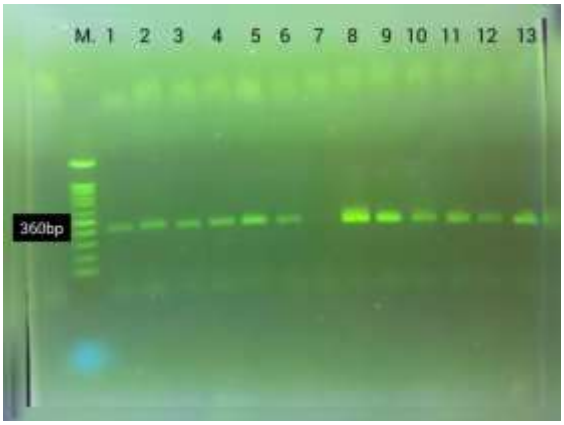

Figure 2: PCR detection of *F. nucleatum* by primers of 16S rRNA-F and 16S rRNA-R to amplify a 360-bp region of the 16S rRNA gene. M: Molecular ladder, No. 1-6 and 9-13: Positive samples, No. 8: Positive control, No. 7: Negative control.

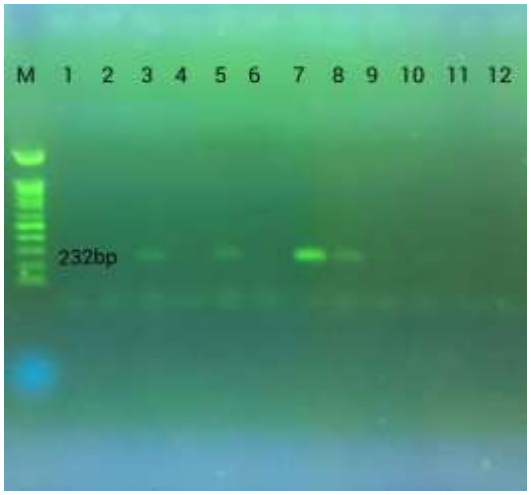

Figure 3: PCR detection of the virulence gene of *F. nucleatum* using the *fad*A primers of *fad*A-F and *fad*A-R to amplify a 232-bp region of the *Fad*A gene from positive samples of *F.nucleatum* with a band of 232 bp. M: Molecular ladder, No. 3, 5, 8, 10 positive samples, No. 7: Positive control, No. 12: Negative control, No. 1, 2, 4, 6, 9, 11: Negative samples.

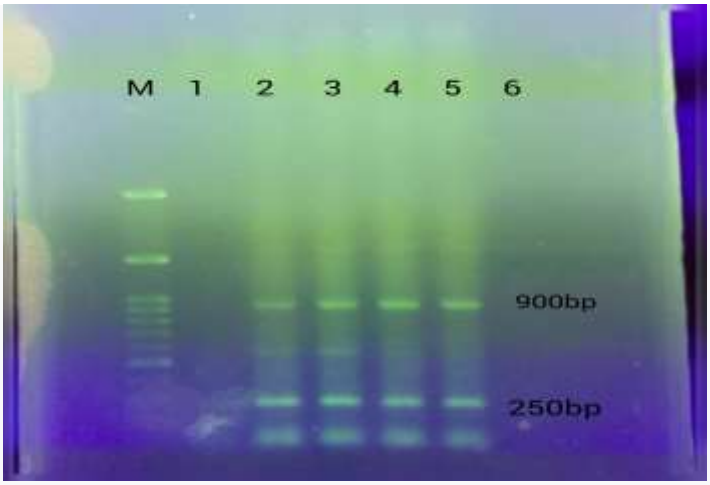

Figure 4: PCR detection of *F. necrophorum necrophorum* and *F. fonduliform necrophorum* species in bands of 250 and 900. M: Molecular ladder, No. 4, 5: Positive *F.* n*ecrophorum necrophorum*, No. 3: Positive *F.* n*ecrophorum fonduliform*. No. 2: Positive control, No. 6: Negative control, No. 1: Negative sample.

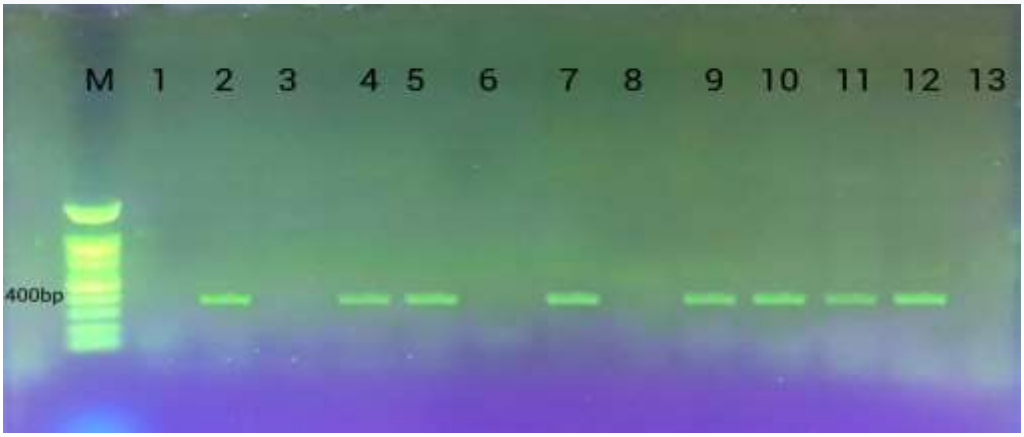

Figure 5: One-step duplex PCR which used to enable the detection and differentiation of *F.necrophorum* subspecies in a single reaction. Primer combinations of TP1–TP2 and WLF2–WLR1 were used to determine of *leukotoxin* virulence gene with 400 bp band. M: 400 bp indicator, No. 2, 4, 5, 7, 9, 10, 11 *F. necrophorum* subspecies that have *leukotoxin* virulence gene Positive *F. nucleatum* DNA, No. 1, 3, 6, 8: Negative samples No. 12: Positive control, No. 13: Negative control.

Table 1: Primer sets and PCR target regions, used in the studied dogs in Ahvaz and Tehran districts

Primers	Primer sequence (5-3)	Bacterial detection/ differentiation	Target (gene fragment)	Size of amplicon	Reference
Fuso$_1$	5'-GAG AGA GCT TTG CGT CC-3'	*Fusobacterium* genus	16S rDNA (position 212e821 in *F. nucleatum*	610	(Nagano et al., 2007)
Fuso$_2$	5'-TGG GCG CTG AGG TTCGAC -3'	*Fusobacterium* genus	16S rDNA (position 212e821 in *F. nucleatum*	610	(Nagano et al., 2007)
F.N$_1$(F)	5`-AGA GTT TGATCC TGG CTC AG -3`	*F. nucleatum*	*F. nucleatum*	360	(Sallum et al., 2004)
F.N$_2$(R)	5`-GTC ATC GTG CAC ACA GAA TTG CTG-3`	*F. nucleatum*	*F. nucleatum*	360	(Sallum et al., 2004)
*fad*A-F	5′-CAC AAG CTG ACG CTG CTA GA -3	*F. nucleatum*	*fadA*	232	(Sallum et al., 2004)
*fad*A-R	5′-TTA CCA GCT CTT AAA GCT TG -3′	*F. nucleatum*	*fadA*	232	(Sallum et al., 2004)
*lkt*A1	5`-AATCGGAGTAGTAGGTTCTG-3`	*Leukotoxin* gene from *F. necrophorum*	*Leukotoxin*	401	(Zhou et al., 2009)
*lkt*A2	5`-CTTTGGTAACTGCCACTGC-30	*Leukotoxin* gene from *F. necrophorum*	*Leukotoxin*	401	(Zhou et al., 2009)

Table 2: Specific primer, used in the present study to detection of *F. necrophorum* subspecies

Primer	DNA sequence (5`–3`)	position	Size of amplicon	Target (gene fragment)	Reference
TP_1 (F)	TCTACGTATGCCTCACGGAT	173–192	900	*rpoB*	(Narongwanichgarn et al., 2003)
TP_2 (R)	AGGAATATGAGGATGAGGAT	1075–1094	900	*rpoB*	(Narongwanichgarn et al., 2003)
WLF_2 (F)	AGGTGCTTCTTCCACAGC	94–111	250	hemagglutinin-related protein gene of *F. n. necrophorum*	(Narongwanichgarn et al., 2003)
WLR_1 (R)	GCACCATTTTGAGCGCGT	323–340	250	hemagglutinin-related protein gene of *F. n. necrophorum*	(Narongwanichgarn et al., 2003)

PCR primers and amplification

16s rRNA-based PCR for the detection of *Fusobacterium* genus

The used primers for 16S rRNA-based PCR were based on the primer designed by Nagano et al, 2007 (Table 1). The primer pair $FUSO_1$ (Forward primer) and $FUSO_2$ (reverse primer) were used to target conserved regions of the 16S rDNA gene for *Fusobacterium* species. PCR reaction system contained 12.5 µl PCR Master Mix, 1 µl (10 pmol/µ) Forward and 1 µl (10 pmol/µ). Reverse primers were included, 1 µl $MgCl_2$, 4 µl DNA template, and 5.5 µl H_2O. Following a 'hot start', the reaction mixtures were subjected to the empirically optimized thermal cycling parameters 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, followed by a final extension at 72°C during 7 minutes.

16S rRNA-based PCR for the detection of *F. nucleatum*

The 16S rRNA-based PCR was used to determine the prevalence of *F. nucleatum*. The PCR was performed on DNA extracts from sub-gingival biofilm samples by using *F. nucleatum* primers of 16S rRNA-F and 16S rRNA-R to amplify a 360-bp region of the 16S rRNA gene (Sallum et al, 2004). For the detection of *F. nucleatum* amplification reaction, it was run in a 20 µl reaction mixture containing 10 µl master, 1 µl $MgCl_2$, 1 µl of each primer (Forward and Reverse primers), 3 µl of Extracted DNA from sub-gingival biofilm samples, and 4 µl H_2O. The 16S rRNA PCR of *F. nucleatum* was carried out for 5 minutes at 94˚C and 30 cycles, with each cycle consisting of denaturation at 94˚C for 30 seconds, annealing at 58˚C for 30 seconds, extension at 72˚C for 1 minute, and final extension for 10 minutes. The amplified products were then electrophoresed on 1.5% agarose gel in Tris-acetate buffer (40 mM Tris-acetate, 1 mm EDTA, pH 8.0).

The 16S rRNA-based PCR and *fad*A specific PCR

The *fad*A primers of *fad*A-F and *fad*A-R were used to amplify a 232-bp region of the *fad*A gene from positive samples of *F. nucleatum* (Liu et al., 2014). Amplification reaction was performed for the detection of *fad*A in a reaction mixture of 25 µl containing 12.5 µl master, 1 µl of each primer 1 µl (10 pmol) Forward and 1 µl (10 pmol) Reverse primers, 3 µl of DNA extracted, 1 µl $MgCl_2$, and 6.5 µl H_2O. The PCR of *fad*A was carried out for 4 minutes at 94˚C and 30 cycles, with each cycle consisting of denaturation at 94˚C for 30 seconds, annealing at 55.8˚C for 30 seconds, extension at 72˚C for 40 seconds, and final extension for 6 minutes.

Determination of *F. necrophorum* species and *necrophorum* and *fundoliform* subspecies

PCR amplification of *F. necrophorum* strains was carried out with 10–23 mer-length random primers. A primer used in the present study was based on Narongwanichgarn et al. (2003). TP_1–TP_2 and WLF_2–WLR_1 were designed based on the nucleotide sequences of each unique band generated with respective random primers D11344 and W1L2. Specific PCR

was performed.to confirm the specificity of the primers to *F. necrophorum*. The PCR was subjected to 30 cycles, with each cycle consisting of 94˚C for 1 minute, 55˚C for 1 minute, and 72˚C for 1 minute. After the final cycle, samples were then heated at 72˚C for 10 minutes for the final extension reaction.

Duplex PCR

One-step duplex PCR was used to enable the detection and differentiation of *F. necrophorum* subspecies in a single reaction. Primer combinations of TP_1–TP_2 and WLF_2–WLR_1 were used in the present survey. PCR was performed in a reaction mixture of 25 µl containing 3 µl of template DNA from *F. necrophorum* tested strains, 4.5 µl H_2O, 1 µl MgL_2, 1 µl primer (WLF_2), 1µl primer (WLR_1), 1µl primer (TP_1), and 1µl primer (TP_2).

Analysis of virulence gene *lkt*A

The primers of *lkt*A1 and *Ikt*A2 were designed based on the study of Zhou et al, (2009). Amplification was performed in a 20 µl reaction containing 3 µl of extracted DNA, 1 µl of each primer 1 µl (10 pmol) Forward and 1 µl (10 pmol) Reverse primers, 1 µl $Mgcl_2$, and 4 µl H_2O. Amplification was carried out in a Master cycler EP thermocycler, and the thermal profile was consisted of denaturation at 94˚C for 2 minutes, followed by 35 cycles of 94˚C for 30 seconds, 60 ˚C for 30 seconds and 72˚C for 40 seconds, with a final extension step at 72˚C for 5 minutes (Tables 1, 2).

Statistical analysis

Obtained data were analyzed by SPSS software version 21 (IBM corporation, USA). Differences were assessed using Independent-T test. A *p* value of less than 0.5 was considered significant. Chi-square test and Univariate logistic regression methods were used for data analysis.

Results

Twenty out of 150 samples (13.33%) had healthy gums, 32 cases (21.33%) periodontitis grade 1, 47 samples (31.33%) periodontitis grade 2, and 51 other cases (34%) periodontitis grade 3. From 150 dental plaque samples that were collected from small breed dogs in Ahvaz and Tehran (75 samples from each district), 27 cases (18%; 95% CI: 11.85-24.15%) (17 cases from Tehran and 10 cases from Ahvaz) were positive in terms of the presence of *Fusobacterium* genus. The percentages of the relative frequency of these bacteria in Tehran and Ahvaz was 21.33 (95% CI: 12.0%-30.6%) and 14.67 (95% CI: 6.6%-22.6%), respectively. The Chi-square test showed that this difference was not statistically significant (P=0.4; df=1; X^2=0.72). Out of 27 samples tested positive for *Fusobacterium* genus, *F. nucleatum* was present in 26 samples (96.3%). Analysis of the *fad*A virulence gene in *F. nucleatum* revealed that out of the 26 samples, 9 tested positive for the *fad*A gene (34%). However, the presence of the *fad*A gene was not statistically significant in relation to periodontitis. (P=0.41; df=1; X^2=0.68). Also, the analysis of these 27 samples in terms of *F. necrophorum* showed that *F. necrophorum* subspecies were present in 17 samples (62.96 %) (14 samples from Tehran and 3 samples from Ahvaz, respectively) and one sample (3%) infected with the subspecies of *F. necrophorum funduliforme* (from Ahvaz). The detection of the *leukotoxin* gene in 18 samples of *F. necrophorum* showed that 11 samples (61.11%) (9 samples from Tehran and 2 samples from Ahvaz) had this gene, and the observed difference, in the presence of this gene, was not statistically significant (P=0.43; df=1; X^2=0.62).

Table 3: Distribution of *Fusobacterium* and its subspecies and virulence genes in relation to different degrees of periodontitis in dogs of Ahvaz and Tehran districts

Bacteria and virulence genes	Healthy gingiva	PD G1	PD G2	PD G3
Fusobacterium genus	3	0	0	24
F. nucleatum	3	0	0	23
F. n. subspecies *necrophorum*	1	0	0	16
F. n. subspecies *fonduliforme*	0	0	0	1
*fad*A	2	0	0	7
leukotoxin	0	0	0	11

Table 4 presents the frequency of *Fusobacterium* infection in both male and female dogs. The survey of *Fusobacterium*-positive samples in Tehran showed that 14 samples were related to male dogs (82.35%) and 3 samples (17.65%) in females and in the Ahvaz, all of the positive samples were in males. The obtained results showed that the relative frequency of positive cases was higher in males than females and this difference was significant ($P<0.001$; df=1; $X^2=16.19$). Univariate logistic regression showed that the chance of infection in male dogs was 10.22 times higher than females (95% confidence interval, 2.92-35.75) ($P<0.001$) and the gender explained 20.6% of the changes for infection by this bacterium (Table 4).

Table 4: Distribution of absolute and relative frequency of *Fusobacterium* infection in dogs of Tehran and Ahvaz districts based on gender

Gender	Total		Positive		Negative	
	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute
F[b]	48	72	4.2	3	95.8	69
M[a]	52	78	30.8	24	69.2	54
Tot	100	150	18	27	82	123

Different lowercase letters indicate significant differences between different gender groups

Table 5 presents the frequency of *Fusobacterium* infection in two different age groups. All of the positive samples in Tehran were above 2 years old and in Ahvaz above 3 years old. The chi-square test showed that there was no significant relationship, between age and infection ($P=0.56$; df=1; $X^2=0.33$). Single-variable logistic regression showed that the chance of infection in dogs 5 years old and older was 1.4 (95% confidence interval, 0.61 - 3.24) and in dogs less than 5 years was 0.7% of changes ($P=0.43$).

Table 5: Distribution of absolute and relative frequency of *Fusobacterium* infection in dogs of Tehran and Ahvaz districts, according to age

Age (Year)	Total		Positive		Negative	
	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute
5[a]>	58.7	88	15.9	14	84.1	74
≥5[a]	41.3	62	21.0	13	79.0	49
Total	100	150	18	27	82	123

Same lowercase letters indicate no significant different between age groups

A survey of the distribution of positive samples for breed in Tehran district showed that three samples (17.65%) were miniature poodles (all females), 7 cases (41.18%) Shih Tzu, 5 samples (29.41%) Yorkshire Terriers and 2 cases (11/76) Pomeranian and in Ahvaz district, three cases (30%) were miniature poodles, 3 items (30%) Yorkshire terriers, 2 samples (20%) Spitz and 2 other samples (20%) white terrier. Table 6 shows the frequency of *Fusobacterium* infection based on the breed. Relative prevalence varied from zero to 57.1%, which was statistically significant (P=0.002). Univariate logistic regression showed that breed explained 36.8% of changes for infection with this bacterium.

Table 6: Distribution of absolute and relative frequency of *Fusobacterium* infection in referred dogs of Tehran and Ahvaz districts, based on breed

Breed	Total		Positive		Negative	
	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute
Poodle	15.3	23	26.1	6	73.9	17
Dachshund	1.3	2	0.0	0	100	2
Shih Tzu	10.0	15	46.7	7	53.3	8
Pomeranian	14.7	22	9.1	2	90.9	20
Yorkshire	9.3	14	57.1	8	42.9	6
Maltese	5.3	8	0.0	0	100	8
Beagle	4.0	6	0.0	0	100	6
Chihuahua	2.0	3	0.0	0	100	3
Cavalier	3.3	5	0.0	0	100	5
Pug	6.0	9	0.0	0	100	9
White Terrier	19.3	29	6.9	2	93.1	27
Jack Russell	1.3	2	0.0	0	100	2
Spitz	5.3	8	25.0	2	75	6
Lhasa Apso	2.0	3	0.0	0	100	3
Basenji	0.7	1	0.0	0	100	1
total	100	150	18	27	82	123

Table 7 shows the frequency of *Fusobacterium* infection based on the periodontitis. All positive samples in Tehran had periodontitis grade 2 and in Ahvaz district, Poodles had gingivitis and white terriers, Yorkshire terriers, and Spitz had periodontitis grade 3. The general assessment of this table showed that the relative frequency of positive cases in dogs with periodontitis grade 3 was higher than grades 1 and 2 and this difference was statistically significant (P<0.001; df=3; X^2 =64/46). The frequency of *Fusobacterium* in dogs with periodontitis grade 3 was significantly higher than periodontitis grades 1 and 2 (P<0.001) or without periodontitis (P=0.02). Univariate logistic regression showed that the type of consumed food explained 49.5% of the changes related to infection with this bacterium. In the studied dogs of Tehran, 11 cases had healthy gums (14.66%), 20 cases gingivitis (26.66%) and 44 cases some degree of periodontitis (58.66%). In dogs of Ahvaz district, 9 cases had healthy gums (12.0%), 12 cases gingivitis (16.0%) and 54 cases some degree of periodontitis (72.0%).

Table 7: Distribution of absolute and relative frequency of *Fusobacterium* infection in referred dogs of Tehran and Ahvaz districts, according to periodontitis

Periodontitis	Total		Positive		Negative	
	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute
Healthy gingiva	13.3	20	15.0	3	85.0	17
grade I	21.3	32	0.0	0	100	32
grade II	31.3	47	0.0	0	100	47
grade III	34.0	51	47.1	24	52.9	27
total	100	150	18	27	82	123

During a study on dog nutrition in Tehran, it was observed that all the dogs included in the research were nourished with either commercially available dry food or homemade meals. These observations revealed indications of poor oral and dental hygiene among the subjects. In Ahvaz district, positive samples were found in dogs that were fed homemade food, and their oral hygiene was very poor. Table 8 shows the frequency of *Fusobacterium* infection based on food consumption. The table revealed that the relative abundance of positive cases in dogs fed with homemade food was significantly higher than in those fed with dry food (P<0.001). The frequency of *Fusobacterium* in dogs fed with homemade food and the combination of dry and homemade was significantly higher than dry (P<0.001). Univariate logistic regression showed that the type of consumed food explained 27.6% of the changes for infection with this bacterium.

Table 8: Distribution of absolute and relative frequency of *Fusobacterium* infection in referred dogs of Tehran and Ahvaz districts, according to the type of food

Food	Total		Positive		Negative	
	Relative (%)	Absolute	Relative (%)	Absolute	Relative (%)	Absolute
dry[b]	34	51	0.0	0	100	61
homemade[a]	32.7	49	20.4	10	79.6	39
both	33.3	50	34.0	17	66.0	33
total	100	150	18	27	82	123

Different lowercase letters indicate the significance between different groups based on nutrition

Multivariable logistic regression showed that age, gender, breed, periodontitis, district, and type of food explained 97.6% of the changes for infection and only gender and periodontitis had a significant effect on infection (P<0.001). Based on the history and blood sample, none of the cases had a history of underlying disease or the administration of corticosteroid drugs.

Discussion

The obtained results of the present study showed that twenty-seven out of 150 samples were infected with *Fusobacterium*. The percentages of the relative frequency of these bacteria were

21.3% and 14.6% in Tehran and Ahvaz, respectively. Survey of *leukotoxin* gene in 18 samples of *F. necrophrum* showed that 11 samples had this gene. The presence of *fadA* gene in *F. nucleatum* isolated from dental plaques of dogs suffering from periodontitis and *leukotoxin* gene in *F. necrophorum* subspecies *necrophorum* were not significant in creating of periodontitis related to two different districts of Tehran and Ahvaz. Most of the dog's populations had gingivitis, which can

progress to periodontitis with increasing of age.

The development of periodontitis is influenced by factors such as age, breed, diet, and oral hygiene. It is important to study the bacteria involved in this disease and the presence of virulence genes for prevention, control, and treatment. *Fusobacterium spp*. plays an important role in causing of periodontitis. Several studies have shown the presence of different bacteria of the sub-gingival samples from dogs with gingivitis or periodontitis (Jamshidi et al, 2005; Stella et al, 2018). In the current study, all positive samples for the presence of the *Fusobacterium* genus were found in dogs with periodontitis. One hundred and thirty out of 150 samples, had some degree of periodontitis, and the prevalence of *Fusobacterium* genus in these was 20%. In this sense, it is consistent with the results of some researchers (Senhorinho et al, 2012). In the present study, the prevalence of *F. nucleatum* was higher than *F. necrophrum*, which confirms the importance of *F. nucleatum* in periodontitis and it is aligned with the results of Zhou et al, (2009); and the lower prevalence rate of *F. necrophorum* and *F. funduliforme* subspecies is consistent with the results of some other researchers indicating that this subspecies is not very pathogenic and it is less important in periodontitis (Antiabong et al, 2013). The presence of *Fusobacterium* genus and its species and even virulence factors were not significant in two districts; probably the geographical factors had no effect on the presence of these bacteria.

According to a survey by Liu et al, (2014), it was reported that the prevalence of *F. nucleatum/fad*A was observed significantly higher in human patients with periodontitis; but in the present study in dogs with periodontitis, this relationship was not significant. This is probably due to the limited number of samples and species differences. The *F. nucleatum* carrying *fad*A may have a higher pathogenicity and can lead to a classification of these strains, which is more closely related to the development of high grade of periodontitis rather than gingivitis.

In the present study, 11 out of 18 tested samples were positive for *F. necrophorum* and carried the *leukotoxin* virulence gene. However, there was no significant correlation between the presence of this gene and the prevalence of periodontitis in these samples. This lack of significance may be due to the specificity of the cytotoxicity of this gene for other species(Nagaraja et al, 2005).

Considering the relationship between the prevalence of *Fusobacterium* infection in gender, according to Table 4, the infection rate was higher in males; so, the gender played an important role in the infection rate with this bacterium. Regarding the relationship between gender and periodontitis and infection with *Fusobacterium*, there was no comprehensive and accurate information available. Some studies had reported that there was no relationship between them, such as the results of some researchers who reported that there was no relationship between periodontitis disease and gender (Carreira et al, 2015; Stella et al, 2018). The previous results have shown that the prevalence of *Enterococci* and *Yersinia* were 30% and 1% respectively, in dogs of Ahvaz district by PCR (Mosallanejad et al, 2023; Mosallanejad et al, 2024). In the present study, the degree of infection had a significant relationship with gender and it was more in males. In old or spayed female dogs, this was probably due to a decrease in estrogen levels and osteoporosis and a decrease in the density of the bone tissue of the mandible and maxilla was observed (Carreira et al, 2015).

In the present study, no significant relationship was observed between the levels of infection in two different districts. We aimed to investigate the influence of geographical and cultural conditions on hygiene and dental care in Ahvaz and Tehran, and it was found that the district had

no significant effect on the prevalence of infection. In Iran, a few studies have been done in this regard. In a study on 300 dogs, referred to the small animal hospital of the Faculty of Veterinary Medicine of the University of Tehran, it was reported that the prevalence of gingivitis and periodontitis was 24% and 12%, respectively (Jamshidi et al, 2005). Unlike the recent results, the obtained data of the present study showed that the prevalence of periodontitis in both districts was higher than gingivitis. This issue is probably due to the fact that dogs over two years of age were selected in both districts and dogs over two years of age are more likely to have periodontitis than gingivitis. It seems that the occurrence of periodontitis disease, the presence of *Fusobacterium spp.* and its virulence genes are independent of the geographical conditions.

As previously reported in companion dogs, increasing of age was associated with increased risk of periodontitis. It has been hypothesized that this can be due to a decline in immune function with getting older (Wallis et al, 2020). Additional studies conducted from 1968–1987 showed an association between increasing of prevalence of periodontitis and increasing of age (Harvey et al, 1993). In the present study, it was also found that the prevalence of periodontitis increased with age and the level of infection with *Fusobacterium* was higher at the age of 5 years and older. It was reported that the prevalence of *Fusobacterium* increases with age and the progression of periodontitis is more in higher grades, and this is probably due to the weakness of the immune system in the elderly due to the production of interleukin I (IL-1) by the lipopolysaccharide (LPS) of periodontopathogenic pathogens such as *Fusobacterium* (Hennet et al, 1991). It also increases the production of lymphokines, including T cell growth factor (IL-2) and osteoclast activating factor (Jewett et al, 2000). According to the present study, the rate of infection with *Fusobacterium* was the highest in periodontitis grade 3. The higher prevalence of *Fusobacterium* in dental plaques in periodontitis grade 3 may be due to the sampling method. Sampling of more severe dental plaques is easier than gum pockets or teeth without plaque, but with gingivitis. Another reason can be that the prevalence of *Fusobacteria* without the *fad*A gene is lower, in more severe periodontitis.

Breed is one of the most influential factors in the prevalence of periodontitis. The previous studies had proven that small breed dogs were more prone to this disease (Stella et al, 2018; Dos Santos et al, 2019; Wallis et al, 2020).

According to Table 6, the most breeds with *Fusobacterium* infection were White Terrier, Poodle, Pomeranian and Shih Tzu. Probably, the prevalence of *Fusobacterium* in these breeds is higher, which makes them prone to periodontitis. In small breed dogs, it may be associated with the challenges related to brushing the teeth of very small dogs, greater reluctance in smaller dogs to accept dental chews and a reputation for fussy eating habits (Mateo et al, 2020). In another survey, on 22,300 dogs, 18 breeds were very susceptible to periodontitis, one of which is poodles (O'Neill et al, 2021). In the obtained results from our study, periodontitis in poodles and the presence of *Fusobacterium* in them was more, which is in agreement with the above results. According to the characteristics of the poodle breed, which is very energetic, it is very difficult for the owners to brush their teeth, and this is one of the reasons for this breed's susceptibility to periodontitis. Perhaps it is better to compare the oral flora of these breeds in the condition of healthy gums and periodontitis in the future studies. In this sense, our study was consistent with some researchers (Wallis et al, 2020). Brachycephalic breeds have 1.25 times the odds of periodontal disease compared with mesocephalic breeds and Spaniel types have 1.63 times the odds compared with non-spaniel types (Wallis et al, 2020). In the

present study, no such results were obtained, due to the low number of this breed.

Nutritional factors have the potential to affect oral tissues and therefore may play an important role in the development of periodontal disease (Wallis et al, 2020). It has been reported that feeding pets with commercial food reduces the incidence of periodontitis (Gorrel, 1998). Daily use of a toothbrush and dental chews is essential for preventing periodontitis in dogs. Homemade food may lack important minerals and vitamins such as zinc, calcium, vitamin C, and B complex, which can also contribute to periodontal disease in dogs (Mateo et al, 2020; Wallis et al, 2020). The present study revealed that dogs fed with homemade food had a significantly higher incidence of *Fusobacterium* infection and periodontitis compared to dogs with commercial diets. Additionally, these dogs exhibited poor oral and dental hygiene, which is a known factor contributing to the increased risk of periodontitis. In conclusion, the presence of *fad*A gene in *F. nucleatum* isolated from dental plaques of dogs suffering from periodontitis and *leukotoxin* gene in *F. necrophrum* subspecies *necrophrum* in two different districts of Tehran and Ahvaz were not significant for periodontitis. In the present study, age, gender, breed, periodontitis, district and type of food explained 97.6% of the changes in *Fusobacterium* infection and only gender and periodontitis had a significant effect on infection. Future work should focus on the relationship between the presences of *Fusobacterium* virulence genes in different degrees of periodontitis and in a larger number of samples. It is also possible to correlate the presence of *Fusobacterium* and *fad*A genes in the dental plaques of dogs and their owners. As dogs age, the probability of periodontitis increases and the owner's emotional dependence on the animal increases also; so, this issue becomes more important for the presence of other zoonotic and pathogenic bacteria between human and dogs.

Acknowledgment

The authors express their gratitude to the Research Council of Shahid Chamran University of Ahvaz for its financial support.

Conflict of Interest

The authors declare that they have no known conflict of interest.

Funding

This research was carried out in the form of a thesis with the financial support of Shahid Chamran University of Ahvaz.

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Received: 29.04.2024
Accepted: 03. 08 .2024

DOI: 10.22055/ivj.2024.454864.2710

تعیین مولکولی گونه‌های فوزوباکتریوم در سگ‌های مبتلا به (یا بدون) ژنژیویت/ پریودونتیت در اهواز و تهران

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تاریخ دریافت: ۱۴۰۳/۲/۱۰ تاریخ پذیرش: ۱۴۰۳/۵/۱۳

چکیده

پریودونتیت، یکی از شایع‌ترین بیماری‌ها، در حیوانات خانگی می‌باشد. باکتری‌های محوطه دهانی، نقش مهمی در ایجاد این عارضه دارند. گونه‌های فوزوباکتریوم، یکی از فاکتورهای مهم باکتریایی است که در پیشرفت پریودونتیت نقش مهمی دارند. هدف از انجام مطالعه حاضر، بررسی ارتباط بین حضور فوزوباکتریوم و تحت گونه‌های آن و ژن‌های حدت آن‌ها (fadA و لکوتوکسین)، در سگ‌های مناطق اهواز و تهران، با یا بدون ژنژیویت/ پریودونتیت می‌باشد. یکصد و پنجاه قلاده سگ (۷۵ سگ از اهواز و ۷۵ سگ از تهران)، بین ۱۱-۲ سال، که ۷۸ سگ نر و ۷۲ قلاده ماده بودند، در طی ۱۰ ماه نمونه‌برداری شدند. نژادهای عمده مورد مطالعه شامل ترییر سفید، پودل، پامرانین، شیتزو، یورکشایر ترییر، پاگ، اشپیتز، مالتیز و بقیه از نژادهای دیگر بودند. آن‌ها از غذای خانگی، خشک و مخلوط تغذیه شده بودند. ۲۰ قلاده سگ (۱۳/۳۳ درصد) لثه سالم ،۳۲ مورد، پریودنتیت درجه ۱ (۲۱/۳۳ درصد) ۴۷ قلاده دیگر، پریودونتیت درجه ۲ (۳۱/۳۳ درصد) و ۵۱ سگ، پریودونتیت درجه ۳ (۳۴ درصد) داشتند. ۲۷ مورد از ۱۵۰ نمونه مورد مطالعه، مبتلا به جنس فوزوباکتریوم بودند (۱۸ درصد؛ ۲۴/۱- ۱۱/۸ درصد= $CI_{\%95}$). فروانی نسبی این آلودگی در تهران و اهواز به ترتیب ۲۱/۳ (۳۰/۶-۱۲/۰= $CI_{\%95}$) و ۱۴/۶ (۲۲/۶-۶/۶ درصد= $CI_{\%95}$) بودند. بررسی ژن لکوتوکسین در ۱۸ نمونه *Fusobacterium necrophorum* نشان داد که ۱۱ نمونه (۶۱/۱۱ درصد) (۹ نمونه از تهران و ۲ نمونه از اهواز) دارای این ژن بودند و این تفاوت مشاهده شده در دو شهر، در حضور این ژن، از نظر آماری معنی‌دار نبود ($p\text{-value}=0.43;\ df=1;\ X^2=0.62$). از این ۲۶ نمونه، ۹ نمونه (۳۴/۶۱ درصد) دارای ژن حدت *fad*A بودند که ارتباط بین وجود ژن *fad*A و درجات پریودنتیت از نظر آماری معنی‌دار نبود ($p\text{-value}=0.41;\ df=1;\ X^2=0.68$). رگرسیون لجستیک چند متغیره نشان داد که سن، جنس، نژاد، پریودونتیت، منطقه و نوع غذا ۹۷/۶ درصد از تغییرات آلودگی را توجیه می‌کند و تنها جنسیت و پریودونتیت تأثیر معنی‌داری برمیزان آلودگی داشتند. حضور ژن *fad*A در *فوزوباکتریوم نوکلئاتوم*، جدا شده از پلاک‌های دندانی سگ‌های مبتلا به پریودنتیت و ژن لکوتوکسین در *فوزوباکتریوم نکروفروم* تحت گونه *نکروفروم*، در پریودونتیت واقع در مناطق مختلف تهران و اهواز معنی‌دار نبود. در قسمت نتیجه‌گیری، میزان شیوع فوزوباکتریوم در پریودنتیت درجه سه، ۱۶ درصد و در لثه‌های سالم ۲ درصد بودند.

کلمات کلیدی: بیماری‌های پریودونتال، *فوزوباکتریوم*، لکوتوکسین، ژن *fadA*، ژنژیویت، سگ

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