

Manuscript Title: **Evaluation of *Helicobacter* spp. and *Wolinella* spp. in the Oral Cavity
of Dogs with Periodontitis and Screening of the Presence of *Helicobacter pylori*
Virulence Genes in *Wolinella*-positive Samples by PCR**

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Abstract

Introduction: Oral *Wolinella* spp. have been reported to be more prevalent than closely related *Helicobacter* spp. in canines. Although *Wolinella succinogenes* is considered nonpathogenic, complete genome analysis has revealed that it carries several genes homologous to sequences associated with virulence or host interaction in *Helicobacter pylori*. This study evaluated the prevalence of the genera *Wolinella* and *Helicobacter*, and *H. pylori* in the canine oral cavity in patients suffering from periodontitis and screened selected *H. pylori*-associated virulence genes *babA* and *cagA* in the *Wolinella*-positive samples.

Materials and Methods: Samples were collected from the oral cavity of sixty-two canine patients with periodontitis. PCR assays targeting 16S rRNA were used to detect *Wolinella* spp. and *Helicobacter* spp. Samples positive for the genus *Helicobacter* were further evaluated for *H. pylori* using the *ureC* gene. *Wolinella*-positive samples were screened for the presence of the *babA* and *cagA* genes.

Results: 52/62 samples were positive for *Wolinella* spp., whereas *Helicobacter* spp. were detected in 36/62 dogs. No *H. pylori* was detected in any *Helicobacter*-positive oral sample. Neither the *babA* nor the *cagA* gene was detected in any *Wolinella*-positive sample.

Conclusion: *Wolinella* spp. showed a high prevalence and were detected more frequently than *Helicobacter* spp. in oral samples from dogs with periodontitis. However, *babA* and *cagA* were not detected in the oral samples examined in the current study. This suggests that these selected *H. pylori*-associated virulence genes are unlikely to explain the putative pathogenic relevance of *Wolinella* spp. in canine periodontitis.

Keywords: Dogs; *Helicobacter*; *Helicobacter pylori*; Virulence factors; *Wolinella*

1. Introduction

The genus *Wolinella* belongs to the family Helicobacteraceae. *Wolinella succinogenes*, known as the lone recognized species of this genus, inhabits the upper gastrointestinal tract, whose epithelium is lined with squamous cells. So far, the organism has not been reported in the canine lower gastrointestinal tract [1-5]. Compared to *Helicobacter* spp, *Wolinella* spp. are more prevalent in the canine oral cavity [3, 5]. Although not conclusively proven pathogenic, a putative novel *Wolinella* species, Candidatus *Wolinella africanus*, has been identified in samples from the upper gastrointestinal tract and might contribute to related diseases in humans and animals [1, 2, 6, 7]. The morphological characteristics of Candidatus *W. africanus* remain unknown due to the inability to isolate and culture it in laboratory conditions. According to the complete genome sequencing of *W. succinogenes*, this “host-associated” organism has a significant number of homologous gene sequences with *H. pylori* in common, including genes that are responsible for encoding the virulence factors like the type IV secretory pathway, adhesins, cytotoxins, and other factors, that contribute to the pathogenicity of *H. pylori* [9]. *Wolinella succinogenes* might have acquired these genomic regions, named as genomic islands, through lateral and horizontal gene transfer [8, 9]. Gene sequences of *W. succinogenes* that are homologous to those in the T4SS of *H. pylori* might be essential for symbiosis and persistence within its ecological niches. However, *Wolinella* might also possess *H. pylori* virulence genes and contribute to a potential pathogenicity [9]. The virulence gene *cagA*, located in the T4SS of the *H. pylori*, is associated with ulcerative gastritis and gastric adenocarcinoma [10]. Although the pathogenic role of *H. pylori* in canine gastrointestinal is not fully clarified, *cagA*-positive genotype strains of this organism have been detected in fecal and gastric biopsies of Iranian dogs [11, 12]. Primers targeting *babA* and *cagA* have been utilized for the molecular detection of *H. pylori* in the upper gastrointestinal tract of humans [13-15]. The high reports of *H. pylori* in the upper aerodigestive tract might be the result of cross-reactivity between targeting primers

and homologous genetic sequences [16, 17]. The current study was designed to evaluate the prevalence of *Helicobacter* spp. and *Wolinella* spp. in the canine oral cavity of patients diagnosed with periodontal disease and to screen *Wolinella*-positive oral samples for the presence of selected *H. pylori*-associated virulence genes *babA* and *cagA*.

2. Materials and Methods

2.1. Ethical issues

The study was approved by the Research Ethics Committees of the Faculty of Veterinary Medicine, University of Tehran (reference number #IR.UT.VETMED.REC.1401.005). During the clinical examination, all samples were collected with the owners' full consent regarding national and international animal welfare protocols.

2.2. Animal and Sampling Procedures

Samples were obtained from sixty-two indoor- and outdoor-living dogs. All dogs were older than two years and were diagnosed with various stages of periodontal disease at the Small Animal Hospital of the Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. Patients were carefully examined, and based on clinical signs, which varied from increased pocket probing depth (>0.3 mm) to alveolar bone loss, were classified into four groups (stages 1-4) [18]. In terms of dental care, we categorized the dogs into three groups: none, poor, and sufficient. A mixture of saliva and biofilm formed on the upper and lower molar teeth was collected using a sterile cytobrush. Samples were suspended in 1 mL of sterile phosphate-buffered saline (PBS) and preserved at -20°C prior to molecular processing.

2.3. DNA Extraction and PCR Assays

DNA extraction from specimens was performed using a SinaPure DNA tissue kit (Sinaclon; Iran) following the instructions provided by the manufacturer. The genera *Wolinella* and *Helicobacter* were detected using their respective 16S rRNA genes. Samples that were positive for *Helicobacter* 16S rRNA were further evaluated for *H. pylori* using the *ureC* gene.

Additionally, samples positive for *Wolinella* 16S rRNA were evaluated for the presence of the *babA* and *cagA* genes. Primer sequences, PCR cycling conditions, number of cycles, and amplicon sizes are summarized in Table 1. PCR assays were performed in a Thermocycler (SensoQuest, Germany). Each reaction mixture contained 3 μ L of extracted DNA, 2 μ L of $\times 10$ PCR buffer (Sinaclon; Iran), 0.5 μ L of dNTP, 0.75 μ L of $MgCl_2$, 0.5 μ L of each forward and reverse primer, 0.25 U of *Taq* DNA polymerase (Sinaclon; Iran), and 17.5 μ L of dH_2O , for a final volume of 25 μ L. Positive controls were included for *Helicobacter* 16S rRNA PCR and *H. pylori ureC* PCR. A negative control of PBS was included in each PCR run. Confirmed positive-control DNA for *babA* and *cagA* was not available; therefore, these assays were performed according to previously published protocols. After PCR, 10 μ L of the PCR products were electrophoresed on a 1.5% (w/v) agarose gel prepared in $1\times$ Tris-borate ethylenediaminetetraacetic acid buffer containing 0.5 μ g/mL ethidium bromide. Bands were visualized and photographed under ultraviolet (UV) transillumination. Amplicon sizes were estimated using a 100 bp DNA ladder.

Table 1. Primer sequences, PCR cycling conditions, number of cycles, and amplicon sizes for the PCR assays used in the present study

Gene name	Primer sequences (5'-3')	PCR condition	Cycle s	Size (bp)	Ref .
16SrRNA (<i>Helicobacter</i> spp.)	F: GCTATGACGGGTATCC R: ACTTCACCCCAGTCGCTG	Initial denaturatio n: 94°C, 10 min Denaturatio n: 94°C, 1 min Annealing: 58°C, 90 sec. Extension: 72°C, 2 min Final extension:	35 $\times$	120 0	[3]

								72°C, 15 min
								Initial denaturation: 95°C, 15 min Denaturation: 95°C, 30 sec Annealing: 58°C, 30 sec Extension: 72°C, 45 sec Final extension: 72°C, 7 min Initial denaturation: 95°C, 3 min Denaturation: 94°C, 1 min Annealing: 52°C, 1 min Extension: 72°C, 1 min Final extension: 72°C, 5 min Denaturation: 95°C, 1 min Annealing: 56°C, 1 min Extension: 72°C, 1 min Final extension:
16SrRNA (<i>Wolinella</i> spp.)	F: AAAGAGCACGTAGGCGGC R: CAGGATTCTATCAATGTCAAGCCC					35 ×	440	[3]
<i>ureC</i> (<i>H. pylori</i>)	F: GGATAAGCTTTTAGGGGTGTTAGG GG R: GCTTACTTTCTAACAATAACGCGC					35 ×	294	[14]
<i>cagA</i>	F: GATAACAGGCAAGCTTTTGAGG R: CTGCAAAAGATTGTTTGGCAGA					35 ×	349	[19]

72°C, 7
min

Initial
denaturatio
n: 95°C, 5
min

Denaturatio
n: 92°C, 1
min

Annealing:
59°C, 1
min

Extension:
72°C, 1
min

Final
extension:
72°C, 5
min

babA
F:
AATCCAAAAAGGAGAAAAAGTAT
GAAA
R:
TGTTAGTGATTTCGGTGTAGGACA

34 × 832 [15
]

2.4. Sequencing and Phylogenetic Tree Construction

The 16S rRNA amplicon obtained from one *Wolinella*-positive oral specimen was subjected to Sanger sequencing (Genomin Co., Iran). The obtained sequence was compared to reference sequences available in the National Center for Biotechnology Information (NCBI) database using BLAST. Closely related sequences were extracted from GenBank for inclusion in the phylogenetic analysis. MEGA7 software was deployed for sequence alignment and phylogenetic analysis. [20]. The phylogenetic tree in this study was constructed using both Neighbor-joining and Maximum-likelihood algorithms with a Tamura-Nei matrix and 1,000 bootstrap replicates [20,21]. After removing all positions with gaps or missing data, the final evolutionary matrix retained a total of 377 positions across 75 nucleotide sequences [21].

2.5. Statistical analysis

Primary data management and descriptive statistic calculations were conducted within Microsoft Excel 2016. Comparative statistical modeling was executed using SPSS version 18.

Fisher's exact test was also performed to analyze the data distribution. The *P*-value below 0.05 was interpreted as statistically meaningful.

3. Results

3.1. PCR results

The 16S rRNA genes specific to the genera *Helicobacter* and *Wolinella* were identified in 36 out of 62 (58.06%) and 52 out of 62 (83.87%) oral samples from dogs, respectively. Among the 57 dogs positive for either *Wolinella* spp. or *Helicobacter* spp. genus, 31 samples were positive for both genera, 21 were positive only for *Wolinella*- specific 16S rRNA, and 5 were positive only for non-*pylori* *Helicobacter* spp. Figure 1 shows genus-specific PCR products for *Wolinella* spp. and *Helicobacter* spp. All *Helicobacter*-positive samples were negative for the *H. pylori ureC* gene. Neither the *cagA* nor the *babA* gene was detected in any *Wolinella*-positive sample.



Figure 1. PCR products for genus-specific 16S rRNA (A) *Wolinella* spp. (440 bp), (B) *Helicobacter* spp. (1200 bp)

3.2. Statistical results

Most dogs in this study were young (2–6 years old), accounting for 74.19% of the statistical population, and a significant portion lived indoors (64.52%). Notably, 88.71% of dogs across various stages of periodontitis did not receive proper dental care. In the preliminary stages of periodontitis (Stages 1 and 2), females were slightly more dominant (55.26%), whereas in

severe stages (Stages 3 and 4), males accounted for a higher proportion (62.50%). Dogs with milder periodontitis (Stages 1 and 2) were predominant, forming 61.30% of the study population. Regarding *Wolinella* positivity, the highest rates were observed in Stage 2 (37.10%) and Stage 3 (25.80%), while lower rates were recorded in Stage 4 (8.06%). There was a slight difference in the *Wolinella*-positivity rate between dogs living indoors and outdoors; 87.50% and 81.80%, respectively. (Table 2)

Table 2. Descriptive analysis of samples (PD: Periodontal disease)

PD stage	Count (%)	Age		Gender		Dental care	
		2-6	>6	Female	Male	None/Poor	Sufficient
1-2	38 (61.30%)	35	3	21	17	31	7
3-4	24 (38.70%)	11	13	9	15	24	0

3.3. BLAST results and Phylogenetic Analysis

BLAST results from partial sequencing of the *Wolinella*-positive PCR product in NCBI revealed that our 16S rRNA had its closest hit (99% identity) with other sequences in the databases. The sequence data were submitted to the GenBank database under accession number ON845539. The phylogenetic tree is presented in Figure 2, illustrating the clustering of the isolate with closely related taxa.

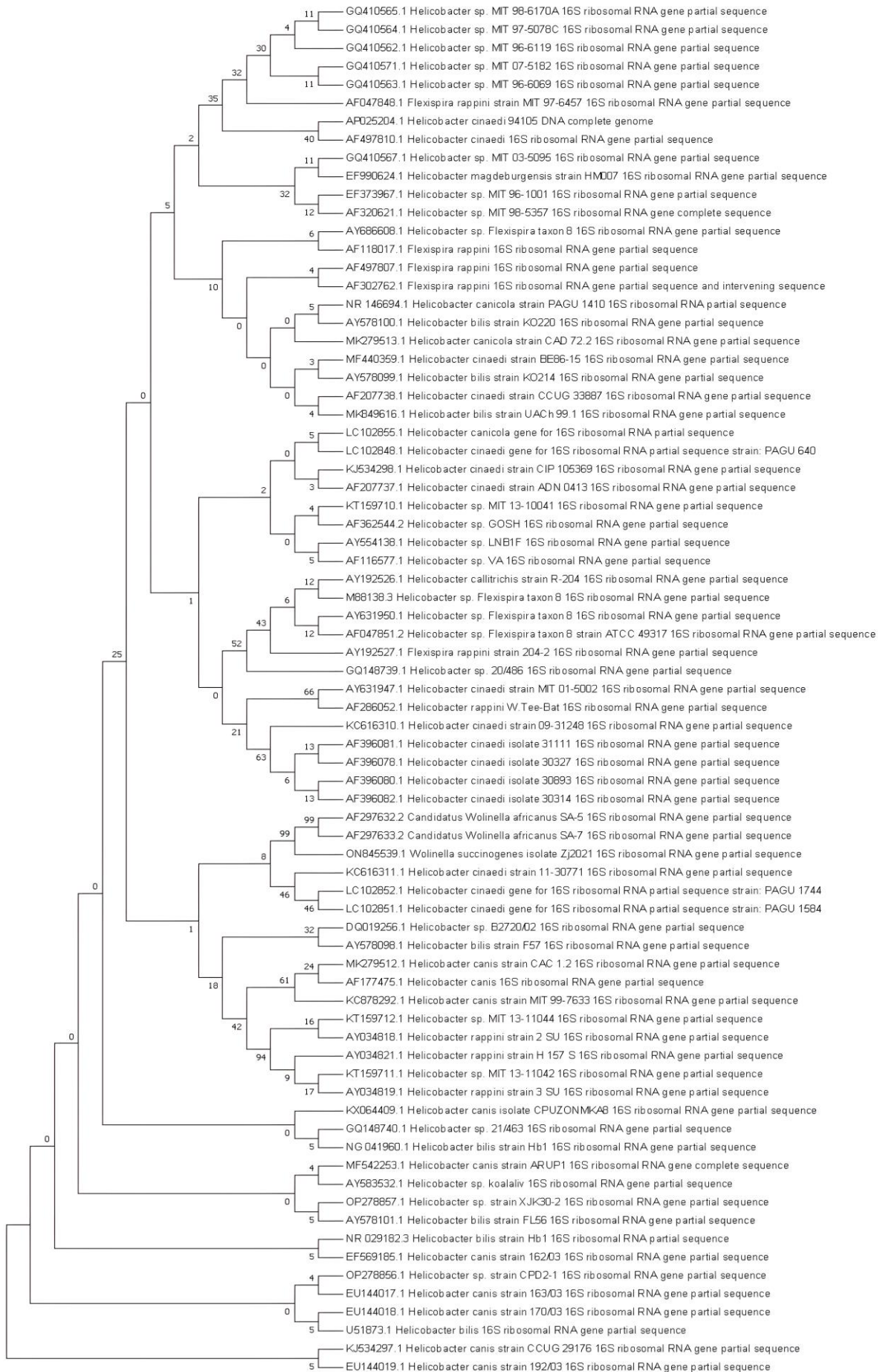


Figure 2. Phylogenetic reconstruction of the *Wolinella* genus based on 16S rRNA gene partial sequencing. As shown in the phylogenetic tree, across both tree reconstruction algorithms, the target 16S rRNA gene sequence (*Wolinella succinogenes* isolate Zj2021) demonstrated a low genetic distance to Candidatus *Wolinella africanus* strain SA-7, followed closely by *Helicobacter cinaedi* strain 11-30771.

4. Discussion

Among the *Helicobacteraceae* population in specimens obtained from the canine oral cavities enrolled in the current study, *Wolinella* spp. (83.87%) were detected more frequently than *Helicobacter* spp. (58.06%). Moreover, positive samples for *Helicobacter* spp. were associated with non-*pylori* species. Our results were in line with former studies [3-5]. In a controlled study including both healthy dogs and dogs with periodontitis, Nowroozilarki et al. reported a significantly higher detection rate of *Wolinella* spp. in dogs with periodontitis than in healthy dogs [5]. However, the present study included only dogs with periodontitis and clinically healthy dogs were not sampled. Therefore, our data cannot determine whether the prevalence of *Wolinella* spp. differs between healthy dogs and dogs with periodontitis, nor can they independently establish an association between *Wolinella* spp. and periodontitis. Moreover, no significant association was observed between *Wolinella* spp. detection and the progression of periodontal disease stages in the present study ($P > 0.05$).

A novel uncultivated taxon of *Wolinella* known as Candidatus *Wolinella africanus* was documented in oral and gastric specimens from clinically asymptomatic Venezuelan cohorts as well as esophageal biopsies from squamous cell carcinomatous patients in Africa [1, 2]. Expanding on veterinary pathology findings, Oxley et al. reported a similar sequence to the *W. succinogenes* in biopsies from gastric ulcers of an Australian sea lion [6]. In an equine medicine practice report, although some stomach samples of thoroughbred horses with gastric ulcers yielded 99% homology to *Helicobacter pylori*, Contreras et al. did not detect the *H. pylori*-

specific genes in any of these samples. Due to a lack of follow-up isolation assays, the precise identification of the organism left unresolved. [22]. Detection of *W. succinogenes*-like organisms in colon samples from pediatric individuals with chronic intestinal diseases, as well as in colonic samples from patients with colorectal polyps, challenges the traditional assumption that *Wolinella* species are entirely nonpathogenic commensals [16, 23]. Bulut *et al.* evaluated the presence of *cagA*-positive *H. pylori* strains in tonsil and adenoid tissues of children suffering from recurrent adenotonsillitis or adenotonsillar hypertrophy, suggesting a potential link between *cagA* genotypes of *H. pylori* and the adenotonsillar hypertrophy [13]. On the contrary, Cedeño *et al.* detected no *H. pylori* among any evaluated specimens from pediatric adenoid tissues utilizing *cagA* and *babA* genes as inclusive markers [14]. Deploying a highly sensitive and specific nested RT-PCR assay evaluating the Family Helicobacteraceae, Hussey *et al.* detected Candidatus *W. africanus* as the sole representative of the family within a hyperplastic adenoid sample from a child with symptoms of extraesophageal reflux. However, the inability to either isolate or culture Candidatus *W. africanus* limited understanding of its morphological characteristics and pathogenic potential [7]. More recently, a Chinese gastric cancer dataset revealed that *Wolinella* emerged as a dominant taxon and served as a core network hub, despite the absence of *Helicobacter*. This network architecture suggests that *Wolinella* may possess functional properties or behavioral parameters similar to those of *Helicobacter* [24]. According to sequence alignment and database BLAST comparison, the representative *Wolinella*-positive 16S rRNA amplicon sequenced in this study was most closely related to *W. succinogenes* rather than Candidatus *W. africanus*. However, a clear limitation of the present study is that only one *Wolinella*-positive 16S rRNA PCR product was sequenced. Consequently, the sequence diversity of *Wolinella* spp. in the examined oral samples could not be fully assessed, and the PCR-positive results should be interpreted at the genus level.

Given the limited data regarding the pathogenic profile of the genus *Wolinella*, the current molecular screening targeted the presence of *babA* and *cagA* loci within *Wolinella*-positive oral samples. This screening was exploratory and does not imply that oral *Wolinella* spp. are expected to contain typical *H. pylori* virulence genes. The rationale for this screening was based on previous comparative genomic report showing that *W. succinogenes* shares several homologous genes with *H. pylori* [9]. These two virulence genes are predominant among Iranian patients presenting with *H. pylori*-associated ulcerative gastritis or gastric malignancies [15]. Moreover, the *cagA* genotype of *H. pylori* was found in gastric biopsies and fecal samples from Iranian dogs with the incidence of gastric ulcers [11, 12]. Despite the high number of homologues shared between *Wolinella* spp. and *H. pylori*, the selected *H. pylori*-associated virulence genes *babA* and *cagA* were not detected in any *Wolinella*-positive sample using the PCR assays applied in this study. It should also be noted that negative PCR results for these genes do not exclude the presence of divergent homologous sequences or other virulence-associated genes in *Wolinella* spp. Therefore, the possible pathogenic potential of oral *Wolinella* spp. may be explained by other mechanisms that require further investigation.

5. Conclusion

The current study showed that *Wolinella* spp. had a high prevalence and were detected more frequently than *Helicobacter* spp. in oral samples from dogs with periodontitis. However, because clinically healthy dogs were not included, these findings should be interpreted as the detection and prevalence of these bacteria in dogs with periodontitis, rather than as evidence of an association or causative link. Because *H. pylori* was not detected among *Helicobacter*-positive oral samples, the *Helicobacter* spp. detected in the current study were likely non-*pylori* *Helicobacter* organisms. The selected *H. pylori*-associated virulence genes *babA* and *cagA* were not detected in *Wolinella*-positive samples using the PCR assays applied in this study.

Further controlled studies are required to clarify the diversity and possible pathogenic relevance of oral *Wolinella* spp. in canine periodontal disease.

Acknowledgment

The authors express their sincere appreciation to Dr. Sepideh Asadi for her technical guidance and exceptional support through the preparation of this study.

Compliance with ethical guidelines

The experimental design and frameworks received official approval from the Institutional Review Board and Research Ethics Committees at the Faculty of Veterinary Medicine, University of Tehran (Reference Number: #IR.UT.VETMED.REC.1401.005).

Data availability

The authors confirm that all data related to this study are provided in this manuscript.

Funding

Institutional resources allocated to the first author's thesis project at the Faculty of Veterinary Medicine, University of Tehran, Iran, supported this work; no external funding was received.

Authors' Contribution

Conceptualization and study design: B.N.F and S.J.; Supervision: B.N.F., S.J., and T.Z.S.; Methodology and investigation: Z.J. and I.A.T.; Data analysis: Z.J.; Original draft preparation: Z.J.; Review and editing: B.N.F., S.J., and T.Z.S. All authors read and approved the final manuscript.

Conflict of interest

There are no conflicts of interest to disclose by any of the contributing authors.

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