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HIGH PREVALENCE OF DNA FROM NON-*H. pylori* HELICOBACTERS IN THE GASTRIC MUCOSA OF VENEZUELAN PET DOGS AND ITS HISTOLOGICAL ALTERATIONS

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SUMMARY

Non-*H. pylori* helicobacters (NHPH) have been demonstrated as gastric spiral-shaped bacteria in specimens obtained from dogs; however, their roles in the pathogenesis of upper gastrointestinal disease have not yet been clearly established. The purpose of this study was to evaluate the prevalence of NHPH DNA in the gastric mucosa of dogs and its association with histopathology. *Helicobacter* was detected through histopathological techniques, PCR, and FISH analysis from fundic biopsies of twenty dogs with or without signs of gastrointestinal disease. PCR and FISH were based on partial 16S rRNA gene sequences. Nineteen dogs showed mild to marked gastritis in the fundus, and only one dog had a healthy gastric mucosa. NHPH DNA was detected in 18 dogs with gastritis and one with normal gastric mucosa. However, there was no significant correlation between the presence of NHPH DNA and the degree of gastritis. These results show a high prevalence of NHPH DNA in the gastric mucosa of dogs from Venezuela. Further studies are necessary to determine a possible association between a specific NHPH species and the degree of gastritis.

KEYWORDS: Gastric NHPH; 16S rDNA; Gastritis; Dogs; Venezuela.

INTRODUCTION

Helicobacter spp. colonizes the stomach and intestine of humans and several animal species[14]. The *Helicobacter* genus currently stands at about 38 formally named members with numerous other putative species under investigation[18]. A variety of gastric non-*H. pylori* helicobacters (NHPH) can infect the stomach (fundus, corpus and antrum) of pets, but their role in the pathogenesis of upper gastrointestinal disease is unclear[24]. *H. felis*, *H. bizzozeronii*, *H. salomonis, H. heilmannii* sensu stricto (s.s.), *H. cynogastricus* and *H. baculiformis* are gastric NHPH species commonly found in dogs and cats, while *H. suis* often colonizes the stomach mucosa of pigs[2,15,27]. All canine and feline associated gastric NHPH have been detected in the human stomach, except for *H. cynogastricus* and *H. baculiformis*, which have not yet been found[14]. Moreover, *H. heilmanni* s.s. and *H. felis* have been associated with gastritis in animals, and mainly in the antral mucosa from humans[12].

The prevalence of gastric NHPH in dogs and cats is high (>70%) and may not correlate with clinical signs and severity of gastritis[16]. However, the majority of these species are difficult to culture and, identification depends mostly on phylogenetic analysis to discriminate between the same species. Therefore, a growing number of gastric NHPH has been detected by genus-specific PCR assays, fluorescence *in situ* hybridization (FISH) targeting the 16S rRNA gene and sequencing of 16S and 23S rRNA-encoding genes and specific genes, such as *hsp60*, *gyrB* and *ureAB*[14]. Although the prevalence of gastric NHPH infection has been reported in dogs from different developed countries, little is known about the presence of NHPH in pet dogs from developing countries[23]. The aim of this study was to evaluate the prevalence of NHPH in the gastric mucosa of dogs from Venezuela and investigate its association with histopathological alterations.

MATERIALS AND METHODS

Animals: Clinical studies and sample collection were carried out at the Unidad de Investigación Quirúrgica Veterinaria de la Universidad Nacional Experimental "Francisco de Miranda" (UNEFM). Twenty pet dogs (nine males and 11 females; range, 0.8-9 years), nine with vomiting and 11 clinically healthy were recruited into this study. None of these dogs had been treated with antibiotics, corticosteroids, or antacids within the three weeks preceding sample collection. Gastric mucosal (fundus) biopsy specimens were taken for histological evaluation and rapid urease testing by Ure-IVIC1[10]. Samples were collected with Olympus endoscopes, GFX II (Japan) with standard 0.8-mm endoscopic biopsy forceps, during routine endoscopic examination of anesthetized dogs. Three mucosal biopsy specimens were obtained endoscopically from the stomach (fundus) of each animal; one was fixed in formaldehyde for histopathology and FISH, one was used for rapid urease testing by *HelicoTest*™ (formerly Ure-IVIC[10]), and one for DNA extraction and polymerase chain reaction (PCR) assays. The two last samples were

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immediately frozen in dry ice and subsequently stored at -80 ºC until experimental procedures were performed at IVIC. Clinical and sample collection protocols were previously approved by the UNEFM Bioethics Committee.

Histopathology: The fundus samples were fixed in formalin and embedded in Paraplast™ medium (Sigma, St. Louis, MO). Sections of each sample, 4 µm in thickness, were obtained and adhered onto Clearcell Thermo Fisher printed slides (Thermo Fisher Scientific, Portsmouth, NH), dewaxed in xylene (3 x 10 minutes), and subsequently rehydrated in 100% (3 x 10 minutes), 70% ethanol, and lastly in phosphate buffer solution pH 7.4 (PBS). The sections were used for FISH, and for staining by hematoxylin-eosin (H&E) and Warthin-Starry's techniques. The histopathological parameters were analyzed according to the World Small Animal Veterinary Association (WSAVA) guidelines[6]. The degree of morphological features and inflammatory changes were graded by using the WSAVA gastrointestinal standardization visual analog scale for normal, mild, moderate, and marked inflammation with a scoring system from 0 to 3, respectively.

Fluorescence-in Situ-hybridization (FISH): FISH was performed as previously described[30]. Briefly, the following treatments were performed before FISH: Immersion of the sections in 264 mM Sodium borohydryde for 20 minutes, 0.01% Triton X100 for 10 minutes, and 25% proteinase K for five minutes. Sections were rinsed three times with PBS at room temperature between steps. Three fluorescent oligonucleotide probes targeting the bacterial 16S rRNA gene were used in this study: two *Helicobacter* genus-specific probes, HEL274 (Cy3-5' labeled) and HEL717 (Cy3-5' labeled) to determine the presence of *Helicobacter* species in the gastric mucosa[5], and a probe for the universal bacteria domain EUB338 (AlexaFluor 488, N-5' labeled) as a positive control and to simultaneously visualize bacteria within intact tissue samples[1]. The probes were commercially synthesized by Integrated DNA Technologies (Dallas, TX). Hybridization was performed at 54 °C for 90 minutes and stringent washing at 56 °C for 10 minutes according to the protocol as described previously[30]. The bacteria were visualized with a Nikon Eclipse E600 epi-fluorescence microscope using cubes B-2A, G-2A and UV-2A for Alexa-488, Cy3 and DAPI respectively: the images were digitally acquired by a Nikon Coolpix 8700 camera. Probe specificity was verified by evaluating positive control (eg, slide prepared from a clinical strain culture of *H. pylori*) and negative control [slide prepared from a strain culture of *Shigella sp*. (ATCC11126)]. The fixation of control strains was performed according to a protocol described previously[26].

PCR assays: DNA from fundic biopsies was extracted using the QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen Inc, Valencia, CA). Helicobacteraceae and *Helicobacter* DNA were detected using family- and genus-specific PCR assays targeting 764 bp and 399 bp fragments of the 16S rRNA gene[4,13]. The presence of *H. pylori* DNA was ruled by using specific assays targeting a 294 bp fragment of the *glmM* gene and a 128 bp fragment of the *cagA* gene[22,25]. PCRs were performed using the Ready To-Go PCR beads kit (Amersham Biosciences Corp., Piscataway, NJ) in a thermal cycler model GeneAMP PCR System 9700 (Applied Biosystems, Hayward, CA). The positive control was *H. pylori* DNA and the negative control was a no-template PCR reaction. The amplicons were visualized by running the reaction mixture in a TBE agarose gel (2.0%), staining with ethidium bromide, and observing them using an UV transilluminator.

16S rRNA gene sequencing and sequence analysis: Helicobacteraceae-specific fragments (764 bp) of the 16S rRNA gene amplified were purified for sequencing using the QIAquick PCR Purification Kit (QIAquick PCR Purification Kit, Qiagen Inc, Valencia, CA), according to the manufacturer's recommendations. The primers used for sequencing of both DNA strands were BohrF (C97-20) and BohrR (H3A-20), as previously described[4]. Both strands of all nineteen purified amplicons were sequenced at the CeSAAN facility (IVIC, Altos de Pipe, Venezuela) with an ABI PRISM™ 3130xl Sequencer (Applied Biosystems, Foster, CA). Sequences were compared with the compilation of 16S rRNA gene sequences available in the GenBank nucleotide library by BLAST searching. The 16S rRNA gene sequences (~600-711 bp) of 19 amplicons were deposited in GenBank under the accession numbers GQ181184-GQ181202.

Phylogenetic analysis: Dog sequences together with closest GenBank matches were aligned in the *greengenes* website (http://greengenes.lbl.gov) using a multiple sequence alignment server for comparative analysis of 16S rRNA genes[8]. Chimera check was performed in the *greengenes* website[9]. The phylogenetic tree was constructed using the neighbor-joining method and the Jukes-Cantor model provided in Molecular Evolutionary Genetics Analysis 2.1 software (MEGA, version 4.0)[29], and the stability of grouping was estimated by bootstrap analysis (500 replications) using the same program.

Statistical analyses: A dog was considered infected if two or more test results were positive. The association between NHPH species detected and gastritis grade was evaluated by Spearman's rank correlation test. This was considered positive when $p \leq 0.05$.

RESULTS

Histopathological observations: Of the 20 dogs studied, only one (5%) presented a normal mucosa whereas 19 (95%) had histopathological changes representative of various degrees of gastritis in the fundic region of the stomach (Table 1). Of these, seven (36.84%) dogs had mild gastritis, which was associated mainly with diffuse intraepithelial lymphocytes infiltration and scarce leukocytes and focal degeneration of the surface epithelium (Fig. 1A). Two dogs (10.53%) had moderate gastritis, evidenced by epithelial degeneration, isolated glandular tissue, showing the presence of bacteria adhering to epithelial cells (Fig. 1B), whereas 10 (52.63%) presented marked gastritis, characterized by necrosis of the epithelium of the gastric pit; nested, atrophic and sparse lobules of glandular tissue, diffused lymphocyte infiltration, and glandular atrophy (Fig. 1C and D). Examination of hematoxylin-eosin (Fig. 2A and B) and Warthin-Starry (Fig. 2C and D) stained sections revealed

Table 1
Degree of gastritis and *Helicobacter* infection in dogs (n = 20)

Degree of gastritis	Non-infected	Infected
Normal	0	1
Mild	0	7
Moderate	0	2
Marked	1	9
Total	1	19

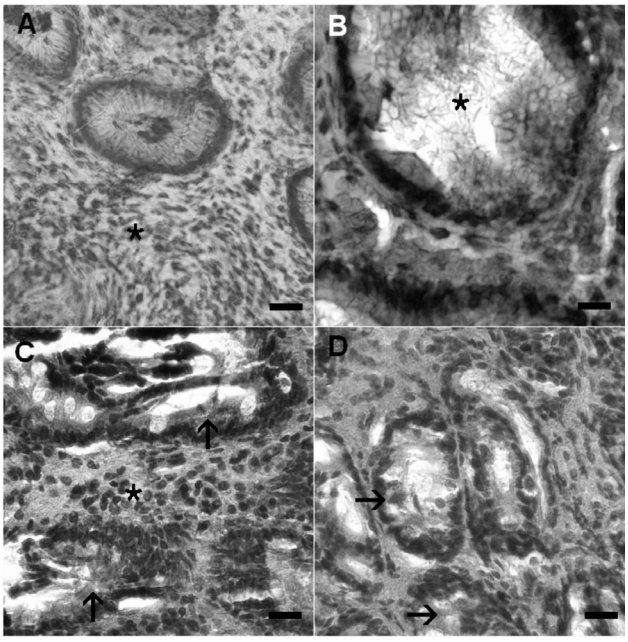

Fig. 1 - Gastric mucosa (fundus) biopsy sections; H&E stained. (A) Mucosa with mild gastritis with diffuse mononuclear cell infiltration (asterisk). (B) Gastric tissue with moderate gastritis showing the massive presence of bacteria (asterisk) adhering to epithelial cells. (C) Gastric tissue section showing marked gastritis with infiltration of inflammatory cells (asterisk) and glandular atrophy (arrows). (D) Necrosis of mucosa in marked gastritis; the arrows indicate destruction of epithelium in an infiltration zone. Bars: A, B, and C: 15 µm; D: 30 µm.

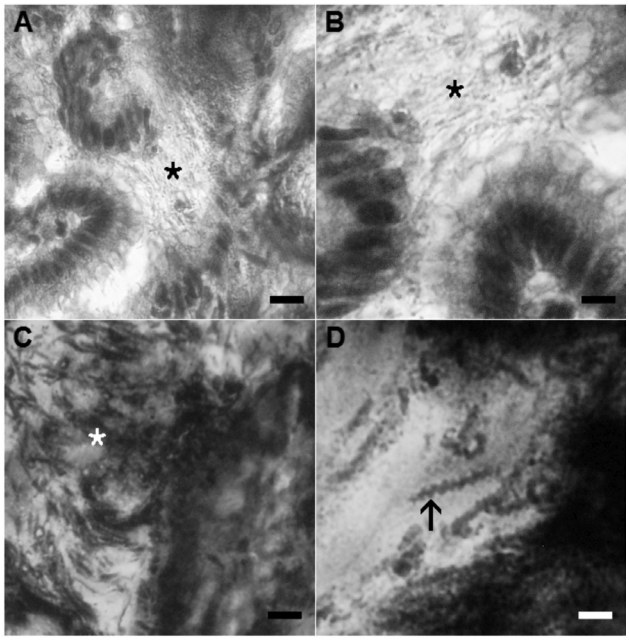

Fig. 2 - Gastric mucosa (fundus) biopsy sections. (A) Gastric tissue section stained by H&E showing massive presence of spiral-shaped bacteria in the gastric mucosa (asterisk). (B) Detail of image in A showing spiral-shaped bacteria. (C) Gastric tissue section stained by Warthin-Starry´s technique showing the presence of spiral-shaped bacteria in the gastric mucosa (asterisk). (D) Detail of image in C, showing the spiral-shaped bacteria (arrow). Bars: A: 25 µm; B, and C: 5 µm; D: 2.5 µm.

spiral-shaped bacteria in 19 out of 20 biopsies examined. In only one case with marked gastritis bacteria were not detected by these methods. However, there was a significant correlation between mild gastritis and the presence of bacteria (r = -0.572; p = 0.0083), but there was no statistical relationship between the degree of gastritis and clinical signs (vomiting) (mild r = -0.0316; p = 0.8948, moderate r = 0.1830; p = 0.4400, marked r = -0.0101; p = 0.9662).

NHPH infection status: Seventeen of the 20 dogs showed positive for NHPH by all four tests used to detect infection (histology, rapid urease test, PCR and FISH). Two out of 20 dogs were urease-negative, but these were positive for histology, PCR and FISH. While one dog showed urease-positive, NHPH were not found by histology, PCR or FISH (Table 2). NHPH DNA was detected by Helicobacteraceae family- and *Helicobacter* genus-specific PCR in 19 out of 20 dogs examined, except in one case where one sample was only PCR-positive for Helicobacteraceae (Table 2). The *cagA* and *glmM* genes were not detected in any of the 19 *Helicobacter* infected dogs. FISH was carried out on all fundic biopsies from 20 dogs to detect a fragment of the 16S rDNA from *Helicobacter* species. The probes HEL274/HEL717 were able to detect *Helicobacter* in 19 out of 20 infected dogs (Fig. 3). *Helicobacter* was observed as red whole-cell with different morphology (spiral-shaped or round organisms) and distributed in the fundus within the luminal area and/or close to the surface of mucus layer covering the secretory epithelium, adhering to epithelial cells, and to mononuclear cells in the submucosa and lamina propia (Fig. 3B and C). FISH was concordant with histology and PCR. Almost all of the animals were NHPH infected (19/20) by these criteria and only one was not infected (1/20); this individual case was insufficient to allow comparisons. NHPH DNA was detected in 18 dogs with gastritis and one with normal gastric mucosa, whereas one dog had gastritis but NHPH infection was not detected (Table 1). There was no statistical relationship between the presence of gastritis and NHPH infected dogs (r = -0.0526; p = 0.8256).

Sequence analysis: The 16S rRNA partial gene sequences obtained in 19 dogs were amplified by PCR using Helicobacteraceae family-specific primers. The length of the sequences obtained ranged from 600 to 711 bp. The sequences were 99-100% identical to 16S rRNA gene sequences of *H. felis* (Genbank accession numbers AY366428, U51871, M57398, AY686607) in 15 dogs (79%), *H. salomonis* (Genbank accession number Y09405) in two dogs (10.5%), and *Helicobacter* sp. (Genbank accession numbers EF217412, AY634577) in two dogs (10.5%). The phylogenetic tree constructed from these sequences showed two main clusters (Fig. 4). Eighteen out of 19 (95%) dog samples clustered mostly with *H. felis* species, *H. salomonis*, and uncultured *Helicobacter* sp., while one dog sample (Dog-1) was the most divergent and clustered with other *Helicobacter* sp. Sequences from dogs were clearly separated from *W. succinogenes* ATCC 29543 (M88159). Of the three NHPH species detected, *H. felis* was most negatively correlated with both *H. salomonis* (r = -0.577; p = 0.007) and/or *Helicobacter* sp. (r = -0.577; p = 0.007).

DISCUSSION

A high prevalence (95%) of gastritis was found in both sick and healthy pet dogs during our study. This agrees with other studies reporting the occurrence of gastritis as a common finding in dogs[17,24]. The gastritis in dogs is characterized by the presence of mild to moderate superficial

Table 2
NHPH infection status as assessed by different tests

No. of dogs with pattern	Urease testing	Presence of Bacteria (H&E and WS)	FISH	Fragment determined by PCR (bp)			
				Helicobacteraceae	*Helicobacter* spp.	*H. pylori*	
				16S rRNA gene (764 bp)	16S rRNA gene (399 bp)	*glmM* gene 294 (bp)	*cagA* gene 128 (bp)
1	-	+	+	+	-	-	-
1	-	+	+	+	+	-	-
17	+	+	+	+	+	-	-
1	+	-	-	-	-	-	-

H&E, hematoxylin-eosin stain; WS, Warthin-Starry stain.

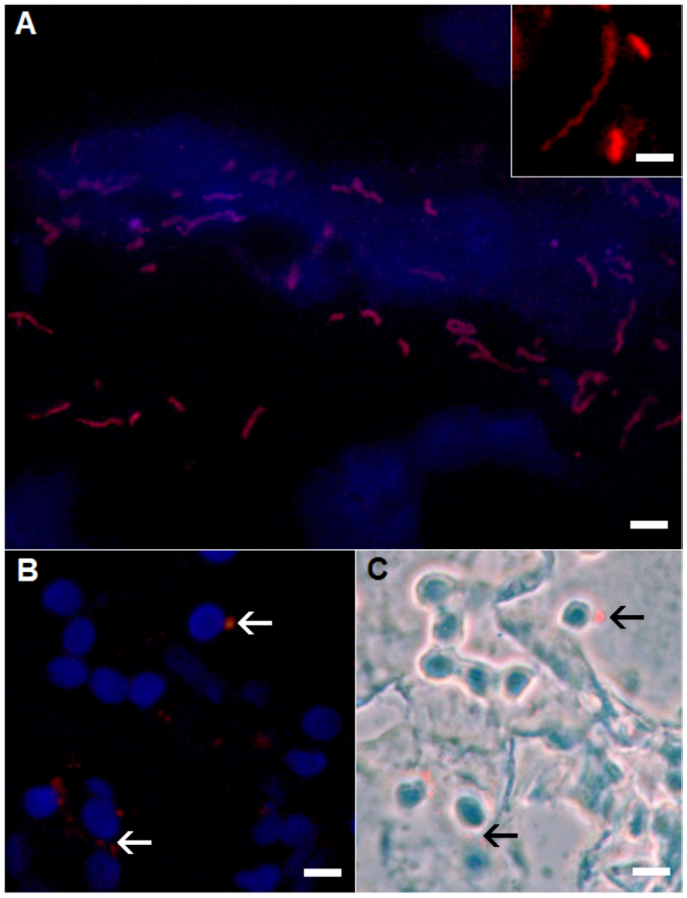

Fig. 3 - Detection of NHPH by FISH in gastric mucosa (fundus) tissue sections. (A) Detection of NHPH in the gastric mucosa (red stained); the nuclei were stained by DAPI (blue). The insert shows various morphologies of NHPH species. (B) Image of Helicobacteraceae adhering to mononuclear cells in the lamina propia (arrows). (C) Merge of fluorescence and phase contrast images, corresponding to the same section as in B; arrow points to the same bacteria as in B. Bars: A: 10 µm; B and C: 5 µm; insert: 2.5 µm.

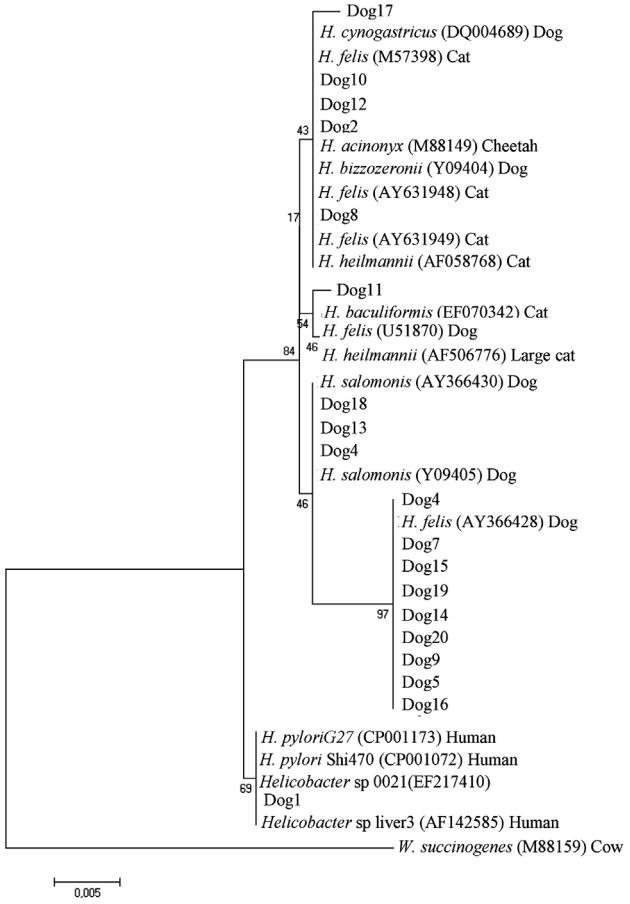

Fig. 4 - Phylogenetic consensus tree showing the genetic relationship of Helicobacteraceae 16S rRNA gene sequences amplified from 19 dogs to other *Helicobacter*. Scale bar represents a 1% difference in nucleotide sequence as determined by measuring the length of horizontal lines connecting any species. Accession numbers of sequences are given in brackets after each isolate name, and the species from which it was isolated except for *Helicobacter* sp. 0021.

lymphoplasmocytic infiltrates, with atrophy and fibrosis[33]. Despite the high prevalence of gastritis in dogs, the cause of this mucosal lesion has not been clearly determined. Several factors for its development have been proposed, as systemic disease, ulcerogenic drugs, and host immune responses to parasites, dietary antigens, or bacterial components all may cause mucosal inflammation[21]. However, examination of fundic biopsies

revealed the colonization of spiral-shaped bacteria, suggesting a possible causal role for the bacteria.

In the present study, the NHPH infection was determined by at least three of four methods (urease test, histology, PCR and FISH). NHPH were identified in 95% of the dogs, which is in agreement with previous studies conducted on pet dogs, with a prevalence ranging from 67 to 86% in healthy animals, and 61 to 100% in those with vomiting[14,19]. NHPH infection was evidenced by Helicobacteraceae-and *Helicobacter*-specific PCR in 19 and in 18 of 20 dogs, respectively. Only one animal (Dog-1) was PCR-positive for Helicobacteraceae and PCR-negative for *Helicobacter* spp. Interestingly, the results of partial 16S rDNA sequencing for Dog-1 is more closely related to *Helicobacter* spp., including *H. pylori* as shown in the phylogenetic tree (Fig. 4). However, the *H. pylori* specific genes *glmM* and *cagA* were never detected in any of the dogs. The *glmM* gene encodes phosphoglucosamine mutase, an enzyme catalyzing the interconversion of glucosamine-6-phosphate into glucosamine-1-phosphate[7]. The *cagA* gene of *H. pylori* is one of the most important virulence factors of this bacterium and is related to cytotoxin production, which is strongly associated with peptic ulcer disease and gastric cancer in humans[32]. This is consistent with another study in which *H. pylori* was not found in dogs, indicating that pet dogs may not represent a source or reservoir of *H. pylori* for the human population[11]. The 16S rRNA PCR was specific and sensitive for detection of NHPH; results obtained with this method were in agreement with the visualization of NHPH in gastric biopsies by FISH. This technique is a powerful tool for the specific detection of *Helicobacter* within gastric tissue. It does not, however, allow identification to the species level of the dog and cat associated NHPH. Sequencing of the 16S and 23S ribosomal RNA encoding genes allows differentiation of *H. suis* from the other gastric non-*H. pylori Helicobacter* species, but it cannot distinguish between *H. felis*, *H. bizzozeronii*, *H. salomonis, H. heilmannii* s.s., *H. cynogastricus* and *H. baculiformis*[14]. Although for differentiation between these species, sequencing of the *hsp60* or *gyrB* gene is useful, sequencing of the *ureA* and *ureB* genes seems currently to be the most suitable method since sequences of these genes are available for all NHPH species[15].

The identity of infecting NHPH species, using PCR with family-specific primers, was determined in 19 of the 20 dogs. They contained approximately 600 to 711 bp amplicons that shared 99-100% sequence identity with the 16S rRNA genes of *H. felis*, *H. salomonis*, and *Helicobacter* sp. as single infections. *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. heilmannii* s.s., *H. cynogastricus* and *H. baculiformis* represent a group of gastric NHPH species commonly found in dogs and cats which have recently been referred to as *Helicobacter heilmannii* s.l.[15,27]. These species are both phenotypically and phylogenetically highly related[3,20,28], and some individual animals can often be infected by multiple gastric NHPH species[28,31].

In summary, these results demonstrate the presence of NHPH DNA in the gastric mucosa of pet dogs from Venezuela. However, there was no correlation between the severity of gastritis and the presence of NHPH. Since the tests used here do not allow distinguishing between *H. felis*, *H. bizzozeronii*, *H. salomonis, H. heilmannii* s.s., *H. cynogastricus* and *H. baculiformis,* a possible association between a specific NHPH species and degree of gastritis remains to be studied.

RESUMEN

Alta prevalencia de ADN de los helicobacteres no-*H. pylori* en la mucosa gástrica de perros domésticos venezolanos y sus alteraciones histopatológicas

Los helicobacteres no-*H. pylori* (NHPH, por sus siglas en inglés) han sido demostrados como bacterias gástricas de forma espiral; sin embargo, sus roles en la patogénesis de la enfermedad gastrointestinal superior no han sido claramente establecidos. El propósito de este estudio fue evaluar la prevalencia de ADN de los NHPH en la mucosa gástrica de perros y su asociación con histopatología. *Helicobacter* fue detectado a través de técnicas histopatológicas, análisis de PCR y FISH en biopsias del fundus gástrico de 20 perros con o sin signos de enfermedad gastrointestinal. La PCR y FISH se basaron en secuencias parciales del gen ARNr 16S. Diecinueve perros mostraron gastritis leve a marcada en el fundus gástrico y sólo un perro tuvo una mucosa gástrica sana. El ADN de los NHPH fue detectado en 18 perros con gastritis y uno con mucosa gástrica normal. Sin embargo, no hubo correlación significativa entre la presencia de ADN de los NHPH y el grado de gastritis. Estos resultados demuestran una alta prevalencia de ADN de los NHPH en la mucosa gástrica de perros de Venezuela. Futuros estudios son necesarios para determinar la posible asociación entre una especie específica de los HNPH y el grado de gastritis.

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