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Evidence of *Helicobacter spp*. in domestic cats from central Rio Grande do Sul State – pre-study

[*Evidências de* Helicobacter spp. *em gatos domésticos na região central do Rio Grande do Sul – pré-estudo*]

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ABSTRACT

Non-Helicobacter pylori Helicobacters (NHPH) account for causing moderate and severe gastritis in humans. Moreover, they have already been documented in the oral cavity of feline animals. The current study aims to investigate *Helicobacter* spp. incidence in necropsied domestic cats from central Rio Grande do Sul. Stomach, liver, and bile samples derived from 30 cats' cadavers were subjected to rapid urease test (RUT), histopathological analysis, PCR, and nucleotide sequencing to investigate the genetic material presence and to identify the main phylogenetic group. Based on RUT results, 64.2% and 53.5% of patients were positive for gastric body and pyloric antrum, respectively. Ten out of these 15 patients were male (70%) and the positive result observed for the pyloric antrum was associated with males. Mild histopathological changes were observed in most gastric and liver samples. Gastric samples subjected to PCR presented positive results in 60.7%, whereas liver samples accounted for positive results in 17.8% and bile samples, in 3.5% of cases. There was high nucleotide identity with NHPH species. This study has found evidence that NHPH can be found in the gastric and hepatic regions, as in the bile of domestic cats, besides emphasizing the zoonotic potential of this disease

Keywords: phylogenetics, cats, liver, bile, *Non-Helicobacter pylori Helicobacters*.

RESUMO

As Helicobacter *não* Helicobacter pylori *(HNHP) são responsáveis por causar gastrite moderada e severa em seres humanos; além disso, são espécies que já são documentadas na cavidade oral de felinos. O objetivo deste estudo é investigar a evidência de* Helicobacter *spp. em gatos domésticos necropsiados, na região central do Rio Grande do Sul. Para tanto, utilizaram-se 30 cadáveres de gatos, cujas amostras de estômago, fígado e bile foram submetidas ao teste rápido de urease (TRU), à análise histopatológica, ao PCR e ao sequenciamento de nucleotídeos, para demonstrar a presença de material genético e identificar o principal grupo filogenético. No TRU, 64,2% dos pacientes foram positivos no corpo gástrico e 53,5% foram positivos no antro pilórico. Destes 15 pacientes, 10 eram machos (70%), sendo o resultado positivo no antro pilórico associado ao sexo masculino. Foram observadas alterações histopatológicas discretas na maioria das amostras gástricas e hepáticas. As amostras gástricas submetidas ao PCR foram positivas em 60,7%; já as hepáticas, em 17,8%; e em 3,5% das amostras de bile. Houve alta identidade de nucleotídeo com as espécies de HNHP. Conclui-se que existem evidências de que as HNHP podem ser encontradas nas regiões gástrica e hepática, incluindo a bile de gatos domésticos, destacando-se o potencial zoonótico da enfermidade.*

Palavras-chave: filogenética, gatos, fígado, bile, Helicobacter *não* Helicobacter pylori

INTRODUCTION

Helicobacter spp. are spiral-shaped, gram-negative and microaerophilic bacteria accounting for colonizing both the stomach and small intestine of several animal species, such as dogs, cats, ferrets, pigs, cheetahs and monkeys (Sousa *et al*., 2019; Hong *et al*., 2016; Rossi *et al*., 2014). Bacterial species *Helicobacter pylori* prevails in humans and accounts for disorders in their upper intestinal and hepatobiliary tracts (Kubota-Aizawa *et al*., 2017; Kusters *et al*., 2006). It was the first species both identified and named after bacterial culture obtained from

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biopsies applied to samples collected from humans affected by peptic ulcers, who underwent endoscopic procedure (Marshall *et al*., 1984). Then, bacterial species *Gastrospirillum hominis* was discovered and it presented features different from those observed for *H. pylori* (Dent *et al*., 1987; McNulty *et al*., 1989), therefore, it was renamed as *Helicobacter heilmanni* (Solnick *et al*., 1993).

Based on sequencing studies applied to the 16s ribosomal RNA (rRNA) gene, *Helicobacter heilmanni* belongs to a wide variety of bacteria, such as *H. salomonis*, *H. bizzozeronii*, *H. felis*, *H. heilmanni* s.s. (strictu sensu), *H. baculiformis* and *H. cynogastricus*, which are isolated from both domestic and wild animals (Haesebrouck *et al*., 2009; O'Rourke *et al*., 2004). Later on, these bacteria were referred to as *Non-Helicobacter pylori Helicobacter* or NHPH (Haesebrouck *et al*., 2009). NHPHs, in their turn, account for causing moderate gastritis in humans, although it can progress to its severe forms, such as gastric ulcers, gastric adenocarcinomas and low-grade lymphoma of mucosa-associated lymphoid tissue (Sousa *et al*., 2019; Tabrizi *et al*., 2010). On the other hand, *Helicobacter heilmanni* is the most prevalent bacteria found in felines. Although this bacterial species was previously identified in felines' stomach, liver, bile duct (Greiter-Wilke *et al*., 2006), pancreas, duodenum (Sjödin *et al*., 2011), feces (Hong *et al*., 2016) and oral cavity (Tabrizi *et al*., 2010), and even in biliary samples (Boomkens *et al*., 2004), it causes little, or no harm at all, to these hosts (Ménard and Smet., 2019; Sousa *et al*., 2019).

Cats licking humans is a social interaction form, be it to show affection or for grooming purposes (Rodan, 2012). However, *Helicobacter* spp. presence was already reported in cats and dogs' saliva (Tabrizi *et al*., 2010; Recordati *et al*., 2007) and it is well known that direct contact with their saliva is a risk factor for infections, regardless of whether it has bile, or not (Tabrizi *et al*., 2015; Kusters *et al*., 2006). Therefore, this succession of factors enables a progressively favorable scenario for the zoonotic aspect of this disease to manifest itself.

The aims of the current study were to investigate *Helicobacter* spp. incidence in domestic cats subjected to necropsy in central Rio Grande do Sul State, based on rapid urease tests, histopathology and on molecular analyses conducted in stomach, liver tissue and bile samples, as well as to identify the main phylogenetic groups found in them.

MATERIALS AND METHODS

Twenty-eight dead cats of different ages, sexes, and breeds were used in the current study. They were provided by the routine clinical care service of the University Veterinary Hospital of a higher education institution, and private clinics in central Rio Grande do Sul State. They were either euthanized or died of different causes during hospitalization and were sent to the Pathology Laboratory of the University Veterinary Hospital at the request of the veterinarian in charge, in agreement with the owners. This hospital is a reference for necropsy procedures. The animals were classified into age groups, namely: pups (up to 12 months), juveniles (1 to 5 years), adults (5 to 7 years), and seniors (7 years and older).

A rapid Urease Test (RUT) was conducted with the aid of the Uretest® kit (Renylab, Barbacena City, Brazil). Two gastric samples were collected (one from the gastric body and one from the pyloric antrum, each one with dimensions of 0.5 x 0.5 centimeters) and aseptically deposited in a test tube in order to have their indicator's color change time assessed. According to the manufacturer's instructions, samples are positive if the kit's yellow color turns pink, at any shade, within 60 minutes. On the other hand, samples are negative when their color change does not occur after 60 minutes.

Right after sample collection was over, gastric (gastric body and pyloric antrum) and liver fragments were placed in 5ml syringes filled with 10% formaldehyde solution, subjected to histopathological analysis, and processed based on techniques described by Esteves *et al*. (2000), Prachsilpchai *et al*. (2007) and Robic *et al*. (2007).

Scores inherent to the degree of structural impairment and inflammatory infiltration of both the epithelium and the lamina propria, in the gastric mucosa of the gastric body and pyloric antrum regions, were set to assess inflammation intensity in the gastric mucosa of each sample, according to consensus elaborated by Day *et al*. (2008).

Similarly, scores were set for liver structural changes by taking into consideration portal infiltrate type and degree, as well as bile duct proliferation and fibrosis degrees, based on studies by Gagne *et al*. (1996).

Samples from the gastric body, pyloric antrum, hepatic and biliary regions were collected and stored - right away - in 3ml Eppendorf tubes filled with 0.9% physiological solution, at -28ºC. DNA extraction from collected samples was carried out based on using the DNeasy Blood and Tissue Kit (Qiagen, Germany); extraction procedure stages followed the methodology by Germani *et al*. (1997). The size of the amplified fragment was 399 base pairs (399 base pairs (bp) for the 16S rRNA gene of the Helicobacter spp.).

DNA material was extracted from each sample and subjected to PCR test. Positive control was obtained from gastric, hepatic, and biliary samples collected from a cat known to be positive for *Helicobacter sp*., whereas negative controls comprised ultrapure water, in all amplifications.

PCR amplification products were subjected to nucleotide sequencing in Prism 3500 Genetic Analyzer (Life Technologies, California, USA), in duplicates, based on the Sanger method. The consensus order of Staden Package software was used to start the nucleotide sequencing (Staden, 1996). Phylogenetic analyses used the consensus order in each amplified sample and *Helicobacter sp*. nucleotide sequence obtained in the Genbank database. Sequences were both edited and aligned in BioEdit Alignment Editor Software, version 7.0.5.3 (Hall, 1999). The phylogenetic tree was built in MEGA X software (Kumar *et al*., 2018). The obtained sequences have been deposited in GenBank under the codes/IDs: OK614040.1, OK614007.1, OK613988.1, OK606115.1, OK606114.1, OK606113.1, OK606112.1, OK606111.1, OK606110.1, OK606109.1, OK606108.1, OK606107.1,OK606106.1, OK606105.1, OK606104.1, OK606103.1, OK606102.1, OK606096.1, OK606095.1, OK606094.1 e OK606093.1.

Statistical analysis was based on absolute and relative frequency calculations applied to qualitative variables (urease, histopathology, and PCR). Association analysis was based on Fisher's exact test. All analyses were carried out in SPSS software v.18.0 (Pasw…, 2009), at 5% significance level.

RESULTS AND DISCUSSION

Two (2) of the 30 assessed cats belonged to the Persian breed (14 males and 16 females) and 28 of them did not have defined breed. Three (10%) of them were puppies up to 1 year old, 10 were young cats in the age group 1-5 years (33.3%), 6 were adult animals in the age group 5-7 years (20%) and 11 were elderly cats over 7 years old (36.7%). Animals presenting positive result in RUT applied to gastric body samples accounted for 66% (2/3) of puppies, 70% (7/10) of young cats, 50% (3/6) of adult cats and for 54.5% (6 /11) of elderly individuals. On the other hand, animals presenting positive urease results in pyloric antrum samples accounted for 40% (4/10) of young and 50% (3/6) of adult cats.

Patients presenting positive urease results in the gastric body accounted for 60% (18/30) of cases. On the other hand, based on histopathological analysis applied to the gastric body of these animals, 50% (9/18) of them did not show surface-epithelium injury, 61.1% (11/18) presented normal gastric fossa and 83.3% (15/18) did not show intraepithelial lymphocytes, or even lymphocytes and plasmocytes, in the lamina propria. In addition, despite being positive for urease, none of these patients presented mucosal atrophy, eosinophils and neutrophils in the lamina propria or follicular lymphoid hyperplasia. Lack of these cell populations is assumingly associated with lack of epithelium due to autolysis identified in certain samples or to surface epithelium without injury.

Based on analysis applied to pyloric antrum samples, 50% of patients (15/30) tested positive for urease (Fig. 1A) and 60% (9/15) of them did not show epithelial damage in the pyloric antrum. In addition, 87% (13/15) of these cats did not present intraepithelial lymphocytes, 80% (12/15) of them did not show lymphocytes or plasma cells in the lamina propria and 87% (13/15) did not present follicular lymphoid hyperplasia. No eosinophils or neutrophils were observed in the lamina propria. However, 10 of the 15 positive animals belonged to the male sex (70%), therefore, positive results observed for pyloric antrum were associated with the male sex (Fig. 1B) (p=0.033).

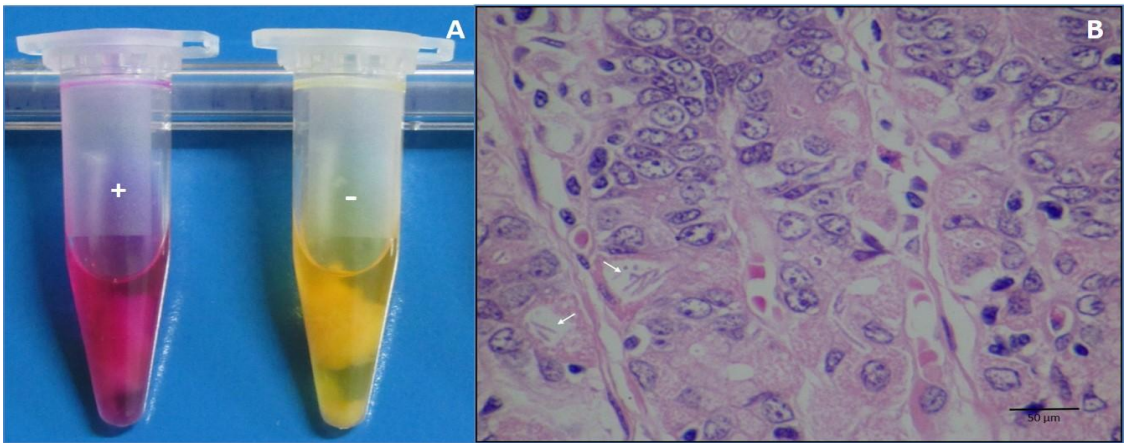

Figure 1. Examples of positive (pink +) and negative (yellow -) urease tests in feline-stomach samples (A). Histological image of the stomach showing multiple spirochete-shaped bacteria (*Helicobacter sp*.) in gastric glands' lumen (arrows). HE, 40x.

Lack of changes and cell populations in gastric body and pyloric antrum samples was assumingly associated with lack of epithelium caused by the autolysis identified in certain samples. It is so, because these leukocyte subpopulations are part of the gastric mucosa's surface region (Day *et al*., 2008).

Based on analysis applied to liver samples, 73.3% (11/15) of patients presenting positive results in urease test applied to the pyloric antrum have shown 5-10 inflammatory cells in the portal space (slight change). This assessment focuses on measuring inflammatory infiltrates' severity in portal areas. Moreover, 60% (9/15) of these patients presented 2 ductulus per portal space (slight change) in the bile duct proliferation assessment, whereas 53% (8/15) of them did not show portal fibrosis. In addition, 66.6% (12/18) of animals who tested positive for urease in the gastric body presented 5-10 inflammatory cells in the portal space (slight change), 55.56% (10/18) of them presented 2 ductulus per portal space (slight change) and 50% (9/18) did not show portal fibrosis.

Furthermore, lymphoplasmacytic infiltrates were the most prevalent type (n=23). Their prevalence in the herein assessed animals was similar to that observed by Gagne *et al*. (1996), according to whom, 27 of 45 assessed cats with inflammatory liver diseases presented lymphoplasmacytic infiltrates. According to them, inflammatory changes were limited to the portal space, and this finding was similar to that observed in the current study. However, difference in bile duct proliferation was herein observed. It was evident and severe in 96.2% (26/27) of cats and in 85.1% (23/27) of patients with portal fibrosis.

If one takes into consideration that all it takes for a given animal to be positive is to have a reactive gastric body and/or pyloric antrum sample in the urease test, the current study had 66.6% (20/30) positive patients, in total. This rate was lower than that found in studies conducted with felines in Switzerland, which recorded 78% (45/58) positive cases (Neiger *et al*., 1998); in research conducted with felines in the United States, which recorded 86% positive patients (47/55) (Otto *et al*., 1994); in study conducted with felines in Niterói City-RJ, which recorded positive urease tests for 78.5% (44/56) of cases (Araujo *et al.*, 2010), as well as in study carried out with dogs in Santa Maria City, Rio Grande do Sul State (Guerra Segundo *et al.*, 2021), which evidenced that 88.5% (31/35) of the assessed dogs were positive for gastric samples in the urease test. Although this last study was not conducted with felines, it took place in the same herein investigated region. According to Macêdo *et al*. 2012), in cats, studies have demonstrated a variation in the prevalence of gastric microorganisms such as Helicobacter, ranging from 76 to 100%. These authors, in their study with felines in the city of Recife, found 82.35% positivity in the urease test, unlike the 66.6% found in our study, a percentage that is not low either. We cannot fail to highlight that the urease test may show positive results in the

presence of other urease-positive bacteria, thus explaining this variation in positivity among species and within the same species, depending on the study location.

Gastric samples subjected to PCR analysis (n = 28) replicated *Helicobacter* spp. material in 60.7% (17/28) of cases. These findings are in compliance with the prevalence found in studies whose rates ranged from 40% to 100% of the assessed cats (Papasouliotis *et al*., 1997). However, this rate was lower than that observed for gastric samples collected from dogs in Londrina City-PR, which comprised 90.9% (30/33) of samples (Takemura *et al*., 2019). A study conducted with 8 human patients without gastritis has shown that 100% of them recorded positive results for PCR (Mapstone *et al*., 1993). In addition, 14.2% (4/28) of positive cases have also shown genetic material in liver tissues. We emphasize that in the present study, 28 samples were validated for PCR, as two of them did not have satisfactory DNA amplification for analysis and therefore were not sequenced.

On the other hand, 17.8% (5/28) of cases investigated in the current study recorded positive PCR results for liver samples. This rate was lower than the one observed for liver samples collected from a group of human patients affected by hepatocellular carcinoma, and from another group affected by other liver diseases, colon carcinoma and uterine myoma. Each group recorded positive results for 60.7% and 72% of cases, respectively (Xuan *et al*., 2006).

Genetic material was found in the bile sample of 3.5% (1/28) of cases. Although this patient recorded *Helicobacter* spp. positive results for the gastric sample, its liver sample recorded negative PCR results. This finding exemplifies the theory of infection ascending from the duodenum to the bile duct and, subsequently, to the bile (Ménard and Smet, 2019). Unlike dogs assessed in Londrina City-PR, this finding was different from that of the study that did not find any replicated bile sample (Takemura *et al*., 2019). However, a study focused on assessing a group of felines affected by lymphocytic cholangitis, and another group of animals affected by another liver disease, observed genetic material replication in bile samples in 26.6% (4/15) and 15.6% (8 /51) of cases, respectively (Boomkens *et al*., 2004). However, this eventual difference was associated with the selection of patients with specific diseases, a fact that increased the likelihood of identifying genetic material deriving from *Helicobacter* spp. in these patients.

There was significant association of qualitative variables between the PCR and urease tests applied to pyloric antrum (p=0.001), at 5% significance level (Table 1). Similarly, a significant association was observed between the PCR and urease tests applied to the gastric body (p=0.014).

Table 1. Association between results recorded for PCR applied to stomach sample and for urease test applied to pyloric antrum* and gastric body** samples

PCR	Urease			Antrum		
	Positive	Negative	Total	Positive	Negative	Total
Positive	13 (76.5%)	4 (23.5%)	17 (100%)	13 (76.5%)	4 (23.5%)	17 (100%)
Negative	1 (9.1%)	10 (90.9%)	11 (100%)	3 (27.3%)	8 (72.7%)	11 (100%)
Total	14 (50%)	14 (50%)	28 (100%)	16 (57.1%)	12 (42.9%)	28 (100%)

* Fisher's exact test value - p=0.001 (5% significance). Horizontal-reading direction.
** Fisher's exact test value - p=0.014 (5% significance). Horizontal-reading direction.

Nucleotides' identity among the herein obtained sequences ranged from 97.89% to 100%. Likewise, they recorded identity rates in comparison to that of sequences deriving from *H. heilmannii* (HE984298.2; 98.97% to 99.23%), *H. bizzozeronii* (FR871757.1; 98.39% to 99.73%), *H. felis* (AY686607.1; 98.71% to 99.47%) and *H. salomonis* (NR_026065.1; 99.47% to 99.73%). Nucleotides' identity ranged from 91.9% to 93.48% in comparison to the sequence obtained from *H. pylori* samples (AY155586) (Fig. 2). Phylogenetic analysis did not determine the *Helicobacter* species detected in the analyzed samples, in separate. However, based on the

closeness between the herein analyzed samples and *Helicobacter* strains, such as *H. heilmannii*, *H. bizzozeronii*, *H. felis* and *H. salomonis*, it was possible implying similarity between the herein investigated samples and these HNHP species.

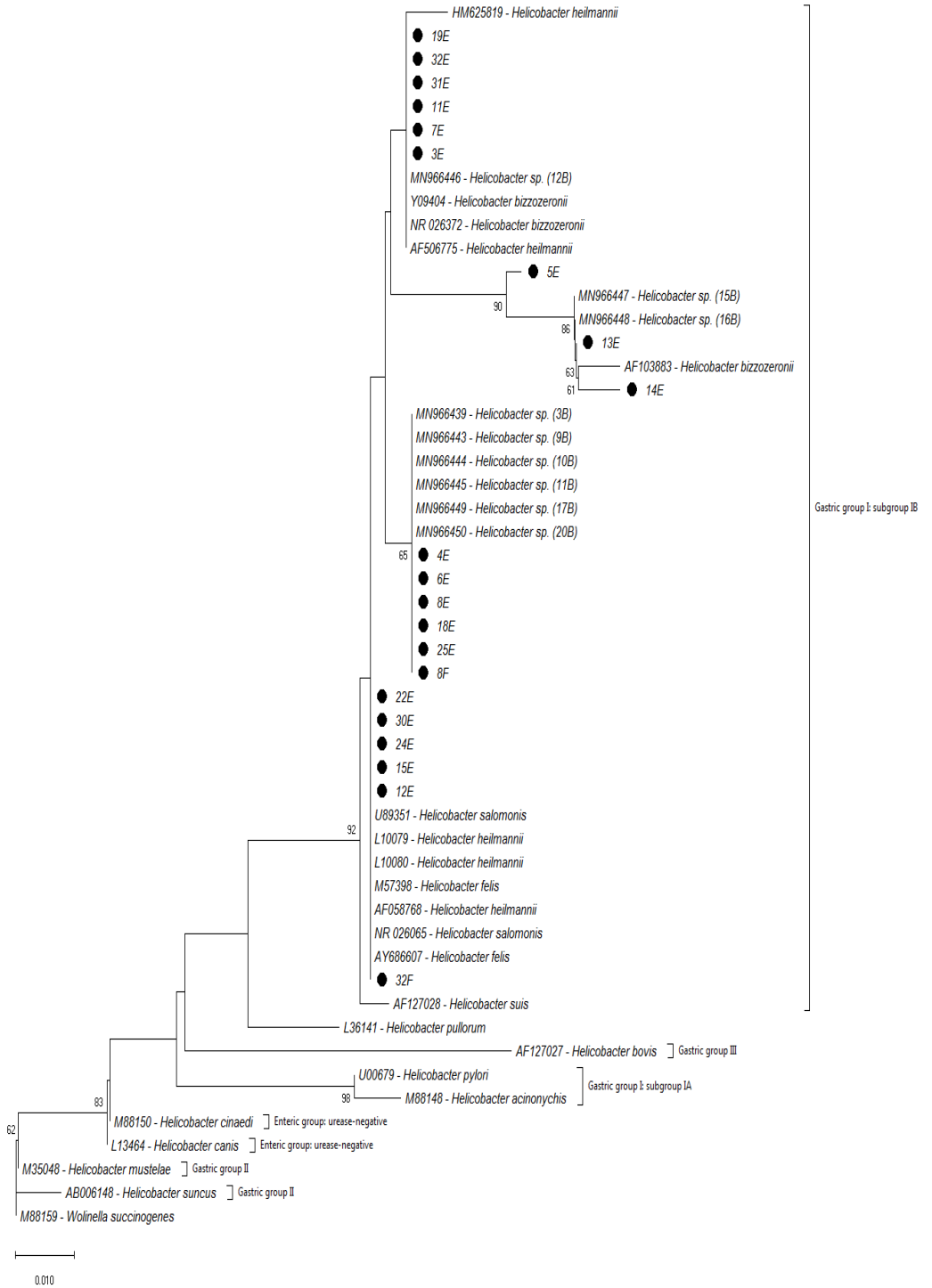

Figure 2. Phylogenetic tree based on the nucleotide sequence of the 16s ribosomal RNA gene specific to *Helicobacter* spp. Values higher than 60% were displayed. The herein obtained sequences are highlighted by a black circle; n.E or n.F, number of the animal/stomach or liver sample.

It is essential to emphasize the current public-health relevance of this disease. Chronic gastritis associated with *Helicobacter* has the potential to cause gastric neoplasms in humans; therefore, our research group is encouraged to carry out further studies like the present one to warn both the scientific community and the overall society about such a risk. Humans' relationship with animals, mainly with dogs and cats, is getting increasingly close; therefore, hygiene care must be taken to avoid and/or control the multiplication of this agent type, mainly in individuals with weak immune systems.

CONCLUSIONS

Results in the current study have evidenced high *Helicobacter* spp. incidence rate through RUT and PCR applied to gastric samples. Positive result in RUT applied to pyloric antrum samples was associated with the male sex. Slight histopathological changes were observed in most gastric and liver samples, and it enabled qualitative definition of the most common changes. Although the rates of liver and bile samples testing positive in the PCR test were lower than those observed in several studies, they were observed, nevertheless. Phylogenetic analysis has determined significant genetic proximity to NHPHs, since these species show close evolutionary relationship with, and genomic similarity to *H. pylori*.

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