Conventional diagnostic methods and immunohistochemistry in the detection of gastric Helicobacter species in dogs with chronic gastropathy

[*Métodos de diagnóstico convencionais e imunoistoquímica na detecção de espécies de Helicobacter gástricas em cães com gastropatia crônica*]

J.E.S. *Paranhos*[1] , J.S. *Leite*[1] , M.G.G. *Souza*[2] , F.G.F. *Padilha*[1] ,
D.A. *Sousa*[1] , A.M.R. *Ferreira*[1]

[1] Universidade Federal Fluminense, Niterói, RJ, Brasil
[2] Policlínica Veterinária Botafogo, Rio de Janeiro, RJ, Brasil

ABSTRACT

An accurate diagnostic test for *Helicobacter* spp. infection in dogs is necessary but no gold standard diagnostic method has yet been established. The purpose of this study was to assess the sensitivity of conventional diagnostic methods as opposed to immunohistochemistry (IHC) in the detection of *Helicobacter* spp. in gastric samples of dogs with chronic gastropathy. Samples of gastric fundus, body and antrum were collected by gastroscopy from 13 domestic dogs presenting chronic vomit and submitted to the rapid urease test (RUT), cytopathology, histopathology with Hematoxylin-Eosin (HE) and Warthin-Starry (WS) stain, and IHC. Cohen's kappa coefficient was carried out to determine the agreement between techniques compared to IHC. Prevalence of colonization detected by IHC was 92% and was higher in the gastric fundus. The sensitivity of each technique in detecting *Helicobacter* spp. in the gastric fundus, body, and antrum, respectively, were as follows: RUT 50%, 70%, 17%; cytology 58%; 70%, 50%; HE 42%; 70%, 50%; WS 67%; 80%, 50%. We found that IHC was the most sensitive method for *Helicobacter* spp. diagnosis revealing that gastric fundus samples have higher presence of bacteria. Squash cytology enhances sensitivity and ancillary staining such as WS should be taken into consideration towards diagnosis.

Keywords: canine, endoscopy, hematoxylin eosin, rapid urease test, Warthin-Starry

RESUMO

É necessário um teste diagnóstico preciso no diagnóstico de Helicobacter *spp. em cães, mas nenhum método padrão-ouro foi estabelecido até o momento. O objetivo foi avaliar a sensibilidade dos métodos diagnósticos convencionais em comparação com a imuno-histoquímica (IHC) na detecção de* Helicobacter *spp. em amostras gástricas de cães com gastropatia crônica. Amostras de fundo, corpo e antro gástrico foram coletadas por gastroscopia de 13 cães com vômitos crônicos, e submetidas ao teste rápido da urease (RUT), citopatologia, histopatologia com coloração de hematoxilina-eosina (HE) e Warthin-Starry (WS), e IHC. O coeficiente kappa de Cohen foi realizado para determinar a concordância entre as técnicas, comparadas ao IHC. A prevalência de colonização detectada na IHC foi de 92%, sendo maior no fundo gástrico. A sensibilidade de cada técnica na detecção de* Helicobacter *spp. no fundo, corpo e antro gástrico, respectivamente, foram: RUT 50%, 70%, 17%; citologia 58%; 70%, 50%; HE 42%; 70%, 50%; WS 67%; 80%, 50%. Concluiu-se que a IHC foi o método mais sensível de diagnóstico de* Helicobacter *spp., revelando que as amostras de fundo gástrico têm maior presença de bactérias. A citologia por* squash *aumenta a sensibilidade, e a coloração auxiliar WS deve ser levada em consideração para o diagnóstico.*

Palavras-chave: cães, endoscopia, hematoxilina e eosina, teste rápido de urease, Warthin-Starry

INTRODUCTION

Helicobacter spp. are spiral shaped gram-negative bacteria that were initially described in the canine stomach by Salomon in 1896. Since then, dogs have been considered natural reservoirs for many *Helicobacter* species, other than *H. pylori* - a well-known cause of chronic gastritis, gastric ulceration, and neoplasia in humans (Robinson and Atherton, 2021). Zoonotic significance has been attributed to the "non-*Helicobacter pylori* helicobacters" (NHPH) (Moussa *et al.*, 2021) and the prevalence of NHPH in canine gastric samples is estimated to be 86-100% in healthy dogs and up to 82% in diseased dogs (Okubo *et al.*, 2017) but its pathogenic role remains unknown (Suárez-Esquivel *et al.*, 2017).

Several studies have been conducted to assess the usefulness of different methods in the diagnosis of canine *Helicobacter* species (Happonen *et al.*, 1996; Kubiak *et al.*, 2017; Ruiz *et al.*, 2017; Guerra Segundo *et al.*, 2021; Husnik *et al.*, 2022) and techniques have gradually evolved, with varying levels of sensitivity and specificity (Sabbagh *et al.*, 2019). Conventional diagnostic methods commonly used include the rapid urease test (RUT), cytology and histologic evaluation of gastric biopsies with routine Hematoxylin-Eosin and ancillary stains, like Warthin-Starry (WS) (Husnik *et al.*, 2022). Advanced diagnostic methods, such as immunohistochemistry (IHC), have high sensitivity and can be used to complement results (Castaneda-Altamirano *et al.*, 2020) and PCR enables sequencing for species identification (Guerra Segundo *et al.* 2021; Husnik *et al.*, 2022).

An accurate diagnostic test for *Helicobacter* spp. infection in dogs is necessary considering that the prescription of antibiotics for NHPH infection treatment in dogs relies on the clinician's interpretation of the diagnostic methods findings and clinical signs observed, being a major concern regarding antimicrobial resistance (Taillieu *et al.*, 2022). Therefore, the aim of this study was to assess the sensitivity of conventional diagnostic methods in comparison to immunohistochemistry in the detection of *Helicobacter* spp. in gastric samples of dogs with chronic gastropathy.

MATERIAL AND METHODS

Ethics statements. Informed written consent was obtained from each owner before procedure and all procedures were in accordance with the ethical standards of the institution or practice where the studies were conducted, being approved by the Ethics Committee on Animal Use (*CEUA, Comitê de Ética no Uso de Animais* of Universidade Federal Fluminense) under the protocol number 5914190721. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Inclusion and exclusion criteria. Dogs presenting primary chronic gastropathy (≥3 weeks of vomiting with or without weight loss) were included and underwent pre-operative screening investigations such as dietary trials, fecal flotation and deworming to discard adverse food reactions and infectious disorders. Furthermore, imaging tests, hematologic and biochemical analysis were done. Dogs with gastrointestinal neoplasia were excluded from this study.

Sample collection. The dogs were anesthetized and placed in left lateral recumbency. Gastroscopy was performed according to the World Small Animal Veterinary Association standards (Washabau *et al.*, 2010). The assessment and collection of gastric mucosal samples were performed using a veterinary endoscope (Ultramedic V 1500) of 8.5mm diameter, with a 2.0mm biopsy channel. Gastric antrum, body, and fundus samples of each animal were collected using biopsy forceps and underwent conventional diagnostic tests for detection of *Helicobacter* spp.

Rapid urease test (RUT). The Renylab Uretest® commercial kit (Renylab Chemicals & Pharmaceuticals) was used and samples of each gastric region were placed into a separate test tube to be evaluated individually. Positivity was defined when the color changed from yellow to magenta within 24 hours.

Cytopathology. Cytopathology was performed using the squash technique by placing a gastric biopsy fragment between two glass slides and spreading out the tissue afterwards. After air-drying, samples underwent immersion in carbol fuchsin for sixty seconds followed by washing in

distilled water. The sample was considered positive when at least one bacterial cell with spiral morphology matching the genus *Helicobacter* was visualized when examining the slide under a 40x objective of a bright field microscope.

Histopathology. After collection, samples were fixed in 10% buffered formalin for 24 hours before processing and paraffin embedding. Histological sections of 4μm thickness were stained with WS silver staining method and HE. Spiral and black microorganisms were counted in five fields of WS staining sections and considered positive in the presence of at least one spiral shaped bacterium whilst positivity in HE stained sections was defined by the presence of basophilic and spiral-shaped microorganisms (Sousa *et al.*, 2017).

Immunohistochemistry. Dewaxed sections of 3-4μm were hydrated and antigen retrieval was performed in a hot water bath at 98°C for 30 minutes with Target Retrieval Solution 10x, concentrated pH 9.0 (DAKO; Glostrup, Denmark). A 10% BSA solution was used to inhibit nonspecific binding and sections were incubated overnight at 4°C with a rabbit polyclonal anti-H. pylori antibody 1:400 (code B0471–Dako Glostrup, Denmark). Afterwards, sections were incubated with EnVisionTM G2System/AP (DAKO Glostrup, Dinamarca). Permanent red was used as chromogen and counterstaining was performed with Harris' hematoxylin. For the positive control, sections of previously confirmed *Helicobacter* spp. canine gastric samples were used, and the primary antibody was replaced with the same immunoglobulin antibody isotype for negative controls. Sections of gastric antrum, fundus and body that presented at least one distinct red labeling in the microscopic evaluation were considered positive for NHPH.

Statistical analysis. To determine the agreement between conventional diagnostic methods and the immunohistochemical technique for the detection of NHPH in canine gastric samples, Cohen's kappa coefficient was carried out. The significance level adopted was p<0.05. Based on the results, the sensitivity of each technique in the diagnosis of NHPH in dogs was determined.

RESULTS

Breeds represented among dogs were Chihuahua (2), Crossbred (4), French Bulldog (1), Labrador Retriever (1), Maltese (1), Pinscher (1), Shih-tzu (1) and West Highland White Terrier (2). The mean age of dogs was 5.3 years (range, 8 months to 13 years).

Rapid Urease Test (RUT). Positivity in the RUT test for each gastric region was 54% (7/13) of fundus and body samples and 15% (2/13) of antrum samples.

Cytopathology. Cytopathology revealed spiral shaped bacteria (Figure 1) in 54% (7/13) of fundus and body samples and 38% (5/13) of antrum samples.

Histopathology. Positivity in HE staining (Figure 2) was 31% (4/13) in fundus samples, 46% (6/13) body samples and 23% (3/13) antrum samples. WS staining (Figure 3) demonstrated 54% (7/13) positivity in fundus samples, 62% (8/13) body samples and 23% (3/13) antrum samples.

Immunohistochemistry. IHC demonstrated positivity (Figure 4) in 92% (12/13) of fundus samples, 77% (10/13) of body samples and 46% (6/13) of antrum samples. Results are described in Table 1.

Statistical analysis. Since twelve animals had, at the minimum, one gastric region positive result in the immunohistochemical study, prevalence of gastric colonization was estimated to be 92% (12/13). The sensitivity of each technique for diagnosing NHPH in the gastric fundus, body and antrum, respectively, when compared to IHC is represented in Table 2 and results were as follows: RUT 50%, 70% and 17%; cytology 58%; 70% and 50%; HE 42%; 70% and 50%; WS 67%; 80% and 50%.
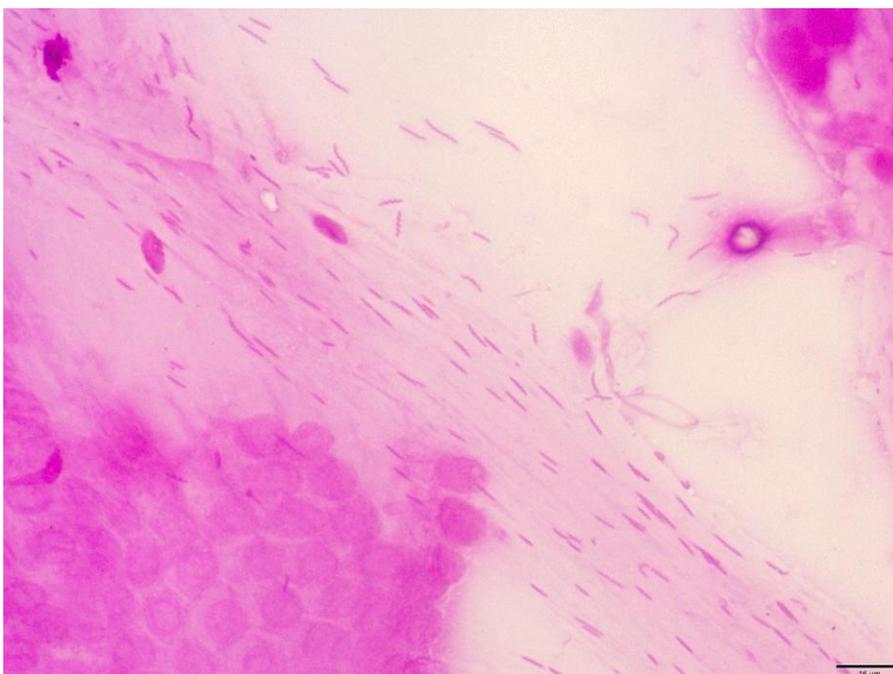
Arq. Bras. Med. Vet. Zootec., v.75, n.6, p.1039-1046, 2023

1041

Figure 1. Cytopathologic examination of a canine gastric sample. Multiple spiral shaped bacteria compatible with NHPH. Carbol Fuchsine. 16 μm.
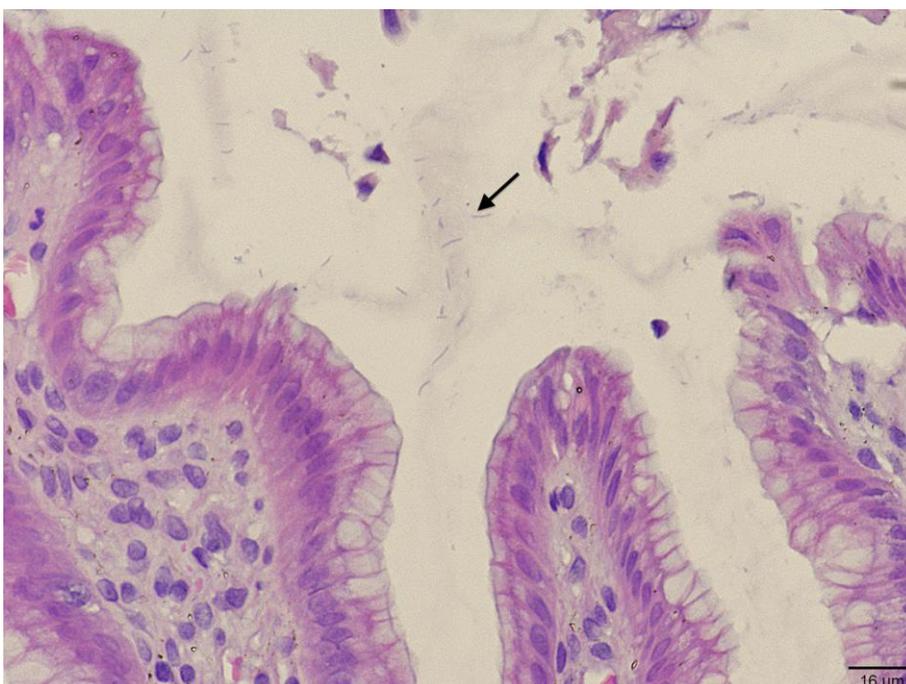

Figure 2: Histologic examination of a canine gastric sample. Multiple spiral shaped bacteria compatible with NHPH (arrow). Hematoxylin-Eosin (HE). 16 μm.
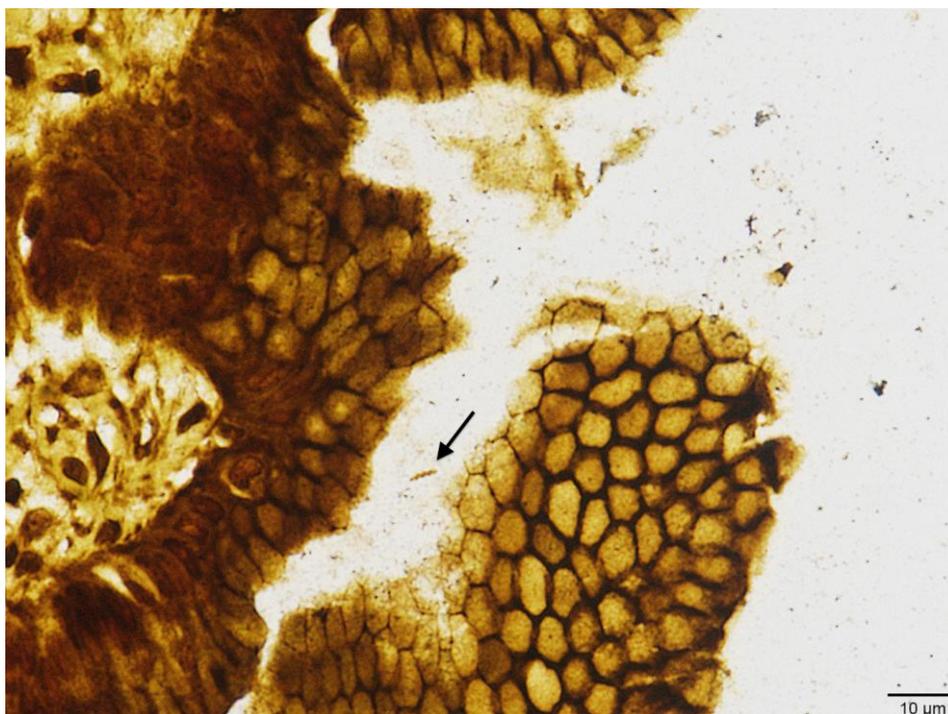
Figure 3: Histologic examination of a canine gastric sample. Spiral shaped bacteria compatible with NHPH (arrow). Warthin-Starry (WS). 10 µm.

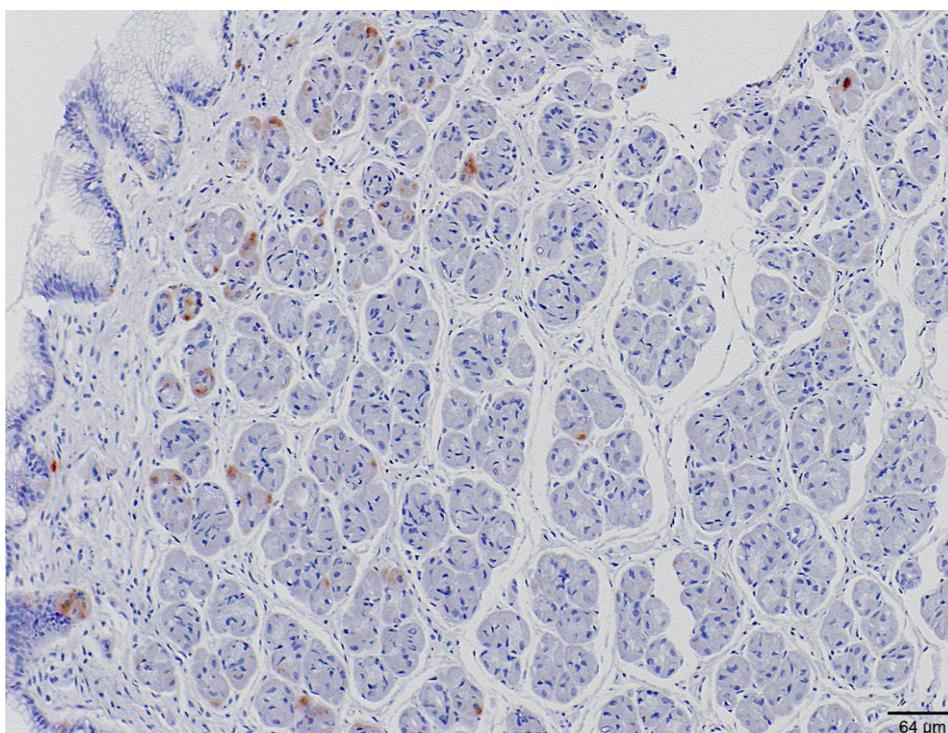Figure 4: Immunohistochemical examination of a canine gastric sample. Red labelling compatible with NHPH. Permanent red. 64 µm.

Arq. Bras. Med. Vet. Zootec., v.75, n.6, p.1039-1046, 2023

1043

Table 1. Detection of *Helicobacter* spp. in canine gastric regions

Methods*	Gastric region Positive percentage and number (N=13)		
	Fundus	Body	Antrum
RUT	54% (7)	54% (7)	15% (2)
Cytology	54% (7)	54% (7)	38% (5)
HE	31% (4)	46% (6)	23% (3)
WS	54% (7)	62% (8)	23% (3)
IHC	92% (12)	77% (10)	46% (6)

*RUT - Rapid Urease Test; HE - Hematoxylin and Eosin; WS - Warthin-Starry; IHC – Immunohistochemistry

The presence of NHPH detected by WS staining had good concordance with IHC detection in the gastric body (kappa 0.649). Moderate concordance (kappa 0.519) was shown between WS and IHC for detection of NHPH in the gastric antrum; between HE and IHC in the gastric body and antrum; between cytology and IHC in the gastric body; and between the RUT and IHC in the gastric body. Other gastric regions and tests evaluated had poor or no concordance between results (Table 2).

Table 2. Sensitivity and Cohen's kappa of conventional diagnostic methods in the detection of *Helicobacter* spp. in canine gastric samples compared to gold-standard immunohistochemistry

Methods*	Gastric region Sensitivity (%) and Kappa		
	Fundus	Body	Antrum
RUT	50 (-0.152)	70 (0.519)$^{\phi}$	17 (0.025)
Cytology	58 (0.177)	70 (0.519)$^{\phi}$	50 (0.217)
HE	42 (0.099)	70 (0.519)$^{\phi}$	50 (0.519)$^{\phi}$
WS	67 (0.235)	80 (0.649)$^{\phi}$	50 (0.519)$^{\phi}$

*RUT - Rapid Urease Test; HE - Hematoxylin and Eosin; WS - Warthin-Starry
$^{\phi}$p<0.05

DISCUSSION

Our study demonstrates that NHPH is present in more than 90% of dogs with chronic gastropathy, which is compatible with other studies findings (Hong *et al.*, 2015; Guerra Segundo *et al.*, 2021) and higher than as described by Okubo and colleagues (2017) in diseased dogs (82%). Besides, there was no predilection for gender, age or breed among dogs that were included in this research, also agreeing with Hong and colleagues (2015).

Furthermore, in this study, sensitivity of the RUT in the detection of NHPH was higher in the gastric fundus (50%) and body (70%) when compared to the antrum (17%). Although the RUT is a simple, popular, and cheap diagnostic test that detects the presence of urease in gastric samples, false negative results may have occurred due to the heterogeneous distribution of *Helicobacter* spp. in the canine gastric mucosa, according to Kubiak and colleagues (2017).

To decrease the chances of false negative results, a protocol suggested by Parihar *et al.* (2015) combining gastric antrum and body samples for the RUT can increase diagnostic accuracy despite a low prevalence of *Helicobacter pylori* in human patients undergoing routine gastroscopy. In dogs, on the other hand, it was observed in our study that the gastric fundus was usually more infected than other gastric regions, followed by the body. Hence, combining these two gastric regions to perform the RUT in dogs increases the technique's sensitivity.

In humans, the RUT seemed to be more sensitive for the detection of *H. pylori* infection compared to histology in patients that used proton pump inhibitors (PPI) and antibiotics (Dechant *et al.*, 2020), since *Helicobacter* spp. may alter its shape to a coccoid form in adverse environmental conditions (Ierardy *et al.*, 2020). In our study, the RUT had higher sensitivity in the detection of *Helicobacter* spp. in the gastric fundus than HE staining but in the gastric antrum HE was superior.

1044

Arq. Bras. Med. Vet. Zootec., v.75, n.6, p.1039-1046, 2023

In the same way, cytopathology is considered a simple and cheap method, with high sensitivity and specificity, but has also limitations in the diagnosis of *Helicobacter* spp. and it is recommended to be associated to the RUT or histopathology for a higher sensitivity (Ruiz *et al.*, 2017). Gastric cytology is considered more sensitive than the RUT or HE in identifying NHPH in dogs (Taulescu *et al.*, 2020) and the method of sampling can also alter the technique's sensitivity, as the squash method provides appropriate samples more often than the imprint technique (Ruiz *et al.*, 2017). In our study, cytopathology was performed using the squash technique and the results showed that it was more sensitive than the RUT and HE in the detection of NHPH in gastric fundus samples.

The WS staining is known to have high sensitivity in the detection of spiral shaped bacteria nevertheless it is a rarely used stain in comparison to HE staining since it is more expensive and time consuming (Ahumada *et al.*, 2020). Despite the disadvantages, in our study the WS staining demonstrated higher sensitivity in the diagnosis of *Helicobacter* spp., as opposed to HE staining in more than one case, in contrast to the findings of Happonen *et al.* (1996) that did not find significant difference when comparing the two staining methods. WS was the most sensitive conventional technique for the detection of NHPH in the gastric fundus and body once the stain highlights bacteria hidden in mucus due the contrast of colors black/brown and yellow.

The low density of bacteria in some samples and staining quality may have affected WS interpretation in contrast to IHC that revealed weak, yet positive, staining of NHPH. The IHC technique was highly specific, therefore, used as the gold standard method for comparison to other techniques, agreeing with previous findings that suggest sensitivity estimated between 98-100% (Ryan and Louri, 2017). The IHC technique demonstrated positivity for NHPH in 92% (12/13) of fundus samples and the only negative sample observed was from a dog that presented negative result in all conventional tests. Disadvantages of the technique included requirement of time, experience, and high costs.

Regarding the microorganism localization, the gastric fundus had a higher percentage of positivity in the gold standard test - IHC - in comparison to antrum and body samples, in agreement with previous studies (Moutinho *et al.*, 2007; Okubo *et al.*, 2017). Oppositely, one study using the histopathology examination revealed that the gastric body and pylorus had higher presence of NHPH compared to other gastric regions, therefore, combining at least two diagnostic techniques has been recommended to obtain reliable results (Vieira *et al.*, 2012).

CONCLUSION

In conclusion, from all methods evaluated, IHC was the most sensitive for *Helicobacter* spp. diagnosis and was proved to be worthwhile to complement ambiguous results. The IHC technique revealed that gastric fundus samples have higher presence of *Helicobacter* spp. as opposed to other gastric regions. Furthermore, squash cytology enhances sensitivity and ancillary staining such as WS should be taken into consideration towards diagnosis.

FUNDING SOURCE

REFERENCES

AHUMADA R.E.; RODRÍGUEZ, G.M.; HIDALGO, P.E. *et al*. Warthin-Starry stain identification of Helicobacter pylori in biopsies of patients who previously tested negative in hematoxylin-eosin staining for follicular gastritis. *Rev. Colomb. Gastroenterol.,* v.35, p.1-7, 2020.

CASTANEDA-ALTAMIRANO, C.A.; CASTILLO, M.; SANCHEZ, J. *et al.* Detection of *Helicobacter pylori* in gastric cancer tissue through histopathology, immunohistochemistry and real-time reverse transcription-PCR. *Futuro Microbiol.*, v.15, p.1131-1137, 2020.

DECHANT, F.X.; DECHANT, R.; KANDULSKI, A. *et al*. Accuracy of different rapid urease tests in comparison with histopathology in patients with endoscopic signs of gastritis. *Digestion*, v.101, p.184-190, 2020.

GUERRA SEGUNDO, D.D.; MELLO, C.B.; CARGNELUTTI, J.F. *et al.* Evidence of helicobacter spp. in saliva and gastric mucosa of domestic dogs in the Central Region of Rio Grande do Sul, Brazil. *Vet. Med. Int.,* v.2021, p.8857231, 2021.

HAPPONEN, I.; SAARI, S.; CASTREN, L. *et al*. Comparison of diagnostic methods for detecting gastric Helicobacter-like organisms in dogs and cats. *J. Comp. Pathol.*, v.115, p.117-127, 1996.

Arq. Bras. Med. Vet. Zootec., v.75, n.6, p.1039-1046, 2023

1045

HONG, S.; CHUNG, Y.; KANG, W.G. *et al*. Comparison of three diagnostic assays for the identification of Helicobacter spp. in laboratory dogs. *Lab. Anim. Res.*, v.31, p.86-92, 2015.

HUSNIK, R.; KLIMES, J.; KOVARIKOVA, S. *et al*. Helicobacter species and their association with gastric pathology in a cohort of dogs with chronic gastrointestinal signs. *Animals*, v.12, p.1254, 2022.

IERARDI, E.; LOSURDO, G.; MILETI, A. *et al*. The puzzle of coccoid forms of Helicobacter pylori: beyond basic science. *Antibiotics*, v.9, p.293, 2020.

KUBIAK, K.; JANKOWSKI, M.; SPUZAK, J. *et al*. An evaluation of the usefulness of invasive and non-invasive methods used to diagnose *Helicobacter* spp. infections in dogs. *Pol. J. Vet. Sci.*, v.20, p.491-499, 2017.

MOUSSA, I.M.; ELJAKEE, J.; BEDER, M. *et al*. Zoonotic risk and public health hazards of companion animals in the transmission of Helicobacter species. *J. King Saud Univ. Sci.*, v.33, p.101494, 2021.

MOUTINHO, F.G.; THOMASSIAN, A.; WATANABE, M.J. *et al*. Prevalência de helicobactérias e alterações na mucosa gástrica de cães saudáveis. *Arq. Bras. Med. Vet. Zootec.*, v.59, p.1080-1883, 2007.

OKUBO, B.M.; RICCI-AZEVEDO, R.; ZOBIOLE, N.N. *et al*. Prevalência de *Helicobacter* spp. em cães de Campo Grande-MS. *Cienc. Anim. Bras.*, v.18, p.e-17286, 2017.

PARIHAR, V.; HOLLERAN, G.; HALL, B. *et al*. A combined antral and corpus rapid urease testing protocol can increase diagnostic accuracy despite a low prevalence of Helicobacter pylori infection in patients undergoing routine gastroscopy. *United Eur. Gastroenterol. J.,* v.3, p.432-436, 2015.

ROBINSON, K.; ATHERTON, J.C. The spectrum of Helicobacter-mediated diseases. *Annu. Rev. Pathol.*, v.16, p.123-144, 2021.

RUIZ, G.; VERROT, L.; LALOY, E. *et al*. Diagnostic contribution of cytological specimens obtained from biopsies during gastrointestinal endoscopy in dogs and cats. *J. Small Anim. Pract.,* v.58, p.17-22, 2017.

RYAN, D.A.; LOURIE, R. The use of *Helicobacter pylori* immunohistochemistry. *Pathology*, v.49, p.S138, 2017.

SABBAGH, P.; MOHAMMADNIA-AFROUZI, M.; JAVANIAN, M. *et al*. Diagnostic methods for *Helicobacter pylori* infection: ideals, options, and limitations. *Eur. J. Clin. Microbiol. Infect. Dis.*, v.38, p.55-66, 2019.

SALOMON, H. Über das Spirillum des Säugetiermagens und sein Verhalten zu den Belegzellen. G. *Fischer*, 1896.

SOUSA, D.A.; CASCON, C.M.; MELLO, M.F. *et al*. Helicobacter spp. in domestic cats: identification and relationship with anatomical and histopathological gastric changes in animals of blood group A. *Pesqui. Vet. Bras.*, v.37, p.1467-1473, 2017.

SUÁREZ-ESQUIVEL, M.; ALFARO-ALARCÓN, A.; GUZMÁN-VERRI, C. *et al*. Analysis of the association between density of *Helicobacter* spp and gastric lesions in dogs. *Am. J. Vet. Res.*, v.78, p.1414-1420, 2017.

TAILLIEU, E.; CHIERS, K.; AMORIM, I. *et al*. Gastric Helicobacter species associated with dogs, cats and pigs: significance for public and animal health. *Vet. Res.,* v.53, p.1-15, 2022.

TAULESCU, M.; AMORIM, I.; WASHABAU, R. Esophagus and stomach. *Veterinary Cytology.* [Ames]:Wiley, 2020. p.380-393.

VIEIRA, F.T.; SILVA, J.P.; VILORIA, M.I.V. *et al*. Frequência e distribuição de Helicobacter spp. na mucosa gástrica de cães. *Rev. Ceres*, v.59, p.25-31, 2012.

WASHABAU, R.J.; DAY, M.J.; WILLARD, M.D. *et al*. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. *J. Vet. Intern. Med*., v.24, p.10-26, 2010.