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Helicobacteriose em cães: diagnóstico molecular e histopatológico

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Resumo

Helicobacteriose é considerada uma das infecções mais comuns em humanos, com uma frequência variando de 20 a 80% de acordo com as condições de saúde de cada país. Em cães, foram relatados altos índices de prevalência desta infecção, porém não foi esclarecida a correlação entre a presença da bactéria e as lesões histopatológicas presente na mucosa gástrica. O objetivo deste estudo foi determinar a frequência e diagnosticar por meio de técnicas histopatológicas e moleculares, helicobacteriose em cães necropsiados. Foram coletadas amostras gástricas de 25 cães oriundos da Unidade de Vigilância Sanitária de Zoonoses do município de São Luis, de diferentes raças e idades, machos e fêmeas, sem histórico clínico prévio de alterações gastrointestinais. Análises histológicas e moleculares foram realizadas e seus resultados confrontados quanto à relação entre a infecção por *Helicobacter* sp. e lesões presentes na mucosa gástrica. Os resultados das análises histopatológicas revelaram que 24 animais (96%) apresentaram pelo menos uma lesão histopatológica no estômago, usando a coloração hematoxilina e eosina, enquanto 21 animais apresentaram organismos semelhantes à bactéria pelo método direto, por meio da coloração Giemsa e 24 animais foram positivas para pelo menos uma das espécies estudadas por análise molecular em tempo real. A coinfecção por *H. felis*, *H. bizzozeronii* e *H. salomonis* esteve presente em 18 dos 25 dos animais. Conclui-se que as lesões histopatológicas, bem como a presença da bactéria foram frequentes em amostras gástricas de cães, no entanto, associando estes resultados, não houve relação significativa. Ainda assim, devido ao alto índice de infecção e considerando seu potencial zoonótico, o tratamento é recomendado para animais, mesmo aqueles que não apresentam sintomas relacionados à infecção, devido ao risco de transmissão entre diferentes espécies.

Palavras chaves: Antropozoonose, *Helicobacter*, saúde pública, gastrite, cão.

Abstract

Helicobacteriosis is considered one of the most common infections in humans, with a frequency ranging from 20 to 80% according to the health conditions of each country. In dogs, high rates of prevalence of this infection have been reported, but the correlation between the presence of the bacteria and the histopathological lesions present in the gastric mucosa has not been clarified. The aim of this study was to determine the frequency and diagnose, by means of histopathological and molecular techniques, helicobacteriosis in necropsied dogs. Gastric samples were collected from 25 dogs from the Sanitary Surveillance Unit of Zoonoses in the municipality of São Luis, of different breeds and ages, male and female, with no previous clinical history. Histological and molecular analyzes were performed and their results compared with the relationship between infection by gender and lesions present in the gastric mucosa. The results of the histopathological analyzes revealed that 24 of the animals (96%) had at least one histopathological lesion in the stomach, using Hematoxylin and Eosin staining, while 84% of the samples had bacteria-like organisms by the right method, using Giemsa and 96% were positive in at least one of the species studied by real-time molecular analysis. Co-infection by *H. felis*, *H. bizzozeronii* and *H. salomonis* was present in 92% of the animals. We concluded that histopathological lesions, as well as the presence of the bacterium, were frequent in gastric samples of dogs, however, associating these results, there was no expressive relationship. Still, due to the high rate of infection and its zoonotic potential, treatment is recommended for animals, even those that do not show symptoms related to the infection, due to the risk of transmission between different species.

Key-words: Anthropozoonosis, Helicobacter, public health, gastritis, dog.

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Lista de Abreviações

°C	Grau Celsius
µg/mL	Micrograma por mililitro
µm	Micrômetro
AC	Agreement coefficient
BID	Duas vezes ao dia
CEEA	Comitê de Ética e Experimentação animal
CO_2	Dióxido de carbono
DNA	Ácido desoxirrubonucleico
EDTA	Ethylenediamine tetraacetic acid
EF	Expected frequency
FC	Carbol-fucsina
FISH	Hibridização fluorescente *in situ*
g	Grama
GM	Giemsa
GoTaq	Taq DNA polymerase
Hbizz	*Helicobacter bizzozzeroni*
HCl	Ácido clorídrico
HE	Hematoxilina e Eosina
Hfel	*Helicobacter felis*
HIV	Vírus da Imunodefinicência Humana
Hp	*Helicobacter pylori*
HpSA	*Helicobacter pylori* stool antigen
Hsal	*Helicobacter salomonis*
HSE	*Helicobacter* spp. enterohepatic
IHQ	Imunohistoquímica
JPEG	Joint Pictures Expert Group
MALT	Tecido Linfoide Associado à Mucosa
MET	Microscopia eletrônica de transmissão
mg/kg	Miligramas por quilograma
min	Minuto
mMol	Milimol

N	Absence of bacteria
N_2	Nitrogênio
Ng	Nanograma
NHPH	*Helicobacter* não *Helicobacter pylori*
O_2	Oxigênio
OF	Observed frequency
OMS	Organização mundial da saúde
P	Presença da bacteria
p	Probabilidade de significância
PCR	Reação em cadeia de polimerase
pH	Potencial hidrogeniônico
PO	Por via oral
qPCR/ RT-qPCR	Reação em Cadeia de polimerase em tempo real
QUID	Quatro vezes ao dia
rpm	Rotações por minuto
S	Segundo
SD	Desvio padrão
sp	Espécie
spp	Espécies
TID	Três vezes ao dia
TRU	Teste Rápido da Urease
UBT	Urea breath test
UEMA	Universidade Estadual do Maranhão
WS	Warthin-Starry
x^2	Elevado ao quadrado

Sumário

1. Introdução

O gênero *Helicobacter* é caracterizado por bactérias espiraladas, Gram-negativas, pertencentes à família *Helicobacteraceae*, da ordem *Campylobacterales*, da classe *Epsilonproteobacteria*, apresentando pelo menos 35 espécies validadas (KAWAMURA et al., 2016). A Organização Mundial da Saúde (OMS) estima a ocorrência desta bactéria em 80% da população humana em países em desenvolvimento e 40% das pessoas em países desenvolvidos (KHODER et al., 2019) e apesar da alta incidência e distribuição mundial, espécies diferentes de *Helicobacter* podem ocorrer de acordo com a localização geográfica (AMORIM et al., 2015).

Há relatos da presença de bactérias do gênero *Helicobacter* em cães e gatos, assim como em outras espécies de animais. Há indícios de relação entre casos da doença em humanos e o convívio com animais portadores da bactéria, revelando a possibilidade de potencial zoonótico. As vias de transmissão podem ser múltiplas, porém ainda não foram esclarecidas. Suspeita-se que os animais transmitem uns aos outros por via oral, lambedura, ingestão de fezes ou alimentos regurgitados (RECORDATI et al., 2007; GHIL et al., 2009), já entre humanos, as vias mais aceitas são a oral-oral, fecal-oral e gastro-oral (ABDEL-RAOUF et al., 2014). Desta forma, cães e gatos são fatores de risco para esta infecção em humanos, sendo transmitida pela saliva (JANKOWSKI et al., 2016a).

Embora a identificação da bactéria *Helicobacter* sp. possa ser realizada por vários métodos, pelo menos dois devem ser realizados e comparados para a obtenção de resultados fidedignos (OKUBO et al., 2017). Os métodos utilizados para o diagnóstico da bactéria são divididos em dois grupos: os invasivos, que utilizam uma amostra tecidual de um órgão, coletado via incisão cirúrgica ou endoscópica, incluindo teste rápido de urease, testes histológicos, microbiológicos e reação em cadeia da polimerase (PCR) e, os não invasivos, dos quais os mais difundidos são o teste respiratório com 13 C-Ureia e o teste de fezes (HpSA), usados somente na medicina humana.

Os testes histológicos são considerados sensíveis e específicos, porém somente a morfologia não é suficiente para caracterizar as bactérias. O isolamento bacteriano é essencial para a investigação do crescimento, teste de suscetibilidade e fatores de virulência, mas também apresenta desvantagens quanto há necessidade de transporte da amostra, meios de cultura, condições de incubação e replicação *in vitro*. A PCR mostra-se como um método

eficiente devido à possibilidade de determinação do DNA da bactéria (LANZONI et al., 2011) e elaboração de *primers* específicos para cada espécie (PATEL et al., 2014).

O tratamento proposto para a resolução de uma gastrite associada à bactéria do gênero *Helicobacter* sp. em humanos, é realizado como uma associação de três fármacos, chamada terapia tríplice, em que são administrados dois antibióticos e um medicamento gastro-protetor, como inibidores da bomba de prótons (BANG; BAIK, 2014) O tempo de tratamento deve ser no mínimo de 14 a 21 dias (ANDRADE, 2002).

Este trabalho será apresentado em forma de capítulos: O primeiro capítulo refere-se à revisão de literatura acerca do tema abordado; o segundo capítulo, refere-se a um artigo de revisão sobre o aspecto zoonótico de *Helicobacter* sp. e o terceiro e último capítulo é constituído de um artigo original, realizado a partir de amostras gástricas de cães, com os quais foram realizados ensaios moleculares e histopatológicos associando à infecção por bactérias do gênero.

A princípio, o intuito do trabalho de tese foi isolar a bactéria em estômago de cães, caracterizá-las quanto a espécies, induzir gastrite em animais de laboratório a partir dessas bactérias e testar métodos terapêuticos alternativos. No entanto, como citado na literatura, este gênero é considerado fastidioso, portanto de difícil isolamento e cultura, o que impossibilitou a realização de algumas etapas do trabalho original. Diante disso, optou-se por identificar as espécies encontradas em mucosa gástrica de cães e associar aos achados histopatológicos.

Ressalta-se a importância desta infecção em cães, ainda que esta não apresente relação com surgimento de sinais clínicos neta espécie, porém, por se tratar de uma doença com alto potencial zoonótico, e estar amplamente associada a gastrite e doenças graves em humanos, o cão, devido a sua íntima relação com seus tutores, pode ter papel importante nesta cadeia de transmissão, sendo veículo desta bactéria, visto que, uma das principais formas de transmissão entre espécies, humanas ou animais, é a oral-oral.

2. Capítulo I

2.1 Revisão de literatura

2.1.1 Gênero *Helicobacter*

O gênero *Helicobacter* sp. possui mais de 35 espécies propostas, identificadas e validadas (MLADENOVA-HRISTOVA et al., 2017) e é caracterizado por bactérias Gram-negativas, pertencentes à família *Helicobacteraceae*, da ordem *Campylobacterales*, inseridas na classe *Epsilonproteobacteria* (KAWAMURA et al., 2016).

Consideradas espécies complexas pela dificuldade para desenvolvimento *in vitro*, devido ao seu crescimento fastidioso, exigindo taxas de umidade atmosférica elevada e meios de cultura recém-preparados, além de cuidados pré-cultivo, incluindo coleta, transporte e tratamentos recentes anteriores à coleta (GUERRA, 2014). São descritas como bacilos curvos, curtos e levemente espiralados, conforme demonstrado na figura 1, variando em espirais largos e movimentando-se por meio de flagelos que podem variar quanto ao número, podendo ser único ou múltiplos em uma das extremidades, localizado bem abaixo da membrana externa no espaço periplasmático e apresentam um citoplasma denso contendo material nuclear e ribossomos (SOLNICK et al, 2006). Medem em torno de 0,1 a 0,5 µm de largura e 3 µm de comprimento, apresentam superfície lisa e extremidades arredondadas. Podem possuir sistema de secreção do tipo IV, que consiste na injeção no citoplasma da célula hospedeira duas proteínas principais, uma vacuolizante e uma citotoxina que atua no processo infeccioso (KONEMAN et al., 2012).

Bactérias deste gênero não oxidam e nem fermentam açucares e são consideradas como microaerofilas (10% CO_2, 5% O_2, e 85% N_2), não esporuladas e mesófilas. As temperaturas ótimas de crescimento variam entre os 37 e 42 °C, podendo ser cultivadas a 37 °C (ON et al., 2017). Reagem positivamente aos testes de urease, catalase e peroxidase (NEIGER; SIMPSON, 2000). São produtoras de enzimas como mucinase, lipase e arginase (ANSELMI et al., 2019).

Uma característica fundamental das bactérias do gênero *Helicobacter* é a capacidade de sobreviver num meio ácido e hostil, graças a produção de uma enzima, a urease, tornando este gênero único no seu modo de ação e patogenia (DUARTE, 2009). A urease atua promovendo a hidrólise da ureia, fisiologicamente presente no suco gástrico, com formação de amônia, que age como aceptor de íons H+, aumentando o pH ao redor da bactéria. Dessa forma, o microrganismo fica protegido, ao menos temporariamente, dos efeitos deletérios do pH ácido do estômago, podendo, assim, ter acesso à camada de muco gástrico (ARAUJO FILHO et al., 2006).

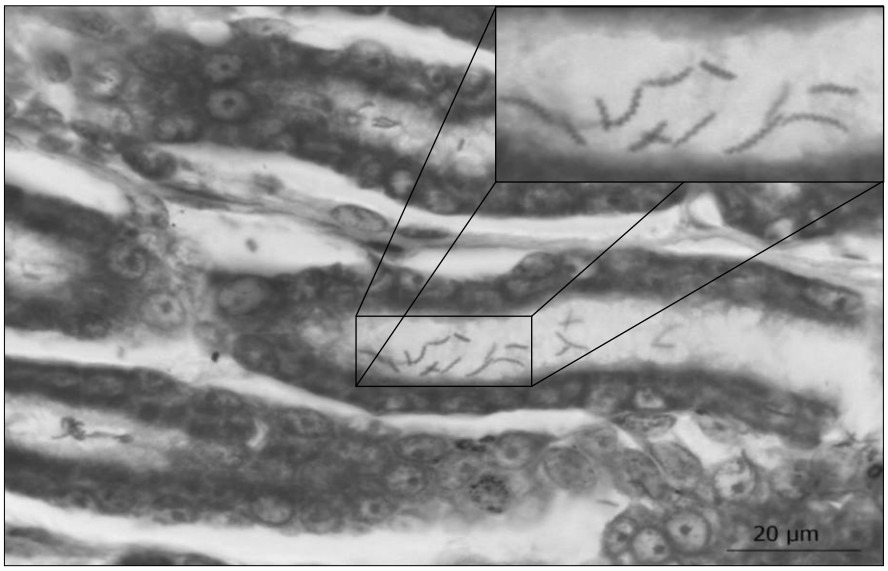

Figura 1. Bactérias morfologicamente compatíveis com *Helicobacter* sp. infectando glândula gástrica de estômago de um cão. GM, obj. 100x. Arquivo próprio.

Dependendo do local de colonização do trato gastrointestinal, o gênero *Helicobacter* sp. é dividido em espécies gástricas e entero-hepáticas. O primeiro grupo inclui espécies que colonizam o trato gastrointestinal superior (estômago e duodeno), enquanto o segundo grupo contém espécies encontradas no trato gastrointestinal inferior (íleo, cólon, reto, ductos biliares e fígado) (MORALES et al., 2010). No quadro 1, estão listados alguns exemplares de espécies pertencentes a cada grupo.

Quadro 1. Espécies de *Helicobacter* sp. de acordo com sua localização no trato gastrointestinal.

REGIÃO	**GÁSTRICAS**	**ENTERO-HEPÁTICAS**
ESPÉCIES	*Helicobacter pylori*	*Helicobacter canis*
	Helicobacter heilmannii	*Helicobacter bilis*
	Helicobacter felis	*Helicobacter rappini*
	Helicobacter salomonis	*Helicobacter hepaticus*
	Helicobacter bizzozeronii	*Helicobacter fennelliae*
	Helicobacter suis	*Helicobacter cinaedi*
	Helicobacter mustelae	*Helicobacter pullorum*

Um grande número de *Helicobacter* não *Helicobacter pylori* (NHPH) já foi reconhecido colonizando seres humanos e animais. Anteriormente, as NHPH incluíam *H. heilmannii, H. suis*, espécie que coloniza os estômagos de suínos e um grupo de espécies conhecidas por colonizar a mucosa de cães e gatos: *H. felis, H. bizzozeronii, H. salomonis, H. cynogastricus, H. baculiformis e H. heilmannii* (HAESEBROUCK et al., 2011).

2.1.2 Vias de transmissão

Há relatos da presença de bactérias do gênero *Helicobacter* em cães e gatos, assim como em outras espécies de animais. Há indícios de relação entre casos da doença em humanos e o convívio com animais portadores da bactéria, revelando a possibilidade de potencial zoonótico. Já foi identificada uma correlação positiva entre a presença do gênero *Helicobacter* em animais de companhia e humanos que mantiveram contato com esses animais, uma vez que este favoreceu a contaminação de tutores com espécies como *H. felis* e *H. bizzozeronii* (CHUNG et al., 2014).

A transmissão zoonótica ainda é controversa e diversas hipóteses (LECOINDRE et al, 2000) foram aventadas com base na evidência de que este grupo de bactérias tem um importante papel na cadeia epidemiológica de doenças crônicas (FOX, 2002) como aquelas consideradas inespecíficas (GILL et al, 2016) e aquelas com sintomatologia bem típica e sugestiva como gastrites, úlceras, além de ser considerada com um fator predisponente para a indução de neoplasias gástricas. Há relatos na literatura de que a bactéria pode ser encontrada em muitas espécies de animais exóticos, silvestres, de companhia e produção, que apontam para transmissão direta e indireta entre estes animais e humanos (MLADENOVA-HRISTOVA et al., 2017) por meio da transmissão oral-oral, beijos (HERRERA, 2004) e lambidas (THOMSON et al., 1994). Tal fato já foi comprovado com a detecção de *Helicobacter* na mucosa gástrica de tutores com úlceras e de seus cães de estimação assintomáticos (DE BOCK et al., 2006).

Embora as vias de transmissão possam ser múltiplas, ainda não estão bem esclarecidas. Provavelmente os animais se infectam por via oral devido o ato de lamber a prole e por meio da ingestão de fezes ou alimentos regurgitados (RECORDATI et al., 2007; GHIL et al., 2009). 2010Para Abdel-Rolf et al. (2014), as vias de infecção mais prováveis são a oral-oral, fecal-oral e gastro-oral. Dessa forma, diante dos hábitos de lambedura dos animais

domésticos domiciliados, que apresentam íntimo contato com os tutores, cães e gatos infectados são fatores de risco em potencial para humanos (JANKOWSKI et al., 2016b).

O ciclo de propagação das bactérias envolve muitos mecanismos, uma vez que colonizam ambientes variados e são passíveis de iserem encontradas em diferentes tecidos (GILL et al., 2016). Casagrande et al. (2010), demonstraram por meio de testes moleculares que fluidos e secreções naturais como saliva e fezes participam ativamente deste processo. Aerossóis e vômitos também são importantes vias de transmissão (RECORDATI et al., 2007; OKUBO et al., 2017).

Água, alimentos de origem vegetal (BELLACK, 2006; QUAGLIA; DAMBROSIO, 2018) e alimentos de origem animal também exercem um papel significativo (ANGELIDIS et al, 2011; QUAGLIA; DAMBROSIO, 2018; HERRERA, 2004) no ciclo de transmissão, o que reforça a ideia de haver potencial zoonótico e possibilidade de disseminação.

2.1.3 Aspectos histológicos das helicobacterioses em animais e humanos

A detecção de *Helicobacter* sp. está associada a doenças do trato gastrointestinal em diversas espécies de animais. *Helicobacter canis* já foi observada em gatos e cães com diarreia e humanos com gastroenterite, bacteremia e hepatite, enquanto *Helicobacter bilis* tem sido identificada em camundongos com enterite (CASTIGLIONI et al., 2012). Gill et al. (2016) detectaram alta taxa de abortamento em ovelhas que pode estar associada à presença de *Helicobacter*, indicando que esta bactéria pode ser uma importante causa de aborto em ovelhas. *H. felis* foi observada em mucosas estomacais ulceradas de tutores, bem como nos seus respectivos animais de companhia, além de outras espécies, como *H. bizzozeronii* e *H. heilmanii* (DE BOCK et al., 2006).

No caso de *Helicobacter* sp. enterohepáticas (HEH), autores descrevem quadros de diarreia, inflamação e neoplasia em diversas espécies de hospedeiros. Em um estudo com 54 casos de carcinoma intestinal não hematopoiético em gatos a HEH esteve presente em 30 dos 54 gatos com carcinoma intestinal e 68% dos gatos com HEH apresentaram tumor em intestino grosso, sendo *H. bílis* mais frequente. Os autores observaram que a infecção por HEH é muito comum em gatos com adenocarcinoma mucinoso (92 variando de 33% em intestino delgado a 68 % em intestino grosso (SWENNES et al., 2016). Kaewpitoon et al. (2016) descrevem a presença de *Helicobacter* sp como possível fator de risco para colangiossarcoma.

Gastrites moderadas a severas podem ser mais frequentes em cães infectados por *H. heilmannii* que em cães negativos. Um estudo sugere que a infecção por *H. heilmannii* pode estar relacionada à severidade do quadro de gastrite em cães. Casos de diarreia crônica em cães positivos para *Helicobacter* sp. tiveram uma frequência significativamente mais alta que em cães negativos. De 144 cães testados, 34,7% estavam infectados com *Helicobacter* spp., sendo que um animal estava infectado por *H. pylori*, apresentando severa gastrite ulcerativa, similar à encontrada em humanos (KUBOTA-AIZAWA et al., 2017).

Sasani et al. (2014) observaram que, de um total de 28 gatos de rua, 75% apresentaram gastrite, sendo 82% positivos para *Helicobacter* spp., 80% positivos pelo teste rápido da urease e 75% positivos pela coloração de Giemsa.

Infecção por *H. pylori* foi associada a alterações hematológicas extragástricas em humanos tais como deficiência específica de ferro, deficiência de cobalamina, trombocitopenia imunomediada e linfoma do tipo MALT (CAMPUZANO-MAYA 2014).

2.1.4 Diagnóstico

Os métodos utilizados para diagnosticar esta bactéria são divididos em dois grupos: invasivos e não invasivos (JANKOWSKI et al., 2016). Os invasivos são aqueles que usam uma amostra de tecido de estômago removida por meio de incisão ou endoscopia, que será submetido ao Teste Rápido da Urease (TRU), testes histológicos, microbiológicos e PCR (SKREBINSKA et al., 2018; MOHAMMADIAN; GANJI 2019). Os testes não invasivos mais difundidos são o respiratório de ^{13}C-ureia e o de fezes (HpSA) (SILVA et al, 2010; EL-SHABRAWI et al., 2018). Embora a determinação da *Helicobacter* spp. possam ser realizada por vários métodos, pelo menos dois métodos devem ser realizados e comparados para a obtenção de resultados fidedignos (OKUBO et al., 2017). Já foi relatado que o método HpSA não é considerado eficiente quando comparado aos demais testes (HONG et al., 2015).

Comparando métodos diagnósticos invasivos de amostras gástricas de 75 cães necropsiados, um estudo revelou que ao exame histopatológico utilizando as técnicas de coloração hematoxilina e eosina (HE) e Warthin-Starry (WS) foram encontrados 17,3% e 46,7% animais positivos, respectivamente. Utilizando imunohistoquímica (IHQ), 30,7% dos animais foram positivos e apenas 10,7% dos cães foram positivos utilizando a PCR. Esses resultados mostram que os métodos histopatológicos diagnosticaram 60% dos cães como

positivos, enquanto a PCR, 10,7 %, demostrando variação em termos de sensibilidade e especificidade entre os testes empregados (PRACHASILPCHAI et al., 2007).

Recordati et al. (2007), investigando amostras de saliva, placas dentárias e mucosa de cães, observaram que 78,9% e 94,7% das amostras estudadas foram positivas, nas técnicas de PCR convencional e Nested PCR, respectivamente. Quando os métodos foram comparados para as amostras de placas dentárias, os autores encontraram percentuais na ordem de 21,1% utilizando PCR convencional e 44,7% utilizando Nested-PCR. Ao histopatológico, usando a coloração WS, 71,1% das amostras foram consideradas positivas.

Em um estudo que objetivou determinar a presença, distribuição regional e localização de *Helicobacter* spp. na mucosa do trato gastrointestinal e no sistema hepatobiliar de cães que apresentaram sinais clínicos compatíveis com helicobacterioses através de PCR e hibridização fluorescente *in situ* (FISH), foi possível detectar 100% de positividade nos animais incluídos na pesquisa. No estômago dos cães foram encontradas as espécies *H. bizzozeronii* e *H. felis*, identificadas pela PCR; no intestino delgado foram encontrados antígenos do gênero por FISH; no cólon e no ceco, o DNA encontrado apresentou similaridade com *H. bilis*, *H. cinaedi* e *H. canis* em todas as amostras. O material genético de *Helicobacter* spp. foi encontrado no intestino grosso, mas nenhum anticorpo foi identificado (RECORDATI et al., 2009).

Em um levantamento epidemiológico sobre o grau de infecção de gatos domésticos e silvestres na Coréia do Sul utilizando a técnica de PCR, resultados substanciais foram encontrados, reforçando a sensibilidade da técnica para o diagnóstico de *Helicobacter* spp. (GHIL et al., 2009).

Em cães necropsiados em Viçosa, MG, Brasil, foi utilizado o método de coloração HE e carbol-fucsina (FC) para identificação e distribuição de *Helicobacter* spp. na mucosa gástrica, onde e 96,7% das amostras foram positivas. 95% das amostras das regiões do corpo do estômago e do piloro foram positivas para *Helicobacter* spp., assim como 91,7% das amostras da região fúndica. Embora os métodos histopatológicos sejam muito sensíveis para este gênero, não são considerados muito específicos (VIEIRA et al., 2012). Em cães da raça Beagle usando métodos histopatológico, IHQ, microscopia eletrônica de transmissão (MET), bem como a presença do DNA da bactéria por PCR, *Helicobacter* sp. foi encontrado em 100% das amostras analisadas. Quanto às espécies, todos os cães estavam co-infectados

pelas espécies *H. bizzozeronii* e *H. felis*, diagnosticadas por PCR e confirmadas por MET (LANZONI et al., 2011).

Anacleto et al. (2011) ao estudarem cães errantes, naturalmente infectados, submetidos ao exame endoscópico, por meio do qual foram coletados fragmentos da mucosa gástrica, seguidos da TRU como teste de triagem, detectaram amostras positivas ao exame histológico usando coloração de Giemsa.

Quanto à determinação de espécies, os testes moleculares têm se mostrado bastante eficientes. Em um estudo conduzido na Suécia, Ekman et al. (2013) utilizaram a PCR multiplex, onde identificaram *H. canis* como a espécie mais comum em saliva e fezes de cães e as espécies *H. salomonis* e *H. bizzozeronii* predominando nas amostras de estômago e duodeno.

Na Polônia, cães que apresentavam úlceras gástricas analisadas por biópsia endoscópica, obtiveram um diagnóstico positivo para *Helicobacter* spp. em 81,6% dos casos, utilizando PCR convencional (JANKOWSKI et al., 2015).

Um estudo realizado em Portugal teve como objetivo identificar espécies de *Helicobacter* spp. na mucosa gástrica de cães. Para isso, utilizaram métodos histológicos, histoquímicos, imuno-histoquímicos e PCR. Os resultados obtidos foram 65,2%, 75,4%, 82,6% e 47,8%, respectivamente, de amostras positivas para o gênero. Dentre as amostras positivas para PCR, foi possível identificar o DNA de *H. heilmannii* em 66,7% dos cães e *H. salomonis* em 51,5%. *H. bizzozeronii* e *H. felis* foram encontrados com menor frequência (AMORIN et al., 2015).

A PCR multiplex é espécie-específico e considerado como o método recomendado para identificação de espécies em amostras fecais quando comparado aos kits de antígeno Helicobacter de fezes (HpSA) e TRU. Nested PCR também tem se mostrado eficiente na identificação de espécies do gênero (JANKOWSKI et al., 2016a).

Patel et al. (2014) apontaram a necessidade de se determinar o padrão-ouro para diagnóstico de *H. pylori* em humanos, expondo vantagens e desvantagens de cada um dos testes. Testes não invasivos como o teste respiratório com 13C-uréia (UBT) e o teste com antígeno fecal de Helicobacter são os mais utilizados na rotina, mas o UBT é tecnicamente muito exigente, além do fato de o estômago estar colonizado por várias bactérias produtoras

de ureia, o que o torna não específico. Embora o teste de detecção de antígeno seja mais específico, algumas pesquisas o descrevem como um teste de baixa sensibilidade. Os testes invasivos, nos quais são utilizados fragmentos de tecido da mucosa, são o TRU, histologia, cultura e PCR. O TRU sofre as mesmas questões que o UBT. Os testes histológicos são considerados sensíveis e específicos, porém somente a morfologia não é suficiente para caracterizar as bactérias. O isolamento bacteriano é essencial para a investigação do crescimento, testes de suscetibilidade e fatores de virulência, mas também existem desvantagens no teste em relação ao transporte da amostra, meios de cultura, condições de incubação e a dificuldade de replicação *in vitro* da bactéria. Desta forma, mais uma vez a PCR mostra-se como um método eficiente devido à possibilidade de elaboração de *primers* específicos para cada espécie. O Nested PCR pode eliminar os falsos negativos obtidos pela PCR convencional, o que possivelmente poderia ocorrer devido à existência de outras espécies que estão afetando o homem, sendo, portanto, considerada o teste padrão ouro.

2.1.5 Tratamento

Em humanos, uma das principais problemáticas do tratamento das gastrites vem da dificuldade de se determinar a concentração de antibióticos ativos na mucosa gástrica. Há uma íntima relação entre a cinética do fármaco e a influência da variação do pH local. Alguns medicamentos entre aqueles que são utilizados para tratar a infecção gástrica são a amoxicilina, metronidazol e claritromicina, que são afetados pelo pH estomacal, o que diminui sua meia vida (ERAH et al., 1997). Além disso, a concentração do medicamento varia dependendo da região gástrica, como no antro, corpo e fundo (MÉGRAUD, 1998).

Devido aos efeitos colaterais, como cólica abdominal, náuseas, vômito, diarreia e disgeusia (BRASIL, 2014), decorrente dos medicamentos combinados que compõem a terapia, ocorre a refratariedade ao tratamento por partes de alguns pacientes. Além disso não existem vacinas específicas para o microrganismo, dificultando a redução nos casos de infecção (ZHANG et al., 2011). Resistência a antibióticos, efeitos adversos, riscos de reinfecção e alto custo de antibioticoterapia são problemas inerentes ao tratamento (AYALA et al., 2014).

Em humanos, o regime padrão para o tratamento de *H. pylori* é a terapia tripla que consiste na combinação de inibidor de bomba de próton, amoxicilina e claritromicina por 14 dias, como tratamento de primeira linha. Alternativas incluem terapia quádrupla com

bismuto por 10 a 14 dias e terapia tripla concomitante por 14 dias. Em casos de falha na terapia tripla com claritromicina ou na terapia quádrupla, as estratégias recomendadas são terapia tripla com levofloxacina ou terapia quádrupla com bismuto, ambas por 10 a 14 dias. Em caso de falha de um dos dois regimes de segunda linha recomendados, deve ser usado como terapia um regime de tratamento de terceira linha. (COELHO et al, 2018). A resistência a antibióticos tem sido um fator decisivo na erradicação e reinfecção por *H. pylori*, reduzindo as taxas de sucesso no tratamento. Regimes terapêuticos devem ser direcionados de acordo com o grau de resistência a antibióticos específicos. Países com altas taxas de reinfecção ou resistência à terapia padrão vêm adotando novos modelos como terapias triplas contendo vonoprazan, terapias quíntuplas, terapias duplas de altas doses e terapias triplas padrão com uso de probióticos (HU et al, 2017).

Na medicina veterinária não há claramente descrita a relação entre a presença de NHPH na mucosa gástrica e gastrite, sendo observada a presença da bactéria com alta frequência de infecção sem o surgimento de sinais clínicos. Dessa forma, questiona-se a necessidade de tratamento em cães diagnosticados com helicobacteriose. No entanto, estudos vêm sendo desenvolvidos e autores defendem o uso de terapias semelhantes àquelas utilizadas em humanos (GARCÍA-ALONSO et al, 2016).

A utilização de clavulanato de amoxicilina a 20 mg/kg PO BID, claritromicina 7,5 mg/kg PO BID e metronidazol 10 mg/kg PO BID durante 14 dias (SIMPSON, 2010) ou de amoxicilina 22mg/kg PO BID, metronidazol 11 – 15 mg/kg PO BID e subsalicilato de bismuto 0,22ml/kg PO TID/QUID durante 21 dias (JERGENS et al., 2009), são protocolos recomendados para tratamento de helicobacteriose em cães e gatos. A utilização de claritromicina, amoxicilina e lansoprazol também se mostrou eficaz na erradicação da *Helicobacter* spp. em cães (ANACLETO et al., 2011).

2.2 Objetivos

2.2.1 Objetivo geral

- Diagnosticar por meio de técnicas histopatológicas e moleculares, helicobacteriose em cães necropsiados na cidade de São Luis – Maranhão, Brasil.

2.2.2 Objetivos específicos

- Diagnosticar gastrite infecciosa causada por *Helicobacter* sp. em cães por meio de testes rápido, histopatológico, microbiológico e molecular;
- Identificar por meio de técnicas moleculares as espécies de *Helicobacter* spp. presentes na mucosa gástrica de cães;
- Caracterizar e classificar lesões inflamatórias na mucosa de cães causada por *Helicobacter* spp.

3. Capítulo II - Artigo de revisão a ser submetido

Helicobacteriosis in humans and nonhumans: Is there a real link?

Anthropozoonotic profile of *Helicobacter* sp.

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ABSTRACT

Studies show the presence of bacteria of the genus *Helicobacter* sp. in the gastrointestinal tract of humans and nonhumans. The variation between species mostly happens due to geographic location, and co-infection can happen in the same individual. Despite the high prevalence and lack of correlation with clinical manifestations in animals, the relationship between the presence of bacteria and pathological findings in the gastric mucosa is being clarified. Transmission pathways between animals can happen through oral-oral and oral-fecal and between animals and humans, mainly through saliva, since the oral cavity and saliva of animals harbor different species of *Helicobacter* sp. It also can happen through contaminated foods of animal origin, water and vegetables. The versatility and ability to adapt to varying environmental seems to be one the key elements to the zoonotic nature of non-pylori gastric *Helicobacter* infections in humans. This review provides evidence of transmission between human and non-human species, different clinical manifestations, epidemiology and prevalence, in addition to propose a cycle of agent dissemination among species and environment.

KEY WORDS:

Antropozoonosis, *Helicobacter*, public health.

INTRODUCTION

Helicobacter spp. is a Gram-negative, microaerophilic bacteria that can survive in acidic environments (Okubo et al., 2017). Gastric *Helicobacter* species can damage the protective barrier of the mucus layer in order to inhibit the acidity of the gastric juice. They are mobile and produce enzymes that help in the breakdown of mucus and its adhesion to the epithelium. The local inflammatory process can culminate in the development of chronic gastritis (Burkit et al. 2017); and although the prevalence of *Helicobacter* spp. is high in animals, the relationship between the presence of bacterium, inflammatory reactions and clinical signs is not well established (Vieira et al., 2012).

In humans, there is a relation between gastric cancer and chronic progressive *H. pylori* infection. This bacterium is associated with gastritis, which varies from chronic to atrophic, intestinal metaplasias, dysplasias, gastric neoplasms (Kusters et al. 2006), neoplasia of the mucosa-associated lymphoid tissue (MALT), non-ulcerative dyspepsia, coronary heart disease and chronic idiopathic urticaria (Gasbarrin et al. 2004). Some studies also suggest the association between this infection and *diabetes mellitus*, metabolic and neurological disorders, cardiovascular, hematological, ophthalmic, dermatological and hepatobiliary disease (Suzuki et al., 2011; Banic et al. 2012).

In dogs and cats, factors such as age, sex, size and race are often not mentioned and, when analyzed, are not associated with *Helicobacter* spp infection. (Castiglioni et al., 2012). In humans, there is a close relationship between *H. pylori* infection and the hygienic sanitary habits practiced, being more frequent in patients with inadequate hygienic habits (Suarez Rivera et al., 2013).

This review aims to compile information related to systematics, epidemiological aspects, transmission pathways and clinical signs of *Helicobacter* spp. infections. in humans and domestic and wild animals, analyzing their zoonotic potential and importance for public health.

METHODS

The research was made in PubMed, from August to September, 2019, using the terms "*Helicobacter* spp.", "*Helicobacter* and animals", "*Helicobacter* and zoonosis", "*Helicobacter* in dogs", "Non-Helicobacter pylori *Helicobacter* spp." and "*Helicobacter* and public health". We used sources published in the past 15 years. Inclusion and exclusion criteria were used based characteristics that disqualify prospective subjects from inclusion in the study. Articles not written in English, Spanish or Portuguese were excluded. Articles that addressed aspects related to the presence of non-*H. pylori* Helicobacter (NHPH) in stomach or other organs of the digestive system of domestic and wild animals and humans were included. Data regarding genus biology, epidemiology, clinical and pathological aspects, transmission pathways and anthropozoonotic relationship were included.

Helicobacter spp.

The genus *Helicobacter* spp. has more than 35 identified and validated species (Mladenova-Hristova et al., 2017). It is a Gram-negative bacteria, spiral shaped, belonging to the family *Helicobacteraceae*, order *Campylobacterales* and class *Epsilonproteobacteria* (Kawamura et al., 2016).

Helicobacter spp. is a curved, short and slightly spiraled bacillus. It is often characterized by sheathed flagella at one extremity of the bacterium, located well below the outer membrane in the periplasmic space and presenting a dense cytoplasm containing nuclear material and ribosomes (Solnick et al., 2006). The size varies between 0.1 to 0.5 µm width and 3 µm length, with a smooth surface and rounded edges.

Bacteria of this genus do not oxidize or ferment sugars and are considered microaerophils (10% CO_2, 5% O_2, and 85% N_2), non-sporulated and mesophilic. Optimum growth temperatures range from 37 to 42 °C and can be grown at 37 °C (On et al., 2017).

A fundamental characteristic of gastric species of *Helicobacter* is the ability to survive in an acidic and hostile environment due to urease production (Duarte, 2009). Urease acts by promoting the hydrolysis of urea, physiologically present in gastric juice, with ammonia formation, which acts as a receptor of H^+ ions, increasing the pH around the bacteria. These adaptations allow the microorganism to remain protected, at least temporarily, from the deleterious effects of stomach acid pH, allowing then to access deeper mucosal layers (Burkitt et al., 2017).

Helicobacter spp. can be divided into gastric and enterohepatic species. The first group includes species that colonize the upper gastrointestinal tract (stomach and duodenum), such

as *H. pylori*, *H. heilmanni*, *H. felis*, *H. salomonis*, *H. bizzozeronii*, *H. suis* and *H. mustelae*. The second group contains species found in the lower gastrointestinal tract (ileum, colon, rectum, bile ducts and liver), such as *H. canis*, *H. bilis*, *H. rappini*, *H. hepaticus*, *H. fennelliae*, *H. cinaedi* and *H. pullorum* (Morales et al., 2010).

Previously, species called NHPH included two groups: *H. heilmannii* and *H. suis*, which were present in swine stomachs. The second group inhabit the gastric mucosa of dogs and cats, such as *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. cynogastricus*, *H. baculiformis* and *H. heilmannii* (Haesebrouck et al., 2011). However, based on PCR identification of the ureaB gene, *H. bizzozeroni* is now the predominant species in the canine gastric mucosa (Van Den Bulck et al., 2005).

TRANSMISSION PATHWAYS

There is variation for transmissions modes. In offsprings, their mother can be the source of infection, which can happen through the act of licking the brood. They can also be infected by ingesting feces or regurgitated food (Recordati et al., 2007; Ghil et al., 2009). The oral-oral, fecal-oral and gastro-oral routes are the most frequent mode of transmission (Abdel-Raouf et al., 2014).

This bacteria can colonize several environments and can be found in many different tissues (Gill et al., 2016) and through molecular tests it was possible to prove that natural fluids and secretions such as saliva and feces actively participate in this process (Casagrande et al., 2010). Aerosols and vomiting are also important transmission path between animals (Recordati et al., 2007; Okubo et al., 2017). Also, water (Bellack, 2006; Quaglia and Dambrosio, 2018) and foods with animal origin play a significant role (Angelidis et al., 2011; Quaglia and Dambrosio, 2018).

CLINICAL ASPECTS OF HELICOBACTERIOSIS

Helicobacter spp. has been associated with a range of diseases in different animal species. Cats and dogs with diarrhea and humans with gastroenteritis, bacteremia, and hepatitis were positive for *H. canis*, and *H. bilis* has already been identified in mice with enteritis (Swennes et al., 2016). In sheep, abortion was associated with *Helicobacter* spp. (Gill et al., 2016).

According to Swennes et al. (2016), *Helicobacter* spp. enterohepatic (HSE) can cause diarrhea, inflammation and neoplasia in several host species. In 55 cases of non-hematopoietic intestinal carcinoma in cats, HSE was prevalent in 30 of 54 cats with intestinal carcinoma and 68% of cats with HSE presented tumor in the large intestine, being *H. bilis* the most common species. It has also been noted that HSE infection is very common in cats with mucinous adenocarcinoma (92%) and may be present in cases of intestinal carcinoma (Swennes et al., 2016); it was also possible to associate the presence of *Helicobacter* sp. with cholangiosarcomas, based on global epidemiological data (Kaewpitoon et al., 2016).

Moderate to severe gastritis is more frequent in dogs infected with *H. heilmannii*. Cases of chronic diarrhea in dogs positive for *Helicobacter* spp. had a significantly higher frequency, with 34.7% of the 144 dogs tested, being *Helicobacter* spp. positive. Only one of these animals was infected with *H. pylori*, showing severe ulcerative gastritis, similar to the one found in humans (Kubota-Aizawa et al., 2017).

There is a high prevalence of *Helicobacter* spp. in dogs in different regions of the stomach and they can be related to gastritis, varied from mild to moderate, existing correlation between the degree of inflammation and the number of helicobacteria, suggesting that these may be responsible for changes found in the gastric mucosa of dogs (Vieira et al., 2012). Gastric species NHPH are mostly opportunistic pathogens in cats and dogs when translocated to the hepatobiliary tract, and cause little or no harm (Ménard and Smet, 2019).

In horses, the most common gastric disease is gastric ulceration (Bell et al., 2007), affecting 53% to 90% of adult horses with clinical signs of colic and weight loss. The specific cause of equine gastric ulcers is not fully elucidated (Andrews, 2005). The helicobacteria was detected in the gastric mucosa of some horses, and *Helicobacter*-like DNA was detected in the gastric mucosa of horses with and without ulcers (Contreras, 2007). In pigs, *H. suis* can cause gastritis and ulcers and can lead to slow growth (Padra et al., 2018).

H. pylori is a strictly human pathogen, currently colonizing the stomach of approximately half of the world's population. In most individuals, it does not lead to serious illness, but gastritis lesions are always present and ulcer can occur in 5 to 10% of the cases. *H. pylori* is considered to be a risk factor for carcinogenesis in gastric adenocarcinoma (Mégraud et al., 1998). *H. pylori* infection is associated with extragastric haematological alterations in humans such as specific iron deficiency, cobalamin deficiency, immune-mediated thrombocytopenia and MALT-like lymphoma (Campuzano-Maya, 2014).

PREVALENCE IN ANIMALS

A study in Thailand using stomach samples from 75 autopsied dogs revealed that 60% were positive for *Helicobacter* spp. (Prachasilpchai et al., 2007). In Italy, *Helicobacter* spp. was identified in saliva, dental plaque and gastric mucosa of 38 dogs of different breeds, male and female, revealing that 78.9% of the dogs investigated were infected by this genus (Recordati et al., 2007).

In Portugal, a percentage of 65.2%, 75.4% and 82.6% of the dogs were positive using histological, histochemical and immunohistochemical methods, respectively. qPCR analysis detected DNA from these bacteria in 47.8% of dogs. *H. heilmannii* was identified in 66.7% of the samples, *H. salomonis* in 51.5% and *H. bizzozeronii* and *H. felis* were found at low frequencies (Amorim et al., 2015).

An epidemiological survey in South Korea to determine the presence of *Helicobacter* spp. in saliva and feces of domestic and wild cats, showed that 56.3% of domestic cats and 91.1% of wild cats were positive (Ghil et al., 2009). Also in South Korean, 62.5% of the samples were positive, being *H. heilmannii* and *H. felis* identified in 37.5% and 25% of dogs, respectively (Hong et al., 2015).

The high percentage of *Helicobacter* spp. in cats found in different studies may be explained by the oral transmission through the habit of licking each other. Up to 80% of stray cats with gastritis were positive for *Helicobacter* spp. (Sasani et al., 2014) and this range can go even higher with the occurrence of *Helicobacter* spp. in more than 90% of mixed-breed cats (Sousa et al., 2017).

A study conducted in Sweden identified species of *Helicobacter* spp. present in saliva, feces, stomach and duodenum of Beagles. All stomach samples were *Helicobacter* positive and *H. canis* was the most common specie in saliva and feces. *H. salomonis* and *H. bizzozeronii* were the most prevalent in stomach and duodenum, and all dogs presented at least two species in the oral cavity (Ekman et al., 2013). *H. bizzozeronii* and *H. felis* have also been found in asymptomatic Beagles (Lanzoni et al., 2011).

In Iran, the cecum and colon of 15 symptomatic and asymptomatic stray dogs were highly colonized. *H. canis* and *H. bizzozeronii* were identified in all samples and *H. bilis* was present in 44% of all samples, *H. felis* in 40%, *H. salomonis* in 80% and *H. pylori* in 7% (et

al., 2014). In Poland, Jankowski et al., (2015) found 81.6% of positive cases for *Helicobacter* spp. in the samples, and Jankowski et al. (2016a) found in 23.3% of the samples.

In Brazil, researchers seeking to evaluate the therapeutic efficacy in stray dogs naturally infected with *Helicobacter* spp. diagnosed 100% of positivity in the sample studied through the rapid urease test, confirmed by histological exam (Anacleto et al., 2011). Also in Brazil, the *Helicobacter* spp. was identified in 96.7% of samples of autopsied dogs, without clinical historic compatible with helicobacteriosis, using histopathological techniques and hematoxilina and eosina and fucsina staining (Vieira et al., 2012).

THE LINK

There are several species of the Helicobacter genus known to infect humans or animals in different tissues. Zoonotic transmission has been suggested due to the presence of these microorganisms in the stomach of dogs, cheetahs, cats, swine, cattle, rats, sheep, birds, foxes, ferrets, guinea pigs, mice, hamsters, marmots, dolphins, beluga whales and monkeys (Carvalho et al., 2008). Exotic, wild, companion and production animals have being reported as *Helicobacter* positive, and the correlation between disease in animals and humans is being studied (Mladenova-Hristova et al., 2017).

One of the key elements in the link between helicobacteriosis in humans and nonhumans seems to be the proximity between pets and humans. Close contact of pets with their owners facilitates transmission and clinical signs are observed in immunocompromised humans. Species such as *H. felis* have been observed in pets owners ulcerated stomach mucosa as well as their respective companion animals. *H. bizzozeronii* (Chung et al., 2014), *H. heilmanii* (De Bock et al., 2006), *H. felis, H. salomonis, H. bizzozeronii*, typically found in the stomach of cats and dogs represent some of the gastric NHPH colonizing the human stomach (Haesebrouck et al., 2009).

However, this association occurs in most cases with a pet dog (Alon et al., 2010). *H. canis* was isolated and identified in a 7-month-old girl with fever who had a dog (Prag et al., 2007), in a 78-year-old man with gastric diffuse large B cell lymphoma with a close exposure to dogs (Alon et al., 2010) and also in a patient with end-stage renal disease physically close to his pet dog (Shakir et al., 2017). The recognition of *H. canis* in immunocompromised patients with dog pet should be considered (Alon et al., 2009). In some cases asymptomatic dogs can be the source of infection to humans (De Bock et al., 2006).

The interesting fact about *H. canis* is its presence also in farm animals. It was identified in fecal sheep samples indicating that these animals may act as reservoirs and potential transmitters for both humans and other animals through derived food from undercooked sheep (Swennes et al., 2014). Therefore, sheep may act as a reservoir for *H. canis* who spreads these bacteria in feces, milk and carcass. The transmission thought this pathway can happen by the ingestion of these inputs (Swennes et al, 2014).

In a domestic cat, *Helicobacter* sp., *H. pylori*, *H. heilmannii* and *H. bizzozeronii* were detected in the fundus, corpus and antrum, *H. pylori* was also found in the antrum, which could represent a new mode of transmission for this anthroponotic disease (Canejo-Teixeira et al., 2014)

In livestock, *H. suis* is a common specie found in the stomach of most pigs, and it is the most prevalent NHPH species found in the stomach of humans. It can cause chronic peptic ulcer disease, gastritis, and MALT lymphoma (Padra et al., 2018). *H. suis* in pigs seems to originate from non human primates, in which infection causes little or no harm. The domestication led to a widespread of this specie in pigs and the infection of humans represents the second most prevalent *Helicobacter* species in the stomach of humans suffering from gastric disease (Flahou et al., 2018). *H. suis* can persist for 48 h in pork meat, and it is suggested that the consumption of raw pork meat can be the source of infection to human (De Cooman et al., 2013).

Infections by *Helicobacter* can be considered occupational diseases, because of cross contamination with caregivers of production animals, evidencing its zoonotic character (Swennes et al, 2014). The contamination of humans thought domestic animals can occur oral-oral, fecal-oral, vectors, water and food of animal origin (Carvalho et al., 2008).

Helicobacter heilmannii sensu lato (*H. heilmannii* s.l.) represents a group of gastric NHPH species that live in the stomach of animals. In humans it can cause mucosa-associated lymphoid tissue lymphoma in gastric tissue. There is evidence that link this bacterium to owners of pet animals, such as the transmission from a cat by direct contact (Van Loon et al., 2003), and also evidence that people who live in contact with cats, cattle or pigs have a higher risk of contamination (Svec et al., 2000).

H. cinaedi has been identified in a HIV (Human Immunodeficiency Virus) positive patient (Mladenova-Hristova et al., 2017) but also can be found in chronic alcoholics and

immunocompetent men and women (Fox et al., 2015). There are many *Helicobacter* spp. species in rodents, with no zoonotic cases, except with hamsters (Whary and Fox, 2006).

According to Schott et al. (2011) the molecular basis of the zoonotic nature of NHPH infections in humans can be explained by metabolic aspects. These species seem to have a big versatility and ability to adapt to varying environmental conditions, which differentiate *H. bizzozeronii* as well as *H. felis* and *H. suis* from *H. pylori*. The phylogenetic and genetic comparisons suggest co-adaptation as well as interspecies periodic transmission (Schrenze et al., 2010).

CONCLUSION

Helicobacteriosis and NHPH diseases are increasingly recognized in domestic and wild animals, as well as in humans. The contact between immunosuppressed patients and contaminated animals may be the key to transmission. The most accepted transmission pathway is oral-oral, through the contact with fomite and by direct contact between people and animals. *Helicobacter* sp. has an important role in epidemiological chain of several diseases which demonstrates its importance for public health, therefore the understanding of the mechanisms mediated by zoonotic transmission is essential for developing strategies for prevention of these infections.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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4. Capítulo III - Artigo submetido para a revista Frontiers in Microbiology

Use of molecular analysis for the identification of *Helicobacter* infective species in dogs and their relationship with the histopathological findings of the disease

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Author contribution statement

AVCAD, APMM, RNMN and DCSCF carried out histopathological and molecular analysis. JRSJ performed statistical analysis. VMN, LGLN and EMS provided reagents for molecular techniques, carried out data analysis, supervised and revised the manuscript. RCC and SAM carried out data analysis, manuscript preparation, revision, and submission of the manuscript. ALAS designed the study, analyzed data, revised the manuscript, and contributed to the study. All authors have read and approved the final manuscript.

Keywords

Helicobacter sp, histopathology, chronic gastrites, Clinical aspects, Dogs

Abstract

Word count: 230

Helicobacteriosis is considered one of the most common infections in humans, with a frequency ranging from 20 to 80% according to the health conditions of each country. Although this bacterium is present in dogs, the correlation between infection and the lesions found in the gastric mucosa is not clear. In order to identify bacteria of the genus *Helicobacter* sp. and associate them with the gross and microscopic morphology of lesions, gastric samples were obtained from 25 dogs, of different breeds, ages and sexes, from the Zoonosis Surveillance Unit of the municipality of São Luis, in the state of Maranhão, Brazil. Samples of five stomach regions were collected for histopathological and molecular analysis. The results of the histopathological analyzes revealed that although 25 animals (96%) had at least one histopathological disorder of the stomach, only 84% of the samples were identified as having the bacteria by direct search, and 96% were positive for one of the species studied via molecular analysis. Co-infection by *H. felis*, *H. bizzozeronii* and *H. salomonis* was present in 92% of the animals. We concluded that the lesions frequently occurred in the gastric samples of dogs, but associating them with infection by *Helicobacter* sp. was not possible. In addition, due to the high rate of infection and its zoonotic potential, treatment is recommended for animals, even those that do not present symptoms related to infection.

Contribution to the field

The Helicobacter genus is present in 80% of the world population. In developing countries, where hygiene conditions seem less adequate, these rates are increasing, with around 90%. This infection is considered a risk factor for chronic gastrointestinal diseases in humans including gastritis, ulcers, dyspepsia and gastric cancer and MALT-type lymphoma. In animals, there is no data to scientific proof the relationship between the presence of the bacterium and gastric disorders. Human-animal transmission was identified as a risk factor for dissemination, being characterized as zoonosis having as forms of transmission, between oral-oral and fecal-oral humans and between animals, oral-oral, oral-fecal, fecal-oral.

Ethics statements

Studies involving animal subjects

Generated Statement: The animal study was reviewed and approved by Animal Ethics and Experimentation Committee of the State University of Maranhão (CEEA/UEMA), under protocol nº 1801-1.

Studies involving human subjects

Generated Statement: No human studies are presented in this manuscript.

Inclusion of identifiable human data

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Data availability statement

Generated Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Use of molecular analysis for the identification of *Helicobacter* infective species in dogs and their relationship with the histopathological findings of the disease

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Keywords: *Helicobacter* **sp, histopathology, chronic gastrites, clinical aspects, dogs.**

Abstract

Helicobacteriosis is considered one of the most common infections in humans, with a frequency ranging from 20 to 80% according to the health conditions of each country. Although this bacteria is present in dogs, the correlation between infection and the lesions found in the gastric mucosa is not clear. In order to identify bacteria of the genus *Helicobacter* sp. and associate them with the gross and microscopic morphology of lesions, gastric samples were obtained from 25 dogs, of different breeds, ages and sexes, from the Zoonosis Surveillance Unit of the municipality of São Luis, in the state of Maranhão, Brazil. Samples of five stomach regions were collected for histopathological and molecular analysis. The results of the histopathological analyzes revealed that although 24 animals (96%) had at least one histopathological disorder of the stomach, only 84% of the samples were identified as having the bacteria by direct search, and 96% were positive for one of the species studied via molecular analysis. Co-infection by *H. felis*, *H. bizzozeronii* and *H. salomonis* was present in 92% of the animals. We concluded that the lesions frequently occurred in the gastric samples of dogs, but associating them with infection by *Helicobacter* sp. was not possible. In addition, due to the high rate of infection and its zoonotic potential, treatment is recommended for animals, even those that do not present symptoms related to infection.

Introduction

The genus *Helicobacter* comprises more than 35 identified and validated species (Kawamura et al., 2017; Mladenova-Hristova et al., 2017) belonging to the *Helicobacteraceae* family, of the *Campylobacterales* order and the *Epsilonproteobacteria* class. They are microaerophilic, Gram-

negative, spiral bacteria, helical in shape, and produce urease, which allows them to survive in acidic environments (Okubo et al., 2017).

Helicobacteriosis is present in 80% of the global human population, with this frequency varying between 20 and 30% in developed countries and reaching 80% in developing countries (Lee and Derakhshan, 2013). Despite the high infection rate, only 10% of those with the condition present clinical signs (Siqueira et al., 2007). Studies have reported infection by non-*H. pylori* helicobacters (NHPH) occurring in up to 86% of healthy dogs and in up to 100% of animals which present chronic vomiting (Recordati et al. 2007; Polanco et al., 2011; Ekman et al., 2013; Jankowski et al., 2016; Jankowski et al., 2016; Nowroozilarki et al., 2017; Suárez-Esquivel et al., 2017).

Several studies have found that transmission routes can be multiple, but the routes themselves remain unclear. Animals can become infected by licking their offspring or eating feces or regurgitated food (Ghil et al., 2009; Recordati et al., 2009). Studies have suggested that the oral-oral, fecal-oral and gastro-oral routes (Abdel-Raouf et al., 2014) are the most important. Thus, infected dogs and cats are potential risk factors for humans (Jankowski et al., 2016b), as there are reports of NHPH species affecting people (Mladenova-Hristova et al., 2017).

The methods used to diagnose this bacterium are divided into two groups: invasive and non-invasive (Jankowski et al., 2016b). Invasive methods are those that use a sample of stomach tissue removed through incision or endoscopy, which is used in the Rapid Urease Test (RUT) and histological, microbiological and molecular tests (Skrebinska et al., 2018; Mohammadian and Ganji, 2019). Non-invasive tests, such as serology or detection of bacterial DNA and antigens in feces, do not require a gastric sample (Silva et al., 2010; El-Shabrawi et al., 2018).

The microbiological identification of this genus can be performed using histopathological techniques, which are considered sensitive and specific, and include – hematoxylin-eosin, toluidine blue, Warthin-Starry and Giemsa, the last of which is considered the technique of choice (Robić et al., 2007). Morphology alone, however, is not enough to characterize the bacteria, and a confirmatory test is therefore required (Patel et al., 2014). Molecular tests are more efficient due to the possibility of creating specific primers for each species, eliminating false negatives through their high specificity and sensitivity, and are therefore considered the gold standard (Patel et al., 2014; Patel et al., 2018).

Therefore, the aim of the present study was to identify species of the *Helicobacter* genus through molecular methods and relate them to the histological findings found in a gastric sample of dogs naturally infected by *Helicobacter* sp.

Material and Methods

Animals

The present study received prior authorization from the **Animal Ethics and Experimentation Committee** of the State University of Maranhão (CEEA/UEMA), under protocol nº 1801-1. A total of 25 adult dogs of no defined breed, with an average weight of 15 kg, which had been euthanized for various reasons by the Zoonosis Surveillance Unit of the City of São Luís, in the state of Maranhão. No euthanasia was performed exclusively for the purposes of this research, and the animals were chosen at random, without any influence on the sample from the researchers. Samples with poor preservation conditions were excluded from the study.

After the animals were euthanized, their stomachs were collected and immediately transported, refrigerated, to the anatomopathology laboratory at UEMA.

Biological samples

Three samples from the cardiac, body, fundus, antrum and pylorus regions of the stomach were collected. One sample of each stomach region was used to identify the bacteria through Giemsa staining. The second sample was used for histopathologic analysis and the samples were stained with H&E. For these two first samples, the material was fixed in a buffered formaldehyde solution, followed by a dehydration process in an increasing series of alcohols, diaphanization in xylol and inclusion in paraffin. The third sample was immersed in a sample-specific lysis buffer with proteinase K to perform quantitative real-time PCR for the detection of *Helicobacter* spp. All samples were examined within 24 hours of collection.

DNA extraction

The third biopsy specimen was placed in 15 mL sterile normal saline and stored at -25°C. Upon analysis of the specimen, 25 mg fragments from the cardiac, body, fundus, antrum and pyloric regions of the stomach were thawed and macerated.

DNA extraction was performed using the total DNA purification from crude lysates method using the DNeasy® Blood & Tissue Kit (QIAGEN), according to the manufacturer's instructions. Briefly, 25 g of the samples were lysed with lysis buffer and proteinase K. The lysate must be acidic (pH <7.0) to obtain maximum binding of DNA to the DNeasy membrane, while the ethanol must not be added to the Buffer AL. A total of 200 µl ethanol (96–100%) was added, and thoroughly mixed again by vortexing, then centrifuged at ≥6000 x g (8000 rpm) for 1 min. The pellets were suspended in 480 µL of digestion buffer (5 mM EDTA, pH 8, 0.05 mol Tris HCl, pH 7.5 and 5% Tween 20) and 20 µL of 100 µg / mL of proteinase K, and incubated at 55ºC overnight. The DNA was extracted twice with a volume of phenol-chloroform, and then precipitated with a double volume of 100% ethanol. Finally, the extracted DNA was resuspended in 100 µL of dilution buffer.

Quantitative real-time PCR for detection of *Helicobacter* spp.

RT-qPCR was performed using the QuantStudioFlex 6 K instrument (Applied Biosystems). The GoTaq® qPCR Master Mix (Promega) gene expression assays listed in Table 1 were used according to the manufacturer's instructions, with cDNA (5 ng) in technical triplicates. The PCR program was as follows: activation (50 °C, 2 min), inactivation (95 °C, 10 min), followed by 40 cycles of denaturation (95 °C, 15 s) and annealing/extension (60 °C, 1 min). The mean Ct values were calculated from the technical triplicates. The primer sequences used in that assay are shown in Table 1 and were designed by DNA Express Biotecnologia LTDA and based on the gene sequences deposited in GenBank.

Specific primers were used to identify *H. felis*, *H. bizzozeronii* and *H. salomonis*, according to the sequences deposited in GenBank, as described in Table 1.

Statistical analysis

Descriptive statistics were calculated and presented as percentages for each variable. To evaluate the significant changes in proportions of the two assessments, McNemar's test was used. For each gastric site, the overall agreement and agreement coefficient were assessed using Gwet's first-order agreement coefficient (AC1) (10). The level of $P < 0.05$ was considered significant for statistical evaluation. Statistical analysis was performed using Stata 12.1 MP4 statistical software.

Data were summarized as means and standard deviations (SD) or percentages, as appropriate.

Results

Histopathological analysis showed that 24 (96.0%) out 25 dogs presented lesions in at least one region. Of the 102 samples obtained, those from 21 dogs were positive. Microscopic analysis

confirmed the occurrence of lesions in 24 out 25 dogs. Regarding the classification of gastritis, six dogs (24 %) showed mild gastritis, characterized by the occurrence of focal inflammatory reaction, composed plasma cells and lymphocytes, with samples presenting hydropic degeneration in the epithelium layer; eleven animals (44%) displayed moderate gastritis, with a multifocal inflammatory reaction associated with the epithelial lining and glandular degeneration; and seven animals (28%) had severe gastritis, with glandular atrophy, fibrosis and necrosis (Figure 01). In 21 animals (84%) microorganisms that morphologically resembled the bacteria of the genus *Helicobacter* were observed, including in animals that did not present any kinds of lesion in the stomach.

In addition to identifying the type of lesion, the distribution of these lesions in the gastric mucosa of dogs was also identified. The region most affected by the lesions was the gastric fundus (84%), followed by the cardia and body regions, present in 80% of the samples, and less frequently, the pyloric antrum region (60%), as can be seen in Figure 02.

In contrast, this microorganism was not found in four animals via the direct search. These animals had lesions in the gastric mucosa, however, and it can therefore be said that the presence of the bacteria does not affect the appearance of lesions in the gastric mucosa. Table 2 shows that the bacteria was most frequent in the antrum, followed by the pylorus ($x^2 = 12.77$; $p = 0.01$).

Figure 03 shows the histopathological changes found in 102 samples in the gastric mucosa using H&E staining. Of the 25 animals analyzed, only one did not present any type of histopathological lesion. The others had chronic gastritis ranging from mild to severe. The fundus was the most affected anatomical region, with 20 of the 26 samples analyzed presenting lesions (Figure 03) (p value <0.01).

Regarding the molecular analyzes, Table 3 shows that 24 out 25 samples were positive for at least one of the species using such analysis. Ten of the 25 samples analyzed were positive for the three species studied in the same individual (*H. felis, H. bizzozeronii* and *H. salomonis*). Only six patients were positive for only one species. Five animals were positive for *H. felis, H. bizzozeronii* and three samples were positive for *H. bizzozeronii* and *H. salomonis*. The numeric and percentage distribution of species is showen in Table 3.

All the results of the histopathological and molecular analyzes are shown in Table 4.

Discussion

Gastritis was present in most of the dogs sampled, as described in previous studies which reported this condition as a common finding (Webb and Twedt 2003; Polanco et al., 2011; Amorim et al., 2016). Despite its high frequency, the presence of the bacteria did not affect the appearance of the gastric lesions found. Studies have reported that gastritis is a common disease in dogs, and, unlike in humans, is a multifactorial disease caused by oxidative stress (Amorim et al., 2016), prolonged anti-inflammatory treatment, ischemia, stroke, iatrogenic trauma (Webb and Twedt 2003; Amorim et al., 2016). In humans, however, *H. pylori* (Hp) is reported as a risk factor for gastritis, dyspepsia, ulcers, and gastric cancer (Webb and Twedt 2003).

Of the 25 animals, 24 exhibited some degree of injury, which varied from mild to severe. In humans and dogs, gastritis is classified based on histopathological characteristics (Washabau et al., 2010; Amorim et al., 2015; Seim-Wikse et al., 2019), with mild cases characterized by the diffuse presence of lymphocytes, rare leukocytes and focal degeneration of the superficial epithelium; moderate including the focal degeneration of the lining of the epithelium and glandular tissue; and the severe form characterized by necrosis of the gastric epithelium (Washabau et al., 2010; Polanco et al., 2011; Amorim et al., 2014; Seim-Wikse et al., 2019).

Although the results of the present study do not relate the presence of the bacteria to sites of the injuries, specialized literature refers to this effect, which can easily explain the major and minor injury frequencies in certain locations in our study. The study by Anacleto et al. (2011) demonstrated the same regularity in the distribution of bacteria in the fundus and antrum regions, as was found in the present study. Amorim et al. (2015) showed that the prevalence of NHPH differs in two regions of the canine stomach, with a higher prevalence in the antrum and a lower incidence in the gastric body. The *in vitro* binding capacity of gastric *Helicobacter* species to the canine gastric mucosa was evaluated, and the authors concluded that *H. heilmannii* was the most adherent species, followed by *H. felis*, *H. bizzozeronii* and *H. salomonis*, which may indicate that the same situation occurred in the present study (Seim-Wikse et al., 2019).

The high frequency of spiral-shaped bacteria (80%) in the gastric mucosa of animals in the present study reinforces the findings of other studies which also found high percentages in histopathological analyzes (Jergens et al., 2009; Lanzoni et al., 2011; Hong et al., 2015; Okubo et al., 2017). In most of the analyzed samples (84%), *H. bizzozeroni* was present. These results agree with other findings described in literature (Okubo et al., 2017), which described this species as one of the most frequent in infection by the genus in dogs.

Similar to the analyzes carried out in the present study, other research has also described (De Bock et al., 2006) a high frequency of *H. felis* infection. The presence of these species of bacteria in the gastric mucosa of dogs, as well as the frequency of the infections they cause, have been widely reported (Jergens et al., 2009; Lanzoni et al., 2011; Hong et al. 2015; Okubo et al., 2017).

Another notable point is the high frequency of co-infection by the species observed herein, which was over 70% of the analyzed samples. Literature has found that these *Helicobacter* species adapt to colonize specific and distinct niches in the gastric mucosa (Lanzoni et al., 2011), and suggests that *H. bizozzeroni* is the species most suited to the canine host, although it has less pathogenic potential (De Bock et al., 2006).

To date, there is no consensus regarding the correlation between histopathological findings of gastritis and the detection of the bacterium in the stomach of dogs as described in humans, with Hp playing an important role in the pathogenesis of gastritis and cancer. Despite scientific evidence of the correlation between chronic lesions and bacteria, only 10 to 20% of the infected population exhibits clinical signs, while the majority of the affected population remains asymptomatic throughout life (Linz et al., 2007). Studies have revealed that the specific clinical manifestation depends on the virulence of the bacteria, the individual characteristics of the host, and environmental factors (Iwańczak et al., 2017), such as adequate sanitary conditions.

This same situation has been reported in studies involving companion animals, mainly dogs, in which high prevalences of infection by bacteria of this genus and the absence of clinical symptoms are described. In addition, in humans, most infections occur from a single species and varied strains, with different degrees of virulence, while co-infection in animals is common, with species exhibiting varying degrees of virulence, which makes it difficult to determine the causes of the lesion in the presence of bacteria.

In addition, Smet et al. (2018) when analyzing the evolution of this genus, it was found that the species that affect domestic animals evolved in parallel with Hp, being considered ecologically similar but genetically distinct. The lack of genetic similarity between Hp and NHPH suggests that, due to their different periods of evolution, an old association between these species and their hosts exists. It is further estimated that *Helicobacter* species associated with pets evolved at the same time, even prior to domestication, making such animals the likely original hosts, explaining the little or no damage to the gastric mucosa in dogs and cats (Haesebrouck et al., 2011).

However, despite the high levels of colonization found in the present study, even with no association with the histopathological findings, the results obtained suggest that the immune system acts as a variable in gastritis caused by *Helicobacter* spp in dogs.

Conclusion

The present study found that infection by the *Helicobacter* sp. genus in dogs does not correspond to the appearance of lesions in the gastric mucosa, and other possible causes should therefore be investigated. Although the q-PCR assay diagnosed *Helicobacter* spp., the normalization of bacterial counts is an important aspect of the standardization of *Helicobacter* spp. q-PCR assays, as it allows the reliable comparison of results between studies. However, domestic animals can serve as a vehicle for the transmission of bacteria of this genus, causing similar harm to that caused by *H. pylori* in humans, in view of their zoonotic potential, thus, the treatment of these patients is required, even when they do not show related clinical signs of infection.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author Contributions

AVCAD, APMM, RNMN and DCSCF carried out histopathological and molecular analysis. JRSJ performed statistical analysis. VMN, LGLN and EMS provided reagents for molecular techniques, carried out data analysis, supervised and revised the manuscript. RCC and SAM carried out data analysis, manuscript preparation, revision, and submission of the manuscript. ALAS designed the study, analyzed data, revised the manuscript, and contributed to the study. All authors have read and approved the final manuscript.

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Ethics approval

This study was approved by the Animal Ethics and Experimentation Committee of the State University of Maranhão, registered under protocol number 12/2017.

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Figure 1 - Canine stomach: A – antrum region showing ectasis gland filled with spirochete-shaped bacteria (Bar = 20µm). B - glands of the antrum in longitudinal section showing large amounts of fibroplasia. E – cardia region showing gland atrophy and intense fibrosis. F – antrum region demonstrating gland hyperplasia with mild fibroplasia. Hematoxylin and Eosin (Bar = 50µm).

Figure 2 - Frequency of observations of histopathological lesions in the cardia, fundus, body, antrum and pylorus regions.

Figure 3 - Histopathological findings from gastric samples from dogs.

Table 1 - Primers used in qPCR based on gene sequences deposited in GenBank.

Table 2 - Frequency of observation of organisms similar to bacteria of the genus *Helicobacter* sp. in stomach samples from dogs, by means of histopathological examination

Table 3 - Helicobacter species in gastric cells from dogs detected by the qPCR method

Table 4 - Results obtained by analyzing gastric samples from dogs using histopathological methods, using Giemsa and Hematoxylin & Eosin and qPCR for the species *H. bizzozeronii, H. felis* and *H. salomoni.* Score 1: Normal; Score 2: Inflammatory process + venous congestion + mucosal edema; Score 3: Inflammatory process + venous congestion + mucosa edema + fibrosis + gland degeneration; Score 4: Inflammatory process + venous congestion + mucosa edema + gland degeneration + fibrosis + necrosis

Table 1

Gene	Primers	Species	References
*Hfel*_Foward -5’	GCT GGT GGC ATC GAT ACG CAT -3’	*H. felis*	26
*Hfel*_Reverse – 5’	TTT TTA GAT TAG CGC GTC CGG GA – 3’		
*Hsal*_Foward -5’	TC TTA TGA GTT GGA CTT GGT GCT CAC CAA T – 3’	*H. salomonis*	
*Hsal*_Reverse – 5’	TTT GCC ATC TTT AAT TCC AAT GTC GGC – 3’		
*Hbizz*_Foward -5’	AAT CTT TGC GTG GGC CCT GCT ACT GAG – 3’	*H. bizzozeronii*	
*Hbizz*_Reverse – 5’	CTG GCA AAT GCT GTG GGG ATT TGT TGG -3’		

385 Table 2

Location		Observation frequency	
		N	P
Antrun pyloric	OF	15	10
	EF	(22.6)	(7.4)
Cardia	OF	20	5
	EF	(22.6)	(7.4)
Body	OF	20	5
	EF	(22.6)	(7.4)
Fundus	OF	21	5
	EF	(22.6)	(7.4)
Pyloric canal	OF	18	7
	EF	(22.6)	(7.4)

386 *x^2= 12.77; p=0.01/ OF – Observed frequency; EF- Expected frequency; N- Absence of bacteria; P – Presence of
387 bacteria.

388 Table 3

Specie	Number	Perce
H. bizzozeronii	21/25	84.0%
H. felis	18/25	72.0%
H. salomonis	12/25	48.0%
H. bizzozeronii + *H. felis* + *H. salomonis*	10/25	40.0%
H. bizzozeronii + *H. felis*	05/25	20.0%
H. bizzozeronii + *H. salomonis*	03/25	12.0%
H. felis + *H. salomonis*	00/25	0.0%

389 Table 4

Dog	Giemsa					H&E	qPCR		
	Cardia	Fundus	Body	Pyloric antrun	Pyloric canal		*H. biz*	*H. fel*	*H. sal*
01						Score 2	X	X	
02		X	X		X	Score 2	X	X	
03	X					Score 4	X	X	
04				X		Score 2	X		
05					X	Score 4	X	X	X
06		X		X	X	Score 4	X	X	X
07	X		X			Score 2	X	X	X
08		X			X	Score 3	X	X	X
09					X	Score 4	X	X	X
10				X	X	Score 4	X		
11				X		Score 3	X	X	
12				X		Score 3	X	X	X
13				X		Score 2	X	X	
15						Score 3	X	X	
16				X		Score 2	X	X	X
17			X	X		Score 3	X		X
18			X			Score 4	X		
19	X					Score 3	X	X	
20						Score 3			
21	X					Score 3	X	X	X
22				X	X	Score 1	X	X	
23			X			Score 3	X	X	
24				X		Score 4	X	X	X
25		X				Score 3	X	X	X
26						Score 3	X	X	X

390

391

Figure 1.JPEG

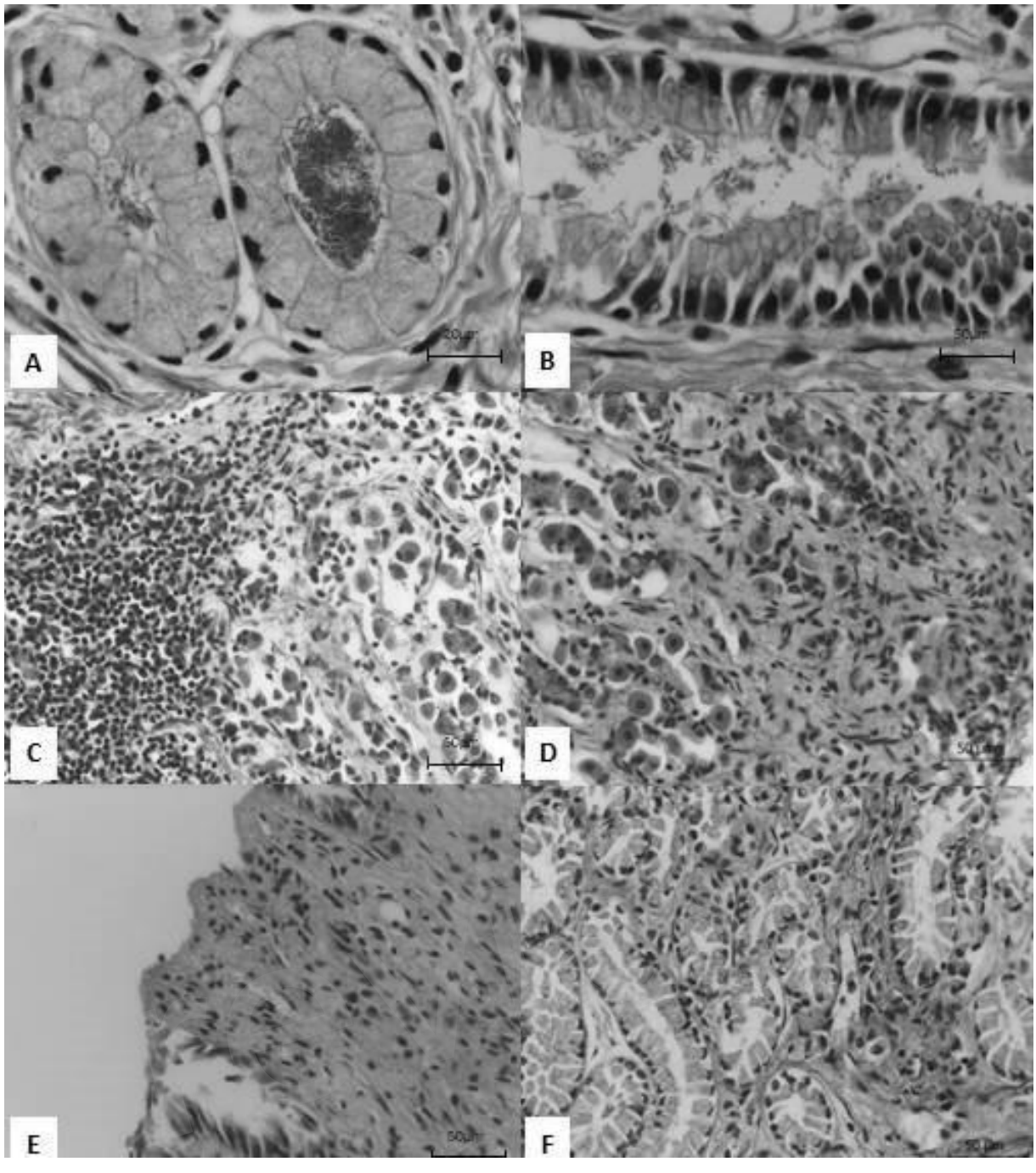


Figure 2.JPEG

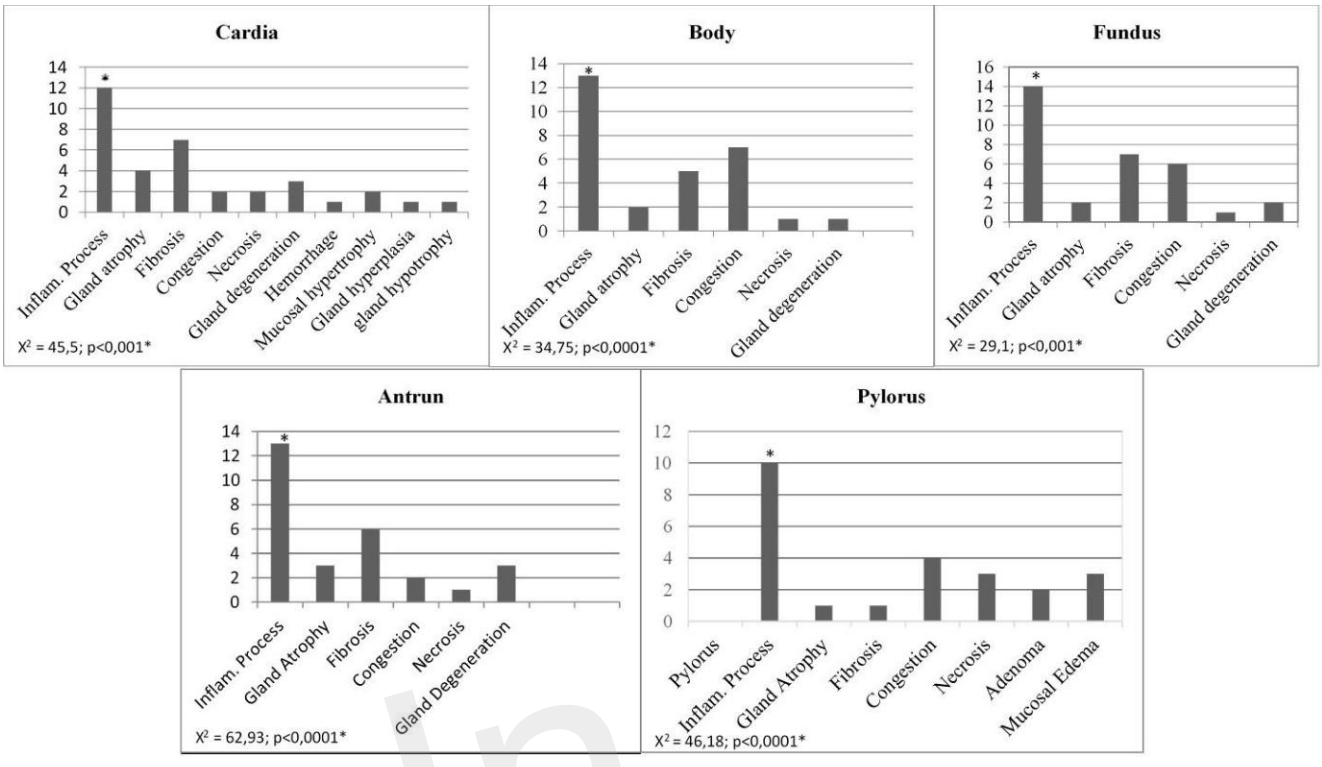
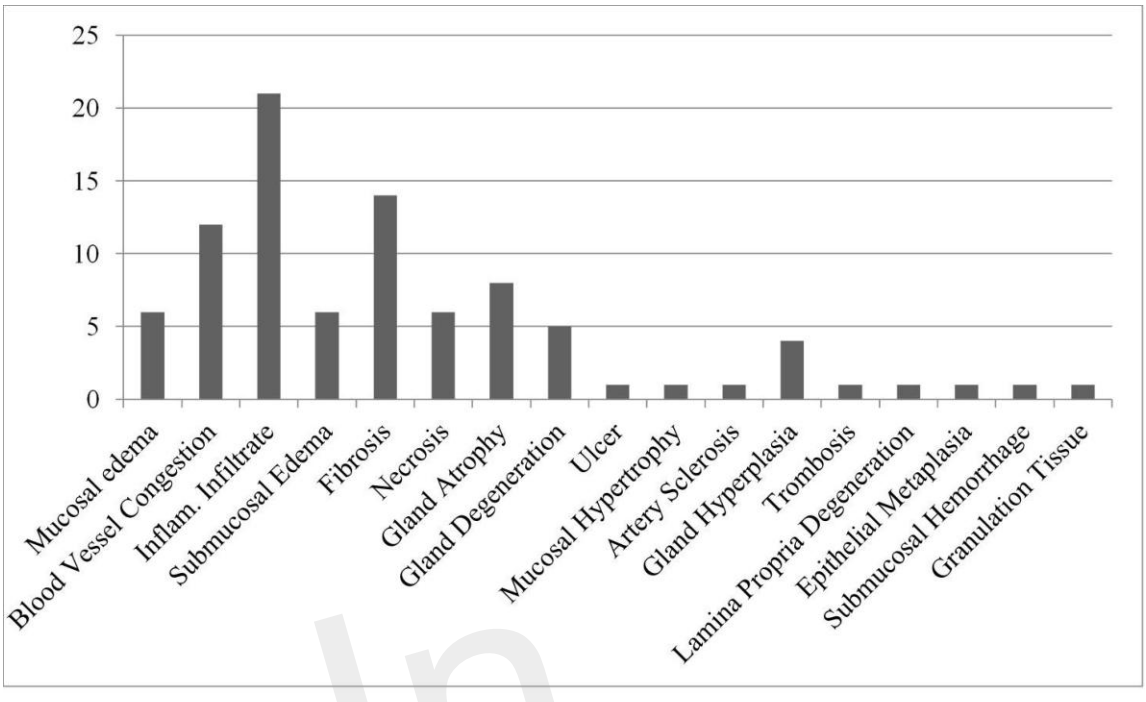


Figure 3.JPEG



5. Conclusão

Diante dos resultados encontrados neste estudo, verificamos que a infecção por *Helicobacter* sp. em cães, pode não estar diretamente associada ao surgimento de sinais clínicos, dependendo, portanto, do grau de virulência da espécie e cepas, características individuais do hospedeiro e fatores ambientais. Porém, por apresentar caráter zoonótico, animais infectados podem ser veículos de transmissão para humanos. Dessa forma, recomenda-se o tratamento em casos positivos de infecção, mesmo que o animal não apresente sinais clínicos ou que estes não tenham relação com a infecção por *Helicobacter* sp. Destaca-se ainda a importância de se realizar estudos direcionados, em cães que apresentem alterações crônicas graves, correlacionando as alterações e a presença da bactéria, bem como realizar estudos randomizados, de longa duração, a fim de se esclarecer esta relação.

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