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Original article

An evaluation of the usefulness of invasive and non-invasive methods used to diagnose *Helicobacter* spp. infections in dogs

M. Jankowski[1], J. Spużak[1], K. Kubiak[1], K. Glińska-Suchocka[1], M. Biernat[2]

[1] Department of Internal Diseases with Clinic for Horses, Dogs and Cats, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Pl. Grunwaldzki 47, 50-366 Wroclaw, Poland
[2] Department of Microbiology, Faculty of Medicine, Wroclaw Medical University, ul. T. Chałubińskiego 4, 50-368 Wroclaw, Poland

Abstract

The aim of this study was to assess the suitability of invasive and non-invasive methods used to diagnose *Helicobacter* spp. in the stomachs of dogs. The study was carried out on 30 dogs of both sexes and different breeds, between one and 15 years old. A histopathologic examination, a microbiological culture, a rapid urease test, a direct bacteriological preparation and a nested PCR assay were carried out. Gastric *Helicobacter* spp. was identified in gastric biopsy specimens from 16 (53.3%) dogs using direct bacteriological preparation, in four (13.3%) dogs based on a culture, in 23 (76.6%) dogs using the rapid urease test and in 21 (70,0%) dogs based on a histopathological assessment of the biopsy specimens. The nested PCR of the gastric biopsy specimens revealed gastric *Helicobacter* spp. in all the dogs (100%). A saliva PCR assay revealed gastric *Helicobacter* spp. in 23 (76.6%) dogs, while stool PCR revealed the bacterium in seven (23.3%) dogs.

We found that invasive methods were more accurate than non-invasive methods in detecting a *Helicobacter* spp. infection in dogs. In addition, the nested PCR method used to evaluate the gastric mucosal biopsy specimens was the most accurate test for detecting *Helicobacter* spp. It was further found that the PCR-based saliva assay was the best non-invasive method for detecting *Helicobacter* spp. However, taking into consideration that most of the diagnostic methods used to detect this bacterium have drawbacks, at least two diagnostic methods should be used to detect *Helicobacter* spp. as is done in human medicine.

Key words: *Helicobacter* spp., invasive and non-invasive methods, dog

Introduction

The discovery of *Helicobacter* (*H.*) *pylori* in the 1980's, followed by the discovery of other species of *Helicobacter* in humans and animals and the identification of the role of *Helicobacter* in the pathogenesis of gastric inflammation, ulcers and neoplasms, has led to the development of various methods enabling its detection (Ogata et al. 2001, Kubiak 2006, Ricci et al. 2007, Al-Ali et al. 2010, Patel et al. 2014).

Correspondence to: M. Jankowski, e-mail: jank1973@tlen.pl

Table 1. Breeds of dogs diagnosed with *Helicobacter* spp. in the stomach.

Breed	Number of dogs
Golden retriever	3
Rottweiler	1
Belgian sheepdog	1
Labrador retriever	4
Boxer	2
Australian cattle dog	1
German Shepherd	3
Dachshund	3
American staffordshire terrier	1
Standard schnauzer	1
Mixed-breed	10

Diagnostic methods used to detect *Helicobacter* spp. can be divided into two types: invasive and non-invasive. Invasive tests require a gastroscopy and a biopsy of the gastric mucosa. They include histopathological examination, a microbiological culture, a direct bacteriological analysis, the rapid urease test and molecular methods. Non-invasive methods do not require an endoscopy or biopsy. They require saliva, exhaled air, stool or blood. These methods include the urea breath test, serological blood tests, stool antigen tests and molecular methods (Tu et al. 1999, Neiger and Simpson 2000, Yanez et al. 2000, Al-Ali et al. 2010).

Despite the development of numerous techniques to diagnose a *Helicobacter* spp. infection, there is no gold-standard test. Each method has advantages and disadvantages and none offers both high sensitivity and specificity. Hence, it is currently recommended to identify *Helicobacter* spp. infections based on at least two diagnostic methods (Yanez et al. 2000, Ramis et al. 2012, Pourakbari et al. 2013).

There are few reports in veterinary literature assessing invasive and non-invasive methods to detect *Helicobacter* spp. infections. Therefore, the aim of this study was to assess the effectiveness of invasive and non-invasive methods in the detection of *Helicobacter* spp. in the stomachs of dogs.

Materials and Methods

The study was carried out on 30 dogs of both sexes (17 males and 13 females) and different breeds (Table 1) from one to 15 years old (mean 5.8 ± 4 years). The study group consisted of animals diagnosed with gastritis based on the clinical symptoms (vomiting, loss or lack of appetite, loss of body mass, *fetor ex ore,* abdominal pain) and the results of the gastroscopy and the histopathologic assessment of sections of the gastric mucosa collected during an endoscopy. These animals were not treated with antibiotics or drugs affecting the gastric acid secretion. Six sections of the gastric mucosa were collected from the body and the pylorus. The samples were collected for histological examination, culture, direct bacteriological analysis, a rapid urease test and a polymerase chain reaction (PCR). In addition, saliva samples were collected for assessment using PCR and stool samples were collected for the detection of *Helicobacter pylori* antigen and assessment using PCR in each dog.

Histopathological examination

The gastric mucosa sections were fixed in 4-10% buffered formalin and embedded in paraffin blocks. The paraffin blocks were cut with a microtome into 5 μm sections, which were then deparaffined and mounted onto Super Frost glass slides. The specimens were then stained with hematoxylin and eosin as well was Giemsa. They were then viewed with a light microscope using a 200x or 400 x magnification. The presence of spiral bacteria indicated a positive test result (Rzeszutko et al. 2006).

Microbiological Culture

Several sections of the gastric body mucosa and the pyloric mucosa were pulverized using a tissue homogeniser and placed on plates containing the following media: Difco Columbia blood agar with 10%

haemolysed horse blood, Oxoid selective Columbia agar with 7% haemolysed horse blood and selective supplements containing 10 mg/l of vancomycin 10 mg/l trimethoprim, 5 mg/l cefsulodin and 5 mg/l amphotericin B, Brucella agar (BA) with 5% horse blood containing a 1% Becton Dickinson IsoVitalex solution and a 1% Sigma hemin solution as well as bioMerieux Campylobacter agar. The test was positive if *Helicobacter* spp. colonies grew on the plates (Megraud and Lehours 2007).

Direct bacteriological preparation

Several sections of the gastric body mucosa and the pyloric mucosa were pulverized using a tissue homogeniser. The tissue sample was placed on a glass slide, and a drop of saline solution was added. The slide was then dried at room temperature and fixed by passing it through a flame. The fixed sample was then stained using Giem's method. The slide was then viewed with a light microscope using a 1000x magnification. The test was positive if spiral-shaped pink bacteria were present (Montgomery et al. 1988).

Rapid urease test

A biopsy specimen of the gastric body and a specimen of the gastric pylorus were placed on the indicator disk developed by the National Food and Nutrition Institute (catalogue no. TU 101). Two drops of saline solution were then added. The results were collected after 15, 30 and 60 minutes. The test was positive if the indicator disc changed colour from yellow to red (Kubiak 2006).

The detection of *H. pylori* antigen in stool

The *H. pylori* antigen was detected in stool samples using an enzyme immunoassay (EIA) with the Oxoid Amplified IDEIA™ Hp StAR™ test. Stool samples were frozen immediately after collection at – 20ºC and stored until needed but no longer than seven days. The stool pellet, which had a diameter of approximately 5-6 mm, was suspended in a 500 µl solution. 50 µl of the faecal suspension and peroxidase conjugated monoclonal antibodies specific for *H. pylori* were added. In addition, positive and negative control solutions provided by the manufacturer were placed in two wells. The plate was incubated with shaking for 60 min at 18-27ºC. The plate was then rinsed five times with a pH 7.4 washing buffer. Subsequently, 100 µl of the substrate was added to each well. The reaction was stopped after 10 minutes by adding 100 µl of an H_2SO_4 solution. The optical density (OD) was measured using a Dynatech MR500 spectrophotometer at 450 nm. According to the manufacturer's instructions, an OD ≥ 0.190 indicated a positive result and an OD ≤ 0.190 indicated a negative result.

Detection of *Helicobacter* spp. DNA in saliva, stool and gastric mucosa biopsy specimens

Saliva samples were collected using a sterile swab, which was placed in a sterile tube and frozen at -20ºC. Rectal swabs were performed in order to obtain a stool sample. The stool was placed in a stool collection container and frozen at -20ºC. Gastric mucosa was collected using biopsy forceps. It was placed in special containers and frozen at -20ºC.

The nested PCR method was used to detect *Helicobacter* and to determine the *Helicobacter* species. This procedure involves performing two consecutive PCR assays. In the first assay, DNA isolated from the sample is used as the matrix, and F and R outer primers are applied. The product of the first reaction forms the matrix of the second reaction, which is activated by adding polymerase and WF and WR outer primers.

Thermo Scientific™ DreamTaq DNA Polymerase (catalogue no. EP0703) was used for the DNA synthesis.

A detailed methodology of the DNA isolation from the biopsy specimens, saliva and stool, the primer sequence and PCR template as well as the conditions of DNA amplification of the chosen species of *Helicobacter* are presented in the following articles: Detection of *Helicobacter* spp. in the saliva of dogs with gastritis (Jankowski et al. 2016a) and The detection of gastric *Helicobacter* spp. in stool samples of dogs with gastritis (Jankowski et al. 2016b).

A test of two proportions based on the chi-square test was used to assess the statistical significance in the proportions of positive results of a *Helicobacter* spp. using different diagnostic techniques between the groups. The statistical significance was set at a 5% level using PQStat Software (version 1.6.2.252). In the tables provided, the presence of the same letter in the columns showing the proportion of positive and negative results for the different methods used to diagnose a *Helicobacter* spp. infection indicates a statistically significant difference in the proportion using these methods.

Table 2. Identification of the species of *Helicobacter* and their incidence in gastric mucosa biopsy samples, saliva and stool samples.

Species *Helicobacter*	Sample		
	sections of the gastric mucosa	saliva	stool
H. heilmannii	29 (96.7%) cases	22 (73.3%) cases	5 (16.6%) cases
H. pylori	2 (6.6%) cases	2 (6.6%) cases	–
H. felis	4 (13.3%) cases	1 (3.3%) cases	–
H. salomonis	11 (36.7%) cases	4 (13.3%) cases	2 (6.6%) cases
H. bizzozeronii	12 (40.0%) cases	3 (10.0%) cases	–

Results

Invasive methods used to detect a *Helicobacter* spp. infection

Helicobacter spp. was identified in 16 dogs (53.3%) using a direct bacteriological preparation. Only four dogs (13.3%) were found to be infected with *Helicobacter* spp. based on a microbiological culture. *Helicobacter* was detected in 76.6% (23 dogs) of the animals using the rapid urease test and in 70.0% (21 dogs) based on the histopathological examination. Nested-PCR proved to be the most effective invasive method to detect gastric *Helicobacter* spp. as it showed that all the studied dogs (100%) were infected with this bacterium. Three dogs (10%) had positive results in all of the five invasive tests. Four positive results were obtained in 12 dogs (40.0%). Three positive tests were recorded in five (16.6%) dogs. Eight dogs (26.6%) had two positive tests, and all the dogs had one positive test result (100%). The mean detection frequency of a *Helicobacter* spp. infection using invasive diagnostic methods was 62.64% (± 32.27%).

Diagnosis of gastric *Helicobacter* spp. using non-invasive methods

The nested-PCR saliva test showed gastric *Helicobacter* spp. DNA in 23 (76.6%) dogs, while the nested-PCR stool test showed that gastric *Helicobacter* spp. DNA was present in seven (23.3%) dogs. All dogs had a negative result in the stool test using peroxidase conjugated monoclonal antibodies specific for *H. Pylori*. None of the dogs obtained a positive result in all three non-invasive tests. Seven dogs (23.3%) had a positive test result in two tests, while 23 dogs had a single positive result (76.6%). The mean detection frequency of a *Helicobacter* spp. infection using non-invasive methods was 33.3% (± 39.27%).

DNA of five *Helicobacter* species was found in the nested-PCR assay of the biopsy specimens of the gastric mucosa, saliva and stool. The *Helicobacter* species and their incidence in each sample are shown in Table 2.

A comparison of the detection of a gastric *Helicobacter* spp. infection using invasive and non-invasive methods

There were no statistically significant differences in the detection rate of *Helicobacter* spp. in the biopsy specimens of the gastric mucosa using the direct bacteriological preparation and the rapid urease test ($p=0.104$). Similarly, there were no statistically significant differences ($p=0.288$) in the detection of *Helicobacter* spp. in the biopsy specimens of the gastric mucosa using the direct bacteriological preparation and the histopathological analysis. There was a statistically significant difference ($p=0.003$) in the detection rate of *Helicobacter* spp. in the biopsy specimens of the gastric mucosa when the specimens were directly analysed bacteriologically, and when a microbiological culture was carried out. There was also a statistically significant difference ($p<0.001$) in the detection rate of *Helicobacter* spp. in the biopsy specimens of the gastric mucosa when analysed bacteriologically and when analysed using a PCR assay. A statistically significant difference ($p<0.001$) in the detection of *Helicobacter* spp. in the biopsy specimens of the gastric mucosa was obtained when using a microbiological culture vs the rapid urease test, and when using the histopathological analysis vs a PCR assay. There was no statistically significant difference ($p=0.770$) in the detection of *Helicobacter* spp. in the biopsy specimens of the gastric mucosa between the rapid urease test and the histopathological examination. There was a statistically significant difference in the detection of *Helicobacter* spp. in the biopsy specimens of the gastric mucosa between the PCR assay and the rapid urease test ($p=0.016$). Similarly, there was a statistically significant difference ($p=0.003$) when *Helicobacter* spp. was detected using PCR and when the detection was based on the histopathologi-

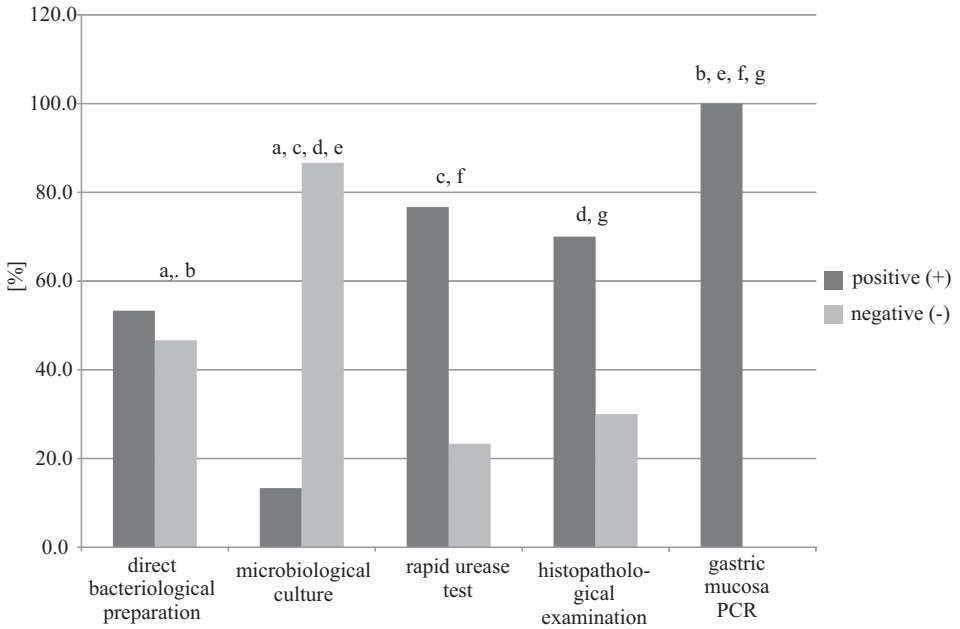

Fig. 1. A comparison of the invasive methods used to detect a *Helicobacter* spp. infection in the gastric mucosa in the studied dogs. The letters a, b, c, d, e, f and g indicate statistically significant differences.

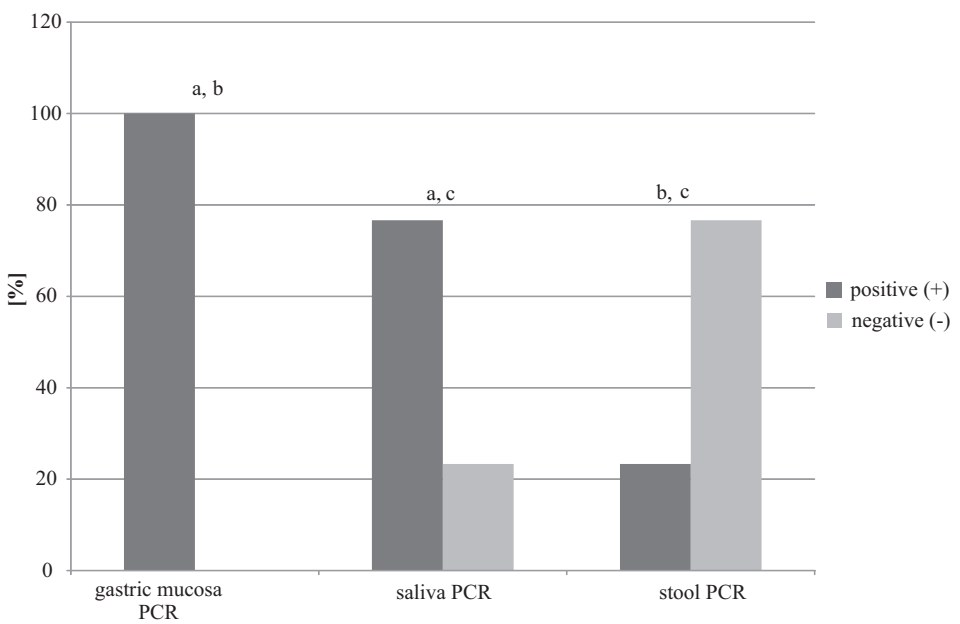

Fig. 2. A comparison of the detection rate of the *Helicobacter* spp. infection using PCR of the gastric biopsy specimen, saliva PCR and stool PCR in the studied dogs. The letters a to c indicate statistically significant differences.

cal examination (Fig. 1). There was a statistically significant difference ($p=0.016$) between the detection of *Helicobacter* spp. in the biopsy specimens of the gastric mucosa using a PCR assay and in PCR-based saliva assay. Furthermore, there was a statistically significant difference ($p<0.001$) between the detection of *Helicobacter* spp. in the biopsy specimens of the gastric mucosa using a PCR assay and in the PCR-based stool test. Likewise, there was a statistically significant difference ($p<0.001$) between the detection of *Helicobacter* spp. in saliva and stool using PCR assays (Fig. 2). Overall, there was a statistically significant difference in the detection of *Helicobacter* spp. using invasive and non-invasive methods ($p<0.001$) (Fig. 3).

Discussion

Currently, a wide array of invasive diagnostic tests (rapid urease test, a histopathological examination,

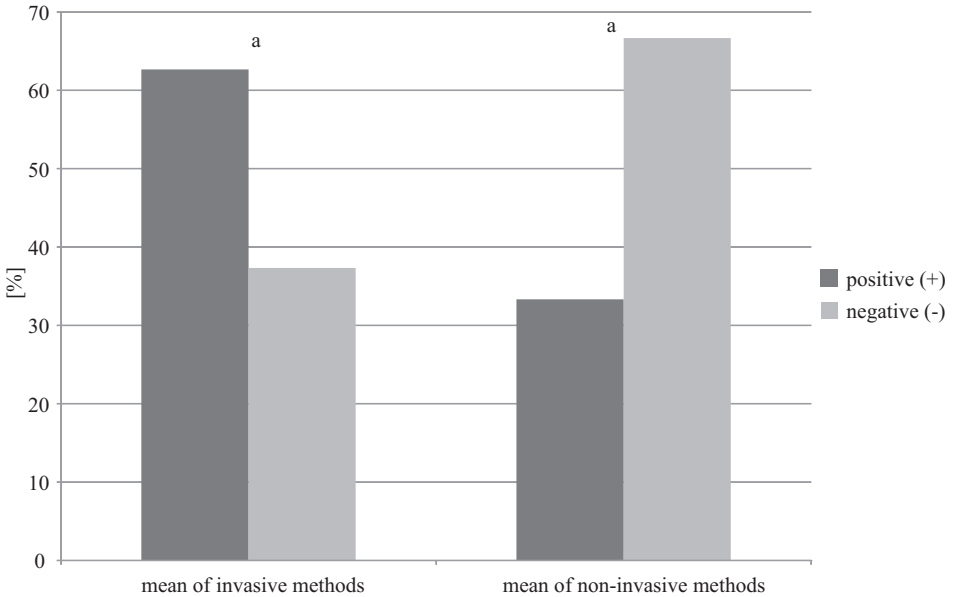

Fig. 3. A comparison of the mean detection rate of the *Helicobacter* spp. infection in the group of studied dogs using invasive and non-invasive methods. The letter "a" indicates a statistically significant difference between the groups.

a direct bacteriological analysis, a microbiological culture, a PCR assay) and non-invasive tests (the urea breath test, a serological test, stool *H. pylori* antigen tests, a PCR assay of the saliva and stool) are used to detect *Helicobacter* in humans and animals (Tu et al. 1999, Neiger and Simpson 2000, Leib and Duncan 2005, Simpson 2005, Patel et al. 2014). Each of the described techniques differs in terms of indications for use, efficacy and cost. Hence, there is no "gold-standard" for the detection of a *Helicobacter* spp. infection, and it is recommended that at least two tests should be performed to confirm the presence of *Helicobacter* spp. (Al-Ali et al. 2010, Ramis et al. 2012, Patel et al. 2014). Our findings support this approach since the detection rate of a *Helicobacter* spp. infection in five invasive tests and three non-invasive tests ranged from 0% to 100%.

In our study, the mean efficacy of the detection of a *Helicobacter* spp. infection using invasive techniques was significantly higher than the mean detection efficacy using non-invasive methods. This suggests the dominance of invasive methods over non-invasive ones. Some authors had similar findings (Tu et al. 1999, Ramis et al. 2012), while others believe that the effectiveness of the invasive and non-invasive diagnostic methods does not vary (Lin et al. 1992, Cutler et al. 1995). Non-invasive methods may be less useful compared to invasive methods in detecting *Helicobacter* spp. infections in dogs based on the fact that: 1) *Helicobacter* spp. constitutes the normal microbial flora of the saliva and stool and may be present in them in low concentrations, 2) there are PCR inhibitors in the stool that may affect PCR assays and 3) the available commercial *H. pylori* antigen tests contain monoclonal antibodies, which do not detect other species of gastric *Helicobacter* occurring much more commonly in dogs than *H. pylori.*

It is currently believed that a microbiological culture is the "gold standard" in the detection of any microorganism. However, this does not seem true in the case of a *Helicobacter* spp. infection. Based on studies in humans, it has been shown that this method is highly specific (94-100%), but has a much lower sensitivity, ranging from 36.4% to 78% (Ogata et al. 2001, Ramis et al. 2012, Patel et al. 2014). In our study, the microbiological culture was the least useful invasive method of detecting a *Helicobacter* spp. infection since the detection rate was less than 14%. These findings are supported by the study of Kubiak (2006), who detected *Helicobacter* spp. on the basis of a microbiological culture in 30% of healthy dogs and 15.3% of dogs with a gastric disease. Similar findings were obtained by Cattoli et al. (1999) and Neiger et. al. (1999), who obtained a positive *Helicobacter* spp. culture in 20% and 9.3% of dogs, respectively. Happonen et al. (1996) reported a slightly higher detection rate of the microbiological culture, which amounted to 37.5%. However, their study was carried out on eight dogs only. Obtaining a *Helicobacter* microbial culture may be challenging due to a number of factors. Firstly, it is difficult to obtain a culture of *Helicobacter* species that occur in dogs. Secondly, no bacteria or low levels of bacteria are present in the biopsy specimens of the gastric mucosa. Thirdly, the administration of antibiotics or other drugs that reduce gastric secretion impede culture growth. In addi-

tion, the presence of coccoid forms or dead bacteria, contamination by other bacteria that inhibit *Helicobacter* spp. growth and inappropriate transport of the collected material to the laboratory all effect the final result of the culture. Therefore, this method is not widely used to detect *Helicobacter* spp. in veterinary medicine. In humans, it is used in cases of failed eradication and to assess the antibiotic susceptibility of the bacteria (Ramis et al. 2012, Patel et al. 2014).

The oldest technique used to detect *Helicobacter* spp. is a histopathological examination. This technique enables the visualisation of the bacteria and the assessment of the lesions in the gastric mucosa (Simpson 2005, Rzeszutko et al. 2006, Ramis et al. 2012, Patel et al. 2014). In human medicine, the sensitivity and specificity of the histopathological examination used to detect *H. pylori* infections ranges from 53 to 100%. In our study, a *Helicobacter* spp. infection was diagnosed in 70% of the dogs using this method. Rzeszutko et al. (2006) reported a similar detection rate (63.5%) of a *Helicobacter* spp. infection in a group of 52 animals (42 dogs and 10 cats). On the other hand, Krstić et al. (2006) reported a lower detection rate of the histopathological examination in a group of 50 dogs, diagnosing a *Helicobacter* spp. infection in 54% of the studied animals. In contrast, Hermanns et al. (1995) and Simpson et al. (1999) detected a *Helicobacter* spp. infection in 80% and 82% of the studied animals, respectively. Such a discrepancy in the results of various authors may be caused by an unevenly distributed colonization of the mucous membrane by the bacteria. The location, number and size of the collected biopsy specimens, the use of antibiotics and drugs reducing gastric secretion, the presence of bacteria morphologically similar to *Helicobacter* and human error on the part of less experienced histopathologists may also affect the results of this technique (Simpson 2005, Rzeszutko et al. 2006, Ramis et al. 2012, Patel et al. 2014). The rapid urease test is another test enabling the detection of a *Helicobacter* spp. infection. This method is based on the detection of urease produced by the gastric *Helicobacter* spp. Due to the fact that this method indirectly detects the *Helicobacter* bacteria, it is used in human medicine as a screening test. Its sensitivity is reported to be between 75 and 100%, while the specificity ranges from 84% to 100% (Ogata et al. 2001, Ricci et al. 2007, Pourakbari et al. 2013, Patel et al. 2014). In our study, the rapid urease test proved to be highly useful in detecting a *Helicobacter* spp. infection. The results of the test indicated that more than 76% of the studied dogs were infected with the bacteria. A similar result was obtained by Kubiak (2006), who used the rapid urease test to diagnose *Helicobacter* in 75% of dogs with dyspepsia. Mirzaeian et al. (2013) reported a higher detection rate since they found *Helicobacter* in 100% of the studied dogs. This discrepancy may be caused by an uneven distribution of the bacteria on the mucous membrane, haemorrhage from the gastrointestinal tract, the presence of intestinal metaplasia, the administration of antibiotics, proton pump inhibitors and H_2 antagonists that give false negative results, and the presence of other urease producing bacteria, such as *Proteus* spp., which give false positive results (Tu et al. 1999, Neiger and Simpson 2000, Ogata et al. 2001, Leib and Duncan 2005, Ramis et al. 2012).

The direct bacteriological preparation is a relatively quick and easy diagnostic method that enables the diagnosis of a gastric *Helicobacter* spp. infection. Using this technique, we detected an infection in more than 53% of the animals. A similar percentage of positive results using this method was obtained in studies on humans carried out by Tzeng et al. (2005) and Al-Ali et al. (2010), who reported *Helicobacter* spp. in 56.75% and 55.2% of the subjects, respectively. A higher detection rate in dogs was reported in the studies carried out by Happonen et al. (1996), Cattolii et al. (1999) and Kubiak (2006). We did not find a statistically significant difference in the detection of *Helicobacter* spp. between the direct bacteriological preparation, a histopathological examination and a rapid urease test. Happonen et al. (1996) reported that the direct bacteriological preparation is more accurate than the histopathological analysis and the rapid urease test. The difference in the detection of *Helicobacter* spp. reported in the cited studies may be caused by an uneven distribution of the bacteria on the gastric mucosa (Tzeng et. al. 2005).

The PCR assay is one of the most modern techniques used to detect a *Helicobacter* spp. infection. The assay may be performed on a biopsy tissue specimen (invasive method) or on saliva or stool (non-invasive method). In human medicine, the sensitivity and specificity of the PCR assay using biopsy tissue specimens ranged from 75% to 100% and from 84% to 100%, respectively (Ricci et al. 2007, Ramis et al. 2012, Patel et al. 2014). These values were lower when saliva (sensitivity 75-98% and specificity 70%-100%) and stool (sensitivity 58%-96% and specificity 67%-100%) were used (Kabir 2001, Aguloelu et al. 2006, Cellini et al. 2010, Smith et al. 2012). We found that the PCR assay of the biopsy tissue specimens was the most accurate technique of all the invasive and non-invasive methods to diagnose a *Helicobacter* spp. infection. Our findings are supported by the results of Neiger et al. (1999) and Kubiak (2006), who diagnosed *Helicobacter* spp. infections using a PCR assay in 100% and 89% of healthy dogs, respectively, and in 89% and 97.1% of sick dogs, respectively. The find-

ings of Hwang et al. (2002) and Mirzaeian et al. (2013) also support our results. They detected a *Helicobacter* spp. infection in all of the dogs in their study using a PCR assay. Van den Bulck et al. (2005) reported a lower detection rate of this method as they detected *Helicobacter* spp. in 71.8% of the studied dogs. We found *Helicobacter* bacteria in the saliva of more than 76% of the dogs using a PCR assay (Jankowski et al. 1016a). A similar result was obtained by Recordati et al. (2007), who reported this bacterium in 71.1% of dogs based on PCR-based testing of saliva, while Ekman et. al. (2013) found the bacterium in 100% of the studied canine population. We found *Helicobacter* in 23% of the studied stool samples using a PCR test (Jankowski et al. 1016b). Hong et al. (2015) reported a higher detection rate, amounting to 62.5%. On the other hand, Ekman et. al. (2013) did not find gastric *Helicobacter* spp. in any of the studied stool samples. These differences may be attributed to different living environments of the dogs and different study material. Some studies used panels of dogs kept in one environment, with a high likelihood of cross-infection between dogs, while others used dogs kept indoors. The main advantage of PCR is that it offers a precise diagnosis and enables differentiation between various species of *Helicobacter*. However, the results of this test do not indicate whether the infection is active or not (Farrugia er al. 2010, Sjodin et al. 2011).

Based on our results, we found that invasive methods are more useful in defining a *Helicobacter* spp. infection in dogs than non-invasive methods. Of all the studied methods, the PCR assay of biopsy specimens of the gastric mucosa is the most effective method of diagnosing a *Helicobacter* spp. infection. PCR-based testing of saliva was the most useful non-invasive method. However, taking into consideration the disadvantages of each method studied, the diagnosis of a *Helicobacter* infection should be based on the results of at least two tests.

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