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Comparison of the non-invasive diagnostic methods, stool antigen test and PCR assay, for *Helicobacter felis* detection in dogs

Sunhwa Hong[1], Hak-Yong Lee[2], Tae-wan Kim[3], Okjin Kim[1]*

[1]*Center for Animal Resources Development, Wonkwang University, Iksan 570-749, Korea*
[2]*Huvet Co. Ltd., Iksan 570-749, Korea*
[3]*College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Korea*

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Abstract

The aim of the present study was to compare the non-invasive methods for the diagnosis of *H. felis* with HpSA kit-based detection method and *H. felis*-specific PCR assay with dog's stool samples without sacrifice. Male Beagle dogs (n=6) were infected with *H. felis* ATCC 49179 (1.0×10^9 CFU/dog) by intragastric inoculation two times at 3-day intervals, and the stool specimens of dogs were collected 1, 3, 5, 7, 14, 21 days after infection to submit to HpSA test and *H. felis*-specific PCR. As the results, the sensitivity of the HpSA and the PCR analysis was 50.0%, 83.3% respectively. Although HpSA test is less sensitive, it could be used for rapid, cheap and easy screening assay for *H. felis* infection in dog and cats. We suggest that the *H. pylori* stool antigen kit, HpSA, is useful and effective for monitoring *H. felis* infection. If HpSA test would be made with *H. felis* antibodies in the future, its sensitivity could be increased. Also, PCR assay could be successfully used to detect the *H. felis* in stools. Applying the *H. pylori* stool antigen kit and PCR assay may be the recommended non-invasive strategy to identify *H. felis* in dog and cats.

Key words : *Helicobacter felis*, *H. pylori* stool antigen kit, Non-invasive, HpSA, Dog

INTRODUCTION

The discovery of the association of *H. pylori* with gastritis, peptic ulcers, and gastric neoplasia has led to fundamental changes in the understanding of gastric disease in humans (Kim et al, 2006; Lee et al, 2007). Like *H. pylori*, other species of *Helicobacter* have also been shown to colonize the stomach and cause disease in animals. Gastric colonizers include *H. felis*, *H. mustelae*, *H. acinonychis*, *H. bizzozeronii*, *H. heilmannii*, *H. salomonis*, and a recently isolated novel *Helicobacter* sp. of dolphins (Hodzic et al, 2001). *H. felis* is a gram-negative, spiral-shaped bacterium originally isolated from the stomachs of cats and dogs (Lee et al, 1990). This organism has been shown by 16S rRNA gene sequencing to be very closely related to *H. pylori* (Fox et al, 1995). Also, *H. felis* has been shown to colonize gnotobiotic and CV mice and elicit host reactions and histopathology which are very similar to those seen in *H. pylori* infections of humans (Lee et al, 1990).

In human, *H. pylori* is a major *Helicobacter* species. *H. pylori* produce large amounts of urease to catalyze urea hydrolysis. Urease neutralizes stomach acid by generating ammonia from urea, which is essential for survival of *H. pylori* in the host (Hu and Mobley, 1990; Eaton et al, 1991). Thus, *H. pylori* diagnosis is based on detecting urease. Several methods have been proposed and used to diagnose *H. pylori* infection. Increasing interest has been directed toward non-invasive tests, compared to endoscopy-based invasive methods (histology and rapid urease test), as non-invasive methods do not require endoscopic assessment

*Corresponding author: Okjin Kim, Tel. +82-63-850-6668, Fax. +82-63-850-7308, E-mail. kimoj@wku.ac.kr

(Mégraud et al, 2012). The ^{13}C-urea breath test (UBT) is the most recommended non-invasive test for detecting *H. pylori* infection and has high sensitivity and specificity (Leal et al, 2011). However, the UBT cannot be applied to animals due to its high cost and the requirement for expensive analytical instruments (Nyan et al, 2004). Thus, many researchers have used polymerase chain reaction (PCR) assays to monitor the infection in stools without biopsy or sacrifice of animals (Santos et al, 2011; Neiger et al, 1998). However, PCR assay need time-consuming and high techniques and high-cost laboratory instrument like as Thermal Cycler (Kim et al, 2006). Furthermore, stool samples remain the most difficult specimens for DNA extraction and amplification (Monteiro et al, 1997). Recently, several companies have been released *H. pylori* stool antigen (HpSA) test kits. HpSA tests are non-invasive diagnostic modules for *H. pylori* infection with human patient's stool samples (Shimoyama, 2013; Patel et al, 2014). However, there was little information about the usefulness of HpSA test on the *H. felis*, which is a major *Helicobacter* species in dogs and cats.

The aim of the present study was to compare the non-invasive methods for the diagnosis of *H. felis* with HpSA kit-based detection method and *H. felis*-specific PCR assay with dog's stool samples without sacrifice.

MATERIAL AND METHODS

Bacterial culture

H. felis (ATCC 49179; American Type Culture Collection, USA) was incubated in a brain-heart infusion broth containing 10% fetal bovine serum at 37°C overnight under a microaerophilic atmosphere and allowed to grow to a density of ~2.0×10^9 colony-forming units (CFU) per 1 ml of culture broth.

Animals and treatment

8-week-old male Beagle dogs (n=6) were obtained from the animal facility of Wonkwang University and housed in a room with constant environmental conditions (22±2°C; 40~70% relative humidity; 12-hour light-dark cycle; 150~300 lux brightness). Pellet feed and purified water were available *ad libitum*. Before the inoculation, preceding PCR assays with their stool specimens indicated that the dogs were free of *H. felis*. All animal experiments were conducted according to Standard Operation Procedures and were approved by the Institutional Animal Care and Use Committee of Wonkwang University, Korea.

Bacterial inoculation

After a 24-hour fast, the dogs were orally inoculated twice at 3-day intervals by oral administration of 1.0×10^9 CFU of *H. felis* suspended in 10 ml of broth.

Stool antigen kit

After *H. felis* inoculation, stool specimens were gathered in days 1, 3, 5, 7, 14, and 21. The *H. felis* antigen was evaluated using the commercially available SD Bioline *H. pylori* Ag kit according to the manufacturer's instructions. Specimens (250 mg) were incubated with diluents solution at room temperature for 30 min and then 100 μl was placed on the Helicobacter Ag examination device. The test results were checked about 15 min later. One red line indicated negative, and double red line indicated Helicobacter positive result (Fig. 1).

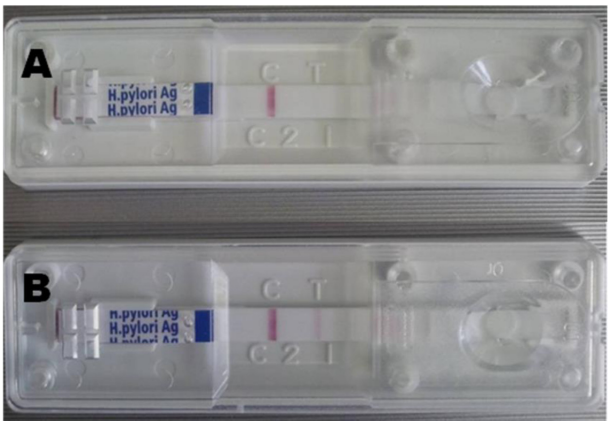

Fig. 1. The results of *H. pylori* stool antigen (HpSA) test stool specimens of the dogs infected with *H. felis* ATCC 49179. (A) One red line indicated negative, (B) Double red line indicated Helicobacter positive result.

Genomic DNA extraction and PCR

After inoculation, stool specimens were gathered in days 1, 3, 5, 7, 14, and 21. Stool samples of *H. felis* infected dogs were homogenized and resuspended in PBS, and DNAs were extracted using the *AccuPrep* Stool DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's instructions. *AccuPrep* Stool DNA Extraction Kit is designed for the rapid, convenient extraction of DNA from fresh or frozen stool, or other samples containing large amounts of material that can inhibit PCR. The kit uses a glass filter, fixed in a column tube that can efficiently bind DNA in the presence of chaotropic salts. Using the spin-column method, contaminants and enzyme inhibitors (such as heparin, bilirubin bile salts, porphyrin) are eliminated and high-purity DNA is obtained, ready for use in a variety of applications (Lee et al, 2007). After washing steps which remove proteins and salt, high-purity DNA is finally eluted using a low-concentration elution buffer. It provides 2～5 μg DNA yields from 100 mg stool (Lee et al, 2007).

DNA was eluted in Tris-EDTA buffer (pH 8.0), and an aliquot was used for PCR amplification. All DNA samples were stored at −20°C until the PCR assays were performed. Previously, the primers were designed to amplify the 16S rRNA gene of *H. felis* (Kong et al, 1996). The sequence of *H. felis* primer HF-L was 5'-ATGACATGCCCTTTAGTTTGGGATAGCCA-3', and that of primer HF-R was 5'-CGTTCACCCTCTCA GGCCGGATACC-3'. This primer set amplified a 169 bp fragment and was used for detection of *H. felis* in samples (Kong et al, 1996).

The template DNA (400 ng) and 20 pmol of each primer were added to a PCR mixture tube (*AccuPower* PCR PreMix; Bioneer) containing 2.5 U of Taq DNA polymerase, 250 μM of each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM $MgCl_2$, and gel loading dye. The final volume was adjusted to 20 μl with distilled water. The reaction mixture was subjected to denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 20 s, 55°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 10 min. Samples were maintained at 4°C until analysis. Reactions were conducted using My Genie 32 Thermal Block PCR (Bioneer). Eight microliters of each sample were mixed with 2 μl of loading buffer, and electrophoretically separated on 2.0% agarose gels stained with 0.5 μg/ml ethidium bromide. DNA bands were observed under ultraviolet light.

Statistical analysis

The statistical analysis was performed using analysis of variance and SPSS for Windows v.12.0 (Chicago, IL, USA). A P-value < 0.05 was considered statistically significant. The positive ratio was measured using MINITAB software and 95% confidential intervals (Minitab Inc., State College, PA, USA). A positive ratio indicated a significant difference.

RESULTS

Stool Ag Kit

After the final inoculation of *H. felis* into the Beagle dogs, we collected stool specimens on days 1, 3, 5, 7, 14, and 21. The stools contained less water. We incubated the stool specimens in diluent solution for 30 min with vortexing every 10 min. Then, we placed 100 μl of sample on the SD Bioline *H. pylori* Ag kit detection device. On day 1, we observed no positive results. But, the positive ratio increased on days 3 [16.7% (1/6)], days 5 [16.7% (1/6)], and days 7～21 [50.0% (3/6)] (Table 1).

PCR of fecal samples

A PCR analysis method using 16S rRNA gene of *H. felis* was employed to detect *H. felis* infection. The 16S rRNA gene of *H. felis* was specifically amplified by PCR with the 16S rRNA gene-specific primers (HF-L and HF-R). The target nucleic acid fragments were specifically amplified by PCR with 16S rRNA primers (Fig. 2). As a result, five of six stool samples were positive by the PCR reaction (5/6, 83.3%) (Table 2).

Table 1. Helicobacter Stool Antigen Test (HpSA) for diagnosing *Helicobacter felis* infected dogs

Day	*H. felis* inoculation	n	Positive reaction[a] (positive percent)
1	Yes	6	0 (0%, CI[b] 0～39.0)
3	Yes	6	1 (16.7%, CI 3.0～56.4)
5	Yes	6	1 (16.7%, CI 3.0～56.4)
7	Yes	6	3 (50.0%, CI 18.8～81.2)
14	Yes	6	3 (50.0%, CI 18.8～81.2)
21	Yes	6	3 (50.0%, CI 18.8～81.2)

[a]A positive reaction revealed Helicobacter colonization, which was observed as a red colored double line.
[b]Incidence percentage (95% confidential interval) was calculated with MiniTab statistical software.

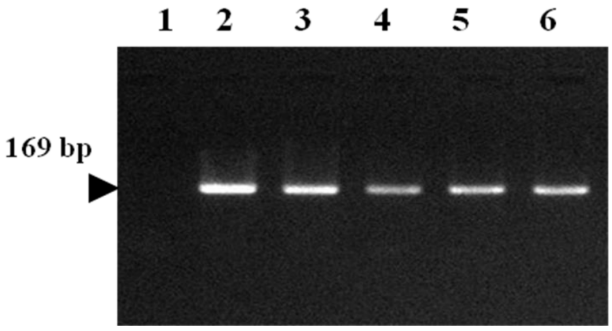

Fig. 2. Amplification with *H. felis*-specific primers in the 16S rRNA gene was identified on a 2.0% agarose gel electrophoresis. Lane 1: Stool sample of No. 1, N: distilled water, LT1: Lung tissue of No. 1 cat, LT2: Lung tissue of No. 2 cat.

DISCUSSION

Diagnosis of *H. pylori* infection can be made with both invasive and non-invasive tests. Invasive tests include histology, culture, and the rapid urease test, which require endoscopy to obtain gastric mucosa biopsies. Non-invasive tests for diagnosis of *H. pylori* infection, which are based on analyzing of samples of breath, blood, or stool, have been developed (Polk and Peek, 2010). However, serological tests are unable to distinguish active from past infections (Vaira et al, 1999). Non-invasive tests require demonstrating the microorganism in gastric biopsy samples; therefore, an endoscopy must be performed. The current gold standard for diagnosing *H. pylori* infection is endoscopic biopsy of gastric tissue for the rapid urease test, histology, and culture. However, such an invasive procedure has major

Table 2. Comparison of the test results of dogs infected with *Helicobacter felis*.

Test	Result							
	1	2	3	4	5	6	Positive ratio	Positive percent
Stool Ag Kit	○	○	●	●	○	●	3/6	50.0 %, CI[a] 18.8～81.2
PCR	○	●	●	●	●	●	5/6	83.3 %, CI 43.7～97.0

○, negative; ●, positive.
[a]Incidence percentage (95% confidential interval) was calculated with MiniTab statistical software.

disadvantages of anesthesia, discomfort, and the possibility for ethical problems (Hoshina et al, 1990). But, noninvasive tests are easy to perform and do not produce significant discomfort and allow a patient to avoid the discomfort and risk of invasive endoscopy. These include serological antibody testing for *H. pylori*, the urea breath test, and the stool antigen assay (HpSA) test (Hoshina et al, 1990).

Among several diagnostic tests, the HpSA test and PCR for diagnosing *H. felis* infection may offer a useful non-invasive method without sacrificing animals during an *in vivo* study. In this study, the sensitivity of the HpSA and the PCR analysis was 50.0%, 83.3% respectively. Despite all the above observation on performance of antigen detection *H. feslis* in stool, it has certain disadvantage: antigen excretion may vary over the time period and antigen may degrade while passing through intestine. Further, use of N-acetylcysteine like mucolytic agent may decrease the accuracy of the diagnosis (Demirtürk et al, 2003). Cut off titer, though difficult to decide but crucial to reach the conclusion by using antigen detection technique. However, stool antigen detection using monoclonal antibody has been recommended by EHSG as it gives equivalent diagnosis accuracy to UBT (Malfertheiner et al, 2012).

In this study, the PCR analysis was more sensitive than the HpSA test. Since the target of HpSA test was *H. pylori* antigens, it may not detect completely whole *H. felis* antigens. Therefore, for non-invasive diagnosis of *H. felis* infection with animal stool samples, the PCR analysis was more sensitive. However, PCR analysis also has been reported several limitations (Kim et al,

2006). There is no single gold standard among the diagnostic tests for *Helicobacter* infection but all of the tests have their pitfalls and limitations (Rautelin et al, 2003). Among noninvasive methods, stool specimens are easy to obtain and consequently of high potential interest for the development of a direct method of *Helicobacter* species detection (Kim et al, 2006; Lee et al, 2007). PCR was successfully used to detect the bacterium in stools (Kim et al, 2006; Lee et al, 2007). Indeed stool samples remain the most difficult specimens for DNA extraction and amplification (Monteiro et al, 1997). The difficulty has been associated with the DNA polymerase inhibitors present in stools, identified as complex polysaccharides (Monteiro et al, 1997). In this study, we used the spin-column method and it eliminated effectively contaminants and enzyme inhibitors (such as heparin, bilirubin bile salts, porphyrin) and high-purity DNA is obtained. It provides 2～5 μg DNA yields from 100 mg stool (Kim and Kim, 2005). Although HpSA test is less sensitive, it could be used for rapid, cheap and easy screening assay for *H. felis* infection in dog and cats. It may be confirmed the positive results using *H. felis*-specific PCR.

We suggest that the *H. pylori* stool antigen kit, HpSA, is useful and effective for monitoring *H. felis* infection. If HpSA test would be made with *H. felis* antibodies in the future, its sensitivity could be increased. Also, PCR assay could be successfully used to detect the *H. felis* in stools. Applying the *H. pylori* stool antigen kit and PCR assay may be the recommended non-invasive strategy to identify *H. felis* in dog and cats.

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