



Usefulness of a *Helicobacter pylori* stool antigen test for diagnosing *H. pylori* infected C57BL/6 mice

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Among several diagnostic tests, a *Helicobacter pylori* stool antigen (HpSA) test may offer a useful non-invasive method for diagnosing infection without sacrificing animals. In this study, male C57BL/6 mice (n=6) were infected with *H. pylori* ATCC 49503 (1×10^8 CFU/mouse) by intragastric inoculation three times at 2-day intervals, and *H. pylori* infected stool specimens were collected 1, 3, 5, 7, 14, 21 days after infection to assess reliability of the HpSA test. Five of six specimens were positive at 5-21 days after infection, and the sensitivity of the HpSA test was 83.33%. The presence of *H. pylori* infection was confirmed by the rapid urease test and genomic DNA polymerase chain reaction (PCR), and showed the same results as the HpSA. However, the rapid urease test and genomic DNA PCR are invasive tests and require animal sacrifice to detect *H. pylori* in gastric biopsy samples. We suggest that an HpSA test kit would be useful and effective for monitoring *H. pylori* in various laboratory animals, as *H. pylori* can be easily monitored without sacrificing animals.

Key words: *Helicobacter pylori*, *H. pylori* stool antigen KIT, non-invasive, CLO, HpSA

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Helicobacter pylori is a Gram-negative, microaerobic, spiral bacterium with polar flagella that lives near the surface of the human gastric mucosa. *H. pylori* has been found in the stomachs of >50% of humans [1,2]. In 1983, Marshall and Warren discovered an unidentified curved bacterium that was present in almost all patients with active chronic gastritis, duodenal ulcers, or gastric ulcers [3]. Spicy food, acid, stress, and lifestyle were considered the major causes of ulcers before 1982, when this bacterium was discovered. Since *H. pylori* was first identified, a complete paradigm shift has occurred in the clinical approach to gastric diseases, and efforts have been invested in the development of noninvasive diagnostic tests for *H. pylori* infection [4].

Modern humans were infected with *H. pylori* before

their migration from Africa, and *H. pylori* seems to have spread from east Africa around 58,000 yr ago [5]. Korea is an area where the *H. pylori* infection rate is high, as these bacteria are found in 60-90% of Korean adult stomachs [6]. Since the discovery of *H. pylori* in 1983, it has become one of the most common bacterial pathogens in humans causing chronic gastritis and peptic ulcer disease and is associated with gastric adenocarcinoma [7,8]. In 1994, the International Agency for Research on Cancer classified *H. pylori* as a definite carcinogenic factor (Group I) in humans [9]. Thus, testing and treatment of patients with *H. pylori* infection has become a familiar part of general practice. Although many researchers have tried to identify the mechanism associating *H. pylori* with gastric carcinogenesis, it is

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poorly understood [10,11].

H. pylori produces 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 [12,13]. Thus, an *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 an endoscopic assessment [14].

The ¹³C-urea breath test (UBT) is the most recommended non-invasive test for detecting *H. pylori* infection and has high sensitivity and specificity [15]. However, the UBT cannot be applied to experimental animals due to its high cost and the requirement for expensive analytical instruments [16]. Thus, many researchers have used polymerase chain reaction (PCR) assays to monitor the infection in feces without biopsy or sacrifice of animals and confirmed the infection with the ¹³C-Urea breath test [17,18].

Mongolian gerbils are an optimal laboratory animal model to study *H. pylori* infection [19]. However, gerbils have several limits such as the limited number of littermates and experimental reagents. Many investigators have recently chosen to use mouse models because of their widespread availability, short breeding cycles, and accessibility to appropriate experimental reagents. The C57BL/6 mice strain has been extensively studied for investigating *H. pylori* [20]. Although there is no single model that is the best for all applications, the mouse is undeniably the most convenient and appropriate model today based on cost and availability of immunological reagents and genome information [20]. Vaccine candidates have been studied in *H. pylori* infected mouse models [21-24]. New therapeutic strategies against *H. pylori* have been investigated in the mouse model [25,26].

The aim of the present study was to evaluate the sensitivity and specificity of a *H. pylori* stool antigen (HpSA) kit-based detection method for monitoring various kinds of laboratory animals without sacrifice. We performed an experiment using the SD Bioline *H. pylori* Ag Kit (SD, Inc., Yongin, Korea) to detect *H. pylori* infection in C57BL/6 mice. We determined the detection limit of the kit in a murine model.

Materials and Methods

H. pylori culture and identification

H. pylori (ATCC 49503) was obtained from the *Helicobacter pylori* Korean Type Culture Collection (Jinju, Korea), and cultured on *Brucella* broth agar with 10% bovine serum in an anaerobic chamber with 10% CO₂, 5% O₂, and 85% N₂ at 37°C with sufficient humidity. The bacteria were identified as *H. pylori* by positive Gram staining, the urease test, and PCR.

Animals and treatment

Male C57BL/6 mice (weight, 20-24 g) were obtained from Samtako Bio Korea (Osan, Korea), 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). Pelleted feed and purified water were available *ad libitum*. All animal experiments were conducted according to standard operating procedures and were approved by the Institutional Animal Care and Use Committee of Wonkwang University, Korea (Approval No. WKU-1201).

H. pylori inoculation

Six pathogen-free male C57BL/6 8-week-old mice were used. After a 24-hour fast, the mice were orally inoculated with *H. pylori* (1×10⁹ CFU/mouse) three times at 2-day intervals.

HpSA kit

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

Biopsy and rapid urease test

The mice were sacrificed 21 days after *H. pylori* inoculation, and their gastric mucosa was biopsied to detect *H. pylori*. Biopsy samples (3×3 cm) from the gastric pylorus were minced and applied to confirm

infection using CLO *Helicobacter*-detection kits (Asan Pharm Co., Ltd., Seoul, Korea) by incubating the biopsy specimens at 35°C for 24 hours to examine urease activity. The reaction (color change) was determined as bright yellow (negative), bright yellow false (partially positive), or bright (dark) red for positive.

Genomic DNA extraction and PCR

Gastric tissue of *H. pylori* infected mice was homogenized, resuspended in PBS, and DNA was extracted from the homogenates. Briefly, genomic DNA was isolated using an *AccuPrep* Genomic DNA Extraction kit (Bioneer Corp., Daejeon, Korea), according to the manufacturer's instructions. 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. Amplification of the CagA DNA was performed with the primers H-cagA-F (5'-ATA ATG CTA AAT TAG ACA ACT TGAA GCG A) and H-cagA-R (5'-TTA GAA TAA TCA ACA AAC ATC ACG CCA T): 298 bp. 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 MgCl₂, 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 95°C for 1 min, 57°C for 30 s, 72°C for 30 s, 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 1.5% 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 (Minitab Inc., State College, PA, USA) and 95% confidential intervals. A positive ratio indicated a significant difference.

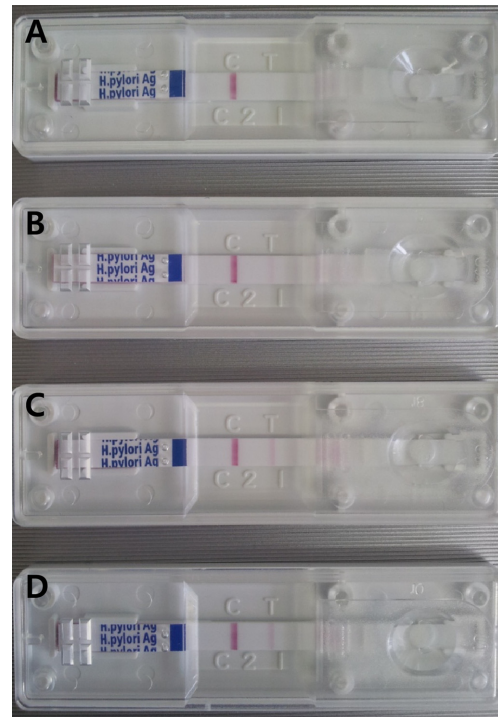


Figure 1. Results of *Helicobacter pylori* Stool Ag Kit based on the number of *H. pylori* ATCC 49503 cells. (A) 1×10^5 CFU/100 μL; (B) 1×10^6 CFU/100 μL; (C) 1×10^7 CFU/100 μL; (D) 1×10^8 CFU/100 μL.

Results

HpSA kit

We used a stool antigen kit to determine the *H. pylori* detection range. We serially diluted the *H. pylori* bacterial stock to 1×10^8 CFU/100 μL and applied the stock solution to the HpSA kit. The detection range of the SD Bioline *H. pylori* Ag Kit was 1×10^6 CFU/100 μL.

After the final inoculation of *H. pylori* into C57BL/6 mice, 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]), 5 (66.7% [4/6]), and 7-21 (83.3% [5/6]) (Table 1).

Rapid urease test

Repeated intragastric inoculation (1×10^9 CFU, three times) of *H. pylori* into C57BL/6 mice revealed a positive reaction (red color) 21 days after the final inoculation, indicating a bacterial infection in the stomach.

Table 1. *Helicobacter pylori* stool Antigen (HpSA) test for diagnosing *H. pylori* infected C57BL/6 mice

Day	<i>H. pylori</i> Inoculation ^{a)}	n	Positive reaction ^{b)} (Positive percent)
1	Yes	6	0 (0%, CI ^{c)} 0-39.3)
3	Yes	6	1 (16.7%, CI 0.42-64.1)
5	Yes	6	4 (66.7%, CI 0.22-99.6)
7	Yes	6	5 (83.3%, CI 35.9-99.6)
14	Yes	6	5 (83.3%, CI 35.9-99.6)
21	Yes	6	5 (83.3%, CI 35.9-99.6)

^{a)}*H. pylori* inoculation was three times every 2 days.

^{b)}A positive reaction revealed *H. pylori* colonization, which was observed as a red colored double line.

^{c)}Incidence percentage (95% confidential interval) was calculated with MiniTab statistical software.

Five of six gastric biopsy specimens were positive by the rapid urease test on day 21. We did not detect a positive reaction from sample no. 1 (5/6, 83.3%) (Table 2).

Genomic DNA RT-PCR of gastric specimens

A RT-PCR analysis method using CagA DNA was employed to detect *H. pylori* infection. The CagA gene (298 bp) was specifically amplified by PCR with the *H. pylori* CagA-specific primers (H-CagAF and H-CagAR). The target nucleic acid fragments were specifically amplified by RT-PCR with CagA DNA primers. As a result, five of six gastric specimens were positive by the PCR reaction. However, we did not detect a positive reaction from sample no. 1 (5/6, 83.3%) (Table 2).

Discussion

H. pylori infection is the main cause of chronic gastritis, which is the most frequent chronic bacterial disease [1,27]. *H. pylori* induces an inflammatory immune response in the gastric mucosa, and many studies have demonstrated a significant correlation between *H. pylori* density and acute and chronic inflammatory grade.

Treatments that suppress *H. pylori* density and virulence could become strategies to prevent *H. pylori*-associated disease in the future. The present study focused on the influence of some factors to eradicate *H. pylori* infection [28]. It has been widely reported over the last decade that the success of *H. pylori* eradication treatment has decreased [29,30].

Diagnosis of *H. pylori* infection can be made with both invasive and noninvasive tests. Invasive tests include histology, culture, and the rapid urease test, which require endoscopy to obtain gastric mucosa biopsies. Noninvasive tests for diagnosis of *H. pylori* infection, which are based on analyzing breath, blood, or stool samples, have been developed [8]. However, serological tests are unable to distinguish active from past infections [31].

Noninvasive 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 disadvantages of anesthesia, discomfort, and possible ethical problems [32]. 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 HpSA test [32].

Among several diagnostic tests, the HpSA test for diagnosing *H. pylori* infection may offer a useful non-invasive method without sacrificing animals during an *in vivo* study. Kazemi *et al.* recently performed a validity comparison of five diagnostic tests including HpSA and rapid urease test in patients [33]. In our study, we carried out experiments to evaluate the utility of the SD Bioline *H. pylori* stool Ag Kit (Standard Diagnostics, Inc., Korea) in a mouse model. As a result, the sensitivity of the HpSA test was 83.33%. We detected the presence of

Table 2. Comparison test results of mice infected with *Helicobacter pylori*

Test	Result						Positive ratio	Positive percent
	1	2	3	4	5	6		
Stool Ag Kit	○	●	●	●	●	●	5/6	83.3%, CI ^{a)} 35.9-99.6
RUT ^{b)}	○	●	●	●	●	●	5/6	83.3%, CI 35.9-99.6
PCR	○	●	●	●	●	●	5/6	83.3%, CI 35.9-99.6

○, negative; ●, positive.

^{a)}Incidence percentage (95% confidential interval) was calculated with MiniTab statistical software.

^{b)}RUT, rapid urease test.

H. pylori infection by HpSA and confirmed the positive results using the rapid urease test and genomic DNA PCR. Confirming results also had a sensitivity of 83.33%. However, the rapid urease test and genomic DNA PCR are invasive tests and require animal sacrifice to detect *H. pylori* in gastric biopsy samples, whereas the HpSA test is noninvasive.

We suggest that the *H. pylori* stool antigen kit is useful and effective for monitoring *H. pylori* in various laboratory animals including mice. Applying the HpSA kit may be the most recommended non-invasive strategy to identify *H. pylori*.

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