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Comparative Assessment of HpSA-LFIA Using a Hemi-Nested 16SrRNA PCR for Diagnosis of *H. pylori* in Human Stool and Feces of Animals

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ABSTRACT

Helicobacter pylori is a zoonotic gut pathogen that affects humans and animals. Half of the world's population is infected with *H. pylori*. *Helicobacter pylori* stool antigen lateral flow immunochromatographic assay is the most common rapid test for disease diagnosis. This study aimed to evaluate the commercial *Helicobacter pylori* stool antigen lateral flow immunochromatographic assay using a gold standard test, a Hemi-nested 16srRNA PCR.

Methodology: 200 stool and fecal samples were collected from dyspeptic humans, dogs, and cats. HpSA-LFIA and 16srRNA Hemi-nested PCR were performed on the collected samples. Statistical analysis was applied to get the sensitivity and specificity of the test under evaluation. The sample size was calculated with a power of 80% (online calculator). The stool and fecal samples were preserved at -20°C until the test performance.

Results: The obtained Se and Sp were 91.89% and 59.51%, respectively.

Conclusion: HpSA-LFIA in our country is a highly sensitive test with low specificity and low accuracy to be the sole test for diagnosis. The test was intended to be used as a screening test and provided a preliminary result which was not enough for precision and final diagnosis.

Keywords: HpSA-LFIA, Nested PCR, Sensitivity, Specificity and 16srRNA.

INTRODUCTION

Helicobacter pylori is present all across the world, with prevalence rates ranging from 25% in wealthier countries to more than 90% in destitute regions. However, not everyone who contracts the germs becomes ill (Ghotaslou et al., 2013).

H. pylori is considered a Gram-negative, microaerophilic bacillus, was detected by Marshall and Warren in 1982. *Helicobacter pylori* is one of the gastrointestinal pathogens. Chronic gastritis and peptic ulcers are linked to the infection, which can progress to stomach cancer, adenocarcinoma and mucosal-associated

lymphoid tissues (MALT) (Thung et al., 2016, Saleh et al., 2020).

Invasive and non-invasive diagnostic methods for detecting infection in the stomach mucosa are now available (Laheij et al., 2000). Although invasive tests such as histology, rapid urease testing, polymerase chain reaction, or culture of gastric biopsy specimens remain the gold standard, non-invasive tests such as the urea breath test, serology, *H. pylori* DNA detection in stool or saliva using polymerase chain reaction, and stool antigen test are becoming more popular (Kisa et al., 2002; Wisniewska et al., 2002; Sabbi et al., 2005; Sen et al., 2005).

Stool antigen assays have recently become accessible. They're thought to be useful for both diagnosing and confirming *H. pylori* infection eradication. Aside from that, they're simple to use and can help you save time and money (Chisholm et al., 2004; Trevisani et al., 2005).

The test for fast lateral flow chromatography can now be purchased. Some scientists have investigated how well it can detect *H. pylori* infection (Blanco et al., 2008; Gisbert De La Morena and Abaira, 2006; Kato et al., 2004; Konstantopoulos et al., 2001; Krausse et al., 2008; Ngom et al., 2010; Oderda et al., 2000). While the utility of enzyme immunoassays (EIAs) using polyclonal or monoclonal antibodies has been well established, the limited data available for the first designed and commercially available fast test (Immuno-Card STAT! HpSA) revealed significant differences between trials (Fox et al., 1998; Den Hoed and Kuipers, 2012; Kato et al., 2004; Konstantopoulos et al., 2001; Krausse et al., 2008), its accuracy is said to be slightly lower than that of monoclonal fecal antigen EIA.

The nested PCR, which is suited for contaminated samples, is the most accurate test for the diagnosis of *H. pylori* in stool samples (Duś et al., 2013).

Studies have increasingly focused on the PCR assay because of its high sensitivity for detecting a small number of organisms present in a clinical specimen (Patel et al., 2014) and ability to amplify the target DNA from coccoid forms of *H. pylori* that are difficult to cultivate and identify histologically. *H. pylori* is currently detected using PCR methods in non-invasive clinical samples such as stool, saliva, and dental plaque (Calvet, 2015; Frenck et al., 2006).

Our study aimed to establish a comparative association between the HpSA-LFIA and nested PCR using a Hemi-nested PCR in 200 human stool samples and 80 fecal samples were collected from dogs and cats.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee of Medicine Cairo University Ref accepted this study, which was carried out following the ideals of Egypt's Declaration of

Independence; the results will be published soon. In addition, owners of dogs and cats signed a written consent form authorizing the collection of feces. Human samples were acquired after patients signed a written consent form.

Patient and animal samples:

Two hundred stool and fecal samples were collected from dyspeptic humans, dogs, and cats. 120 stool samples from human patients went to the clinical laboratory for stool Ag detection according to their physician recommendations with written and assigned consent from the patients. The samples were recruited from Menofia, Benha, Giza governorates in Egypt. The participants ranged in age from 3 to 55 years old. Fecal samples from 80 dogs and cats were collected from different pet clinics. The sample size was calculated with a power of 80% (online calculator). The stool and fecal samples were preserved at -20°C until the test performance.

Lateral flow immunochromatography assay (LFIA):

The samples were subjected to the Right sign® stool Ag quick test as directed in the brochure (LFI). Polyclonal anti-HP antibodies are used in this approach. There were three stages to the procedure: The first stage (1) is sample processing, which comprises stabbing 50 mg of stool sample from three separate spots in solid samples at random. If the sample is in the form of a liquid, 80 ul of the sample was held in an extraction buffer. (2) Pour 2 drops of the extracted sample onto the cassette (about 80 ul). (3) Reading the findings and then the results after a 10-minute incubation time at RT (Abdelmalek et al., 2021).

Polymerase chain reaction:

The QIAamp DNA Mini kit (Cat. No. 51604, Qiagen, Germany) was used to extract DNA from stool according to the manufacturer's procedure. 25 liters of Emerald Amp GT PCR master mix (TAKARA), 1 liter of each primer (20 pmol), 4.5 liters of nuclease-free water, and 6 liters of DNA template were used in the amplification processes. Table 1 shows the primer sequences in detail. Two runs were performed on the PCR reaction mixture: Initial denaturation at 94°C/3min, 35 cycles at 94°C/30sec., annealing at 58°C/30sec., and extension at 72°C/30 sec., and final extension at

72°C/5min. Second run: Initial denaturation at 94°C for 3 minutes, 20 cycles at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 5 minutes, followed by electrophoresis on a 1% agarose gel. The gel was stained with ethidium bromide and examined under a UV transilluminator for the presence of the amplified DNA (Image Quant 400, GE Healthcare) (Waheeb et al., 2022).

Statistical analysis:

The SPSS TM software, version 25 was used to analyze all of the data (IBM corporation). The percentages of sensitivity and specificity were computed. When a sample gave a positive result for both ELISA and PCR tests, it was deemed true positive (TP), and when a sample gave a positive result for ELISA but a negative result for PCR, it was considered false positive (FP). True negative (TN) means the sample is negative in both ELISA and PCR, whereas false negative (FN) means the sample is negative in ELISA but positive in PCR.

$$\text{Sensitivity} = \frac{TP}{TP + FN} \times 100$$

$$\text{Specificity} = \frac{TN}{TN + FP} \times 100$$

RESULTS

The stool samples collected from dyspeptic patients and animals were preserved till the LFIA was performed. The test technicians are blind to the patients' data to avoid test bias. The

results were read by different technicians to ensure the accuracy of the results.

The results of LFIA in all samples are 100 positive (human and animals) (80 and 20, respectively). The negative samples are 100 (40 human samples and 60 animal samples).

The stool samples were tested by 16srRNA using specific primers after DNA extraction. The expected 1024 bp (first run) and 250 bp (second run) fragments were found after nested PCR amplification of genomic DNA (Figure 1). HpSA-LFIA positive results were 100 out of 200 stool and fecal samples with a 50% incidence rate, whereas nested PCR positive results were 37 out of 200 specimens with an 18.5 percent incidence rate (Table 2).

The results of both LFIA and 16srRNA PCR were analyzed statistically by SPSS software to get the diagnostic performance of HpSA-LFIA, the commercially available and most common test for *H.pylori* using the gold standard test (PCR).

HpSA-LFIA and PCR: the number and percentage of false positives, false negatives, true positives, and true negatives for HpSA-LFIA are determined by the PCR results (Table 3). In the diagnosis of *H. pylori*, crosstabulation and comparative evaluation of HpSA-LFIA based on the results of PCR demonstrated a sensitivity and specificity of 91.89 percent and 59.51 percent, respectively (Table 4).

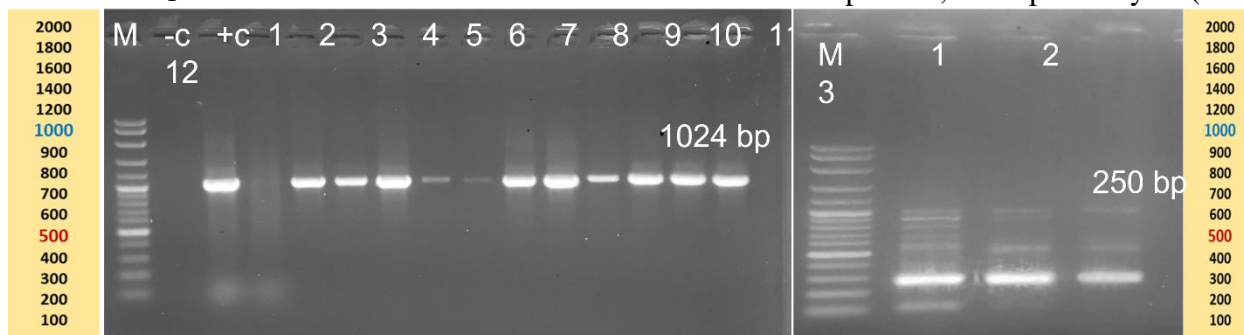


Figure (1): Agarose gel electrophoresis (1.5%) of 16sRNA using a Hemi-nested PCR showing; first run of Hemi-nested PCR using outer primers showing 1024 bp product and second run PCR using Hemi-nested primers, 250 bp product.

Table (1): Oligonucleotide primers and PCR conditions:

Run	Primer sequence (5' → 3')	PCR fragment	Reference
1 st Run	CTGGCGGCGTGCCTAATAC	1024 bp	Qin et al., 2016
	CTCACGACACGAGCT GAC		
2 nd	CTGGCG GCGTGCCTAA TAC	250 bp	

Run	ACCCTCTCAGGCC GGATACC		
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Table (2): Result of *H. pylori* in human stool and animal fecal samples using HpSA-LFIA and PCR:

Sample Result	Human and animal samples	
	HpSA-LFIA	PCR
Positive	100	37
Negative	100	163
Total	200	200

Table (3): Results of HpSA-LFIA and PCR of *H. pylori* in both human and animal samples:

Type of sample	Human and animal samples
False positive	66
False negative	3
True positive	34
True negative	97

Table (4): Result of sensitivity and specificity of HpSA-LFIA and PCR using SPSS:

	Sensitivity	Specificity
Humans and animal samples	91.89 %	59.51 %

DISCUSSION

H. pylori is a Gram-negative bacillus that colonizes the stomach mucus layer and the upper region of the small intestine in humans (duodenum). It's the most common cause of peptic ulcers and the main cause of stomach cancer. The vast majority of those affected (more than 70%) have no symptoms or warning signs. As people get older, *H. pylori* infection becomes more common (Anto et al., 2005; Chisholm et al., 2004).

There are two types of stool antigen tests for detecting *H. pylori* infection: one based on enzyme immunoassay (EIA) and the other based on immunochromatography (LFIA). In both types of investigations, monoclonal and polyclonal antibodies can be employed. Despite being similarly sensitive and specific, the EIA-based tests appear to be more accurate than the LFIA-based tests. In contrast, ICA-based tests do not require any special equipment, are simple to perform, and can be used to promptly diagnose *H. pylori* infection (Shimoyama, 2013; Calvet et al., 2010).

In comparison to other invasive and non-invasive diagnostic procedures, molecular diagnostics using polymerase chain reaction (PCR) is easier, more accurate, and more feasible (Gatta et al., 2004; Cheng and Hu, 2004; Veijola et al., 2005). *H. pylori* can be identified using polymerase chain reaction (PCR). Gastrointestinal mucosa, feces, saliva, tooth plaque, and other environmental samples all contain *Helicobacter pylori* DNA (Atherton, 1997). Extragastric digestive cancers such as hepatic carcinoma, bile duct cancer, pancreatic cancer, and colon cancer are all linked to the Infection with *Helicobacter pylori*. (Kalach et al., 2005; Dunn et al., 1997).

Molecular diagnostic approaches based on PCR, particularly nested PCR, will be the gold standard in helicobacter diagnosis (Patel et al. 2014). They are gaining a lot of momentum in the medical industry, even though they are now primarily utilized in research (Calvet, 2015). As a result, for comparison and evaluation, the PCR was chosen as the gold standard test.

The diagnostic values (Sensitivity and Specificity) of the most commonly used non-invasive test are evaluated and reported in this study. A heminested PCR was compared to the Rightsign® *Helicobacter pylori* stool Antigen fast test. The latter is regarded as a highly specialized and delicate master examination. The results of the HpSA-LFIA showed that it had a low specificity (59.51%) but a high sensitivity (100%) (91.89%). Our data demonstrated a considerable reduction in test specificity, which did not match the product attributes of the Rightsign® *H. pylori* Ag fast test, which didn't match with the manufacturing instructions (Abdelmalek et al., 2021)

HpSA-LFIA Se and Sp were found to be 93.75 and 59.76 %, respectively, by Abdelmalek et al. which is consistent with prior findings (Abdelmalek et al., 2021). According to Da Silva, Kato et al., and others, HpSA-LFIA has a sensitivity of 52.5-94.6 percent and a specificity of 55.5-98.4 percent. Small sample sizes were used in other studies, such as the ImmunoCard STAT! (65 stool samples). The sensitivity and specificity of the HpSA test were 77.8% and 79.3%, respectively (Kato et al., 2004; da Silva et al., 2010).

The HpSA-LFIA was reported to be able to detect *H. pylori* infection by Karakus, Salih, and Kato et al., but our findings disputed their assertions. With a Se of 93% and anSp of 91%, HpSA-LFIA was reported to be exceptionally accurate. They discovered that the HpSA-LFIA had a sensitivity of 93% and a specificity of 100% in a 5-year follow-up investigation. (Kato and colleagues, 2004). The sensitivity was 90–100 percent (average 95%) and the specificity was 80–100 %, according to Karakus and Salih (average 96 %) (Karakus and Salih, 2013).

Helicobacter diagnostic methods depending on invasive procedures yield better results than those depending on non-invasive methods. Therefore, for a more reliable diagnosis, it is recommended not to depend only on the non-invasive *H. pylori* testing procedures

CONCLUSION

HpSA-LFIA is a highly sensitive test with low specificity and low accuracy to be the sole test for diagnosis. The test was intended to be used as a screening test and provided a preliminary result which was not enough for precision and final diagnosis.

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