OPEN ACCESS
Advances in Animal and Veterinary Sciences

Research Article

Comparative Evaluation of Elisa Using A Semi-Nested 16SrRNA Pcr as a Master Test for Detection of Helicobacter Pylori Antigen in Human Stool and Feces of Dogs and Cats

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Abstract | *Helicobacter pylori (H. pylori)* is one of the most prevalent infectious agents in the world which causes a variety of gastrointestinal diseases including gastritis, peptic ulcer, and gastric carcinoma. Laboratory diagnosis of *H. pylori* infection is made by invasive and non-invasive methods. Invasive methods require endoscopy which is uncomfortable and unacceptable for most patients. Therefore, non-invasive methods particularly serological tests are easier and comfortable for patients. The objective of this study was to comparatively evaluate noninvasive enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) in the diagnosis of infection with *H. Pylori* in human stool and fasces of dogs and cats. Two hundred stool samples from humans and eighty-eight fecal samples from dogs and cats were collected with gastric disorders. The presence of *H. Pylori* infection in stool and fecal samples were tested by ELISA and PCR methods. In ELISA the test utilizes *H. pylori* antibodies to selectively detect *H. pylori* Antigen in human stool and fasces of pet animals, and PCR by using 16srRNA primers through a semi-nested PCR technique.

Keywords | ELISA, PCR, 16srRNA

Received | October 07, 2021; **Accepte**d | October 17, 2021; Published | January 15, 2022
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Citation | Waheeb N, Marouf S, Nasr E, Abdelmalek S (2022). Comparative evaluation of elisa using a semi-nested 16srrna pcr as a master test for detection of helicobacter pylori antigen in human stool and feces of dogs and cats. Adv. Anim. Vet. Sci. 10(3): 466-471.
DOI | http://dx.doi.org/10.17582/journal.aavs/2022/10.3.466.471
ISSN (Online) | 2307-8316

INTRODUCTION

Helicobacter pylori has a worldwide distribution with a prevalence range from 25% in developed countries and sometimes reach more than 90% in developing areas, but not all infected individuals developed the disease. (Ghotaslou et al., 2013).

H. pylori is a Gram-negative bacillus, a microaerophilic bacterium that was discovered in 1982 by Marshall and Warren. *H. Pylori* is one of the most common human-specific pathogens which colonizes the gastric mucosa. *H. Pylori* infection is always associated with chronic gastritis and peptic ulcer which can be developed to gastric cancers such as adenocarcinoma, gastric lymphoma, or benign mucosal-associated lymphoid tissues (MALT). (Thung et al., 2016, Saleh et al., 2020).

The appearance of symptoms of *H. Pylori* is depended on the strains of *H. Pylori* and also the interaction between bacterial and host factors. The less virulent strains mostly lead to asymptomatic cases. (Yamaoka et al., 2010, Moussa

et al., 2021).

H. Pylori releases several pathogenic proteins like cytotoxin-associated antigen (Cag A) and vacuolating cytotoxin antigen (VacA). The cytotoxin-producing strains of Helicobacter which are isolated from a patient with gastritis contain the cag A gene (type I strains), So, the detection of cag A is important for identifying infection with harmful strains. (Jones et al., 2010).

There are several methods currently available for the detection of *H. pylori* infection. The endoscopic biopsy (invasive method) for detection of *H. pylori* infection by histological examination, culture, rapid urease test (RUT), and polymerase chain reaction. (Garza-Gonzalez et al., 2014).

The non-invasive tests used for *H. pylori* diagnosis include the urea breath test (UBT), serological tests, and *H. pylori* stool antigen (HpSA) tests. (McNulty et al., 2011).

The UBT and stool antigen test (SAT) are considered as the best methods to determine active infection for H. Pylori, while the indirect antibody-based tests, especially serology, are widely available and relatively insensitive and their specificity is low. Guidelines indicated that a single test can't be considered a gold standard for the diagnosis of *H. pylori*. (Miftahussurur and Yamaoka, 2016).

The diagnostic assays for *H. pylori* infection are designed by mixing the recombinant and synthetic peptides of *H. pylori* antigens. Recently, the most common immunological diagnostic methods used as a screening tool for *H. pylori* detection are enzyme immunoassays (EIAs). These methods are characterized by very cheap, comfortable, easily applied, and noninvasive methods of diagnosis. It is a reliable method to diagnose an active infection and confirm effective treatment. (Shimoyama et al., 2011).

Urea breath tests and stool antigen tests are used to detect *H. Pylori* active infection while serological test does not differentiate between active infection and old infection with *H. Pylori*. (Peng et al., 2009).

Several stool antigen tests using monoclonal antibodies (Mabs) have been established. These tests are characterized by having high sensitivity and specificity if compared with other tests like the urea breath test. (Calvet et al., 2010).

The *H. pylori* antigen immunoassay is the main method for the qualitative and quantitative detection of *H. pylori* antigens in human stool. The polyclonal stool antigen test was firstly identified in 1997 which has an 88.8% sensitivity and 94.5% specificity used in patients before medication and/or patients following up after treatment. It was followed by using a monoclonal test. (Mégraud et al., 2007).'

PCR is the accurate method used for detecting the *H. Pylori* DNA by using several gene targets such as urease operon genes, cag A and Hsp60. Although PCR could be performed even with a trace of bacterial DNA, it is mainly considered an invasive method that needs a biopsy. (Patel et al., 2014).

The selection of appropriate antigens is very critical. The selected antigens should have conserved sequences to cover different genotypes of *H. pylori*. Among different *H. pylori* virulence factors, Cytotoxin-associated gene A (CagA) as oncoprotein which can affect the host cell biological pathways, which affect on the gastric epithelium cell tight junction and change the cytoskeleton, affecting the proliferation and differentiation of cells, and causing the inflammatory responses. (Jones et al., 2009).

The use of the multiplex PCR method in routine diagnosis of *H. pylori* infection is recommended as this method is more helpful for detection of *H. pylori* in samples with non-cultivable organisms and mild inflammation cannot be detectable by other methods. (Fadilah et al., 2016).

The present study aimed to evaluate and compare enzyme-linked immunosorbent assay with a polymerase chain reaction as a master test for the diagnosis of infection with *H. Pylori* in human stool and feces of dogs and cats.

MATERIALS AND METHODS

Animal ethics

This study was carried out according to the principles of the Declaration of Egypt and approved by Medicine Cairo University Institutional Animal Care and Use, The Committee Ref; will be available soon. In addition, a written consent approving the collection of the fecal sample was carried out by the owners of dogs and cats.

Samples

Stool samples of 200 human patients with gastric disorders and88 dogs and cats participated in this study. The study population samples were collected from January 2019 to January 2021. Stools should be stored at low temperatures (-5 to -25°C) if not tested in a short period below seven days. Moreover, the samples should be stored at -80°C to maintain the antigen for long-time storage (Shimoyamaet al., 2011).

Enzyme-Linked Immunosorbent Assay

Three semiquantitative double sandwich Commercial ELISA kits (Perfect Ease Biotech, Chemux Bioscience, and ACON Laboratories) were performed according to the

manufacturer's instructions for each (Megraud et al., 1989, Kim et al., 2002). Briefly, The *H. pylori* Antigen EIA Test Kit is a solid-phase enzyme immunoassay based on the double sandwich principle for semiquantitative detection of *H. pylori* antigen in human stool. The microwell plate is coated with monoclonal anti-*H. Pylori* antibodies. During testing, the antigens are extracted out with extraction solution and added to the antibodies coated microwell plate with the enzyme-conjugated antibodies to *H. pylori* and then incubated at 37°C for 2 hours. If the specimens contain *H. pylori* antigens, they will bind to the antibodies coated on the microwell plate and simultaneously bind to the conjugate to form immobilized antibody-*H. Pylori* antigen-conjugate complexes. If the specimens do not contain *H. pylori* antigens, the complexes will not be formed. After initial incubation, the microwell plate is washed to remove unbound materials. Substrate A and substrate B are added and then incubated to produce a blue color indicating the amount of *H. Pylori* antigens present in the specimens. A sulfuric acid solution is added to the microwell plate to stop the reaction producing a color change from blue to yellow. The color intensity, which corresponds to the amount of *H. pylori* antigens present in the specimens, is measured with a microplate reader at 450/630-700 nm or 450 nm.

Table 1: Oligonucleotide primers (16srRNA gene)

Run	Primer sequence (5' → 3')	PCR fragment	Reference
1st Run	CTGGCGG-CGTGCCTAATAC	1024 bp	Qin et al., 2016
	CTCACGACAC-GAGCT GAC		
2nd Run	CTGGCG GCGTGCCTAA TAC	250 bp	Qin et al., 2016
	ACCCTCTCAGGCC GGATACC		

Polymerase chain reaction

DNA extraction from stool was done by using the QIAamp DNA Mini kit (Cat. No. 51604, Qiagen, Germany) according to the manufacture's protocol. The amplification reactions were 25µl containing 12.5µl of Emerald Amp GT PCR master mix (TAKARA), 1µl of each primer (20 pmol), 4.5µl of nuclease-free water, and 6µl of DNA template. Primer sequences are clarified in Table 1. The mixture of PCR reactions was subjected to 2 runs:

First run: Initial denaturation at 94 c°/3min., 35 cycles at 94c° /30sec. & annealing at 58c°/30sec. & extension at 72c°/30 sec. and Final extension at 72c°/5min.

Second run: Initial denaturation at 94c°/ 3min, 20 cycle at 94c°/30sec. & annealing at 58/30sec. & extension at 72c°/30sec. and Final extension at 72 c°/5min,

Then analyzed by 1% agarose gel electrophoresis. 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).

Statistical analysis

All data were analyzed using the SPSS TM software, version 25 (IBM corporation). Sensitivity and specificity percentages were calculated.

Results were considered true positive (TP) when the sample give a positive result for both ELISA and PCR tests and considered false positive (FP) when the sample give a positive result for ELISA and negative for PCR. While true negative (TN) means that the sample gives negative in both ELISA and PCR and false-negative (FN) means that ELISA gives negative while PCR is positive.

Sensitivity = [TP | (TP + FN)] × 100
Specificity = [TN| (TN+ FP)] × 100

RESULTS

Nested PCR amplification of genomic DNA revealed the expected 1024 and 250 bp fragments (Figure 1). The positive results of ELISA were 27 out of 200 human specimens with a percentage of 13.5 % and 18 out of 88 dogs and cats specimens with a percentage of 20.5 % while the positive results of nested PCR assay were 36 out of 200 human specimens with a percentage of 18 % and 20 out of 88 dog's and cat's specimen with percentage 22.7 % (Table 2).

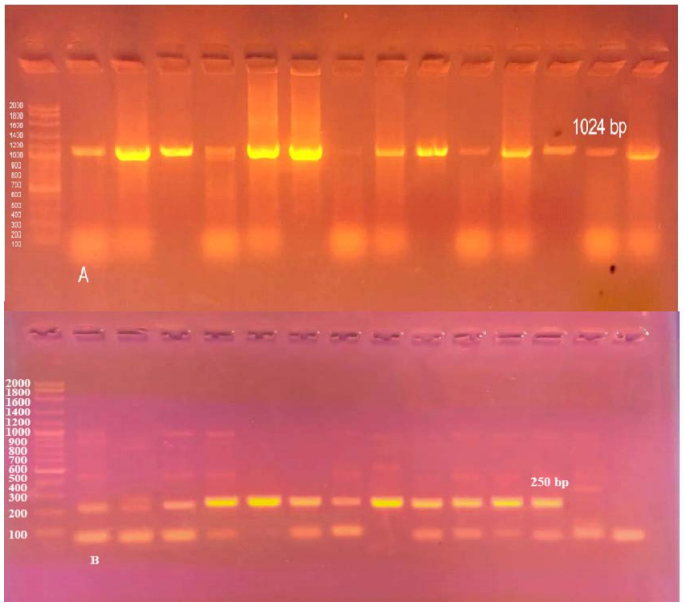

Figure 1: Agarose gel electrophoresis (1%) of 16sRNA using nested PCR showing; first run of heminested PCR using outer primers showing 1024 bp product and second run PCR using heminested primers. 250 bp product.

Table 3 illustrate the comparison between the result of

Table 2: Result of detection of *H. Pylori antigen* in human stool and animal fecal samples using ELISA and PCR.

Sample type / Result	Human stool (200 sample)				Dogs and cats faeces (88 sample)			
	ELISA		PCR		ELISA		PCR	
	No.	%	No.	%	No.	%	No.	%
Positive	27	13.5	36	18	18	20.5	20	22.7
Negative	173	86.5	164	82	70	79.5	68	77.3

Table 3: Results of ELISA and PCR for detection of H. *Pylori antigen in* both human and animal samples.

Type of sample / Result	Human stool	Animal faeces (Dogs and cats)	Total	
			No.	%
False positive	3	1	4	1.4
False negative	12	3	15	5.2
True positive	24	17	41	14.2
True negative	161	67	228	79.2

Table 4: Result of sensitivity and specificity of ELISA compared to PCR in detection of *H.pylori* antigen.

	Sensitivity	Specificity
Human samples	64.86 %	98.16 %
Animal samples	85.71 %	100.00

ELISA and PCR; the number and percentage of false positive, false negative, true positive and True negative for ELISA depend up on result of PCR.

Crosstabulation and comparative evaluation of ELISA depending upon the result of PCR in the diagnosis of *H. pylori* revealed a sensitivity and specificity of 64.86% and 98.16% respectively depending on the data of human samples, where the animal's data reveals the sensitivity of 85.71% and specificity of 100% (Table 4).

DISCUSSION

Helicobacter spp. can affect the gastrointestinal tract mucosa of humans, wild animals as monkeys, and domestic animals (Moussa et al., 2021). Some Helicobacter species are non-cultivable. Serological tests for veterinary application to detect Helicobacter species are not yet clinically available, however, the detection of fecal *H. pylori* antigens is possible. PCR assay is a non-invasive, faster, simple, specific, and sensitive diagnostic test that will help recognize Helicobacter infection in humans and companion animals (Ford and Moayyedi, 2014).

These serological techniques were reported to use for companion animals samples including feces as well as human stool (Hu et al., 2017).

Most of the immunoassay methods are depending on the detection of *H. pylori* antibodies in serum. In the case of active infection, IgM antibody levels are detectable followed by a rise of IgG and IgA antibodies which remain constantly high until the infection is eliminated. Consequently, such serological tests are not reliable to differentiate between active and non-active old infection, In addition, the serological tests that detect H. Pylori IgG antibodies could also lead to false negatives due to low sensitivity. In these tests, decreasing of antibody titer during *H. Pylori* progression is the main cause associated with the false diagnosis in the laboratory assays. (She et al., 2009; Imanieh et al., 2014)

The present investigation gives data about the evaluation of sensitivity and specificity of some used serological techniques depending on detection of *H.pylori* antigen in fecal samples using ELISA in comparison to PCR as a master test based on human and companion animal samples.

Our result regarding the sensitivity and specificity for detection of fecal *H. pylori* antigen by ELISA and nested PCR revealed a sensitivity of 64.86% and 85.71% and a specificity of 98.16% and 100% for human stool and dogs and cats feces respectively. This indicates that ELISA based on monoclonal antibody reveal good sensitive and high specific results for detection of fecal *H.pylori* antigen in feces of dogs and cats and human stool samples.

In harmony with our result, Sabbagh et al. (2019) reported that the detection of human stool *H. pylori* antigen using ELISA was more accurate and reliable especially by using

monoclonal antibodies based testes. Also, Razaghr et al. (2010) concluded that detection of human stool *H. pylori* antigen by ELISA was a non-invasive economical method with a sensitivity and specificity of 96% and 80% respectively and the method was considered a reliable and valid alternative test.

Jalalypour et al. (2016) reported that ELISA yielded 90.20 % sensitivity and 61.11% specificity, respectively. Compared to PCR, ELISA presented higher sensitivity and lower specificity. Sensitivity is an important parameter where the test is used to identify a serious but treatable disorder. Therefore, despite lower specificity, ELISA could be considered as a first-line method for the detection of *H. pylori* infection. To accurately diagnose disorders, it is recommended to subject the initially positive patients with «high sensitivity/ low specificity» tests to a second line-test with «low sensitivity / high specificity». In this way, the majority of false positives will be identified as disease negative. (Lalkhen et al., 2008)

However, Nevoa et al. (2017) found that the detection rate of *H. pylori* by the PCR was significantly higher when compared to the rapid urease test and ELISA.

CONCLUSION

The results of the present study indicated that noninvasive ELISA which detects *H. Pylori* Ag using monoclonal antibodies is a highly sensitive test for first-line detection of *H. Pylori* infection and also a highly specific test. PCR could be considered for the determination of *H. Pylori* eradication in patients subjected to antimicrobial treatments.

ACKNOWLEDGEMENTS

The author would like to thank the MICROBIOLOGY laboratory unit in the Microbiology Department, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt for providing the facilities for conducting this study. The authors did not receive any funds.

CONFLICT OF INTEREST

The authors declare that the present study has no conflicts of interest or financial ties to disclose.

AUTHOR CONTRIBUTIONS

The present study conceptualization was performed by SherifMarouf, Neveen Nagy Waheeb, and Shaymaa Abdelmalek collected the samples, made laboratory and statistical analysis, and draft the manuscript. Shaymaa Abdelmalek was supervisor of the study and participated in its design and interpretation of the data. .Naser, E.A. approved the final revision.

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