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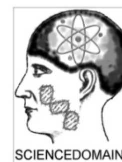

Evaluation of Real Time PCR, ELISA and Direct Strip Tests for Detection of *Helicobacter pylori* Infection in Patients with Gastrointestinal Illnesses

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Authors' contributions

This work was carried out in collaboration between both authors. Author FSM designed the study, wrote the protocol and wrote the first draft of the manuscript. Author AYE managed the literature searches, performed the methodology of study and statistical analyses of results. Both authors read and approved the final manuscript.

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ABSTRACT

Helicobacter pylori (H. pylori) infection is very common worldwide. The infection causes chronic gastritis which significantly increases the risk of developing gastric or duodenal ulcer, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma. This study was undertaken for the detection of *cagA* gene in biopsy specimens from patients with gastrointestinal diseases by real time PCR test, in addition to serological detection of anti *H. pylori* IgG and IgM antibodies by ELISA and direct strip tests. A total of 50 antral biopsy and serum specimens were subjected to real time PCR test conducted together with ELISA test for serological diagnosis. Stomach cancer comprised the lowest frequency by admission diagnosis, 1(2%). PCR test was positive in 45 (90%) of study group. Specimens from patients with gastric ulcer, gastritis & dudenitis, stomach cancer, and multiple polyps recorded 100% positive PCR test. Out of total, ELISA-IgM was positive in 16 (32%) of study group, whereas IgG was positive in 23 (46%) of study group. Direct

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strip test was positive in 18 (36%) of study group. Our study suggested that stomach cancer is unusually rare in Iraq, despite the high prevalence rate of *H. pylori* infection in this developing country. ELISA-IgM and IgG and direct strip tests showed a low sensitivity and specificity for the diagnosis of *H. pylori* infection in a group of Iraqi patients. Polymerase chain reaction is highly specific and may be more sensitive than other biopsy-based diagnostic techniques. Although PCR is a time consuming and expensive procedure with need for highly trained staff performing it, our study demonstrated that using PCR methods for detection of *H. pylori* have a high diagnostic accuracy rate. Further research is needed to study virulence markers and genetic heterogeneity of *H. pylori* in patients with gastrointestinal illnesses.

Keywords: Helicobacter pylori; GIT; biopsy; PCR; ELISA; strip.

1. INTRODUCTION

H. pylori are a Gram-negative bacterium which causes chronic gastritis and plays important roles in peptic ulcer disease, gastric carcinoma, and gastric lymphoma. *H. pylori* have been found in the stomachs of humans in all parts of the world. In developing countries, 70 to 90% of the population carries *H. pylori*. In developed countries, the prevalence of infection is lower, ranging from 25 to 50% [1]. Despite the geographical proximity, the gastric cancer rate varies from very low in Iraq and Egypt to intermediate in Israel and Turkey to high in Iran. It was shown that prevalence of gastric cancer and cagA status among *H. pylori* strains isolated in the Saudi Arabia was ($5.7/10^5$) and (52%), respectively as compared with Kuwait which was $4.8/10^5$ and (41%), respectively. These figures were highly different in Iran ($26.1/10^5$) and (76%), respectively, whereas in Iraq was ($4.5/10^5$) and (71%), respectively [2].

H. pylori infection has been proved to be of great relevance to public health in unindustrialized countries, especially in low socioeconomic groups. Poor hygiene, deficient sanitation, and crowded conditions have been reported as risk factors for this infection [3].

H. pylori bacteria induce gastric inflammation in virtually all hosts, and such gastritis increases the risk for gastric and duodenal ulceration, or gastric malignancy. Many studies published have demonstrated that *H. pylori* isolates possess substantial phenotypic and genotypic diversity, rendering differential host inflammatory responses that influence clinical outcome. These investigations of the mechanisms of *H. pylori* pathogenesis will help to define colonized persons bearing the highest risk for disease, and support physicians to appropriately focus diagnostic testing and eradication therapy [4]. It was shown that molecular docking studies of five sesquiterpenoids showed that these natural compounds are potential ligands to inhibit the peptide deformylase activity and this study could pave the ways in discovery of new lead compounds and molecular targets as potential anti-*H. pylori* [5].

The accurate detection of *H. pylori* is essential for managing infected patients and for eradicating the bacteria. Since the discovery of *H. pylori*, several diagnostic methods have been developed for the aim of accurate detection of this organism. These tests include noninvasive method—serology, urea breath test, or stool antigen test—and invasive methods, such as, culture, histological examination, and rapid urease test, which require upper gastrointestinal endoscopy to obtain gastric biopsy samples [6]. PCR detection of *H. pylori* has been reported using a variety of clinical samples including gastric biopsy, gastric juice, saliva, dental plaque, and stools as well as environmental samples [7]. No single test can be relied upon to diagnose *H. pylori* infection, and a combination of two tests is more optimal. The choice of tests depends on the clinical symptoms, age of the patient, the cost-effectiveness of testing strategy and the availability of the tests [8]. The accuracy of these tests is influenced by factors such as the prevalence as well as specific individual technical or method inherent factors. In addition, treatment factors affecting the *H. pylori* infection e.g. proton pump inhibitor might influence the bacterial distribution within the stomach and inhibit urease activity [9].

Several genes have been proposed as possible virulence determinants of *H. pylori*: *cagA, vacA, oipA, iceA, dupA, jhp0562, and β-(1,3) galT*. It was shown that *cagA* is the major *H. pylori* virulence factor [10]. Infections involving *H. pylori* strains that possess the virulence factor *cagA* have a worse clinical outcome than those involving *cagA*-negative strains. It is remarkable

that *cagA*-positive *H. pylori* increase the risk for gastric cancer over the risk associated with *H. pylori* infection alone [11].

In Iraq, published information is sparse concerning evaluation of different diagnostic methods for detection of *H. pylori* infection, and clinicians need to get a better understanding of this pathogen and gastro-duodenal diseases.

The principal aim of our study was for the detection of pathogenic *cagA* gene in biopsy specimens from patients with gastrointestinal diseases by real time PCR test, in addition to serological detection of anti *H. pylori* IgG and IgM antibodies in study patients by ELISA and direct strip tests.The secondary aim was to compare these tests for more diagnostic accuracy.

2. MATERIALS AND METHODS

2.1 Study Patients and Samples

This study has been done at Endoscopy Unit / Baqubah Teaching Hospital/Baquba City from the period of July 2012 to November 2012. A total of 50 patients (29 female and 21 male, mean age 39.11 years) were enrolled according to the following study criteria:

- Patients with chronic strong epigastric pain, melena, haematemesis, family history of CA stomach were included.
- Patients with long-term use of (NSAIDs) drugs, *anti-H. pylori* therapy or bismuth containing drugs were excluded.

All patients had been referred to endoscopy unit after specialist physician requests. Endoscopic examination and antral biopsy collection from all study patients was done by specialist physicians, regardless of normal or abnormal findings. Consent forms were obtained from all patients. Blood samples were collected for complete blood picture, and serum obtained for direct strip test which were conducted at Laboratory Unit / Baqubah Teaching Hospital/ Baquba City. Biopsy specimens were subjected to real time PCR test which was conducted together with ELISA test at Pharma Laboratory in Erbil City. A detailed history of each patient was obtained concerning, age, gender, address, job, smoking & alcoholism, chronic disease, treatment, admission diagnosis of all study patients. Demographic and clinical characteristics of study patients are shown on Table 1.

Table 1. Demographic and clinical characteristics of study patients

Characteristics	(No.)	(%)
1- Gender		
• Male	21	42%
• Female	29	58%
2- Age group in years		
• 10-20	6	12%
• 21-40	22	44%
• 41-60	15	30%
• > 60	7	14%
Diagnosis		
- Gastroesophageal reflux disease (GERD)	10	20%
- Dudenitis	5	10%
- Gastritis & Dudenitis	11	22%
- Gastric ulcer	2	4%
- Stomach cancer	1	2%
- Multiple polyps	1	2%
- Normal	20	40%

2.2 Methods

2.2.1 Real time polymerase chain reaction (RT-PCR)

The assay was based on detection and amplification of the selected region of pathogenic DNA (*cagA* gene). The *cag A* gene is a marker of pathogenicity of strains identified as *H pylori* previously, and it is usual to be used in combination with other genetic markers such as *vac A*, *glmM*, 16SrRNA genes. We selected *cagA* gene only, because it is the major *H. pylori* virulence factor, furthermore the principal aim of our study was for the detection of pathogenic *cagA* gene in biopsy specimens from patients with gastrointestinal diseases. The amplification of the selected DNA fragment (consisting of repeated cycles: thermal denaturation, hybridization of primers with complementary sequences, extension of polynucleotide sequences from these primers with Taq DNA polymerase) was recorded in real time mode (Real line *H. pylori* fla-format, Bioron Diagnostics, Germany) according to manufacturer's instructions. The following primers were used for PCR detection of *H. pylori*:

2.2.1.1 Realline H. pylori (Fla-format) kit

The Real time PCR is based on the detection of the fluorescence, produced by a reporter molecule, which increases as the reaction

Table 2. Primer sets used for *H. pylori* DNA by PCR

Target gene	Amplicon size (bp)	Primer sequences	Annealing temperature	References
cagA F +	189	(5-TTGACCAACAACCACAAACCGAAG-3)	62℃	van Doorn et al. 1998 [12]
cagA R -		(5-CTTCCCTTAATTGCGAGATTCC-3)		

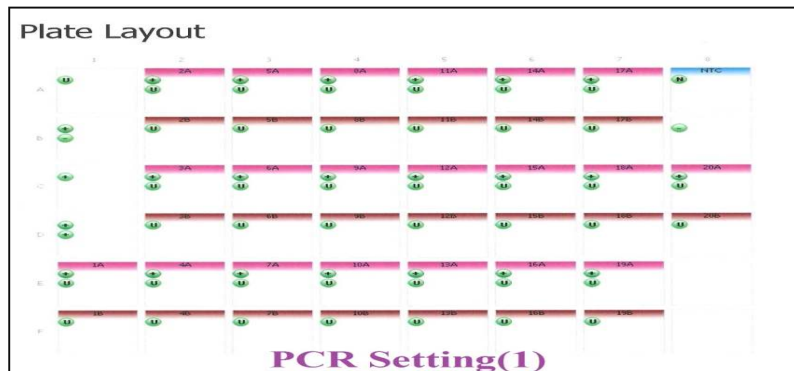

Fig. 1. Plate layout of PCR setting

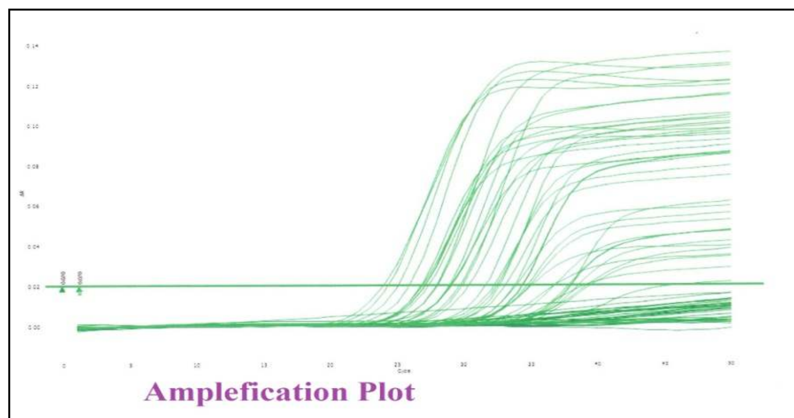

Fig. 2. Amplification plot of targeted gene

proceeds. Reporter molecule is dual-labeled DNA probe, which specifically binds to the target region of pathogen DNA. Dual-Labeled Probes are the most common probe type for RT-PCR and are often referred to as hydrolysis probes. A Dual-Labeled Probe is a single-stranded oligonucleotide labeled with two different dyes. A reporter dye is located at the 5' end and a quencher molecule located at the 3' end. The quencher molecule inhibits the natural fluorescence emission of the reporter by fluorescence resonance energy transfer (FRET). Fluorescent signal increases due to the fluorescent dye and quencher separating by Taq DNA-polymerase exonuclease activity during amplification. PCR process consists of repeated cycles: temperature denaturation of DNA, primer annealing and complementary chain synthesis. The Fla-format Kit contains 5 vials with the lyophilized Mastermix, each vial with 10 reactions, for volume of 50 µl per reaction. The kit contains reagents required for 50 tests, including the positive control samples.

RealLine *H. pylori* (Fla-format) assay kit is designed to detect *H. pylori* DNA isolated from clinical specimens using extraction kits:

> RealLine DNA-Extraction 2. The assay procedure is performed according to manufacturer's instructions [13].

2.2.1.2 DNA extraction

Antral biopsy specimens were received and technique consisted of grinding the tissue to release the bacteria and bacterial lysis by boiling at 100℃ in a lysis buffer. The "real line DNA-extraction 2"kit is designed for DNA extraction from clinical specimens of biopsy material. Nucleic acids isolation principle was a temperature treatment of specimens with multicomponent lysis reagent that destructs nucleoprotein complex, followed by precipitation of nucleic acids magnetic particles with alcohol, wash procedures and elution. The sample was then ready for PCR reaction.

2.2.1.3 Real-time PCR

An appropriate number of 0.2 ml tubes for PCR were prepared. Each tube for each specimen and control was labeled. A quantity of 25 µl of prepared Master Mix was added to each 0.2 ml tube. A quantity of 25 µl of corresponding isolated DNA solution was added to each tube using a separate pipette tip with filter, mixed up, and then 20 micron was added to the plate for the real-time PCR system (Eco Real-Time PCR System (Illumina, USA) according to manufacturer's instructions [13].

2.2.2 Serological tests

- Direct strip test: is a lateral flow chromatographic immunoassay based on the principle of the double antigen-sandwich technique for the detection of *H. pylori* antibody. This test was applied by using *H. pylori* Ab combo rapid test (Biotech, USA) according to manufacturer's instructions.
- ELISA is one of the acceptable serological tests from easiness and fineness in measuring the range of specific antibodies IgM or IgG for diagnosis of *H. pylori* infection. These tests were applied by using ELISA kite IgM, IgG, Monobind, inc (USA). According to manufacturer's instructions.

For diagnostic purposes, *H. pylori* IgM ELISA results should be used in conjunction with other test results and overall clinical presentation. The sample diluent has been formulated to resolve specific IgG and rheumatoid factor interference. However interference by these antibodies cannot be excluded in samples with very high levels.

2.3 Statistical Analysis

Descriptive statistics was used in terms of Frequency, percentage and central tendency. Computed Sensitivity, Specificity, Positive and Negative Predictive Values were used to compare ELISA-IgM, ELISA-IgG and direct strip methods for the diagnosis of *H. pylori* infection. For all analysis, P-value ≤ 0.05 was considered statistically significant. Data analysis was performed by using SPSS (Statistical Package for Social Sciences) version 16.

3. RESULTS

The results show that female patients were higher than male patients within the study group, 58% versus 42%. The age group of 21-40 years comprised the highest number and percentage, 22(44%) within study group. PCR test was positive in 45 (90%) of study group. Stomach cancer and multiple polyps comprised the lowest frequency in admission diagnosis, 1(2%) for each. Specimens from patients with gastric ulcer, gastritis & dudenitis, stomach cancer, and multiple polyps recorded 100% positive PCR test as shown in Table 3. It was shown that 17 (85%) of the 20 antral biopsies with normal histological findings were detected with positive PCR test for *H. pylori*.

Frequency of positive ELISA-IgM and IgG antibodies in serum specimens according to age and gender is shown in Table 4. Out of total, ELISA-IgM was positive in 16 (32%) of study group. It was clearly demonstrated that age group 21-40 years showed the highest number and percentage of positive ELISA-IgM (7, 14%). Positive ELISA-IgM in female patients was higher than that observed with male patients, 14 (28%) versus 2 (4%). Out of total, ELISA-IgG was positive in 23 (46%) of study group. Age group 21-40 years showed the highest number and percentage of positive ELISA-IgG (10, 20%). Positive ELISA-IgG in female patients was higher than that observed with male patients, 14 (28%) versus 9 (18%).

Frequency of positive direct strip test for *H. pylori* according to age and gender is shown in Table 5. Out of total, direct strip test was positive in 18(36%) of study group. It was detected that positive direct strip was higher in age group 41-60 years 9(18%) than that observed with other age groups. Female patients showed the highest number and percentage in positive direct strip 11(22%).

Sensitivity, specificity, positive and negative predictive values of ELISA-IgM, ELISA-IgG and direct strip methods for the diagnosis of *H. pylori* infection are shown in Table 6. Sensitivity is the

Table 3. Positive PCR test for *H. pylori* according to admission diagnosis of study patients

Admission diagnosis	No.	Positive PCR test	
		No.	Percentage
Dudenitis	5	4	80%
Gastric ulcer	2	2	100%
Gastritis & dudenitis	11	11	100%
Gastroesophageal reflux disease	10	9	90%
Stomach cancer	1	1	100%
Multiple polyps	1	1	100%
Normal	20	17	85%

Table 4. Frequency of positive ELISA-IgM and IgG antibodies in serum specimens according to age and gender

Age in years and gender	Positive ELISA-IgM		Negative ELISA-IgM		Positive ELISA-IgG		Negative ELISA-IgG	
	(No.)	(%)	(No.)	(%)	(No.)	(%)	(No.)	(%)
Age group 10-20	2	4%	4	8%	1	2%	5	10%
Age group 21-40	7	14 %	15	30%	10	20%	12	24%
Age group 41-60	6	12%	9	18%	9	18%	6	12%
Age group > 60	1	2%	6	12%	3	6%	4	8%
Male gender	2	4%	19	38%	9	18%	12	24%
Female gender	14	28%	15	30%	14	28%	15	30%
Total	16	32%	34	68%	23	46%	27	54%

Table 5. Frequency of positive direct strip test for *H. pylori* according to age and gender

Age and gender	Positive direct strip		Negative direct strip	
	(No.)	(%)	(No.)	(%)
Age group 10-20 years	0	0%	6	12%
Age group 21-40 years	5	10%	17	34%
Age group 41-60 years	9	18%	6	12%
Age group > 60 years	4	8%	3	9.37%
Male gender	7	14%	14	28%
Female gender	11	22%	18	36%
Total	18	36%	32	64%

Table 6. Sensitivity and specificity of ELISA-IgM, ELISA-IgG and direct strip methods for the diagnosis of *H. pylori* infection

Diagnostic method	Sensitivity	Specificity	Positive predictive value	Negative predictive value
ELISA-IgM	34.8%	50%	88.9%	50%
ELISA-IgG	50%	50%	92%	8%
Direct strip	30.4%	50%	87.5%	12.5%

ability of a test to correctly classify an individual as 'diseased'. The ability of a test to correctly classify an individual as disease-free is called the test's specificity.

Sensitivity = No. of true positive / Total with disease

Specificity = No. of true negative/Total without disease

It was clearly demonstrated that ELISA-IgG recorded the best sensitivity among this test panel (50%). However, the specificity of these tests was equally recorded as (50%). Positive predictive value of ELISA-IgG was higher than that observed in comparison with ELISA-IgM and direct strip methods, 92%.

4. DISCUSSION

It was shown that patients with gastritis & dudenitis had the highest number and percentage, 11 (22%), whereas only one (2%) patient was with stomach cancer. This result was in accordance with the previous studies [13]. In Iraq, despite the early acquisition of *H. pylori infection*, gastric cancer is unusually rare. The mild pathology and antral-predominant gastritis help explain the low cancer rate in Iraq [14].

Our study revealed that 17 (85%) of the 20 antral biopsies with normal histological findings were detected with positive PCR test for *H. pylori*. Gottrand, F. reported that *H. pylori* infection can be associated with a normal gastric histology in children [15]. Detection of *cagA* gene in 100% of antral biopsy specimen by our study confirmed the strong association of *H. pylori* infection with gastric ulcer, gastritis & dudenitis and stomach cancer. The *H. pylori cagA* gene is a major virulence factor that plays an important role in gastric pathologies. The false negative results of *cagA* gene that may be encountered cannot be only explained by gene polymorphism; our assumption for using these primers was that they were previously reported as high sensitivity and specificity methods [16]. According to literature review, it was known that the size variation of *cagA* gene contains one or more Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs and *cagA* multimerization (CM) motifs. It was shown that the structure of the 3' region of the *cagA* gene in Iraqi strains was Western type with a variable number of EPIYA-C and CM motifs. A significant association was found between increase in number of EPIYA-C motifs and GU compared with gastritis indicating predictive association with the severity of the disease [17]. As compared with neighbor countries, it was shown that gastric adenocarcinoma is common in Iran but not in Iraq. It was hypothesized that more virulent *H. pylori* strains may be found in Iran than in Iraq and so compared established and newly described virulence factors in strains from these countries. *CagA* was found in similar proportions of strains from both countries (76% in Iran versus 71% in Iraq) and was significantly associated with peptic ulcer disease in Iraq but not in Iran. *CagA* alleles encoding four or more tyrosine phosphorylation motifs were found in 12% of the Iranian strains but none of the Iraqi strains [18].

Our study revealed that ELISA-IgM was positive in 16(32%) of study group, whereas IgG was positive in 23(46%) of study group. Direct strip test was positive in 18(36%) of study group. PCR test was positive in 46(92%) of study group. Serological tests that detect anti-*H. pylori* IgG antibodies could also lead to false-negatives. False-negative results may occur for new infection when the antibody levels are not sufficiently elevated [19].

Low sensitivity and specificity of ELISA-IgM and IgG and direct strip tests for the diagnosis of *H. pylori* infection were observed. The diagnostic accuracy of ELISA kits made in Western countries has been reported to be lower in other countries [20]. Therefore, every serology tests must have been evaluated with indicated study population and the choice of the antigen is critical [21].

Methods based on molecular biology are considered highly specific and sensitive tests, and many PCR-based assays have been developed to detect *H. pylori* DNA in gastric biopsies, saliva and stool samples. However, this technique is able to detect specific fragments but not viable bacteria, and its sensitivity also depends on several factors [22].

Previous research studies confirm that there is no single test can be undertaken as a reference gold standard for the evaluation of other tests in the diagnosis of *H. pylori* infections. Advantages and disadvantages of these tests should be considered. Although cultural methods of *H. pylori* has their advantages in antibiotic sensitivity testing, but still need selective transport and growth requirements and long incubation period before colonies to appear.

5. CONCLUSIONS

Our study suggested that stomach cancer is unusually rare in Iraq, despite the high prevalence rate of *H. pylori* infection in this developing country. ELISA-IgM and IgG and direct strip tests showed a low sensitivity and specificity for the diagnosis of *H. pylori* infection in a group of Iraqi patients. Polymerase chain reaction is highly specific and may be more sensitive than other biopsy-based diagnostic techniques. Although PCR is a time consuming and expensive procedure with need for highly trained staff performing it, our study demonstrated that using PCR methods for detection of *H. pylori* have a high diagnostic accuracy rate.

Further research is needed to study virulence markers and genetic heterogeneity of *H. pylori* in patients with gastrointestinal illnesses.

CONSENT

Consent was obtained from our study patients.

ETHICAL APPROVAL

The present study was carried out with approval and agreement of Ethical & Medical Committee in College of Health & Medical Technology/Baghdad.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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