Evaluation of Non Invasive versus Invasive Methods for Diagnosis of *Helicobacter pylori* Infection among Patients with Gastroduodenal Disorders

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H. pylori, **a spiral gram-negative bacterium, is associated with gastroduodenal diseases. All *H. pylori* diagnostic assays have limitations. Cytotoxin-associated gene A (*cag A*), a *v*irulence marker, can be identified by PCR. We evaluated *H. pylori* diagnostic methods, invasive: rapid urease test (RUT), and histopathological examination (HE), and serology as non-invasive method. Positive cases were studied for presence of *cag A* gene. Upper endoscopies and gastric biopsies were performed on 67 dyspeptic patients for RUT, HE and PCR. Anti *H. pylori*IgG were measured by ELISA. Of 67 dyspeptic patients, 23 (34%) had more than one endoscopic finding, 46 (68.7%) were *H.pylori* positive by HE, and 21(31.3%) were negative with variable grades of mucosal antral neutrophil infiltration. Of the 46 HE positives, PCR detected *CagA* in 22 (47.8%). Using HE as the gold standard test, the sensitivity of ELISA and RUT was 93.48% and 86.96%, respectively; and the specificity was 85.71% and 47.62%, respectively. In conclusion, IgG detection by ELISA is a suitable screening test for diagnosis of *H. pylori* associated gastroduodenal diseases. Histopathology should be performed in ELISA negative cases to exclude infection.**

Helicobacter pylori (*H. pylori*) is the microorganism responsible for the most frequent and persistent bacterial infection worldwide. *H. pylori* infection affects nearly half of the world's population. In developing countries, the prevalence of infection is as high as 90%, whereas in developed countries, excluding Japan, the prevalence is below 40% (Tonkic *et al.*, 2012).

H. pylori, a gram-negative urease producing bacterium, has been associated with many benign forms of gastritis, peptic ulcer disease and malignancies as gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma in adults (Bayedröffer*et al.*, 1992).

H. pylori urease converts urea from gastric juices into bicarbonate and ammonia, thereby neutralizing stomach acid around *H. pylori* for its survival. Subsequently, a fraction of *H. pylori* strains became more virulent by acquiring the ability to produce and secrete a protein called cytotoxin-associated gene A *(CagA*). *CagA*is encoded by the *cagA* gene, one of ~30 genes present in a 40 kp DNA segment termed the *cag* pathogenicity island (*cag* PAI) (Fischer, 2011).

Several methods are currently available to detect the presence of *H. pylori;* each has its own advantages and limitations. A classic way to categorize the methods is according to whether or not an endoscopy is necessary. Biopsy-based tests include histological evaluation, culture, polymerase chain reaction (PCR), and the rapid urease test (RUT), all of which are performed on tissue obtained during endoscopy. Alternatively, the urea

breath test (UBT), serology test, and stool antigen test (SAT) can be performed as non-invasive procedures (Garza- González *et al.,* 2014).

Histologywas the first method used for the detection of *H. pylori.* Detection of typical bacteria along with the inflammatory reaction in the tissue slides has been the diagnostic test for *H. pylori* infection (Patel *et al*., 2014).Histology is considered the standard method for the diagnosis of *H. pylori* infection. It provides additional and essential information on the status of the mucosa such as the presence of acute or chronic inflammation, lymphoid aggregates, intestinal metaplasia and glandular atrophy (Hirschl and Makristathis, 2007).

Since the application of polymerase chain reaction (PCR) to detect *H. pylori* infection, PCR has been extensively used for the diagnosis of *H. pylori* from different specimens including: gastric biopsy specimens, saliva, stool, and gastric juice specimens (Momtaz *et al*., 2012).

Serology was one of the first methods used for diagnosis of *H. pylori* infection. Anti-*H. pylori* IgG antibodies in patients sera is usually detected by ELISA (Burucoa *et al*., 2013).

Other tests also detect the presence of IgA in the saliva or IgG in the urine. The commercially available serological tests can be performed in most hospital or clinic laboratories (Ricci *et al.,* 2007).

The present study was designed to evaluate different diagnostic methods for detection of *H. pylori* infection in patients with gastroduodenal disorders. These include: histopathological examination, rapid urease test, and detection of anti-*H. pylori* IgG by ELISA. Subsequently, detection of the virulence marker *cag* A gene by PCR was attempted in *H. pylori* positive cases.

Patients and Methods

Subjects

Sixty-seven patients, with dyspeptic symptoms selected from the Tropical Medicine Department, Al-Azhar University Hospital, were enrolled in this study during the period October 2014 to December 2015. Patients who had received anti-microbial agents, H_2 receptor antagonists, proton-pump inhibitor within 4 weeks prior to endoscopy and those who had gastric surgery were excluded from the study.

Methods

The Clinical Research Committee of the hospital approved the study and it was performed according to well-known ethical procedures. A written informed consent was obtained from each patient for the participation in the study. All the cases were subjected to full history taking and thorough clinical examination. For the detection of *H. pylori* infection, endoscopic gastric biopsies were obtained to carry out RUT, histopathological examination and PCR. Sera were obtained to perform ELISA for detection of anti *H. pylori* IgG antibodies.

- Gastric biopsy

Three antral biopsies were taken from each patient within about 2cm of the pyloric channel (Abdulqawi *et al.,* 2012); using (Olympus GIF P; 230 Olympus Optical Co., Tokyo Japan) fibre-optic endoscope. Two biopsies were sent immediately for both RUT and histopathological examination. The third biopsy specimen was kept in normal saline and stored at -70°C for molecular analysis.

- Blood collection

Five ml blood sample was collected within three days of endoscopy and before initiation of any therapy against *H. pylori*. The blood was kept at room temperature for at least 1h, followed by centrifugation at 450 xg for 10 minutes. Serum samples were divided into aliquots and stored in cryovials at -70°C (Abdulqawi *et al*., 2012).

- Rapid Urease Test (RUT)

Immediately after collection, one biopsy was tested for detection of urease activity using AMA RUT 1 (Association of Medicine and Analytics Company Limited; AMA Co Ltd, Russia). The biopsy specimen was placed on a special slide containing urea, provided by the kit, using a dry clean forceps. The principle of AMA RUT 1 is based on the color change of the indicator disk after biopsy specimen has been placed on its surface (shades of blue within 3 minutes).

- Serum anti *H. pylori* antibodies ELISA testing

Serum samples were tested for the presence of anti *H. pylori* IgG using the SERION ELISA classic *H. pylori* IgG kit, (Serion Immundiagnostica GmbH, Germany) according to the manufacturer's instructions, a cutoff point of >50 U/ml was provided by the kit. The optical density (OD) was read using a microplate reader Metertech 960 (Metertech, Taipei, Taiwan) within 60 minutes, from adding the color reagent, at 405 nm against substrate blank and reference wavelength between 620 nm and 690 nm. Results were interpreted as reactive if the absorbance value was >50 U/ml, non-reactive if value was <35 U/ml and in borderline range if value was between 35-50U/ml.

- Histopathological evaluation

Gastric biopsy specimens were fixed immediately after collection in a tube containing 5ml of 10% formalin (freshly prepared) (Sharma *et al.,* 2013). The specimen was then paraffin-embedded and sections of 4µm-thick were cut, spotted onto glass slides and fixed by incubation overnight at 55°C (Rüssmann *et al.,* 2003).

Sections were stained with hematoxylin and eosin (H&E), and Giemsa stain. Light microscopic examination was carried out specifically looking for *H. pylori* bacterium, intestinal metaplasia and infilteration of gastric mucosa by mononuclear cells and polymorphonuclear leukocytes. Grading of gastritis was conducted according to the updated Sydney system which records the presence of inflammation (mononuclear cell infiteration), activity (neutrophil infilteration), atrophy and intestinal metaplasia; using visual analog scale for normal mild, moderate, and marked; with a scoring system from 0 to 3, respectively (Dixon *et al.*, 1996).

- Polymerase Chain Reaction for detection of *cag A* gene

The DNA was extracted from biopsy specimens from the gastric antrum using WTZARD genomic DNA purification kit (Promega, Germany) according to manufacturer's protocol. According to the method of Brito *et al*., 2003 and Marie, 2012, the total DNA was amplified with the following primers *CagA*-F (GATAACAGGCAAGCTTTTGAGG) and *CagA*-R (CTGCAAAAGATTGTTTGGCAGA). Amplification was performed in a volume of 25µl containing 1µl forward and reverse primers, 1µl template patient DNA, and 10 ml of 2X of Master Mix (Bioline, UK). PCR was performed using a thermal cycler (Biometra, Germany) under the following cycling parameters. An initial denaturation at 94^0C for 5 minutes and 35 cycles of 94^0C for 1 minute, 55^0C for 1 minute and 72^0C for 1 minute. This was followed by a final extension of 72^0C for 5 minutes. The gene product was 349bp. The amplified PCR products were separated by 1.5% agarose gel electrophoresis(Biometra-Agarose gel mini, Germany), stained with ethidium bromide (1µl/ml) for 30 minutes under 100V in 1X borate EDTA (TBE) buffer and visualized by ultraviolet trans illuminator (Hero Lab UVT-20M, Germany). PCR amplicon sizes were calculated by a comparison with 100 bp to 1kb molecular weight DNA ladder (marker) DNA ladder 100 bp (Thermo Scientific, USA) 100 bp to 1kb linear scale ready-to-use solution.

Statistical Analysis

Data entry and analysis were done using SPSS version 19 (Statistical Package for Social Science). Results were presented as number, percentage, mean, standard deviation for quantitave variables and number (percentage) for qualitative variables. Differences in proportion were assessed by using Chi-square test. P-value is considered statistically significant when $P < 0.05$. MedCalc statistics were used to calculate sensitivity, specificity, positive and negative predictive values, accuracy and area under the Roc curves (AUC). Kappa index was done to study agreement between qualitative items.

Results

Among 67 patients undergoing upper gastro endoscopy, 33 were males and 34 were females. Their ages ranged from 19-84 years with a mean age 45.27 ± 15.11 years. Based on the presence of abnormal endoscopy findings, 23 patients (34.3%) had more than one endoscopic finding (Table 1).

Table 1. Distribution of 67 patients according to the frequency of endoscopic findings

Upper endoscopy	No. of patients	%
Normal	1	1.5
One endoscopy finding	43	64.2
More than one endoscopy finding	23	34.3

The common endoscopic change in the studied patients was duodenal ulcer in 40 patients (59.7%) followed by gastro oesophygeal reflux disease (GERD) in 21 patients (31.3%), then gastritis in 20 patients

(29.9%) and portal hypertensive gastropathy (PHG) in 18 patients (26.9%) (Table 2).

Table 2. Endoscopic findings in 67 patients with dyspeptic symptoms

Endoscopy finding	No. of patients	%
Gastritis	20	29.9
Duodenal ulcer	40	59.7
Gastro-oesophygeal reflux disease	21	31.3
Portal hypertensive gastropathy	18	26.9

In our study, patients were considered to be *H. pylori* infected when histopathology was positive for *H. pylori* bacterium. Histopathological examination was done for detection *H. pylori* as a spiral bacterium, grading of activity and presence of intestinal metaplasia. Of the 67 studied patients, 46 patients (68.7%) were positive for *H. pylori* bacterium and 21 (31.3%) were negative. Variable grades of mucosal antral infiltration by neutrophils (activity) and chronic inflammatory cells were present. Intestinal metaplasia was detected in six of *H. pylori* positive cases. Sections of gastric antrum in *H. pylori* infected patients showed a rod shaped organism stained blue by Giemsa, and red by H&E. Chronic gastritis was evident histopathologically by the presence of chronic inflammatory cells (Figure 1).

A total of 51 patients (76.1%) were RUT positive while anti *H. pylori* IgG antibodies were present in sera of 46 (68.7%) patients (Table 3).

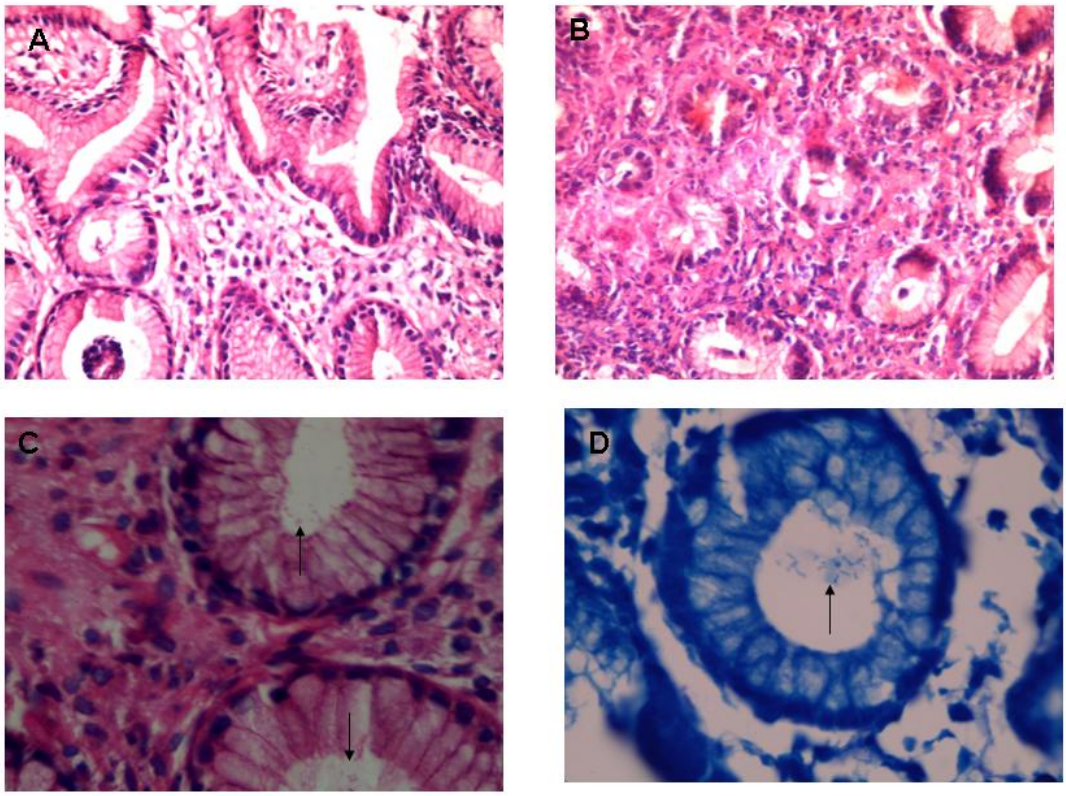

Figure 1. Photomicrograph showing *H. pylori* associated chronic gastritis. A: mild inflammatory cell infiltrate. B: Marked inflammatory cell infiltrate. C: *H. pylori* organism as seen in H&E stained sections (arrows). D: *H. pylori* organism by Giemsa stain (arrow). (A, B: H&Ex400, C: H&Ex1000, D: Giemsa stainx1000).

Table 3. Diagnostic methods for detection of *H. pylori* infection

Method For Diagnosis	No. of patients	%
Histopathological identification (*H. pylori*)		
Positive	46	68.7
Negative	21	31.3
Rapid urease test		
Positive	51	76.1
Negative	16	23.9
ELISA:		
Positive	46	68.7
Negative	21	31.3

H. pylori bacterium was identified histopathologically in (82.5%) of duodenal ulcer patients, 70% of gastritis patients, 71.4% of patients with GERD and 44.4% of patients with PHG. Of duodenal ulcer patients 87.5% were RUT positive, 80% of gastritis, 76.2% of GERD and 61.1% of PHG. Serum IgG was positive in 75% of each of duodenal ulcer and gastritis patients; and 71.4% and 50% of GERD and PHG, respectively (Table 4).

Table 4. Relation of endoscopy findings to results of various diagnostic tests for *H. pylori* detection

Endoscopy finding	Histopathology				Rapid urease test				ELISA			
	positive		negative		positive		negative		positive		negative	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Gastritis (n= 20)	14	70.0	6	30.0	16	80.0	4	20.0	15	75.0	5	25.0
Duodenal ulcer (n= 40)	33	82.5	7	17.5	35	87.5	5	12.5	30	75.0	10	25.0
GERD (n= 21)	15	71.4	6	28.6	16	76.2	5	23.8	15	71.4	6	28.6
PHG (n= 18)	8	44.4	10	55.6	11	61.1	7	38.9	9	50.0	9	50.0

When the histopathological identification of *H. pylori* was used as the gold standard test, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of both serum *H. pylori* antibodies level (cut-off values > 50 U/m) and RUT were calculated and are shown in Table (5). The serology test (ELISA) achieved the highest sensitivity (93.48%) and specificity (85.71%) followed by RUT (86.96%) and (47. 62%), respectively. In our study, an agreement of 0.79 by Kappa statistic was

calculated between the ELISA serology test and histopathology while an agreement of 0.37 was found between RUT and histopathology (Table 5). Receiver operating characteristics (ROC) curves for both ELISA and RUT are presented in Figures (2) and (3), respectively.

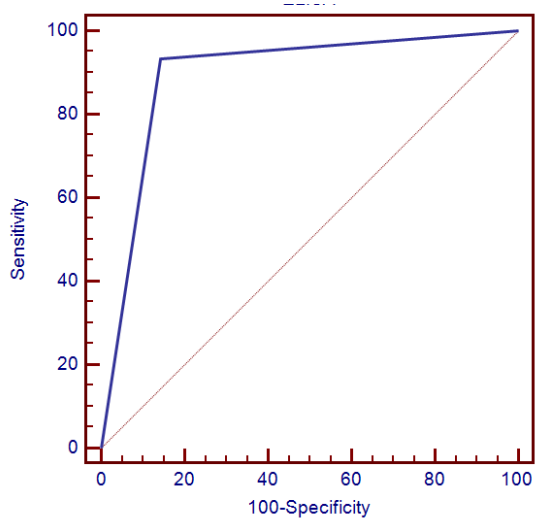

Figure 2. ELISA ROC curve.

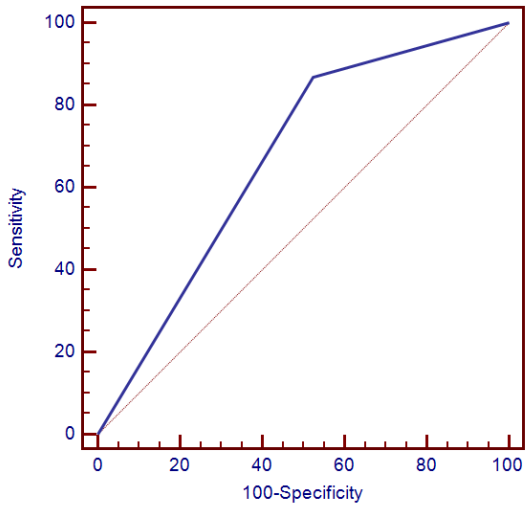

Figure 3. Rapid urease test ROC curve.

Of the 46 *H. pylori* positive cases, the virulence marker *cagA* was identified in 22 (47.8%) cases by PCR (Figure 4).

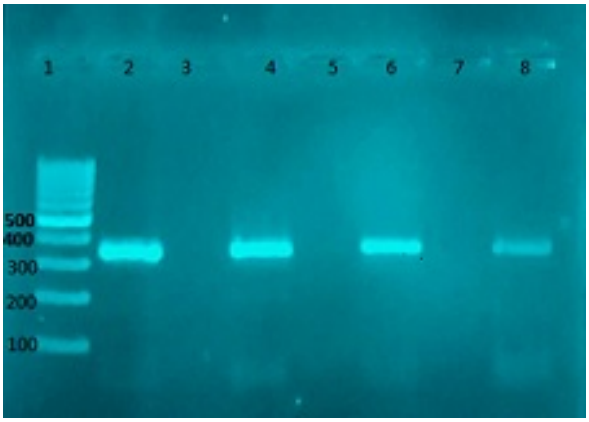

Figure 4. A photograph of ethidium bromide stained 1.5% agarose gel showing the product of a representative PCR experiment for *cagA* genotype of *H. pylori.* Lane 1:100 bp DNA ladder, lanes (2, 4, 6 and 8) show the PCR product of *cagA* (349 bp). Lanes (5 and 7) show no PCR product of *cagA*. Lane 3: negative control.

Among the *H. pylori* positive subjects, *cagA* was detected in 16 (48.5%) of duodenal ulcer patients, in eight (57.1%) of gastritis patients, in nine (36.4%) of GERD patients and in four (50%) of PHG patients, respectively. The difference in the prevalence of *cagA* in the studied patient groups did not reach statistical significance (Table 6). The relation between the grades of mucosal antral infilteration by neutrophils and presence of *cagA* gene is shown in Table (7). Seven *cagA* positive cases and fifteen *cagA* negative cases were grade 0 of activity (P =0.037), while in grade 2 of activity, seven positive cases for *cag A* were detected (*P* =0.010). Four cases of intestinal metaplasia were positive for *cag A* gene.

Table 5. Performance characteristics of ELISA and RUT for detection of *H. pylori*

	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC	95% CI	Kappa index of agreement
ELISA	93.48	85.71	93.5	85.7	91.04	0.896	0.797-0.957	0.79
RUT	86.96	47.62	78.4	62.5	74.6	0.673	0.547-0.783	0.37

PPV: Positive Predictive Value; NPV: Negative Predictive Value; AUC: Area under the Roc curve; 95% CI: 95% Confidence Interval

Table 6. Distribution of *cag A* according to the endoscopic findings

Endoscopy finding (*H. pylori* positive cases)	*cag A*				*P. value*
	positive		negative		
	No.	%	No.	%	
Gastritis (n= 14)	8	57.1	6	42.9	NS
Duodenal ulcer (n= 33)	16	48.5	17	51.5	
GERD (n= 15)	9	60.0	6	40.0	
PHG (n= 8)	4	50.0	4	50.0	

P>0.05 is not significant (NS).

Table 7. Correlation between histopathological changes and *H. pylori* cagA

Histopathological examination	*cagA*				**P. value*
	Positive (n= 22)		Negative (n= 24)		
	No.	%	No.	%	
Activity(neutrophil infilteration)					
Grade 0	7	31.8	15	62.5	0.037
Grade 1	7	31.8	9	37.5	NS
Grade 2	7	31.8	0	0.0	0.010
Grade 3	1	4.5	0	0.0	NS
Intestinal metaplasia:					
Positive	4	18.2	2	8.3	NS
Negative	18	81.8	22	91.7	

**P*>0.05 is not significant (NS).

Discussion

H. pylori causes gastro intestinal diseases such as gastritis and peptic ulcer in adults and children (Tan & Goh, 2012). In most people, *H. pylori* infection is largely restricted to the gastric antrum (Chuan*et al*., 2005). Accurate diagnosis of *H. pylori* infection involves the combined knowledge, effort and research of laboratories, gastroentrologists and pathologists (Lopes *et al*., 2014).

In the present study, the prevalence of *H. pylori* infection by histopathological examination was 68.7% that correlated well with findings in Indian studies. For instance, Sharma *et al.,* 2013 in Mumbai, India and Sodhi *et al*., 2013 in Kashmir, India reported 57.14% and 58%, respectively. In addition, data of Trowell *et al.*, 1987 (56%) and, Joshi *et al.* 1997 (54.4%) were close to ours. However, higher prevalence rates were reported by Kang *et al*., 1991 in Korea (98%) and, Sandikci *et al.,* 1993 (86%). On the other hand lower prevalence rates were documented by Cheong *et al.,* 1990 (34%) and Kunz *et al*., 1990 in Germany (47.9%). Differences in prevalence of *H. pylori* infection could be explained according to Eusebi *et al*., 2014 as significant differences in the prevalence of infection exist within and between countries and people in developing nations and residents of developed countries with low socio-economic status have a higher prevalence of infection. Bruce and Maaroos, 2008 concluded that the prevalence of *H. pylori* infection significantly differs by ethnicity, geographic areas, and socioeconomic status.Graham *et al*., 2009 reported that all over the world, a decreasing incidence of *H. pylori* infection inversely parallels the improvement of living standards.

H. pylori was histopathologically positive in 70% of gastritis patients. Various workers reported that the *H. pylori* is positive in 47-92% of gastritis indicating its importance as a common agent of gastritis (Sharma *et al*., 2013).

This study showed a substantial agreement (0.79) between the gold standard test for *H. pylori* (histopathological examination) and serology testing for anti *H. pylori* IgG antibodies by ELISA and a diagnostic accuracy of 91.04%. This is in concordance with Iqbal *et al*., 2013 who found 0.72 agreement between serology testing for *H. pylori* IgG antibody by ELISA and histopathology results and a good diagnostic accuracy of serological testing (86%) was observed for the diagnosis of *H. pylori* infection.

The sensitivity and specificity results of *H. pylori* IgG antibodies by ELISA test were 93.48% and 85.71%, respectively using a cutoff level of > 50 U/ml with AUC of 0.896 at the ROC curve. In agreement with this study, Alam El-Din *et al*., 2013 found that diagnosis of *H. pylori* infection by non-invasive methods including the serum antibody test revealed a sensitivity and positive predictive value of 88.9% and 94.2%, respectively. The presence of IgG antibodies against *H. pylori* denotes active infection because once an individual is colonized, the infection continues throughout life unless a course of appropriate eradication therapy is instituted (Rajindrajith *et al*., 2009). Serological tests are commercially available, easy to perform and inexpensive and therefore have been recommended for the diagnosis of *H. pylori* in adults (Veijola and Oksanen, 2010).

The current study demonstrated that RUT had 86.96% sensitivity and 47.62% specificity for the diagnosis of *H. pylori* infection. This finding was in contrast to that reported by Haruma *et al*., 1992 as sensitivity and specificity of RUT with histopathology as

gold standard were 93.9% and 98.0%, respectively. Another study by Roy *et al*., 2016 showed that RUT had 97.22% sensitivity and 94.04% specificity when histopathological demonstration of *H. pylori* was taken as standard for diagnosis. However, it is known that in RUT, false negative results may occur due to irregular distribution of bacteria in gastric mucosa or the use of anti microbials or proton pump inhibitors (Mégraud and Lehours, 2007). False positive results can occur if other urease containing organisms are present in sufficient quantity or if one allows contact of the specimen and the media for a prolonged period, typically longer than 24 hours, approximately 10^5 bacteria must be present in the biopsy sample for a positive result (Mégraud *et al.,* 2014). Anything that reduces the bacterial density such as the use of antibiotics, bismuth-containing compounds, or proton pump inhibitors may result in false-negative results (Graham *et al*., 2003).

Cag A is a 120-kDa protein of *H. pylori* with high immunogenicity. Its gene (*CagA*) is contained in the pathogenicity island (PAI) of *H. pylori* chromosome. Individuals infected with *cagA* positive strains tend to have more sever gastritis, a higher likelihood of developing gastric atrophy and intestinal metaplasia, and a higher incidence of duodenal ulcer and intestinal – type gastric cancer (Kiesslich, *et al*., 2005). The detection of virulence determinants, such as *cagA* is an area of particular interest (Lopes *et al*., 2014).

In our study, the prevalence of *cag A gene* was not statistically different according to endoscopic findings of studied patients. In contrast, Yakoob *et al*., 2013 found H. *pylori CagA* was strongly associated with duodenal ulcer and gastric carcinoma compared with non-ulcer dyspepsia in Afghan patients. However, in a study by Sedaghat *et al.,* 2014 there was no significant difference between the existence of *cag A* gene with peptic ulcer diseases and non ulcer dyspepsia groups of patients. In addition, Watada *et al*., 2011 reported that the prevalence of *cag A* was not different between patients with duodenal ulcer, gastritis and gastric carcinoma, and they hypothesized that the presence or absence of novel factors that accompany the presence of *cagA* could lead to severe clinical outcomes.

In conclusion, our study showed a considerable agreement between serology and histopathology results to detect *H. pylori* infection. The ELISA test to detect anti *H. pylori* IgG antibodies is "comparatively" non-invasive, easy to perform and can be performed as a first screening test. Nevertheless, one should not underestimate the prevalence of *H. pylori* infection in subjects with negative IgG serology in clinical practice. Thus, histopathology is indicated for excluding infection in such circumstances. It is accepted that *cagA* is an important virulence factor of *H. pylori*, but further studies are still required to resolve possible association of the existence of *cagA* in different disease pathological groups.

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