Research Article

WESTERN BLOT METHOD EVALUATION FOR DETECTION OF *HELICOBACTER PYLORI* INFECTIONS AGAINST H. PYLORI AG IN STOOL ENZYME-LINKED IMMUNOASSAY IN ADULT EGYPTIAN PATIENTS

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Abstract

Helicobacter pylori infection is tremendous medical burden especially in developing countries. Various immunological tests are available for diagnosis of *H. pylori* infection. Western blot method is proven to be promising for Precise, easy reading, sensitive and specific detection of *H. pylori* infections, besides it also permits the detection for the different virulence factors of CagA / VacA positive strains (type I). The objective of this study is to evaluate the diagnostic value of commercial Western Blot (WB) method in the serological diagnosis of *H. pylori* infections against the *H. pylori* Ag in stool (HpSAg) using commercial enzyme-linked immunoassay (ELISA) in adult dyspeptic Egyptian patients. Also we investigated the prevalence of virulence factors, cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) in the infected patients. Samples from 46 adult dyspeptic Egyptian patients were tested by the two methods. WB test gave accurate confirmed result with (82.6% accuracy and 89.5% sensitivity) compared to HpSAg test. Also the results indicated a high seroprevalence of cagA- and vacA-positive virulent *H. pylori* type I strains in adult infected population indicate that such strains may be common in this population and responsible for the majority of *H. pylori* infection among adult Egyptians. We concluded that WB method could be useful for the confirmatory detection of antibody profiles to *H. pylori* antigens and virulence factors in adult Egyptian patients.

Keywords: *H. pylori;* Western blot; cytotoxin-associated gene A; vacuolating cytotoxin A; Egypt

Introduction

Helicobacter pylori (*H. pylori*) is a recognized gram-negative, spiral-shaped gastrointestinal bacterium pathogen with world-wide distribution. According to the Centers for Disease Control and Prevention (CDC), about two-thirds of the world's population harbors the bacterium, with much higher infection rates in developing countries than in developed nations. *H. pylori* is associated with a variety of severe gastric diseases and considered as a major cause of chronic gastritis and risk factor for peptic ulcer disease and is responsible for the majority of ulcers of the stomach and upper small intestine such as gastric and duodenal ulcer, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (MALT) (Parsonn *et al.*, 1991; Parsonnet *et al.*, 1994; Uemura *et al.*, 2001). Classified by World Health Organization (WHO) as a class I carcinogen, *H. pylori* is considered a serious medical issue especially in developing countries. In Egypt, several studies indicated that *H. pylori* infection is highly endemic in Egypt with higher rate in rural areas and increasing trend in older ages. Also the studies suggested the relation between the *H. pylori*

infection and progression of liver fibrosis due to Hepatitis C virus (HCV) (Ragheb *et al.*, 2012; Rocha *et al.*, 2005; Mohammad *et al.*, 2008).

The virulence factors and pathogenesis, which influence the clinical outcome of *H. pylori* infection, have not fully elucidated yet. However, many studies showed that the disease causation by *H. pylori* is due to the various virulence factors where *H. pylori* strains vary in the degree of virulence (Atherton, 1997; Rudi *et al.*, 1998; Farshad *et al.*, 2009). *H. pylori* strains are divided into Type I and Type II, respectively based on the presence and absence of cagA, vacA and iceA genes which are considered to be the putative virulence factors of *H. pylori* (Censini *et al.*, 1996; Covacci *et al.*, 1997; Yamaoka *et al.*, 1999). In particular cytotoxin-associated gene A (cagA) and the vacuolating cytotoxin (vacA) are the two main *H. pylori* virulence factors found among the bacterial markers associated with pathogenesis of different strains (Farshad *et al.*,, 2009). This toxin CagA, produced by cagA gene, alters the structure of stomach cells and allows the bacteria to attach to them more easily. Long-term exposure to the toxin causes chronic

inflammation(Bagnoli *et al.*, 2005). VacA cytotoxin induces large host cell vacuoles when *H. pylori* filtrates (Leunk *et al.*, 1988). Both CagA and VacA cytotoxins are highly immunogenic proteinsassociated with type I strains which are responsible for severe forms of gastrointestinal diseases (Xiang *et al.*, 1995; Weel *et al.*, 1996; Enroth *et al.*, 2000; Farshad *et al.*,, 2009).

The standard primary diagnostic procedures for *H. pylori* infection, including endoscopy and histology, or bacterial culture of biopsied specimen are endoscopic invasive and not available in most clinics in the developing countries. While the non-invasive breath test are useful in the diagnosis of *H. pylori* but expensive due to the need for expensive mass spectrometers and the availability of ^{13}C-urea limits the use of the breath test in the developing countries. On the other hand, the indirect immunological tests are commercially available, accurate and cheap and measure the immune system response to an infectious agent and the components of the agent itself.

We therefore aimed first to evaluation of western blot (WB) method for serologic diagnosis of *H. pylori* infections against the common enzyme-linked immunosorbent assay (ELISA) by detection of antibodies to *H. pylori* virulence factors such as CagA and VacA using commercial immunoblotting test compared to *H. pylori* Ag in stool as a gold standard. We also investigated the frequency of occurrence of serological markers of *H. pylori* virulence factor(s) of type I *H. pylori* strains in adult Egyptian patients.

Patients and Methods

This study was conducted including 46 of dyspeptic individuals (32 males and 14 females) suspected to be infected with *H. pylori*

infection. The range of the patients' age varied from 20 to 70 years old with a median age of 40 years old (40.5 males and 40 females) see Table 1.

Blood specimens for serological testing was drawn, where 5ml venous blood was taken and collected in dry tube, after clotting, the sera were separated by centrifugation for (10 minutes at 3000 rpm) divided into aliquots that may be refrigerated at 2–8 °C for up to seven days or frozen for up to six months till used. Samples of stools were collected in dry clean tubes and stored at (-20ºC) until used. ELISA measurement of *H. pylori* Antigen (HpSAg) in human stools was performed and the results were interpreted using *H. Pylori* Antigen E32-320 manufacturer's instructions (Immunospec, USA).While serum samples were examined for *H. pylori* antibodies to six virulent *H. pylori* antigens by Wester blot test using the commercial *Helicobacter pylori* LINE IgG / IgA Line (*H. pylori* line) immunoblot kit WE243G96 (Sekisui Virotech, Japan). Qualitative detection of *H. pylori* antibodies in human serum to the following six virulent antigens: cagA (cytotoxin-associated geneA), vacA (vacuolating cytotoxin A), UreA (urease A), p30 (OMP), p25 (OMP) and p19 (OMP), according to the manufacturer's instructions. Additional *H. pylori* IgA antibody test ((HpIgA) was performed for 20 randomly selected samples using *H. pylori* IgA E30-274. Assay values were calculated according to the manufacturer's instruction (Immunospec, USA) as shown in Table 2.

Statistical Analysis

Statistical analyses were performed using SPSS software (version 16). Sensitivity, specificity, predictive values of positive and negative results were calculated in accordance with standard methods suggested by Fleiss (Fleiss *et al.*, 2003). Chi-square test for significance used to compare proportions between qualitative parameters. The result considered significant if the probability (P-value) was < 0.05.

Table 1: The distribution of the participated cases

Ages	Males		Females		Total	
	Number	Percent	Number	Percent	Number	Percent
<= 30	5	15.6 %	2	14.3 %	7	15.2 %
31 - 40	11	34.4 %	6	42.9 %	17	37 %
41 - 50	11	34.4 %	2	14.3 %	13	28.3 %
51 - 60	3	9.4 %	2	14.3 %	3	10.9 %
61+	2	6.3%	2	14.3%	4	8.7
Total	32	69.6 %	9	30.4 %	46	100%

Table 2: The parameters used for interpretation of the results according to the manufacturer's instructions

	H. Pylori Antigen (HpSAg)	*H. pylori* line	*H. pylori* IgA (HpIgA)
Negative	<15 ng/ml	No band or only one band of p30, p19 CagA	< 0.9* > 1.1*
Positive	>20 ng/ml	or Arise from ≥2 bands of the following: VacA, p30, UreA, p25, p19	
Borderline	15-20 ng/ml	Only one band of VacA, UreA, p25	0.9 - 1.1*

*ratio between the average absorbance value of sample and that of the cut- off.

Results

All specimens were tested for with (HpSAg) and *H. pylori* line for *H. pylori* infection was diagnosed by HpSAg as a gold standard in 38 patients (82.61%), also *H. pylori* line gave the same detection percentage as shown in Fig. 1. However, the results were not matched as both tests detected the infection in 73.9% (positive result) and clear 8.7% from being infected (negative results) as shown in Table 3. The sensitivity and positive predictive value were 89.47%, while the specificity and negative predictive value were 50% as exhibited in Table 4. The *H. pylori* line exhibited significant association with the standard method HpSAg HpSAg (p < 0.01) which showed that the *H. pylori* line could be a reliable method to detect the *H. pylori* infection. Also there was no significance difference in results of *H. pylori* line test neither between different genders nor among different ages, see Fig. 2. For the HpIgA results of the 20 randomly samples, there was no statistically agreement between their result neither with the *H. pylori* line nor with HpSAg (p > 0.05). Only 2 (10%)

cases shared positive HpIgA results with the other tests, HpIgA results was aligned with HpSAg test in 35% of samples (15% negative and 20% positive), while the percentage decreased with *H. pylori* line test to 20% (5% negative and 15% positive) as presented in Fig. 3.
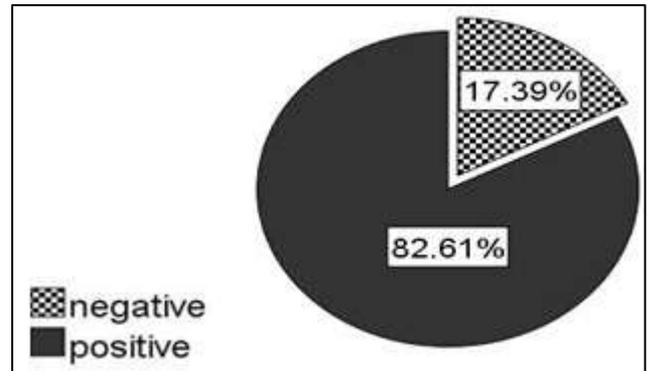

Fig. 1: laboratory results of HpSAg and *H. pylori* line tests for *H. pylori* infection. Both tests gave the same total percentage results regarding all the specimens that was tested

Table 3: Comparison between HpSAg and *H. pylori* line in diagnosing *H. pylori* infections

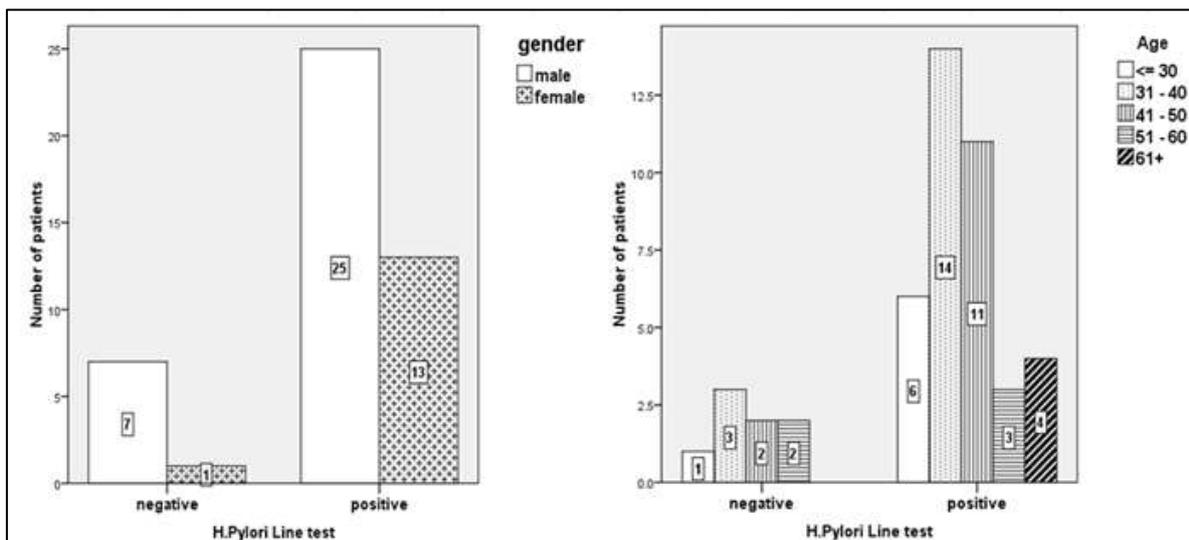|			*H. Pylori* Line		Total
			negative	positive	
H. pylori Ag in stool	negative	Count	4	4	8
		% of Total	8.7%	8.7%	17.4%
	positive	Count	4	34	38
		% of Total	8.7%	73.9%	82.6%
Total		Count	8	38	46
		% of Total	17.4%	82.6%	100.0%

Fig. 2: The distribution of the results of *H. pylori* line test regarding the gender and the ages among 46 dyspeptic patients tested for *H. pylori* infection.
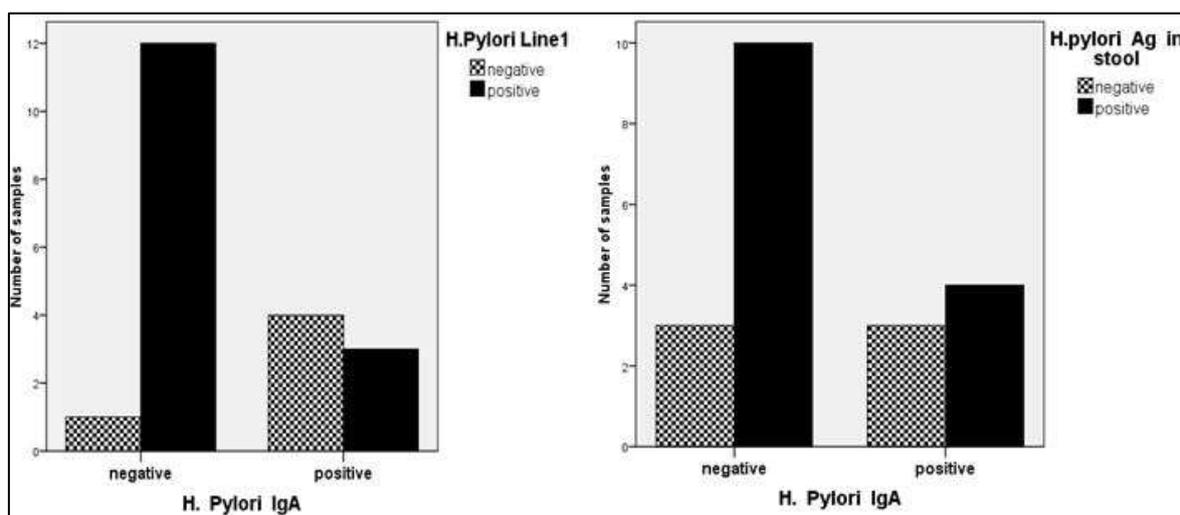
Fig. 3: the agreement of the results of HpIgA test with HpSAg and *H. pylori* line in diagnosing *H. pylori* infections

Table 4: Diagnostic evaluation of *H. pylori* line test against HpSAg test (as a gold standard) in the detection of *H. pylori* infection.

Sensitivity	89.47%
Specificity	50%
positive predictive value	89.47%
Negative predictive value	50%
Accuracy	82.61%
Prevalence	82.61%

Higher number of antibodies to all six virulent antigens of *H. pylori* was present in positive cases tested by the *H. pylori* line and HpSAg test as shown in Fig. 2. Antibodies to CagAa VacA and UreaA were predominated with 54.3%, 30.4 % and 37%, respectively which indicated the high prevalence of virulent strain type I. Although the CagA antigen was confirmed in positive HpSAg test with the highest rate up to 55.3% but there was no significant association between the presence of CagA and positive result gave by HpSAg test. On the other hand, there was a strong relation between the positive results of *H. pylori* line test and presence of CagA antigen. About 65.8% of positive results of *H. pylori* line test were due to the presence of CagA antigen. The prevalence of VacA antigen did not change in the positive results of both tests, as 36.8% of the positive results showed presence of VacA antigen. CagA antigen was detected with VacA in 10.9% of the samples tested, with other antigen than VacA in 26.09% of the cases. While VacA band associated with other bands (p30, UreaA, p25, p19) in 13.04% of the cases. While only 6.52% of the positive results by *H. pylori* line test was due to the presence 2 bands other than CagA and VacA as in Table 5.
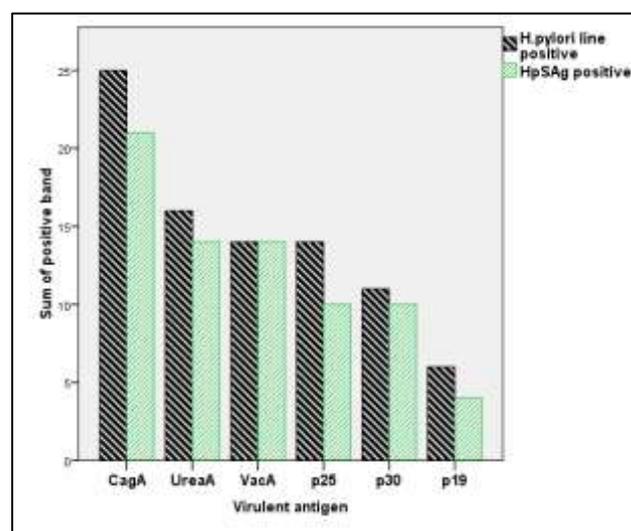

Fig. 4: The antibodies to six virulent antigens of *H. pylori* in positive patients by *H. pylori* line and HpSAg test

Table 5: Distribution of the associated positive bands in the samples of 46 dyspeptic patients using *H. pylori* line test

Positive bands	Number of cases	Percent %
CagA + VacA	5	10.9 %
CagA + other antigen other than VacA	12	26.09%
VacA + other antigen other than CagA	6	13.04%
Two bands of p30, UreaA, p25, p19	3	6.52

Discussion

According to the Centers for Disease Control and Prevention (CDC), people should be tested for *H. pylori* when they have active gastric or duodenal ulcers or a documented history of ulcers. While in developing countries where poor hygiene and sanitation conditions could easily facilitate the transmission of the infection. *H.*

pylori infection is a potential threat especially when associated with other endemic chronic disease as in elder people (Ragheb *et al.*, 2012; Rocha *et al.*, 2005). Early treatment can counteract the development of the serious complications such as gastric carcinoma. Therefore diagnosis of *H. pylori* infection as soon as possible should be attempted. Detection methods of *H. pylori* infection can be classified into invasive and non-invasive methods. The invasive methods where the pathogen is found in biopsies include the rapid urease test, histology, culture and PCR. The non-invasive tests include the urea breath test, the stool antigen test and detection of antibodies in the serum. While invasive methods is the accurate standard diagnostic methods, their expenses hinder their application in the developed countries. On the other side, non-invasive tests are not absolutely accurate on their own but are simple, commercially available and easy to be performed by provincial health laboratories especially immunological tests. Immunochemical tests for *H. pylori* vary in different populations, largely due to their socioeconomic status; the *H. pylori* strain heterogeneity and variations in antigenic preparations. Therefore, the usages of these tests need to be confirmed in the target populations. Both ELISA and Western blot is commonly wide spreading methods for immunological tests. There are several ELISA commercial kits for detection of either antigen or antibodies against *H. pylori*. Also the presence of antibodies against *H. pylori* antigens can be evaluated by immunoblotting as Western blot (WB) (Figueiredo *et al.*, 2001; Oleastro *et al.*, 2002). Regarding being expensive and time-consuming, Western Blot is less likely to give false positive results as it can effectively distinguish *H. pylori* antibodies and other antibodies. Finally immunoblotting allows the detection of antibodies to *H. pylori* virulence factors such as CagA and VacA (Figueiredo *et al.*, 2001). This study aimed to evaluate immunoblotting technique for detection of *H. pylori* infection using commercial Western blot kit (*H. pylori* line) compared to *H. pylori* Ag in stool (HpSAg) by ELISA method as gold standard. HpSAg was proven to be of high specificity and sensitivity test for diagnosis of *H. pylori* infection or whether treatment for an *H. pylori* infection has been successful (Ohkura *et al.*, 2000; She *et al.*, 2009; Rashed, 2015). A total of 46 adult Egyptian dyspeptic patients (age range 20-70 years old) were tested for *H. pylori* infection by both HpSAg and *H. pylori* line methods. The *H. pylori* line gave the same general percentage results as HpSAg with remarkable sensitivity ($\approx$ 90%) and accuracy ($\approx$ 83%) with no significant difference between the results of the two tests. The specificity and negative predictive value was remarkably low (50%) due to the small proportion of uninfected patients evaluated in these current study. Compared with HpSAg, *H. pylori* line correctly identified about 74% of the positive results detected by HpSAg. About 9% was positively detected by *H. pylori* line while showed negative results by HpSAg,

which may related to the nature of method itself as the Western blot test seems to be more sensitive than ELISA especially with low levels of antibodies as individual bacterial proteins are better exposed (Nilsson *et al.*, 1997). On the other side, the positive results of HpSAg which were undetected by *H. pylori* line, could be explained that the *H. pylori* infection was due to strains other than type I, as Pylori line test specifically detect only the virulent factors of *H. pylori* type I strains. The same explanations could be adapted for the unassociated results of HpIgA test for 20 randomly samples with *H. pylori* line results, as there was only 20% agreements between the results of the two tests. However HpIgA is known to be less sensitive to rely on for detection of *H. pylori* infection as observed in other study done also on adult Egyptian patients (Rashed, 2015). Collectively, these results showed that most of the *H. pylori* infection was caused by type I strains of *H. pylori*.

Based on these observations, we also investigated the prevalence of serological markers of *H. pylori* virulence factor(s) especially CagA and VacA of type I *H. pylori* strains among infected adult Egyptian patients. *H. pylori* line used for qualitative detection of *Helicobacter pylori* specific IgG and IgA-antibodies for 6 high specific antigens expressed by type I strain. Phenotype 1 *H. pylori* strain is believed to be more pathogenic than phenotype 2. It was observed that virulent type I has a correlation with higher risk of carcinoma development (Roesler *et al.*, 2014). Phenotype 1 *H. pylori* strain is characterized by the presence of both cagA and vacA genes which are responsible for secretion of CagA and VacA toxins, respectively. CagA protein is a highly immunogenic protein which is responsible for severe forms of gastrointestinal complications such as gastric adenocarcinoma and duodenal ulcer (Blaser *et al.*, 1995; Nomura *et al.*, 2002). There was more than 50% of CagA-positive *H. pylori* in the patients infected by *H. pylori* which reflect the high prevalence of CagA-positive *H. pylori* infections. These results were different than a study done on rural and semi-rural Egyptian citizens, as the study reported that CagA positive strains are not common in the tested subjects, however, the study observed also that seropositive for anti-CagA is significantly increased with increasing ages (EL Dine *et al.*, 2008) which could explaine the strong significant association between the higher frequency of seropositive CagA and ages over 30 years. While there was no relation between the CagA positive results of the different genders. VacA is the second most important toxin in the array of *H. pylori*'s virulence factors. Gastric epithelial cell injury is caused by a vacuolating cytotoxin (VacA), which induces host cell vacuolation and, finally, cell death. Our results showed also a high seropositive VacA (40%) in the infected patients. slightly less than 50% of the positive results had either CagA or VacA combined with different virulent factor.

Conclusion

One immunological test (detecting specific antibody or bacterial antigen) could not be reliable to give accurate determination of *H. pylori* infection. Our finding indicated the western blot is a reliable method which could be used as a confirmatory accurate test to diagnose *H. pylori* infection especially to determine seropositivity anti-CagA, VacA virulence factor status of adult Egyptian dyspeptic patients. Also our study showed high prevalence of phenotype 1 strains in positive detected *H. pylori* infected adult Egyptian patients. However, other studies are still needed to confirm the genotypes of *H. pylori* strains including larger patient populations and control groups, in our country.

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