RESEARCH ARTICLE

Seroreactivity to *Helicobacter pylori* Antigens as a Risk Indicator of Gastric Cancer

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Abstract

Background: Multiple etiologic factors are suspected to cause gastric cancer, the most important of which is infection with virulent types of *Helicobacter pylori*. Materials and Methods: We have compared 102 gastric cancer patients with 122 non-ulcer, non-cancer dyspeptic patients. Gastric specimens were evaluated for *H. pylori* infection by tissue-based detection methods. Patient sera underwent antigen-specific ELISA and western blotting using a Helicoblot 2.1 kit and antibody responses to various *H. pylori* antigens were assessed. Results: The absolute majority (97-100%) of both groups were *H. pylori* seropositive. Multivariate regression analysis demonstrated serum antibodies to the low molecular weight 35kDa protein to be protective and reduce the risk of gastric cancer by 60% (OR:0.4; 95%CI:0.1-0.9). Conversely, seroreactivity to the 89kDa (VacA) protein was significantly higher in gastric cancer patients (OR:2.7; 95%CI:1.0-7.1). There was a highly significant association (p<0.001) between seroreactivity to the 116kDa (CagA) and 89kDa (VacA) proteins, and double positive subjects were found at nearly five fold (OR:4.9; 95%CI:1.0-24.4) enhanced risk of gastric cancer as compared to double negative subjects. Conclusions: Seroreactivity to *H. pylori* low (35kDa) and high (116kDa/89kDa) molecular weight antigens were respectively revealed as protective and risk indicators for gastric cancer.

Keywords: Helicoblot - gastric cancer - 35kDa - VacA - CagA

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Introduction

Infection with *Helicobacter pylori*, a gastric pathogen, is mostly acquired during childhood and unless eradicated, tends to persist chronically and induce various degrees of gastritis. However, only a small fraction of the infected subjects develop more severe gastric complications such as peptic ulcers, atrophic gastritis, intestinal metaplasia and ultimately gastric adenocarcinoma (Sachs and Scott, 2012). Gastric cancer is a highly prevalent and mortal cancer worldwide. It is the second most incident cancer in Iran with a mortality rate of 14.1 ASR per 100,000 (Nagini, 2012). A combination of host, pathogen and environmental susceptibility factors are believed to contribute to the risk of gastric cancer development (Nagini, 2012). It is, therefore, essential to screen for various susceptibility factors to identify *H. pylori*-infected individuals at risk of gastric cancer. Gold standard screening methods are based on invasive, costly, biopsy-based measurements. But devising non-invasive screening methods will allow for a wider population coverage at lower costs, of which serology has obtained the highest application and credibility (Herbrink and Van Doorn, 2000). Serologic screening of subjects for the type of infecting strain enables categorization of infected subjects into high and low risk categories. High risk individuals should be monitored for early signs of neoplastic changes and advised for possible life style changes. Accordingly, serologic screening for antibodies against *H. pylori* antigens has been made possible individually (Baiocchi et al., 2002) and collectively (Veijola et al., 2008; Michel e al., 2009) and their associations with gastric cancer have been evaluated. We have, hereby, assessed serum antibody responses to a collection of *H. pylori* antigens including CagA, VacA, 37kDa, 35kDa, 30kDa (UreA) and 19.5kDa proteins and evaluated their association with gastric cancer in an Iranian population.

Materials and Methods

Study population

Our study population constituted of 122 NUD (non-ulcer dyspeptic) and 102 GC (gastric cancer) patients. NUD patients were defined as those with dyspeptic

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symptoms having been referred for endoscopy with no signs of peptic ulcers or gastric cancer. GC patients were defined as those with histologically confirmed gastric adenocarcinomas. The demographic characteristics of the two mentioned groups are listed in Table 1. Fasting blood samples and gastric biopsies were obtained from each subject to undergo diagnostic tests. Every subject provided written informed consent for study participation which was approved by the National Ethical Committee for Medical Research.

Bacterial culture

Gastric biopsies which were obtained through endoscopy (in NUD subjects) or gastric surgery (in GC subjects) underwent homogenization and cultured on specific peptone agar medium (HPSPA) (Mohammadi et al., 2012) plates containing 7% defibrinated sheep blood. Plates were incubated at 37 ºC in a microaerophilic condition (10%CO_2, 5%O_2, and 85%N_2) for up to 7 days. Grown bacteria were identified as *H. pylori* by routine microbiological assays including urease and catalase tests. Subjects with contaminated cultures (N=25) were excluded from further statistical analyses.

Histology

Gastric mucosal biopsy specimens from the antrum, incisura angularis and corpus were fixed in formalin, paraffin embedded and sectioned. Giemsa stained slides were studied for the presence of *H. pylori* organisms in the gastric tissue. Subjects with unreadable histologic slides (N=91) were excluded from further statistical analyses.

Rapid Urease Test (RUT)

Equivalent gastric specimens were placed into the RUT medium (Chemenzyme Co. Tehran, Iran) containing urea and phenol red indicator. The urease produced by the existing *H. pylori* organisms in the gastric homogenate hydrolyzes urea to ammonia and raises the pH resulting into a yellow to pink color conversion within 4 hours.

ELISA

Sera were isolated from fasting blood samples and kept at -70ºC until further analysis. *H. pylori*-specific IgG antibodies were detected through a locally developed ELISA assay (IPI™) according to the previously described protocol (Mohammadi et al., 2008). Subjects with borderline titers (N=10) were excluded from further statistical analyses.

Table 1. Demographic Characteristics of the Studied Subjects

Categories		GC (n=102)	NUD (n=122)	P values
Gender	Male	75 (73.5%)	58 (47.5%)	<0.01
	Female	27 (26.5%)	64 (52.5%)	
Age Groups	<40	5 (5%)	64 (52.5%)	<0.01
	41-50	10 (10%)	21 (17.2%)	
	51-60	32 (31.4%)	18 (14.8%)	
	61-70	26 (25.5%)	9 (7.4%)	
	>70	26 (25.5%)	9 (7.4%)	
	ND	3 (2.9%)	1 (0.8%)	
Ethnicity	Fars	21 (20.5%)	51 (41.8%)	<0.01
	Non-Fars	73 (71.6%)	66 (54.1%)	
	ND	8 (7.8%)	5 (4.1%)	

Western blotting

Western blotting of patient sera against *H. pylori* bacterial lysate was performed using Helicoblot 2.1 kit (Genelabs Diagnostics, Singapore), in which nitrocellulose membrane strips contain electrophoretically separated protein plus a recombinant antigen as a current infection marker (CIM). The assay was done according to the manufacturer's instructions. Test strips contained different *H. pylori* antigens, including the 116kDa (CagA), 89kDa (VacA), 37kDa, 35kDa, 30kDa (UreA), 19.5kDa antigens and CIM. The recommended criteria for determining a sample as *H. pylori* seropositive was defined by the manufacturer as the Helicoblot "interpretation" as follows: 1) 116kDa (CagA) positive plus one or more of the following bands: 89kDa (VacA), 37kDa, 35kDa, 30kDa and 19.5kDa together, or with CIM; 2) presence of any one band at 89kDa, 37kDa or 35kDa, with or without CIM; 3) Presence of both 30kDa and 19.5kDa with or without CIM.

Statistical analysis

Data analysis was performed using Stata/SE 10.0 software (Stata Statistical Software, College Station, TX). The rate of agreement between detection methods was determined by Kappa Cohen Coefficient. The detection criteria of Helicoblot test including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were calculated against tissue-based gold standard methods using an online calculator (http://www.vassarstats.net/clin1.html). Chi square and Fisher's exact tests were used to measure the association between seroreactivity to different Helicoblot antigens and the clinical diagnosis. Logistic regression model was used to estimate the odds ratios (OR) and their corresponding 95% confidence intervals (CI), measuring the risk associated with seroreactivity to different Helicoblot bands and gastric cancer. Multivariate logistic regression was used for the adjustment of potential confounders (age, gender and ethnicity) and variables with P values less than 0.2 in the crude model. The differences were considered significant if P values were less than 0.05.

Results

There was an almost perfect agreement between the Helicoblot kit-instructed interpretation and *H. pylori*-specific serum ELISA (Kappa value=0.9, 95%CI=0.8-1.0).

The efficiency of Helicoblot interpretation in detection of *H. pylori* infection was assessed against gold standard tissue-based methods, by which subjects with two or more positive tests were considered positive and those with all three negative results were assigned as negatives. Having done this, we found a high rate of sensitivity and low rate of specificity for the method of Helicoblot against gold standard methods in detection of *H. pylori* infection (Table 2). The low specificity rate was due to ten subjects with 3

Table 2. Helicoblot Detection Criteria Based on Gold Standard Tissue-based Methods

Helicoblot Interpretation	Tissue-based Tests 3 tests* Neg	≤2 tests Pos	Detection Criteria	Rate (Confidence Interval)
Negative	3	4	Sensitivity	97% (0.91-0.99)
Positive	10	117	Specificity	23% (0.06-0.54)
Total	13	121	PPV	92% (0.85-0.96)
			NPV	43% (0.12-0.80)
			Accuracy	90%

*Culture, histology and rapid urease test

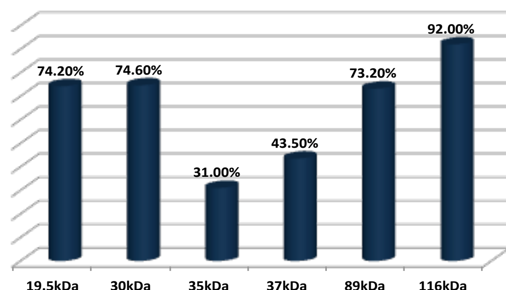

Figure 1. Seroreactivity to Helicoblot Bands

negative tissue-based tests but positive Helicoblot results. All of whom, except one, belonged to the gastric cancer group.

According to the kit recommended "interpretation", the absolute majority of our study population including 100% of GC and 97% of NUD patients were *H. pylori* seropositive.

The total frequency of serum antibody responses to Helicoblot 19.5kDa, 30kDa, 35kDa, 37kDa, 89kDa and 116kDa bands are depicted in Figure 1. On average, the highest serum response was observed in reaction to the 116kDa (CagA) protein and the lowest (31%) to the 35kDa band.

Risk analysis using logistic regression model demonstrated that seroreactivity to *H. pylori* 35kDa or 37kDa protein bands were protective and reduced the risk of gastric cancer by approximately fifty percent. On the other hand, serum antibodies against Helicoblot 116kDa (CagA) band amplified the risk by 4.62 folds (OR:4.6; 95%CI:1.3-16.5). Serum antibodies to the 19.5kDa, 30kDa and 89kDa bands showed no statistically significant difference between the two clinical groups (Table 3). Adjustment for age, gender, ethnicity and other statistically significant bands retained and enhanced the protective role of the serum antibodies to 35kDa by 10 percent. It also amplified the pathogenic roles of serum antibodies to the 89kDa band up to 2.7 (OR:2.7; 95%CI:1.0-7.1) (Table 3). In the adjusted model, the obtained point estimates for the 37kDa and 116kDa did not reach statistical significance.

Due to the highly significant positive association between seroreactivity to the 116kDa (CagA) and 89kDa (VacA) protein bands (Table 4), subjects were categorized according to their variations in CagA/VacA serotypes: 1) CagA+/VacA+ (N=162, 72%), 2) CagA+/VacA- (N=44, 20%), 3) CagA-/VacA+ (N=4, 2%), and 4) CagA-/VacA- (N=14, 6%). It was, then revealed that the majority (72%) of subjects were CagA/VacA double seropositive (Tox+)

Table 3. GC Risk Analysis using Logistic Regression Model

	Crude OR	Crude Lower CI	Crude Upper CI	Crude p value	Adjusted* OR	Adjusted* Lower CI	Adjusted* Upper CI	Adjusted* p value
Helicoblot bands								
19.5kDa	0.9	0.5	1.7	0.83	1.20	0 .5	3.0	0.64
30kDa	0.8	0.5	1.5	0.50	0.57	0.2	1.4	0.17
35kDa	0.5	0.3	0.9	0.02	0.40	0.1	0.9	0.03
37kDa	0.5	0.3	0.8	0.01	0.80	0.3	1.8	0.54
89kDa	1.6	0.9	3.0	0.10	2.70	1.0	7.1	0.04
116kDa	4.6	1.3	16.5	0.01	2.10	0.4	10.8	0.35

*adjusted for age, gender, ethnicity and other Helicoblot bands

Table 4. Association between Seropositivity to CagA and VacA

		89kDa (VacA) Neg	Pos	Total	Kappa (P value)
116kDa (Cag A)	Neg	14	4	18	0.27 (<0.001)
	Pos	46	160	206	
	Total	60	164	224	

Table 5. GC Risk Analysis of CagA/VacA Double Positive Subjects in Reference to Double Negative Ones

	Crude OR	Crude Lower CI	Crude Upper CI	Crude P value	Adjusted* OR	Adjusted* Lower CI	Adjusted* Upper CI	Adjusted* P value
Helicoblot bands								
116kDa & 89kDa	3.7	1.0	13.6	0.05	4.9	1.0	24.4	0.05

*adjusted for age, gender, ethnicity and other Helicoblot bands

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Hel[illegible] t 2.[illegible] s a [illegible] ative[illegible] ern blotting assay p[illegible] sly [illegible] y se[illegible] grou[illegible] investigators (Vilaichone et al., 2003; Rocha et al., 2004; Yang et al., 2006; Simán et al., 2007; Mitchell et al., 2008; Chomvarin et al., 2009) for assessment of *H. pylori* infection as well as serologic typing of the infecting strains. Having used this assay in our comparative study, we found the absolute majority of our study population to be *H. pylori* seropositive. Over ninety percent of whom were CagA (116kDa) seropositive in accordance with our previous genotyping studies on Iranian *H. pylori* strains (Talebkhan et al., 2008) as well as serology findings from other developing countries such as Northeastern Thailand (Chomvarin et al., 2009). In agreement with the latter study and others (Hoang et al., 2006), we found the least seropositivity toward the 35kDa protein.

Immunoreactivity to the above mentioned *H. pylori* proteins including high and low molecular weight proteins has been demonstrated to be associated with age, gender (Jaber, 2005) and ethnicity (Hoang et al., 2006; Epplein et al., 2011). We have, therefore, analyzed our data by multivariate logistic regression model adjusting for the three mentioned potential confounder and found

immunoreactivity towards *H. pylori* 35kDa (low molecular weight) protein drastically reduced the risk of GC by 60 percent. Accordingly, in a northeast Thai population, absence of reactivity to this protein in the presence of anti 19.5kDa protein was reported as a risk marker for gastric cancer (Chomvarin et al., 2009). Conversely, in another study from Thailand (Suriani et al., 2008) a risk inducing role was reported for *H. pylori* low molecular weight (35 and 37kDa) antigens in the development of gastrointestinal complications including gastric ulcers and cancer respectively. These low molecular weight antigens were also previously reported by other investigators (Yamaoka et al., 1998) to be closely associated with higher gastric mucosal IL-8 production and peptic ulcers. Considering the fact that the identity of the Helicoblot 35kDa protein is not defined, we have searched the potential matches for the *H. pylori* 35kDa (±0.5%) molecular weight proteins using Expasy Swiss Prot software and found 17 matches which mostly carboxyl and methyl transferases.

On the other hand, we found that joint possession of antibodies against CagA and VacA, in other words, infection with Tox-positive strains increases the risk of gastric cancer by nearly five folds compared to those harboring Tox- negative strains. This finding is supported by previous reports including those from Turkey (Sezikli et al., 2006), Italy (Suriani et al., 2008), Mexico (Garza-González et al., 2004) and Chile (Figueroa et al., 2002). Noteworthy is a recent report from highly prevalent countries like China recommend addition of other proteins like OMPs, HP0305, HyuA, and HpaA to CagA and VacA to the risk profile in order to increase the chance of detecting high risk individuals (Epplein et al., 2011). Similarly, a study on Lithuanian subjects with and without gastric cancer demonstrated that Tox-positive subjects remain at higher risk of gastric cancer, to which addition of *H. pylori* 94 and 30kDa increases the differential screening power (Janulaityte-Günther et al., 2007).

The advantages of non-invasive serologic screening methods as compared to the invasive biopsy-based detection methods include their simplicity, cost-effectiveness and subject compliance making them highly suitable for developing countries with limited resources. The advantages of *H. pylori* multiplex western blotting assays include their capacity to simultaneously identify antigen-specific antibody responses which can replace biopsy-based methods for virulence profiling of the infecting *H. pylori* strain. Hence, multiplex western blotting becomes a particularly attractive method for application in children (Ögünç et al., 2003; Treepongkaruna et al., 2006) and the elderly (Simán et al., 2005) population for whom endoscopy-based methods are not advised.

Evaluation of the efficiency of Helicoblot revealed an almost perfect agreement with *H. pylori* routine serum ELISA. Similarly, the detection criteria based on gold standard tissue-based methods demonstrated a high sensitivity and PPV but a low specificity and NPV which were mostly (9/10) contributed by gastric cancer patients. It is believed that the atrophic changes in the gastric mucosa disrupts the nutritious niche for *H. pylori* colonization (Fox and Wang, 2007), subsequent to which the bacteria is either lost or reduced to sub-threshold levels of detection. Hence the persistence of serum antibodies in gastric cancer patients may indicate past infections as also previously speculated (Rocha et al., 2004; Yang et al., 2006; Mitchell et al., 2008).

Therefore, an added advantage of the western blotting method is their capacity in detecting and typing of past infections which may eventually lead to more severe complications such as gastric cancer despite their lack of detection by biopsy-based methods which only detect current infections (Mitchell et al., 2008). Furthermore, serum anti-CagA antibodies due to their longer lasting nature are able to farther trace past infection and clarify false negative serologic results (Rocha et al., 2004; Simán et al., 2005; Ye et al., 2005) which may underestimate the associated cancer risk.

In conclusion, in this study *H. pylori* low molecular weight (35kDa) protein behaved as a protective marker, absence of which may increase gastric cancer risk. Conversely, joint possession of serum antibodies to *H. pylori* CagA (116kDa) and VacA (89kDa) proteins were identified as striking risks factor for gastric cancer. However, due to the high prevalence of CagA-positive *H. pylori* strains in our population, the application of CagA and VacA serologic typing for cancer-associated risk screening require further optimization. On the other hand, due to the low prevalence of low molecular weight antigens (<40%) amongst our population of infecting strains, screening *H. pylori* infected subjects for antibodies against 35kDa may help assign risk associated with gastric cancer in order to help recognize individuals in need of follow-up management. It is preferable, however, to re-evaluate this hypothesis in a case-control study where the control subjects would represent the source population from which the cases have risen, but the limitation of performing endoscopy (for tissue-based test) on the control group remains an ethical limitation.

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