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 et 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...
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%CO₂, 5%O₂, and 85%N₂) 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.

**Western blotting**

Western blotting of patient sera against *H. pylori* bacterial lystate 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.

**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 |            |             | <0.01    |
| <40        | 5 (5%)     | 64 (52.5%)  |          |
| 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  |            |             | <0.01    |
| Fars       | 21 (20.5%) | 51 (41.8%)  |          |
| Non-Fars   | 73 (71.6%) | 66 (54.1%)  |          |
| ND         | 8 (7.8%)   | 5 (4.1%)    |          |
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Table 2. Helicoblot Detection Criteria Based on Gold Standard Tissue-based Methods

| Helicoblot Interpretation | Tissue-based Tests | Detection Criteria (Confidence Interval) |
|---------------------------|-------------------|----------------------------------------|
| Negative                  | 3                 | 97% (0.91-0.99)                        |
| Positive                  | 10                | 23% (0.06-0.54)                        |
| Total                     | 13                | 92% (0.85-0.96)                        |

*Culture, histology and rapid urease test

Table 3. GC Risk Analysis using Logistic Regression Model

| Helicoblot bands | Crude OR Lower CI Upper CI p value Adjusted^* OR Lower CI Upper CI p value |
|------------------|--------------------------|--------------------------|--------------------------|
| 19.5kDa          | 19.5kDa                  |                          |                          |
| 30kDa            | 30kDa                    |                          |                          |
| 35kDa            | 35kDa                    |                          |                          |
| 37kDa            | 37kDa                    |                          |                          |
| 89kDa            | 89kDa                    |                          |                          |
| 116kDa           | 116kDa                   |                          |                          |

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

Table 4. Association between Seropositivity to CagA and VacA

| 89kDa (VacA) | Kappa (P value) |
|--------------|-----------------|
| Neg          | Pos             |
| 116kDa (CagA)| 116kDa          |

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

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

| CagA/VacA | Crude OR Lower CI Upper CI p value Adjusted^* OR Lower CI Upper CI p value |
|-----------|--------------------------|--------------------------|--------------------------|
| 116kDa & 89kDa | 116kDa & 89kDa |                          |                          |

Discussion

Helicoblot 2.1 kit is a qualitative western blotting assay previously used by several groups of 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 (Talebkhani 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; Eppelein 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 transfersases.

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 further 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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