Immunodominant antigens of *Helicobacter pylori* strains isolated from patients with different gastroduodenal diseases

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ABSTRACT

Objective: To detect the immunogenic proteins in *Helicobacter pylori* (*H. pylori*) strains isolated from patients with different gastric diseases.

Methods: We performed this study in the Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, during July 2003 to September 2004. Total proteins of *H. pylori* strains isolated from the gastric biopsies of 3 groups of patients were separated by 1D-SDS-PAGE and then blotted with the sera of their respective hosts.

Results: In SDS-PAGE the members of each group showed high correlation according to similarity in their patterns, resulting in considering them in the same cluster. The patterns of immunoblots differed from that of Coomassie Brilliant Blue stained gels. The blotting method did not recognize some of the protein bands in the SDS-PAGE. Only the bands of 106 and 45 kDa from *H. pylori* strains isolated from patients with gastric cancer were significantly ($p<0.05$) recognized specifically with the sera of their respective patients, and the band of 13 kDa was recognized specifically ($p<0.05$) with the sera of nonulceric patients. With the exception of these bands, in the patterns of blotting of the sera from all patients no significant differences were observed.

Conclusion: By using 1D blotting methods we could find 2 antigenic protein bands (106 and 45 kDa) for *H. pylori* strains isolated from cancerous patients, and one (13 kDa) for the strains isolated from nonulceric patients, which were specifically recognized with their respective host.

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Helicobacter pylori (*H. pylori*) is one of the most prevalent causes of infection in human beings worldwide. It persists in the human gastric mucus layer for decades and possibly for life, despite an antibody mediated immune response being produced, and causes different gastroduodenal diseases such as gastritis, peptic ulcer, and cancer.[1-4] Host, bacterial or environmental factors might determine the outcome of a long-term infection.[5-10] Additionally, the high genetic diversity and differences in expression of virulence factors between different strains of *H. pylori* might contribute to the outcome of infection.[11-13] With the increasing emergence of antibiotic-resistant *H. pylori* strains, development of an effective vaccine may represent an alternative means of controlling, or even preventing *H. pylori* infection.[14-16] Given the

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enormous heterogeneity of *H. pylori* strains, there is a great need for additional vaccine candidates conserved between strains and antigen of diagnostic value. To find these candidates, *H. pylori* specific serologic markers indicative for a clinical status should be determined, if they exist. The comparison of the antigen recognition patterns of sera from *H. pylori* infected patients that developed either one of the clinically divergent conditions of ulceric (Ul), nonulceric (Nul) or gastric cancer (GC) should be the most promising approach to identify such markers. The aim of the present study was to find immunogenic proteins in *H. pylori* strains isolated from different gastric diseases by immunoblotting with the sera of their respective hosts, thus providing information regarding potential protein antigens for use in serological diagnosis and vaccination.

Methods. ***Patients and Samples.*** A total of 144 patients undergoing endoscopy, from July 2003 to September 2004, at the Endoscopy Ward of Nemazee Hospital of Shiraz University of Medical Sciences in Shiraz, Southern Iran, were included in this study. The diagnosis of *H. pylori* infection and the confirmation of gastric disease by histology was established by a central study pathologist. From each patient, 2 samples from body and antrum were taken and transferred to the laboratory in transfer media (Brain heart infusion broth supplemented with 20% glucose). Blood samples from patients were obtained at the time of clinical diagnosis and sent to the laboratory to separate the sera. The sera were stored at -20^{O}C until further usage. General exclusion criteria for patients' recruitment to the study were previous attempts to eradicate *H. pylori*, use of antibiotics, proton pump inhibitors or bismuth compounds within the last 2 weeks before endoscopy, and previous gastric surgery.

Isolation of H. pylori strains. Biopsy samples from patients were gently homogenized and cultured on rapid urease test media and Brucella agar base (Merck, Germany) supplemented with 10% lysed horse blood and antibiotics of Amphotericin B (2 mg/L), Trimethoprim (5 mg/lit), and Nalidixic acid (10 mg/lit). The cultures were kept in a microaerophilic atmosphere (6% O_2, 7.1% CO_2, 7.1% H_2, 79.8% N_2) at 37^{O}C for 5-10 days. The samples were also evaluated for the presence of *H. pylori* by positive oxidase, catalase and rapid urease tests.

Whole cell proteins preparation. The *H. pylori* cells were collected from the surface of solid medium, and a suspension of 6×10^{10} colony forming unit (CFU)/ml was prepared in sterile phosphate buffer saline (PBS). This suspension was washed twice with ice-cold PBS (50 mM sodium phosphate, 0.15 M sodium chloride, pH 7.2) containing one mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Sigma, USA). The pellet was then suspended in extraction buffer consisting of 0.75% Tris, 2% sodium dodecyl sulfate, 5% dithiothreitol, 10% glycerol, and 0.1% bromophenol blue.[17] The homogenate was heated for 5 minutes in a boiling water bath and frozen at -20^{O}C until use.

SDS-PAGE and Immunoblotting. The fractions of whole cell proteins were separated by one-dimensional-Sodium Dodecyle Sulphate-Polyacrylamide Gel Electrophoresis (ID-SDS-PAGE) on Hoefer SE 600/SE 660 (Amersham Pharmacia Biosciences, San Francisco, USA) using a 10% gel prepared according to the method of Laemmli.[18] After electrophoresis, the gels were further processed in parallel by Coomassie Brilliant Blue G-250 staining or immunoblotting, according to the Sambrook method.[19] The stained gels were scanned and the molecular weights of the relevant bands were determined according to the protein molecular weight marker. For blotting, the proteins were transferred to polyvinylidene fluoride (PVD) membranes using a Semi-dry blotting system (Hoefer TE 77, Amersham Pharmacia Biosciences, San Francisco, USA). Blotting time was 2 hours at 1 mA/cm^2. After blocking for 45 minutes in PBS containing 2% Bovine Serum albumin at room temperature, the membranes were washed with PBS-T (PBS buffer containing 0.05% Tween-20) for 3-5 minutes. The membranes were then successively incubated at room temperature with patient's sera diluted 1/80 for 2 hours, peroxidase conjugated goat anti-human polyvalent IgG antiserum diluted 1/1000 for 2 hours. Washings between incubation periods were performed with PBS-T. After 3 washes, the blots were developed by incubation at room temperature in a solution of PBS containing 0.1% (wt/vol) 4-chloro-1-naphtol and 4-7 μl H_2O_2. The reaction was stopped by washing in distilled water. Blotting was performed for each *H. pylori* strain isolated from different patients with all patients' serum.

Results. ***Patients group.*** During the study period, 144 patients with different gastric diseases were enrolled. According to the pathology and clinical findings, the patients were categorized to 3 groups: Ul (n=37), Nul (n=77), and GC (n=30).

Helicobacter pylori isolation. Totally, from body and antrum of Ul patients 28 and 30, Nul patients 31 and 35, cancer cases 11 and 13 *H. pylori* were isolated. The presence of *H. pylori* organisms in these samples was also confirmed by pathology findings. Characteristics of the patients including their ages, genders and infection with *H. pylori* organisms are shown in **Table 1**.

Table 1 - Characteristics of patients whose biopsy samples and sera were used for this study.

Patients groups	Age range	Average age (SD)	No. of males	No. of females	No. of positive cases for *Helicobacter pylori*			
					Culture		Pathology	
					antrum	body	antrum	body
Nonulceric (n=77)	16 - 80	48 ± 15	32	45	35	31	37	31
Duodenal ulcer (n=37)	17 - 74	45.5 ± 14	27	10	30	28	31	30
Gastric ulcer (n=30)	29 - 75	52 ± 14	18	12	13	11	14	11

Protein profiles in SDS-PAGE. Protein profiles of different strains isolated from patients with different diseases, generated by denaturing polyacrylamide gel electrophoresis revealed some differences in expression pattern. The members of each group showed high correlation according to similarity in their patterns, resulting in considering them in the same cluster. **Figures 1a-1c** show protein patterns of some representative strains from 3 groups of the patients. The highest number of bands was observed in the cancer (20 bands) group, and the lowest was seen in the Nul group (14 bands). The bands specific for the members of each group were 106, 61.5, 45, 34 and 30 kDa for Ca, 22 kDa for Ul and 13 kDa for Nul group. No significant differences between the strains isolated from body and antrum of the same patient were observed.

Immunoblot analysis. In order to find an antigenic proteins candidate for a diagnostic test or vaccination, proteins of clinical isolates of *H. pylori* strains recognized by sera from their respective infected hosts suffering from either duodenal ulcer or GC were compared with those of the patients suffering from Nul gastric disease. The patterns of immunoblots (**Figures 2a-2c**) differed from that of Coomassie Brilliant Blue stained gels (**Figures 1a-1c**) and some of the protein bands in the SDS-PAGE were not recognized by the blotting method. Statistical analysis by Chi square test showed that only the bands of 106 and 45 kDa from *H. pylori* strains isolated from patients with GC were significantly ($p<0.05$) recognized specifically with the sera of their respective patients (80%), and the band of 13 kDa was recognized specifically ($p<0.05$) with the sera of Nul patients (84%). With the exception of these bands, in the patterns of blotting of sera from all patients no significant differences were observed. The bands of 160, 94, 63, 60, 58.5, 47, 44, and 14 kDa were the most common antigenic proteins among *H. pylori* strains, which were recognized commonly with the sera of their respective hosts.

Discussion. The gram negative bacterium *H. pylori* is a human pathogen which infects the gastric mucosa and causes an inflammatory process leading to gastritis, ulceration, and cancer.[1] Several studies have been carried out to find antibody responses to *H. pylori* proteins expressed in different gastric clinical status by probing immunoblots containing proteins of one *H. pylori* strain with serum from different patients.[20-25] Here, to find marker antigens correlated with specific manifestations of *H. pylori* associated diseases, we analyzed antigenic patterns of clinical *H. pylori* isolates obtained with the sera of their respective human hosts. Immunoblot analysis is highly sensitive and specific; it only reveals bands that are recognized by antibodies contained in the patient serum. In SDS-PAGE the protein pattern of the members of each group showed high correlation according to similarity in their patterns, resulting in considering them in the same cluster. The highest number of bands was observed in the cancer (20 bands) group, and the lowest was seen in the Nul group (14 bands). The bands specific for the members of each group were 106, 61.5, 45, 34 and 30 kDa for GC, 22 kDa for Ul, and 13 kDa for Nul group. Although some slight differences between the strains isolated from the body and antrum of the same patient were observed, there did not appear to be any significant difference in production of proteins that could be attributed to the location of *H. pylori* isolation from stomach. However, in the blotting, only the bands of 106 and 45 kDa from *H. pylori* strains isolated from patients with GC were significantly ($p<0.05$) recognized specifically with the sera of their respective patients (80%), and the band of 13 kDa was recognized specifically ($p<0.05$) with the sera of Nul patients (85%). With the exception of these bands, in the patterns of blotting of the sera from all patients there were not significant differences. This finding indicated some proteins in the SDS-PAGE of 3 groups of *H. pylori* strains were not immunogenic for their hosts. Therefore, 2 proteins of 106 and 45 kDa for GC strains and one protein of 13 kDa for Nul strains could be contributed to the pathogenic potential of *H. pylori* strains in each category of diseases. Using 2D immunoblotting, Kimmel et al,[23]

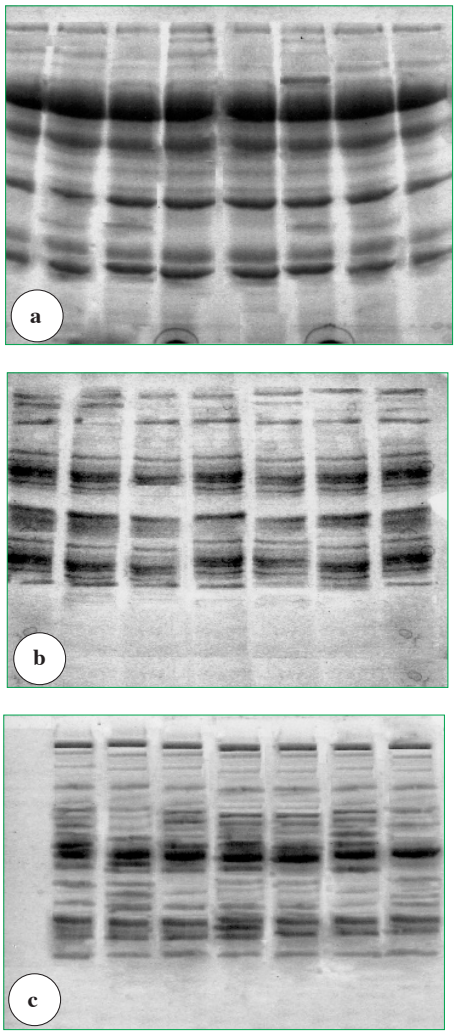

Figure 1 - One-dimensional-Sodium Dodecyle Sulphate-Polyacrylamide Gel Electrophoresis of total protein from 7 representative *Helicobacter pylori* strains isolated from patients suffering from **a)** nonulceric gastric disease **b)** duodenal ulcer and **c)** gastric cancer.

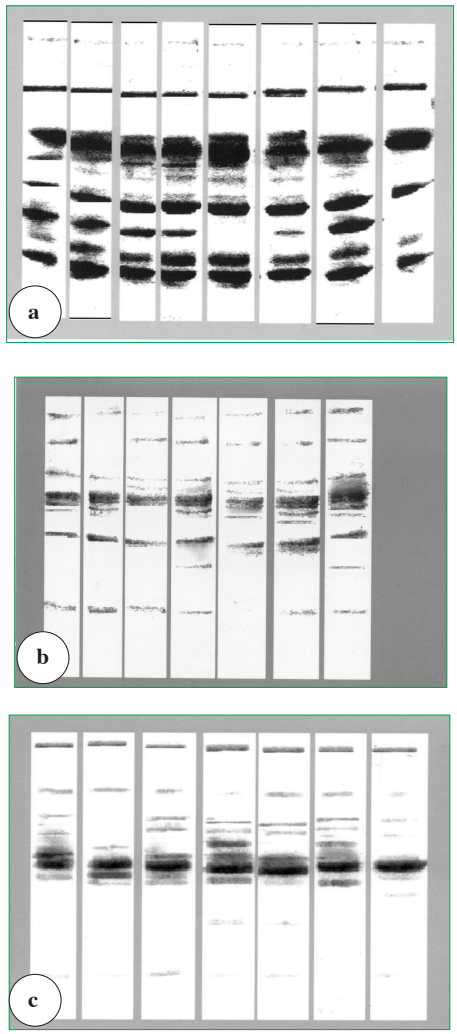

Figure 2 - Antigenic proteins of clinical isolates of *Helicobacter pylori* strains recognized by sera from their respective infected hosts suffering from **a)** nonulceric gastric disease, **b)** duodenal ulcer, and **c)** gastric cancer.

did not find association of *H. pylori* G27 antigens with antibodies in patients with particular gastroduodenal pathologies. While, another study has reported the recognition of 14 antigenic protein spots, which differed significantly between GC and Ul diseases.[22] To determine the exact role of these proteins in giving specific virulence traits to *H. pylori* strains, accurate in vivo and in vitro studies are required. However, most of the immunogenic proteins proved to be housekeeping proteins.[24] The fact that the detected immunodominant proteins represent mainly housekeeping functions may indicate a general limitation, since only proteins that are abundant in the cell could be detected. Furthermore, the scope of specific antigens detectable by 1D SDS-PAGE method is restricted by the fact that the proteins with different function or properties, but similar molecular weights line up in a same single band. For example, antibodies against virulence and housekeeper proteins with similar molecular sizes detect only one band in blotting for different bacterial strains. These limitations might partially be overcome by using 2D- SDS-PAGE or IEF methods.

Although the immunoblotting technique proved to be a useful approach for the identification of immunogenic proteins, the detection of marker antigens correlated with a particular gastroduodenal clinical manifestation could have failed for several reasons. 1. Host immune response: in some cases due to immunodeficiency statue, host immune system can not respond to some of the bacterial antigenic

proteins and so, no hybridization will be seen in blotting. 2. Genetic factors: it has been assumed that genetic factors of both the bacterium and the host can affect the clinical outcome of *H. pylori* infection. 3. Expression of virulence factors: in vitro culture conditions can suppress the expression of some virulence factors, which is depended on certain in vivo stimuli. 4. Age at infection and their duration: on average GC patients are older than Ul patients and hence may have carried *H. pylori* for longer periods of their life (**Table 1**).

In conclusion, by using 1D blotting methods we could find 2 antigenic protein bands (106 and 45 kDa) in *H. pylori* strains isolated from cancerous patients and one (13 kDa) in the strains isolated from Nul patients, which were specifically recognized with their respective host. Further studies will have to show if the antigens described here are of predictive value for certain clinical states.

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