

IgG IMMUNE RESPONSES TO DIFFERENT PROTEINS OF HELICOBACTER PYLORI AS DEFINED BY IMMUNOBLOT ASSAY

M. Raeiszadeh¹, A Massoud¹, F. Siavoshi² and R. Malekzadeh³

(1) Department of Immunology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran

(2) Department of Microbiology, Faculty of Science, Tehran University, Tehran, Iran

(3) Digestive Diseases Research Center, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran

ABSTRACT - *Helicobacter pylori* (H.pylori) is an etiologic factor for chronic gastritis and peptic ulcers. Serological testing of H.pylori infection is common in Iran, as other parts of the world. There are geographical variations in the humoral immune response to various H.pylori strains in different parts of the world. We studied the immunogenic proteins of H.pylori by means of an Immunoblot assay with antigens of H.pylori strains isolated in Iran. Sera of 64 patients suffering from dyspepsia were analyzed to determine antibodies which were good marker of infection and the antibody patterns associated with peptic ulcer. 54 out of 64 dyspeptic patients were infected by H.pylori based on positive culture or positive results of both rapid urease test and direct examination. 14 out of fifty-four had peptic ulcers and the rest were categorized as patients with non-ulcer dyspepsia. Some of them had multiple erosions in the gut or duodenum. Twenty-two major bands were identified by Immunoblot. Of these, IgG antibodies against 10 proteins were significantly more frequent in sera of the infected patients, and they produced immunoreactive bands at 14, 16, 22, 26, 32, 35, 44, 87, 92, 120 KDa. Antibody patterns were not identical in the patients. The presence of at least one band at 14, 16, 22, 26, 32, 35 KDa was the best marker of infection (sensitivity, 90% and specificity, 80%). Major serological cross reactions were found at moderate molecular weight bands (50, 52, 54, 60, 66 KDa). The presence of antibodies to 120 KDa protein (Cag A) and 87 KDa protein (Vac A) were not associated with the presence of peptic ulcers. These were in contradiction to results obtained across Europe and U.S. but in agreement with Asian studies. However the presence of at least one band at either 32 or 35 KDa was more frequent in the sera of peptic ulcer patients and non-ulcer dyspeptic patients with erosions ($P < 0.05$). These results could be applicable to design new serological kits in Iran and could also be used to identify new putative virulence factors for H. pylori.

Acta Medica Iranica 38 (2): 92-99 ; 2000

Key Words: H.pylori, immunoblot, peptic ulcer, nonulcer dyspepsia, IgG response

INTRODUCTION

Helicobacter pylori (H.pylori) organisms are gram negative bacteria that cause gastritis and have a major role in the pathogenesis of peptic ulcers and gastric cancers (1). These bacteria infect 70-90% of humans in developing countries (2) and 85-90% of the male population in Iran (3). H.pylori infections are diagnosed by means of invasive (direct) and noninvasive (indirect) methods (4). The invasive methods include urease tests, histology, culture and polymerase chain reaction (PCR) technique. The noninvasive methods obviate the need for endoscopy and include serology and urea breath test. Serological tests which are rapid and easy to perform, have been used in epidemiological studies and have been recommended for initial pre-endoscopy or pre-treatment screening in dyspeptic patients. These tests are, mostly, enzyme immunoassays (EIAs) with several different antigenic preparations (4,5). Initially, crude sonicates and supernatants of ultracentrifuged lysates of H.pylori were used as antigens of solid phase for EIA. The sensitivities of these kits were remarkably good, but they lacked high specificity due to antigenic cross reactivity with other bacterial species (6). In recent years, new generations of EIA kits with optimal specificities have been produced. More purified antigens such as acid glycine extract, urease and 120 KDa protein (Cytotoxin associated gene A, Cag A) have been used in the new kits (5). However there is no consensus regarding the best antigenic preparation to be used for H.pylori serology (7).

Immunoblot assay provides a good opportunity to search antibody reactivity against different proteins of a complex antigen. This method has been used to identify antigens of H.pylori which are markers of infection. Also, by means of this method, antigens which associate with peptic ulcers and gastric cancers were identified (8,9). Some of the most important antigens of H.pylori

with identified functions are Vac A (vacuolating cytotoxin A, 87 KDa)(10), HspB (Heat shock protein B, 60KDa), HspA (13 KDa) (11), two subunits of urease: Ure A(30 KDa) and Ure B (66 KDa) (12), a 29 KDa protein specific to the flagella sheath (13), outer membrane proteins range from 31 to 80 KDa(14) Hp - NAP (H.pylori neutrophil activating protein, 150 KDa) (15), a 51 KDa flagellin protein (16), CagA protein acts as a pathogenic marker of H.pylori. The function of this protein (with molecular weight range from 110 to 140 KDa)(17) had not been identified, but it is strongly associated with the presence of peptic ulcers and gastric cancers.

There is no published data about antibody responses of Iranian dyspeptic patients to antigens of H.pylori. It is important to know as to which antigens have the least cross reactivity and thus must be considered in the use of serological tests. By using an Immunoblot (Western blot) technique, we evaluated the frequencies of the IgG antibodies to major antigens of H.pylori in the sera of 64 clinically documented patients. We sought to determine the antibodies which are the best markers of infection and the antibody patterns associated with the presence of peptic ulcers.

MATERIALS AND METHODS

Patients : A total of 64 consecutive patients (26 males and 38 females) examined in the Endoscopy Division, Dr. Shariati Hospital were included in the study in 1998. The median ages were 50 years (range, 25 to 74 years) and 44 years (range, 13 to 70 years) for males and females, respectively. The patients presented with dyspeptic syndrome and underwent an upper gastroduodenal endoscopy with multiple antral biopsies. They had received neither antimicrobial nor antacid therapies during the previous 3 months. The presence of an ulcer was noted during the endoscopic-examination. Sera were collected on the day of the endoscopy, were aliquoted and frozen at -20 °C.

Bacteriology : One of the biopsies from each patient was tested for rapid urease reactions (Chemenzyme, Iran). Urease reaction were recorded after 1 h of incubation at 37 °C. Another biopsy specimen was placed into sterile 0.15 M NaCl solution and transported to the laboratory (Dept. of Microbiology, Faculty of Science) within 2h. Each biopsy specimen was grounded and inoculated into a selective medium, consisting of brucella blood agar (as base), 1% starch, 10% sheep blood, Vancomycin 1 mg/L, Polymixin B 0.25 mg/L, Amphotericin B 2mg/L, and Trimethoprim 5mg/L. The plates were incubated at 37 °C under microaerobic condition (5% CO₂) for 4 days. Colonies were identified as H.pylori by morphology, urease production, positive catalase and

oxidase reaction. A part of the ground specimen was smeared and Gram stained for direct examination of spiral bacteria.

H.pylori strains and antigen preparation

H.pylori ATCC 43504 was used as the reference strain. This strain produced Vac A and Cag A proteins. 9 isolated strains from patients and the reference strain were separately cultured in brucella broth with 5% fetal bovine serum (Sigma, US) at 37°C under microaerobic condition for 48 h. Bacterial cells were sedimented by centrifugation (4,000 xg for 20 min). The pellets of strains were resuspended in sterile water.

The 9 isolated strains were pooled and reached to a concentration of 1 g (wet weight) per ml of sterile water. Whole cell preparations were broken by sonication at 10 micro amplitude for 20 s. This process was repeated 3 times. The preparations were cooled during sonication by immersion in ice water. The sonicated suspensions were centrifuged (10000 × g for 20 min) and the supernatants were collected and protein concentration determined.

SDS-PAGE : By using a vertical electrophoresis equipment (Akhtarian, Iran) we performed sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS - PAGE) of bacterial extracts under reduction condition, as described by Laemmli (18). Proteins were separated in a 10% gel with a 5% stacking gel. Prior to electrophoresis, the extracts (2 mg/ml) were diluted with equal volumes of 2X SDS sample buffer (0.5 M Tris - HCl [pH 6.8], 1% bromophenol blue, 20% glycerol, 4% SDS, 2% 2-mercaptoethanol) and the mixture heated at 90 °C for 5 min. After cooling, the proteins were loaded onto the gel and were separated at 110 V until the bromophenol blue dye migrated out of the gel. Molecular size standards (Sigma) that included proteins ranging from 29 KDa to 205 KDa were treated similarly and loaded onto each gel.

Relative molecular weight of proteins were determined with the calibration curve. The method was reproducible and the coefficients of variation (i.e., the ratios of standard deviation (SD) to means) of the migration distances for molecular size standards were <12%.

Coomassie brilliant blue staining was performed as described by Weber and Osborne (19). Silver staining was performed using a silver kit (Pharmacia - Biotech, Sweden).

Immunoblot assay after SDS PAGE

The proteins were transported electrophoretically from polyacrylamide gel to prewetted Immobilon nitrocellulose membranes (Millipore, Bedford) by using a tank blotting apparatus (Aktarian). The wet transfer was performed for 16h under a constant current of 15 V in Tris - hydrochloride (25mM) - glycine (0.192 M, pH 8.4) containing-methanol (4.9 M). Following the protein transfer, the membranes were cut

into strips. The strip corresponding to the molecular size standard was stained with Ponceau red (Sigma) and kept for calibration purposes. The remaining binding sites on the strips were blocked by incubation with 0.1% Tween 20 (Sigma) in Tris -buffered saline (TBS) (pH 7.5) for 1 h. The blot strips were then incubated for 1h at 20°C with the patient's sera diluted 1: 150 in 0.1% Tween 20-TBS. The strips were then washed three times in 0.1% Tween 20-TBS and then incubated for 1h at 20°C with alkaline phosphatase conjugated goat anti human IgG (Zymed, US), diluted 1:3000 in the above mentioned buffer. The washing was repeated as described above and the strips were incubated in the solution contained 5-bromo-4-chloro-3-indolylphosphate (BCIP)(Sigma) as the substrate and nitroblue tetrazolium (Sigma) as the chromogenic indicator. The reactions were stopped after 30 min by washing the strips thoroughly with water.

Statistics: Fisher's exact probability test was used to evaluate the results.

RESULTS

Status of patients: Infected patients were identified as patients who were positive by culture of *H. pylori* or patients who were positive for spiral bacteria by direct examination of biopsy specimens and who were negative by culture, direct examination and rapid urease test. 54 (84%) of 64 patients were infected by *H. pylori*. Among the infected patients, 14 had peptic ulcers (12 patients with duodenal ulcers and two with gastric ulcers). The rest were categorized as the patients with non ulcer dyspepsia (NUD). The endoscopic findings in the NUD patients were patchy antral or duodenal erythema. 12 out of 40 patients with NUD had multiple erosions in the antrum and deodenum. These patients were categorized with peptic ulcer patients in the one group.

Among the ten noninfected patients, eight were females and only one patient (male) had gastric ulcer (Table 1)

SDS - PAGE results: a) Coomassie brilliant blue staining revealed 17 bands from extracts of pooled strains and 12 bands from ATCC 43504.

The molecular weight of bands were 14, 18, 32, 35, 44, 54, 61, 66, 74, 80, 87, 96, 110, 115, 120 and 140 KDa and 18, 28, 45, 48, 55, 60, 66, 75, 87, 97, 110 and 150 KDa for isolated strain and ATCC 43504, respectively (Fig. 1. b) Silver staining method had high sensitivity and thus more band were identified. The proteins of isolated strains included :14, 18, 22, 24, 26, 29, 32, 35, 40, 44, 48, 52, 54, 60, 66, 74, 87, 96, 110, 120, 130, 135, 150 and 210 KDa and the proteins of ATCC 43504 were 18, 22, 24, 26, 29, 31, 32, 42, 48, 52, 55, 60, 66, 75, 87, 90, 97, 100, 110, 125, 135, 150 and

210 KDa (Fig. 2).

Immunoblot assay and IgG responses : Sera from infected patients tested by Immunoblot technique with extract of isolated strains revealed 5 to 19 bands (average 10 bands), while sera from noninfected patients revealed 0 to 15 bands (average 6 bands). A set of blots with sera from infected and noninfected patients is shown in Figure. 3.

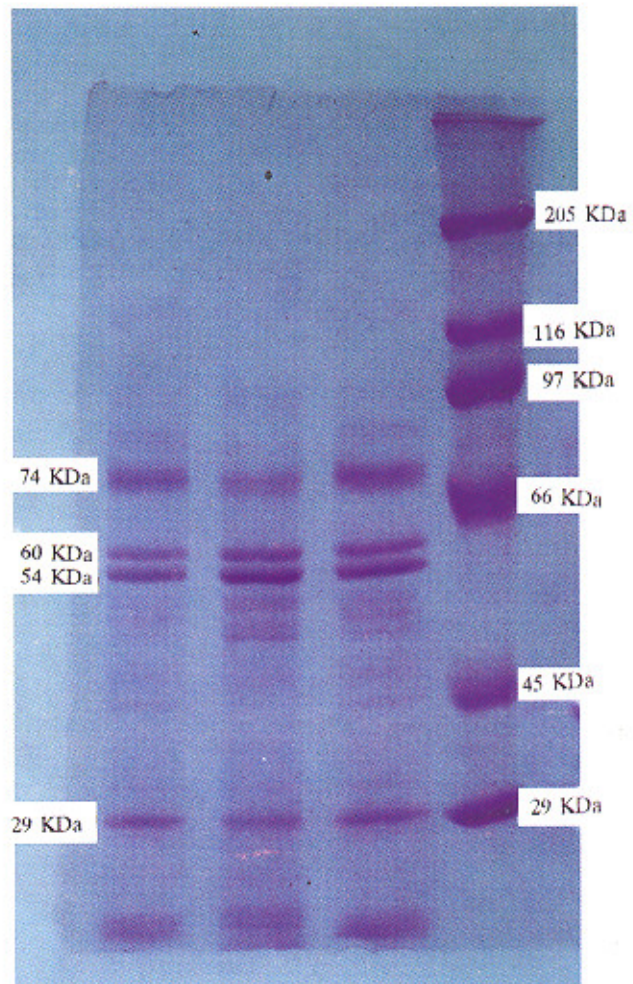


Fig. 1. Protein profile of sonicated strains of *H. pylori* have been revealed by coomassie brilliant blue staining. Lane 1 and 3, repeat of same sample (pooled of isolated strains from Iran). Lane 2, ATCC 43504 and lane 4, molecular weight standards (include: from up to down: 205, 116, 97, 66, 45, 29 KDa proteins). The protein profile of *H. pylori* strains is very similar and major bands of them include: 74 for ATCC 43504) 60, 54, 29 KDa. Some of the minor bands (e.g: 120 KDa) are not obvious in the Figure.

Twenty - two different bands were identified on the 64 blots. Three bands out of 22, (60, 66, 74 KDa) presented in the blots of the majority of the

patients (Table 2). These bands and the nine other bands (18, 20, 30, 40, 50, 52, 54, 82, 95 KDa) were disregarded because their frequencies were not significantly different in the infected and noninfected patients (Fisher's exact test $P > 0.05$). The frequencies of 10 remaining bands were statistically different in the infected and noninfected patients (Fisher's exact test $P < 0.05$) (Table 3).

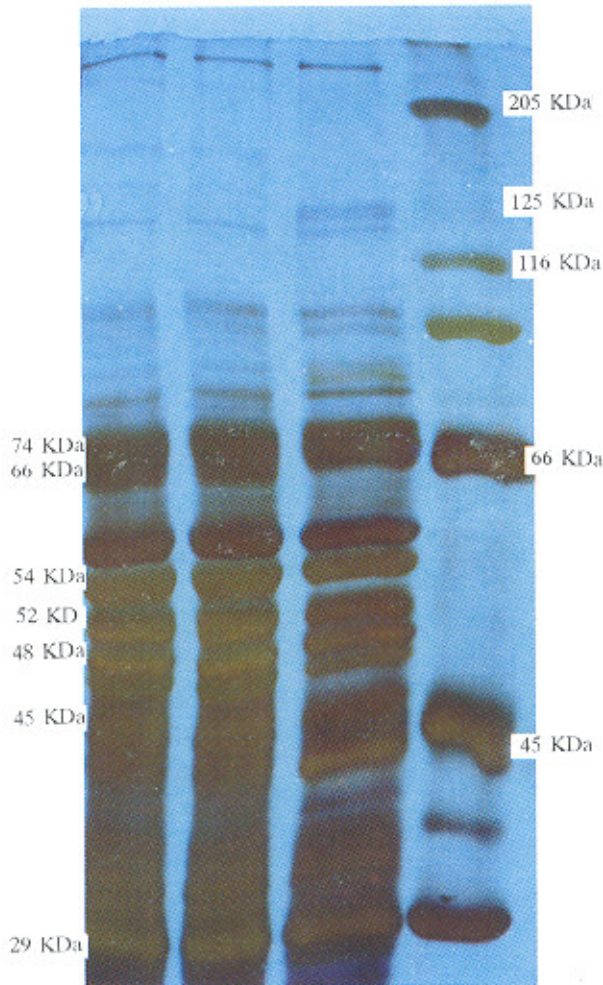


Fig. 2. Protein profile of sonicated strains of *H. pylori* as have been revealed by Silver-staining. Lane 2, repeat of same sample (ATCC 43504); Lane 3, pooled isolated strains from Iran, Lane 4, molecular weight standards (include: from up to down: 205, 116, 97, 66, 45, 29 KDa proteins). 125 KDa band is one of the minor bands some of the major bands are: 74, 66, 60, 54, 52, 48, 45, 29 KDa proteins.

We determined specificity, sensitivity, positive predictive value, negative predictive value and efficiency for the presence of each band.

In this regard; results of culture, direct examination

and urease test were used as gold standard. The best results for both specificity and sensitivity were obtained with bands at 44 and 87 KDa. The best efficiency (the efficiency is the percentage of patients correctly classified as colonized or noncolonized by the presence or the absence of the specific band) for the presence of one band was obtained with a band at 87 KDa (80%) (Table 3).

The presence of at least one immunoreactive band at 14, 16, 22, 26, 32, 35 KDa, predicts colonization with 90% sensitivity, 80% specificity and 89% efficiency. The presence of at least one band at 87, 92, 120 KDa, predicts infection with 85% sensitivity, 70% specificity and 83% efficiency.

Peptic ulcers and antibody patterns: We sought to determine whether the presence of ulcers could be correlated with the presence of certain antibody patterns. We evaluated the frequencies of the ten antibodies (listed in the Table 3) in the patients with peptic ulcers and patients with NUD. We failed to show any relationships between the presence of IgG against pathogenic markers of *H. pylori* including 87 KDa (Vac A) and 120 KDa (Cag A) antigens.

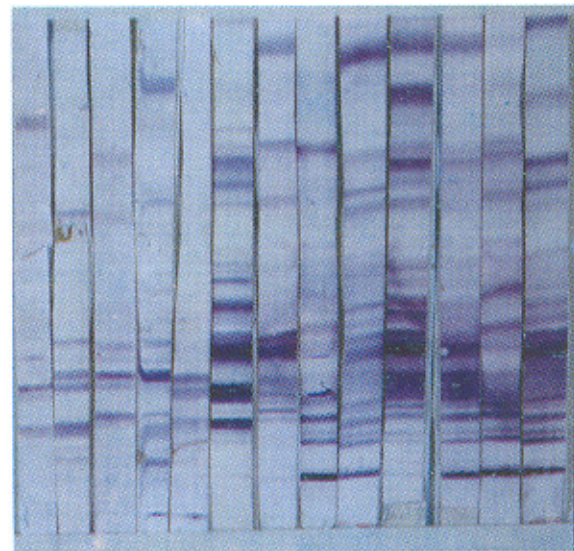


Fig. 3. Immunoblot patterns obtained with a sonicated extract from isolated strains of *H. pylori* in Iran and with 8 sera from patients infected with *H. pylori* (Lanes 1 to 8) and with 5 sera from noninfected patients (Lanes 9 to 13). Molecular masses are indicated on the left. The number of immunoreactive bands with sera of infected patients are more than the bands with sera of noninfected patients. Bands at 120 KDa are obvious in lanes 1,2,3,5,6 and bands at 35 KDa are seen in Lanes 4,5.

Table 1. Characteristics of 64 patients with dyspepsia

Endoscopic diagnosis	Infected patients ^a (No. 54)		Noninfected patients (No.:10)	
	male	female	male	female
	Peptic ulcer	10	4	1
Non-ulcer dyspepsia with erosions	6	6	-	-
Non-ulcer dyspepsia without erosions	8	20	1	8
Total	24	30	2	8

a: Based on urease assay, direct examination or culture.

Table 2. Frequencies of 12 IgG antibodies to H. pylori in 64 human sera.

Immunoreactive band (KDa)	No. of reacting sera from	
	infected patients ^a (n=54)	noninfected patients ³ (n=10)
95	35	3
74	54	8
66	46	7
60	53	8
54	35	4
-	32	6
50	29	4
40	19	2
30	32	5
20	7	1
18	19	3

The presence of these antibodies were not different between infected and noninfected patients (Fisher's exact test, P> 0.05)

a: Based on urease assay, direct examination or culture.

Table 3. Frequencies of 10 IgG antibodies^a to H.pylori in 64 human sera and their abilities to predict H.pylori infection

Immunoreactive band (KDa)	No. of reacting sera from		Sensitivity ^b	specificity ^b	Positive ^b predictive value	Negative ^b predictive value	Efficiency ^b
	infected patients (n = 54)	noninfected patients (n = 10)					
120	38	3	70	70	92	30	70
92	37	3	69	70	92	29	69
87	44	3	81	70	93	41	80
44	39	2	72	80	95	35	73
35	24	0	44	100	100	25	53
32	25	0	46	100	100	25	54
26	30	1	56	90	97	27	61
22	22	0	41	100	100	24	50
16	16	0	30	100	100	21	41
14	30	1	56	90	97	27	61

a : The antibodies against the 10 antigens listed were significantly more frequent in the infected patients than in the noninfected patients (Fisher's exact test P<0.05)

b : These parameters were calculated based on the results of culture, direct examination and rapid urease test as gold standard for definition of true infected and noninfected patients.

Table 4. Number of H.pylori infected patients^a with at least one IgG antibody^b against either 32 or 35 KDa antigens and ability of the antibody to predict peptic ulcers

Patients	No. (%) of patients	Efficiency with antibodies
Peptic ulcer and non-ulcer dyspepsia with erosions	20 (77)	(62)
non-ulcer dyspepsia without erosions	14 (50)	

a: Based on urease assay, direct examination or culture.

b: These antibodies were significantly more frequent in the sera of peptic patients with ulcers and non-ulcer dyspeptic patients with erosions (Fisher's exact test; P<0.05)

We used ATCC 43504 as a source of antigens to confirm the results obtained with 120 KDa and 87 KDa antigens. We carried out Immunoblot assay with some selected sera showing immunoreactive bands at 87 KDa and 120 KDa and also negative sera these bands. All the selected sera for having antibodies to 87 KDa and 120KDa antigens, showed an immunoreactive bands at 135 KDa (Cag A) and at 87 KDa (VacA), with the extract from ATCC 43504.

Conversly, the 87 and 135 KDa bands were absent when the negative sera were tested by Immunoblot assay with ATCC 43504.

Among the other antibodies, only the presence of at least one immunoreactive band at either 32 or 35 KDa was more frequent in the sera of peptic ulcer patients and NUD patients with multiple erosions (P < 0.05)

(Table 4).

DISCUSSION

H. pylori possesses a number of antigens inducing an immune response in infected persons. Application of Immunoblot technique to identify antigenic proteins of *H. pylori* has begun since 1985 (20). So far, many papers have been published and at present Immunoblot kits are commercially available. Variation in methodology and the use of different bacterial strains have led to some differences in the reported molecular weight for antigenic proteins. Of course, a general agreement can not be reached unless these proteins have been purified and their functions characterized. By means of bacterial sonication, we studied superficial and released proteins from Iranian strains of *H. pylori* which included the most important antigens of *H. pylori*, in regard to a standard strain. In Iran, there is only one other study that has searched antigens of *H. pylori* (21). In that study, the antigens were identified by means of pooled positive sera from infected patients as : 20, 27, 29, 32, 35, 54, 60, 66, 75, 85, 92, 115, 170, 220 KDa. Some of these antigens have reacted with pooled negative sera, too. (75,66,60,54,32 KDa). These results are similar to our findings. Some, but not all, of our results are in agreement with previous works (6,22,23,24). Andersen and coworkers have found strong correlation between *H. pylori* infection and the presence of IgG antibodies to 19, 25, 27, 30, 36, 47, 50, 56, 60, 75, 120 KDa antigens (22). They have found polypeptides with molecular weights between 19 and 36 KDa as the most specific proteins for the diagnosis of *H. pylori* infection. This is in agreement with our results. Faulde and coworkers have showed the presence of IgG to 19,25,30,67,75,90,120,180 KDa antigens in the sera of infected patients (23). Nilsson and coworkers have found seven antigens of 26,29,30,31,33 and 110 to 120 KDa as the most immunogenic proteins during *H. pylori* infection (6). Fauchere and coworkers have showed IgG antibodies against twelve antigens as significantly more frequent in sera from colonized patients. Those antigens include 14, 16, 30, 35, 42, 46, 48, 54, 66, 74, 87, 125 KDa proteins (24).

Cross - reactive antigens in this study were the medium size proteins (50-66 KDa), since these antigens reacted with sera from non *H. pylori* infected patients. This finding is nearly in agreement with previous data (6,22,23,25). We didnot determine the characteristics of cross-reactive proteins, but previous data have revealed some of them as cross -reacting flagellin (66 KDa) and flagellar sheat protein (51 KDa). The specificity of high (87 to 120 KDa) and low molecular weight proteins (14 - 44 KDa) in the serodiagnosis of *H. pylori* infection confirmed in our study as well in most of other studies

(6,21,22,24). However, it is important to mention that immunoblot findings of different authors are not identical. This fact may be due to both the diversity of the technical conditions and the use of different strains as the source of antigens. There are also ethnic differences in the immune response (26) and this may lead to variation in the profile of antibody response against *H. pylori*. The present work is the first step to identify immunogenic proteins from Iranian strains of *H. pylori*. Undoubtedly, complete identification of these antigens is very important for the preparation of serodiagnostic kits (such as ELISA kits) and the production of related vaccines. *H. pylori* infection can lead to a variety of diseases. All of the *H. pylori* strains are not pathogenic. Pathogenic strains of *H. pylori* have specific virulence determinants that the outcome of infection. To date, *H. pylori* studies have primarily focused on two groups of potential bacterial virulence factors including the Cag pathogenicity island (for which Cag A is a marker) and the vacuolating cytotoxin, Vac A (27,28). Strains that possess the Cag pathogenicity island are associated with increased interleukin - 8 (IL - 8) production (28), with peptic ulceration (8,17) and with gastric cancer (9). In Asia (Japan, Korea, China) (29,30) and in new studies from the United States (31) Cag A - gene positive, vacuolating cytotoxin - producing strains are the predominant type circulating and the prevalence of these factors is similar among those with asymptomatic gastritis or peptic ulcer. Our findings, also confirmed the Asian results. We didnot find any correlation between the presence of antibodies (IgG) to 120 KDa (Cag A) or 87 KDa (Vac A) and presence of peptic ulcer. One of us (S. Massarat) in another study has obtained similar findings about the association of anti Cag A antibody and peptic ulcer in Iran (unpublished data). Thus new data have shown that the presence of these antigens have not lived up to hope that they would provide a reliable marker predicting outcome of an infection. Nevertheless, antibodies to other antigens may be better markers for predicting severe diseases. In the present study, we found that the presence of at least one antibody to either 32 or 35 KDa were more frequent in the sera of patients with peptic ulcer and non- ulcer dyspeptic patients with multiple erosions. The correlation between the presence of IgG against 35 or 33 KDa has been reported in the recently published papers (24,32,33). Fauchere has showed that the anti - 35 KDa antibody is the best marker of ulcer and predicts, with 66% efficiency, predisposition to ulcers. In our study, this efficiency was 62%. Lamarque et al found higher rate for seroreactivity against Cag A and 35KDa antigens in European and African patients with peptic ulcers(32).

In Asia, it seems there is only association between the presence of antibody to antigens in the range of 33 (perhaps our 32 KDa) - 35 KDa and increased levels of

IL - 8 and the presence of peptic ulcers (33). The current study can not answer whether our 32 - 35 KDa antigens are identical to 33 or 35 KDa antigens reported by three other studies or not. This remains unanswered until purification of these antigens are done. In conclusion, the failure of the original observations of an association between Gag A and Vac A and outcome of infection has led to a search for new putative virulence factors and 32 - 35 KDa antigens are potential candidates.

Acknowledgements

We are grateful to Dr. M. Gorgani (Institute of Biochemistry and Biophysics, Tehran University) for providing the reference strain of *H. pylori* and helpful technical advice. We also thank Mr. M. Hashemi for isolating of *H. pylori* strains from biopsy samples.

This study was supported by grants from Tehran University of Medical Sciences.

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