

# Discrimination of Dominant *H. pylori* Strains Isolated from Patients with Different Gastroduodenal Pathologies by Protein Profiling

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## ABSTRACT

It is not clear what factors determine divergent outcomes of infections caused by *H. pylori*. In the present study, the protein profiles of different strains of *H. pylori*, isolated from three groups of patients with ulcerative disease, non-ulcerative gastritis and cancer disease, were analyzed using 1D-SDS-PAGE. The patterns of different *H. pylori* strains were highly divergent. About 30.76% (7 bands) of the 26 observed protein bands were common in all strains isolated from 3 groups of the patients. While the similarity for the strains inside each group were 75% (15 from 20), 76.47% (13 from 17) and 78.57% (11 from 14) for cancerous, ulcerative and nonulcerative group, respectively. Some of the observed bands were significantly specific for each group. Therefore, we speculated that some *H. pylori* strains might be more associated with a specific disease than others, giving the clustering of some, but not all, strains within each disease group. In conclusion, this study showed that protein profile can be a characteristic in discrimination of dominant strains in different gastric clinical status. Specific and dominant proteins of different strains isolated from three groups of patients under study were candidates for further exploration for laboratory tests, which analyze disease-specific *H. pylori* strains, and for diagnosis of the different diseases and outcomes associated with this widespread bacterium. *Iran. Biomed. J.* 10 (2): 85-91, 2006

Keywords: *Helicobacter pylori*, SDS-PAGE, Protein profile

## INTRODUCTION

*Helicobacter pylori*, presumably the widespread agent causing chronic bacterial infection in humans [1], is a major cause of inflammations leading to dyspepsia, duodenal or gastric cancer (GC) or gastric mucosa associated lymphoid tissue lymphoma [2]. In 1994, the WHO declared *H. pylori* to be a definitive carcinogen [2]. It is estimated that 90-95% of duodenal ulcers (Du) in Europe originates from a *H. pylori* infection [3]. Infection is also associated with a 2.7-12 fold increased risk of developing GC. Despite the fact that *H. pylori* is a common pathogenic factor for Du and GC, both diseases appear to be clinically divergent, i.e., Du patients face a lower risk to

develop GC than the normal population [4, 5].

Host, bacterial or environmental factors might determine the outcome of a long-term infection. Strain diversity among *H. pylori* isolates from different patient groups has been extensively studied [6-10]. Whether there exist good or bad strains of *H. pylori* is still under discussion [11]. Several genetic markers of pathogenic characteristics for different *H. pylori* strains have been described [12]. Polyacrylamide gel electrophoresis (SDS-PAGE) is a laboratory technique with a wide variety of applications for a range of bacterial infections. It can be used to link certain gel patterns to specific diseases or modification of specific marker proteins. This technique can also be applied to answer molecular

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epidemiological question in medical microbiology [13].

The aim of our study was to discriminate *H. pylori* strains isolated from patients with cancer disease (CD), ulcerative disease (UD), and non-ulcerative disease (NUD), according to their protein profiles provided by SDS-PAGE method. We also searched for disease-specific protein bands, protein marker candidates, which could be useful for *H. pylori* strain characterization.

## MATERIALS AND METHODS

**Patients.** A total of 144 patients undergoing endoscopy, from July 2003 to September 2004, at Endoscopy Ward of Nemazi Hospital of Shiraz University of Medical Sciences (Shiraz, Iran) were included in this study. The diagnosis of *H. pylori* infection and the confirmation of gastric disease by histology were established by a central study pathologist. From each patient, 2 samples from body and antrum were taken and transferred to the lab in transfer media (Brain heart infusion broth supplemented with 20% glucose). 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 prior to endoscopy, and previous gastric surgery. Characteristics such as age, job and sex of the patients were recorded.

**Cultivation of *H. pylori*.** 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/lit), trimethoprim (5 mg/lit) and nalidixic acid (10 mg/lit). The cultures were kept in a microaerophilic atmosphere (6% O<sub>2</sub>, 7.1% CO<sub>2</sub>, 7.1% H<sub>2</sub>, 79.8% N<sub>2</sub>) (ANOXOMAT Mark II, Mart Microbiology BV, Netherlands) at 37°C for 5-10 days. The samples were also evaluated for presence of *H. pylori* by positive oxidase, catalase, rapid urease tests and direct gram staining.

**PCR for confirmation of *H. pylori* isolates.** DNA of *H. pylori* isolates was extracted using phenol chloroform protocol. A previously established Multiplex PCR method was performed to confirm all isolates as *H. pylori* [14]. Briefly, 10 µl of each DNA sample was suspended in 40 µl of a reaction mixture containing 50 µl comprising 50 pmol each

primer, 10 µL of chromosomal DNA extracted from *H. pylori* isolates, 2 U of Taq DNA polymerase, 0.2 mM deoxynucleoside triphosphates and 2 mM MgCl<sub>2</sub> in a gradient thermal cycler (Eppendorf, Germany). Two sets of primer (TIB MOLBIOL, Syntheselabor GmbH, Germany) were used in this method. A pairs of primer on the basis of 16S rRNA gene sequence of *Helicobacter* genus were with the sequences of 5'-GT A AAG GCT CAC CAA GGC TAT-3' and 5'-CCA CCT ACC TCT CCC ACA CTC-3' to amplify a fragment of 389-bp [15]. The second set of primers based on an isocitrate dehydrogenase gene sequence of *H. pylori* species, were with the sequences of 5'-ATG GCT TAC AAC CCT AAA ATT TTA CAA AAG CC-3' and 5'-TCA CAT GTT TTC AAT CAT CAC GC-3' to amplify a fragment of 1200-bp [16]. The cycle profiles were set as follows: initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and 2 min extension at 72°C. The samples were amplified for 30 cycles followed by 10 min at 72°C. PCR products were analyzed by electrophoresis of a 10 µl aliquot using a 1.5% (wt/vol) agarose gel. The sizes of the PCR products were estimated by comparison with 100-bp DNA size markers (MBI, Ferments, Lithuania). After electrophoresis, the gel was stained with ethidium bromide and video images were obtained by a gel documentation system (Unitec, EEC, USA). DNA from pure *H. pylori* isolates that were confirmed by their morphology upon gram staining and by positive oxidase, catalase and rapid urease tests was extracted using phenol chloroform protocol to use as a positive control in our PCR. Negative control reactions with distilled water were performed with each batch of amplification to exclude the possibility of contamination.

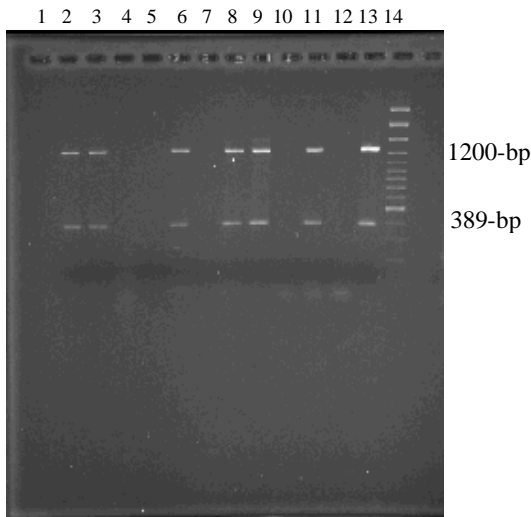
**Whole cell protein preparation.** *H. pylori* cells were collected from the surface of solid medium and a suspension of 6 × 10<sup>10</sup> CFU/ml was prepared. This suspension was washed twice with ice-cold phosphate buffered saline (50 mM sodium phosphate, 0.15 M sodium chloride, pH 7.2) containing 1 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 min in a boiling water bath and frozen at -20°C until use.

**SDS-PAGE.** The fractions of whole cell proteins were separated by SDS-PAGE on Hoefer SE 600/SE 660 (Amersham Biosciences) using a 10% gel prepared according to method of Laemmli [18]. After electrophoresis, the gels were stained by Coomassie Brilliant Blue G-250 based on Sambrook method [19], scanned and the molecular weights of the relevant bands were determined according to the protein molecular weight marker.

## RESULTS

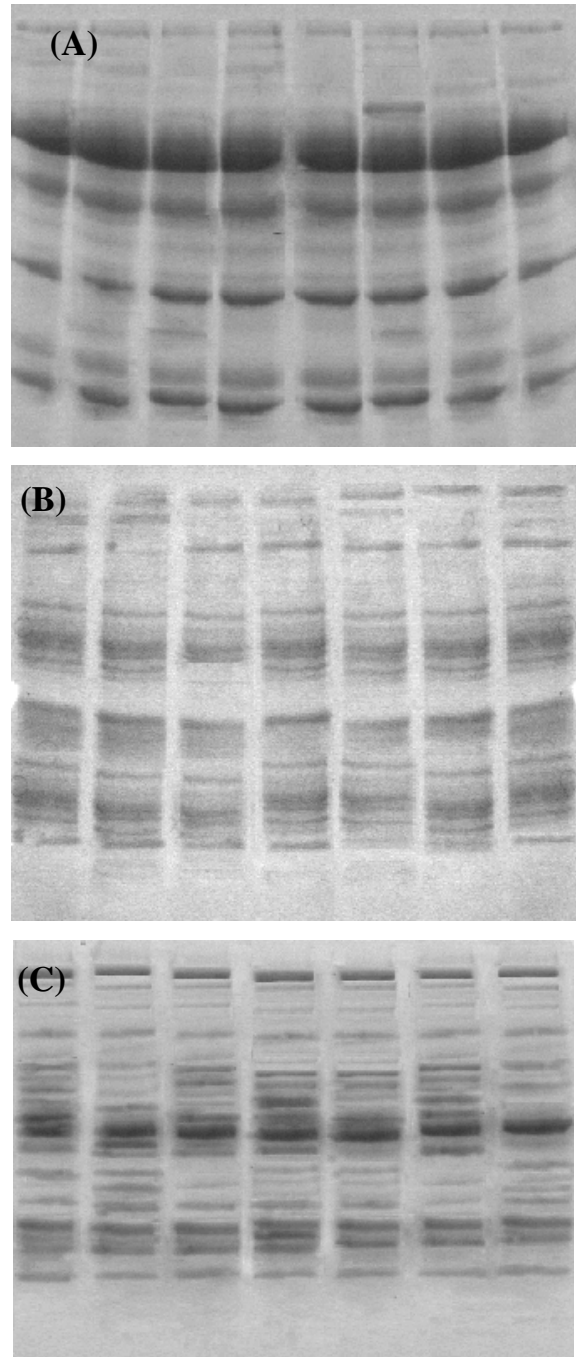
**Patients groups.** During the study period, 144 patients with different gastric diseases were enrolled. According to the pathology and clinical findings, 3 groups of patients with UD (n = 37), NUD of gastritis (n = 77) and CD (n = 30) were categorized.

***H. pylori* isolation and confirmation.** Totally from body and antrum of patients with UD 28 and 30, NUD 31 and 35, CD 12 and 13 *H. pylori* were isolated. From all DNA preparations of *H. pylori* isolates, two fragments of the expected sizes of 389-bp and 1200-bp specific for *Helicobacter* genus and *H. pylori* species, respectively, were amplified using our Multiplex PCR (Fig. 1).



**Fig. 1.** Agarose gel electrophoresis of amplification products of 16S RNA region (389-bp) and isocitrate dehydrogenase gene (1200-bp) of *H. pylori*. Lanes 2, 3, 6, 8, 9, 11, *H. pylori* isolates; lanes 1, 4, 5, 7, 10, non-*H. pylori* isolates; lane 12, negative control; lane 13, positive control; lane 14, molecular size marker (100-bp plus ladder).

**Protein profiles of *H. pylori* isolates in SDS-PAGE.** Protein profiles of different strains of *H. pylori* isolated from patients with different diseases



**Fig. 2.** 1D-SDS-PAGE of total protein from some representative *H. pylori* strains isolated from non-ulcerative (A), ulcerative (B) and cancerous (C) patients. Protein profiles of different strains of *H. pylori* isolated from patients with different diseases revealed some differences in the expression pattern. The highest number of bands observed in the CD group (C) and the lowest was seen in the NUD group (A).

revealed some differences in expression pattern. Figure 2 shows protein patterns of some representative strains from 3 groups of the patients.

The members of each group showed high correlation according to similarity in their patterns, resulting in considering them in the same cluster (Tables 1 and 2). The highest number of bands was observed in the CD group (20 bands) and the lowest was seen in the NUD group (14 bands). The largest band with the molecular weight of 160 kDa was common in all strains and the smallest one (13 kDa) was specific for the members of NUD group. The bands specific for the members of each group were 106, 61.5, 45, 34 and 30 kDa for CD, 22 kDa for UD and 13 kDa for NUD group. Although some slight differences between the strains isolated from 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.

## DISCUSSION

Although *H. pylori* is known to be a causative agent of different gastric diseases such as Du and gastric carcinomas, it is not clear what factors determine these divergent outcomes of infection. However, the realization that *H. pylori* strains differ in virulence has created a continuing need for new and improved methods of diagnosis and treatment of infection. In the present study, the protein profiles of different strains of *H. pylori* isolated from three groups of the patients were analyzed using 1D-SDS-PAGE. The patterns of the different *H. pylori* strains were highly divergent. About 30.76% (7 bands) of the 26 observed protein bands were common in all strains isolated from 3 groups of the patients. The similarity for the strains inside each group was 75% (15 from 20), 76.47% (13 from 17) and 78.57% (11 from 14) for CD, UD and NUD group, respectively. Some of the observed bands were significantly specific for each group (Table 2). The bands of 106, 61.5, 45, 34, 30 kDa

were seen only in CD group. The bands of 22 and 13 kDa were specific for UD and NUD groups, respectively. By this, we speculate that some *H. pylori* strains might be more associated with a specific disease than others, giving the clustering of some but not all strains within each disease group.

Our findings correspond to the results of the study has been done by Enyroth *et al.* [13], but differ from some other results [7]. According to the numbers of the bands have been observed in our strains, it seems that there is an increase in protein expression potential from NUD to UD to CD group. It can be the result of diversity in the genomic contents of 3 groups' strains. Clinical isolates of *H. pylori* display a high inter strains variation at the genomic level [7, 13, 20, 21]. However, a high divergence at the genomic level does not imply functional differences among strains because of the occurrence of silent mutations, e.g., mutations in non-coding regions or in the third codon. Mutations giving rise to amino acid changes are more likely to be of functional and selective nature. Such changes may explain some, but not all, of the divergence in the protein profile patterns observed in this study. However, according to the current view that the presence of the respective genes in *H. pylori* does correlate with the clinical status [22], the effect of the factors related to the patient or disease status on protein expression of the strains with similar genomic fingerprints should not be ruled out.

Genomic diversity among the strains isolated in this study is under investigation in our laboratory. In the present study, no patient's parameter as sex or age showed correlated bands at the level of significance. Age was of particular relevance at the time of diagnosis for CD patients who were on average older than UD patients and hence may have carried *H. pylori* for longer periods of their life. There are different results in this view. In one study using 2D SDS-PAGE, the recognition of the protein of GroES was correlated with age [23] but another

**Table 2.** Identification of specific and common bands in different *H. pylori* strains groups.

Different <i>H. pylori</i> strains groups	Number of total bands	Common bands (kDa)	%	Specific bands (kDa)	%
Cancer disease	20	160, 106, 94, 63, 61.5, 60, 58.5, 47, 45, 44, 34, 30, 27, 26.5, 14	75.00	106, 61.5, 45, 34, 30	25
Ulcerative disease	17	160, 124, 94, 63, 60, 58.5, 53, 47, 44, 38, 27, 22, 14	76.47	22	5.88
Non-ulcerative disease	14	160, 94, 63, 60, 58.5, 53, 47, 44, 26.5, 14, 13	78.57	13	7.14
Total groups	26	160, 94, 63, 60, 58.5, 47, 44, 14	30.76	-	-

**Table 1:** Frequency of 26 most relevant protein bands of the SDS-PAGE pattern in different *H. pylori* strains isolated from 3 different gastric patient groups.

Presence (pos <sup>1</sup> ) or absence (neg <sup>2</sup> ) of protein bands in different groups	Protein bands (kDa)																										
	160	145	124	106	105	94	90	85	82	63	61.5	60	58.5	53	47	45	44	38	34	30	27	26.5	22	20	14	13	
CD <sup>3</sup>	pos Count	13	12.0	0	13	13	13	7	0	10	13	13	13	10	13	13	13	0	13	13	13	13	3	0	13	0	
	%	100	92.3	0	100	100	100	54	0	77	100	100	100	100	75	100	100	100	0	100	100	100	100	23	0	100	0
	neg Count	0	2.0	13	0	0	0	6	13	3	0	0	0	3	0	0	0	13	0	0	0	0	10	13	0	13	
	%	0	7.6	100	0	0	0	46	100	23	0	0	0	25	0	0	0	100	0	0	0	0	77	100	0	100	
UD <sup>4</sup>	pos Count	30	0	30	0	25.0	30	0	15	20.0	30	0	30	30	30	30	0	30	30	0	0	30	0	30	13.0	30	0
	%	100	0	100	0	83.3	100	0	50	66.7	100	0	100	100	100	100	0	100	100	0	0	100	0	100	43.3	100	0
	neg Count	0	30	0	30	5.0	0	30	15	1.0	0	30	0	0	0	30	0	0	30	30	0	30	0	17.0	0	30	
	%	0	100	0	100	16.7	0	100	50	33.3	0	100	0	0	0	100	0	0	100	100	0	100	0	56.7	0	100	
NUD <sup>5</sup>	pos Count	35	0	6.0	0	0	35	0	0	8.0	35	0	35	35	35	35	0	35	0	0	0	35	18.0	0	35	35	
	%	100	0	17.1	0	0	100	0	0	22.9	100	0	100	100	100	100	0	100	0	0	0	100	51.8	0	100	100	
	neg Count	0	35	29.0	35	35	0	35	35	27.0	0	35	0	0	0	35	0	35	35	35	35	0	17.0	35	0	0	
	%	0	100	82.9	100	100	0	100	100	77.1	0	100	0	0	0	100	0	100	100	100	100	100	0	48.6	100	0	0

<sup>1</sup>, Positive; <sup>2</sup>, Negative; <sup>3</sup>, Cancer Disease; <sup>4</sup>, Ulcerative disease; <sup>5</sup>, Non-ulcerative disease

study did not confirm this finding [24]. It is clear that the differential recognition of the proteins described here could be a consequence of the diseases rather than being correlated with their potential cause. Our study was not designed to discriminate between these possibilities, but with the identified candidates this will be possible in the future. Expression of virulence factors, age at infection and their duration, immune response, level of acid secretion, and environmental factors have all been proposed to influence the outcome of *H. pylori* infection [25-27], but their relative contributions are not yet clear.

In conclusion, this study showed that protein profile can be characteristic in discrimination of dominant strains in different gastric clinical status. Specific and dominant proteins of different strains isolated from three groups of patients are candidates for further exploration for laboratory tests which analyze disease-specific *H. pylori* strains, and for diagnosis of different diseases and outcomes associated with this widespread bacterium.

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#### REFERENCES

- Ernst, P.B. and Gold, B.D. (2000) The disease spectrum of *Helicobacter pylori*: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. *Annu. Rev. Microbiol.* 54: 615-640.
- IARC working Group on the Evaluation of Carcinogenic Risks to Humans (1994) Schistosomes, liver flukes and *Helicobacter pylori*. *IARC. Monogr. Eval. Carcinog. Risks. Hum.* 61: 1-241.
- Labenz, J., Meining, A., Tillenburger, B. and Stotle, M. (1999) *Helicobacteriose*: update. *Leber. Magen. Darm.* 29: 80-92.
- Parsonnet, J., Friedman, G.D., Vandersteen, D.P., Chang, Y., Vogelstein, J.H., Orentreich, N. and Sibley, R.K. (1991) *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Engl. J. Med.* 325: 1127-1131.
- Uemura, N., Okamoto, S., Yamamoto, S., Matsumura, N., Yamaguchi, S., Yamakido, M., Taniyama, K., Sasaki, N. and Schlemper, R.J. (2001) *Helicobacter pylori* infection and the development of gastric cancer. *N. Engl. J. Med.* 345: 784-789.
- Blaser, M.J. (1997) Not all *Helicobacter pylori* strains are created equal: should all be eliminated? *Lancet* 349: 1020-1022.
- Hazell, S.L., Andrews, R.H., Mitchell, H.M. and Daskalopoulos, G. (1997) Genetic relationship among isolates of *Helicobacter pylori*; evidence for the existence of a *Helicobacter pylori* species-complex. *FEMS. Microbiol. Lett.* 150: 27-32.
- Jiang, Q., Hiratsuka, K. and Taylor, D.E. (1996) Variability of gene order in different *Helicobacter pylori* strains contributes to genome diversity. *Mol. Microbiol.* 20: 833-842.
- Salaun, L., Audibert, C., Le Lay, G., Burucoa, C., Fauchere, J.L. and Picard, B. (1998) Panmictic structure of *Helicobacter pylori* demonstrated by the comparative study of six genetic markers. *FEMS. Microbiol. Lett.* 161: 231-239.
- Van der Ende, A., Rauws, E.A.J., Feller, M., Mulder, C.J.J., Tytgat, G.N. and Dankert, J. (1996) Heterogeneous *Helicobacter pylori* isolates from members of a family with a history of peptic ulcer disease. *Gastroenterology* 111: 638-647.
- Blaser, M.J. (1999) In a world of black and white, *Helicobacter pylori* is grey. *Ann Intern. Med.* 130: 695-697.
- Censini, S., Lang, C., Crabtree, J.E., Ghiara, P., Borodovsky, M., Rappuoli, R. and Covacci, A. (1996) Cag A pathogenicity island of *Helicobacter pylori*, encodes type I- specific and disease - associated virulence factors. *Proc. Natl. Acad. Sci. USA* 93: 14648-14653.
- Enroth, H., Akerlund, T., Sillen, A. and Engstrand, L. (2000) Clustering of clinical strains of *Helicobacter pylori* analyzed by two-dimensional gel electrophoresis. *Clin. Diagn. Lab. Immunol.* 7: 301-306.
- Farshad, S.H., Rasouli, M. and Alborzi, A. (2004) Simultaneous detection of *Helicobacter* genus and *Helicobacter pylori* species using a multiplex PCR method. *Iran. Biomed. J.* 8: 205-209.
- Choi, Y.K., Han, J.H. and Joo, H.S. (2001) Identification of Novel *Helicobacter* species in pig stomachs by PCR and partial sequencing. *J. Clin. Microbiol.* 39: 3311-3315.
- Argyros, F.C., Ghosh, M., Huang, L., Masubuchi, N., Cave, D.R. and Grubel, P. (2000) Evaluation of a PCR primer based on the isocitrate dehydrogenase gene for detection of *Helicobacter pylori* in feces. *J. Clin. Microbiol.* 38: 3755-3758.
- She, F.F., Su, D.H., Lin, J.Y. and Zhou, L.Y. (2001) Virulence and potential pathogenicity of coccoid *Helicobacter pylori* induced by antibiotics. *World J. Gastroenterol.* 7: 254-258.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227: 680-685.

19. Sambrook, J. and Russel, D.W. (2001) Molecular cloning a laboratory manual. 3<sup>rd</sup> edition, Vol. 3, pp. A8.40-A8.51.
20. Enroth, H., Nyren, O. and Engstrand, L. (1999) One stomach-one strain: does *Helicobacter pylori* strain variation influence disease outcome? *Dig. Dis. Sci.* 44: 102-107.
21. Taylor, D.E., Eaton, M., Chang, N. and Salama, S.M. (1992) Construction of a *Helicobacter pylori* genome map and demonstration of diversity at the genome level. *J. Bacteriol.* 174: 6800-6806.
22. Graham, D.Y. and Yamaoka, Y. (2000) Disease-specific *Helicobacter pylori* virulence factors: the unfulfilled promise. *Helicobacter* 5: S3-S9.
23. Iaquinto, G., Todisco, A., Giardullo, N., D'Onofrio, V., Pasquale, L., De Luca, A., Andriulli, A., Perri, F., Rega, C., De Chiara, G., Landi, M., Taccone, W., Leandro, G. and Figura, N. (2000) Antibody response to *Helicobacter pylori* Cag A and heat-shock proteins in determining the risk of gastric development. *Dig. Liver Dis.* 32: 378-383.
24. Krah, A., Muhlke, S., Pleissner, K.P., Zimny-Arndt, U., Kiesch, C., Lehn, N., Meyer, T.F., Jungblut, P.R. and Aebischer, T. (2004) Identification of candidate antigens for serologic detection of *Helicobacter pylori*-infected patients with gastric carcinoma. *Int. J. Cancer* 108: 456-463.
25. Graham, D.Y. (1997) *Helicobacter pylori* infection in the pathogenesis of duodenal ulcer and gastric cancer: a model. *Gastroenterology* 113: 1983-1991.
26. Farthing, M.J., Fitzgerald, R. and Zhang, Z.W. (2001) Acid, *Helicobacter* and immunity: a new paradigm for oesophagogastric cancer. *J. Physiol. (Paris)* 95: 423-427.
27. Calam, J. and Baron J.H. (2001) ABC of the upper gastrointestinal tract: pathophysiology of duodenal and gastric ulcer and gastric cancer. *BMJ* 323: 980-982.