

Immunoblotting for the Serodiagnosis of *Helicobacter pylori* Infection in Brazilian Patients with and without Gastric Carcinoma

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We evaluated the performance of a commercial immunoblotting in the serodiagnosis of *Helicobacter pylori* infection in Brazilian patients. The presence of anti-*H. pylori* antibodies was also investigated in a group of 20 duodenal ulcer patients after successful treatment. One hundred and ninety one patients were studied. Among the 164 infected patients, 46 had gastric carcinoma. The duodenal ulcer patients were treated with antimicrobial drugs and the eradication of the microorganism was confirmed in all of them one month after the end of the treatment by the ¹³C-urea breath test. Sera were assayed for *H. pylori* antibodies using the Helicoblot 2.0 (Genelabs Diagnostics, Singapore). The sensitivity, specificity, positive, and negative predictive values of the test were 93.9%, 92.6%, 98.7%, and 71.4%, respectively. The sensitivity of the test was similar in patients with (93.5%) and without (95.7%) gastric carcinoma. Twenty-four months after the end of the treatment, the band of 116 kDa was still detected in one of the patients. In conclusion, the Helicoblot 2.0 is an accurate test to diagnose *H. pylori* infection and although it can not be employed to monitor the bacterium eradication, it may be useful for diagnosing past infection, especially in gastric carcinoma patients.

Key words: *Helicobacter pylori* - *H. pylori*/diagnosis - immunoblotting

Helicobacter pylori infection is predominantly acquired in childhood (Parsonnet 1995, Bardhan 1997) and in most subjects its long-term course is without complications. Nevertheless, a small percentage of infected subjects develops peptic ulcer disease, gastric carcinoma or mucosa-associated lymphoid tissue lymphoma (NIH 1994, IARC 1994, Wotherspoon 1998). Infected individuals develop an immune response and IgG antibodies to *H. pylori* can be detected in serum (Mégraud 1996, Rocha et al. 1998).

The presence of antibodies against *H. pylori* antigens can be evaluated by immunoblotting (Mitchell et al. 1996, Aucher et al. 1998, Yamaoka et al. 1998, Figueiredo et al. 2001, Park et al. 2002). Although expensive and time-consuming, the test seems to be more sensitive than ELISA, especially when the levels of antibodies are low. This is probably due to the fact that the individual bacterial proteins are better exposed, allowing antibodies to bind more easily (Nilsson et al. 1997). The immunoblotting also allows the detection of antibodies to *H. pylori* virulence factors such as CagA and VacA (Yamaoka et al. 1998, Figueiredo et al. 2001, Park et al. 2002).

Commercial serological tests for *H. pylori* diagnosis have different performances in different populations, in part because of the variations in geographical distribution of the strains and in the antigenic preparations of the

assays (Aucher et al. 1998, Yamaoka et al. 1998, Leung et al. 2001, Figueiredo et al. 2001). Thus, validating serological assays for each population in order to achieve more reliable results is recommended. The aim of the present study was to evaluate the performance of Helicoblot 2.0 in Brazilian patients. We also investigated the presence of anti-*H. pylori* antibodies in a group of 20 duodenal ulcer patients after successful treatment.

PATIENTS AND METHODS

This project was approved by the Ethics Committee of Hospital das Clínicas, Universidade Federal de Minas Gerais (UFMG), Brazil, and informed consent was obtained from all patients.

We studied 191 patients (105 male and 86 female; mean age, 46.3 years \pm 15.5; range from 17 to 95 years), 164 with and 27 without *H. pylori* infection. Among the infected patients, 48 had duodenal ulcer, 46 gastric carcinoma and 70 had gastritis only. All patients without gastric carcinoma were selected among those referred to the Digestive Endoscopy Unit of the University Hospital, UFMG, for evaluation of symptoms related to the upper gastrointestinal tract. Those who had received antibiotic therapy for the eradication of *H. pylori* or antimicrobial drugs during the 2 months before endoscopy, who were taking H₂ receptor antagonists or non-steroidal anti-inflammatory drugs or who had portal hypertension, coagulation disorders, or anatomical obstacles preventing endoscopy were not included in the study. The patients with gastric carcinoma were selected among those who had been treated at the Surgical Clinic of University Hospital, UFMG, at Luxemburgo Hospital, and at Mário Penna Hospital of Oncology.

We also evaluated a group of 20 duodenal ulcer *H. pylori*-positive patients (13 male, 7 female, mean age 48.5

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years \pm 15.6, range from 27 to 76 years) treated with clarithromycin (500 mg, bid), furazolidone (200 mg, bid) plus omeprazole (20 mg, om). The eradication of the microorganism was confirmed in all of them one month after the end of the treatment by the ^{13}C -urea breath test. To evaluate the antibody response, sera were collected 6 months after the treatment from 12 patients. From the other 8 patients, sera were obtained 1 year ($n = 4$) and 2 years ($n = 4$) after the end of the treatment.

At endoscopy, biopsy specimens were obtained from the antral and oxyntic gastric mucosa for microbiological and histological study. In patients with gastric carcinoma, gastric mucosa fragments from the antrum and body were obtained from the stomachs removed by gastrectomy. For urease test, one antral fragment was placed in a tube containing Christensen's 2% urea agar and examined within 24 h of incubation at 37°C for urea hydrolysis. For culture, fragments from the antrum and body were kept in thioglycolate broth (Difco Laboratories, Detroit, MI, US) at 4°C for no longer than 1 h before processing. The tissue specimens were ground in a tissue homogenizer and plated onto Petri dishes containing Belo Horizonte medium (Queiroz et al. 1987) and the plates were incubated at 37°C in a microaerobic environment for up to 7 days. Colonies of *H. pylori* were identified by spiral Gram-negative appearance, positive oxidase and catalase tests, and a rapidly positive urease test. For histology, one fragment of the antral mucosa and one fragment of the oxyntic mucosa were fixed in formalin, dehydrated in alcohol and xylene and embedded in paraffin. Five- μm thick sections were obtained for the preparation of slides which were stained with hematoxylin-eosin for histological examination and with carbofuchsin for *H. pylori* identification.

Patients were considered to be *H. pylori*-positive if both urease test and carbofuchsin were positive or if the culture was positive, and negative if the three test results were negative.

Venous blood samples (5 ml) were drawn from each patient at the time of endoscopy or surgery. The serum was separated, divided into aliquots and stored at -20°C before testing.

Sera were assayed for *H. pylori* antibodies using the commercial immunoblotting Helicoblot 2.0 (Genelabs Diagnostics, Singapore). Helicoblot 2.0 is a qualitative assay used for the detection of IgG antibodies specific for different antigens of *H. pylori* in human serum or plasma. The antigen is prepared from a lysate of a strain of *H. pylori* isolated from a patient with peptic ulcer. The proteins from the lysate are electrophoretically separated and transferred to nitrocellulose. The strips contain different *H. pylori* antigens including: 116 kDa (CagA), 89 kDa (VacA), 35 kDa, 30 kDa, 26.5 kDa, and 19.5 kDa. The assay was performed in the Autoblots System 36 (Genelabs) according to the recommendations of the manufacturer of the kit. Briefly, the strips were washed with wash buffer and each strip was incubated for 1 h with the diluted sample 1:100 in blocking buffer. A serum-positive control and a serum-negative control were used. Following the incubation period, the sera were aspirated and the strips washed three times. Then, the strips were incubated with an anti-human IgG antibody conjugated with alkaline phos-

phatase for 1 h. After this step, the conjugate solution was aspirated from each well, the strips were washed three times and incubated with a solution of 5-bromo-4-chloro-3-indoyl-phosphate and nitroblue tetrazolium for 15 min. The reaction was stopped by rinsing the strips several times with distilled water. Finally, the strips were removed from the wells and dried before mounting. Western blotting was considered to be positive for *H. pylori* if any one band at 116 kDa, 89 kDa, 35 kDa, or if any two bands from among bands of 30 kDa, 26.5 kDa or 19.5 kDa were present. The analysis of the test was done by two investigators who were unaware of the *H. pylori* status of the patients.

For the urea breath test, one breath sample was obtained, after a 6-h fast, and an oral dose of urea comprising 75 mg ^{13}C -urea was administered to the patient with 100 ml of orange juice. The other sample was collected 30 min after the ingestion of the substrate. The breath samples were analyzed by an isotope selective nondispersive infrared spectrometer (NDIRIS; Wagner Analytical Systems, Bremen, Germany). The results were considered positive when delta over baseline (DOB) was $> 4\%$.

The performance of the immunoblotting was evaluated by determining the sensitivity, specificity, and positive and negative predictive values with 95% confidence intervals. Statistical analysis was performed by using the χ^2 or Fisher's exact test for non-parametric values and the Student's t test for comparison of the mean age between the groups. The level of significance was set at $p < 0.05$.

RESULTS

The characteristics of the patients studied are listed in Table I. A higher frequency of male patients ($p = 0.005$) and a higher mean age ($p < 0.001$) were observed in the gastric carcinoma group when compared with the other groups.

Anti-*H. pylori* antibodies were detected in 154 of 164 *H. pylori*-positive patients (44/48 with duodenal ulcer, 43/46 with gastric carcinoma and 67/70 without duodenal ulcer or gastric carcinoma) and in 2 of 27 non-infected patients. The sensitivity, specificity, positive and negative predictive values of the blot for the diagnosis of the infection were 93.9% (95% CI: 88.8-96.9%), 92.6% (74.2-98.7%), 98.7% (95-99.8%) and 71.4% (53.5-84.8%), respectively.

The sensitivity of the test was similar in patients with duodenal ulcer (91.6%), gastric carcinoma (93.5%), and in those without the diseases (95.7%) ($p = 0.44$). No difference was observed in the frequency of the bands of 19.5 kDa ($p = 0.59$), 30 kDa ($p = 0.31$), 35 kDa ($p = 0.14$) and 89 kDa ($p = 0.29$) among the three groups, but the band of 116 kDa was more frequently ($p < 0.01$) observed in patients with duodenal ulcer and gastric carcinoma than in patients without the diseases. In regard to the 26.5 kDa band it was more frequent in patients with gastric carcinoma than in those with gastritis ($p = 0.03$) (Table II). Although the difference ($p = 0.07$) did not reach statistical significance, the frequency of this band was also higher in patients with gastric carcinoma than in those with duodenal ulcer.

False-positive results were due to the detection of antibodies to the 116 kDa antigen in one patient and to

TABLE I
Characteristics of patients studied

	Patients			
	Hp-positive			Hp-negative
	DU	GC	Gastritis	
Number	48	46	70	27
Gender (male/female)	29/19	34/12	31/39	11/16
Mean age \pm SD (years)	44.8 \pm 12.5	60.0 \pm 14.9	40.3 \pm 13.2	41.4 \pm 13.4
Age range (years)	24-76	33-95	17-78	21-73

Hp: *Helicobacter pylori*; DU: duodenal ulcer, GC: gastric carcinoma

TABLE II
Frequency of immunoreactive bands in the serum of patients with positive result in the immunoblotting

<i>Helicobacter pylori</i> positive patients	Immunoreactive bands (kDa) (% positivity)					
	19.5	26.5	30	35	89	116
With duodenal ulcer (DU) (n = 44)	14 (32)	34 (77)	18 (41)	25 (57)	22 (50)	41 (93) ^b
With gastric carcinoma (GC) (n = 43)	13 (30)	40 (93) ^a	21 (49)	33 (77)	16 (37)	37 (86) ^b
Without DU and GC (n = 67)	26 (39)	50 (75) ^a	38 (57)	41 (61)	24 (36)	40 (60) ^b

a: p = 0.02; b: p < 0.001

the 30 kDa and 35 kDa antigens in the other.

The ¹³C-urea breath test was negative in all treated patients. The result of the immunoblotting was positive in all 12 patients in the 6 months follow-up. One year after the end of the treatment only the band of 116 kDa (CagA) could be detected in the sera of the 4 patients evaluated at that time. Sera of the other 4 patients were studied 24 months after the treatment and in only one of them the band of 116 kDa was detected.

DISCUSSION

Since *H. pylori* infection elicits a systemic, strong and polymorphic humoral immune response (Herbrink & van Doorn 2000), serological assays have been successfully used for the diagnosis of the infection. Although ELISA is an accurate test, some authors have demonstrated that immunoblotting is more sensitive to detect recent (Mitchell et al. 1996) and previous *H. pylori* infection (Sörberg et al. 1997), especially in the case of sera with low levels of antibodies (Nilsson et al. 1997). In addition, as the antibodies react with defined antigens, the test allows the simultaneous detection of bacterium virulence factors. However, as there is a high degree of genetic heterogeneity among *H. pylori* strains and, consequently, in the proteins that are surface-exposed or secreted (Go et al. 1996, Herbrink & van Doorn 2000), the assay must be validated for the population being studied (Mitchell et al. 1996, Aucher et al. 1998, Yamaoka et al. 1998, Figueiredo et al. 2001).

In the present study, employing three tests as gold standard, we validated a commercial blot to diagnose *H. pylori* infection in Brazilian population. Similarly to that we have previously observed for children (Rocha et al. 2000), the assay showed high sensitivity (92.8%) for the diagnosis of the infection in adults. Other authors, em-

ploying the same kit (Yamaoka et al. 1998, Malaty et al. 2002, Park et al. 2002) or in-house techniques (Faulde et al. 1993, Klaamas et al. 1996, Karvar et al. 1997, Nilsson et al. 1997, Holtmann et al. 1998) also reported high sensitivity for the test. In addition, we observed that the sensitivity was similar in patients with duodenal ulcer, gastric carcinoma, and gastritis only. It is an important finding because as demonstrated by Queiroz et al. (1999), the current methods used to diagnose *H. pylori* infection are less sensitive in gastric carcinoma patients, being recommended the association of at least a direct and an indirect test in these patients. In fact, as the infection may have disappeared by the time the cancer is diagnosed, the detection of antibodies, especially anti-CagA, can be the unique positive method (Ekström et al. 2001).

Similarly to the data of other authors employing the same kit (Yamaoka et al. 1998, Malaty et al. 2002) or in-house blot (Nilsson et al. 1997), we observed 2 false-positive results. This may be due more to the use of antimicrobial drugs for other purpose with a resulting decrease in bacterial load or spontaneous bacterium elimination with persistence of antibodies than detection of cross-reacting antibodies. This hypothesis is strengthened by the detection of the 116 kDa band (CagA) in the serum of 1 of the 2 patients, once this antibody lasts longer after bacterium eradication. On the other hand the specificity we observed was higher than that described by Karvar et al. (1997) (specificity of 71%) and Aucher et al. (1998) (specificity of 82%), employing in-house techniques. These differences may be due to the gold standard used and the criteria adopted to establish negativity. We employed 3 methods for diagnosing infection and rigid criteria for negativity, which limits erroneous classification of the patients as *H. pylori* negative. Although the exact antigen composition of the Helicoblot 2.0 was not provided

by the manufacturer, the assay is based on Western-blot analysis of whole-cell *H. pylori* strain NCTC 11916 antigens (Figueiredo et al. 2001, Park et al. 2002) and differences in preparation of the antigen can also justify the differences observed in the specificity of the tests.

Although the positive predictive value of the assay was high (98.7%), the negative predictive value was not (71.4%). This finding may be due to the small proportion of uninfected patients evaluated in the present study.

In agreement with the results obtained in other western populations (Crabtree et al. 1993, Klaamas et al. 1996, Holtmann et al. 1998), in the present study, serum anti-CagA antibodies were detected more frequently in gastric carcinoma and duodenal ulcer patients than in *H. pylori*-positive patients without these diseases. Infection with *cagA*-positive strains has been associated with higher degree of gastric mucosa inflammation, a risk for the development of the diseases associated with infection.

We also observed a higher frequency of the band of 26.5 kDa, a species-specific protein antigen of *H. pylori* (O'Toole et al. 1991), in serum from gastric carcinoma patients when compared with those without the disease. This result is in accordance with that reported by Wang et al. (1998) in Taiwanese patients, who suggested that the 26 kDa antigen may be used to screen gastric carcinoma patients. Other authors such as Yamaoka et al. (1998) and Holtmann et al. (1998) did not observe differences in the frequency of the serum low molecular weight bands between patients with and without gastric cancer. Since Mitchell et al. (1996) showed that an antibody response to this protein was one of the first to appear after infection with *H. pylori* in children, we may speculate that antibody to 26 kDa protein could be a marker of prolonged infection, an essential step in the development of gastric carcinoma.

In regard to the duodenal ulcer patients submitted to the antimicrobial therapy, the results of the present study are in agreement with those obtained by Sörberg et al. (1997) and Kist et al. (1999) who detected anti-CagA antibodies in the patients' sera 32 and 12 months after successful treatment, respectively. In our study, 100% of patients had anti-CagA antibodies 1 year after eradication of the bacterium and antibodies could still be detected in 1 of 4 (25%) of the patients evaluated 2 years after the end of the treatment. This result reinforces that serology is not adequate for monitoring treatment.

In conclusion the Helicoblot 2.0 is an accurate test to diagnose *H. pylori* infection and although it cannot be employed to monitor the bacterium eradication, the test may be useful for diagnosing past infection, especially in gastric carcinoma patients.

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