Use of *Helicobacter pylori*-specific antibodies in the evaluation of intestinal metaplasia and gastric dysplasia

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ABSTRACT

It is believed that *Helicobacter pylori* acts mainly during the initial phases of gastric carcinogenesis. Therefore, this study aims to assess the usefulness of *H. pylori* diagnosis in patients with chronic gastritis (CG), intestinal metaplasia (IM) and dysplasia – conditions that are associated with gastric cancer. A cross-sectional study of 94 patients was performed, which involved endoscopic biopsy and determination of specific serum anti-*H. pylori* antibodies (IgA, IgG and IgM) by enzyme-linked immunosorbent assay (ELISA). Biopsies were taken from the gastric antrum and corpus, and from endoscopic lesions. Two specimens per patient were used for bacterial culture. *H. pylori* infection status, used as the gold standard, was based on culture results. Validity measures were determined and receiver operating curve (ROC) was used to determine the best cut-off for serum antibody levels. Histopathological evaluation (n=160) was performed independently by two pathologists. Lesions consistent with CG were found in 86 patients (91%), consistent with IM in 69 patients (73%) and with dysplasia in five patients (5%). In the 86 patients with CG, 38 (44%) were infected by *H. pylori*, as were 26 (38%) and one (20%) with IM and dysplasia, respectively (P=0.039). Area under the curve (AUC) was 0.40 (95% confidence interval [CI]: 0.28–0.51) for IgM, 0.69 (0.58–0.80) for IgA and 0.83 (0.74–0.92) for IgG for the diagnosis of *H. pylori* infection. Best cut-off was 41 u/mL for IgG, with a sensitivity (95% CI) of 90% (84–96%) and a negative predictive value (NPV) of 91% (85–97%). For IgA the results were 22 u/mL, 74% (65–83%) and 77% (68–86%), respectively. Prevalence of *H. pylori* appeared to decrease with increasing severity of the gastric lesion. In conclusion, it is suggested that non-invasive serological evaluation of anti-*H. pylori* (IgG) status after eradication therapy for peptic ulcer disease could be extended, after proper assessment of cut-off values and their validation, to the follow-up of patients with CG and IM.

KEY WORDS: Antibodies. Gastritis. Helicobacter pylori. Metaplasia. Precancerous conditions.

Introduction

Helicobacter pylori, first isolated by Warren and Marshall in 1983, acts mainly during the initial phases of gastric carcinogenesis. Its microbial mechanisms, used to survive in the gastric environmental, destroy gastric cells.[1-6] *H. pylori* is frequently acquired during childhood[7] and infection of the gastric mucosa[8] persists unless the organism is eradicated by appropriate treatment.

As a result of host and agent factors, infected individuals may be prone to develop gastric cancer, particularly intestinal-type carcinoma,[9-12] through the development of multifocal atrophic gastritis.[1-8] In addition, infection may cause peptic ulcer disease in both adults and children.[17-26] Conversely, *H. pylori* has been reported only infrequently in areas of intestinal metaplasia.[9, 13-16]

Colonisation of the gastric mucosa by *H. pylori* can be diagnosed by invasive methods such as isolation by culture or through histopathological diagnosis with special stains (e.g., modified Giemsa, Warthin–Starry, silver stains) or an immunohistochemical technique using antibodies to *H. pylori* antigens.[7-9] Although highly sensitive and specific, these invasive methods only provide information about infection in that part of the gastric mucosa sampled or biopsied.[9]

H. pylori diagnosis can also be made by non-invasive methods that measure specific anti-*H. pylori* antibodies present in patients' sera. Also, in recent years, antibody-mediated agglutination of bacterial cell antigens has been exploited by commercial systems[17] that involve the detection of specific immunoglobulins.

Immunoglobulin M (IgM) can be detected shortly after *H. pylori* infection is acquired, while IgA and IgG appear after colonisation.[9,17-26] Some studies suggest that *H. pylori* antibody reflects the current state of infection in the gastric mucosa.[9]

Such serological tests show an acceptable level of sensitivity and specificity and have been used for the diagnosis of *H. pylori* infection.[17-27] In addition, they are more comfortable for the patient and can be easily performed in the laboratory.

Previous study has focused on the use of these non-invasive methods in post-treatment prevention and management of peptic ulcer disease.[23] However, the purpose of the present study is to assess the validity and consistency

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of a non-invasive method to detect *H. pylori* infection in a group of patients with previously diagnosed chronic gastritis (CG), intestinal metaplasia (IM) and dysplasia, and its value in patient management.

Material and methods

Participants

A cross-sectional study was performed in 94 adults (median age: 60 years [range: 26–80]; 34 males, 60 females) previously diagnosed with a range of gastric lesions. These patients had been on follow-up for at least a year and had not been treated for *H. pylori* infection. The study was approved by the ethics committee of the Portuguese Oncology Institute (IPO), Porto, Portugal.

Endoscopic technique

After giving informed consent, each patient underwent upper gastrointestinal endoscopy, using chromoendoscopy with methylene blue (1%) and magnification.[26] Biopsies were taken from the gastric antrum and corpus, and from any lesions identified. Two specimens per patient were used for bacterial culture. Two pathologists evaluated the histopathological lesions independently in all other specimens (n=160).

Histopathological evaluation

Histopathological evaluation was performed on formalin-fixed, paraffin wax-embedded biopsy samples stained with haematoxylin and eosin (HE), modified Giemsa (2%) and an immunohistochemical technique using anti-*H. pylori* antibody. Histopathological classification was performed on HE-stained sections, and modified Giemsa and immunohistochemistry were used to confirm the presence of *H. pylori*.

For the immunohistochemistry technique, polyclonal rabbit antibodies were diluted in phosphate-buffered saline (PBS; Biochrom AG) containing 1% bovine serum albumin (BSA; Biomedia). Following removal of paraffin wax and rehydration, sections were placed in 3% (v/v) H_2O_2 for 30 min at room temperature, followed by incubation in 0.1% trypsin for 20 min at 37°C. Sections were washed with PBS and H_2O_2, and then covered with normal serum (1 in 100 dilution) in BSA (100 µL/slide). Excess was removed after 20 min and replaced with rabbit polyclonal antibody to *H. pylori* (Novocastra Laboratories), diluted 1 in 30 in normal serum (100 µL/slide), and incubated overnight at 4°C.

Sections were then incubated with universal biotinylated anti-rabbit secondary antibody (Vectastain Elite ABC kit [Vector Laboratories]) diluted 1 in 50 in normal serum (100 µL/ slide) for 30 min. Sections were then incubated with ABC complex (Vectastain Elite ABC kit) for 30 min. Subsequently, colour was developed using diaminobenzidine tetrahydrochloride (DAB; Merck) for 7 min, and the sections were counterstained with haemotoxylin, dehydrated, cleared and mounted.

In order to obtain uniform classification, two pathologists reviewed the biopsies for the following histopathological features: (i) presence of bacteria, (ii) mononuclear inflammatory cells (iii) degree of atrophy, (iv) IM (complete or incomplete), and (v) presence of dysplasia.

Bacterial culture

Gastric samples were preserved for two to four hours in transport medium (Portagerm Pylori; bioMérieux) until inoculation. Biopsies were homogenised under aseptic conditions in 1.5 mL sterile broth (brain–heart infusion [BHI]; BioGerm). A loopful of homogenised tissue was streaked on to both non-selective (Columbia agar, with 5% sheep blood; bioMérieux) and selective (Pylori agar; bioMérieux) media using previously described techniques.[18-19]

Subsequently, agar plates were incubated under microaerophilic conditions (10% CO_2, 10% O_2; Genbag, bioMérieux) at 37°C for 5–10 days. Normally, *H. pylori* colonies appear small, grey and translucent,[18] and such colonies were studied.

Biochemical analyses were performed for catalase, oxidase and urease activity. Gram stain was used to study morphology.

Definition of infection

Infection was considered to be present when typical colonies of *H. pylori* were seen in the selective medium (Pylori agar, bioMérieux), non-selective culture medium (Columbia agar, bioMérieux) or in both, and confirmed by biochemical reactions and Gram stain.

Serological evaluation

Prior to endoscopy, two venous blood samples (10 mL) were obtained from each patient. The serum was separated (3000 rpm, 5 min, 20°C), divided into aliquots and stored at –20°C prior to testing. All sera were tested for IgG, IgA and IgM using enzyme-linked immunosorbent assay (ELISA; Virion/Serion ELISA Classic *Helicobacter pylori*, Germany). The assays were performed following the manufacturer's instructions and without knowledge of individual *H. pylori* status.

Briefly, serum samples were diluted in serum dilution buffer (1 in 100 dilution). To individual wells, coated with inactivated *H. pylori* antigens, were added 100 µL dilution buffer, calibrator serum samples (negative and cut-off) and the diluted serum samples, and these were then incubated for 60 min at 37°C in the dark. Plates were passed through the washing solution (1 in 10 dilution) and then 100 µL alkaline phosphatase-conjugated anti-human IgG/IgA/IgM was added to each well.

Plates were incubated for 30 min at 37°C in the dark. After washing, 100 µL substrate solution (*p*-nitrophenil phosphate) was added to the wells and incubated for 30 min at 37°C). Finally, the enzyme reaction was blocked by the addition of 100 µL 1 mol/L NaOH.

Absorbance was read at 405 nm (A_{405}). Immunoglobulin titres (IgA, IgG and IgM) for each adult were interpreted from a graph obtained from semi-logarithmic axis analysis of the manufacture's standard curve. Quantitative determination of each immunoglobulin titre (IgG, IgA and IgM) was calculated by an equation acquired from the standard curve. All titres were considered positive if ≥ 30 u/mL.

Statistical analysis

All analyses were performed using the Statistical Package for Social Sciences (SPSS, version 10.1) package. The level of significance was set at $P<0.05$. χ^2 test was used to compare prevalence of infection according to gastric lesions. Receiver operating curve (ROC) was used to obtain the optimal

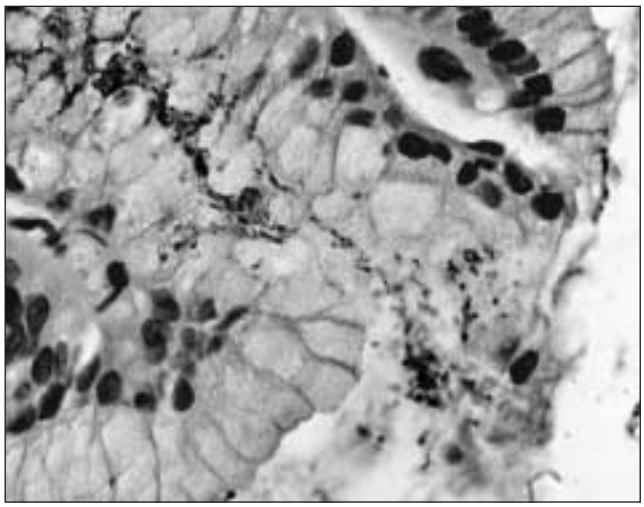

Fig. 1. Immunohistochemical staining of *H. pylori* colonising superficial gastric mucosa (original magnification x1000).

cut-off. Best cut-off point was obtained as the tangent point to the curve, parallel to 50% diagonal line. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using culture as the gold standard or reference test.

Results

Prevalence of H. pylori

Thirty-eight patients (40%) were *H. pylori* culture-positive. *H. pylori*-like organisms were identified in 40 biopsies (90%) by immunohistochemistry (Fig. 1) and in 35 (100%) using the modified Giemsa method (Table 1). Prevalence of infection detected by histopathological study is shown in Table 2. No age (P=0.654) or gender (P=0.745) distribution differences were found.

CG lesions were found in 86 patients (91%), IM lesions in 69 patients (73%) and dysplasia in five patients (5%). Most patients showed no colonisation.

No age or gender differences were apparent among the histopathological lesions. Patients with CG had a median age of 61 years (range: 36–70), while those with IM and dysplasia had median ages of 60 (range: 26–75) and 64 (25–75), respectively (P=0.412, Mann-Whitney test).

IgG, IgM and IgA determination

Area under the curve (AUC) (95% CI) for *H. pylori* diagnosis was 0.40 (0.28–0.51) for IgM; 0.69 (0.58–0.80) for IgA and 0.83 (0.74–0.92) for IgG. Best cut-ff was 41 u/mL for IgG, with a sensitivity (95% CI) of 90% (84–96%) and NPV of 91% (85–97%); and 22 u/mL for IgA, with a sensitivity of 74% (65–83%) and NPV of 77% (68–86%).

IgG and IgA positivity for *H. pylori* in patients with no lesions, CG, IM and dysplasia is shown in Table 3.

Discussion

Colonisation of the gastric epithelium by *H. pylori* results in an inflammatory reaction, promoting the development of different gastric pathologies. *H. pylori* is known to be

Table 1. *H. pylori* infection diagnosis by culture, immunohistochemical and modified Giemsa methods (n=94).

H. pylori infection determined by culture	*H. pylori* infection determined by histopathological methods				
n (%)	*n* (%)				Total
	IHC		MG		
	Negative	Positive	Negative	Positive	
Negative	52 (96)	4 (10)	56 (95)	0 (0)	56 (60)
Positive	2 (4)	36 (38)	3 (5)	35 (100)	38 (40)
Total	54	40	59	35	94

IHC: immunohistochemistry

MG: modified Giemsa

Table 2. Prevalence of culture-detected *H. pylori* infection among gastric lesions found in endoscopic biopsies (n=160).

	Patients *n*	*H. pylori* infection *n* (%)
Chronic gastritis	86	38 (44)
Non-atrophic CG	52	26 (50)
Atrophic CG	34	12 (35)
Intestinal metaplasia	69	26 (38)
Complete IM	13	5 (39)
Incomplete IM	56	21 (38)
Dysplasia	5	1 (20)
		P=0.039

associated with the development of gastritis [1-11, 20-27] and, if not treated, may result in CG and atrophy, a precursor to the development of gastric carcinoma.[2,20-25] More severe lesions such as IM and dysplasia predispose to the development of gastric adenocarcinoma.[20-25, 31-33]

Studies of the Portuguese population have verified that *H. pylori* infection affects 80–90% of the adult population[8,22] and 50% of the paediatric population.29 In the present study, this prevalence was not verified because only 38 (40%) of the 94 patients presented with gastric infection. This may have been related to our selection criteria of patients with severe gastric lesions that predispose to gastric carcinogenesis, or perhaps because biopsies were not representative of the infected gastric area.

Of the 94 patients studied, five showed no lesion in the gastric mucosa. The remainder presented with CG, IM and dysplasia. We were able to verify that all *H. pylori*-positive cases had CG, most of which also showed mucosal atrophy. This is consistent with the fact that *H. pylori* colonisation causes cell damage, resulting in gastritis and more severe lesions if untreated.

Some 38% of patients with IM (all with gastritis) were infected. In other studies, however, *H. pylori* was not found in association with IM, as these lesions are not thought to provide an appropriate environment for survival of the microorganism.[32, 36-38] El-Zimaity *et al* reported [36] that in IM areas the gastric mucosal phenotypes are substituted by the intestinal mucosal phenotypes, resulting in pH change and

Table 3. IgG and IgA positivity for *H. pylori* in patients with no lesions, chronic gastritis (CG), intestinal metaplasia (IM) and dysplasia.

		No lesions	CG	IM	Dysplasia
	IgG (u/mL)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Manufacturer*	Pos (>30)	3 (60)	63 (74)	46 (67)	5 (100)
	Neg (<20)	2 (40)	15 (17)	15 (21)	0 (0)
In-house cut-off	Pos (>41)	2 (40)	47 (55)	36 (52)	2 (40)
	Neg (<41)	3 (60)	39 (45)	33 (48)	3 (60)
	IgA (u/mL)				
Manufacturer*	Pos (>30)	1 (20)	52 (61)	39 (57)	4 (80)
	Neg (<20)	4 (80)	26 (30)	24 (35)	1 (20)
In-house cut-off	Pos (>22)	1 (20)	48 (56)	36 (52)	1 (20)
	Neg (<22)	4 (80)	38 (44)	33 (48)	4 (80)

* Manufacturer data results considered as borderline were excluded.

eradication of *H. pylori*. However, anti-*H. pylori* antibodies were detected in most patient by serological means, indicating previous infection.

The present study verified that *H. pylori* prevalence decreases with the severity of the gastric lesion. However, some workers have reported the microorganism in individuals with IM,[34] especially when differential staining methods are used (modified Giemsa, Warthin–Starry, silver stain). Clearly, histopathological analysis can be influenced by factors such as bacterial density, mucosal location and the pathologist's experience.[36-37]

H. pylori infection of the gastric mucosa results in immunological responses, with elevation of specific IgG and IgA levels in the serum,[10-16] and elevated levels of secretory IgA and IgM in the stomach.[16] IgA is considered to be a specific local immunoglobulin because the number of secretory gastric cells increases when *H. pylori* is present.[29-31]

IgG is a secondary antibody, appearing in chronic *H. pylori* infection of the gastric mucosa.[1] In support of previous work,[16,17-24] we verified that the majority of infected adults present with IgG antibodies. In the absence of treatment, antibody levels remain high but IgG and IgA tend to decrease after eradication. However, Kosunen *el al*.[41] showed that IgG titres can remain elevated for quite some time after eradication, which might explain the higher IgG prevalence found in the present study.

The study by Li *et al*.,[42] in patients with precancerous lesions (CG, IM and dysplasia), reported that both serum anti-*H. pylori* IgG and IgA titres were higher in *H. pylori*-positive patients. In the present study, however, we showed that IgG titres dropped as the severity of the lesion increased. This supports the work of Osawa *et al*.,[9] who reported a negative correlation between the extent of metaplasia and anti-*H. pylori* titre.

Some authors suggest that different immunological responses may be due to a difference in bacterial species between the human stomach and those used to produce the serological detection kits.[18-20] Sensitivity and specificity of serological test kits depend on the antigen preparation used. Also, there are differences in the antigenicity of the various *H. pylori* strains, so different immunological responses should be expected.[1] As IgG and IgA are more specific antibodies, testing for both with new cut-off values would appear to permit more accurate identification of positive patients.

In conclusion, we suggest that non-invasive serological evaluation of anti-*H. pylori* (IgG) status after eradication therapy for peptic ulcer disease[15,16] could be extended, after proper assessment of cut-off values and their validation, to the follow-up of patients with CG and IM. □

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