Western Blotting of Total Lysate of *Helicobacter pylori* in Cases of Atrophic Body Gastritis

ROBERTA MINI, BRUNO ANNIBALE, EDITH LAHNER, GIULIA BERNARDINI, NATALE FIGURA, and ANNALISA SANTUCCI

**Background:** Atrophic body gastritis is considered the first important step in the histogenesis of gastric carcinoma, a multistep process starting from chronic gastritis and progressing through chronic atrophic gastritis, intestinal metaplasia, and dysplasia. *Helicobacter pylori* is involved in the induction of atrophic body gastritis, but documentation of *H. pylori* infection is difficult because of the progressive disappearance of the bacterium. Our study aimed to detect past *H. pylori* infection in patients with atrophic body gastritis.

**Methods:** We used Western blot analyses of whole bacterial protein lysate of 2 different strains to probe sera from 143 patients. All sera were analyzed by ELISA (Bio-Rad), and results of gastric histology were available for all patients.

**Results:** Among 111 patient sera previously classified as negative for *H. pylori* infection by ELISA, 106 (95.5%) were positive when assayed by immunoblotting.

**Conclusions:** Commercial diagnostic reagent sets may fail to detect *H. pylori* infection. Western blotting of whole bacterial protein extracts could provide the basis of a noninvasive serology tool able to assess previous infection with *H. pylori* in patients with atrophic body gastritis.

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Atrophic body gastritis (ABG), a disorder that increases the risk for gastric cancer (1), is characterized by chronic inflammation of oxyntic mucosa that leads to progressive atrophy of the oxyntic glands with consequent hypochlorhydria (2). ABG is considered the first important step in the histogenesis of gastric carcinoma (3), which develops in a multistep process starting from chronic gastritis and progressing through chronic atrophic gastritis, intestinal metaplasia, and dysplasia (3). This sequence is usually triggered by *Helicobacter pylori* infection and is affected by a variety of genetic and environmental factors that may act synergistically (4). *H. pylori* is involved in the induction of ABG (5, 6), and longstanding uncured *H. pylori* antral gastritis could promote, as a sequel, atrophy of the body mucosa (7).

Treatment of *H. pylori* infection that could lead to major gastric pathology is recommended (8). By promoting healing of the gastric mucosa, pharmacologic treatments may reduce the risk of gastric cancer (9). In patients with ABG, diagnosis of an active *H. pylori* infection is difficult because of the progressive disappearance of the bacterium (10). Discrepancies between the detection of *H. pylori* antibodies and the presence of the microorganism in biopsy specimens from patients with advanced atrophic gastritis are common as a consequence of the gradual reduction of *H. pylori* colonization of the gastric mucosa during the progression of atrophic gastritis and development of hypochlorhydria (6, 11). In patients with ABG, anti-*H. pylori* antibody concentrations may be increased without histologic evidence of infection (12–14). In accordance with this finding, similar posttreatment healing of the corpus mucosa was observed in patients with histology-negative, serology-positive *H. pylori* infection and in those with positive histology and serology (15). In a subset of ABG patients, *H. pylori* infection was detected only with serologic tests (16, 17), which cannot differentiate between active and past infection (18). A reliable diagnostic test for active *H. pylori* infection in patients with ABG is therefore needed.

No currently available diagnostic tests for current or past infection with *H. pylori* can be considered, in clinical
practice, as the gold standard method. A single test cannot reliably detect the presence of *H. pylori*; thus, a combination of at least 2 tests is recommended (8), particularly for clinical identification in ABG patients with active *H. pylori* infection (9, 13, 19) and the risk of gastric neoplasia (20).

The use of single recombinant bacterial proteins in most conventional current diagnostic serology systems limits the detection of the whole anti-*H. pylori* immunoglobulin repertoire. In this study, we used immunoblotting with the total lysate of 2 different strains of *H. pylori* to probe the serostatus of samples from 143 patients with histologically and serologically defined *H. pylori* infection.

Materials and Methods

Bacterial Strains and Culture Conditions

*H. pylori* strains 10K [cagA* A* subtypes, vacA* s1/m1 subtype] and 4Cb ([cagA* C subtype, vacA* s1/m2 subtype] were isolated from biopsy samples of patients with intestinal-type gastric carcinoma (strain 10K) and diffuse-type gastric carcinoma (strain 4Cb). Biopsy tissue was streaked on the surface of Columbia agar containing 50 mL/L horse blood, 10 mg/L vancomycin, 5 mg/L trimethoprim, 20 kIU/L polymyxin B, and 5 mg/L cefazolin. Plates were incubated in a microaerobic environment obtained by use of an anaerobic jar with a gas generator that produced a microaerophilic atmosphere (10% CO₂, 6% O₂, 84% N₂; Oxoid Camp GasPak; Oxoid). Colonies resembling *H. pylori* were identified by Gram stain and by oxidase, catalase, and urease tests.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

We performed sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions with a MiniPROTEAN® 3 Cell (Bio-Rad) (21), using a 4% stacking gel and a 12% separating gel. After sonication for 20 min, the bacterial suspension was denatured in sample buffer for SDS-PAGE. All samples were heated for 5 min in boiling water, cooled to room temperature, and subjected to electrophoresis (100 mg of protein loaded in 1 large well) with a constant voltage of 150 V until the dye front reached the bottom of the gel. At the end of the run, proteins were detected with Coomassie Brilliant Blue R250, or alternatively, electropherograms were transferred to nitrocellulose membranes.

2-Dimensional PAGE

The sonicated *H. pylori* bacterial suspension was denatured in the buffer for the first dimension of 2-dimensional PAGE consisting of a solution containing 8 mol/L urea, 40 g/L CHAPS (3-[cholamidopropyl]dimethylamino]-1-propanesulfonate), 40 mmol/L Tris, 65 mmol/L dithioerythritol, and a trace of bromphenol blue. We performed 2-dimensional PAGE according to procedures detailed elsewhere (22) and applied 50 μg of protein sample to an Immobiline strip (Immobilon pH Gradient; Amersham Pharmacia Biotech) consisting of a nonlinear pH 3.5–10 gradient, previously rehydrated. Isoelectric focusing was carried out on a Multiphor II (Amersham Pharmacia Biotech). The voltage was linearly increased from 300 to 3500 V during the first 3 h and then stabilized at 5000 V for 22 h (total, 110 kV h). The Immobilized pH Gradient strip was then equilibrated in 6 mol/L urea, 300 g/L glyceral, 20 g/L SDS, 0.05 mol/L Tris-HCl (pH 6.8), and 20 g/L dithioerythritol and then with 25 g/L iodoacetamide. We performed electrophoresis in the second dimension on a 9%–16% polyacrylamide linear gradient gel (18 cm × 20 cm × 1.5 mm) at a constant current of 40 mA. Analytical gels were stained with silver nitrate as described previously (23). Digitized images were obtained by scanning the gels with a laser densitometer (Molecular Dynamics) and then were analyzed qualitatively and quantitatively with Melanie II 2-D PAGE software (Bio-Rad). The spot intensities were obtained in pixel units and normalized to the total absorbance of the gel.

Patients and Human Sera

We analyzed available sera from 143 patients with ABG diagnosed according to the updated Sydney system (24). Patient sera were classified into groups on the basis of *H. pylori* detection by histologic (Giemsa stain) and serologic [anti-*H. pylori* IgG antibodies; determined by a commercial ELISA (Bio-Rad)] analysis, as reported previously (18). The reported specificities and sensitivities of the assays were 94% (range, 88%–97%) and 79% (range, 65%–90%), respectively (25) (Table 1). Group I consisted of 31 sera from patients both histologically and serologically negative for *H. pylori* infection (negative/negative); group II consisted of 80 sera from patients histologically negative and serologically positive for *H. pylori* infection (negative/positive); and group III consisted of 32 sera

<table>
<thead>
<tr>
<th>Table 1. Characteristics of patients.</th>
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<tr>
<td><strong>Group I</strong> (Negative/Negative)</td>
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<td>n</td>
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<tr>
<td>M/F, n</td>
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<tr>
<td>Median (range) age, years</td>
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<tr>
<td>Mean (SE) body mucosa Sydney score/Atrophy</td>
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* Negative/Negative, Giemsa stain and ELISA serology both negative; Negative/Positive, Giemsa stain negative and ELISA serology positive; Positive/Positive, Giemsa stain and ELISA serology both positive.
from patients both histologically and serologically positive for *H. pylori* infection (positive/positive).

We used as positive controls anti-CagA, anti-VacA, anti-HspB, anti-UreA, and anti-UreB rabbit polyclonal antibodies kindly provided by Rino Rappuoli (Chiron Vaccines, Siena, Italy). We used as negative controls sera obtained from 5 patients without ABG and that were histologically and serologically (commercial ELISA and Western blotting tests) negative for *H. pylori* infection.

**WESTERN BLOTTING**

Proteins were electrotransferred from gels to nitrocellulose sheets by semidry blotting with a Novablot semidyblot cell and a transfer buffer containing 50 mmol/L Tris, 39 mmol/L glycine, 1 mmol/L SDS, and 200 mL/L methanol (26). The electrotransfer time was 45 min with a current/area of 0.7 mA/cm². The membranes were washed in phosphate-buffered saline (PBS), blocked for 1 h in 30 g/L bovine serum albumin in PBS, and then again washed 3 times for 10 min in PBS. The membranes were incubated for 2 h at room temperature with human sera from patients affected by ABG, with polyclonal antibodies, or with positive and negative pooled sera at a dilution of 1:1000. Immunodetection of IgG was revealed with goat anti-human IgG horseradish peroxidase–conjugated immunoglobulin (Bio-Rad) at a dilution of 1:80,000 (by volume) for human sera and with goat anti-rabbit IgG horseradish peroxidase–conjugated immunoglobulin (Bio-Rad) at a dilution of 1:80,000 for polyclonal antibodies. Before and after each step, the membranes were washed extensively 3 times for 10 min in PBS. Enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) was used for the final detection.

**STATISTICAL ANALYSIS**

Data are reported as the mean (SD). Statistical comparisons were performed with ANOVA with the Bonferroni post hoc test. Differences between groups were considered statistically significant at *P* < 0.05.

**Results**

An example of the results obtained is shown in Fig. 1 (the entire collection of strip images is available on request).

**SEROREACTIVITY TO ANTIGENS OF STRAIN 4Cb**

Immunoblotting data obtained with strain 4Cb indicated that 138 of 143 sera were positive for *H. pylori* infection (Table 2 in the online Data Supplement), regardless of their previous group designation. We considered as positive all sera containing IgG recognizing 2 to 5 known antigen markers. The mean number of reactive known antigens per group of patients indicated that all of the groups were positive for *H. pylori* infection [group I, 3.45 (1.09); group II, 3.62 (1.14); and group III, 4.19 (1.09)]. Statistical analysis of the data indicated homogeneity in immunopositivity among sera groups (*P* = 0.015).

In group I (negative/negative patients), immunoblotting analysis showed only 1 serum negative for *H. pylori* infection. Specifically, 96.8% of patients had anti-VacA IgG, 93.5% had anti-UreA, 80.6% had anti-UreB, 35.4% had anti-CagA, and 32.2% had anti-HspB IgG (Table 2). Only 4 of 80 sera of group II (negative/positive) were negative for *H. pylori* infection: 93.7% of sera had anti-UreA IgG, 90% had anti-VacA, 87.5% had anti-UreB, 48.7% had anti-CagA, and 41.2% had anti-HspB IgG (Table 2).

In accordance with the results obtained with other tools used for diagnosis of *H. pylori* infection and patient classification, all sera of group III were positive by immunoblotting analysis: 100% of sera had anti-VacA IgG, 93.7% had anti-UreB, 87.5% had anti-UreA, 71.8% had anti-CagA, and 68.7% had anti-HspB IgG (Table 2).

Each group had strong seroreactivity against the standard marker antigens probed. Moreover, negative sera of both adopted strains had similar immunoreactive responses. R129 (group I) and R123 (group II) sera were negative for the whole antigenic pattern for both strains; Q663 (group II) serum was negative for strain 10K but was immunoreactive against the VacA antigen of strain 4Cb. Analogously, serum Q78 (group II), negative for the 4Cb strain, immunorecognized VacA antigen in strain 10K.
We observed strain specificity by comparing the seroreactivity to CagA in 2 different strains. Although each serum showed similar reactivity to most of the antigens tested in this study, the immunoreactivity to CagA was very different for strains 4Cb and 10K. This difference could be related to differential expression of the cagA gene in these strains, as deduced from the comparison of the proteome maps of H. pylori strains 10K and 4Cb. Each 2-dimensional gel was obtained by 2-dimensional PAGE using a nonlinear pH 3–10 gradient in the first dimension and a linear 9%-16% polyacrylamide gradient covering the molecular mass range 200 000 to 8000 Da in the second dimension. The relative volume percentage was quantified for the spot corresponding to CagA for both maps; it was 0.836 for strain 10K and 0.134 for strain 4Cb, thus showing a 6.24-fold increase for 10K with respect to 4Cb. CagA was identified on a nitrocellulose replica of the 2-dimensional gel by immunoblotting (data not shown).

Table 2. Seropositivity of group I, II, and III sera against 5 markers on both strains.

<table>
<thead>
<tr>
<th></th>
<th>Strain 10K</th>
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<th>Strain 4Cb</th>
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<tbody>
<tr>
<td></td>
<td>Group I</td>
<td>Group II</td>
<td>Group III</td>
<td>Group I</td>
</tr>
<tr>
<td>CagA</td>
<td>80.6</td>
<td>68.7</td>
<td>87.5</td>
<td>35.4</td>
</tr>
<tr>
<td>UreB</td>
<td>74.2</td>
<td>73.7</td>
<td>87.5</td>
<td>80.6</td>
</tr>
<tr>
<td>HspB</td>
<td>19.3</td>
<td>32.5</td>
<td>65.6</td>
<td>32.2</td>
</tr>
<tr>
<td>VacA</td>
<td>87.1</td>
<td>86.2</td>
<td>96.9</td>
<td>96.8</td>
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<tr>
<td>UreA</td>
<td>87.1</td>
<td>83.7</td>
<td>93.7</td>
<td>93.5</td>
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</tbody>
</table>
by use of the same anti-CagA rabbit polyclonal antibodies adopted for 1-dimensional Western blotting under the same experimental conditions.

**Discussion**

Our immunoblotting analysis results indicated that 138 of 143 patients affected by ABG had anti-\textit{H. pylori} antibodies, confirming the hypothesis that \textit{H. pylori} infection plays a specific role in ABG. Moreover, seroreactivity to the \textit{H. pylori} antigens was similar among the 3 sera groups for both \textit{H. pylori} strains. Statistical analysis of immunoreactivity results against the 5 marker antigens showed strong positivity, and we found homogeneity of positivity among the 3 groups, thus negating any previous classification of sera based on commercial \textit{H. pylori} infection tests.

Remarkably, we found positive sera among samples from patients classified as negative according to histologic assays. This result may be related to ABG stage and patient age, both of which affect \textit{H. pylori} disappearance, which in turn leads to reduction/disappearance of antibodies other than those related to the immunologic memory. The spontaneous disappearance of anti-\textit{H. pylori} antibodies in patients with advanced ABG has been documented by ELISA tests (27) and could explain the negative/negative cases found in this study. Because of the high genetic variability of \textit{H. pylori} and because long-term infection is accompanied by a decrease of circulating antibodies, negative serology may also be related to inadequate sensitivity of ELISA tests that use single-bacterium recombinant proteins.

The 2 different \textit{H. pylori} strains used in this study, 10K and 4Cb, have individual features relevant to immunoblotting applications. The 2 strains were from the same geographic area and thus autochthonous with respect to the sera probed, but they were derived from 2 different pathologic specimens of gastric adenocarcinoma, the intestinal histotype and the diffuse histotype. These histologically distinct variants differ pathologically both in origin and evolution. The intestinal type progresses through a stepwise transition from healthy mucosa to metaplasia and then to a potentially malignant final destiny. In contrast, the diffuse type appears in healthy gastric mucosa without any apparent association with metaplasia (28). Moreover, the use of 2 different \textit{H. pylori} strains with different protein and antigenic (data not shown) repertoires also allowed us to investigate the importance of the specificity of single-protein antigen immunorecognition. Even a well-known immunodominant bacterial antigen such as CagA, widely considered the most reliable \textit{H. pylori} antigen because anti-CagA immunoglobulins are the most long-lived circulating antibodies (29), can yield false-negative results for anti-\textit{H. pylori} immunoglobulins if the strain is \textit{cagA}-negative or has poor \textit{cagA} expression (such as strain 4Cb compared with 10K). False-negative results can also occur when single recombinant proteins, often reproducing only portions of native proteins or missing important bacterial posttranslational modifications (30), are used in \textit{H. pylori} infection tests.

At least 4 of the 5 antigens we focused on are undoubtedly specific for \textit{H. pylori} infection. The 2 virulence factors CagA and VacA, although not expressed by all strains, are proteins coded exclusively by the \textit{H. pylori} genome. In regard to the specificity for \textit{H. pylori} in Western blotting assays, a recently published exhaustive comparative study (31) classified CagA, VacA, and UreA as “HS, highly specific” for \textit{H. pylori} infection, whereas HspB was classified as “S, specific”. Only UreB was considered not specific for \textit{H. pylori} by those authors, but in our experience the presence of the \textit{a} subunit of urease depends exclusively on reduction of the disulfide bridge linking the \textit{a} to the \textit{b} subunit (SDS-PAGE under reducing conditions); therefore, it is impossible to have the first without the second. Analogously, a comparative Western blotting study carried out at the proteome level confirmed the specificity of those 5 antigens for \textit{H. pylori} infection (32). Moreover, CagA, VacA, and urease are well-known \textit{H. pylori}-specific virulence factors; thus, all 3 are considered equally viable candidates for a vaccine against such a bacterium.

Three procedures were important to the success of our approach in revealing \textit{H. pylori} infection: (a) the use of a whole bacterial protein lysate, a technique that probes almost the entire protein repertoire and is particularly relevant for the specific identification of low–molecular-mass (32–14 kDa) bacterial antigens important for the detection of infection, particularly for ongoing infection, and the evaluation of eradication (33); (b) the use of more than 1 strain of \textit{H. pylori}, thus covering a qualitatively and quantitatively wider range of antigens to be tested and providing a more reliable control on results; and (c) the use of one of the most sensitive detection systems, enhanced chemiluminescence. As a consequence, our study provides the basis for an improved diagnostic system for \textit{H. pylori} infection, at least in cases of ABG, for which an efficient test is required for both diagnosis of infection and verification of eradication.

A sensitive and reliable diagnostic test is important in ABG patients because \textit{H. pylori} disappears as the disease progresses As a consequence, biopsy-based diagnostic tests might give false-negative results, especially if \textit{H. pylori} infection is patchy or if the bacterial colonization is low. In clinical practice, the diagnosis of active \textit{H. pylori} infection in ABG patients is generally made after histologic examination of antral and body mucosa specimens with Giemsa and Genta tissue stains, in addition to the routine hematoxylin and eosin, during the diagnostic work-up of ABG (20, 34). It has been shown that anti-\textit{H. pylori} antibody concentrations are increased in patients with ABG without histologic evidence of infection (12–14). On the other hand, according to some authors (15), serology is an elective tool for \textit{H. pylori} diagnosis in ABG.
patients. Lahner et al. (35) investigated the role of the $^{13}$C-urea breath test and the $H. pylori$ stool antigen test as additional diagnostic tools for active $H. pylori$ infection in patients with ABG, but neither of these tests was considered a gold standard method in clinical practice. Moreover, in patients with ABG, determining the efficacy of the pharmacologic treatment to eradicate $H. pylori$ represents a problem. Eradication of the microorganism itself in these patients could reduce the risk of gastric neoplasia because ABG is an important step of the process leading to intestinal adenocarcinoma. In patients with atrophic gastritis, the eradication of $H. pylori$ led to substantial long-term reduction of symptoms of nonulcer dyspepsia with fundic atrophic gastritis (36). In patients with non-atrophic gastritis, ELISA has been considered a valid tool for the diagnosis of $H. pylori$ and may be helpful in verifying the eradication of bacterium (37, 38) because the decrease in anti-$H. pylori$ antibody titer after treatment is related to the healing of gastric inflammation (39). Serology alone may not be a valid tool to assess the efficacy of the eradication therapy in patients with ABG, however, because in most of these patients, the decrease in immunoglobulin titer does not coincide with the reduction in gastric inflammation (18).

Unfortunately, our study results cannot have any direct implication in clinical routine work at the diagnostic level because the whole system is quite expensive and time-consuming and is not commercially available. Nevertheless, our results can provide physicians with an important clinical “take-home-message”, underlined by other authors (40), that can be extremely useful for correct interpretation of seroepidemiologic studies.

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References