Cross-Reactivity between Immune Responses to Helicobacter bilis and Helicobacter pylori in a Population in Thailand at High Risk of Developing Cholangiocarcinoma*

Paola Pisani1,7,* Mark T. Whary,2 Ingrid Nilsson,3 Supannee Sriamporn,4 Torkel Wadström,3 James G. Fox,2,5,† Åsa Ljungh,3,† and David Forman6,†

Descriptive Epidemiology Group, International Agency for Research on Cancer, 150 Cours Albert-Thomas, 69372 Lyon Cedex 03, France;1 Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Massachusetts2; Department of Laboratory Medicine, Division of Medical Microbiology, University of Lund, Lund, Sweden3; Department of Epidemiology, Faculty of Public Health, Khon Kaen University, Khon Kaen, Thailand4; Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts5; Centre for Epidemiology and Biostatistics, Leeds Institute of Genetics, Health and Therapeutics, University of Leeds, Leeds, United Kingdom6; and Cancer Epidemiology Unit, University of Oxford, Oxford, United Kingdom7

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Helicobacter bilis DNA has been detected in human tissue and is a candidate for etiologic investigations on the causes of hepatic and biliary tract diseases, but reliable serologic tests need to be developed in order to pursue such investigations. The scope of this study was to assess the specificity of two assays for H. bilis immune response allowing for H. pylori, and their cross-reactivity in a population in Thailand at high risk for cholangiocarcinoma. Plasma samples from 92 Thai volunteers were independently tested in two laboratories (Massachusetts Institute of Technology [MIT] and Lund). MIT performed three analyses of H. pylori and H. bilis based either on (i) outer membrane protein (OMP) with no preabsorption or on antigens derived from whole-cell sonicate before (ii) or after (iii) preabsorption with H. pylori sonicate protein. Lund used cell surface proteins from H. pylori and H. bilis as antigens. Testing for H. bilis was preabsorbed with a whole-cell lysate of H. pylori. More than 80% of the samples were positive for H. pylori in both laboratories. As tested by MIT, 58.7% (95% confidence interval, 47.9 to 68.9%) were positive for H. bilis by OMP and 44.5% (34.1 to 55.3%) were positive for H. bilis sonicate protein, but only 15.2% (8.6 to 24.2%) remained positive after preabsorption with H. pylori sonicate protein. Lund found 34.5% of the samples positive for H. bilis (22.0 to 41.0%), which was statistically compatible with all three MIT results. Serologic responses to OMPs of the two bacteria coincided in 66 and 45% of the samples in the MIT and Lund assays, respectively. We found high cross-reactivity between the immune responses to H. pylori and H. bilis antigens. More-specific H. bilis antigens need to be isolated to develop serologic tests suitable for epidemiological studies.

An increasing number of Helicobacter species (other than Helicobacter pylori) that colonize the enterohpatic tract of animals and humans have been identified in recent years (8). Some cause hepatitis and hepatocellular carcinoma in animal models (10) and bacteremia in both immunocompromised and immunocompetent hosts (19, 24). The documented role of H. pylori in developing duodenal and gastric ulcer disease and stomach cancer in humans (22) justifies the hypothesis that some of these bacteria may have a role in human enterohpatic diseases.

The presence of H. bilis DNA, one of the eight species identified in humans, was first described in the bile and gallbladder tissue of Chilean patients affected by cholecystitis in a region where the incidence of gallbladder cancer was high (9). The DNA of enteric species, including H. bilis, H. hepaticus, and H. pullorum, has been identified by PCR in neoplastic and normal tissues of cancer patients (29), in pediatric liver tissue (45), and in various chronic hepatic disorders, including malignancy and intra- and extrahepatic biliary diseases (2, 6, 20, 23, 37). In particular, H. bilis DNA was more common in Thai and Japanese patients affected by malignancies of the biliary tract than in patients affected by nonmalignant disorders (23). Because H. bilis has been associated with chronic hepatitis (11, 12), inflammatory bowel disease (3, 13, 40), and cholecystitis (25) in mouse models, potential H. bilis infection in humans appears to be suitable for epidemiological investigations.

Epidemiological studies require, however, the availability of noninvasive tests to compare large numbers of cases and suitable controls.

In the context of a long-term prospective investigation on cholangiocarcinoma in rural Thailand (42), we assessed whether individuals from the resident population expressed detectable plasma immunoglobulin G (IgG) to H. bilis antigens that can be discriminated from antibody responses to H. pylori infection. To establish reliable biomarkers based on minimally invasive tests is a necessary prerequisite to the development of epidemiological studies on the etiologic relevance of different Helicobacter species.

MATERIALS AND METHODS

Plasma samples of 92 subjects from a cohort of 24,000 volunteers recruited in northeastern Thailand between 1990 and 2001 were retrieved from the cohort...
biobank. At recruitment, each volunteer signed informed consent and donated a blood sample that was divided into several aliquots and stored at −20°C (42). The 92 subjects were selected to represent the cohort: 32 were men, the mean age of the group was 55.0 ± 10.0 years (range, 20 to 70 years), and 19.6% were positive for Opisthorchis viverrini, a liver fluke endemic in this population. Infection with hepatitis B and C viruses was not assessed in this set of subjects. However, the prevalences of participants positive for hepatitis B surface antigen and core and surface antigens to hepatitis C in a random sample tested at baseline were 10 and 4%, respectively (unpublished data).

The 92 vials of plasma were thawed, split into two aliquots (0.2 to 0.4 ml), and shipped in dry ice to the two laboratories at the Massachusetts Institute of Technology (MIT) in Cambridge, MA, and the University of Lund (Lund) in Sweden. The laboratories conducted the analyses independently and were blinded with respect to any characteristics of the participants.

**Laboratory assays at MIT: bacterial strains and culture conditions.** The MIT laboratory performed two analyses for antibody titers to H. pylori and H. bilis, one based on outer membrane proteins (OMPs) with no preabsorption and the second on antigens derived by whole-cell sonication (51). In the second assay, comparisons were made between IgG levels before and after preabsorption with H. pylori sonicate protein. To prepare OMPs, H. pylori and H. bilis were cultured in brucella broth containing 5% fetal bovine serum for 24 h under microaerobic conditions. After 3 washes in phosphate-buffered saline (PBS) and examination for bacterial contaminants using Gram staining and phase microscopy, the pellet was resuspended in 4 ml of 1% N-octyl-β-glucopyranoside (Sigma, St. Louis, MO) for 30 min at room temperature. Insoluble material was removed by ultracentrifugation at 100,000 × g for 1 h. After dialysis against PBS for 24 h at 4°C, supernatant protein concentration was measured by the Lowry technique (175784). Then, the suspensions of both bacterial cultures were resuspended in sterile PBS and sonicated on ice (Artect Sonic Dismembranator; Artect Systems, Framingdale, NY). Sonication was for four cycles of 30 s on and 30 s off at a duty cycle of 50% and with power applied slowly to 60 W. After sonication, the mixture was examined by phase-contrast microscopy to confirm the absence of intact bacteria, followed by determination of the protein concentration as described above. OMP antigens for the first analysis consisted of OM proteins extracted from five clinical isolates of H. pylori (Hp1018, Hp1010, NQ366, NQ1725, and NQ1708) mixed in equal amounts based on protein concentration. The ATCC type strain of H. bilis (ATCC S6130) was used. Sonicate antigens for the second analysis were prepared from four of the five previously listed clinical isolates of H. pylori (Hp1018, Hp1010, NQ366, and NQ1708), also mixed in equal amounts based on protein analysis. H. bilis sonicate was prepared using the same ATCC type strain as described above.

For all assays, a checkerboard titration of reagents was performed to identify the optimal enzyme-linked immunosorbent assay (ELISA) conditions. Sera from eight confirmed H. pylori-positive (four from Louisiana and four from Colombia) and five confirmed negative patients (two from Louisiana and three from Colombia) were used as controls (based on PCR, culture, and histology). Seroconversion to H. pylori was defined as ELISA values that exceeded the mean plus three standard deviations from the ELISA optical density of the negative sera obtained from the confirmed negative H. pylori sera. Seroconversion to H. bilis could not be proven and was based on ELISA values generated from the samples from the five confirmed H. pylori-negative patients.

**MIT laboratory, first analysis.** For plasma IgG measurement, 96-well Immunolon II plates (ThermoLab Systems, Franklin, MA) were coated with 100 g/ml concentrations of antigen. To preabsorb plasma with H.9261 azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt substrate (Sigma) was used. A pool of 10 sera, determined to be negative by ELISA for H. pylori and H. bilis, was included as a negative control, and a polyclonal rabbit antisem to the two Helicobacter species was applied as a positive control. After a washing step, an alkaline phosphate-conjugated anti-human IgG (diluted 1/30,000; Sigma, St. Louis, MO) or an anti-rabbit IgG antibody (diluted 1/500; DacoCytomation AS, Glostrup, Denmark) was used as a secondary antibody, and the plates were again incubated for 1 h at 37°C. Antibody absorbance was visualized by adding a substrate buffer containing 1 mg of p-nitrophenyl phosphate (Sigma) per ml in a diethanolamine buffer (pH 9.8). The absorbance was measured using an ELISA reader (model 2000) at 405 nm after 35 min of incubation. The absorbance was measured at 600 nm before and after incubation. The results are expressed as the corrected mean absorbance values in percentages of the reference standard and are presented as the relative antibody activity (RAA). The cutoff value for seropositivity to H. pylori is based on sera from patients with culture-positive and -negative biopsy specimens, sera from children, and blood donors. An RAA value of >35 was regarded as positive, and an RAA value between 25 and 35 regarded as a low-positive (borderline) result.

For H. bilis (RAA > 35), the cutoff values were established based on comparisons of the ELISA results of 90 blood donors (Swedish) corresponding to the upper limit for the 95th percentile level.

**iv) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting.** Extracted CSPs of H. pylori and H. bilis were separated under reducing conditions using Criterion gel electrophoresis equipment (Bio-Rad) in 8 to 16% gradient gels with a 4% stacking gel (Criterion precast gel; Bio-Rad). Proteins were blotted onto polyvinylidene difluoride membranes (Micron Separations, Westborough, MA) for antibody detection, using semidyed electrophoretogram equipment (Anoc, Vigs, Denmark). The transfer time was 90 min at a constant current of 0.8 mA/cm². The membranes were blocked in a blocking solution containing 0.15% gelatin, polivinylpyrrolidone, Tween 20, ethanolamine, and glycine; cut into strips; probed with serum samples diluted 1/100 in a Tris buffer containing gelatin hydrolysate, sodium chloride, and Tween 20 (pH 8.7) (24); and then incubated for 1 h at 8°C with gentle shaking. After repeated washings, horseradish per...
TABLE 1. MIT laboratory: within-subject association between *H. pylori* and *H. bilis* immune response

<table>
<thead>
<tr>
<th>Antigen and <em>H. bilis</em> detection result</th>
<th>No. of samples (% positive) with result for <em>H. pylori</em> detection with OMP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Agreement</th>
<th>Kappa</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMP (no preabsorption)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>49 (58.7)</td>
<td>66.3</td>
<td>0.243</td>
<td>0.003</td>
</tr>
<tr>
<td>Negative</td>
<td>26</td>
<td>12</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>75 (81.5)</td>
<td>17</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Sonicate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>38 (44.5)</td>
<td>56.5</td>
<td>0.186</td>
<td>0.007</td>
</tr>
<tr>
<td>Negative</td>
<td>37</td>
<td>14</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>17</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Preabsorbed sonicate&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>14 (15.2)</td>
<td>33.7</td>
<td>0.0786</td>
<td>0.03</td>
</tr>
<tr>
<td>Negative</td>
<td>61</td>
<td>17</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>17</td>
<td>92</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent positive values are indicated in parentheses.

<sup>b</sup> That is, the *H. bilis* sonicate after preabsorption with the *H. pylori* sonicate.

oxidase-labeled anti-human IgG antibodies (DakoCytomation) diluted 1:600 were added, and the strips were incubated for 2 h at 8°C. Bound antibodies were detected by adding a 50 mM sodium acetate buffer (pH 5.0) containing 0.04% 3-aminoo-9-ethylcarbazole (Sigma) and 0.015% H<sub>2</sub>O<sub>2</sub>. For each of the immunoblot assays, a polyclonal rabbit antiserum to the *Helicobacter* species was included as a reference (50). All sera giving positive or borderline EIA results were analyzed by immunoblotting. Stained protein patterns were documented by visual inspection, and very weakly stained bands were ignored and considered nonspecific binding. For *H. pylori*, a serum was deemed blot positive if found to contain antibodies reacting with the CagA protein (120 kDa) and/or at least two of the low-M<sub>r</sub> proteins (26, 29, 30, and 33 kDa) considered *H. pylori* specific (32, 47). Antibody responses to the 20- to 22-kDa proteins of *H. bilis* are considered *H. bilis* specific (21) and were used as criteria for positive.

**RESULTS**

The prevalence of *H. pylori*-positive individuals was high in both laboratories: 75 of 92 samples analyzed by MIT (Table 1), yielding a prevalence of 81.5% (95% CI = 72.1 to 88.9%), and 77 of 88 analyzed by Lund (Table 2; four vials did not contain enough material for all assays), yielding a prevalence of 87.5% for *H. bilis* and *H. pylori* immune response<sup>a</sup>.

TABLE 2. Lund laboratory: within-subject association between *H. pylori* and *H. bilis* immune response<sup>a</sup>

<table>
<thead>
<tr>
<th><em>H. pylori</em> cell surface protein</th>
<th>No. of samples with result for <em>H. bilis</em> cell surface proteins after preabsorption with <em>H. pylori</em> lysate</th>
<th>Agreement</th>
<th>Kappa</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>29</td>
<td>48</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>59</td>
<td>88&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The percentages of samples with a positive response to *H. pylori* and *H. bilis* were 87.5% and 22.7%, respectively.

<sup>b</sup> Percent agreement, 45.5; kappa, 0.136; P < 0.005.

<sup>c</sup> There were four missing values for *H. pylori*.

According to MIT (Table 1), 54 of the 92 samples were positive for *H. bilis* using OMP (58.7%; CI = 47.9 to 68.9%), and 41 were positive using the sonicate protein (44.5%; CI = 34.1 to 55.3%), but only 14 remained so after preabsorption of the samples with *H. pylori* sonicate, reducing the prevalence to 15.2% (CI = 8.6 to 24.2%). The proportion of *H. bilis*-positive samples, confirmed as positive after preabsorption with *H. pylori*, was 34.0% (CI = 20.1 to 50.6%).

The proportion of *H. bilis*-positive reported by the Lund laboratory (Table 2) was intermediate between the MIT assays: 29 of 92 samples, corresponding to a prevalence of 31.5% (CI = 22.0 to 41.0%), which was statistically compatible with all three MIT results. Correlation of data from MIT indicated that antibody responses to OMPs of the two bacteria coincided for 66% of the subjects (Table 1), while the concordance was 45% by the Lund laboratory (Table 2).

Table 3 shows the agreement between the Lund and the three MIT assessments of the immune responses to *H. bilis*. Concordance between the two laboratories was statistically significant only between the Lund and the two MIT sonicate assays; however, this was due to a greater proportion of discordant negatives rather than positives. The proportion of discordant positive samples was the same by Lund (CSPs, preabsorbed plasma samples) and either MIT OMP or sonicate without preabsorption (20/92 [21.7%]; CI = 13.8 to 31.6). The MIT assay that was most comparable with the Lund method (sonicate with preabsorption) detected 7 positives (7.6%) and 56 negatives (60.9%). The remaining are considered undetermined.

Four of the twenty-nine *H. bilis* EIA-positive sera were confirmed by immunoblotting in the Lund laboratory. The sera reacted strongly to the 20- and 22-kDa proteins, previously shown to have high specificity (31). Three were EIA positive by the MIT laboratory using OMPs as antigen, but none remained positive after preabsorption with *H. pylori*.

Altogether, the proportion of discordant outcomes was high for *H. bilis*, ranging from 31.5% (sonicate preabsorbed) to (CI = 78.7 to 93.6%). The difference was not statistically significant.
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46.7% (OMP). In epidemiological studies, such a level of misclassification can reduce a true odds ratio of 10 (magnitude of the association between H. pylori and gastric cancer as an example) to an observed value of 2.4 to 4.3 (46).

**DISCUSSION**

This study confirmed high cross-reactivity between the immune responses to antigens obtained from H. pylori and H. bilis to an extent that infection with the two species cannot be distinguished serologically in most subjects given the high prevalence of H. pylori in the population studied. Cross-reactivity between bile-tolerant species and H. pylori has been documented previously (1). Several aspects of the study design limit the interpretation of the results: the two laboratories used different strains of H. pylori, with none originating from the Thai population investigated. Further, cutoff points for positivity were based on different reference distributions. Despite these methodological differences, the two laboratories showed nonrandom agreement in the characterization of the immune response to the H. bilis strain used, suggesting that the detected antibodies were not entirely explained by infection with H. pylori. Moreover, concordance between the two laboratories after preabsorption with H. pylori remained high (68%) and statistically greater than would be expected by chance. A further indication of a genuine presence of the enteric H. bilis in this population comes from the confirmation by immunoblotting of H. bilis-specific antibodies in four subjects. Our preliminary results justify further investigations designed to better control the confounding effect of H. pylori infection and improve the specificity of the assays. These studies would be particularly relevant in this population due to the very high incidence of liver-fluke related cholangiocarcinoma, the low risk of gastric cancer, and the high prevalence of H. pylori infection. Ideally, the outcome of ELISAs should be compared to a gold standard such as an immunoblotting. ELISA OMP antigens were prepared by different techniques but were optimized for each laboratory. The OMP preparations used for H. pylori EIA-based assays were found to be superior in sensitivity and specificity compared to antigen preparations based on acid glycine extracts, crude cell lysates, high-molecular-weight urea preparations, and flagellum extracts (35). Similar banding patterns derived from OMP preparations of four H. bilis strains isolated from different species were shown to be distinct from those of H. pylori (14). Immunoblotting demonstrated that OMPs from H. bilis and H. pylori have little cross-reactivity, except for their flagellins, and that at least two of the immunogenic polypeptides identified in H. bilis OMP had no N-terminal sequence homology with protein sequences available in public databases. Although the significance of H. bilis OMPs in terms of virulence is not known, these unique H. bilis antigens may be useful as targets in serologic assays.

The ability to characterize the infection status of individuals with enterohepatic helicobacters described in the last 20 years would open entirely new perspectives for investigations into the causes of chronic and malignant diseases of the biliary tract in human beings. Cancers of the gallbladder and biliary tract show 10-fold variation worldwide (36), being generally rare but with areas of an exceptionally high incidence in the Andean region in South America, Thailand, Japan, central Europe (36), and northeastern India (28). Established risk factors for the disease such as cholelithiasis, obesity, and parity seem to account for only a small degree of variation, with endemic liver fluke infection in Thailand being the only exception (17). The risk attributed to the parasite, however, could be confounded by enterohepatic Helicobacter infection as a cofactor contributing to biliary carcinogenesis, similar to the role of H. pylori infection in the development of gastric cancer.

Based on molecular evidence that enterohepatic Helicobacter species infect humans (8, 9, 19, 24), it remains unknown how commonly these infections develop and which human populations are at greatest risk. The geographical distribution of H. bilis or other enteric Helicobacter species in humans is unknown but suggests that their prevalence may be low in populations at low risk for nonviral hepatitis and biliary duct malignancies (4, 5, 30, 37, 38, 48) but could contribute to the unexplained increase of extra- and intrahepatic cancer inci-

### Table 3. H. bilis immune response: joint distributions according to the two laboratories

<table>
<thead>
<tr>
<th>Antigen and H. bilis detection result (MIT)</th>
<th>No. of samples (% positive) with result for H. pylori detection with OMP (Land)**</th>
<th>% Agreement</th>
<th>Kappa</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>OMP (no preabsorption)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>20</td>
<td>34</td>
<td>54 (58.7)</td>
<td>53.3</td>
</tr>
<tr>
<td>Negative</td>
<td>9</td>
<td>29</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>63</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Sonicate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>20</td>
<td>21</td>
<td>41 (44.6)</td>
<td>67.4</td>
</tr>
<tr>
<td>Negative</td>
<td>9</td>
<td>42</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>63</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Preabsorbed sonicate&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>7</td>
<td>7</td>
<td>14 (15.2)</td>
<td>68.5</td>
</tr>
<tr>
<td>Negative</td>
<td>22</td>
<td>56</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>29 (31.5)</td>
<td>63</td>
<td>92</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent positive values are indicated in parentheses.

<sup>b</sup> That is, the H. bilis CSPs after preabsorption with H. pylori lysate.

<sup>c</sup> NS, not significant.
idence in North America and Europe (18, 39, 44) and the wide geographical variation in the incidence of gallbladder and extrahepatic bile duct cancers observed in other regions (36). Enterohemorrhagic Helicobacter infection could also be a factor in the development of cholesterol gallstones and intrahepatic cholelithiasis, as suggested by recent studies in animal models (25–27).

In order to increase the probability of detecting positive subjects, investigators have analyzed patients affected by a variety of disorders of the liver and biliary tract. When serological markers of infection were assessed, more patients were generally found positive compared to blood donors, depending on the specific disease (31). The hepatic disease may, however, modify responses to infection as is well documented for H. pylori and atrophy of the gastric mucosa (15, 33, 41). The nature of a putative association between the enterohemorrhagic helicobacters and hepatic diseases in humans will therefore eventually clarified by epidemiological studies that can discriminate subjects harboring active infection from negative subjects, hopefully preceding the diagnosis of clinical disease. It is therefore important to develop inexpensive but accurate serological markers of infection for use in large-scale epidemiological studies. The design of future descriptive investigations of the kind we have presented here will require improved control of the confounding effect of H. pylori infection (16, 34) and the adoption of a highly reproducible diagnostic standard. Assays based on antigens derived from the population in which the study is performed are more sensitive and specific. A significant limitation to date is that gastric, liver, and lower-bowel samples suitable for PCR and culture have not been commonly used, and the interpretation of epidemiological studies such as those presented here must acknowledge the inability to definitively demonstrate H. pylori or H. bilis infection.

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