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

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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 enterohepatic 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 enterohepatic 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 etiological 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

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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 *Opistorchis 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 based 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 (Sigma). For sonication, bacterial pellets from broth cultures were resuspended in sterile PBS and sonicated on ice (Artek Sonic Dismembranator; Artek 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 OMP extracts harvested 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 51630) 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 of the ELISA optical densities obtained for the five 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 Immulon II plates (ThermoLab Systems, Franklin, MA) were coated with 100 μl per well of 1-μg/ml concentrations of *H. pylori* or *H. bilis* OMP in carbonate buffer (pH 9.6) overnight at 4°C. Sera were diluted 1:1,000 and incubated on wells for 1 h at 37°C. The biotinylated secondary antibody was goat anti-human IgG (Southern Biotechnology Associates, Birmingham, AL), which was used at a 1:10,000 dilution for 1 h at 37°C. Incubation with extravidin peroxidase for 30 min at 37°C (Sigma) was followed by a 30-min incubation with ABTS [2,2′ azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] diammonium salt substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for color development at room temperature. Absorbance (optical density) at 405/562λ was recorded by an ELISA plate reader (Dynatech MR7000; Dynatech Laboratories, Inc., Chantilly, VA).

MIT laboratory, second and third analyses. The ELISA conditions were identical to the first analysis except that sonicate proteins were used as coating antigen. To preabsorb plasma with *H. pylori* antigens to minimize cross-reactive IgG to *H. bilis*, the amount of sonicate protein used was standardized by adjusting the concentration to achieve and optical density of 0.15 at a wavelength of 540 nm when mixed with 1 ml of plasma diluted to 1:1,000. Tubes were placed on a mixing platform for 1 h at room temperature and then overnight at 4°C. A paired sample was incubated with PBS instead of protein and subjected to the same incubation conditions. Nonabsorbed and preabsorbed samples were then plated against *H. pylori* or *H. bilis* sonicate antigens for the ELISA. The data generated on serial days of assay were normalized by including a set of the same three samples on every plate and adjusting the values for interday variability.

Lund laboratory. (i) Bacterial strains and culture conditions. A reference strain of *H. pylori* (17874 obtained from the Cell Culture Collection, University of Gothenburg, Gothenburg, Sweden [CCUG]) and an *H. bilis* strain (CCUG 38995) were cultured on brucella blood agar (Becton Dickinson, Franklin Lakes, NJ) supplemented with 5% horse blood, 10% inactivated horse serum, 1% IsoVitalex (Becton Dickinson), and 1% hemin (ICN, Aurora, OH) for 3 to 4 days at 37°C in a microaerobic atmosphere (Anoxomat; MART Microbiology, Lichtenvoorde, The Netherlands). Cells of the two species were harvested, washed twice in PBS (pH 7.2; 0.02 M sodium phosphate, 0.15 M NaCl) and used for subsequent protein extraction.

(ii) Extraction of CSPs. An acid glycine extraction of cell surface proteins (CSPs) of the two *Helicobacter* species was performed as follows. Washed cells were resuspended in 0.2 M glycine hydrochloride (pH 2.2; 4 g of cells/100 ml) supplemented with protease inhibitors (Complete; Roche Diagnostics, Mannheim, Germany) and stirred magnetically for 15 min at 20°C. Cells were removed by centrifugation at 12,000 × *g* for 15 min at 8°C, and the supernatants were neutralized with NaOH and dialyzed for 18 h at 8°C against PBS. Protein was quantified by the Bradford method using the Bio-Rad protein assay (Bio-Rad, Richmond, CA) and bovine serum albumin as a standard. Protein extracts were divided into aliquots and kept frozen at −22°C until use.

(iii) Serology. To remove potentially cross-reacting antibodies between *H. pylori* and the enteric *H. bilis*, a preabsorption step was performed. Plasma samples to be tested for antibodies to antigens of *H. bilis* by enzyme immunoassay (EIA) and immunoblotting were absorbed with a whole-cell lysate of *H. pylori* (CCUG 17874). Then, 10 μl of serum was added to 1 ml of cell lysate (with the density adjusted at A_{540} to 1.5), followed by incubation for 2.5 h at 22°C under constant shaking. Cells were removed by centrifugation at 12,000 × *g* for 15 min, and the supernatants were used for the serology (21, 49).

CSPs of the *Helicobacter* species were used as the coating antigens, diluted to a concentration of 5 μg/ml in carbonate buffer (pH 9.8), and 100 μl/well was added in duplicate to the microtiter plates (Maxisorp Immunoplates; Nunc, Roskilde, Denmark). Plates were incubated for 16 h at 8°C, washed with PBS-T (PBS plus 0.05% Tween [pH 7.2]), and blocked for 1.5 h at 22°C with 2% bovine serum albumin in PBS-T. Samples, diluted 1/800, were applied and incubated for 1 h at 37°C. As a reference standard, human gamma globulin (Pharmacia & UpJohn, Stockholm, Sweden) was used. A pool of 10 sera, determined to be negative by EIA for *H. pylori* and *H. bilis*, was included as a negative control, and a polyclonal rabbit antiserum to the two *Helicobacter* species was applied as a positive control. After a washing step, an alkaline phosphatase-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. Bound antibodies were 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 in a spectrophotometer at 405 nm after 35 min of 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 EIA 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 cell electrophoresis equipment (Bio-Rad) in 8 to 16% gradient gels with a 4% stacking gel (Criterion precast gel; Bio-Rad). Proteins were diluted in a standard sample buffer (Bio-Rad), loaded onto the gels (120 μg/gel), and separated at 200 V for 90 min. The M_rs of the separated proteins were established using marker proteins (Bio-Rad) (32, 47).

After electrophoresis, the gels were transferred to polyvinylidene difluoride membranes (Micron Separations, Westborough, MA) for antibody detection, using semidry electroblotter equipment (Ancos, Vig, Denmark). The transfer time was 90 min at a constant current of 0.8 mA/cm^2. The membranes were blocked for two periods of 15 min each in a blocking buffer containing hydrolyzed gelatin, polyvinylpyrrolidone, 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 16 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

Antigen and *H. bilis* detection result	No. of samples (% positive) with result for *H. pylori* detection with OMP[a]			% Agreement	Kappa	*P*
	Positive	Negative	Total			
OMP (no preabsorption)						
Positive	49	5	54 (58.7)	66.3	0.243	0.003
Negative	26	12	38			
Total	75 (81.5)	17	92			
Sonicate						
Positive	38	3	41 (44.5)	56.5	0.186	0.007
Negative	37	14	51			
Total	75	17	92			
Preabsorbed sonicate[b]						
Positive	14	0	14 (15.2)	33.7	0.0786	0.03
Negative	61	17	78			
Total	75	17	92			

[a] Percent positive values are indicated in parentheses.
[b] 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-amino-9-ethylcarbazole (Sigma) and 0.015% H_2O_2. 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_r 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 positively.

Statistical analyses. Subjects were classified as positive or negative for infection. Comparisons were carried out by means of contingency tables. Associations were tested by the kappa statistics, which test deviations from the expected joint distribution given the marginal distribution of the two variables, corrected for chance (7). Statistically significant kappa values indicate nonchance associations. Exact 95% confidence intervals (CI) of proportions were calculated based on the binomial distribution. Statistical computing was carried out by using STATA software, version 9 (43).

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% (CI = 78.7 to 93.6%). The difference was not statistically significant.

TABLE 2. Lund laboratory: within-subject association between *H. pylori* and *H. bilis* immune response[a]

H. pylori cell surface protein	No. of samples with result for *H. bilis* cell surface proteins after preabsorption with *H. pylori* lysate		
	Positive[b]	Negative	Total
Positive	29	48	77
Negative	0	11	11
Total	29	59	88[c]

[a] The percentages of samples with a positive response to *H. pylori* and *H. bilis* were 87.5% and 22.7%, respectively.
[b] Percent agreement, 45.5; kappa, 0.136; $P < 0.005$.
[c] 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%), 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 concordant negatives rather than positives. The proportion of concordant 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

TABLE 3. *H. bilis* immune response: joint distributions according to the two laboratories

Antigen and *H. bilis* detection result (MIT)	No. of samples (% positive) with result for *H. pylori* detection with OMP (Lund)[a]			% Agreement	Kappa	*P*
	Positive	Negative	Total			
OMP (no preabsorption)						
Positive	20	34	54 (58.7)	53.3	0.122	NS[c]
Negative	9	29	38			
Total	29	63	92			
Sonicate						
Positive	20	21	41 (44.6)	67.4	0.321	<0.001
Negative	9	42	51			
Total	29	63	92			
Preabsorbed sonicate[b]						
Positive	7	7	14 (15.2)	68.5	0.151	0.05
Negative	22	56	78			
Total	29 (31.5)	63	92			

[a] Percent positive values are indicated in parentheses.
[b] That is, the *H. bilis* CSPs after preabsorption with *H. pylori* lysate.
[c] NS, not significant.

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 urease 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-

dence 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). Enterohepatic *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 enterohepatic 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 available, particularly in high-risk populations. Thus, 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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