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Helicobacter bilis-Associated Hepatitis in Outbred Mice

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Although *Helicobacter bilis* infects mice worldwide, it is not known whether *H. bilis* causes enterohepatic disease in outbred Swiss Webster (SW) mice. Intestinal and liver specimens from four groups of 39 SW mice, five of which were treated with creatine in the drinking water, were obtained for culture for the presence of *H. bilis* and were analyzed as to whether infection status was associated with *H. bilis* seroconversion and/or hepatitis. *Helicobacter bilis* was isolated from the colon of all 27 mice of groups I–III, but only from the liver of one 12- to 13-month-old female mouse. Ten of 27 livers were *H. bilis*-positive based on polymerase chain reaction (PCR) analysis; 8 of 10 (80%) of the positive results were for older mice. Results of PCR analysis for *H. bilis* were negative, and *H. bilis* was not isolated from 12 control mice (group IV). Irrespective of treatment group or controls, severity of histologic lobular and periportal chronic inflammatory lesions in the liver of *H. bilis*-infected outbred mice ranged from minimally to moderately severe. *Helicobacter bilis* infection was associated with increased portal inflammation in group III mice, compared with age-matched, helicobacter-free, group IV mice ($P < 0.03$). A comparison of potential sex effects in group III mice indicated that *H. bilis*-infected female mice developed more severe portal inflammation than did *H. bilis*-infected male mice ($P < 0.01$). On the basis of results of an ELISA, 8 of 11, 6- to 8-month-old *H. bilis*-infected mice of group III seroconverted to *H. bilis* outer membrane antigen. *Helicobacter bilis* infection is associated with hepatitis in SW mice and can confound experimental results.

Microaerophilic, spiral-to-curved bacteria isolated from the stomach of humans and animals have become a subject of intense research because of their association with gastric disease (9, 29). *Helicobacter pylori*, the type species of the genus, causes chronic gastritis, peptic ulcer, and gastric cancer disease in humans (18, 27, 29).

More recently, there has been considerable interest in enterohepatic *Helicobacter* spp. (EHS) of mice because of the association with hepatitis and inflammatory bowel disease (IBD) (4, 13, 14, 41-43). *Helicobacter hepaticus*, an intestinal helicobacter that causes persistent hepatitis in A/JCr mice, has been linked with hepatitis and hepatic tumors in several strains of mice and has been proposed as a model of *Helicobacter*-induced tumorigenesis (13, 14, 41-43). *Helicobacter hepaticus* was first identified in a long-term toxicology study in which the control group was found to have an extremely high incidence of hepatic tumors (11, 43). *Helicobacter bilis* also was identified as a novel *Helicobacter* species colonizing the bile, liver (with hepatitis and hepatoma), and intestine of aged inbred mice (15). It is important to determine the pathogenic potential of these novel EHS, to determine their usefulness as models, and to assess the degree of interference they may cause in research using infected mice. Importantly, many of these EHS are widespread in commercial and academic rodent colonies and may substantially confound interpretations of some research studies (6, 33).

Anecdotally, mixed infection of *H. bilis* and *H. rodentium* (35) has been associated with severe, necrotizing, proliferative typhlocolitis and diarrhea in a colony of immunodeficient mice (36). Experimentally, *H. bilis* causes IBD in *scid* mice with defined flora and confirms a pathogenic role for *H. bilis* in mice (15, 33). In addition, *H. bilis* has been used experimentally to induce IBD in mdr$^{-/-}$ and IL-10$^{-/-}$ mice (3). However, to the authors' knowledge, there are no studies to date describing naturally acquired *H. bilis*-associated disease in outbred mice. Thus, the purposes of the study reported here were to determine whether natural *H. bilis* infection in outbred mice is associated with enterohepatic disease and whether its presence was confounding interpretation of experimental results.

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Case History

A study was designed to determine whether long-term oral supplementation with creatine, used by athletes in training, causes histologic lesions in selected internal organs of mice. Outbred Swiss Webster (SW) female mice were purchased at the age of 15 weeks and were randomly assigned to either control or two experimental groups that consumed an average of either 0.025 or 0.05 mg of creatine/g of body weight/d administered in drinking water. Creatine monohydrate was 99.9% pure HPLC grade manufactured by Pfanstiehl Laboratories, Inc. (Waukegan, Ill.), and distributed by PowerStar, Inc. (Pinehurst, N.C.), a subsidiary of Dexter Sport Science (Carthage, N.C.). Mice were housed in wire mesh stainless steel cages and were fed a commercial rodent diet. Water was provided ad libitum, and water consumption and body weight were monitored throughout the 10-month study. In a routine histologic review of the liver by one of the authors (SK), creatine-supplemented and control mice developed hepatic lesions. Specimens from control and treated mice of sub-

sequent experiments were submitted to the Massachusetts Institute of Technology (MIT) for further analysis.

The procedures followed were approved by the Springfield College Animal Use Review Committee and adhered to the policy outlined in the *Guide for the Care and Use of Laboratory Animals* (24).

Materials and Methods

Animals. Four groups of SW mice totaling 39 animals were analyzed at MIT for the presence of *H. bilis*, and the gastrointestinal tract and liver were examined histologically for inflammatory lesions. The first two groups of mice were housed in the investigators' laboratory. Group I consisted of four control females; two were 7 months old, and two were 13 months old. In group II, 11- to 14-month-old females were surveyed; six were controls, and five received the high-dose creatine treatment in the drinking water. The third group of mice was directly shipped from the commercial vendor (commercial source 1) and consisted of four male and eight female retired breeders, approximately 6 to 8 months of age, and specified as viral antibody free (VAF), and free of ecto- and endoparasites and selected murine bacterial pathogens but not helicobacter free. The last group, group IV, consisted of six male and six female 6- to 8-month-old retired breeders shipped from commercial source 2 (commercial source 1 but different geographic location) that were specified as helicobacter free and VAF, as well as free of other murine pathogens.

Control sera positive for *H. hepaticus* antibody were obtained from four VAF SW mice (Taconic Farms, Germantown, N.Y.) that were experimentally infected with *H. hepaticus* by use of oral gavage at 6 weeks of age. Blood samples were obtained at postinfection week 8 by use of a published protocol (4).

Bacterial isolation. Briefly, the cecal, colon, and liver tissue was obtained aseptically, ground by use of a tissue grinder, and plated on TVP (trimethoprim-vancomycin-polymyxin; Remel Laboratories, Lenexa, Kans.) plates for microaerobic isolation. Cultures were incubated under microaerobic conditions in vented jars containing N_2, H_2, and CO_2 (80:10:10) at 37°C on Columbia blood agar plates (Remel Laboratories) for 3 days. Plates without growth were kept for 21 days before assignment of negative growth (34).

Extraction of DNA and PCR analysis. The DNA was extracted from the cultured bacterial isolates and from the liver tissues by use of the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, Ind.). *Helicobacter* genus-specific primer pairs were used to generate 16S rRNA amplicons of 1,200 bases (10, 21). Ten microliters of the DNA preparation was used for PCR analysis. The PCR amplification conditions have been described (10). The *H. bilis*-specific primers C62 (forward primer, AGAACTGCATTTGAAACTACTTT) and C12 (reverse primer, GTATTGCA TCTCTTTGTATGT) amplified a 640-bp product.

Restriction fragment length polymorphism analysis. The DNA fragments of 1.2 kb from all cultured bacterial isolates were subjected to restriction fragment length polymorphism (RFLP) analysis using restriction endonucleases *Alu*I and *Hha*I (New England Biolabs, Beverly, Mass.) as previously published (34). Each reaction contained 10 U of either *Alu*I or *Hha*I, 2 µl restriction buffer (New England Biolabs) and 16 µl of PCR product. Reactions were incubated at 37°C for 2 h. The products were examined by electrophoresis through a 6% Visigel separation matrix (Stratagene, La Jolla, Calif.), stained with ethidium bromide, and viewed under short wavelength UV illumination.

Amplification of 16S cistrons by use of PCR analysis and purification of PCR products from cultured bacteria. The rRNA cistrons from two culture isolates from two mice in two shipments (MIT No. 01-2124-1a [liver] and 01-6639 [cecum]) were amplified using universal bacterial primers F24 and F25 for 16S rRNA (10). Hot-start PCR analysis was performed in thin-walled tubes using a thermocycler (Model 9700, Perkin-Elmer, Norwalk, Conn.). One microliter of the DNA template was added to a reaction mixture (50 µl final volume) containing 20 pmol of each primer, 40 nmol of deoxynucleoside triphosphates, and 1 U of *Taq* 2000 polymerase (Stratagene, La Jolla, Calif.) in buffer containing Taqstart antibody (Sigma, St. Louis, Mo.). In a hot-start protocol, samples were preheated at 95°C for 8 min, followed by amplification under the following conditions: denaturation at 95°C for 45 sec, annealing at 60°C for 45 sec, and elongation for 1.5 min, with an additional 5 sec for each cycle. A total of 30 cycles were performed, followed by a final elongation step at 72°C for 10 min. The PCR amplicons were examined by use of electrophoresis in 1% agarose gel. The DNA was stained with ethidium bromide and visualized under short wavelength UV light. The PCR products were purified by use of the QIA Quick PCR purification kit (Qiagen, Valencia, Calif.).

Sequencing of the 16S rRNA gene and data analysis. Purified DNA from PCR analysis was sequenced by use of an ABI prism cycle-sequencing kit (BigDye Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase FS; Perkin-Elmer). The primers used for sequencing have been described (10). Quarter-dye chemistry was used with 80 µM primers and 1.5 µl of PCR product in a final volume of 20 µl. Cycle sequencing was performed using an ABI GeneAmp PCR System 9700 with 25 cycles of denaturation at 96°C for 10 sec and annealing and extension at 60°C for 4 min. Sequencing reactions were run on an ABI 3100 DNA sequencer. Sequence data were entered into RNA, a program set for data entry, editing, sequence alignment, secondary structure comparison, similarity matrix generation, and dendrogram construction for 16S rRNA (5). Our database contains over 1,000 sequences obtained in our laboratory and over 500 retrieved from GenBank for 16S rRNA and 50 23S rRNA sequences of *Helicobacter* spp.

Histologic examination. Sections of liver and gastrointestinal tract tissues were fixed in neutral-buffered 10% formalin. Formalin-fixed tissues were embedded in paraffin, sectioned at 5-µ thickness, and stained with Warthin-Starry silver to visualize bacteria in liver tissue. Hematoxylin and eosin (H&E) also was used to stain 5-µ sections.

Liver sections representing replicate specimens from each lobe were examined by a comparative pathologist blinded to sample identity and were graded on a 0 to 4 scale for lobular histologic activity and periportal activity using criteria established by Scheuer (32). Lesion scores were analyzed using the Mann-Whitney and Kruskal-Wallis nonparametric tests, with significance set at $P < 0.05$.

Serologic testing. Sera were obtained from four mice of group I and were screened by use of an ELISA for evidence of infection with murine viruses, including mouse hepatitis virus, minute virus of mice, mouse parvovirus, Sendai virus, epizootic diarrhea of infant mice virus, reovirus type 3, Theiler's encephalomyelitis virus, K virus, mouse adenovirus, ectromelia virus, polyomavirus, murine cytomegalovirus, mouse thymic virus, and

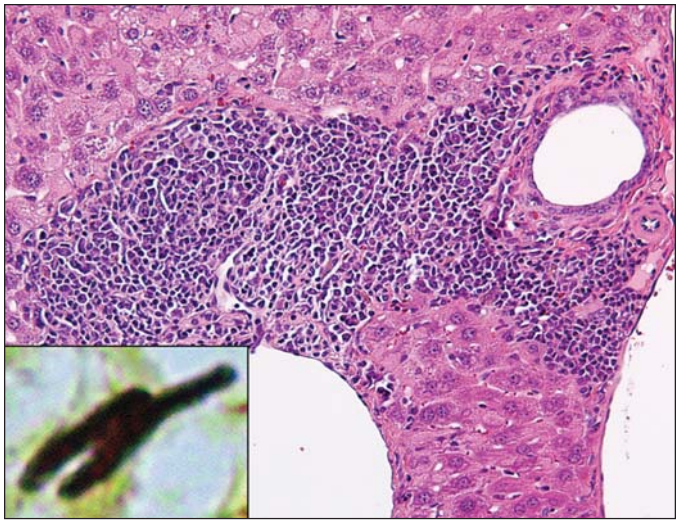

Figure 1. Photomicrograph of a section of liver from a mouse infected with *Helicobacter* sp.. Notice bridging portal hepatitis comprising chiefly mononuclear cells. H&E stain; magnification, 400×. Inset: Intracanalicularly situated, approximately 2- to 7-µm, argyrophilic, spiral bacteria consistent with *H. bilis* morphology. Warthin-Starry stain; magnification 1,000×.

lymphocytic choriomeningitis virus. To determine the usefulness of serologic testing to confirm infection with *H. bilis*, sera for an ELISA were obtained at necropsy from group III (commercial source 1, n = 11) and group IV (commercial source 2, n = 11) mice. Outer membrane antigens of the type strains of *H. bilis (*ATCC 51630) and *H. hepaticus (*ATCC 51449*)* were prepared and used in the ELISA along with standard methods as described (44). Seroconversion to *H. bilis* or *H. hepaticus* antigens was defined as an ELISA optical density (OD) value that exceeded the mean plus 3 SD of the OD measured for samples collected from the group IV mice that had negative results for all *Helicobacter* spp. on the basis of PCR analysis and culture. Comparison between groups was made using the Student's *t* test, and the sensitivity, specificity, and positive and negative predictive values of the ELISA were calculated as described (44).

Results

Histopathologic findings. Outbred mice infected with *H. bilis* had liver lesions ranging in severity from minimal to moderate (Fig. 1, Table 1). Most mice had lobular and portal histologic activity, indicating a chronic ongoing inflammatory process. Lesions were similar to those we described in inbred strains of aged mice infected with *H. bilis* (15). Random foci of lobular hepatocellular coagulative necrosis were rimmed by macrophages with fewer neutrophils and/or lymphocytes. Portal inflammation consisted of predominantly lymphocytes, with a few interspersed larger monocytic cells. Portal infiltrates remained confined to triad boundaries, with only selected mice having mild interface hepatitis characterized by inflammatory extension through the hepatic limiting plate (Fig. 1). Secondary hepatic changes such as foci of altered hepatocytes, fibrosis, and hyperplasia and dysplasia, were not evident. The focal nature of liver disease in this group of mice as a whole could be attributed to low-to-undetectable hepatic bacterial colonization, as documented by results of culture and/or PCR analysis. However, in the 12- to 13-month-old mouse where *H. bilis* was isolated from the liver, the fusiform, slightly curved organisms were recognized in bile canaliculi by use of the Warthin Starry stain (Fig. 1).

Generalized inflammatory responses of unspecified origin were evident in some *H. bilis*-infected mice, including amyloidosis of the small intestine and renal arcuate perivascular and subcalyceal interstitial lymphoplasmacytic infiltrates. Histologic changes in the colon or cecum were not observed in *H. bilis*-infected mice. Minimal or no changes were evident in the liver of helicobacter-free mice. In selected mice, focal lesions, when noted, consisted of discrete foci of 5 to 10 mononuclear cells in hepatic parenchyma. One mouse had a few small foci of coagulative necrosis, with a minimal inflammatory component.

The first group of SW mice submitted for pathologic review (see case history) were 7- and 13- month-old female control mice in a creatine study (group I). These mice had developed lesions of hepatitis, with a lobular and portal distribution consistent with inflammatory lesions associated with *H. hepaticus* (14) or *H. bilis* (15) infection. The liver lesions in these mice were similar to those observed in the slightly older group II control and creatine-treated female mice. Creatine administered long term in drinking water did not have an additive effect on lobular or portal inflammation in these age-matched mice naturally infected with *H. bilis* ($P = 0.40$, $P = 0.92$, respectively). Importantly, because control and creatine-treated mice were infected with *H. bilis*, the interpretation of potential adverse effects of creatine on liver lesions was confounded.

To determine whether the case study mice (groups I and II) may have acquired *H. bilis* infection prior to purchase, retired breeders from the same vendor were evaluated and found to be *H. bilis* infected (group III). *Helicobacter bilis* infection in this group was associated with increased portal inflammation compared with minimal or no lesions in age-matched, helicobacter-free, group IV mice obtained from a second commercial source ($P < 0.03$). The effect of *H. bilis* infection on lobular distribution of inflammation was lower ($P = 0.05$). *Helicobacter bilis*-infected female mice of group III developed more severe portal inflammation than did *H. bilis*-infected male mice ($P < 0.01$). Scores for lobular distribution of inflammation in group III female and male mice were similar ($P = 0.09$).

Isolation of *H. bilis* and 16s RNA sequences. Microaerobic culturing yielded gram-negative fusiform bacteria from all intestinal samples of mice of groups I–III, but negative results for group IV mice. The bacteria were urease, catalase, and oxidase positive. On the basis of these criteria, all 27 mice had *H. bilis* isolated from the large intestine. In contrast, *H. bilis* was isolated from the liver of one mouse only. All isolates were PCR positive using *H. bilis*-specific primers. The full 16S rRNA sequencing performed on the liver and cecal bacterial isolate yielded results identical to each other, as well as to the *H. bilis*-type strain ATCC 51630. For this series of four mouse shipments, including the control group, we did not isolate any other *Helicobacter* species either from the intestine or liver.

Polymerase chain reaction analysis. All 27 cecal and colon DNA specimens from the three *H. bilis*-positive shipments (groups I–III), were positive using 1,200-base pair *Helicobacter* genus-specific primers and *H. bilis*-specific PCR primers (Fig. 2). Results of PCR analysis for *H. bilis* was positive for 10 of 27 liver specimens compared with only one *H. bilis* isolation from the liver (1/27) (Table 2). The oldest mice in the survey, 13 to 14 months, had the highest number of positive results (8/13) compared with the 6- to

Table 1. Mean and range of severity scores for hepatic lobular and portal inflammation attributable to *Helicobacter bilis* infection

Group	Source of mice	Age (months)	Infection status	Creatine treatment	Sex	Median (range) of hepatic inflammation scores	
						Lobular	Portal
I	Investigator colony	7-13 mo	*H. bilis*	Control	4 F	0.5 (0-2)	1.25 (0.5-1.5)
II	Investigator colony	11-14 mo	*H. bilis*	Control	6 F	0.5 (0-2)	1 (0-1.5)
		11-14 mo	*H. bilis*	High dose[1]	5 F	0.5 (0-0.5)	1 (0-2.5)
III	Commercial colony 1	~6-8 mo	*H. bilis*[2]	NA	8 F	0.75 (0-2.5)	0.5 (0-1)[3]
					4 M	0 (0-0.5)	0 (0)
IV	Commercial colony 2	~6-8 mo	Helicobacter-free	NA	6 F	0 (0-0.5)	0 (0)
					6 M	0 (0-0.5)	0 (0-1)

[1]Creatine had no effect on lobular ($P = 0.40$) or portal ($P = 0.92$) inflammation in age-matched, *H. bilis*-infected female mice of group II.
[2]*Helicobacter bilis* infection was associated with increased portal inflammation in group III mice, compared with age-matched, helicobacter-free, group IV mice ($P < 0.03$). The effect of *H. bilis* infection on lobular distribution of inflammation was lower ($P = 0.05$).
[3]*Helicobacter bilis*-infected female mice of group III developed more severe portal inflammation than did *H. bilis*-infected male mice ($P < 0.01$). Scores for lobular distribution of inflammation in group III female and male mice were similar ($P = 0.09$).
F = Female; M = male.
NA = Not applicable.

Table 2. Recovery of *H. bilis* from three groups of outbred Swiss Webster mice by use of culture or proof of infection by use of PCR analysis

Group	Age	No. of *H. bilis*-positive samples/total no. of mice			
		Cecum/colon		Liver	
		Culture	PCR	Culture	PCR
I	~7 mo	2/2	2/2	0/2	0/2
	12-13 mo	2/2	2/2	1/2	1/2
II	14 mo	11/11	11/11	0/11	7/11
III	6-8 mo	12/12	12/12	0/12	2/12
Total		27/27	27/27	1/27	10/27
IV*	~6-8 mo	0/12	0/12	ND	ND

*Control helicobacter-free mice.
ND = Not done.

7-month-old mice (2/14). In the group IV SW mice ordered from a helicobacter-free commercial source, all 12 mice were negative for the *Helicobacter* genus-specific PCR primers.

Restriction fragment length polymorphism analysis. The PCR reaction amplified a 1,219-bp PCR product from all of the *H. bilis* isolates (Fig. 2). The PCR reaction using *Aln*I and *Hha*I had a pattern consistent with that of *H. bilis* (Fig. 3) (34).

Serologic testing. Sera from group I mice were negative for antibodies to a standardized panel of 14 viral antigens. Results of the *H. bilis* ELISA indicated that 8 of 11 mice of group III (commercial source 1, confirmed to be *H. bilis* infected by PCR and culture results) seroconverted to *H. bilis* antigen (Fig. 4). Four of the eight seropositive mice had robust antibody responses to *H. bilis*. Three of these eight mice had low but technically positive antibody responses, and the remaining three *H. bilis*-infected mice did not seroconvert. Notably, these three seronegative mice did not have any relevant liver lesions (scores of zero). All 11 mice of group IV, confirmed to be helicobacter-free, were seronegative to *H. bilis* antigen ($P < 0.0001$). Two of the four SW mice experimentally infected with *H. hepaticus* to provide serum antibody controls developed low but technically seropositive responses to *H. bilis* antigens, indicating a false-positive reaction, most likely from cross-reactivity to shared antigens, between *H. bilis* and *H. hepaticus*. Sera from all four SW mice experimentally infected with *H. hepaticus* had high titer of antibody to *H. hepaticus* antigen. One of the 11 mice of group III infected with *H. bilis* had a false-positive reaction to *H. hepaticus* antigen; the OD value for *H. hepaticus* antigen was similar to the OD generated from the assay using *H. bilis* antigen (0.84 and 0.82, respectively). This mouse had developed lobular and portal hepatitis score that was equivalent to the median score for its respective group III mice.

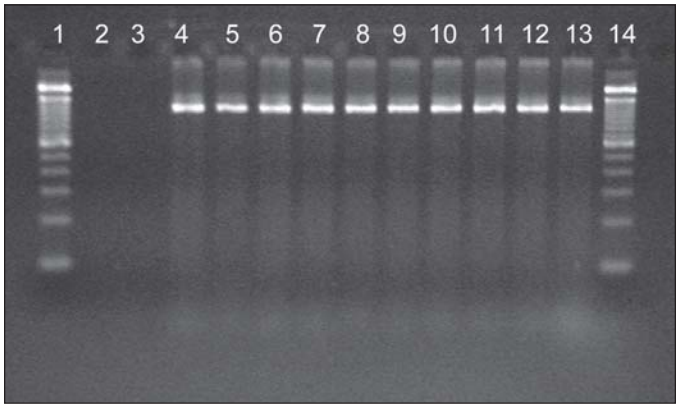

Figure 2. The 1.2-Kb polymerase chain reaction (PCR) products of isolates using helicobacter-specific primers CO5 and C97. Lanes: 1 and 14, 100-bp DNA ladders; 2 and 3, 03-7307 and 03-7304 (negative cecal tissues); 4, 01-6644; 5–10: 01-6646, 01-6637, 01-6636, 02-585, 02-582, and 02-580 (*H. bilis* cecal isolates); 11, 01-2124 (*H. bilis* isolate from the liver), and 12 and 13, *H. bilis*^{T} positive controls.

On the basis of PCR and culture results for group III and group IV mice that confirmed their *H. bilis* infection status, the *H. bilis* ELISA was 73% sensitive and 100% specific for identifying infected mice, yielding a positive predictive value of 100% and a negative predicative value of 80%. Using the four *H. hepaticus*-infected SW mice as positive controls, the same analysis applied to the *H. hepaticus* ELISA results for groups III and IV indicated that the assay was 100% sensitive and 96% specific, yielding a positive predictive value of 80% and a negative predicative value of 100%.

Discussion

In A/JCr, AXB, and B6C3F1 mice, *H. hepaticus* is associated with chronic persistent hepatitis, hepatoma, and hepatocellular carcinoma (13, 19, 23, 43). *Helicobacter hepaticus*, the morphology of which is similar to that of several other intestinal helicobacters, is a long, slightly curved to spiral bacterium with bipolar sheathed flagella (11). While examining aged, inbred mice with chronic hepatitis for infection with *H. hepaticus*, organisms with a different ultrastructural morphology were noted (15). These organisms, now classified as *H. bilis*, were fusiform to slightly curved rods, had periplasmic fibers, and had 3 to 14 bipolar sheathed flagella. *Helicobacter bilis* was isolated from the intestine of these aged strains of inbred mice, some of which had chronic hepatitis and hepatomas. Of the four strains examined, *H.*

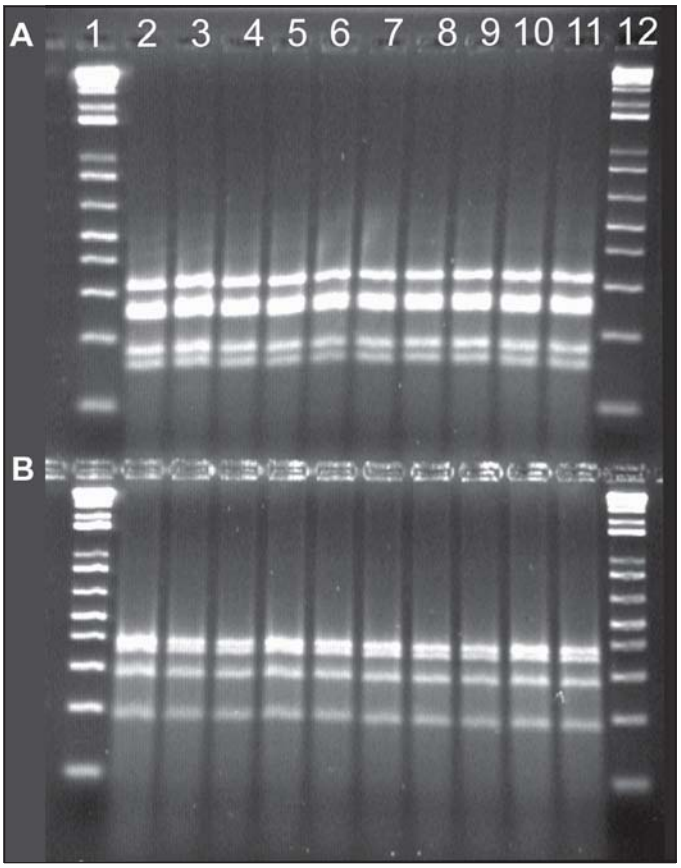

Figure 3. Restriction length polymorphism pattern of isolates using *Alu*1 (A) and *Hha*1 (B), and 1.2-Kb PCR products from helicobacter-specific PCR analysis. Lanes: 1 and 12, DNA 100-bp ladder; 2, *H. bilis*^{T}; 3–9: 01-6649, 01-6646, 01-6637, 01-6636, 02-585, 02-582 and 02-580 (cecal *H. bilis* isolates); 10, 01-2124 (*H. bilis* isolate from the liver); and 11, *H. bilis*^{T}.

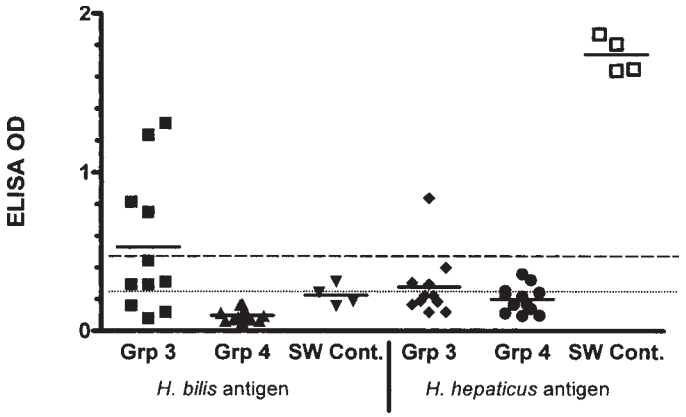

Figure 4. Mean and individual ELISA values for antibody titer to *H. bilis* (left) and *H. hepaticus* (right) antigens in sera from mice confirmed to be *H. bilis* infected (Grp 3), confirmed to be helicobacter-free (Grp 4), or experimentally infected with *H. hepaticus* as positive controls (SW Cont.). Seroconversion to *H. bilis* or *H. hepaticus* was evident by values exceeding the mean plus 3 SD of values measured in sera from helicobacter-free mice (lower dotted line is *H. bilis* antigen; upper dotted line is *H. hepaticus* antigen).

bilis was isolated from the liver of DBA/2, C57BL/6 and CBA/CA, but not BALB/c mice (15). Thus, the study of outbred mice reported here supports earlier findings in inbred mice indicating that *H. bilis* not only efficiently colonizes the gastrointestinal tract but, in addition, colonizes the liver and is associated with hepatitis, particularly in aged mice (15). Although hepatomas were not observed in outbred mice infected with *H. bilis*, the diagnosis was made in three inbred mice: two male CBA/CA mice, and one male BALB/c mouse (15). *Helicobacter bilis*-like bacteria were identified at the periphery of the hepatic lesions in DBA/2, CBA/CA, and C57BL/6 mice, and *H. bilis* was isolated from the liver and/or bile, as it was in the SW mouse of this study that had multifocal hepatitis. Liver lesions in the three strains of inbred mice of the aforementioned study were similar to those in outbred mice of this study. In the outbred and inbred mice, *H. bilis*-associated liver disease varied from no lesions to disseminated focal aggregates of leukocytes located in close proximity to portal triads, central and collecting veins, or both, and were randomly distributed in the parenchyma. The cell infiltrates were composed principally of lymphocytes and macrophages (15).

The types of hepatitis associated with *H. hepaticus* and the liver lesions observed in the SW and aged inbred mice with *H. bilis* infection have some similar features. In the liver of *H. hepaticus*- and *H. bilis*-infected SW and inbred mice, the lesions consisted of multifocal hepatitis with a mixed-cell inflammatory infiltrate. However, livers from *H. bilis*-infected mice did not exhibit the oval cell hyperplasia and vasculitis that were observed in some strains of mice with *H. hepaticus*-associated hepatitis (13, 43). It should also be noted that oval cell hyperplasia and karyomegaly observed in male A/JCr mice infected with *H. hepaticus* were not as pronounced in outbred germfree female mice associated with *H. hepaticus* mono-infection (14).

As noted earlier with specific-pathogen-free inbred and axenic outbred mice and confirmed in this study, focal idiopathic necrotic hepatitis in the absence of *H. bilis* or *H. hepaticus* infection does occur (14, 15, 39). The genesis of the lesion appears to be the result of microvascular thrombosis and infarction. However, unlike *Helicobacter*-associated hepatitis, a liver with this idiopathic vascular lesion has little or no portal triad inflammation. Careful culture or PCR analysis for helicobacters and determining the susceptibility of select mouse strains to *Helicobacter*-associated hepatitis as well as idiopathic liver lesions are important considerations in arriving at a correct diagnosis. Importantly, in this study, *H. bilis* observed by silver stain in the liver of the one SW mouse also was isolated in culture from diseased hepatic parachyma. Liver lesions also were present in the liver where *H. bilis* DNA was identified by use of PCR analysis, and *H. bilis*-infected mice had significantly more severe hepatitis than did age-matched, uninfected controls. This does not prove that *H. bilis* is the cause of hepatitis in these mice; however, its presence in a liver with hepatitis certainly indicates that further studies are warranted to prove or disprove its association with hepatitis.

Although there were only four male mice in our study population, comparison of potential effects of sex in group III mice indicated that *H. bilis*-infected female mice had significantly more severe portal inflammation than did *H. bilis*-infected male mice. This contrasts with the effect of sex on increased disease susceptibility in males that was observed in several studies of *H. hepaticus*-associated hepatitis (13, 14).

In this study, sera from 3 of 11 *H. bilis*-infected mice yielded false-negative ELISA results for *H. bilis* infection. These mice were 6 to 8 months old at necropsy, and likely acquired *H. bilis* infection prior to weaning. Lack of seroconversion may be related to

the absence of hepatitis in these mice, or alternatively, acquisition of natural infection with *H. bilis* may have been more recent, with insufficient time for an antibody response to develop, as others have reported (22, 44). Previous studies by our group that measured seroconversion of outbred sentinel mice exposed to *H. bilis*, *H. hepaticus*, and *H. rodentium* by dirty bedding transfer established that this ELISA protocol, using outer membrane antigens, detected seroconversion with sensitivity of 98 to 100% across the three *Helicobacter* spp (44). However, only a low number of mice acquired *H. bilis* infection and not until after 4 months of dirty bedding exposure. Analysis of the ELISA data reported here for *H. bilis* and *H. hepaticus* infection in a more limited number of mice, indicated that the sensitivity for detecting *H. bilis* antibody was 73%, and was 100% sensitive for *H. hepaticus* infection. Specificity of both assays was high (100 and 96%, respectively), potentially biased by mono-infection with *H. bilis* or *H. hepaticus*.

Because serologic testing, using currently available methods, is clearly limited in sensitivity for detecting seroconversion to *H. bilis*, recombinant immunodominant proteins of *H. bilis* membrane extracts have been used in an attempt to improve sensitivity and specificity for serodiagnosis of *H. bilis* infection in mice (25). On the basis of results from testing 76 mice naturally infected with *H. bilis* (n = 41), *H. hepaticus* (n = 27), or an unspeciated helicobacter, (n = 8) the assay sensitivity using outer membrane extracts to each respective helicobacter, was similar to our results (76 and 73%, respectively). However, the assay based on recombinant antigens to each of the helicobacters yielded lower sensitivity (51 to 62%). The recombinant approach enhanced specificity to 96% compared with 87%, for outer membrane extracts (25). Our findings that three *H. bilis*-infected, outbred SW mice, representing 27% of group III mice, did not seroconvert, reinforces current data that positive results of helicobacter species-specific PCR analysis or recovery by culture remain definitive evidence of infection.

The finding of *H. bilis* infection and associated disease in a genetically heterogeneous outbred strain of mice is consistent with the ability of *H. bilis* to infect other mammals with heterogeneous genetic backgrounds. Indeed, to date, this species of helicobacter ranks near the top in terms of its wide host range. *Helicobacter bilis* has also been isolated from the stomach of dogs and intestine of gerbils, and from nude rats with typhlocolitis (20). The organism is also capable of experimentally inducing typhlocolitis in nude rats, ICR SCID mice, mdr1a$^{-/-}$ mice on a FVB background, and IL-10$^{-/-}$ mice on a C57BL background (3, 20, 28, 37). In addition to typhlocolitis, male CB17 SCID mice also develop hepatitis when experimentally infected with *H. bilis* (16). Importantly, *H. bilis* has been identified by molecular means in the gallbladder tissue of humans with chronic cholecystitis and from the liver of humans with primary sclerosing cholangitis PSC (8, 10). Most recently, *H. bilis* DNA was identified in the liver of a high percentage of Thai and Japanese patients with hepatocellular carcinoma and of Japanese patients with hepatobiliary cancer (17, 30). Seropositivity to *H. bilis* has also been linked to chronic liver and autoimmune liver disease in humans (1, 31).

There is precedent as well for other *Helicobacter* species causing hepatitis in other mammals under certain circumstances. *Helicobacter bilis* belongs to a taxa of closely related helicobacters (5). One of these with *H. bilis* morphology is '*H. rappini*' ('*Flexispira rappini*'), which can cross the placenta of pregnant sheep, induce abortions, and cause acute hepatic necrosis in sheep fetuses. This organism also can experimentally induce abortion in guinea pigs; the fetuses have hepatic necrosis (2, 26). Also, an organism with "*F. rappini*" morphology (possibly *H. bilis*) was observed in the common bile duct of rats experimentally infected with the liver fluke *Fasciola hepatica* (7). The authors speculated that the fluke infection altered the biochemical properties of the rats' bile and allowed the bacteria to colonize this normally bacteriostatic milieu. In addition, another EHS, *H. pullorum,* has been isolated from the liver of chickens with hepatitis and from humans with gastroenteritis (38). *Helicobacter canis* has been isolated in culture from the liver of a puppy with hepatitis (15). Most recently, *H. cinaedi* has been isolated from the inflamed liver and intestine of a rhesus macaque with clinically debilitating, idiopathic colitis (12).

In this study, infection of the outbred mice with *H. bilis* made it difficult to interpret whether creatine administered to these animals had a pathologic effect on the liver. Others who have studied hepatic lesions in mice dosed with creatine have also speculated that unidentified microorganisms in mice may be acting as co-promoters of inflammation in the liver of creatine-treated mice (40). Clearly, although *H. hepaticus* is well established as a pathogen in certain strains of mice, further studies are needed to define the role that *H. bilis* and other EHS may play in hepatic and biliary disease in mice, and in other animals and humans with naturally acquired gastrointestinal diseases. Additional studies in animals and humans, screening for *H. bilis* and other EHS should be done by use of culture and PCR analysis of specimens of diseased hepatobiliary tissue; the absence of the organism in healthy tissue also should be confirmed. Serologic assays should be developed and field tested to conduct epidemiologic studies in humans and other animals to ascertain association and possible causality of EHS with liver disease and whether aging predisposes the host to more severe disease due to EHS. Finally, to understand the pathogenesis of this emerging pathogen, continued investigations in *H. bilis*-infected animal models should remain a priority.

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