Serodiagnosis of *Helicobacter hepaticus* Infection in Mice by an Enzyme-Linked Immunosorbent Assay

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Helicobacter hepaticus is a newly recognized bacterium associated with chronic active hepatitis, hepatic carcinoma, and inflammatory bowel disease in mice. Currently, fecal or tissue PCR, fecal culture, or histologic examination of silver-stained liver sections is used to diagnose *H. hepaticus* infection. In this report, we describe an enzyme-linked immunosorbent assay (ELISA) for serodiagnosis of *H. hepaticus* infection in mice with a membrane digest preparation of *H. hepaticus* as the antigen. Sera from mice positive for *H. hepaticus* by PCR or histologic examination ($n = 88$), positive for *Helicobacter bilis* by PCR ($n = 13$), positive for other helicobacters (not identifiable to species level) by PCR ($n = 25$), or negative for all *Helicobacter* species by PCR ($n = 162$) were used to evaluate the ELISA. Results indicated that ELISA provided 93.2% sensitivity, 94% specificity, 87.2% positive predictive value, and 96.9% negative predictive value. Cross-reactive antibodies were detected in some mice infected with helicobacters not identifiable to species level. To further define ELISA sensitivity and specificity, groups of 10 C57BL/6 mice were inoculated per os with *H. hepaticus*, *Helicobacter muridarum*, or *H. bilis*. Sera were collected and examined by the ELISA. *H. hepaticus*-infected mice seroconverted by 2 weeks and maintained ELISA reactivity throughout the 18-week study, while mice infected with *H. muridarum* and *H. bilis* were negative by ELISA. These results indicate that this reported ELISA is highly sensitive and specific for the serodiagnosis of *H. hepaticus* infection in mice.

Helicobacter hepaticus is a gram-negative, spiral-shaped, microaerobic, motile bacterium that was first identified in mice in 1992 (3). Infection with *H. hepaticus* has been associated with chronic active hepatitis and hepatic carcinoma in a variety of mouse strains (3, 4, 10, 13) and with inflammatory large bowel disease in immunodeficient mice (11). Although *H. hepaticus* infections are often subclinical, the potential for this bacterium to adversely affect or confound data obtained from infected mice is substantial. Liver and intestinal lesions may invalidate data from studies involving these organ systems, and rectal prolapse, as a sequela to inflammatory bowel disease, may necessitate euthanasia of mice involved in experimental investigations prior to completion of the project. In addition, studies of infected mice have demonstrated expression of heat shock protein 70 (12), elevated levels of serum alanine aminotransferase (4), altered bile acids (10), and production of anti-*Helicobacter* antibodies (4, 10, 12, 13), indicating that *H. hepaticus* infection may perturb homeostasis. Diagnosis of infected mice is paramount because of the profound effects that *H. hepaticus* may have on research.

Diagnostic methods currently used to detect *H. hepaticus*-infected mice include histopathologic examination of silver-stained sections of liver to visualize organisms (10), culturing the liver or feces to recover organisms (4), and PCR of cecal tissue or feces to detect specific *H. hepaticus* DNA (1, 2, 8). Histopathology is a relatively specific test but requires euthanasia of the animal, lacks sensitivity, and requires trained personnel for interpretation. Culture is both specific and sensitive but has several disadvantages: (i) *H. hepaticus* is fastidious and requires strict environmental growth conditions, making cul-

ture relatively expensive; (ii) the intestinal tract is the preferred site to culture *H. hepaticus*, and contamination with other enteric bacteria can be problematic; and (iii) growth of *H. hepaticus* may require 5 to 7 days. PCR is a highly sensitive and specific test, and results can be obtained within 24 h of collection. However, this test is labor-intensive and requires specialized equipment and expensive reagents.

Experimental investigations using enzyme-linked immunosorbent assay (ELISA) as a diagnostic test have demonstrated that *H. hepaticus*-specific immunoglobulins are produced in infected mice, but seroconversion was not evident until about 6 months of age (4). In this report, we describe a highly specific and sensitive ELISA which detects *H. hepaticus*-specific immunoglobulins as early as 2 weeks after exposure to the bacterium.

MATERIALS AND METHODS

Bacteria and cultivation. An *H. hepaticus* isolate was obtained from an adult male A/JCr mouse from a colony in which infection was endemic (10, 13) by a filtration and culture technique previously described (4). Briefly, fecal pellets were placed in phosphate-buffered saline (PBS) (14.5 mM Na_2HPO_4 , 108 mM Na_2HPO_4 , 1.37 M NaCl [pH 7.4]), disrupted by vortexing, and centrifuged at $10,000 \times g$ for 20 min. The supernatant was removed and passed through a 0.45- μm -pore-size filter, and the filtrate was placed onto moist 5% sheep blood agar plates. Plates were incubated for 5 to 7 days at 37°C in a microaerobic environment created by flushing a vented jar with 90% N_2 –5% H_2 –5% CO_2 for 45 s. The isolate was identified as *H. hepaticus* by ultrastructural morphology, urease activity, and DNA sequence analysis (8). For the ELISA antigen, *H. hepaticus* was grown in brucella broth (Difco Laboratories, Detroit, Mich.) supplemented with 5% fetal calf serum (Sigma Chemical Co., St. Louis, Mo.). Cultures (50 ml) were vigorously agitated with a stir bar in 250-ml Erlenmeyer flasks and incubated for 24 h at 37°C in the microaerobic environment. Type strains of *Helicobacter bilis* (ATCC 51630) and *Helicobacter muridarum* (ATCC 49282) were obtained from the American Type Culture Collection (Rockville, Md.) and cultured on blood agar plates as described above.

Antigen preparation. Bacteria were pelleted, washed twice by centrifugation for 10 min at $10,000 \times g$ in sterile PBS, and frozen at -20°C . Stored pellets were combined and resuspended in PBS containing 1% *n*-octyl- β -D-glucopyranoside (Sigma) for 30 min at 25°C to release membrane proteins (7). Insoluble material

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was pelleted by centrifugation for 20 min at $10,000 \times g$. To remove detergent, the supernatant was decanted and dialyzed overnight at 4°C against PBS through a 12,000- to 14,000-Da membrane (Spectrum Medical Industries, Inc., Houston, Tex.). The protein concentration of the membrane digest antigen preparation was determined by a bicinchoninic acid procedure (9), and aliquots were frozen at -20°C .

ELISA. Antigen was diluted to 0.25 μg of protein per ml in coating buffer (0.1 M NaHCO_3 , pH 9.6), and 200 μl was added to each test well of Falcon Pro-bind 96-well plates (Becton Dickinson and Company, Lincoln Park, N.J.). Antigen was allowed to bind to plates for 48 h at 4°C . The antigen solution was discarded; 200 μl of blocking buffer (PBS with 0.5% nonfat dry milk and 0.05% Tween 20) was added to each well, incubated for 30 min at room temperature, and discarded; and 200 μl of serum diluted 1:100 in fresh blocking buffer was added to duplicate wells. After incubation at 37°C for 2 h, plates were washed three times in PBS with 0.05% Tween 20 (PBS-Tween). Two hundred microliters of alkaline phosphatase-labeled secondary antibody [F(ab')₂ fragment goat anti-mouse immunoglobulin G (H + L); Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.] diluted 1:7,000 in PBS-Tween was added to each well and incubated at 37°C for 2 h. Plates were washed five times with PBS-Tween. One hundred microliters of phosphatase substrate solution (1 mg of Sigma 104 phosphatase substrate [Sigma] per 1 ml of substrate buffer [2 mM MgCl_2 , 27.5 mM Na_2CO_3 , 22.5 mM NaHCO_3]) was added to each well and incubated in the dark at room temperature for 1 h. The optical density (OD) of each well was measured (kinetic reader EL312E; Bio-Tek Instruments, Inc., Winooski, Vt.) and automatically calculated as the OD at 405 nm minus the OD at 490 nm to correct for optical interference. Two wells not coated with antigen, but subjected to all other steps of the ELISA, served as controls. The cutoff OD value at 405 nm was set at 3 standard deviations above the mean of 162 serum samples from mice negative for *Helicobacter* infection by PCR (2, 8).

Animals and sample collection. Sera from multiple strains of immunocompetent mice sent to the University of Missouri Research Animal Diagnostic and Investigative Laboratory (Columbia) from a variety of sources were used to evaluate the ELISA. Sera from 88 *H. hepaticus*-positive mice, 13 *H. bilis*-positive mice, 162 *Helicobacter*-negative mice, and 25 mice infected with helicobacters not identifiable to species level were tested by the ELISA. *Helicobacter*-infected mice were identified by amplification of *Helicobacter* genus- or species-specific DNA from cecal tissue (8) or feces (2) by PCR as previously described or by visualization of argyrophilic bacteria morphologically consistent with *H. hepaticus* on histopathologic examination of silver-stained liver sections. Mice were considered free of *Helicobacter* infection if *Helicobacter* genus-specific DNA was not amplified from cecal tissue or feces by PCR. For PCR evaluation, DNA was extracted either from voided fecal pellets or from fecal pellets or cecal tissue aseptically harvested from euthanized mice. Blood was collected by cardiocentesis from euthanized mice, and the serum was removed and stored at -20°C . For histopathologic examination, the liver was collected in neutral buffered 10% formalin, processed into 5- μm sections, and stained with hematoxylin and eosin and Steiner silver stains.

To evaluate anti-*H. hepaticus* antibody production following exposure to the bacterium and to determine if cross-reactive antibodies were generated by infection with other well-characterized murine helicobacters, groups of 10 3-week-old C57BL/6 mice documented to be free of *Helicobacter* infection by PCR were orally gavaged with 10^7 viable organisms of either *H. hepaticus* (MU isolate), *H. muridarum* (ATCC 49282), or *H. bilis* (ATCC 51630). Five C57BL/6 mice orally gavaged with 0.5 ml of PBS served as negative controls. At 2, 4, 6, 11, and 18 weeks postinoculation, blood was collected by retro-orbital sinus puncture from each mouse under methoxyflurane anesthesia. Serum was recovered and tested by the ELISA. To confirm *Helicobacter* intestinal colonization, a fecal pellet was collected from each mouse at 2, 6, and 18 weeks postinoculation and subjected to PCR evaluation for *Helicobacter*-specific DNA.

ELISA performance. The sensitivity, specificity, negative predictive value, and positive predictive value of the ELISA for detecting *H. hepaticus*-infected mice were calculated with the following formulas: sensitivity = $(\text{TP}/(\text{TP} + \text{FN})) \times 100$, specificity = $(\text{TN}/(\text{TN} + \text{FP})) \times 100$, positive predictive value = $(\text{TP}/(\text{TP} + \text{FP})) \times 100$, and negative predictive value = $(\text{TN}/(\text{TN} + \text{FN})) \times 100$ where TP (true positive) = *H. hepaticus* PCR or histopathology positive and ELISA positive, TN (true negative) = *H. hepaticus* PCR negative and ELISA negative, FP (false positive) = *H. hepaticus* PCR negative and ELISA positive, and FN (false negative) = *H. hepaticus* PCR or histopathology positive and ELISA negative.

RESULTS

ELISA. To establish a baseline OD value to classify sera as positive or negative, sera from mice negative for *Helicobacter* genus-specific DNA by PCR ($n = 162$) were tested by ELISA. The mean OD value of these sera was 0.057 with a standard deviation of ± 0.060 . The baseline for the ELISA was arbitrarily established as the mean OD value of the *Helicobacter* PCR-negative population plus 3 standard deviation units. On this basis, sera with OD values >0.237 were classified as pos-

TABLE 1. *H. hepaticus* ELISA results from mice free of *Helicobacter* infection or infected with *H. hepaticus*, *H. bilis*, or helicobacters not identifiable to species level

ELISA result	No. (%) with <i>Helicobacter</i> infection status:			
	<i>Helicobacter</i> free	<i>Helicobacter hepaticus</i>	<i>Helicobacter bilis</i>	<i>Helicobacter</i> not identifiable to sp. level
Positive	3 (2)	82 (93)	0 (0)	9 (36)
Negative	159 (98)	6 (7)	13 (100)	16 (64)

itive for *H. hepaticus* and those with OD values <0.237 were classified as negative for *H. hepaticus*. Based on this criterion, 159 of 162 serum samples from PCR-negative mice were negative by ELISA.

The ELISA performance was evaluated with sera from mice known to be infected with *H. hepaticus*, *H. bilis*, or other helicobacters not identifiable to species level (Table 1). The ELISA was 93.2% sensitive and 94% specific in identifying *H. hepaticus*-infected mice and had an 87.2% positive predictive value and a 96.9% negative predictive value.

Sera from C57BL/6 mice experimentally infected with *H. hepaticus*, *H. bilis*, or *H. muridarum* were used to further evaluate the sensitivity and specificity of the *H. hepaticus* ELISA and to determine when serum antibody to *H. hepaticus* was first detectable. *H. hepaticus*-gavaged mice developed anti-*H. hepaticus* antibodies by 2 weeks postinoculation and maintained ELISA-positive antibody levels throughout the 18-week study (Fig. 1). Sera from mice orally gavaged with *H. muridarum* or *H. bilis* were not ELISA positive at any time. To document intestinal colonization with *Helicobacter* following experimental inoculations, the feces of all mice were subjected to *Helicobacter* PCR assay at the end of the study. *Helicobacter*-specific DNA was amplified from the feces of *H. hepaticus*- and *H. muridarum*-gavaged mice but not from the feces of mice gavaged with *H. bilis*. Because intestinal colonization with *H. bilis* could not be confirmed by fecal PCR, 10 additional C57BL/6 mice were inoculated intraperitoneally with 10^7 viable *H. bilis* organisms. Sera collected from these mice at 3, 6, and 9 weeks postinoculation did not react in the *H. hepaticus* ELISA.

FIG. 1. Serum antibody response of C57BL/6 mice following oral inoculation with 10^7 *H. hepaticus*, *H. bilis*, or *H. muridarum* organisms. Control mice were orally inoculated with PBS. Each point represents the mean of 10 serum samples. Error bars represent standard errors of the means.

DISCUSSION

In this report, we describe an ELISA that detects anti-*H. hepaticus* antibodies in the sera of mice colonized with *H. hepaticus*. The data indicate that the ELISA is highly sensitive for the diagnosis of *H. hepaticus* and has a high negative predictive value. The ELISA also had a high specificity, although cross-reactive antibodies were detected in some mice infected with murine helicobacters not identifiable to species level. The ELISA has advantages over PCR, culture, and histopathology as a primary diagnostic test to detect *H. hepaticus*-infected mice because numerous samples can be tested in a short period of time at a minimal expense.

The ELISA was highly sensitive (93.2%) for identifying mice naturally infected with *H. hepaticus* and was comparable to fecal PCR or histopathology. The high sensitivity of the ELISA allowed us to detect anti-*H. hepaticus* antibodies in the sera of mice orally gavaged with the bacterium as early as 2 weeks postinoculation. This is in contrast to a previously reported *H. hepaticus* ELISA that did not detect anti-*H. hepaticus* antibodies in the sera of A/J mice with endemic infection until about 6 months of age (4). Some of the difference between the detection times of the two ELISAs may be because we used sera from experimentally infected mice. However, in other studies in our laboratory, mice free of *Helicobacter* infections that were exposed to dirty cage bedding from *H. hepaticus*-infected mice tested positive by our ELISA within 4 weeks (6). The higher sensitivity of our ELISA may also be due to the method of antigen preparation. The previously reported ELISA used a whole-cell sonicate of *H. hepaticus* as the antigen, while our ELISA used a membrane digest preparation. In pilot studies using a limited number of serum samples, the ratio of OD values from *H. hepaticus*-positive sera and OD values of negative sera (positive-negative ratio) was determined with both whole-cell sonicate and membrane digest preparations. The positive-negative ratio of sera tested on membrane digest preparations was approximately 19-fold higher than the positive-negative ratio of the same sera tested on whole-cell sonicate preparations (6). The reason for this increased sensitivity is unknown but may be the preservation (14) or concentration of immunogenic epitopes of *H. hepaticus* membrane proteins during extraction with the nonionic detergent *n*-octyl- β -D-glucopyranoside.

The specificity of the ELISA for detecting anti-*H. hepaticus* antibodies was high when sera from mice free of all *Helicobacter* infections were tested, suggesting that cross-reactive immunoglobulins directed against common bacterial flora of mice are infrequently identified by this ELISA. When sera from mice naturally or experimentally infected with *H. bilis* or experimentally infected with *H. muridarum* were tested by ELISA, no cross-reactive antibodies were detected, indicating limited cross-reactive epitopes among these species. However, sera from several mice naturally infected with *Helicobacter* sp. other than *H. hepaticus* or *H. bilis* tested positive by the ELISA, suggesting that mice infected with these helicobacters not identifiable to species level can produce cross-reactive antibodies. It is well known that many bacteria share cross-reactive antigens, with the degree of antigen cross-reactivity increasing among genetically related bacteria (5). Therefore, it was not unexpected that cross-reactive antibodies were generated in mice infected with some of the uncharacterized murine *Helicobacter* spp.

To exploit the strengths of the ELISA, we propose that it be utilized as a screening test to identify *H. hepaticus*-infected mice. With the negative predictive value of the ELISA being 96.9%, *H. hepaticus* infection can be ruled out with a high

degree of confidence in mice serologically negative by ELISA. Also, the ELISA was highly sensitive for detecting *H. hepaticus*-infected mice (93.2%), making it likely that *H. hepaticus*-infected mice would be identified by serologic monitoring. Because some mice infected with uncharacterized murine *Helicobacter* sp. developed cross-reactive antibodies, sera testing positive for *H. hepaticus* by ELISA should be confirmed by other tests to determine if the antibody response is the result of infection with *H. hepaticus* or some other *Helicobacter* sp. PCR diagnosis of *Helicobacter* infections is an optimal secondary test for mice seropositive by ELISA since it is a highly sensitive and specific test capable of identifying individual species of the *Helicobacter* genus (8).

In summary, serologic diagnosis of *H. hepaticus* infection in mice by ELISA provides a rapid and relatively inexpensive screening test for *H. hepaticus* compared to the current diagnostic methods of histopathology, culture, and PCR. The high negative predictive value of the test allows accurate identification of mice free of *H. hepaticus* infection, while high test sensitivity allows *H. hepaticus*-infected mice to be readily identified. Thus, the ELISA is an ideal test to screen mouse colonies for *H. hepaticus* infection.

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