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Use of the P167 Recombinant Antigen for Serodiagnosis of *Helicobacter bilis*

Lon V. Kendall, DVM, PhD,* Sunlian Feng, PhD, Emir Hodzic, DVM, PhD, Kimberly Freet, and Stephen W. Barthold, DVM, PhD

***Helicobacter bilis* is widespread among research mouse colonies. Serodiagnosis of *Helicobacter* infections involves use of bacterial lysates or membrane antigen preparations that lack specificity, necessitating the need to identify a specific and sensitive antigen. A previously reported recombinant protein (P167) was evaluated for use as an *H. bilis*-specific antigen for serologic testing. Seventy-six mice naturally infected with *Helicobacter* spp. were identified from commercially bred or sentinel mice. Infection was confirmed and speciated by use of cecal specimen culture and fecal polymerase chain reaction (PCR) analysis, followed by restriction enzyme digest of the amplicon. Forty-one mice were determined to be monoinfected with *H. bilis*, 27 mice were determined to be monoinfected with *H. hepaticus*, and eight mice were infected with another species of *Helicobacter*. Serum was diluted 1:100 to evaluate the immunoreactivity to enzyme-linked immunosorbent assay preparations of *H. bilis* membrane extract and the immunodominant C and D fragments of the *p167* gene. The sensitivity was greatest for the membrane extract preparation (76%), whereas sensitivity to the P167C and D recombinants was lower (62 and 51%, respectively). However, the specificity of the membrane extract preparation was low (87%), compared with the much improved specificity of the recombinant P167C and D fragments (96 and 96%, respectively). These findings suggest that the recombinant P167C and D fragments of the *p167* gene product from *H. bilis* can be used as specific reagents in the serodiagnosis of *H. bilis* infection in mice.**

Helicobacters are a diverse group of enterohepatic, commensal, opportunistic bacteria that infect laboratory mice. The growing list of helicobacters isolated from mice includes *H. hepaticus* (8), *H. bilis* (11), *H. rodentium* (23), *H. muridarum* (16), *H. typhlonius* (13), *H. ganmani* (21), and others yet to be named (3). *Helicobacter bilis* is among the most common, and was first isolated from the bile, liver, and intestine of aged inbred strains of mice (11). Infections are often subclinical, but can induce hepatic and enteric disease in some strains of mice, particularly mice with immune deficiencies (12). Despite being recognized for several years, *H. bilis*-infected mice continue to be identified in research mouse colonies (15, 20, 25), with potentially adverse effects on research results. It is, therefore, important to include helicobacters in murine health-monitoring programs.

Several diagnostic methods, including culture (11) and molecular (1, 4, 20, 25), and serologic (15, 25) techniques, are available to identify *H. bilis*-infected mice. Because health-monitoring programs rely principally on use of serologic methods for detection of other murine pathogens, it would be advantageous to include *H. bilis* in the battery of tests. However, the currently available serologic assays for detecting *Helicobacter* infections in mice rely on either bacterial lysates or various types of membrane antigen preparations (9, 14, 18, 25). Both of these are antigenically complex, with presence of cross-reactive antigens resulting in decreased specificity (25). In addition, these antigen preparations do not detect low level antibody titers in naturally infected mice, and is not useful for detecting the early stages of infection (10, 18). Recombinant antigens offer the advantage of increased sensitivity and specificity. A novel *H. bilis* gene, *p167*, has recently been identified from an *H. bilis* genomic expression library that was immunodominant and specific for *H. bilis* (7). This recombinant protein was evaluated for the use as an *H. bilis*-specific antigen in the serodiagnosis of mice naturally infected with *H. bilis*.

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Center for Comparative Medicine, Schools of Medicine and Veterinary Medicine, University of California, Davis, Davis, California 95616.
**Corresponding author.*

Materials and Methods

Mice. Virus antibody- and *Helicobacter*-free, three- to five-week-old C3H/HeN (C3H) mice were purchased from the Charles River Hollister facility (Charles River Laboratories, Wilmington, Mass.). A complete list of agents screened is available on-line at: http://www.criver.com/health/indexstrain.jsp. The C3H mice were entered into the sentinel monitoring program at this institution and were housed in breeding pairs in accordance with the *Guide for the Care and Use of Laboratory Animals*, and the study reported here was approved by the University of California institutional animal care and use committee.

Sentinel mice were exposed to dirty bedding from various populations of colony mice for a period of eight to 10 weeks. At that time, a male and female were weaned to establish a new sentinel breeding pair, and the original adults and the remaining weanlings were submitted for serologic monitoring. Samples were collected after the mice were euthanized by use of carbon dioxide narcosis. Blood was collected by cardiocentesis, and the serum was diluted 1:5 and stored at –20°C for future analysis. Fresh fecal pellets were collected and stored at –20°C for future *Helicobacter* speciation by use of polymerase chain reaction

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(PCR) analysis. Sentinel mice were found to be seronegative to ectromelia virus, epizootic diarrhea of infant mice virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse encephalomyelitis virus, mouse hepatitis virus, mouse parvovirus, pneumonia virus of mice, reovirus, Sendai virus, and *Mycoplasma pulmonis*. Bacterial pathogens were not detected from culture of the cecal or nasopharyngeal specimens, and endo- or ectoparasites were not observed.

In addition, naturally infected mice were purchased from commercial vendor colonies with documented *Helicobacter* infections of unknown duration. According to health-monitoring reports, these mice were seronegative to ectromelia virus, *Encephalitizoon cuniculi*, epizootic diarrhea of infant mice virus, Hantaan virus, K virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse adenovirus, mouse cytomegalovirus, mouse encephalomyelitis virus, mouse hepatitis virus, mouse parvovirus, mouse thymic virus, *Mycoplasma pulmonis*, pneumonia virus of mice, polyoma virus, reovirus, and Sendai virus. Other than the identification of the *Helicobacter* species described later in this report, there were no pathogens isolated from cecal or nasopharyngeal specimen culture and parasites were not reported. Mice purchased included adult female and male C3H/HeN inbred mice infected with *H. bilis* (Charles River Laboratories, Wilmington, Mass.); Hsd:NSA (CF-1) outbred mice infected with *H. hepaticus* (Harlan Sprague Dawley, Indianapolis, Ind.); and CF-1 mice infected with *Helicobacter* spp. (Sasco Inc., Wilmington, Mass.). On arrival, mice were euthanized and samples were collected as described previously.

***Helicobacter* speciation.** Fresh fecal pellets were collected and suspended in one milliliter of phosphate-buffered saline (PBS: 0.55*M* sodium chloride, 5.8 m*M* sodium phosphate monobasic anhydrous, and 4.3 m*M* sodium phosphate dibasic anhydrous, pH 7.4). The suspension was centrifuged at 700 ×*g* for five minutes, and 60 μl of the suspension was combined with 140 μl of PBS. Purified DNA from the fecal supernatant was obtained following the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, Calif.) protocol for blood according to the manufacturer's instructions. Speciation of the infecting *Helicobacter* was performed by use of fecal PCR analysis with *Helicobacter* genus-specific primers and published methods (1). Amplicons were digested separately, using the restriction endonucleases *Cfo* I (Roche Applied Science, Indianapolis, Ind.) and *Nde* II (Promega, Madison, Wis.), and were analyzed by use of gel electrophoresis. The restriction digest of amplicons allowed differentiation among *H. hepaticus*, *H. bilis*, and *H. muridarum*, but could not distinguish other *Helicobacter* spp. (20). *Helicobacter* speciation was confirmed by use of PCR amplification of fecal DNA with *H. bilis* species-specific primers (20) or *H. hepaticus* species-specific primers (22) as described.

At the time of necropsy, cecal scraping specimens and contents were taken and placed in one milliliter of PBS. The mixture was passed through a 0.8-μm filter and plated directly onto a *Brucella* blood agar plate supplemented with trimethoprim, vancomycin, and polymixin B antibiotics to prevent overgrowth of bacterial contaminants. The culture was incubated at 37°C under microaerobic conditions. Cultures were checked daily for the presence of bacteria with morphology consistent with that of *Helicobacter* species. Once the organisms were identified, the culture plates were washed with PBS to suspend the bacteria. The DNA was isolated from the suspensions, and *Helicobacter* speciation was confirmed by use of PCR analysis and restriction enzyme digestion. The culture results from vendor mice were in agreement with fecal PCR results.

Antigen preparation. The *H. bilis* membrane extracts were prepared as described for use as an antigen in a serodiagnostic test (18). Briefly, *H. bilis* was cultured for two days at 37°C in *Brucella* broth with 5% fetal bovine serum in a shaker incubator under microaerobic conditions. Bacterial cells were pelleted by centrifugation, washed with cold PBS, and resuspended in PBS with 1% n-octyl-b-D-glucopyranoside (Sigma Diagnostics, Inc., St. Louis, Mo.) to release membrane proteins. Insoluble proteins were removed by centrifugation, and supernants were dialyzed in PBS to remove the detergent.

Recombinant *H. bilis* proteins were also prepared as described (7). Briefly, an *H. bilis* genomic expression library was created, using the Strategene λZAPII Library system (Strategene, La Jolla, Calif.). The P167 protein was identified as an immunodominant protein following screening with immune sera from mice experimentally infected with *H. bilis*. Five overlapping fragments of p167 encoding five proteins were designed, and each fragment was amplified by use of PCR analysis. Amplified DNA fragments were cloned in frame with the glutathione-S-transferase (GT) gene into a pGEX-2T expression vector (Pharmacia, Pistacaway, N.J.). The recombinant proteins were purified on glutathione columns and freed of their GT fusion partner by use of thrombin cleavage as described (6). The *H. bilis*-specific immunodominant protein fragments were identified as fragments P167C and P167D (7).

Enzyme-linked immunosorbent assay. Optimal coating concentrations of the P167C and P167D recombinant proteins and the *H. bilis* membrane extract were determined in an ELISA by use of checker board titration. Antigen was placed in 96-well plates (Enhanced Binding Microplates, Thermolab Systems, Helsinki, Finland) at concentrations ranging from 1 to 5 μg/ml in carbonate coating buffer (15 m*M* sodium carbonate and 33 m*M* sodium bicarbonate, pH 9.6). Proteins were allowed to bind to plates for 24 h at 4°C. Wells were emptied, incubated with 250 μl of blocking buffer (0.5% nonfat dry milk in PBS) for 30 min at room temperature, then were washed. Control serum from mice experimentally infected with *H. bilis* was diluted 1:100 in blocking buffer, and 50 μl was added to duplicate wells coated with the various antigen concentrations. A working dilution of 1:100 was chosen to maintain consistency with other serodiagnostic tests performed under the health surveillance program. Plates were incubated for 60 min at 37°C, followed by five washes with PBS-Tween (PBS with 0.05% Tween). Fifty microliters of horse-radish peroxidase-labeled secondary goat anti-mouse IgG (Charles River Laboratories, Wilmington, Mass.) diluted 1:10,000 in conjugate buffer (3% bovine serum albumin [BSA] in PBS with one milligram of gentamicin sulfate) was added to each well, and the plate was incubated for 60 min at 37°C. Plates were washed five times, and 100 μl of ATBS substrate solution (Roche Diagnostics, Indianapolis, Ind.) was added to each well, and the plate was incubated for 20 min at room temperature. Optical density (OD) values at 405 nm were read using a Kinetic Microplate Reader (Molecular Devices, Sunnyvale, Calif.). The optimal coating concentration used in further studies was determined to be 1 μg/ml for each of the three antigens: P167C, P167D, and *H. bilis* membrane extract. The baseline OD value for each of the three antigens was determined

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Table 1. Confirmation of *Helicobacter bilis* colonization by use of fecal polymerase chain reaction (PCR) analysis in sentinel C3H mice and commercially available mice

Mice	*H. bilis*	*H. hepaticus*	*Helicobacter* sp.	*Helicobacter* sp. negative
Sentinel C3H	9/75	8/75	0/75	58/75
Commercial mice	32/65	19/65	8/65	6/65

Data are expressed as No. of mice positive/No. of mice tested.

by evaluation of 46 serum samples from *Helicobacter*-free mice, verified by results of fecal PCR analysis. Antibodies were considered present when absorbance exceeded three standard deviations from the mean value for control serum from uninfected mice. The performance of the recombinant-protein-based ELISA was directly compared with that of the *H. bilis* membrane extract ELISA.

The probability that a test result is positive when evaluating infected mice (sensitivity) was determined by the number of true-positive results divided by true-positive plus false-negative results. The probability that the uninfected mice tested negative (specificity) was determined by the number of true-negative results divided by true-negative plus false-positive results. True-positive and true-negative results were based on PCR results as this assay is more sensitive than is culture (15).

Results

***Helicobacter* colonization status.** Seventy-five C3H sentinel mice were evaluated for *Helicobacter* colonization by use of fecal PCR analysis. *Helicobacter bilis* was identified as colonizing nine mice, *H. hepticus* was identified as colonizing eight mice, and 58 mice were not colonized by any species of *Helicobacter*. A total of 65 commercially available mice reportedly infected with *H. hepaticus*, *H. bilis*, or another *Helicobacter* species were purchased from companies with mice naturally infected with helicobacters on the basis of vendor health reports. *Helicobacter* colonization was confirmed on arrival at the animal facility by use of fecal PCR analysis and culture of cecal specimens. Of the 65 mice examined, *H. bilis* was confirmed in 32 mice, *H. hepaticus* was confirmed in 19 mice, *Helicobacter* spp. was confirmed in eight mice, and six mice were determined not to be infected. A summary of the number of mice tested and the number of mice positive for *H. bilis* is provided in Table 1. These results corroborated the PCR results obtained from culture of cecal specimens from the commercially available mice.

Comparison of recombinant antigen-based ELISA with membrane extract ELISA. The sensitivity and specificity of each ELISA were determined by evaluating sera from the mice following identification of their *Helicobacter* status. Table 2 indicates the performance of the three ELISAs. The specificity of each assay was high. The specificity of the membrane extract was 87%, and the recombinant protein antigens yielded specificity of 96 and 96% for P167C and P167D, respectively. However, the recombinant protein antigens were less sensitive at detecting reactive antibody in mice naturally infected with *H. bilis*. The membrane extract had sensitivity of 76% compared with that of the recombinant proteins, which had sensitivity of 62 and 51% for P167C and P167D, respectively, but this did not rule out non-specificity of the membrane extract. The results from the data suggest that P167C is a better antigen for the serodiagnosis of *H. bilis*.

Table 2. Performance of the recombinant P167 *H. bilis* proteins in an ELISA

ELISA[a]	ELISA result	*Helicobacter free*[b]	*H. bilis*[c]	*H. hepaticus*[d]	Other *Helicobacter* sp.[e]
P167C	Positive	3 (6)	21 (63)	0 (0)	0 (0)
	Negative	49 (94)	13 (37)	20 (100)	8 (100)
P167D	Positive	4 (6)	21 (51)	0 (0)	0 (0)
	Negative	60 (94)	20 (49)	27 (100)	8 (100)
Membrane extract	Positive	6 (9)	31 (76)	6 (22)	0 (0)
	Negative	58 (91)	10 (24)	21 (78)	8 (100)

[a]Purified recombinant proteins P167C and P167D were used as antigens in an ELISA and were compared with the membrane extract. Sera from mice free of *Helicobacter* infection and naturally infected with *H. bilis*, *H. hepaticus* or other *Helicobacter* species were tested.
[b]*Helicobacter* status was determined by use of fecal PCR analysis, followed by restriction endonuclease and/or species-specific primers.
[c]Mice naturally infected with *H. bilis* from sentinel mice or commercial mice.
[d]Mice naturally infected with *H. hepatiucs* from sentinel mice or commercial mice.
[e]Mice naturally infected with another *Helicobacter* species from commercial mice.
Data are expressed as No. (%) of mice with the indicated *Helicobacter* infection status.

Discussion

Since their identification, helicobacters have continued to pose substantial challenges to the research community. The role that *H. bilis* and *H. hepaticus* plays in the pathogenesis of hyperplastic typhlocolitis is well documented in genetically engineered mice with deficiencies in interleukin 10, T cell alpha and beta receptors, and others (2, 5, 19). Therefore it is important to determine the *Helicobacter* status in mice.

Diagnosis of *Helicobacter* infections in laboratory mice relies on culture, molecular diagnostics, serologic testing, or histologic examination. *Helicobacter bilis* is a fastidious bacterium that requires microaerobic conditions and several days to weeks for positive culture results; its growth can be inhibited by the presence of other enteric bacteria (11). Molecular diagnostics, such as fecal PCR analysis (1, 20) and fluorogenic nuclease PCR analysis (4) offer the advantage of increased sensitivity and specificity for fecal or tissue specimens; however, the reaction may be perturbed by inhibitors in feces, such as hemoglobin or bile pigment, and PCR analysis is costly and not universally available (15). With the advent of molecular diagnostics, the insensitive method of examination of silver-stained histologic sections of colon and/or liver has virtually been eliminated. Serologic diagnosis is a practical means for diagnosis of *H. bilis* infection; however the current antigens (i.e., whole-cell lysate and membrane extract) (14, 25) are insensitive, with low specificity as a result of the antigenic complexity and cross-reactive antigens. Additionally, these preparations generally detect only high titers of serum from naturally infected mice, and are not useful for detecting early infections (15).

Recombinant protein antigens offer the advantage of improved sensitivity and specificity over more crude antigen preparations, such as membrane extracts. In the study reported here, the previously identified P167C and P167D fragments of the immunodominant *p167* gene were evaluated for their usefulness in a serodiagnostic assay for *H. bilis*. The results of this study indicate that the recombinant proteins have increased specificity. However, the sensitivity of the recombinant proteins was less than that of the membrane extract antigen. These findings are similar to previous reports of an *H. hepaticus* recombinant outer membrane protein that was used in the serodiagnosis of *H. hepaticus* infection in mice. The reasons behind the de-

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creased sensitivity are likely similar (17). The cross reactivity of antibodies to other bacteria and other *Helicobacter* species may increase the sensitivity of the membrane extract assay. A limited number of antigenic epitopes may be present in the recombinant form of the protein. In addition, the in vivo expression of the P167 protein during the course of infection may be delayed, which could result in a delayed or attenuated immune response. This may be attributable to the protein being a secretory protein that has not been secreted during the course of infection (7), or the gene may be preferentially expressed during the course of infection. Additionally, the mice may not have had time to develop antibody responses to the recombinant proteins, as the immune response appears to be prolonged in naturally infected mice (10, 15). The duration of infection in these naturally infected mice from commercial vendors was unknown, but retired breeders were used in the evaluation. The sentinel mice were exposed for a period of 10 to 12 weeks before testing. A longer period of infection may have resulted in a more pronounced antibody response and improved sensitivity.

Other factors that may play a role in the decreased sensitivity are the sex and strain variability of the mice examined. A serodiagnostic assay cannot be developed for individual mouse strains because of its limited use. However, strain variability in response to *Helicobacter* infections in mice is well known and this may influence the serologic responses. For example, male A/J strain mice are prone to developing hepatic tumors associated with *H. hepaticus* infections (24), and C3H mice appear to be more susceptible to disease than are C57BL/6 mice (15). In this report, an inbred strain and outbred stocks of mice of both sexes were used to assess the usefulness of the recombinant protein-based ELISA, and these genetic differences may have resulted in the decreased sensitivity observed in association with the recombinant proteins.

One method used to bolster the sensitivity of the assay is to combine recombinant antigens in the assay. When P167C and P167D antigens were combined in the sample ELISA well, the assay performed with sensitivity and specificity equivalent to that when the antigens were used singly (data not shown). However, other yet to be identified recombinant proteins could be added to the P167 recombinant antigen to improve the sensitivity.

In conclusion, the P167C recombinant proteins out-performed the P167D protein and the *H. bilis* membrane extract, with sensitivity and specificity of 62 and 96%, respectively. Although lacking in sensitivity, the P167C protein is a serodiagnostic assay that is useful in identifying *H. bilis*-infected mice, and future assays that exploit this specificity may be developed.

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