Recombinant *Helicobacter bilis* Protein P167 for Mouse Serodiagnosis in a Multiplex Microbead Assay

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Infection of mice with *Helicobacter bilis* is widespread in research and commercial mouse colonies. Therefore, sensitive, specific, and high-throughput assays are needed for rapid and accurate testing of mice in large numbers. This report describes a novel multiplex assay, based on fluorescent microbeads, for serodetection of *H. bilis* infection. The assay requires only a few microliters of serum to perform and is amenable to a high-throughput format. Individual microbead sets were conjugated to purified, *H. bilis*-specific, recombinant proteins P167C and P167D and bacterial membrane extracts from *H. bilis* and *Helicobacter hepaticus*. For detecting *H. bilis* infection in the microbead multiplex assay, P167C and P167D provided significantly higher sensitivities (94 and 100%, respectively) and specificities (100 and 95%, respectively) than membrane extract (78% sensitivity and 65% specificity). Microbead multiplex assay results were validated by enzyme-linked immunosorbent assay. Purified recombinant proteins showed low batch-to-batch variation; this feature allows for ease of quality control, assay robustness, and affordability. Thus, recombinant antigens are highly suitable in the multiplex microbead assay format for serodetection of *H. bilis* infection.

**Helicobacter bilis** was initially identified as a mouse pathogen in 1995 (6) and is now grouped with enterohepatic helicobacters of rodents, including *H. hepaticus* (5), *H. muridarum* (11), *H. rodentium* (19), *H. typhlonius* (7), and others. *H. bilis* infection is widespread among research and commercial mouse colonies (8, 14). The infection is often subclinical, but in some genotypes of mice, particularly those with immune deficiency, this bacterium can produce liver and enteric diseases (8). In addition, *H. bilis* has been isolated from dogs, gerbils, rats, and cats (8). Importantly, *H. bilis* has also been isolated from humans and may be linked to disease (14). Human sera from patients with chronic liver disease contained antibodies to *H. bilis* (1).

At present, three types of diagnostic assays are used for the detection of *Helicobacter* infection: isolation and identification by bacterial culture, enzyme-linked immunosorbent assay (ELISA), and DNA amplification by PCR. For the detection of serum or plasma antibodies, the current ELISA protocols use bacterial membrane extracts as antigens. Membrane extract is not sufficiently sensitive for the detection of low levels of antibody in the early stage of infection (6, 7, 12). In addition, ELISA with membrane extract shows limitations in specificity, frequently leading to cross-reactivity among *Helicobacter* species (20). DNA amplification by PCR is sensitive and specific, particularly for diagnosis of early-stage infection. However, this method is prone to yield false-negative data because of PCR inhibitors in blood and/or fecal samples. Therefore, diagnostic assays with improved sensitivity and specificity are needed for testing animal samples. Once developed, accurate and high-throughput serodiagnostic assays for the detection of infection by *H. bilis* and other murine *Helicobacter* species may also be useful for diagnosing infection in other animal species, including humans.

We recently identified P167, an immunoreactive recombinant protein of *H. bilis* that was utilized as an antigen for testing antibody in mouse sera by ELISA (4). Among five overlapping peptides of P167 that were generated, only P167C and P167D were reactive to the *H. bilis* immune serum (4). P167C and P167D peptides were expressed in *Escherichia coli*, purified, and tested by ELISA for antigenicity against sera from mice infected with *H. bilis* and *H. hepaticus*. In this small trial, both P167C and P167D proved to be more sensitive and specific antigens than *H. bilis* membrane extracts (4).

In the present study, a multiplex serodetection assay was developed to detect *Helicobacter* infection in mouse serum samples by using the recombinant antigens P167C and P167D of *H. bilis*. This assay utilizes instrumentation and microbeads developed by Luminox Corp. (Austin, Tex.). The multiplex capability of this system is based on unique sets of fluorescent polystyrene microbeads (5.6 μm in diameter) (10, 15). Individual bead sets are coded by internal labeling with a specific ratio of red and orange fluorophores. Each bead set is conjugated with one of several target analytes (e.g., an antibody or antigen) and assembled into one reaction container (2). Thus, multiple analytes can be simultaneously detected in a single sample. In contrast, the conventional ELISA format is limited to detection of a single analyte. In addition to P167C and P167D, the multiplex assay also included *H. bilis* bacterial membrane extract for comparison and *H. hepaticus* membrane extract as a control for specificity. The multiplex assay not only conserved the sample but also reduced assay time and labor. Results described here also show that in the multiplex assay...
format, purified recombinant H. bilis antigens P167C and P167D are superior in sensitivity and specificity to bacterial membrane extracts as antigens for serodetection.

MATERIALS AND METHODS

Mice. C3HHeN (C3H) mice and C3H.Smn.ClerHsd-Pkrd−/− mice were purchased from the National Cancer Institute Animal Production Program (Fredrick Cancer Research Center, Frederick, Md.) and from Harlan Sprague Dawley (Indianapolis, Ind.), respectively. To confirm that the mice were free of Helicobacter infection, fecal pellets from all mice were tested by bacterial culture and PCR amplification upon arrival (9, 18). The mice were maintained as previously described (4).

The University of California, Davis, laboratory animal care program is fully accredited by the Association for Assessment and Accreditation of Laboratory Care, and this study was reviewed and approved by the Institutional Animal Care and Use Committee. All procedures and the use of mice were in compliance with the Public Health Service Guide for the Care and Use of Laboratory Animals.

Bacteria and native bacterial antigens. H. bilis (ATCC 51630) and H. hepaticus (ATCC 51448) were obtained from the American Type Culture Collection (Gaithersburg, Md.) and from the National Cancer Institute Animal Production Program (Fredrick, Md.) and cloned by plasmid transfection (7, 9). The species identity of each clone population was verified by PCR amplification with species-specific primers (9). Membrane antigen extracts of H. bilis or H. hepaticus were prepared as previously described (12).

Sera. Immune sera for H. bilis and H. hepaticus were generated in two steps as previously detailed (4, 9). They were used as positive controls for each species. Briefly, both H. hepaticus strains were grown under microaerobic conditions in brucella broth for 3 days at 37°C. The bacteria were adjusted to 10^8 CFU/ml and cultured on gelatinized culture plates for 7 days (9). The species identity of each clone population was verified by PCR amplification with species-specific primers (9). Membrane antigen extracts of H. bilis or H. hepaticus were monitored weekly by PCR amplification of fecal samples and culturing. At 6 months postinoculation, blood was collected and immune serum samples were harvested from infection-positive mice.

C3H mice, naturally infected with H. bilis or H. hepaticus and confirmed to be free of infection by other Helicobacter spp., were obtained from commercial sources. Eighteen mice, infected with only H. bilis, were purchased from Charles River (Portage, Mich.) colony PO3. These included 9 weanlings (9 males) and 9 retired breeders (5 females and 5 males). Helicobacter species (H. bilis or H. hepaticus) verification was performed for these two types of mice upon arrival. Fecal samples from all 18 mice were subjected to PCR using both sets of species-specific primers (9) and PCR with Helicobacter-specific primers followed by restriction fragment length analysis (16). Both methods confirmed that H. bilis-infected mice were infected with H. bilis and free of H. hepaticus and that H. hepaticus-infected mice were infected with H. hepaticus and were free of H. bilis. In addition, bacterial cultures of mucosal scrapings from the cecum and colon of these mice were analyzed to confirm infection by the respective Helicobacter strains (9, 16). All mice were necropsied, and serum samples were collected for testing.

Expression and purification of recombinant proteins. The two H. bilis-specific peptides, P167C and P167D, were cloned in pMx, a pGEX-2T vector, in frame with the glutathione S-transferase gene (Pharmacia, Piscataway, N.J.) containing a modified polylinker (3, 17). Recombinant proteins were expressed and purified on glutathione columns and freed of their glutathione S-transferase fusion partner by thrombin cleavage (3).

ELISA. Immunol flat-bottomed microtiter plates (Thermo Labsystems, Franklin, Mass.) were coated with recombinant P167C and P167D antigens or membrane extracts from H. bilis and H. hepaticus. Antigens were diluted to a final optimized concentration of 0.33 lg/ml in a coating buffer of Hanks balanced salt solution containing 0.375% sodium bicarbonate. Antigens (100 lg/well) were added to plates and incubated overnight at 4°C. The plates were washed with wash buffer (0.1% Tween 20 in phosphate-buffered saline [PBS], pH 7.4) and blocked with BLOTTO (5% nonfat powdered milk in wash buffer) for 1 h at room temperature. After blocking, the plates were washed, and 100 ll of mouse serum diluted 1:200 in BLOTTO was added. The plates were allowed to incubate at room temperature for 1 h and washed again with wash buffer. Detection antibody, biotinylated goat anti-mouse immunoglobulin G (IgG; Kirkegaard and Perry), was diluted 1:10,000 in BLOTTO and added to ELISA plates (100 ll/well). The plates were incubated with the detection antibody for 30 min at room temperature. After washing the plates again, 100 ll of Vectastain ABC detection reagent containing horseradish peroxidase H (Vector Laboratories, Burlingame, Calif.) was added according to the manufacturer’s instructions. The plates were washed and developed by adding 100 ll of 3.5:5.5-tetramethyl-
ylbenzidine color development reagent (Sigma Chemicals, St. Louis, Mo.)/well. The plates were developed at room temperature for 10 to 12 min, and the reaction was stopped by adding 50 ll of 1 M sulfuric acid/well. The color development was read at 450 nm in an ELISA plate reader (Bio-Rad, Hercules, Calif.). Cutoff values were determined for all four antigens; an average of ELISA readings in three separate pools of normal mouse sera was calculated, and three times the standard deviation value was added to the average. The samples were analyzed in duplicate. A minimum of two separate assays were performed for each antigen. Sera were designated positive or negative for antibodies to H. bilis and H. hepaticus based on the ELISA cutoff value.

Coupling proteins to Luminex microbeads. Microbeads were purchased from Luminex Corp. The beads were coated with various proteins by chemical cross-linking according to the manufacturer’s instructions. The bead stock was resuspended in vortexing and sonication (15 to 30 s). An aliquot of 2.5 10^5 beads was removed and centrifuged at 12,000 3 g for 2 min. Beads were resuspended in 80 ll of activation buffer (100 mM monobasic sodium phosphate, pH 6.3) by vortexing and sonication (15 to 30 s).

To activate the beads for cross-linking to proteins, 10 ll of 50-mg/ml sulfo-N-hydroxysuccinimide (Pierce, Rockford, Ill.) was added, and the beads were mixed by vortexing. Then 10 ll of 50-mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce) was added to be mixed by vortexing. The bead mixture was shaken on a rotator at room temperature for 20 min and centrifuged at 12,000 3 g for 2 min. The beads were washed twice with 1 ll of 50 mM morpholinoethanesulfonic acid (MES) buffer (pH 6.0). To coat with proteins, pelleted beads were resuspended in the relevant protein solution, diluted in 50 mM MES buffer (pH 6.0). Optimization of the antigen coating on microbeads was performed by coating different microbead sets with a protein concentration of each antigen at 200 llg/ml. The coated beads were washed four times and used to test against standard mouse sera positive for antibodies to either H. bilis or H. hepaticus. Microbead sets that provided the strongest signal specific for each antigen against the standard mouse sera were selected. The optimized protein concentrations for each antigen are as follows: 100 llg/ml for P167C, P167D, and H. bilis membrane extract and 25 llg/ml for H. hepaticus membrane extract. Beads were also coated with a control antibody (biotin-conjugated goat IgG, 100 llg/ml) and bovine serum albumin (BSA) as a control protein (100 llg/ml) diluted in 50 mM MES buffer (pH 6.0). The mixtures of activated beads and proteins were incubated by shaking on a rocker for 2 h at room temperature for coupling. After being coated with proteins, the beads were washed twice with wash buffer (0.1% Tween 20 in PBS, pH 7.4) and resuspended in 1 ll of blocking buffer (1% BSA, 0.1% Tween 20 in PBS [pH 7.4], 0.05% sodium azide). One bead was placed on a rocker at room temperature for 1 h before performing reactions.

After blocking, the beads were washed twice in 1 ll of blocking buffer. Finally, antigen-coated beads were resuspended in 1 ll of blocking buffer and stored at 4°C for up to 1 week. For long-term storage, the beads were kept frozen at 70°C for several months.

Multiplex detection of antibodies in mouse sera. Immunoreactions were set up in 96-well, filter-bottomed plates designed for high-throughput separations (1.2-mm-pore-size MultiScreen; Millipore Corporation, Bedford, Mass.). Typically, up to 2,500 beads for each individual bead set, coated with a known protein, were added per well. For example, for a six-target assay, 2,500 beads from each set coated with a known antibody were mixed to provide a total of 15,000 beads. Mouse serum was diluted 1:200 on 5% BLOTTO, and 50 ll of diluted serum was mixed with the six-target bead mixture per well, in a 96-well plate. The mixture of beads and serum was then incubated on a shaker for 1 h at room temperature. After incubation, the liquid was drained from the bottom of the plate in a vacuum manifold designed to hold 96-well plates (Millipore Corporation). The beads were washed three times successively by adding 100 ll of wash buffer per well and draining under a vacuum. For detection of mouse IgG, biotinylated goat anti-mouse IgG was used (Vector Laboratories) at a 1:1,000 dilution in wash buffer, and 100 ll was added per well. The beads were mixed as before and incubated at room temperature for 30 min. Following incubation with the second antibody, the beads were washed three times as before. To detect biotinylated IgG, 100 ll of streptavidin conjugated to R-phycocerythrin (streptavidin-PE; CalTag, Burlingame, Calif.) was added at a dilution of 1:500 in wash buffer. The contents of each well were mixed and incubated at room temperature for 15 min. The beads were washed three times with wash buffer. The washed
beads were finally resuspended in 100 μl of wash buffer per well and analyzed in a Luminex-100 instrument equipped with an XY-Platform for automatic reading of a 96-well plate.

**Luminex-100 instrument operation and data analysis.** The Luminex-100 instrument was manufactured by Luminex Corp. and purchased from Upstate USA (Lake Placid, N.Y.). The instrument was used at default settings, set by the manufacturer for routine applications, as directed by the user’s manual. Data were acquired by use of the Luminex Data Collection software (version 1.7). This software package allowed routine operation of the instrument, data acquisition, and data analysis. Calibration beads supplied by the manufacturer were used to adjust the instrument settings for bead set identification and for the detection of reporter (phycoerythrin). Events were gated to exclude doublets and other agglomerates. A minimum of 100 independent gated events were acquired for each bead set. The median fluorescence intensity (MFI) of 100 events (beads) was used as a measure of antibody detection. After acquisition by Luminex software, the data were further processed with Microsoft Excel software. For each assay, cutoff values were determined for all four antigens conjugated at optimal protein concentration. An average MFI for each bead set in three separate pools of normal mouse serum was calculated, and three times the standard deviation value was added to the average. For routine analysis, each experiment also contained a positive (immune) control serum sample. The samples were analyzed in duplicate, and a minimum of two separate multiplex experiments were performed. Serum samples were designated positive or negative for antibodies to *H. bilis*, based on the MFI readings.

**RESULTS**

Detection of serum antibodies by ELISA. ELISA plates were coated with purified *H. bilis* recombinant proteins P167C and P167D. In addition, ELISA plates were coated with membrane extracts prepared from *H. bilis* and *H. hepaticus* to determine the sensitivity and specificity of the assay. Each ELISA plate also contained a positive immune serum control. Two groups of *H. bilis*-infected mice were included in this analysis. Mice 1596-3 to 1596-11 were weanlings, and mice 1597-5 to 1597-19 were retired breeders. ELISA data for *H. bilis* showed good agreement between detection of serum antibodies and detection of *H. bilis* infection by PCR and bacterial culture (Table 1). However, there were a few exceptions in the weanling group. Though all weanlings were positive for *H. bilis* by PCR, two (1596-7 and 1596-11) did not show detectable reactivity to P167C, and one (1596-4) did not react to *H. bilis* membrane extract (Table 1). These results suggest that the three serum samples were probably taken early in infection, when the antibody response levels were still low. Because these mice were weanlings, it was possible that they had only a recent exposure to infection. In contrast, retired breeders (mice 1597-5 to 1597-19) showed strong levels of reactivity to all the *H. bilis* antigens as well as cross-reactivity to *H. hepaticus* membrane extract (Table 1). These mice probably had a much longer exposure to infection. For the weanling group, which showed moderate antibody responses, all sera reacted to purified recombinant antigen P167D. This antigen produced lower ELISA signals in most of the samples than P167C and *H. bilis* membrane extract (Table 1). In the case of sera from the *H. bilis*-infected retired breeder mice, all samples reacted strongly to all three antigens, but the ELISA signal with P167D antigen was lower than with P167C and *H. bilis* membrane extract (Table 1).

ELISA results for sera from animals infected with *H. hepaticus* are also shown in Table 1. The first group of 10 animals (mice 1582-11 to 1582-20) consisted of weanling mice, and the second group of 10 mice were retired breeders (mice 1583-6 to 1583-15). Accordingly, all of the retired breeder mice showed very strong antibody responses to membrane extract from *H. hepaticus*. In contrast, only 4 of 10 weanling mice were positive for antibodies. In terms of specificity, none of the sera from *H. hepaticus*-infected weanling mice reacted against any of the three *H. bilis* antigens. However, sera from retired breeder mice (1583-6 to 1583-15) with high antibody titers displayed higher cross-reactivity to *H. bilis* membrane extract than the two recombinant antigens, P167C and P167D (Tables 1 and 2). Both P167C and P167D were more specific (75% specificity) than *H. bilis* membrane extract (65% specificity) (Table 2).

**Detection of serum antibodies by multiplex microbead assay.** For direct and accurate comparison between the ELISA and the multiplex microbead assay, both assays were performed under the same experimental conditions, e.g., using the same buffers, antibodies, secondary detection antibodies, and temperature. The same biotin-conjugated secondary antibody, i.e., goat anti-mouse IgG, was used in both assay formats. However, the detection reagent used in the multiplex analysis (streptavidin-PE) was different from that used in the ELISA (streptavidin-conjugated horseradish peroxidase). The optimal concentration of each antigen for conjugation to beads was determined as described in Materials and Methods. For the three *H. bilis* antigens (P167C, P167D, and membrane extract), 100 μg of total protein/ml was optimal; for *H. hepaticus* membrane extract, 25 μg of protein/ml was optimal.

The results of the multiplex analysis of the sera tested by ELISA are shown in Table 1. The microbead set coated with BSA served as a control for nonspecific serum reactivity. For all mouse sera tested, these beads displayed MFI values of 15 to 20. The microbead set coated with biotinylated goat IgG served as a positive control for the detection reagent, streptavidin-PE; these beads displayed MFI values of 3,500 to 4,000. The multiplex results demonstrated a good correlation between serodetection and infection status. For *H. bilis* antibody detection, there were two clear differences between the two assay formats. First, there was an overall improvement in sensitivity and specificity in the multiplex microbead assay with P167C and P167D peptide antigens compared to that with the *H. bilis* membrane extract. Second, purified recombinant antigens in the ELISA yielded a higher sensitivity than *H. bilis* membrane extract in the multiplex microbead assay. For example, in weanling mice that might have low antibody levels (1596-3 to 1596-11), microbeads coated with either one of the two recombinant antigens, P167C or P167D, detected *H. bilis* antibodies in all nine sera (Table 2). On the other hand, two weanling mice (1596-8 and 1596-9) and two retired breeders (1597-8 and 1597-10) that were found by ELISA to be positive for *H. bilis* antibodies against *H. bilis* membrane extract were negative for a reaction to microbeads coated with the same antigen (Table 1). In addition, when microbeads coated with *H. bilis* membrane extract did detect *H. bilis* antibodies, the sensitivity was much lower than that of the beads coated with P167C and P167D (Table 1). The simplest explanation for this lower sensitivity in the multiplex assay with membrane extract is that microbeads have a much smaller surface area than ELISA wells, so the number of specific antigen molecules per bead is also lower. As discussed below and shown in Tables 2 and 3, purified recombinant antigens P167C and P167D were both highly sensitive (94 and 100%, respectively) and specific (100 and 95%, respectively) compared to *H. bilis* membrane extract.
### TABLE 1. Detection of serum antibodies by ELISA and multiplex microbead assay

| Serum sample or animal no. | P167C ELISA | P167C Multi | H. bilis extract ELISA | H. bilis extract Multi | H. bilis PCR | H. bilis culture | H. hepaticus PCR | H. hepaticus culture |
|---------------------------|-------------|-------------|------------------------|------------------------|-------------|-----------------|-----------------|---------------------|
| H. bilis immune serum     | 5+          | 5+          | 4+                     | 5+                     | 5+          | +               | 5+              | 5+                  |
| H. hepaticus immune serum | --          | --          | +                      | --                     | --          | --              | --              | --                  |
| H. bilis-infected weanling mice |            |             |                        |                        |             |                 |                 |                     |
| 1596-3                    | 2+          | 3+          | 2+                     | 4+                     | +           | +               | +               | +                   |
| 1596-4                    | 2+          | 3+          | +                      | 4+                     | --          | +               | +               | +                   |
| 1596-5                    | 3+          | 4+          | +                      | 3+                     | 2+          | +               | --              | +                   |
| 1596-6                    | 4+          | 4+          | +                      | +                      | 2+          | 2+              | --              | +                   |
| 1596-7                    | --          | +           | 2+                     | +                      | --          | +               | --              | +                   |
| 1596-8                    | 2+          | 3+          | +                      | 2+                     | --          | --              | +               | +                   |
| 1596-9                    | 3+          | 4+          | +                      | 3+                     | --          | --              | +               | +                   |
| 1596-10                   | +           | +           | 2+                     | +                      | 2+          | +               | --              | +                   |
| 1596-11                   | --          | +           | +                      | 2+                     | --          | --              | +               | --                  |
| H. bilis-infected retired breeder mice |            |             |                        |                        |             |                 |                 |                     |
| 1597-5                    | 4+          | 4+          | 2+                     | 4+                     | 3+          | +               | +               | +                   |
| 1597-7                    | 4+          | 4+          | 2+                     | 4+                     | 3+          | +               | 2+              | +                   |
| 1597-8                    | --          | +           | 3+                     | --                     | --          | --              | --              | ND                  |
| 1597-10                   | 4+          | 4+          | 2+                     | 3+                     | 4+          | 2+              | --              | +                   |
| 1597-13                   | 4+          | 4+          | 3+                     | 3+                     | 4+          | 3+              | 2+              | +                   |
| 1597-14                   | 4+          | 4+          | 3+                     | 3+                     | 4+          | 3+              | 3+              | ND                  |
| 1597-17                   | --          | 4+          | 4+                     | 5+                     | 4+          | 2+              | 2+              | 3+                   |
| 1597-18                   | 2+          | 3+          | 2+                     | 4+                     | 4+          | 3+              | 2+              | 4+                   |
| 1597-19                   | 4+          | 5+          | +                      | 2+                     | 4+          | 3+              | 2+              | 3+                   |
| H. hepaticus-infected weanling mice |            |             |                        |                        |             |                 |                 |                     |
| 1582-11                   | --          | --          | --                     | --                     | --          | --              | --              | --                  |
| 1582-12                   | --          | --          | --                     | --                     | --          | --              | --              | --                  |
| 1582-13                   | --          | --          | --                     | --                     | 4+          | 3+              | --              | +                   |
| 1582-14                   | --          | --          | --                     | --                     | +           | +               | --              | +                   |
| 1582-15                   | --          | --          | --                     | --                     | --          | --              | --              | ND                  |
| 1582-16                   | --          | --          | --                     | --                     | --          | --              | --              | ND                  |
| 1582-17                   | --          | --          | --                     | --                     | --          | --              | --              | ND                  |
| 1582-18                   | --          | --          | --                     | --                     | 4+          | 3+              | --              | +                   |
| 1582-19                   | --          | --          | --                     | --                     | 5+          | 4+              | --              | ND                  |
| 1582-20                   | --          | --          | --                     | --                     | --          | --              | --              | +                   |
| H. hepaticus-infected retired breeder mice |            |             |                        |                        |             |                 |                 |                     |
| 1583-6                    | 2+          | --          | 2+                     | 3+                     | 5+          | 5+              | 5+              | 5+                  |
| 1583-7                    | --          | --          | --                     | --                     | ++          | 5+              | 5+              | 5+                  |
| 1583-8                    | +           | --          | --                     | ++                     | 5+          | 5+              | 5+              | 5+                  |
| 1583-9                    | +           | --          | --                     | ++                     | 5+          | 5+              | 5+              | 5+                  |
| 1583-10                   | --          | --          | --                     | ++                     | 5+          | 5+              | 5+              | 5+                  |
| 1583-11                   | --          | --          | --                     | ++                     | 5+          | 5+              | 5+              | 5+                  |
| 1583-12                   | +           | --          | --                     | ++                     | 5+          | 5+              | 5+              | 5+                  |
| 1583-13                   | --          | --          | --                     | ++                     | 5+          | 5+              | 5+              | 5+                  |
| 1583-14                   | +           | +           | +                      | ++                     | 5+          | 5+              | 5+              | 5+                  |
| 1583-15                   | +           | +           | +                      | ++                     | 5+          | 5+              | 5+              | 5+                  |

*All samples were tested in duplicate at a 1:200 dilution (the sample from mouse 1596-4 was diluted 1:100 for ELISA with H. bilis extract), and two separate ELISA and multiplex microbead assays were performed for each antigen. The cutoff point for each antigen, in both assay formats, was determined as described in Materials and Methods. ELISA plates were read for OD at 450 nm. Results are presented as negative if below the cutoff point and positive if above the cutoff point. The number next to a symbol indicates the OD value as follows: +, 2+, 3+, 4+, and 5+ indicate OD values between the cutoff point and 0.3, between 0.3 and 0.7, between 0.7 and 1.0, between 1.0 and 1.5, and greater than 1.5, respectively. Multiplex microbead assay (Multi) results are reported as MFI values. Results are presented as negative if below the cutoff point and positive if above the cutoff point as follows: +, 2+, 3+, 4+, and 5+ indicate MFI values between the cutoff and 200, between 200 and 500, between 500 and 1,000, between 1,000 and 2,000, and greater than 2,000, respectively. For comparison, results of PCR and bacterial cultures for the detection of H. bilis and H. hepaticus infection in mouse tissue are also presented. ND, not done.

To detect serum antibodies against H. hepaticus, microbeads coated with H. hepaticus membrane extract were also included in the bead mixture used in the multiplex assay (Table 1). As in the ELISA, sera from all the H. hepaticus-infected retired breeders (1583-6 to 1583-15) showed the strongest antibody reaction to H. hepaticus membrane extract-coated microbeads. On the other hand, sera from only 5 of 10 H. hepaticus-infected weanling mice were positive for antibodies. Thus, H. hepaticus membrane extract is similar in sensitivity in both ELISA and the multiplex microbead assay for the detection of antibodies extract (78% sensitivity and 65% specificity) in the detection of serum antibodies to H. bilis.
against *H. hepaticus*. However, as shown in Table 1, *H. hepaticus* membrane extract was less specific in the detection of antibodies against *H. hepaticus* than *H. bilis* membrane extract was in the detection of antibodies to *H. bilis*, in both the ELISA and multiplex microbead formats.

**DISCUSSION**

Serdetection in a multiplex assay format enables simultaneous detection of antibodies to several antigens in a small amount of a single sample. Thus, a major advantage over other immunoassays is the ability to analyze a small sample volume; this feature is a critical consideration for species such as mice. The multiplex technology allows flexibility in assay configuration; conjugated bead sets can be mixed as needed. This feature avoids waste of important reagents. In addition, a key strength of the multiplex format is the ability to incorporate internal controls in the test sample (13). For example, beads coated with control antigens are mixed with beads coated with test antigens. This built-in system of controls enables the direct and simultaneous measurement of specificity and sensitivity in analysis of immune responses to multiple antigens (13). The multiplex microbead assay provides a larger dynamic range for analysis than ELISA; this feature allows for accurate analysis of antibody levels without additional dilution of sera with high antibody titers.

In this study, the multiplex microbead methodology was used to test mouse sera for antibodies to four different *Helicobacter* antigens and to detect simultaneously antibodies to two different species of *Helicobacter*. To validate results obtained by the multiplex microbead assay, ELISA was performed on all the serum samples. A summary of the results from the two assays is presented in Tables 2 and 3. Mice infected with *H. bilis* (18 mice) and *H. hepaticus* (20 mice) were used. In each category, one half of the mice were weanlings, representing animals likely to have had a shorter exposure to bacterial infection or containing maternal antibodies, and the other half were retired breeder mice, which were likely to have had a longer exposure to bacterial infection (Table 1).

For *H. bilis*, the membrane extract produced consistent results in the ELISA, with 94% sensitivity (Table 2); this extract was less sensitive in the microbead assay (78%) (Table 3). Among the two recombinant antigens, P167D showed the highest sensitivity (100%) in both the ELISA (Table 2) and the microbead assay (Table 3). P167C demonstrated higher sensitivity in the microbead assay (94%) (Table 3) than in the ELISA (88%) (Table 2). In addition, both recombinant antigens demonstrated higher specificity than the *H. bilis* membrane extract in the ELISA as well as the multiplex microbead assay. In particular, the specificity for the *H. bilis* recombinant antigens was significantly higher (100% for P167C and 95% for P167D) than for the *H. bilis* membrane extract (65%) in the microbead assay (Table 3). In the ELISA, the specificities of *H. bilis* recombinant antigens P167C and P167D were both 75%, whereas *H. bilis* membrane extract showed 65% specificity (Table 2).

In both immunoassay formats, *H. hepaticus* membrane extract was the least sensitive (70% in the ELISA and 75% in the microbead assay) and specific (50% in the ELISA and 39% in the microbead assay) antigen in the detection of antibodies against *H. hepaticus* (Tables 2 and 3). Thus, recombinant antigens for *H. hepaticus*, like those for *H. bilis* (P167C and P167D), may be necessary for use in serodetection.

Taken together, the above results show that P167C and P167D recombinant antigens are better suited for the detection of *H. bilis*-specific antibodies in mouse sera than the membrane extract by both the microbead assay and the ELISA. The two recombinant antigens detected antibodies in all the *H. bilis*-infected retired breeder mice. More importantly, the two recombinant antigens together provided a highly sensitive assay for serodetection in animals with low levels of *H. bilis* antibodies, i.e., weanling mice. The added advantage of P167C and P167D recombinant antigens over the *H. bilis* membrane extract is a higher specificity in testing mice that may be coinfected with other species of *Helicobacter*. Thus, P167C and P167D together provide a highly sensitive and specific assay. Because the multiplex microbead method is

### TABLE 2. Summary of ELISA results

| Antigen | ELISA | PCR |
|---------|-------|-----|
|         | P167C | P167D |
| Sera from *H. bilis*-infected mice | 16/18 (88) | 17/18 (94) |
| Sera from *H. hepaticus*-infected mice | 5/20 (25) | 7/20 (35) |

**H. bilis**

**H. hepaticus**

* Primers were designed based on *H. bilis* 16S ribosomal DNA.

* Primers were designed based on *H. hepaticus* 16S ribosomal DNA.

### TABLE 3. Summary of multiplex assay results

| Antigen | ELISA | PCR |
|---------|-------|-----|
|         | P167C | P167D |
| Sera from *H. bilis*-infected mice | 17/18 (94) | 14/18 (78) |
| Sera from *H. hepaticus*-infected mice | 0/20 (0) | 7/20 (35) |

**H. bilis**

**H. hepaticus**
ideally suited for testing the seroreactivities of multiple antigens simultaneously, the multiplex assay reported here will enhance the ability to reliably detect *H. bilis* infection in mice.

Purified recombinant proteins like P167C and P167D offer a significant advantage in the control of batch-to-batch consistency of antigen purity and yield. The importance of these properties in the development of a new assay is manifold: (i) assay robustness, (ii) ease of quality control, (iii) affordability, and (iv) shelf life. In contrast, whole-membrane extracts provide not only inconsistent purity but also a poor yield, making antigen preparation difficult at a small scale and production unfeasible at a large scale. Therefore, membrane extracts are not well suited for an assay intended for widespread use.

A main advantage of the multiplex technology is its ability to perform simultaneous detection of several analytes in a small volume of test sample. We estimate that under our present research laboratory conditions, approximately 300 to 400 serum samples can be analyzed per day. ELISA tests of 400 sera with four antigens would require several times more labor, time, and resources (e.g., plates and detection reagents). Because of the high-throughput capability of the multiplex microbead format, we will continue to expand this assay by adding capabilities for serodetection of additional *Helicobacter* species, e.g., *H. hepaticus*, *H. muridarum*, *H. rodentium*, and *H. typhlonius*.

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