

# Containment of *Helicobacter hepaticus* by Use of Husbandry Practices

Mark T. Whary, Jennifer H. Cline, Amy E. King, Charlotte A. Corcoran, Shilu Xu and James G. Fox

*Helicobacter* spp. colonize the cecum and colon of mice and are enzootic in mouse colonies unless specific steps are taken to prevent introduction and dissemination (1, 2). *Helicobacter*-associated disease is dependent on interaction between host factors of age, sex, genetics and immune competence and bacterial virulence factors that either are known or are suspected to influence tissue tropism and host immune responses (2). Experimentally induced and naturally acquired infections of mice with *Helicobacter* spp. have been documented to cause chronic active hepatitis, hepatic tumors, and inflammatory bowel disease (IBD), which in immunodeficient mice is a substantial clinical problem characterized by diarrhea and rectal prolapse associated with typhlitis, colitis, and proctitis (3, 4). Typhlitis has been attributed to *H. hepaticus* in a variety of immunocompetent mouse strains (5, 6). Persistent infection of mice with helicobacters may confound research objectives due to the presence of concurrent disease associated with chronic inflammation. *Helicobacter hepaticus* has received the most attention because it was the first *Helicobacter* sp. recognized as a cofactor for hepatic carcinogenesis (5).

Before the impact of *Helicobacter* infection on the colony health of research mice was appreciated, the diagnostic laboratory at the MIT Division of Comparative Medicine frequently identified *Helicobacter*-infected mice from commercial and academic sources (1). Most commercial vendors now supply mice that are free of *H. hepaticus* and *H. bilis* and, by use of the same production methods (i.e., cesarian section, embryo transfer), these mice may also be free of other *Helicobacter* spp. such as *H. rodentium*. Natural infection with several murine *Helicobacter* spp. remains common in conventional mouse colonies because of horizontal transmission through probable fecal-oral contact. Fecal-oral spread is the most likely route of natural acquisition of infection with helicobacters in rodents and is supported by reports that *H. hepaticus* and other rodent helicobacters can be transferred on soiled bedding (7, 8).

The level of biocontainment necessary to prevent horizontal transmission of *H. hepaticus* within a mouse colony is unknown. In germ-free Swiss Webster mice housed in an isolator, Fox et al. documented transmission of *H. hepaticus* between experimentally infected and uninfected mice housed in cages equipped with wire bar lids (9). Space and economic constraints may dictate that an *H. hepaticus*-free colony may have to be housed in close proximity to infected mice or, more commonly, in proximity of mice with unknown *Helicobacter* status. In the study reported here, we evaluated a husbandry protocol that included use of microisolator caging, forceps to transfer mice, and a cage

change order of uninfected mice before experimentally infected mice to provide a barrier to horizontal transmission of *H. hepaticus*. For 15 weeks, cages of helicobacter-free A/JCr and C57BL/6 mice were housed alongside cages of mice experimentally infected with *H. hepaticus*. These mouse strains were compared because A/JCr mice chronically infected with *H. hepaticus* develop hepatitis and typhlitis (6) and C57BL/6 mice are resistant to lesion development (10); it is unknown whether these strains differ in susceptibility to infection. At the conclusion of the experiment, mice were tested for evidence of *H. hepaticus* infection by serologic testing and by culture and polymerase chain reaction (PCR) analysis of cecal scrapings, using *H. hepaticus*-specific primers. Animal use was approved by the MIT Committee on Animal Care.

Six-week-old female A/JCr (n = 90) and C57BL/6 (n = 90) mice (Jackson Laboratory, Bar Harbor, ME) were randomly assigned to the uninfected experimental group (12 cages of each strain housing 5 mice) or to a group experimentally infected with *H. hepaticus* (6 cages of each strain housing 5 mice). The source colony was free of murine ectoparasites, endoparasites, antibody to murine viral pathogens and known bacterial pathogens, including *H. hepaticus*, *H. bilis*, *H. muridarum*, and '*H. rappini*.' The *Helicobacter*-free status of 12 mice randomly selected from each strain was confirmed by serologic testing on each mouse and by culture and PCR analysis of pooled feces, using primers that detect all known *Helicobacter* spp. Mice were housed in one 80 ft<sup>2</sup> cubicle under negative pressure within an AAALAC-approved animal facility under environmental conditions of 22°C, 40 to 70% humidity, 15 air changes/h, and a 12:12-hour light:dark cycle. Heat-treated hardwood was used for bedding (Sanichips®, PJ Murphy Inc., Montville, NJ) and pelleted diet (RMH 3000®, Purina Mills Inc., Richmond, IN) and water produced by reverse-phase osmosis were provided ad libitum. Polycarbonate microisolators (7.5 x 11.5 x 5 in.) with filter tops (Lab Products, Inc., Seaford, DE) were positioned on two 4-tier racks and labeled in numerical sequence to direct the cage change sequence of uninfected mice before mice experimentally infected with *H. hepaticus* (Figure 1). Cages were changed twice per week within the cubicle without the use of a biosafety hood. Changing only one cage at a time, mice were transferred from dirty to clean caging, using 10-in. metal forceps dipped briefly between cages in Quatracide PV® (Pharmaceutical Research Laboratories Inc., Naugatuck, CT) that was made fresh at a concentration of 700 ppm at the start of cage changing. Personnel were required to wear ¾ length laboratory coats, disposable shoe covers, face masks, head covers, and examination gloves. Dirty cages were emptied of soiled bedding in the cagewash facility and washed in a tunnel washer (Model SW 2500, Scientek,



**Figure 1.** Position of 6 cages of mice infected with *Helicobacter hepaticus* (shaded boxes) and 12 cages of uninfected mice (clear boxes) housed 5/cage on a 4-tier rack. Cage positions were kept constant throughout the 15-week study in case physical proximity to an infected cage would have an effect. Numbers indicate the order in which cages were changed to ensure that cages of mice that started the experiment *Helicobacter*-free were changed before cages of mice that were infected with *H. hepaticus*. The A/JCr and C57BL/6 mice were housed on separate racks for a total of 36 cages containing 180 mice.

Richmond, BC, Canada) using detergent (Clout, Pharmacal Research Laboratories Inc.) and 180°F rinse water, temperature of which was verified daily, using temperature indicator tapes (Temp-Tape® 180°, Pharmacal).

For experimentally induced infection, the type strain of *H. hepaticus* (ATCC 51448) was grown as described (11). Briefly, cultures were first established under microaerobic conditions at 37°C on trypticase soy blood agar (Remel Laboratories, Lenexa, KS) and inoculated into *Brucella* broth containing 5% fetal bovine serum. After 48 hours of incubation on a rotary shaker (New Brunswick Scientific, Edison NJ), the culture was centrifuged at 10,000 RPM (Sorvall® RC-5B Centrifuge with SS-34 Rotor, Du Pont Instruments, Newton, Conn.) for 20 minutes at 4°C. After examination for bacterial contaminants, using Gram staining and phase microscopy, the pellet was resuspended in *Brucella* broth containing 30% glycerol to a concentration of approximately 10<sup>8</sup> organisms/ml as confirmed by spectrophotometry. Mice received 0.2 ml of fresh inoculum by oral gavage every other day for 3 doses. The inoculum was subcultured on blood agar to confirm the purity of the broth culture. Infection with *H. hepaticus* was confirmed 1 month later by culture and PCR analysis of pooled feces collected from each cage of experimentally dosed mice.

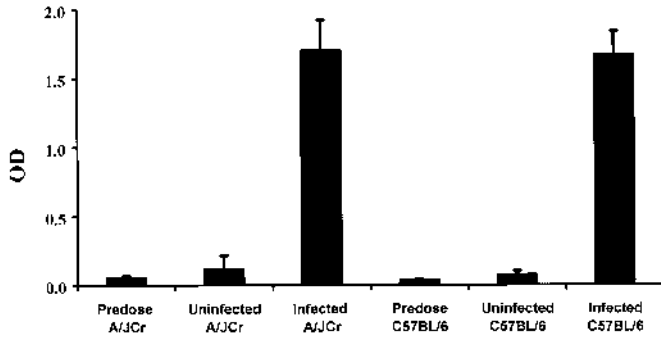
Fecal samples pooled by cage were collected from the uninfected mice every 2 weeks for the first 10 weeks of the experiment and were submitted for culture for *H. hepaticus*. All mice were necropsied at 15 weeks. Serum was obtained for measuring IgG to *H. hepaticus* antigens by use of ELISA, all major abdominal organs were inspected for gross abnormalities, and cecal scrapings were collected aseptically for *H. hepaticus* culture and PCR analysis. To re-isolate *H. hepaticus*, fecal samples pooled by cage and cecal scrapings obtained from individual mice at necropsy were cultured as described (11). Feces were suspended in phosphate-buffered saline (PBS), and the slurry was passed through a 0.45-µm filter. Filtered slurry and cecal scrapings were streaked onto blood agar plates (Remel Laboratories), which were incubated at 37°C under microaerobic conditions for up to 3 weeks if no growth was detected. Feces collected from cages of experimentally infected mice at 4 weeks were tested for *H. hepaticus* by PCR amplification of bacterial DNA extracted by use of the QIAamp Tissue Kit (Qiagen Inc., Valencia, CA), following the kit protocol for the isolation of nucleic acids from blood. Individual cecal scrapings obtained from all mice at necropsy were tested for *H. hepaticus* by PCR analysis,

using the Boehringer-Mannheim High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, IN), following the kit protocol for the isolation of nucleic acids from tissue. The primer sequences used were specific for *H. hepaticus* (5'-GCA TTT GAA ACT GTT ACT CTG-3' [C68] and 5'-CTG TTT TCA AGC TCC CC-3' [C69]), which yielded an amplification product of 414 bp (1).

For measurement of *H. hepaticus*-specific serum IgG antibody responses by ELISA, an outer membrane antigen preparation of *H. hepaticus* was obtained by use of described methods (6). Briefly, *H. hepaticus* was cultured in broth as detailed previously. After 3 washes in PBS and examination for bacterial contaminants, using Gram staining and phase microscopy, the pellet was resuspended in 4 ml of 1% N-octyl-β-glucopyranoside (Sigma Chemical Co., St. Louis, MO) for 30 minutes at room temperature. Insoluble material was removed by ultracentrifugation at 100,000 x g for 1 hour. After dialysis against PBS for 24 hours at 4°C, supernatant protein concentration was measured by use of the Lowry technique (Sigma Chemical Co.). Immulon II- 96-well plates (Dynax Technologies, Chantilly, VA) were coated (100 µl/well) with 1 µg of *H. hepaticus* protein/ml of carbonate buffer (pH 9.6) overnight at 4°C. Plates were blocked with PBS-2% bovine serum albumin (BSA) for 2 hours at 37°C. Serum was diluted 1:100 and applied to coated wells for 1 hour at 37°C. Biotinylated secondary antibody was goat-anti-mouse IgG (Sigma Chemical Co.) diluted 1:2000 and applied for 1 hour at 37°C. Incubation with extravidin peroxidase (Sigma Chemical Co.) for 30 minutes at 37°C was followed by addition of ABTS substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for color development. After 30 minutes at room temperature, optical density (OD) development at 405λ was recorded by use of an ELISA plate reader (Dynatech MR7000, Dynatech Laboratories, Inc., Chantilly, VA).

Culture of fecal samples pooled by cage from experimentally infected mice 4 weeks after inoculation confirmed colonization and shedding of viable *H. hepaticus*. None of the fecal samples collected during the first 10 weeks from cages containing mice that started the experiment *Helicobacter*-free were culture positive for helicobacters. All mice remained clinically normal during the 15-week study, and at necropsy, all major abdominal organs were free of lesions. Analysis by PCR of individual cecal scrapings collected at necropsy yielded positive results for *H. hepaticus* in the experimentally infected mice and negative results for mice of the uninfected group. A subset of cecal samples negative for *H. hepaticus* by PCR (n = 16) were cultured for *H. hepaticus*, and there was no growth from any samples. Serum IgG concentration in experimentally infected mice were significantly greater at 15 weeks after inoculation, compared with that in uninfected mice at necropsy ( $P < 0.01$ ) (Figure 2). The magnitude of the antibody response was similar in experimentally infected A/JCr and C57BL/6 mice. Seroconversion was not detected in any uninfected A/JCr or C57BL/6 mice.

Inadvertent horizontal transmission of *H. hepaticus* is a substantial risk unless adequate husbandry precautions are closely followed. Naturally acquired *Helicobacter* infections of mice are persistent, and chronic shedding of the organism in feces occurs (12). The shedding status of the experimentally infected mice was confirmed at 4 weeks after infection on the basis of culture and PCR results for feces and the persistence of infection was documented by PCR of cecal scrapings at necropsy. Presence of



**Figure 2.** Serum IgG concentrations to *H. hepaticus* in experimental groups of mice as measured by ELISA. Serum antibody responses were reported as the mean (solid bars) and 1 SD (error bars). An antibody response against *H. hepaticus* was considered to have occurred if the OD value was significantly higher than the mean measurement for sera from uninfected mice, using a one-tailed Student's *t*-test for comparison of normally distributed data.

infection was supported by positive ELISA serologic results for *H. hepaticus* antigens; the ELISA is a test with high sensitivity, although only moderate specificity (8, 13). All uninoculated mice that were at risk for acquisition of *H. hepaticus* infection remained uninfected, as indicated by the absence of seroconversion, and negative culture and PCR results for *H. hepaticus*. Thus, this study indicates that *H. hepaticus*-free mice can be economically maintained in the uninfected state when housed in close proximity to mice experimentally infected with *H. hepaticus*.

The protocol we tested for prevention of horizontal transmission of *H. hepaticus* consisted of three key components that were not evaluated independently; microisolator caging, use of forceps to transfer mice, and adherence to the practice of opening one cage at a time in the order of clean to dirty cages. Microisolator caging was designed to prevent aerosol transmission of infective agents, but has been evaluated under controlled conditions for only a few murine pathogens. Microisolator caging was an effective aid in preventing transmission of mouse hepatitis viral infection (MHV) (14) and *Pneumocystis carinii* in rats (15). Microisolators have also been used to successfully rederive mice from dams that were antibody positive for MHV and mouse encephalomyelitis virus (16). We added the additional safeguards of handling mice with forceps dipped in disinfectant after each cage change to prevent transmission by potentially contaminated gloves and by changing only one cage at a time in the order of *Helicobacter*-free mice to mice known to be experimentally infected with *H. hepaticus*. Although Quatracide PV® is reported by the manufacturer to be bactericidal against a broad range of gram-positive and gram-negative bacteria, there are no reports of its activity against *Helicobacter* spp. Use of forceps by the husbandry staff to transfer mice is a universal practice at our institution, and we find that it is atraumatic and fast and believe it minimizes risk of transmitting infective agents. Opening only one cage at a time minimized but would not entirely prevent potential aerosol exposure to bacteria that are likely to be air-borne. We purposely did not use a biosafety hood to change cages, acknowledging that use of a hood would provide additional insurance against horizontal transmission of *H. hepaticus* between cages of mice by aerosol. Contaminated bedding was removed from the cages in the cagewash area, which is important for preventing aerosol exposure of animals and personnel.

Use of microisolator caging, forceps to transfer mice, and adherence to the practice of changing one cage at a time in a sequence that considers potential differences in the *Helicobacter* infection status of different groups of mice will prevent horizontal transmission of *H. hepaticus*. Whether this protocol would be effective in preventing transmission of other rodent *Helicobacter* spp. between cages of mice that differ in health status requires further study. However, given the similarities in their purported spread by a fecal-oral route, this procedure should also minimize transmission of other murine *Helicobacter* spp.

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