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Evaluation of *Helicobacter hepaticus* Bacterial Shedding in Fostered and Sex-Segregated C57BL/6 Mice

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Neonatal fostering has been evaluated as a means of eliminating *Helicobacter hepaticus* infection in laboratory mouse colonies. The purpose of the present study was to evaluate cross-fostering of neonatal C57BL/6 pups from experimentally infected dams after male-absent parturition and to determine the effects of sex and housing strategy on *H. hepaticus* populations. Approximately 20 C57BL/6 mice (age, 1 to 4 days) were fostered daily. In all fostered mice, fecal samples collected at 21 and 42 days of age and cecal samples collected at 42 days of age tested negative for *H. hepaticus* by polymerase chain reaction analysis. Our results demonstrate that removal of the male prior to parturition extends the fostering period to yield *Helicobacter*-free mice. In a second experiment, the effects of time of infection, housing strategy, and sex on fecal *H. hepaticus* shedding and cecal colonization were evaluated. Neither time nor housing strategy affected bacterial shedding. In contrast, fecal and cecal bacterial loads were higher in male mice versus female mice. A novel predictive algorithm was developed to predict cecal bacterial colonization levels in light of fecal bacterial loads. Our findings likely will prove useful in *Helicobacter* eradication efforts and in studies designed to further elucidate the role of *H. hepaticus* in disease.

Since the discovery of *Helicobacter pylori* in humans, several other naturally occurring *Helicobacter* spp. have been isolated and characterized (14). One of these, *H. hepaticus*, is an enterohepatic helicobacter first discovered in A/JCr mice (15, 50). *H. hepaticus* primarily is transmitted through the fecal–oral route; however, transmission by xenograft and the transplacental route have been suggested (20, 25, 28, 53). Techniques successfully used to eliminate *H. hepaticus* from infected mouse colonies include rederivation by embryo transfer, early weaning, neonatal cross-fostering, and cesarean section (8, 42, 47, 52). The success in using the latter two methods provides anecdotal evidence for a low or negligible rate of transplacental transmission in immunocompetent mice (42, 47). Transmission of *H. hepaticus* as a result of coprophagy has been demonstrated clearly (8). However, the bacterium may also be spread by other means such as contaminated bedding and foodstuffs, or as a result of husbandry practices (28, 52). Several virulence factors such as urease, cytolethal distending toxin, granulating cytotoxin, and the putative pathogenicity island HHGI1 have been proposed to contribute to its pathogenicity (1, 5, 43, 45, 56, 57). Recently, CDT has been shown to be necessary for persistent colonization in Swiss Webster mice (18). Infected susceptible mice develop chronic infections that can lead to typhlocolitis, hepatitis, proctitis, and neoplasia (10, 15-17, 22, 25, 49, 50). Several of the disease characteristics found in mice with *H. hepaticus* closely resemble those of *H. pylori* in humans (37). Accordingly, *H. hepaticus*-infected mice often are used as an animal model of chronic inflammation.

H. hepaticus colonization in mice may be influenced by sex-based differences. For example, Ge and coworkers recently reported that the amount of cecal or colonic *H. hepaticus* (expressed as genomic copies per µg of mouse DNA) was ~1000-fold lower in female Swiss Webster mice than males by 16 weeks postinoculation (18). These observed gender effects appeared related to interferon γ and interleukin 10 production (18). Livingston and coworkers evaluated cecal immune responses to *H. hepaticus* in chronically infected A/JCr mice (27) and reported that female mice had greater Th1 responses to *H. hepaticus* than did male mice. This increased response accompanied increased lesion severity scores in the cecum, suggesting a predilection in female A/JCr mice for intestinal disease (27). In contrast, Ward and coworkers found that in certain strains of *Helicobacter*-positive mice, males developed more severe hepatic lesions than did females (49, 50). Similar reports by others support this conclusion (16, 22, 25, 27, 36). Gender may also influence *Helicobacter*-associated hepatocellular and gastrointestinal neoplasia (11, 22, 36, 37). Taken together, these reports suggest that male and female mice respond differently to infection with *H. hepaticus*, and highlight the need for increased understanding of the mechanisms behind these differences.

Infection of laboratory rodents with *H. hepaticus* is particularly problematic, because marked alterations in host physiology can lead to confounding interpretation of experimental results. Earlier research in our laboratory showed that successful cross-fostering of C57BL/6 pups from *H. hepaticus*-infected breeder mice must be performed within 24 h of birth, when both parents remain in the birthing cage (42). In that

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study we also demonstrated a sex effect on fecal *H. hepaticus* shedding. The percentage of fecal samples positive for *Helicobacter* by polymerase chain reaction (PCR) analysis among male C57BL/6 mice consistently peaked prior to that of female mice when evaluated over time (41). In light of these findings, we sought to examine the possible influence of sex on neonatal fostering as a method of eradicating *H. hepaticus* from a mouse colony. The specific aims of the current experiment were to: 1) determine whether removal of the C57BL/6 *H. hepaticus*-infected sire prior to parturition would extend the time allowable for neonatal cross-fostering beyond 24 h postpartum; 2) evaluate the effects of housing arrangement and gender on bacterial colonization and shedding in *H. hepaticus*-infected C57BL/6 mice; and 3) examine cecal bacterial colonization in relation to cecal tissue histopathology.

Materials and Methods

Animals. The study was approved by the Louisiana State University Institutional Animal Care and Use Committee and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*. The university's animal program and facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. We purchased 78 male and 78 female 7- to 8-week-old C57BL/6NHsd mice from one commercial vendor (Harlan, Indianapolis, Ind.) and eight male and 20 female 7- to 8-week-old BALB/c mice from another (Charles River Laboratories, Portage, Mich.). Vendor reports indicated that upon arrival all animals were free of common viral, parasitic, mycoplasmal, and bacterial pathogens, including *Helicobacter* spp. For all mice, the *Helicobacter*-free status was verified after arrival by fecal PCR using genus-specific primers as described in a later section. Husbandry practices were as previously described except as noted below in regard to glove changing and disinfectant type (42).

Briefly, mice were housed in static polycarbonate Micro-Isolator™ caging with filter tops (Lab Products, Inc., Seaford, Del.) using corncob bedding (Bed-O'Cobs, Anderson's Inc. Maumee, Ohio). Breeder C57BL/6 mice in Experiment 1 and all mice in experiment 2 were given regular tap water and Purina Lab Diet 5001 ad libitum in non-autoclaved caging materials, whereas BALB/c mice and fostered C57BL/6 pups in experiment 1 were given ad libitum autoclaved tap water and diet (Purina Lab Diet 5010, PMI Nutrition International, Inc., Brentwood, Mo.) by using autoclaved caging materials. Cages were changed twice weekly by using a 1:128 dilution of quaternary ammonia-based disinfectant (Super HDQ Neutral, Spartan Chemical Company, Inc., Maumee, Ohio) to spray caging and contact surfaces between cages. Gloves used to handle mice during change-outs were changed between cages to prevent bacterial cross-contamination for all mice except for mice in the experiment 1 C57BL/6 breeder group. In that case, the handler's gloves were changed only as needed during the change-out process. A 12:12-h light:dark cycle was used with an environmental temperature of 22 to 23°C and a humidity range of 40 to 60%. Euthanasia was performed by CO_2 asphyxiation for all mice in experiments 1 and 2.

Bacterial culture and inoculation. Culture, preparation, and inoculation of bacteria were as previously described (42). Briefly, commercially supplied lots of *H. hepaticus* (no. 51449, American Type Culture Collection, Manassas, Va.) were reconstituted according to manufacturer's instructions. Cultures were grown on trypticase soy agar with 5% sheep blood (Remel, Lenexa, Kans.) and suspended in *Brucella* broth (Remel, Lenexa, Kans.) with 5% fetal bovine serum for inoculation. Bacteria were quantified spectrophotometrically at 660 nm. C57BL/6 mice were inoculated by gavage with 0.2 ml of a suspension of *H. hepaticus.* Mice were inoculated every other day, for a total of three doses. All doses were administered at the same time of day on the first (day 0) and subsequent (days 2 and 4) days of infection.

Design of experiment 1. (i) Timed breeding and infection. C57BL/6 mice were gavaged with *H. hepaticus* suspensions of optical densities 1.187, 1.191, and 1.089 Å on infection days 0, 2, and 4, respectively. To confirm infection status, C57BL/6 and BALB/c mice were tested bimonthly by fecal PCR, beginning 2 weeks from the date of the last inoculation and continuing throughout the experiment. Male and female C57BL/6 breeder pairs were arranged thereafter by placing one *Helicobacter*-positive male into a cage containing a single *Helicobacter*-positive female for a period of 14 days. Subsequently, pregnant females were each removed to a clean cage for single-housing until the next timed breeding event. A minimum of two cage changes occurred prior to parturition. After parturition, female mice remained single-housed for approximately 2 to 3 weeks before the next paired mating cycle. Mice were paired multiple times until the animal needs of the study were met.

(ii) Fostering. C57BL/6 pups from *Helicobacter*-infected parents were fostered onto lactating *Helicobacter*-free BALB/c dams when the pups were 1 to 4 days of age, as previously described (42). Only the pups of C57BL/6 dams with recent (within the past 7 days) evidence of *H. hepaticus* shedding, as determined by fecal PCR, were fostered. C57BL/6 pups that were 0 to 24 h old were classified as day 1 pups. A total of 23 C57BL/6 pups were fostered on day 1, 20 pups each on days 2 and 3, and 19 pups on day 4. At the time of cross-fostering, BALB/c pups were euthanized. However, in order to encourage maternal acceptance of the fostered litter, a single BALB/c pup was allowed to remain with the fostered litter until weaning of the C57BL/6 pups at 21 days of age. All C57BL/6 pups fostered onto each BALB/c foster dam were from the same *Helicobacter*-positive C57BL/6 mother. Each BALB/c foster dam was used as a foster parent only once.

(iii) Specimen collection. At 21 days of age, fostered C57BL/6 pups were weaned and fecal specimens were collected as previously reported by our laboratory (42). Simultaneously, fecal samples were obtained from the corresponding BALB/c dam and remaining BALB/c pup, which then was euthanized. At 42 days of age, C57BL/6 mice and their BALB/c foster dams were euthanized, and terminal cecal and fecal samples were collected. Specimens were processed as previously described (42). All tissue samples obtained at euthanasia were collected under sterile conditions. Separate sterile individual instrument sets were used for manipulating skin, abdominal muscle, ceca, and feces. One longitudinal half of the cecum was collected for PCR analysis, while the remaining half was preserved in 10% neutral buffered formalin. Fecal pellets collected from the terminal colon, or from the rectum prior to euthanasia, were processed as described above for day 21 fecal samples.

(iv) DNA extraction and PCR analysis. A single fecal pellet from each mouse was individually tested at each collection time point. Fecal DNA extraction was performed using the hot sodium hydroxide and Tris (HotSHOT) method as described elsewhere (42, 46). DNA extraction from mouse cecal tissue was performed

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using the QIAamp DNA Mini Kit, according to manufacturer's instructions (Qiagen, Valencia, Calif.). Extracted DNA from the fecal and cecal samples was stored at –20°C until PCR analysis was performed. The genus-specific *Helicobacter* primers (which amplify a highly conserved region of the 16S rRNA gene) and reaction conditions used were as previously reported by our lab (42) with minor modifications. Briefly, after amplification, 15 µl of PCR product from each reaction was mixed with 5 µl 1× Type I gel loading solution (Sigma, St. Louis, Mo.). Results were recorded after electrophoresis on and ethidium bromide staining of 1 to 2% agarose I gels (Amresco, Solon, Ohio). Positive controls consisted of DNA extracts of *H. hepaticus*-spiked cecal tissue for the cecal assay or from pure bacterial culture for the fecal assay. Negative (no template) controls were used in all PCR reactions.

Design of experiment 2. (i) Housing arrangements. To evaluate potential sex- and housing-associated effects related to *H. hepaticus* colonization and shedding over time, C57BL/6 mice were assigned randomly to five different housing groups (a through e) two days prior to inoculation with *H. hepaticus*. Housing category assignments remained permanent throughout the study and were as follows: (a) paired male mice; (b) single male mice; (c) paired female mice; (d) single female mice; and (e) male–female paired mice. All mice were inoculated with *H. hepaticus* as described for experiment 1. Spectrophotometric optical densities for the three doses were 0.401 Å for day 0, 0.697 Å for day 2, and 0.559 Å for day 4 of infection.

(ii) Surgical vasectomy procedure. Male C57BL/6 mice in group e were anesthetized with isoflurane gas and vasectomized under sterile conditions 11 days prior to pairing. The vas deferens were transected bilaterally between two ligatures through a 1.0-cm ventral midline lower abdominal incision (24). A standard two-layer closure was performed. Buprenorphine (0.075 mg/kg) was administered subcutaneously as an analgesic 30 min prior to surgery and as needed postoperatively for pain relief.

(iii) Specimen collection. An approximately equal number of mice from each housing group (a through e) were euthanized at 4, 8, and 12 weeks postinoculation (WPI; Table 1). Fecal samples were collected from all remaining mice every 2 weeks postinfection. Cecal tissue was collected at 4, 8, and 12 WPI. Fecal and cecal specimens were collected as described for experiment 1. Fecal and cecal specimens were placed separately in sterile collection tubes, flash-frozen in liquid nitrogen, and stored at –80°C until processed.

(iv) DNA extraction and quantitative PCR analysis. DNA was extracted from cecal tissue by using the QIAamp DNA Mini Kit tissue protocol as described for experiment 1. For fecal assay, DNA was extracted from a single fecal pellet of variable mass by using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol for pathogen detection; this test kit is designed by the manufacturer to reduce fecal PCR inhibitors. All quantitative PCR (QPCR) assays were performed at the Massachusetts Institute of Technology (Cambridge). Extracted fecal and cecal specimens were processed by fluorogenic QPCR using the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, Calif.) as described previously and using the thermocycler settings recommended by the manufacturer (19). For each sample, both the copy number of *H. hepaticus* and the amount of mouse DNA were quantified as previously described (19). All samples were run in duplicate, with a maximal allowable cycle threshold difference of 1.0 for inclusion in the study.

Table 1. Housing assignment and sample collection points of male and female C57BL/6 mice in experiment 2

Housing assignment category (per cage)	Sample collection points (weeks postinoculation): Terminal cecal collection	Fecal samples	Group size at euthanasia
a) paired male	4	2 and 4	6
b) single male	4	2 and 4	5
c) paired female	4	2 and 4	6
d) single female	4	2 and 4	5
e) male–female pair	4	2 and 4	12
a) paired male	8	2, 4, 6, and 8	6
b) single male	8	2, 4, 6, and 8	5
c) paired female	8	2, 4, 6, and 8	6
d) single female	8	2, 4, 6, and 8	6
e) male–female pair	8	2, 4, 6, and 8	12
a) paired male	12	2, 4, 6, 8, 10, and 12	6
b) single male	12	2, 4, 6, 8, 10, and 12	6
c) paired female	12	2, 4, 6, 8, 10, and 12	6
d) single female	12	2, 4, 6, 8, 10, and 12	7
e) male–female pair	12	2, 4, 6, 8, 10, and 12	12

Reagents were supplied commercially (Applied Biosystems). Positive controls consisted of DNA extracts of pure *H. hepaticus* bacterial culture. Negative (no template) controls were used in all QPCR runs.

In a 25-µl reaction volume, QPCR conditions for *H. hepaticus* consisted of 200 nM of forward and reverse cdtB primers (19), 100 nM probe, 12.5 µl TaqMan 2× PCR Master Mix, and 5 µl of DNA template. The primers and probe sequences were based on the *H. hepaticus cdtB* gene (19). Ten-fold serial dilutions of DNA (from 10^5 to 10 pg) extracted from the ceca of a C57BL/6 *Helicobacter*-negative sibling mouse pair were used to create the standard curve for the host DNA reaction. QPCR for the host DNA contained: 1.25 µl of 20× 18S rRNA gene-based primer–probe mixture (Applied Biosystems), 12.5 µl TaqMan 2× PCR Master Mix, and 5 µl of DNA template. The results are reported as number of copies of the *H. hepaticus* genome per µg of mouse DNA.

(v) Histologic examination and intestinal lesion scoring. Formalin-fixed, longitudinal, paraffin-embedded cecal sections were sectioned at 5 µm, stained with hematoxylin and eosin, and evaluated for pathologic lesions in a blinded fashion by a veterinary pathologist. The ceca were examined and scored according to a previously described system (27), which defines the extent of cecal pathology in regard to degree of inflammation and hyperplasia. The total score was derived using the sum of the inflammation scores (subtracting two in order to correct for scale comparison) plus the hyperplasia score.

Statistical analysis. Data from experiment 1 were not analyzed for statistical significance. For experiment 2, QPCR data were analyzed using SAS (version 8.2, SAS Institute Inc., Cary, N.C.) and graphed using SigmaStat and SigmaPlot 2000 (version 2.03, Systat Software Inc., Point Richmond, Calif.). Mean replicate values obtained from QPCR were expressed as the number of copies of *H. hepaticus* genome per µg host DNA. Raw data were $\log_{10}$-transformed prior to analysis. The threshold for statistical significance was $P < 0.05$ for all of the following tests.

One-way analysis of variance (ANOVA) in a repeated measures design was used to evaluate fecal shedding of *H. hepaticus.* The main effects were housing arrangement, individual mouse identification, and time postinoculation. Two-way interactions between mouse sex and time postinoculation were evaluated for

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their effects on *H. hepaticus* fecal shedding by using a second repeated-measures ANOVA. Tukey's Studentized range tests were used for pairwise mean comparisons for significant main effects. Least-square means were used to make pairwise comparisons with *t* tests when overall interaction effects were statistically significant. An ANOVA using a non-repeated measures design was used to evaluate cecal histologic lesion scores based on gender and time postinoculation (irrespective of housing group). Pearson product moment correlation coefficients were evaluated for correlation of cecal lesion scores with gender, time postinoculation, and number of copies of *H. hepaticus* genome per µg host DNA.

A factorial ANOVA in a non-repeated measures design was used to evaluate fecal and cecal *H. hepaticus* copy number for each sex, regardless of treatment group, for the euthanasia time points of 4, 8, and 12 WPI. Between these time points, fecal and cecal specimens were from different cohorts of mice, whereas within each time point, specimens were from the same cohort. The 2 × 2 × 3 factorial arrangement allowed assessment of specimen type, sex, and time postinoculation (4, 8, and 12 weeks). When interaction effects were statistically significant, least-squares means were evaluated with pairwise *t* tests. Pearson product moment correlation coefficients and regression analyses were performed to evaluate relationships between fecal and cecal samples with regard to the copy number of *H. hepaticus* per µg of host DNA at 4, 8, and 12 WPI.

Results

Experiment 1. A total of 83 C57BL/6 pups (46 females and 37 males) were fostered. No pups fostered between 1 and 4 days of age tested positive for *H. hepaticus* by fecal PCR at 21 or 42 days of age. All cecal specimens from these mice likewise tested negative. All fecal and cecal specimens collected from BALB/c foster dams tested negative for *H. hepaticus*. None of the fecal pellets collected from the natural litter BALB/c pups left with the dams tested positive at weaning. No *Helicobacter*-positive reserve BALB/c breeder male or female mice were detected at any time during the bimonthly fecal testing. Initial testing of fecal pellets excreted by C57BL/6 sires and dams revealed shedding by all breeders. Among C57BL/6 dams, the percentage of mice with *Helicobacter*-positive fecal PCR results gradually declined over time postinoculation. The decrease in the percentage of *Helicobacter*-positive female fecal samples did not appear to closely follow patterns in the percentage positivity of male fecal PCR samples over time. These data (Fig. 1) comprise the combined PCR fecal test results from an earlier study from our laboratory (41, 42) merged with those of the current study which were obtained from similarly inoculated mice. The number of observations made for each of the two studies are shown separately (Table 2).

Experiment 2. Neither time postinoculation nor housing strategy affected fecal shedding of *H. hepaticus* in C57BL/6 mice. In contrast, gender affected shedding, independent of housing arrangement (Fig. 2). Time postinoculation was not a significant main effect, although the two-way interaction of time and gender did affect bacterial shedding. In addition, *t* test comparisons of least-square means detected statistical significance ($P < 0.05$) at 6, 10, and 12 WPI.

The factorial ANOVA revealed significant main effects for sex and specimen type (feces or cecal tissue) but not for time postinoculation. However the time postinoculation × gender interaction

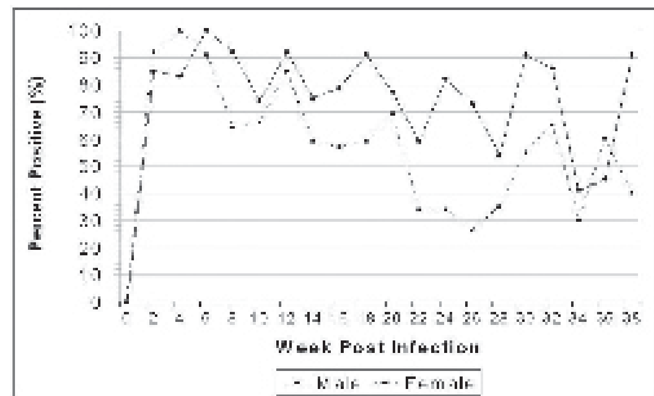

Figure 1. Polymerase chain reaction analysis was used to determine fecal shedding of *Helicobacter hepaticus* in adult C57BL/6 mice after experimental inoculation. The solid line represented by circles illustrates the percentage of male mice shedding the bacterium. The broken line indicated by squares represents the percentage of female mice shedding the bacterium.

term was statistically significant ($P < 0.05$). Tukey's Studentized range test confirmed overall significance ($P < 0.05$) for the effects of gender and specimen type. At 12 WPI, least-square mean comparisons found the combined fecal and cecal bacterial load greater for male mice than for female mice. In addition, the combined fecal and cecal bacterial load for female mice was significantly ($P < 0.05$) less at 12 WPI than at either 8 or 4 WPI. Therefore the combined fecal and cecal bacterial loads in female mice decreased over time postinoculation. Regarding specimen type, overall cecal bacterial load was significantly ($P < 0.05$) greater than overall fecal bacterial load for both sexes when evaluated without respect to time postinoculation. Likewise, male cecal and fecal specimens contained higher bacterial loads than did the corresponding female specimens when compared overall without respect to time postinoculation.

When all mice were compared regardless of gender, housing strategy, or time postinoculation, Pearson's correlation coefficient detected a statistically significant ($P < 0.001$) and moderately strong correlation ($r = 0.68376$) between average fecal and cecal *H. hepaticus* bacterial levels. Regression analysis yielded significant parameter estimates for slope (m) and intercept (b; [$P < 0.0001$] for both) so that the equation model $y = mx + b$ was generated. For this model, the dependent variable (y) is average number ($\log_{10}$) of cecal *H. hepaticus* genome copies/µg host DNA, the independent variable (x) is the average number ($\log_{10}$) of fecal *H. hepaticus* genome copies/µg host DNA, $m = 0.60545$, and $b = 2.98005$.

Histologically, inflammation of the cecum was generally mild and consisted of multifocal aggregates of lymphocytes within the basal mucosa. Lesions with increased severity were seen occasionally in some mice but did not follow a predictable trend. These lesions were characterized by increased number and size of the inflammatory foci. Hyperplasia of the gut-associated lymphoid tissue and mucosal epithelium was seen in association inconsistently with the inflammatory infiltrates. Neither gender nor time postinoculation affected cecal lesion scores, except at 12 WPI. In that case, correlation analysis in male mice revealed a significant negative correlation between cecal lesion score and cecal $\log_{10}$ average copy number of *H. hepaticus*/µg host DNA ($r = –0.53791$, $P = 0.0175$).

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Table 2. Percentage of adult breeder male and female C57BL/6 mice testing positive by fecal PCR for *Helicobacter* spp. per week postinoculation (WPI) in two studies

	Singletary and coworkers (ref.41)			Current study		
WPI	Male mice	Female mice	Total	Male mice	Female mice	Total
2	NA	NA	NA	11/13 (84.62)	11/12 (91.67)	22/25 (88.00)
4	NA	NA	NA	10/12 (83.33)	12/12 (100)	22/24 (91.66)
6	11/16 (68.75)	9/17 (52.94)	20/33 (60.61)	11/11 (100)	10/11 (90.91)	21/22 (95.45)
8	14/15 (93.33)	8/16 (50.00)	22/31 (70.97)	10/11 (90.91)	7/9 (77.78)	17/20 (85.00)
10	14/15 (93.33)	7/16 (43.75)	21/31 (67.74)	6/11 (54.55)	8/9 (88.89)	14/20 (70.00)
12	14/15 (93.33)	13/16 (81.25)	27/31 (87.10)	10/11 (90.91)	7/8 (87.50)	17/19 (89.47)
14	13/15 (86.67)	12/15 (80.00)	25/30 (83.33)	7/11 (63.64)	3/8 (37.5)	10/19 (52.63)
16	10/15 (66.67)	7/15 (46.67)	17/30 (56.67)	10/11 (90.91)	4/6 (66.67)	14/17 (82.35)
18	12/12 (100)	6/12 (50.00)	18/24 (75)	9/11 (81.82)	4/6 (66.67)	13/17 (76.47)
20	9/11 (81.82)	6/11 (54.55)	15/22 (68.18)	8/11 (72.73)	5/6 (83.33)	13/17 (76.47)
22	6/11 (54.55)	2/11 (18.18)	8/22 (36.36)	7/11 (63.64)	3/6 (50.00)	10/17 (58.82)
24	7/11 (63.64)	0/11 (0)	7/22 (31.82)	11/11 (100)	4/6 (66.67)	15/17 (88.24)
26	7/11 (63.64)	2/11 (18.18)	9/22 (40.91)	9/11 (81.82)	2/6 (33.33)	11/17 (64.71)
28	7/11 (63.64)	4/11 (36.36)	11/22 (50.00)	5/11 (45.45)	2/6 (33.33)	7/17 (41.18)
30	10/11 (90.91)	6/10 (60.00)	16/21 (76.19)	10/11 (90.91)	3/6 (50.00)	13/17 (76.47)
32	8/11 (72.73)	8/10 (80.00)	16/21 (76.19)	11/11 (100)	3/6 (50.00)	14/17 (82.35)
34	6/11 (54.55)	6/10 (60.00)	12/21 (57.14)	3/11 (27.77)	0/5 (0)	3/16 (18.75)
36	5/11 (45.45)	6/10 (60.00)	11/21 (52.38)	NA	NA	NA
38	10/11 (90.91)	4/10 (40.00)	14/21 (66.67)	NA	NA	NA

NA, not available.
Data are presented as the number of mice testing positive for the bacterium/the total number in the group (percentage). A graphical representation of the merged data by sex from the two studies is shown in Fig. 1.

Discussion

H. hepaticus is an important murine pathogen. Infection may be associated with significant pathologic changes (10, 22, 25, 30, 37, 49), altered disease pathogenesis (6), and genetic dysregulation (3, 27, 32, 33) in susceptible animals. Infection can result in confounding effects on research involving the immune and enterohepatic systems of mice (22, 54). Therefore, rapid and effective methods to detect and eradicate the bacterium from infected mice are needed (7). Traditional medical therapies for *Helicobacter* spp. can be expensive, time-consuming, and subject to failure (13, 44, 48). In addition, drug administration typically requires either frequent handling of animals or alteration of water or feed (12, 38). Physical manipulation and gavage can increase animal stress, alter reproductive performance, and introduce variability into research (4, 23, 26, 31, 34). Previous work in our laboratory has shown that cross-fostering of neonates performed within 24 h of birth is a reliable, reproducible, and cost-effective alternative to anti-*Helicobacter* medical regimens and other rederivation approaches (42). The current study refines the fostering paradigm to better accommodate caretaker schedules and minimize the potential for transmission to newborns.

Our results indicate that fostering of C57BL/6 pups from infected dams can be extended through day 4 of age if the sire is removed from the birthing cage approximately 1 week prior to parturition. This finding is important from an animal husbandry standpoint as it allows greater flexibility in developing a *H. hepaticus* eradication program. In addition, from these findings we conclude that increased cage population at parturition will likely increase *H. hepaticus* infection rate in offspring through increased exposure to contaminated feces. Hence, the use of harem breeding schemes, where multiple adult breeder mice are group-housed at parturition, may be contraindicated in instances where elimination of *H. hepaticus* is attempted.

Fecal PCR inhibitors found in stool samples can complicate PCR assay interpretation by creating false-negative test results (2, 29, 40). The HotSHOT method of fecal DNA extraction used in experiment 1 did not involve a processing step designed to reduce fecal PCR inhibitors. It is therefore possible that the DNA extraction technique may have affected the experimental outcome of the fecal PCR testing. In the methods described herein, fecal and cecal samples were assayed independently by PCR for *Helicobacter* spp. to determine a combined test result. The finding that both the fecal and cecal samples for each mouse were negative lessens the likelihood that our experimental outcome was reached by false-negative results due to fecal PCR inhibitors.

The findings of the current study are in agreement with a previous study by our laboratory which suggested that adult male mice more frequently shed *H. hepaticus* in feces than do adult female mice (41, 42). These results highlight the contribution of the male to cage contamination and the resulting potential for pathogen exposure to offspring. When the sire remains in the cage at parturition, he may contribute to grooming and socialization of the pups, thereby increasing horizontal bacterial transfer. To the best of the authors' knowledge, the amount and type of parental behavior of the male C57BL/6 mouse has not been quantified; however, several studies indicate that male mice do participate in neonatal rearing to various degrees depending on both mouse strain and housing strategy (21, 39, 55). Transmission of *H. hepaticus* is thought to occur primarily via the fecal–oral route through ingestion of contaminated feces (8, 28, 53), but because mice are coprophagic, paternal grooming could facilitate transfer of transient oral bacteria to the areas being groomed on the pups if the sire had recently consumed infected fecal pellets. Therefore, although it is unknown whether the C57BL/6 sire's parenting behavior influenced the results of this earlier study, this does represent one possible difference in the care given to the pups between the current study and that of Singletary and coworkers, where both parents were present in the birthing cage at parturition (41, 42).

Additional studies are underway to accurately determine the day by which C57BL/6 pups must be fostered to prevent infection with *H. hepaticus*. Age-dependent neonatal coprophagic behavior after 4 days of age may contribute to eventual neonatal infection. In one study, male ICR mice demonstrated coprophagy at 17 days of age (9). To the best of the authors' knowledge, it is unknown when neonatal coprophagic behavior begins in the

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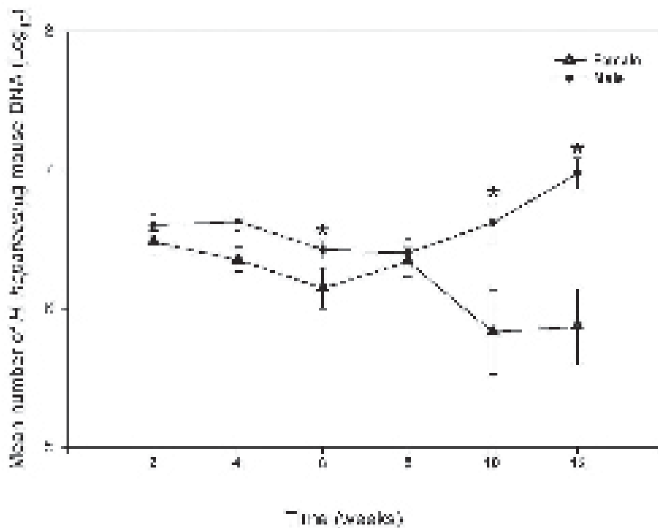

Figure 2. Quantitative polymerase chain reaction analysis was used to generate these data which show $\log_{10}$ numbers of copies of fecal *Helicobacter hepaticus* genome per µg mouse DNA (mean ± standard error of mean) in C57BL/6 mice at 2, 4, 6, 10, and 12 weeks postinfection, with treatment groups merged. Within each time point, significantly different values are indicated (*, $P < 0.05$ by least-square means).

C57BL/6 mouse or when it reaches levels sufficient to facilitate *Helicobacter* transfer. It is therefore possible that when the sire is absent at parturition, C57BL/6 pups can be fostered beyond 4 days of age without *Helicobacter* infection. It is also possible that the ability to foster beyond 4 days postpartum may apply to other species of *Helicobacter*.

Several other factors may affect *H. hepaticus* transmission to neonates. One potential variable may be the frequency of bedding litter changes. More frequent cage changes will certainly reduce the quantity of infected fecal pellets present within the cage. In our study, cages were changed twice weekly, and a minimum of two cage changes occurred in the absence of the sire and prior to birth of the C57BL/6 pups. Although *Helicobacter* DNA remains stable in feces for as long as 5 days (2, 7), it is unknown how long *H. hepaticus* in fecal pellets remains infective and whether a reduction in the frequency of cage changes would have increased transmission to the C57BL/6 offspring. In comparison, frequent cage changing is stressful to mice and has been associated with increased pup mortality (35). The type of caging and bedding used also might affect bacterial transmission by introducing differences in bedding moisture content, which may affect bacterial survival. Nevertheless, the fostering method we used in this study proved effective in the elimination of *H. hepaticus* from C57BL/6 pups derived from infected parents through fostering on or before day 4 of age.

Rogers and coworkers reported that bacterial loads in liver tissue of *H. hepaticus*-infected A/JCr mice were positively correlated with hepatic lesion severity and were greater for male mice than female mice (36). Others have reported an inverse relationship between liver disease and cecal colonization with *H. hepaticus* when comparing A/JCr with C57BL/6 mice (51). Livingston and coworkers reported that female A/JCr mice infected with *H. hepaticus* had greater cecal lesion scores than did male mice (27). Recently, it has been demonstrated that colonization of *H. hepaticus* in the ceca and colons of Swiss Webster mice was significantly lower in females than males (18). We expected to find that female C57BL/6 mice, with lower cecal *H. hepaticus* burdens, would have increased cecal lesion scores relative to males. Unexpectedly, this was not the case and may reflect mouse strain-associated differences in response to infection.

The experimental design as well as the DNA extraction and PCR methods differed in experiments 1 and 2, but the strain, age, and dates of infection of and amount of inoculum given to the mice were similar. When Fig. 1 and 2 are compared, more animals tested negative by PCR for *Helicobacter* spp. (Fig. 1) than would have been predicted from Fig. 2, where the QPCR results were positive at all time points for all animals. A possible explanation may be that the gel-based PCR assay has a lower threshold for detection than does the QPCR assay. QPCR has been demonstrated to detect as few as approximately 14 copies of *H. hepaticus* (19). In addition, differences in the fecal DNA extraction methods between the two experiments may have caused false-negative results due to fecal PCR inhibitors in experiment 1. If this were the case, it would not have been possible to distinguish true-negative PCR results from false-negative results due to PCR inhibition. Presumably, if the mice in experiment 2 had been followed with fecal QPCR out to 38 WPI as in experiment 1, a greater difference in bacterial quantity between the sexes may have been observed, which would have more closely corresponded with the data of Fig. 1.

Correlation and regression analyses proved useful in creating a mathematical algorithm for predicting cecal bacterial load from fecal bacterial load. However, it should not be assumed that all mouse strains shed equal numbers of *H. hepaticus* in their feces. Hence these findings should be extrapolated with caution to other strains of mice, which may shed more or less *H. hepaticus* than the C57BL/6 mice we used in this study. Similarly, the findings we report pertain to the inbred strains of mice used in this study, but these results may extend to other stocks and strains. We recently conducted a large field trial involving several strains of mice, using day 1 postpartum as the time by which fostering was performed. In that study, mouse strain did not appear to affect the success of neonatal fostering for deriving *Helicobacter*-free mice (41, 42). Finally, the findings that pertain to fostering and *H. hepaticus* may be applicable to other *Helicobacter* species as well.

In conclusion, our results demonstrate that fostering C57BL/6 mice through 4 days postpartum can be used to eliminate *H. hepaticus* from experimentally infected mice. This finding will facilitate rederivation efforts by allowing for a greater number of days by which fostering can occur. Studies are underway to evaluate fostering of C57BL/6 mice past 4 days postpartum with paternal separation prior to parturition.

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