

# *Helicobacter* Infection Decreases Reproductive Performance of IL10-deficient Mice

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Infections with a variety of *Helicobacter* species have been documented in rodent research facilities, with variable effects on rodent health. *Helicobacter typhlonius* has been reported to cause enteric disease in immunodeficient and IL10<sup>-/-</sup> mice, whereas *H. rodentium* has only been reported to cause disease in immunodeficient mice coinfecting with other *Helicobacter* species. The effect of *Helicobacter* infections on murine reproduction has not been well studied. The reproductive performance of C57BL/6 IL10<sup>-/-</sup> female mice intentionally infected with *H. typhlonius*, *H. rodentium*, or both was compared with that of age-matched uninfected controls or similarly infected mice that received antihelicobacter therapy. The presence of *Helicobacter* organisms in stool and relevant tissues was detected by PCR assays. *Helicobacter* infection of IL10<sup>-/-</sup> female mice markedly decreased pregnancy rates and pup survival. The number of pups surviving to weaning was greatest in noninfected mice and decreased for *H. rodentium* > *H. typhlonius* >> *H. rodentium* and *H. typhlonius* coinfecting mice. *Helicobacter* organisms were detected by semiquantitative real-time PCR in the reproductive organs of a subset of infected mice. Treatment of infected mice with a 4-drug regimen consisting of amoxicillin, clarithromycin, metronidazole, and omeprazole increased pregnancy rates, and pup survival and dam fecundity improved. We conclude that infection with *H. typhlonius*, *H. rodentium*, or both decreased the reproductive performance of IL10<sup>-/-</sup> mice. In addition, antihelicobacter therapy improved fecundity and enhanced pup survival.

**Abbreviation:** qPCR, qualitative real-time PCR

*Helicobacter rodentium* and *H. typhlonius*<sup>5</sup> are gram-negative, urease-negative, microaerophilic flagellated bacteria.<sup>6,20</sup> Numerous *Helicobacter* species have been identified in various rodent organ systems, including portions of the gastrointestinal tract, liver, and associated biliary system.<sup>5</sup> Although they often are found in the intestinal tract of immunocompetent mice without clinical disease, various *Helicobacter* species have been shown induce enteric disease in immunodeficient mice.<sup>6,20</sup> This propensity has been a useful tool in developing mouse models to study inflammatory bowel disease and colon cancer.<sup>7,13,14</sup>

Murine fecal samples submitted from a variety of institutions to the University of Missouri Research Animal Diagnostic Laboratory (Columbia, MO) between November 2001 and October 2002 showed 17% positivity for *H. typhlonius* and 10% positivity for *H. rodentium*.<sup>11</sup> *H. typhlonius* has been reported to cause significant enteric disease in immunodeficient and IL10<sup>-/-</sup> mice.<sup>4</sup> In contrast, *H. rodentium* has only been reported to cause disease in immunodeficient mice coinfecting with other *Helicobacter* species.<sup>16,20,21</sup> Because these agents cause disease, they are best considered to be rodent pathogens, despite the frequency of their detection in clinically normal mice. Although most murine *Helicobacter* infections are subclinical, infection with *H. rodentium* and *H. typhlonius* may affect experimental studies in vivo under some circumstances. In addition, *Helicobacter* infections can influence murine reproduction, although this effect has not been well studied.

The gastric-infecting species *H. pylori* influences murine pregnancy by increasing the number of fetal resorptions and producing lower fetal weights compared with those of noninfected controls.<sup>18</sup> Induction of Th1-type responses at the endometrial level was a possible mechanism suggested for these phenomena but not further investigated. Whether intestinal-infecting *Helicobacter* species such as *H. rodentium* and *H. typhlonius* affect murine pregnancy in wild-type or genetically modified mice, particularly those with mutations that affect immune function, has not been determined.

Mice deficient in IL10 (IL10<sup>-/-</sup> mice) mount an exaggerated and prolonged inflammatory response resulting from their lack of circulating IL10, a cytokine that normally functions to limit inflammatory processes. Thus IL10<sup>-/-</sup> mice may be at greater risk of adverse effects after *Helicobacter* infection due to their lack of IL10 to inhibit Th1 immune responses. In a breeding colony of IL10<sup>-/-</sup> mice, those housed in a facility where *H. rodentium* or *H. typhlonius* (or both) infections were endemic appeared to have less reproductive success than those that were housed in a facility free from *Helicobacter* spp. These observations lead to this study to specifically determine the effect of infection with *H. rodentium* and *H. typhlonius* on the fecundity (potential reproductive capacity) of IL10<sup>-/-</sup> mice. Because antihelicobacter drug therapy might provide a viable alternative to embryo rederivation for some strains, particularly relative to the risk and resource commitment involved with rederivation, we also investigated whether reproductive performance could be improved by the administration of commercially available antihelicobacter wafers as a method of *Helicobacter* eradication.

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## Materials and Methods

**Animal studies.** Specific pathogen-free IL10<sup>-/-</sup> male and female mice on the C57BL/6 background (B6.129P2-Il10<sup>tm1Cgn</sup>/J; stock number, 002251) were obtained from Jackson Laboratories (Bar Harbor, ME) and used for breeding at 8 to 10 wk of age. Mice were housed in a common room in an AAALAC-accredited animal facility in polycarbonate microisolation caging in ventilated isolation units or on individually ventilated racks under Biosafety Level 2 conditions, with access to food (LabDiet 5001, Purina, Framingham, MA) and water ad libitum. The room was maintained on an automatic timer for a 12:12-h light:dark cycle with regulated room temperature (22 ± 2 °C) and relative room humidity (30% to 70%). Stringent husbandry techniques, including a strictly enforced order of cage handling and scrupulous attention to environmental sanitization, were followed to prevent cross-contamination. A laminar flow hood was used when animals were weighed and to change cages. These studies were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*<sup>17</sup> and approved by the Duke University Institutional Animal Care and Use Committee.

**Health surveillance of animals.** Sentinel mice housed on soiled bedding from the cohorts of mice studied were examined by the Duke Veterinary Diagnostic Laboratory for the presence of endo- and ectoparasites. Tape tests, skin scrapes, and fecal flotations were negative for all endo- and ectoparasites. Serum was collected and submitted to the Research Animal Diagnostic Laboratory (Columbia, MO). The sample was negative for 17 murine viral pathogens, cilia-associated respiratory bacillus, *Encephalitozoon cuniculi*, and *Mycoplasma pulmonis* by serology. Feces from each mouse studied were analyzed for the presence of *Helicobacter* spp. by PCR (see *PCR detection of Helicobacter organisms*). All control (noninfected) mice were negative in this assay. Infected animals demonstrated positivity for the species with which they were infected intentionally and were negative for other *Helicobacter* species.

**Infection and breeding schemes.** Virgin female mice were infected on day 0 with *H. typhlonius* (clinical isolate DU01), *H. rodentium* or both by oral gavage of a single 500- $\mu$ l dose of culture (approximately 5 × 10<sup>7</sup> organisms). Noninfected controls received sterile *Brucella* broth or PBS by gavage. Because preliminary experiments showed frequent failure of sustained *H. rodentium* infection when *H. rodentium* and *H. typhlonius* were given simultaneously (data not shown), mice in the dual-infection group received an additional dose of *H. rodentium* on day 2. Infection was confirmed at 1 wk after infection and every 4 wks thereafter by PCR analysis of freshly passed stool pellets. Pellets were assayed for the presence of *Helicobacter* 16S rDNA sequences by using primers specific for both genus and species<sup>3</sup> or semiquantitative real-time PCR.

Breeding triads consisting of 1 male and 2 female mice typically were formed on day 0, immediately after infection. The male mice used in this study were virgin and were exposed only to the 2 female mice of their triad. Mice were weighed 3 times each week throughout the study, with the control and *H. rodentium*-infected groups weighed on Mondays, Wednesdays, and Fridays, and the *H. typhlonius* and dual (*H. rodentium* + *H. typhlonius*)-infection groups weighed on Tuesdays, Thursdays, and Saturdays. Detection of vaginal plugs was not possible due to the 48-h interval between handling sessions for each group of animals. When rapid and sustained maternal weight gain indicated imminent parturition, female mice were removed and housed in individual

cages. Days to conception for each female mouse were calculated by the subtracting date of conception (estimated to be 21 d before the date of birth) from the total number of days exposed to a male mouse. The number of pups born and number of pups surviving to weaning (day 21 after birth) were determined for each successful pregnancy.

Mice were euthanized by CO<sub>2</sub> asphyxiation followed by exsanguination through the inferior vena cava in accordance with the American Veterinary Medical Association Guidelines on Euthanasia<sup>1</sup>. Portions of the uterus and ovaries were collected for PCR and histologic analyses. Tissue for PCR analysis was harvested with clean instruments prior to collection of other tissues to minimize potential contamination. Tissues from uninfected control mice collected at the same time were analyzed along with tissues from infected mice as an additional control for ex vivo contamination of samples.

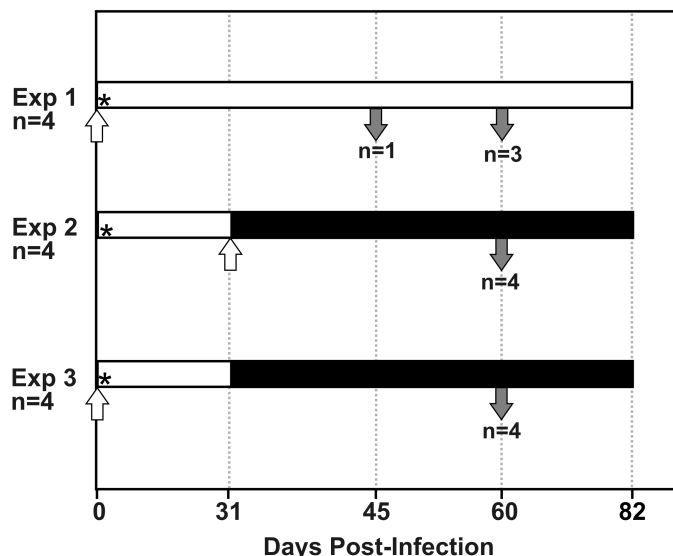
**Study design.** Experiment 1 (Figure 1) was designed to test the hypothesis that *Helicobacter* infection has a negative effect on fecundity of IL10<sup>-/-</sup> mice. Female mice were infected on day 0. Breeding triads were established immediately after infection and maintained for 45 to 60 d, with 4 female mice per infection condition: control, *H. rodentium* infection, *H. typhlonius* infection, and dual infection (*H. rodentium* + *H. typhlonius*). Male mice were removed on day 45 or 60, and all mice were observed until euthanasia for tissue harvest on day 82 after initial infection.

Experiment 2 (Figure 1) was designed to test the hypothesis that treatment with antihelicobacter therapy ameliorates reduced fecundity in *Helicobacter*-infected IL10<sup>-/-</sup> mice. Female mice (n = 4 per group) were infected on day 0. These mice and age-matched noninfected female mice were exposed to male mice in breeding triads beginning on day 31 after infection. Treatment also began on day 31 and consisted of replacing their standard diet with a commercially available diet that contained 4 medications: 3 mg amoxicillin, 0.5 mg clarithromycin, 1 mg metronidazole, and 20  $\mu$ g omeprazole in each per 5-g wafer (Mouse *Helicobacter* MDs, Bio-Serv, Frenchtown, NJ). Control and infected mice were maintained on antihelicobacter therapy until euthanasia for tissue harvest on day 82 after initial infection.

Experiment 3 (Figure 1) was designed to determine whether antihelicobacter therapy could restore fecundity in infected female mice that did not become pregnant after exposure to male mice in breeding triads for 30 d. These female mice received feed containing antihelicobacter medications beginning on day 31 and remained in breeding triads for an additional 30 d. Male mice were removed on day 60 after infection. The female mice were maintained on antihelicobacter therapy until they were euthanized for tissue harvest on day 82 after infection.

Days to conception, number of pups born, and number of pups surviving to weaning were compared between infection groups in the presence and absence of antihelicobacter treatment. Treatment-related changes in excretion of *Helicobacter* organisms were determined by quantitative real-time PCR (qPCR) of feces. Fecal qPCR was performed on feces from pups at 1 wk after weaning to determine the effect of maternal antihelicobacter treatment on vertical transmission of *Helicobacter* infection.

**Bacterial culture.** The clinical isolate of *H. typhlonius* (DU01) strain used was obtained by using a previously described culture technique<sup>12</sup> and identified by morphology and sequence analysis of the 16S rRNA and cytolethal distending toxin (*cdtB*) genes. *Helicobacter rodentium* (isolate MIT 95-1707; also called ATCC type



**Figure 1.** Experimental design. Each experiment (Exp) consisted of 4 female mice in each of 4 groups (control [noninfected], *H. rodentium*-infected, *H. typhlonius*-infected, and *H. rodentium*- and *H. typhlonius*-coinfecting). Mice were infected on day 0 (indicated by an asterisk), which serves as the reference for all date calculations, recorded as days postinfection. The point at which male mice were added is indicated by a white arrow; the point at which they were removed is indicated by a gray arrow. The duration of maternal treatment with the commercial diet containing antihelicobacter drugs is indicated by the black bar.

strain 700285)<sup>20</sup> was obtained from the American Type Culture Collection (Manassas, VA). For inoculation, both strains were grown in *Brucella* broth (Becton Dickinson, Franklin Lakes, NJ). Cultures were agitated with a stir bar in a 250-ml Erlenmeyer flask and were incubated for 24 h at 37 °C in a microaerobic environment with 90% N<sub>2</sub>-5% H<sub>2</sub>-5% CO<sub>2</sub>.

**PCR detection of *Helicobacter* organisms.** The DNA was extracted from frozen uterus, ovary, and feces with the DNeasy Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Briefly, each sample was thawed in 180 µl ATL buffer, 20 µl of proteinase K was added, and samples were incubated overnight at 56 °C. Next, 200 µl AL buffer was added to the tube, the sample was vortexed, and 200 µl ethanol was added. Finally, the sample was loaded onto the silica-gel column, washed, and eluted in 100 µl elution buffer.

Primers designed to amplify segments of the *Helicobacter* Ni/Fe hydrogenase (fwd, 5' CAT TCT GTG AGT TTT GTG GTT 3'; rev, 5' CCA AAA CAT ATG CAA AGG CTT 3') and *cdtB* (fwd, 5' CAC TTG GAA TCT TGG CTC ATC 3'; rev, 5' CCA ATG CGA ATA CCA AGC AC 3'<sup>15</sup>) genes were used to quantify the relative concentrations of *H. rodentium* and *H. typhlonius*. Absolute numbers of organisms per sample were obtained by comparison of the cycle threshold of each sample with that obtained from a standard curve. This curve was generated by using serial dilutions of known number of organisms obtained from pure cultures of *H. rodentium* and *H. typhlonius*. Estimates of the number of *Helicobacter* genome copies in the standard were based on a genome size of 1.8 Mb and a molecular mass of 1.09 × 10<sup>9</sup> Da. The absolute numbers of organisms present in each sample then were normalized to provide the number of *Helicobacter* genomes per milligram

of feces or tissue. The PCR reactions and melting curves were performed in a 20-µl volume that contained 0.5 µl of a 20-µM solution of each primer, 10 µl SYBR Green PCR Master Mix (Stratagene, La Jolla, CA), and 4 µl DNA derived from a precisely weighed (20 to 50 mg) sample of feces or tissue. The PCR reaction was incubated at 95 °C for 15 min to activate the polymerase followed by 40 cycles consisting of 15 s denaturing at 94 °C, 20 s annealing at 58 °C, and 30 s extension at 72 °C. Fluorescence was monitored at the end of each extension phase. After amplification, melting curves were generated to verify PCR product identity. The sensitivity of this PCR assay was between 10<sup>1</sup> and 10<sup>3</sup> organisms per reaction volume.

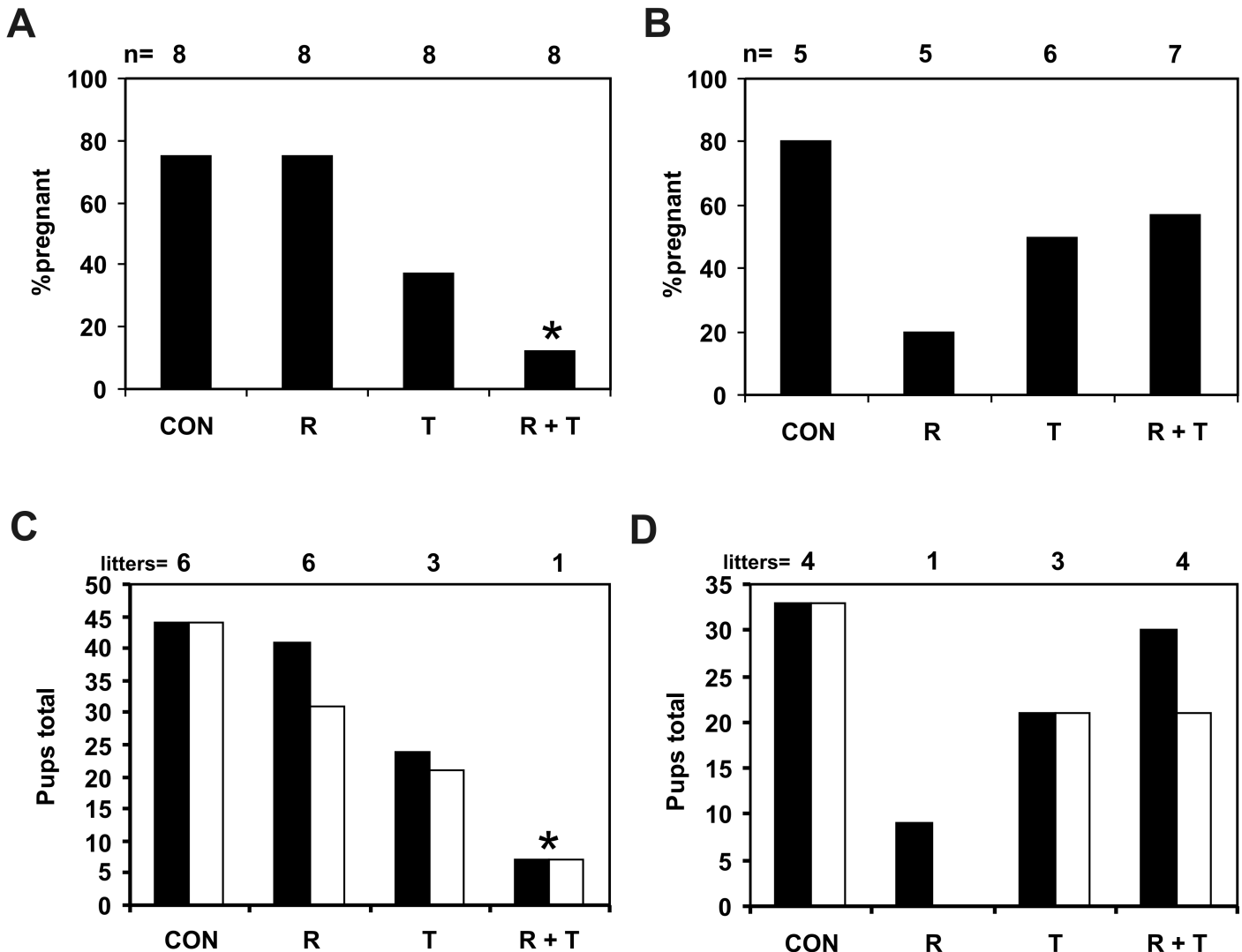
**Statistical analysis.** Pregnancy rates were defined as the number of dams that became pregnant and gave birth divided by the total number of dams exposed to male mice per infection group. Differences in pregnancy rates between groups were determined by using the Fisher exact test. Differences in days to conception and number of pups produced per female mouse in each group were determined by using ANOVA followed by the least significant differences method for multiple comparisons. SAS Version 8.2 (Cary, NC) was used for all analyses. *P* values of less than or equal to 0.05 were considered to represent significant differences.

## Results

***Helicobacter* infection decreases reproductive success of IL10<sup>-/-</sup> mice.** Although female mice were cohoused with male mice for as long as 60 d in experiment 1, all pregnancies occurred within the first 30 d of exposure to male mice. The data from this experiment were combined with those of experiment 3 to determine the reproductive success of mice exposed to male mice from day 0 to 30 in absence of antihelicobacter treatment. A lower percentage of mice became pregnant among those infected with both *H. typhlonius* and *H. rodentium* (pregnancy rate, 13%; *P* < 0.02), with a trend toward a lower percentage of pregnancy in mice infected with *H. typhlonius* alone (38%; 0.05 < *P* < 0.10) when compared with the control and *H. rodentium* groups (both 75%; Figure 2 A). However, among those mice that delivered litters, the number of days to conception (mean ± SEM) was similar between groups: control and *H. typhlonius* groups, 4 ± 1 d; dual-infection group, 5 d; and *H. rodentium* group, 8 ± 4 d.

All groups had normal litters containing 7 to 9 pups per litter. All pups in the control and dual-infection groups survived to weaning, whereas the *H. rodentium* and *H. typhlonius* groups lost 10 and 3 pups, respectively. Therefore, given the differences in pregnancy rates between the groups, the total number of pups that survived to weaning (Figure 2 C) was greatest in the control group (total, 44 pups; mean, 6 pups per dam), followed in decreasing order by *H. rodentium* (31 pups; mean, 4 pups per dam), *H. typhlonius* (21 pups; mean, 3 pups per dam), and dual infection (7 pups; mean, 1 pup per dam; *P* < 0.05 compared with control value).

**Antihelicobacter therapy may improve fecundity of *Helicobacter*-infected IL10<sup>-/-</sup> mice.** In experiment 1, no additional pregnancies occurred in infected mice after day 30 of exposure to male mice. Therefore in experiment 2, infected female mice were housed separately from male mice for the first 30 d after infection. On day 31, antihelicobacter therapy was begun, and breeding triads were established with these animals. Female mice in experiment 3 that did not become pregnant during the first 30 d of exposure to a male mouse received antihelicobacter therapy beginning on day 31 and remained in established breeding groups.



**Figure 2.** Effect of *Helicobacter* infection on reproductive success in IL10<sup>-/-</sup> mice. (A) Pregnancy rates for female mice that did not receive antihelicobacter therapy are shown as a function of infection status. (B) Pregnancy rates for female mice that received antihelicobacter treatment on day 31 and continued for the duration of the experiment, shown as a function of infection status. (C) The numbers of pups born (black bars) and surviving to weaning (white bars) are shown as a function of infection status for females that did not receive antihelicobacter therapy. (D) The numbers of pups born (black bars) and surviving to weaning (white bars) are shown as a function of infection status for female mice that received antihelicobacter therapy starting on day 31 postinfection for the duration of the study. \*,  $P < 0.05$  compared with value for noninfected control dams. CON, control (noninfected); R, infected with *H. rodentium*; T, infected with *H. typhlonius*; R + T, dually infected with *H. rodentium* and *H. typhlonius*.

Pregnancy rates for dams that received antihelicobacter therapy in the control, *H. typhlonius*, and dual-infection groups were not significantly different from those of the corresponding nontreated dams (control group, 80% versus 75%; *H. typhlonius* group, 50% versus 38%; and mixed infection group, 57% versus 13%; Figure 2 B). Twenty percent of mice ( $n = 5$ ) that were infected with *H. rodentium* and received antihelicobacter therapy became pregnant compared with 75% of untreated *H. rodentium*-infected dams ( $n = 8$ ).

In all 4 groups of treated mice, females that delivered litters had 7 to 9 pups per litter. All pups from control and *H. typhlonius*-infected dams survived to weaning, but the *H. rodentium* and dual-infection dams lost 9 and 8 pups, respectively. The total number of pups surviving to weaning was numerically greatest in the control group (33 pups), followed in decreasing order by

*H. typhlonius* and dual-infection groups (21 pups each) and *H. rodentium* group (0 surviving pups; Figure 2 D).

**Effect of maternal antihelicobacter treatment on vertical transmission of *Helicobacter* infection.** Fecal samples from dams and pups were analyzed by quantitative real-time PCR to evaluate transmission of *Helicobacter* horizontally (between members of the same species but not in a parent-child relationship; that is, for example, between littermates) and vertically (from dam to pup peri- or postnatally). Prior to receiving antihelicobacter therapy, dams infected with *H. typhlonius* and *H. rodentium* excreted  $10^6$  and  $10^8$  bacterial organisms per gram of feces, respectively. All dams had undetectable *Helicobacter* DNA in their stool 7 d after beginning therapy.

Table 1 contains results of quantitative real time-PCR of feces collected at 1 mo of age from pups born to treated dams that were

**Table 1.** Presence of *Helicobacter* spp. in stool from 1-mo-old pups

Maternal infection <sup>a</sup>	Maternal treatment with antihelicobacter wafer?	<i>H. rodentium</i> <sup>b</sup>	<i>H. typhlonius</i> <sup>b</sup>
None	no	0/17	0/17
None	yes	0/5	0/5
<i>H. typhlonius</i>	no	0/18	11/18
<i>H. typhlonius</i>	yes	not done	4/19 <sup>c</sup>
<i>H. rodentium</i> + <i>H. typhlonius</i>	no	0/7	5/7
<i>H. rodentium</i> + <i>H. typhlonius</i>	yes	2/19	2/19 <sup>c</sup>

<sup>a</sup>Dams were inoculated with approximately  $5 \times 10^7$  bacterial organisms in *Brucella* broth prior to breeding.

<sup>b</sup>No. of infected pups by quantitative PCR analysis /no. of total pups

<sup>c</sup> $P < 0.05$  versus value for nontreated dams

previously infected with *H. typhlonius* or both *H. typhlonius* and *H. rodentium*. None of the pups born to *H. rodentium*-infected mice that received therapy survived to weaning, and therefore that group could not be evaluated. *H. rodentium* was not detected in stool of pups born to nontreated dams infected with both *H. rodentium* and *H. typhlonius*. The feces of pups born to dams in the nontreated *H. typhlonius* and dual-infection groups were positive for *H. typhlonius* DNA as follows: 11 of 18 pups (61%) in the *H. typhlonius* group and 5 of 7 pups positive (71%) in the dual-infection group.

As expected, pups born to noninfected, treated dams of the control group had no detectable *H. rodentium* or *H. typhlonius* DNA in their stool. Among pups from infected dams, maternal antihelicobacter therapy reduced the proportion with *Helicobacter* DNA in the stool. In the *H. typhlonius* group, 21% (4 of 19) of pups from treated dams were positive for fecal excretion of *H. typhlonius* as compared with 61% of pups from untreated dams ( $P < 0.01$ ). In the dual-infection group, 11% (2 of 19) treated pups compared with 71% without treatment ( $P < 0.01$ ) had positive fecal PCR results for both *H. rodentium* and *H. typhlonius*.

**Helicobacter infection status of reproductive organs.** *Helicobacter* species typically infect organs of the gastrointestinal tract, including stomach, intestine, and liver. Our detection of different pregnancy outcomes in infected mice raised the question of whether *Helicobacter* organisms also infect the reproductive tract. To address this possibility, qPCR was performed on uterine and ovarian tissues collected on day 82. Analysis was performed on 3 mice per infection group. One of the 3 uterine samples from mice that had been infected with both *H. typhlonius* and *H. rodentium* and had not received therapy was positive for *H. typhlonius*. This mouse had not become pregnant throughout the course of the experiment. Testes samples from male mice, which became infected due to exposure to the infected females, were negative for *Helicobacter*.

In a separate experiment, tissues from 5 female IL10<sup>-/-</sup> mice, infected in a similar fashion with both *H. rodentium* and *H. typhlonius*, were analyzed 1 wk after inoculation. Quantitative PCR showed high content of DNA from both organisms in the uterus in 4 of 5 mice. The mean numbers of bacterial genomes present were  $1.0 \times 10^3$  and  $4.5 \times 10^5$  per mg of uterine tissue for *H. rodentium* and *H. typhlonius*, respectively.

## Discussion

This study shows that *Helicobacter* infection of IL10<sup>-/-</sup> mice reduces their reproductive performance by decreasing pregnancy rates and the number of pups surviving to weaning, particularly in mice coinfecting with *H. typhlonius* and *H. rodentium*. Antihelicobacter therapy improved reproductive performance by increasing pregnancy rates and pup survival in the dual-infection group. *Helicobacter*-infected dams that received antihelicobacter therapy had less vertical transmission of *H. typhlonius*. The uterus of 1 dam that was infected with *H. typhlonius* was positive for *H. typhlonius* DNA on day 82 after infection, suggesting that local infection of the reproductive tract may contribute to vertical transmission. DNA from both *H. typhlonius* and *H. rodentium* was present in the uterus of 4 of 5 infected females when tested one week after infection, suggesting a possible mechanism by which infection may affect conception and/or early pregnancy. Overall, antihelicobacter therapy improved the reproductive success of *Helicobacter*-infected IL10<sup>-/-</sup> dams, most notably in the groups infected with *H. typhlonius* only or *H. typhlonius* and *H. rodentium*.

Noninfected IL10<sup>-/-</sup> mice had a pregnancy rate of 75% to 80% throughout all experiments, with litters of average size and all pups surviving to weaning. Because the pups that were born survived to weaning, the data suggest that any impact of *Helicobacter* on fecundity is related to effects on initial conception or early termination of pregnancy, which would not have been distinguishable in this study. Additional studies are needed to further investigate this point.

Our studies showed that reproductive success decreases when IL10<sup>-/-</sup> mice are infected with *H. rodentium* and *H. typhlonius*. Despite the differences in pregnancy rates, all litters were of average size, with 7 to 9 pups per litter, regardless of infection status. However, there was a trend toward a decline in the number of pups born and those surviving to weaning declined in infected mice, with the control group having the highest number born and surviving to weaning, followed by *H. rodentium*, *H. typhlonius*, and the dual-infection group. Figure 2 A, C highlights how the combination of decreased pregnancy rates and decreased pup survival results in an overall marked decrease in reproductive success of IL10<sup>-/-</sup> mice infected with both *H. rodentium* and *H. typhlonius*. Although untreated dams had moderate to severe inflammatory bowel disease on histologic analysis of tissues collected on day 82 after infection<sup>2</sup>, colitis likely did not affect their breeding performance because the dams were placed in mating groups immediately after inoculation, such that time was not sufficient for the development of colitis. Dams that received antihelicobacter therapy were clinically similar to untreated dams, with no significant weight loss noted in either group. Additional studies are necessary to explore the mechanism of this decreased reproductive performance, with focus on how *Helicobacter* infection affects the uterine microenvironment.

Due to limited space and funding, this study used small group sizes to determine whether further investigation was warranted. Larger group sizes might support or refute the trend data. Despite this limitation, the data raise concern that the breeding performance of strains not typically considered immunodeficient might also be impaired by *Helicobacter* infection. This outcome could profoundly affect multiple areas of research, in light of the many transgenic lines being generated and maintained and the high prevalence of *Helicobacter* spp. within animal facilities.

Rederivation and antihelicobacter treatment typically are used to eliminate *Helicobacter* infection from rodent colonies.<sup>9</sup> A previous study compared rederivation by in vitro fertilization, embryo transfer, and ovary transplantation to determine whether transfer from potentially infective reproductive tissues of *H. typhlonius*-infected immunodeficient mice to negative surrogate dams results in vertical transmission of disease to offspring. All 3 methods resulted in recipient dams that remained negative until at least 7 to 8 wk postoperatively and in pups that tested negative at weaning.<sup>19</sup>

In our study, a commercially available feed containing amoxicillin, clarithromycin, metronidazole, and omeprazole was used to treat *Helicobacter* infection. A medicated diet formulated with these drugs was shown to eradicate *H. pylori*, *H. bilis*, *H. rodentium*, and *H. typhlonius* infections in rats.<sup>8</sup> The mouse formulation eliminated *H. bilis* and *H. hepaticus* infection in various transgenic lines of mice as well.<sup>9</sup> Accordingly, we tested whether this therapy was a viable alternative to rederivation for eradication of *Helicobacter* spp. in infected mice. Reproductive success generally improved when antihelicobacter therapy was administered to IL10<sup>-/-</sup> mice infected with *H. rodentium*, *H. typhlonius*, or both organisms. Although only 1 mouse in the *H. rodentium* group became pregnant, this animal became pregnant sooner than did those that did not receive antihelicobacter therapy (2 d versus 8 ± 4 d). The reason for the lack of pregnancy in the remainder of the group infected with *H. rodentium* is still unclear. The *H. typhlonius* and dual-infection groups showed trends toward higher pregnancy rates with antihelicobacter therapy compared with those of the nontreated groups (Figures 2 A, B). *H. typhlonius* pregnancy rate improved from 38% to 50% and mixed infection from 13% to 57% after administration of antihelicobacter therapy. The number of pups born and surviving to weaning for both the *H. typhlonius* and dual-infection groups also increased with administration of antihelicobacter therapy (Figure 2 C, D). On the basis of our data and observations, IL10<sup>-/-</sup> mice infected with *H. typhlonius* or both *H. typhlonius* and *H. rodentium* benefit most from antihelicobacter therapy to improve reproductive success. The effect on the mixed infection group was important information for us because this type of coinfection is detected during routine rodent health surveillance in some of our animal facilities. Although not uniformly successful in our study, antihelicobacter treatment may be a viable alternative to rederivation of these animals with careful monitoring.

Dams infected with *H. rodentium* had a high incidence of pup death in both the treated (9 of 9 pups) and untreated (10 of 41 pups) groups. The reason for this high number of deaths is unclear. All pups appeared to be healthy when born, and no overt signs of clinical disease were noted on pups that were found dead, although many appear to have been cannibalized. More studies are needed to investigate potential phenotypic changes in mice infected with *H. rodentium* to determine the mechanisms that cause decreased fecundity and increased cannibalism.

Fecal samples taken from pups at 1 mo of age indicate that *Helicobacter* can be vertically transmitted (Table 1). In groups that did not receive antihelicobacter therapy, this transmission might occur postnatally (via a maternal fecal-pup oral route of infection) in addition to any vertical (perinatal) transmission, in light of positive stool samples from the dam. However, postnatal transmission is not supported for those pups that tested positive but were born to dams that received antihelicobacter therapy.

Fecal samples collected from female mice 7 d after initiation of antihelicobacter therapy had no detectable *Helicobacter* organisms by qPCR. These female mice remained negative on subsequent monthly stool analyses for the duration of the study. How the systemic distribution of *Helicobacter* organisms differs at different time points postinfection and whether infections cleared from nongastrointestinal organs are cleared naturally or due to administration of antihelicobacter therapy are unknown. These variables would affect vertical transmission of infection and require additional studies.

Antihelicobacter therapy significantly decreased, but did not eliminate, vertical transmission in *H. typhlonius* and mixed infection groups. Because all positive mice were cohoused with mice that tested negative, horizontal transmission between cohoused pups is evidently not highly efficient during the first month of life. Contamination during animal handling is another possible explanation for positive stool samples in these pups, although unlikely given the experimental design. Pups were handled a maximum of 2 times: during routine cage changing that occurred once every 2 wk and at the time of weaning when pups were separated from their dams and cohoused in groups no more than 5 pups per cage. Additional studies would be needed to determine if antihelicobacter therapy of pups after weaning would further reduce *Helicobacter* infection in pups from infected dams.

Uterine and ovarian tissues harvested on day 82 immediately after euthanasia of a subset of mice were analyzed by qPCR. The uterine sample of 1 mouse in the *H. typhlonius* group that did not receive antihelicobacter therapy was positive for *H. typhlonius* on day 82 after infection. The best time for analysis of systemic *Helicobacter* distribution is unknown. However, we found large numbers of *Helicobacter* organisms in the uterus 7 d after infection of a separate group of mice not used for breeding. Our observations confirm previous work that documented the presence of *Helicobacter* DNA in reproductive organs.<sup>19</sup> This previous study used nested PCR to document the presence of *Helicobacter* DNA associated with oocytes and embryos carried by infected female mice and with sperm of infected male mice, although no infected embryos resulted when these tissues were used for assisted reproduction and embryo transfer.<sup>19</sup> In contrast, *H. hepaticus* was cultured from tissues of unborn pups of infected dams late during gestation, supporting vertical transmission in utero by means of the placenta.<sup>10</sup> Further studies are needed to investigate the variables involved with vertical transmission of *Helicobacter* species.

The effects of *Helicobacter* infection followed by antihelicobacter therapy on reproduction may vary depending how long animals are treated before mating and subsequent parturition. Further studies are needed to determine whether prolonged treatment prior to mating further improves reproductive success by increasing pregnancy rates and pup survival. The time point at which pups born to *H. typhlonius*- and dual-infected, treated dams became infected and thus whether prolonged antihelicobacter therapy prior to mating could possibly lessen vertical transmission remain to be determined. In addition, whether mice infected with *Helicobacter* spp. neonatally have decreased reproductive outcomes when they reach reproductive age is unknown currently. Further studies are needed to investigate these scenarios.

The use of virgin 'unproven' male mice is a possible limitation of our study. However, the 2 control and 2 *H. rodentium*-infected female mice that did not become pregnant despite exposure to male mice for 30 to 60 d (Figure 2 A) were from different tri-

ads, and their male partners were of proven fertility. Therefore female factors are the most likely explanation for the less than 100% pregnancy rates in these groups. The *H. typhlonius*-infected and mixed infection groups had 1 and 2 triads, respectively, that did not yield pups. The study design does not allow us to determine whether male or female factors were involved in these cases. Although initially uninfected, all male mice eventually become infected when cohoused with infected female mice. Perhaps *Helicobacter* infection affects male fertility. However, some of the infected female mice that failed to produce litters had patterns of weight gain and loss suggestive of early pregnancy losses rather than failure to impregnate (data not shown). Similarly, a previous study showed increased numbers of fetal resorptions and decreased fetal weights in CD1 female mice infected with *H. pylori*; these mice showed increased lymphocyte infiltration into uterus and increased endometrial expression of interferon  $\gamma$ .<sup>18</sup> Additional studies are required to determine whether similar mechanisms decrease fecundity in IL10<sup>-/-</sup> mice infected with *H. typhlonius*, *H. rodentium*, or with both organisms.

This study focused specifically on how *Helicobacter* infection affects the reproductive performance of IL10<sup>-/-</sup> mice. Further studies are needed to determine whether other strains similarly have decreased reproductive success when infected with these commonly acquired organisms. For example, mice with genotypes that affect immune function may be particularly prone to the reproductive sequelae of *Helicobacter* infection. If this notion is correct, fertility defects that have previously been attributed to genotype may instead be related to presence or absence of infection. This scenario could pose a serious problem for researchers, in light of the prevalence of these infections which have been considered to be innocuous. Given the variety of *Helicobacter* species that have been used to generate models of inflammatory bowel disease and inflammation-associated colonic neoplasia in IL10<sup>-/-</sup> and other immunodeficient mice,<sup>7,13,14</sup> *Helicobacter* infection could have far-reaching implications on a variety of research studies involving reproduction and inflammatory changes. This potential and the effect of maternal infection on pup yield provide convincing arguments for eradicating these infections in laboratory animal facilities.

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