

Eradication of *Helicobacter* spp. from a Rat Breeding Colony

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Although *Helicobacter* spp. have been viewed as bacteria with low pathogenicity, many investigators have shown that these low-grade pathogens have the potential to become a severe threat in immunocompromised, inbred, and transgenic animals. Therefore the presence of *Helicobacter* spp. in experimental animals is considered to be an unacceptable variable. In this study a formulation of medicated feed was designed and tested in an attempt to eradicate *Helicobacter* spp. from an infected rat breeding colony. Two feeding protocols were used: 1) treating *Helicobacter*-infected pregnant dams to produce clean offspring and 2) treating infected adult animals long enough to eliminate the organisms. Bacterial DNA was extracted from feces and amplified using primers that recognized the *Helicobacter* spp.-specific region of the 16S rRNA gene. Fecal samples from the weanlings from protocol 1 tested negative for *Helicobacter* spp. at 1 week before and 2 and 12 weeks after weaning. Infected adult rats from protocol 2 tested negative after three cycles of 2 weeks on and 2 weeks off the medicated feed. Animals from both protocols have remained *Helicobacter*-free for 8 months.

Since Marshall first described *Helicobacter pylori* in 1982 (15), several other gastric and enterohepatic species of *Helicobacter* have been isolated from a variety of mammalian species, including rodents (7, 8, 9, 10, 21, 22). There are at present at least 26 formally named species, which have been extensively reviewed by Whary and Fox (26). New species of this bacterium are continually being identified and have yet to be reported, making it difficult to fully understand the role that *Helicobacter* spp. may have in health and disease. These bacteria have become of interest not only as human and animal pathogens but also for their value as models of human disease including inflammatory bowel disease (IBD; 24). The pathogenesis of IBD has not been clearly defined; however, it is generally agreed that genetic, environmental, and immunological factors are involved and that microbial flora are important. IBD may result from either an abnormal immunological response to enteric flora or from a normal response to a specific pathogen (2, 12). In the presence of a dysfunctional immune system or in a host with an impaired mucosal barrier, microbial antigens (commensal or pathogenic) may initiate and promote inflammation (2, 9).

Symptoms resulting from *Helicobacter* infection are also dependent on environmental, genetic, and immunological factors and may have different effects depending on the strain, age, or sex of the animals. For example, *H. felis*, when used as a model of gastric carcinoma, causes severe gastritis in C57BL/6 mice, whereas the inflammation induced from the same organism in Balb/c mice is mild. *Helicobacter hepaticus* infection in immunocompetent mice produces only mild inflammation and epithelial cell hyperplasia, whereas the same bacterium causes marked typhilitis and colitis in immunodeficient SCID mice (24). Gender also may have an effect on the severity of symptoms caused by *Helicobacter* spp. infection. Court and colleagues (3) have shown that female C57BL/6 mice infected with *H. felis* show an earlier onset and increased severity of chronic gastric inflammation than do their male counterparts, even though the density of the bacteria was similar in both genders.

There is limited information on the effect of *Helicobacter* infection

in rats, although it has been shown that there is a 19% prevalence in research rats, consisting of 3 to 9% *H. bilis*, 10% *H. typhlonius*, 5% to 8% *H. hepaticus*, and 2% *H. rodentium* and unidentified species (26). As in mice, *H. felis* has also been used as a model of *H. pylori* infection in rats (24). Similarly, infection in rats can pose a threat to immunocompetent animals or those with a dysfunctional mucosal barrier; for example, *H. bilis* has been shown to cause ulcerative typhlocolitis and proctitis in immunodeficient rats (20). *H. trogonium* has been isolated from the colon and *H. muridarum* from the intestinal mucosa of Holtzman and Wistar rats, but the pathogenicity has not yet been clarified (27).

There is an increasing awareness of the importance of enteric flora in research animals (13). Several treatments to eradicate *Helicobacter* have been tried with varied success in mice. Amoxicillin in drinking water for 2 weeks eradicated *Helicobacter hepaticus* from weanling mice but did not eradicate the infection from older mice with an established colonization (20). A combination of amoxicillin, metronidazole, and bismuth given by gavage to mice 3 times daily for 2 weeks was successful in eradicating *H. hepaticus*, but this was a very labor-intensive protocol (5). There is also a commercially available medicated dosing system (Bio-Serv, Frenchtown, N.J.) designed for treating *Helicobacter hepaticus* in mice, which combines amoxicillin, metronidazole, and bismuth into a feed tablet (6, 16).

Despite the lack of evidence of the pathogenicity of *Helicobacter* in rats, the potential importance of these organisms cannot be overlooked. Unintentional *Helicobacter* infections have confounded interpretation of results from established models of IBD in mice (9), and further examination of rat models could show the same. We became concerned about the *Helicobacter* status of our colony of rats as our research involves models of intestinal inflammation and intestinal mucosal barrier function. Fecal samples from five cages of animals in our breeding colony of mast-cell-deficient rats tested positive for both *Helicobacter* spp. and *H. typhlonius*. We therefore set out to attempt to eradicate the infection from the colony. An initial pilot study was carried out to test the efficacy of the existing Bio-Serv feed in treating the infected rat colony. The existing formulation was not effective in treating the infected rats; therefore a plan was developed to formulate a new combination of medications.

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Materials and Methods

Animals. The breeding colony of rats (McMaster University, Hamilton, Ontario, Canada) consisted of W^s/W^s intestinal mast-cell-deficient rats, which have a 12-base deletion in the tyrosine kinase domain of the *c-kit* gene, $+/+$ (Norwegian Brown) controls, and $W^s/+$ heterozygote (17). In addition, a small secondary study was conducted on a colony of Flinders Sensitive (FLS) rats. All rats used in all studies were between 3 and 6 months of age. Ethical approval for these studies was obtained from the Animal Care Committee at McMaster University.

During the course of the feeding studies, the sentinel animals tested negative (by enzyme linked immunosorbent assay) for Sendai virus, mouse poliovirus, sialodacryoadenitis virus, Kilham rat virus, H-1 virus, reovirus 3, *Mycoplasma pulmonis*, and rat parvovirus (Charles River Diagnostics, Wilmington, Mass.).

Animal husbandry. The animal facility at McMaster adheres to guidelines set by the Canadian Council on Animal Care (CCAC) in accordance with the Animals for Research Act.

The breeding room has restricted access to research investigators and is maintained at positive pressure, on a 12:12-h light:dark cycle, at an average temperature of 23°C, with 12 air changes per hour. All staff wore cap, mask, gloves, gown, and booties upon entering the room. All cages, bedding (Corn Cob Bedding, Harlan, Madison, Wis.), and water were sterilized by autoclaving. Procedures such as feeding, weaning, and changing of cages were done in a biological safety cabinet (model 1104 S/N, Forma Scientific, Marietta, Ohio). Animals were housed in cages with isolator lids. Before any contact with the rats, the outside of the cages and the technician's gloves were sprayed with a 1:125 dilution of Mikro-Quat (Ecolab, Mississauga, Ontario) before the cages were opened inside the hood. In the initial pilot study, which involved only three cages, cages were changed daily. This frequency was not practical on a larger scale, and therefore cages were changed every other day in the larger study. Gloves were changed between every cage. The rats involved in the feed study were handled before the rest of the colony.

Pilot study with Bio-Serv Mouse *Helicobacter* MDs. In the initial study to test the efficacy of the existing medicated feed (Mouse *Helicobacter* MDs, Bio-Serv, Frenchtown, N.J.) three breeding pairs of $W^s/+$ rats were used. Prior to the beginning of the study, all rats tested positive for *Helicobacter* spp., and four animals were positive for *H. typhlonius*. The samples were tested both in our laboratory and at an outside testing facility (RADIL, MU Research Animals Diagnostic Laboratory, Columbia, Mo.). One week after the animals were paired for breeding, their normal rat chow (S-2335 Mouse Breeder Sterilizable Diet 7904, Harlan-Teklad, Madison, Wis.) was replaced with medicated feed ad libitum. The male rats were removed 2 weeks after pairing and not included in the rest of the study. The female animals remained on the medicated diet for the remainder of the gestation period and for the 21 days prior to weaning of the pups. Fecal samples were collected from the dams and pups at the time of weaning. All samples tested positive for *Helicobacter* spp.

Feed formulation. The new formulation contained 0.07 mg omeprazole, 3.3 mg metronidazole, 6.7 mg amoxicillin, and 1.7 mg clarithromycin, incorporated into a 5-g nutritionally complete grain-based, bacon-flavored tablet (Bio-Serv, Frenchtown, N.J.). Final drug concentrations (omeprazole, 0.88 mg/kg; metronidazole, 39.6 mg/kg; amoxicillin, 80.0 mg/kg; clarithromycin, 20.0 mg/kg) in the feed tablets reflected the assumption that the normal adult rat consumes 10 to 15 g of food per day.

Feeding protocols. Two feeding protocols were designed. Protocol 1 involved treating infected pregnant rats with the new formulation of medicated feed in an attempt to produce *Helicobacter* spp.-free offspring. Pregnant dams were started on the medicated feed (4

tablets per day) on day 7 of gestation and were kept on the feed until the pups were weaned at 3 weeks of age. Feed consumption was monitored daily, and estimated consumption was recorded. The pups were weaned and housed two animals per cage and fed normal rat chow (S-2335 Mouse Breeder Sterilizable Diet 7904, Harlan-Teklad). Fecal samples from dams and weanlings were taken 1, 3, and 6 months post weaning. At the time of weaning, the dams were put back on normal rat chow.

In the second protocol, adult male, *Helicobacter* spp.-positive rats were fed the medicated feed for 2 weeks, put back on normal rat chow for 2 weeks, and then tested for the presence of *Helicobacter* spp. This cycle of 2 weeks on followed by 2 weeks off the medications and subsequent testing was repeated until all animals in the study tested negative. These animals then were tested 1, 3, 6, and 8 months after the cessation of the last feeding cycle.

Sample collection. *Helicobacter* spp. testing was done on fresh fecal samples, although the same results could be obtained from frozen samples. Fecal samples (five pellets from each animal) were collected aseptically into sterile centrifuge tubes by using sterile forceps. The forceps were sterilized by autoclaving.

***Helicobacter* spp. analysis.** The polymerase chain reaction (PCR) assay to detect *Helicobacter* spp. was set up at our own facility in order to obtain immediate results throughout the course of the study. Initial fecal samples from each study as well as the 1-month post-treatment samples were sent to an outside laboratory (RADIL) for confirmation.

Bacterial DNA was extracted from the feces by using a Qiagen Stool Mini Kit, in which the initial lysis is carried out at 70 to 95°C in order to lyse the bacterial cells (18). This protocol also includes a step through which enzymes are adsorbed that can degrade DNA and thus inhibit downstream PCR reactions. DNA was amplified using a modified method of Beckwith and colleagues (1). The primers, 5'-TATGACGGGTATCCGGC-3' and 5'-ATTC-CACCTACCTCTCCCA-3', recognize a specific region of the 16S rRNA gene and were synthesized on site (MOBIX McMaster University). PCR products were run on a 1.5% agarose gel stained with ethidium bromide. Positive samples were visualized under UV light at the 375-bp band. DNA standards (100-bp DNA Ladder, Fermentas, Burlington, Ontario), to confirm the size of the DNA product, as well as a known *Helicobacter* spp.-positive fecal sample and a no-template control were run along with the samples.

Results

We conducted a small initial study using three *Helicobacter* spp.-positive pairs (two $+/+$ and one W^s/W^s) and feeding protocol 1. The rats were treated with the new formulation of medicated feed (4 tablets per day) starting on day 7 of gestation, and they continued on the feed until the pups were weaned at day 21. There was an initial concern over whether the rats would eat the feed, as they had consumed only 0.58 ± 0.6 of a feed tablet on the first day. However, this was not the case and by day 3 the rats were consuming on average 2.5 ± 1.25 tablets per day. Fecal samples were taken from the dams and pups at 2 weeks and 3 and 6 months post weaning. All samples tested negative for *Helicobacter* spp. at all time points. Samples were analyzed both in house and by RADIL. In light of these results, we decided to attempt a similar study on a larger scale. We increased the population to nine pairs, three of each genotype in our breeding colony (W^s/W^s , $+/+$, $W^s/+$). There were no adverse effects on litter size or health status of the pups in the treated groups. Dams and pups were tested 1 and 3 months after weaning. All samples tested negative at all time points. Even though the primary objective was not to "clean up" the females, they also tested negative after being on the feed for a total of 5 weeks.

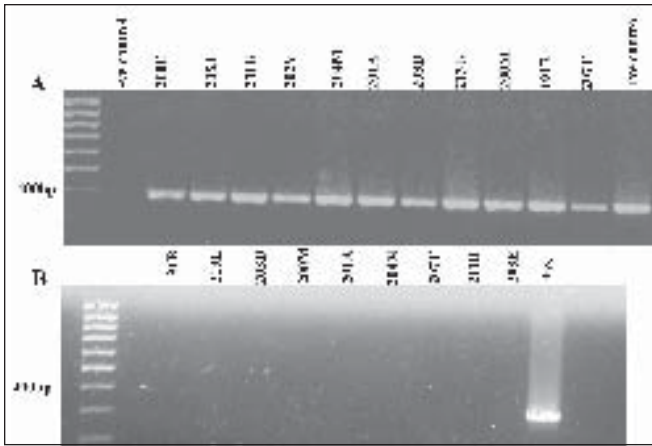


Figure 1. Ethidium bromide-stained agarose gel. Lane 1, 100-bp DNA ladder; lane 2, negative control; the last lane in each panel shows a known *Helicobacter* spp.-positive fecal sample. The genotype of rats 191B, 213K, and 203D is $W^s/+$; that of 200M, 201A, and 204M is $+/+$; and that of 207T, 211B, and 208E is W^s/W^s . (A) *Helicobacter* spp.-positive rat fecal samples from 11 adult male rats screened for use in protocol 2. (B) Fecal samples taken 6 months after the third treatment cycle from the nine male rats on protocol 2 are free of *Helicobacter* spp.

In an attempt to eradicate *Helicobacter* spp. from adult animals, we designed feeding protocol 2. In this portion of the study, nine *Helicobacter* spp.-positive male rats (3 W^s/W^s , 3 $+/+$, and 3 $W^s/+$) were put on the medicated feed for 2 weeks followed by 2 weeks on normal rat chow. The cycle of 2 weeks on the feed followed by 2 weeks on normal chow was repeated three times, with testing done after each cycle. All animals tested negative (according to RADIL) after the third cycle. These animals have remained *Helicobacter* spp.-free for 6 months (Fig. 1).

Protocol 2 was repeated in another strain of inbred rats (Flinders Sensitive) at McMaster. In this study, nine adult (five female and four male) animals, positive for *H. bilis* and *H. rodentium* (RADIL), were treated with the medicated feed. These animals tested negative after only two cycles on the feed and have remained *Helicobacter*-free for 8 months.

Discussion

In this study we designed and tested a new formulation of medicated feed to eradicate *Helicobacter* spp. from an infected colony of rats. The new formulation was developed based on a formulation used successfully to eradicate *H. pylori* in nonhuman primates (4). It was suggested that the dose used in nonhuman primates be doubled for rodents.

Protocol 1 was designed with the goal of producing *Helicobacter*-free offspring and avoiding the lengthy rederivation process. With the success of protocol 1, we proceeded to test whether we also could treat infected adults effectively. A potential problem with antibiotic treatment is the possibility that the treatment only suppresses the bacterial growth without fully eradicating it, which then could lead to a reemergence of the infection. It has also been reported that *H. bilis* is shed intermittently leading to false negative results (14). In an attempt to eliminate the possibility of a reemerging infection, the second protocol was designed, in which the animals had a 2-week break from the medications. The treated animals were followed for 8 months after testing negative, as we were concerned about the findings of Whary (25), who observed that there was a delayed and inconsistent transmission of infection (when tested by fecal PCR analysis) to sentinel mice when they were exposed to bedding from *H. bilis*- and *H. rodentium*-infected animals. We were successful in reproducing

the favorable results of protocol 2 with another infected strain of rats (FSL), and this reproducibility suggests that this treatment may be effective in many strains of rats. The FSL animals were known to be infected with both *H. bilis* and *H. rodentium*, and some of the $+/+$ animals were identified also to be infected with *H. typhlonius*; this infection pattern suggests that we have a formulation of feed that targets a variety of species.

As stated above, the female rats from protocol 1 also tested negative for *Helicobacter* when the pups were weaned. These animals had been on the feed for a total of 5 weeks and then returned to the normal rat chow, indicating that a continuous feeding protocol may also be effective. A continuous feeding protocol may prove to be more effective, as the cyclical nature of protocol 2 might lead to the acquisition of resistance to the medications.

There was also the concern that, if the infection was dormant in these animals and not fully eradicated, the subsequent stress of breeding and pregnancy might lead to re-emergence of the infection. Fortunately this was not the case, and the “clean” offspring from protocol 1 have since been used as breeders and have become the parent generation of the new colony. We have been screening breeders routinely, and all have tested negative for *Helicobacter* spp.

Helicobacter has become one of the most prevalent pathogens in mice used for biomedical research (27), and thus the implications of a successful eradication program in mice are wide-ranging. A small study using 10 female Nep Def mice that are infected with *H. hepaticus* and *H. rodentium* is now in progress at McMaster. The mice were fed the medicated feed for a total of 9 weeks. Although the animals were still weakly positive at 4 weeks, they all tested negative at 9 weeks. Further studies are in progress at other institutions with the 9-week continuous feeding protocol in mice.

PCR for the detection of *Helicobacter* was set up at McMaster to facilitate rapid testing. Our PCR assay was not species-specific, and we can only conclude that bacterial DNA levels were below the detection level of our assay. We therefore sent duplicate samples to an independent laboratory for testing. We might have further verified our results by intestinal tissue PCR and bacterial culture. Direct PCR from feces can be impaired by inhibitory substances in the feces which destroy DNA (11) and thus may result in false negative results. This potential problem was avoided by using the Qiagen Stool Mini Kit, in which these inhibitory components are removed through an extra step during the extraction protocol.

As we learn more about the effects of enteric flora, we become aware of the importance of both animal husbandry and environment when interpreting results of animal experiments. In fact experimental results may change with setting; for example genetically engineered mice with a pro-inflammatory genotype may develop inflammation in some facilities but not in others (25).

The success of the present study was to a large extent dependent on the excellent husbandry practices of the animal health technician. It is interesting to note that there also were cages of *Helicobacter* spp.-positive animals in the room where the colony is housed.

The pathogenicity of *Helicobacter* spp. is varied, and most species of *Helicobacter* have only a low-grade potential to cause disease. However in animals with a compromised immune system, any bacteria (including commensal organisms) can become a threat. Similarly animals with a dysfunctional intestinal epithelial barrier may become susceptible to these nonpathogens, which might then be able to penetrate the epithelium and gain access to the internal milieu (23). Differences in experimental outcome may well be attributed to the differences in the endogenous intestinal microflora (11, 19).

Most commercial vendors of laboratory animals are working to provide *Helicobacter* spp.-negative mice. It is not always possible or feasible to purchase *Helicobacter*-free animals in order to “clean up”

a facility; for example, in closed colonies of inbred animals, the only ways to eliminate the bacteria are to rederive clean offspring or treat existing infections in animals. The treatment protocol used with the adult animals may become an invaluable tool in treating expensive transgenic or knockout animals infected with *Helicobacter*. With the success of treating infected adult animals with the new formulation of medicated feed in the second protocol, we conclude that the lengthy rederivation of an infected colony is not necessary for the W^s/W^s and FLS strains of inbred rats.

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