Comparative Medicine
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Vol 55, No 3
June 2005
Pages 265-268

Eradication of Enteric Helicobacters in Mongolian Gerbils is Complicated by the Occurrence of *Clostridium difficile* Enterotoxemia

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Outbred Mongolian gerbils from a United States commercial source were examined for colonization with naturally occurring enterohepatic *Helicobacter* spp. *Helicobacter* spp. were identified in the cecum and colon by culture and by using genus-specific primers in polymerase chain reaction (PCR) assays. Nutritionally balanced triple-antibiotic wafers (containing amoxicillin, metronidazole, and bismuth) used previously to eliminate helicobacter infections in mice were administered in an attempt to eradicate the naturally occurring novel helicobacters in the gerbils. After 7 days of antibiotic treatment, two of the experimental animals died due to *Clostridium difficile*-associated enterotoxemia. However, at 3 weeks after antibiotic cessation, the surviving three animals had no *Helicobacter* spp. in the cecum or colon according to PCR analysis. Eradication of *Helicobacter* spp. using dietary administration of antibiotics was complicated by the presence of toxin-producing *C. difficile*. An alternate method to develop helicobacter-free gerbils (such as Caesarian rederivation) may be necessary.

Gerbils, like other laboratory rodents, are colonized by *Helicobacter* spp. (8). The presence of these organisms may confound experimental studies of helicobacter infection and other types of research in these animals (16). One potential strategy for the eradication of enteric helicobacter species from gerbils is antibiotic treatment of breeding animals. Short-term administration of antibiotics has been used successfully to treat experimental infections with *H. pylori* in Mongolian gerbils and naturally occurring infections by other enterohepatic helicobacters in mice (5, 13). These protocols involve oral gavage and extended treatment periods, which are cumbersome and impractical for large groups of animals. Nutritionally balanced antibiotic wafers that can be used in place of the regular diet have been developed for eradication of helicobacter infections in mice (5). We used these wafers in an attempt to eradicate naturally occurring enteric helicobacter infections in Mongolian gerbils.

Complications can occur with the use of antibiotics. In particular, antibiotic administration can result in growth inhibition of the normal intestinal flora and overgrowth of toxin-producing *Clostridium difficile. C. difficile* may be present in the environment and can exist commensally in low numbers in the intestine. Overgrowth results in the rapid onset of clinically significant, potentially life-threatening enterotoxemia. Antibiotic-associated diarrhea has been reported to occur in humans and a variety of animals, most notably hamsters and guinea pigs (7, 9, 12). The purpose of the present report is to document how the presence of toxin-producing *C. difficile* in experimental gerbils precluded the use of dietary antibiotic administration as an effective method to eliminate enteric helicobacters from the lower bowel of gerbils.

Received: 12/21/04. Revision requested: 1/12/05. Accepted: 1/13/05.
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Materials and Methods

Animals and housing. Ten, 10-week-old outbred Mongolian gerbils [CRL(Mon)] were obtained from Charles River Laboratories (Wilmington, Mass.). All gerbils were housed in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, and under environmental conditions of 22°C, 40 to 70% humidity, 15 complete fresh air changes/h, and a 12:12-h light:dark cycle. Heat-treated hardwood was used for bedding (Sanichips, PJ Murphy Inc., Montville, N.J.). Water was provided ad libitum. Bedding in all cages was changed twice per week. Serologic assessment performed by the vendor indicated the gerbils were obtained from a colony that were antibody-free for lymphocytic choriomeningitis virus and *C. piliforme*. In addition, animals were culture-negative for *Salmonella* spp. *Bordetella bronchiseptica*, and *Pasteurella pneumotropica*. The gerbils were considered positive for *Helicobacter* spp. according to PCR assay. Three animals were euthanized on arrival for characterization of endogenous *Helicobacter* spp. infection status by PCR and culture. The remaining animals were housed two to three per cage in polycarbonate cages measuring 10.5 × 18.5 × 8 in. (ca. 26.7 × 342.2 × 20.3 cm). Five animals were fed nutritionally balanced wafers containing antibiotics as described below. Two control animals were fed a commercially available purified diet (Basal Diet, Purina TestDiet, Richmond, Ind.). This experiment was approved by the Animal Care Committee at Massachusetts Institute of Technology.

Antibiotic wafers. The antibiotic-treated animals were given commercially available, nutritionally balanced triple-antibiotic wafers containing 3 mg amoxicillin, 0.69 mg metronidazole, and 0.185 mg bismuth subsalicylate per tablet (Helicobacter MDs, BioServe; Frenchtown, N.J.). Each tablet weighed 3 g, and the animals were provided with 2 tablets per animal per day of the study. Dietary intake was estimated at 1 tablet per animal per day. The initial study protocol consisted of antibiotic administration to the experimental group for 2 weeks. The animals then

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were to be euthanized at 3 weeks post-treatment.

Necropsy. Animals were euthanized using CO_2 asphyxiation. Gerbils then were necropsied, and tissues were collected. Animals that died before the completion of the study were necropsied, and their tissues submitted for histopathology. Gross evaluation was performed on all tissues, and the gastrointestinal tract was harvested for histopathology and PCR.

Histopathology. The gastrointestinal tract was divided into stomach, small intestine, cecum, and colon and was fixed in neutral buffered 10% formalin. Tissues were processed, embedded in paraffin, and sectioned at 5 µm. Sections were stained with hematoxylin and eosin (H&E) and Brown and Brenn's Gram stain.

DNA isolation. Sections of the cecum and colon were harvested aseptically and frozen at –70°C until DNA isolation. DNA was isolated from the tissues by using a commercial kit (High Pure PCR Template Preparation kit, Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions.

PCR. PCR analysis was performed in a 100-µl reaction volume containing 1× buffer, 0.5 mM each of Helicobacter genus-specific primers CO5 and C97 (6), 200 µM each dNTP, 200 µg/ml bovine serum albumin, 2.5 U *Taq* polymerase (Roche Diagnostics Corporation, Indianapolis, Ind.), and 1 U of the polymerase enhancer Perfect Match (Statagene, La Jolla, Calif.); each reaction was covered with 75 µl mineral oil. The 1200-bp *Helicobacter* genus-specific PCR product was separated electrophoretically in a 1% agarose gel according to a previously published protocol (6). PCR products were visualized by ethidium bromide staining.

Microbiological culture for *Helicobacter* spp. Longitudinal sections approximately 1 cm in length of the cecum and colon were collected aseptically, and their contents were scraped into 1 ml aliquots of *Brucella* broth with 5% fetal calf serum and 15% glycerol. These aliquots were frozen at –70°C. For bacterial isolation, the aliquots were thawed and homogenized in 0.5 ml sterile phosphate buffered saline (PBS) in sterile glass tissue grinders. One-third of each homogenate was pipetted onto cefoperazone/vancomycin/amphotericin agar (CVA, Remel, Lenexa, Kans.), one-third was pipetted onto selective agar (1.5% agar, 5% blood, 100 µg/ml vancomycin, 3.3 µg/ml polymyxin B, 50 µg/ml amphotericin B, and 200 µg/ml bacitracin), and one-third was filtered through a 0.45-µm filter onto a blood agar plate (Remel). The plates were incubated at 37°C for 1 week under microaerobic conditions (80% N_2, 10% H, 10% CO_2, and trace O_2). Plates were examined for growth twice weekly for 3 weeks. The identity of the organisms was confirmed as *Helicobacter* spp. by colony morphology, Gram staining, phase-contrast microscopy, biochemical reactions, and PCR with genus-specific primers.

***Clostridium difficile* detection.** Samples were analyzed for the presence of toxin-producing *C. difficile* by using culture, cytotoxicity assay, and enzyme-linked immunosorbent assay (ELISA). For culture, cecal contents from animals suspected of *C. difficile* infection were plated on pre-reduced pre-reduced cycloserine/cefoxitin/fructose agar (CCFA, Remel), and trypticase soy agar medium (Remel) and incubated at 37°C in a Coy anaerobic chamber.

For cytotoxicity assay, 100 µl of 5×10^4 HeLa cells in Eagle's Minimum Essential Media (EMEM, Sigma Chemical Company, St. Louis, Mo.) with 5% fetal calf serum (Sigma Chemical Company) were grown to near-confluency in microtiter plates at 37°C in 5% CO_2. Supernatant from cecal homogenate was filter-sterilized and serially diluted 10-fold in EMEM with 5% fetal calf serum.

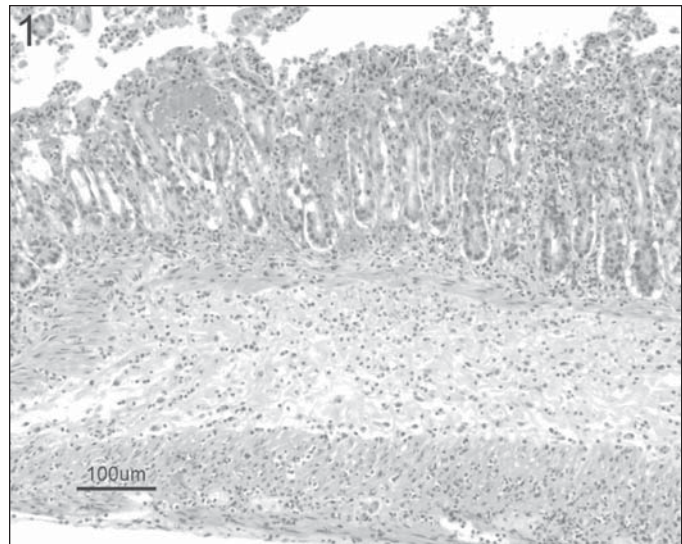

Figure 1. Necrohemorrhagic typhlocolitis in a 4-month old Mongolian gerbil treated with antibiotics for 7 days. Note the necrosis and sloughing of the superficial enterocytes, lamina propria hemorrhages, marked submucosal edema, and transmural mixed inflammatory cellular infiltrates. H&E stain; bar, 100 µm.

A 25-µl aliquot of each dilution was inoculated per well and incubated overnight at 37°C in 5% CO_2. The titer of cytotoxin was indicated by the highest dilution showing typical actinomorphic changes. The next lowest dilution was used in a neutralization assay. A 25-µl aliquot of this sample and 25 µl PBS was added to cells as a positive control. To a second well, 25 µl of sample and 25 µl of antiserum to *C. difficile* toxin B (TechLab, Blacksburg, Va.) was added. The plate was incubated overnight, and neutralization of actinomorphic changes was considered confirmation of the presence of *C. difficile* toxin B.

A commercial ELISA kit that detects the presence of *C. difficile* toxins A and B was used according to the manufacturer's instructions (*C. difficile* Toxin AB Test, Techlab). Briefly, cecal and colonic contents were diluted and applied to wells containing immobilized polyclonal goat anti-toxin A and anti-toxin B antibodies. A conjugate consisting of monoclonal mouse antibody against toxin A and polyclonal goat antibody against toxin B, both conjugated to horseradish peroxidase, then was added to the wells. Detection was by addition of the color substrate tetramethylbenzidine. Color absorbance was read at 450 nm on a microplate ELISA reader (Bio-Tek Instruments, Inc., Winooski, Vt.).

Results

Necropsy findings. After 7 days of antibiotic therapy, two of the treated animals were found dead. No premonitory signs had been noted, and body weights of the antibiotic-treated animals were comparable to those of the nontreated control animals. At necropsy, both gerbils had distended colons and watery ingesta in their ceca. One of the gerbils had reddened colonic mucosa with multifocal diphtheritic membranes. The remaining animals in the study were taken off the antibiotic wafers at 7 days post-treatment and placed on the control basal diet.

Histopathology. The histological findings of the colon and cecum were consistent with necrohemorrhagic typhlocolitis. There was segmental to diffuse sloughing and effacement of the superficial enteric epithelium. The underlying lamina propria

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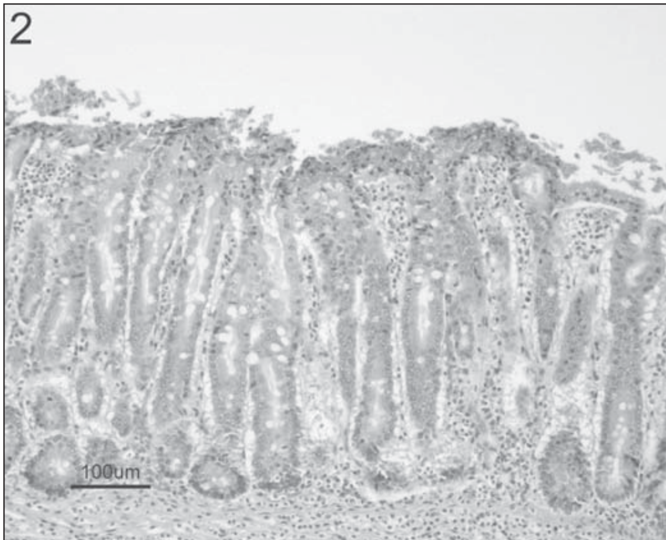

Figure 2. Photomicrograph of the colon of a 4-month old Mongolian gerbil treated with antibiotics for 7 days. Note superficial necrosis and denudation of the colonocytes and increased separation of the crypts of Lieberkuhn by edema and mixed inflammatory cellular infiltrates. There is also moderate glandular hyperplasia. H&E stain; bar, 100 µm.

contained hemorrhages admixed with fibrin, edema, infiltrates of degenerate and viable neutrophils, and intralesional bacillary gram-positive bacteria (Fig. 1 and 2). In many segments, the intestinal lumina were distended with proteinaceous exudate, sloughed necrotic enterocytes, and short bacillary bacteria. The submucosa and tunica muscularis were variably edematous with multifocal perivascular neutrophilic infiltrates and occasional macrophages and lymphocytes (Fig. 1 and 2). Both gerbils demonstrated mild to moderate neutrophilic peritonitis often as an extension of the transmural intestinal inflammation. There were no other significant lesions in the tissues examined.

Histopathology performed 3 weeks after antibiotic cessation revealed no differences between the surviving animals that had received antibiotic and the controls (data not shown).

Identification of C. difficile enterotoxins in animals with colitis. Anaerobic cultures for *C. difficile* were negative. *C. difficile* toxin B was demonstrated using tissue culture assay of cecal contents from one of the animals that had died. An ELISA performed on cecal contents detected *C. difficile* toxins A and B in both of the animals that had died and in none of the unaffected or control animals.

PCR amplification of sequences from naturally occurring enteric helicobacters before antibiotic therapy. Three animals were euthanized at the initiation of the study. These animals were shown to be positive for Helicobacter species by PCR.

PCR assays using *Helicobacter*-specific primers were done to determine the helicobacter infection status of the animals surviving the antibiotic study (Fig. 3). *Helicobacter* spp. were detected in the cecum and colon of untreated animals as well as in the two treated animals that died. The three surviving treated animals, however, were PCR-negative for *Helicobacter* spp. in the cecum and colon 3 weeks after cessation of antibiotic treatment.

Morphological and biochemical characteristics of the *Helicobacter* spp. A microaerobic bacteria was isolated from the cecal or colonic contents of only one of the three animals euthanized at the initiation of the study. In this animal, filtered cecal homogenate plated on blood agar grew a spreading colony of motile, gram-negative, fusiform to slightly spiral bacteria, which on phase-contrast microscopy were morphologically similar to *H. bilis*. Biochemically, the microaerophilic bacteria from the gerbil were spiral to fusiform in morphology and were oxidase-, catalase-, and strongly urease-positive. There was no growth from any of the cecal samples on CVA or the other selective agar containing antibiotics.

Discussion

There are currently 26 formally named species in the genus *Helicobacter*. Numerous clinical isolates identified by our group and others represent partially characterized novel species isolated from a variety of hosts (16). Identification of naturally occurring enteric *Helicobacter* spp. colonizing the outbred gerbil cecum and colon was not surprising. *H. hepaticus* has been cultured in three out of four inbred MGS/Sea gerbil colonies examined for helicobacter infection status in Japan (8). In our laboratory, we previously identified a novel helicobacter with close taxonomic relatedness to *H. bilis* (2) in the cecum and colon of outbred gerbils from the same supplier as the animals in this study. In subsequent shipments of gerbils from the same commercial vendor, we have isolated and identified the presence of this same *Helicobacter* sp., indicating that the organism is endemic within this particular gerbil colony. These findings confirm the health surveillance profile regarding the presence of *Helicobacter* spp. provided by the vendor. In addition, we isolated *H. rodentium* in the cecum and colon of inbred MGS/Sea gerbils purchased from Japan (3). Therefore, it appears that naturally occurring intestinal helicobacter infections are common in gerbils, and their helicobacter status by using both PCR- and culture-based assays should be ascertained when using these animals for helicobacter infection studies or other studies that may be compromised by the presence of these enterohepatic organisms (16).

The attempt to use antimicrobials to eradicate intestinal *Helicobacter* spp. in the present study was complicated by the occurrence of *C. difficile*-induced enterocolitis in two of the experimental animals. *C. difficile* is a common cause of antibiotic-associated diarrhea in both humans and animals (7). This organism produces two toxins. Toxin A is an enterotoxin that is the primary cause of disease (15). Toxin B produces cytopathic effects in cell lines, a property that is used in diagnosis and that may be synergistically involved in promoting the effects of toxin A (7).

Use of the cell-culture cytotoxin assay to detect toxin B of *C. difficile* has been considered the diagnostic "gold standard." Although it is very sensitive, this assay is associated with some potential limitations (1). Sensitivity of cell lines, dilution of fecal specimens, and interfering substances in the feces may present unwanted variables in interpretation of results (10). The discrepancy between the negative cytotoxin assay and the positive toxin A/B ELISA may have been caused by several factors. The samples for ELISA were processed shortly after collection. The samples then were stored frozen and later thawed and processed. It is possible that biological activity was lost on storage, freezing, and thawing. However, using both assays, we were able to verify the presence of *C. difficile* toxin.

In our study, amoxicillin in the commercial wafers was the likely cause of the *C. difficile*-associated enterocolitis, because beta-lactam antibiotics previously were associated with the disease (7). Although amoxicillin has been administered to gerbils

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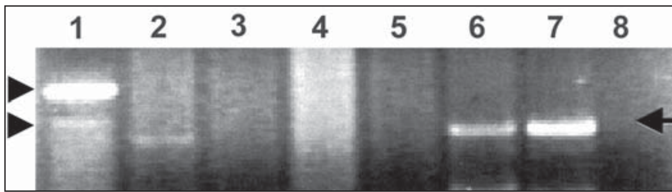

Figure 3. PCR detection of helicobacter infection after antibiotic treatment. A 1200-bp PCR product (arrow) indicative of helicobacter infection (lanes 2, 6, and 7) was detected in DNA prepared from the colonic contents of untreated animals and those that died of *C. difficile* enterocolitis. No helicobacter was detected similar samples from treated animals unaffected by enterocolitis. Lane 1, 100-bp DNA ladder (Invitrogen, Carlsbad, Calif.). The top arrow indicates the 2072-bp marker; the second arrow indicates the 1500-bp marker; lane 2, untreated animal; lanes 3 through 5, animals treated for 7 days and unaffected by colitis; lanes 6 and 7, animals treated for 7 days and that died of colitis; lane 8, negative control.

without ill effects (12), the use of antibiotic wafers and the duration of treatment may have provided the opportunity for growth of *C. difficile*, toxin production, and subsequent development of disease. Ad libitum feeding of antibiotic wafers results in continuous exposure of the gastrointestinal tract microbial flora to antibiotics and offers less control over the total dose given to individual gerbils. It is possible that this form of oral antibiotic therapy is more detrimental than is intermittent exposure when antibiotics are delivered by gavage.

Successful eradication of experimental infection with *H. pylori* has been reported in gerbils dosed with amoxicillin by oral gavage or mucoadhesive microspheres (13). However in these studies, amoxicillin was only given for 3 days, and the animals were examined for *H. pylori* infection 1 day after antibiotic treatment ended. The results thus obtained may indicate that bacterial numbers decreased below the limit of detection, but prolonging the post-treatment interval would be necessary to determine if true eradication had been achieved. In standard mouse protocols, treatment periods of 2 weeks or more usually are necessary to eradicate naturally occurring enteric *Helicobacter* spp. (4, 5). In our study, a novel *Helicobacter* sp. was undetectable in the surviving animals 3 weeks after the 7-day treatment period had ended. This finding is supportive of the efficacy of shorter treatment protocols, but it remains to be seen whether a further abbreviated treatment duration would be as effective. Whereas a shorter treatment duration may decrease the risk of *C. difficile* enterotoxemia, it may not be sufficient to eradicate naturally occurring helicobacter infection. Another potential approach to antibiotic eradication would involve increasing the dose of metronidazole available in the wafers. Metronidazole usually is effective against *C. difficile* overgrowth, and an increased dose may prevent the occurrence of enterotoxemia.

To our knowledge, ours is the first report of *C. difficile*-associated toxigenic typhlocolitis in gerbils. The presence of *C. difficile* in these animals complicated our attempt to eradicate helicobacters in gerbils by using antibiotic wafers. However, several other methods also might be considered. *C. difficile*-free gerbils could be identified by PCR for use in an antibiotic-treatment regimen. Alternately, the pathogenic effects of existing *C. difficile* infections could be blocked through competitive inhibition by nonpathogenic bacterial strains. In hamsters, pretreatment of *C. difficile*-colonized animals with nontoxigenic clindamycin-resistant *C. difficile* strains protects the hamsters from developing antibiotic-associated colitis during subsequent clindamycin administration (11). Another strategy, which would not involve antibiotics, is Caesarian rederivation and crossfostering onto mice. *C. piliforme*, the causative agent of Tyzzer's disease, has been eliminated from gerbil colonies by using this method (14). Although labor-intensive initially, Caesarian rederivation may provide the most reliable permanent solution for elimination of naturally occurring helicobacter infection because this method avoids the risks of treatment failure and antibiotic-induced disease.

Acknowledgments

This work was supported by grants RR 07036, R01CA67529, R01 AI37750 and NIEHS P30ES02109 (to JGF) from the National Institutes of Health.

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