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Evaluation of Antibiotic Therapies for Eradication of *Helicobacter hepaticus*

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The newly recognized murine pathogen *Helicobacter hepaticus* is known to colonize the ceca and colons of several strains of mice from a variety of commercial suppliers. Additionally, the organism persistently infects mice, causes a chronic hepatitis, and is linked to hepatic tumors in the A/JCr inbred mouse strain. For this reason, eradication of the organism from infected mouse colonies is desirable. Treatment modalities for eradication of *H. hepaticus* from the gastrointestinal system consisted of oral administration of various antibiotic combinations previously evaluated for eradication of experimental *H. felis* gastric infection in mice. A/JCr mice (8 to 10 weeks old) naturally infected with *H. hepaticus* were divided into six treatment groups of 10 animals each. Animals received monotherapy of amoxicillin, metronidazole, or tetracycline or triple therapy of amoxicillin-metronidazole-bismuth (AMB) or tetracycline-metronidazole-bismuth (TMB). All medications were administered by oral gavage three times daily for 2 weeks. One month after the final treatment, mice were euthanatized and livers, ceca, and colons were cultured for *H. hepaticus*. All untreated control animals had *H. hepaticus* isolated from the cecum and/or colon. *H. hepaticus* was not recovered from the livers, ceca, or colons of the AMB or TMB treatment groups. All animals receiving the various antibiotic monotherapies had *H. hepaticus* isolated from the cecum and colon. We conclude that at the doses and the route evaluated, AMB and TMB triple therapies are effective for eradication of *H. hepaticus* in 8- to 10-week old A/JCr mice.

Bacteria belonging to the genus *Helicobacter* are isolated from humans and a variety of mammals (5, 13). The type species, *Helicobacter pylori*, causes gastritis and peptic ulcer disease in humans and has been linked to the development of gastric adenocarcinoma as well as mucosa-associated gastric lymphoma (7, 13, 14, 16, 19). Additional *Helicobacter* species colonize the lower intestinal tracts of mammals and birds. Most recently, *H. hepaticus* was isolated from the livers of mice with active chronic hepatitis, and in one inbred strain of A/JCr mice, the organism is associated with the development of hepatic tumors (4, 18). Furthermore, this helicobacter, like the other known murine helicobacters, "*H. rappini*," *H. muridarum*, and *H. bilis*, is an efficient colonizer of the cecum and colon (6, 10, 17).

Experimentally, *H. felis*, which naturally colonizes dogs and cats, causes a persistent gastritis when inoculated into mice (3, 9). This model has been used to study helicobacter-related gastric disease and has proven to be a practical model for the in vivo screening of potential anti-*H. pylori* agents (2). In this mouse model, proven antimicrobial agents effective in eradicating *H. pylori* in humans are equally efficacious in eradicating *H. felis*. The purpose of this study was to ascertain whether a similar treatment strategy would successfully eradicate *H. hepaticus*, a new murine pathogen from naturally infected A/JCr mice.

MATERIALS AND METHODS

Animals. Male A/JCr mice (8 to 10 weeks old) naturally infected with *H. hepaticus* were obtained from the National Cancer Institute, Frederick, Md. Animals were housed in solid-bottom, polycarbonate microisolator cages within a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Standard commercially available rodent chow and water were provided ad libitum. The lighting cycle consisted of a 12-h light-dark cycle. Corncob cage bedding was changed twice weekly. Animals were housed five per cage. Verification of *H. hepaticus* infection was done by culture of pooled fresh fecal samples collected from all animals for each study group. Additionally, one representative animal was euthanatized and culture of liver, cecum, and colon was performed.

Treatment. Animals were randomly divided into six groups of 10 animals each. Daily treatments for each group were divided and given by oral gavage of 0.1 ml of drug suspended in physiologic saline three times daily for 2 weeks (Table 1). Doses were as follows: amoxicillin (AmoxiDrops; Smith Kline and Beecham), 1.5 mg/30-g mouse per day; tetracycline (Polyotic; American Cyanamid) 1.5 mg/30-g mouse per day; metronidazole (Schein Pharmaceutical), 0.69 mg/30-g mouse per day; and bismuth (De-Nol; Parke-Davis), 0.185 mg/30-g mouse per day. Group 1 received triple therapy of amoxicillin-metronizadole-bismuth (AMB). Group 2 received triple therapy of tetracycline-metronizadole-bismuth (TMB). Groups 3 through 5 received monotherapy of amoxicillin (1.5 mg/30-g mouse per day), tetracycline (0.15 mg/30-g mouse per day), and metronidazole (0.69 mg/30-g mouse per day), respectively. Group 6 consisted of untreated control animals (Table 1).

Two weeks following the final treatment, pooled fresh fecal samples were collected from all animals in each study group. Pooled fecal samples from each study group were cultured for the presence of *H. hepaticus*. All animals were euthanatized by CO_2 overdose 1 month following the final antibiotic dosing. Livers, ceca, and colons were aseptically collected and placed in sterile phosphate-buffered saline (PBS) for culture or frozen at −70°C for PCR.

Isolation of *H. hepaticus*. *H. hepaticus* organisms were isolated from pooled fecal samples by filtering a fecal slurry through a 0.45-μm-pore-size filter followed by streaking of the filtered solution onto TVP (trimethoprim sulfa, vancomycin, polymixin) blood agar microaerophilic plates (Remel Labs, Lenexa, Kans.) and incubating at 37°C under microaerophilic conditions (GasPak system; BBL Microbiology Systems, Cockeysville, Md.). Culture of liver, cecum, and colon tissue 1 month following the final treatment was performed by streaking filtered tissue homogenate onto TVP on blood agar plates and incubating as described above. *H. hepaticus* organism was characterized by size (0.45-μm-pore-size filtration), typical colony morphology, Gram stain reaction, and urease, catalase, and oxidase activity as previously described (4). Cultures were held for 3 weeks to verify negative status.

PCR evaluation of ceca was performed to confirm infection with *H. hepaticus*. Ceca from all animals in group 1, AMB triple-therapy animals, and 3 of 10 untreated control animals from group 6 were evaluated.

DNA extraction. DNA was isolated from frozen mouse tissue of infected and noninfected animals. The samples were washed twice in PBS and centrifuged at 13,000 × *g* for 10 min, at which time they were resuspended in STET buffer (8.0% sucrose, 50 mM Tris HCl [pH 8.0], 50 mM EDTA, and 0.1% Triton X-100). Lysozyme (from chicken egg white; Boehringer Mannheim, Indianapolis, Ind.) was added to a final concentration of 3 mg/ml, and the solution was incubated for 12 min at 37°C. Sodium dodecyl sulfate (SDS) and RNase (bovine

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TABLE 1. Dosages of chemotherapeutic agents

Treatment group and chemotherapeutic agent(s)	Total daily dose (mg)[a]
1	
Amoxicillin	1.5
Metronidazole	0.69
Bismuth	0.185
2	
Tetracycline	1.5
Metronidazole	0.69
Bismuth	0.185
3 (amoxicillin)	1.5
4 (tetracycline)	0.15
5 (metronidazole)	0.69
6 (no treatment)	

[a] Per 30-g mouse per day. All treatments were divided and given three times daily.

pancreas; Boehringer Mannheim) were added to final concentrations of 1.0% and 50 μg/ml, respectively, and the mixture was incubated for 1 h at 37°C. Pronase and proteinase K (Boehringer Mannheim) were added to final concentrations of 0.8 and 0.5 mg/ml, respectively. Samples were incubated overnight at 37°C. The DNA was then extracted in an equal volume of phenol:chloroform (1:1) and precipitated overnight at 20°C in the presence of 0.3 M sodium acetate and 2.1 volumes of absolute ethanol. The DNA was precipitated by centrifugation at 16,000 × *g* for 30 min and allowed to air dry. The pellet was then redissolved in sterile distilled water.

PCR amplification. Primer sequences chosen for amplification recognized an *H. hepaticus*-specific region of the 16S rRNA gene (rDNA). These two oligonucleotides, 5′ GCA TTT GAA ACT GTT ACT CTG 3′ (positions 633 to 653 in the *E. coli* numbering scheme) and 5′ CTG TTT TCA AGC TCC CC 3′ (positions 1032 to 1047 in the *E. coli* numbering scheme), produced an amplified product of 416 bp. Between 0.1 and 5 μg of each DNA preparation was added to a 100-μl (final volume) reaction mixture containing 1× *Taq* polymerase buffer (2.25 mM $MgCl_2$, 50 mM KCl, Tris [pH 8.42]), each primer at 0.5 μM, deoxynucleotide at 250 μM, and 2.0 mg of bovine serum albumin per ml. Samples were heated to 95°C for 5 min to denature the DNA, briefly centrifuged, and cooled to 61.0°C, at which time 2.5 U of *Taq* polymerase enhancer (Perfect Match; Stratagene, La Jolla, California) was added, followed by an overlay of 100 μl of mineral oil. The following conditions were used for amplification; denaturation at 95°C for 1 min, annealing at 61°C for 1.30 min, and elongation at 72°C for 1.30 min. A total of 33 cycles were performed, followed by an elongation step of 7 min at 72°C. Purity of the sample was determined by electrophoresis on a 3% Visigel separation matrix (Stratagene), followed by staining with ethidium bromide and viewing by UV illumination.

Southern blots. Aliquots were taken from both the positive and negative PCR reaction samples. These were electrophoresed on a 3.5% SeaKem genetic technology grade agarose gel (FMC, Rockland, Maine). The DNA on the resultant gel was then denatured in 0.5 M NaOH–1.5 M NaCl, neutralized in 1.5 M NaCl–1.0 M Tris-HCl (pH 8.0), transferred onto a 0.45-μm-pore-size positively charged nylon membrane (Hybond-N; Amersham, Arlington Heights, Ill.) by capillary action, and baked at 80°C for 2 h as described elsewhere (11). PCR product, to be used as a probe, was isolated from the *H. hepaticus* type strain directly labeled with the enzyme horseradish peroxidase and hybridized at 42°C to the blot by using the ECL direct nucleic acid labeling detection system as outlined by the manufacturer (Amersham) and exposure onto Hyperfilm (Amersham).

RESULTS

One animal each from groups 1 and 5 was euthanatized because of unrelated causes, leaving nine animals each in these two groups.

***H. hepaticus* isolation. (i) Predosing.** Culture of pooled fecal samples from groups 1 through 6 confirmed infection of animals with *H. hepaticus*. The single representative mouse euthanantized had *H. hepaticus* isolated from the liver, cecum, and colon.

(ii) Two weeks after antibiotic therapy. Culture of pooled fecal samples for *H. hepaticus* from each group at two weeks posttreatment indicated that *H. hepaticus* persisted in group 3 whereas in groups 2, 4, and 5 culture results were negative for *H. hepaticus* isolation. Evaluation was not possible in group 1 because of enteric microbial contamination of the cultured fecal specimen (Table 2).

TABLE 2. Eradication of *H. hepaticus* following therapy

Treatment group	*H. hepaticus* culture results		% Eradication
	2 weeks posttreatment[a]	4 weeks posttreatment[b]	
1	NR[c]	0/9	100
2	Negative	0/10	100
3	Positive	10/10	0
4	Negative	10/10	0
5	Negative	9/9	0
6	Positive	9/10	NA[d]

[a] Based on pooled fecal culture.
[b] No. of positive cultures/total no. of cultures. Based on liver, cecal, or colon culture.
[c] NR, not recorded because of bacterial contamination.
[d] NA, not applicable.

(iii) One month after antibiotic treatment. Tissue samples from liver, cecum, and colon of each mouse analyzed from group 1 (AMB triple therapy) and group 2 (TMB triple therapy) were negative by culture for *H. hepaticus*. Colons of 10 animals, 9 of 10 ceca, and 1 of 10 livers in group 3 (amoxicillin monotherapy) were *H. hepaticus* positive by culture. Ceca of 10 animals and colons of 6 animals of group 4 (tetracycline monotherapy) and 9 of 9 colons and 8 of 9 ceca in treatment group 5 (metronidazole monotherapy) were positive by culture for *H. hepaticus. H. hepaticus* was isolated from 9 of 10 ceca, 8 of 10 colons, and 2 of 10 livers from the control mice (Table 2).

PCR evaluation. PCR analysis of DNA isolated from the cecal tissue of all nine animals from group 1 (AMB triple therapy) (Fig. 1) and six animals evaluated from group 2 (TMB) (data not shown) did not amplify a product with the *H. hepaticus*-specific 16S rDNA primers. However, a product was obtained with the cecal DNA isolates of three group 6 (untreated control) mice (Fig. 1).

Southern blots. In addition, as seen from the more sensitive and specific Southern blot shown in Fig. 2, no reactions were obtained with DNA isolated from the ceca of the nine mice given AMB triple therapy. Hybridizations were obtained with the DNA isolated from the three nontreated mice, and the *H. hepaticus* probe hybridized to itself as well.

DISCUSSION

It is now known that *H. hepaticus* is prevalent in commercially available mouse colonies and is associated with chronic

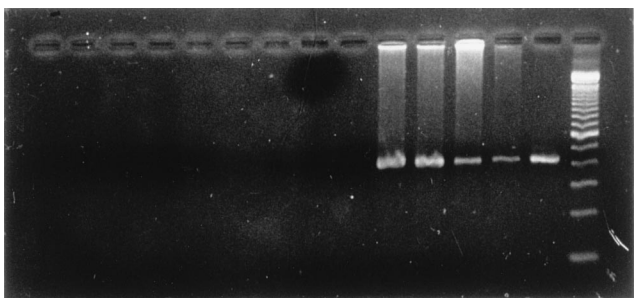

FIG. 1. PCR detection of *H. hepaticus* in mouse cecal tissue. Agarose gel electrophoresis of amplified PCR products obtained from cecal DNA extracted from selected A/JCr mice. Lanes: 1 to 9, cecal tissue from AMB triple therapy-treated mice; 10 to 12, cecal tissue from infected but untreated mice; 13, DNA isolated from *H. hepaticus* type strain (3Bl); 14, DNA isolated from another *H. hepaticus* strain; lane 15, 100-bp molecular size markers (BRL, Gaithersburg, Md.).

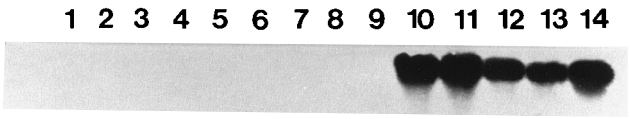

FIG. 2. Corresponding Southern hybridization of gel shown in Fig. 1. The PCR product obtained with the *H. hepaticus* type strain (3Bl) was used as a probe. Lanes: 1 to 9, cecal tissue from AMB triple therapy-treated mice; 10 to 12, cecal tissue from infected but untreated mice; 13, DNA isolated from *H. hepaticus* type strain 3Bl; 14, DNA isolated from another strain of *H. hepaticus*.

hepatitis in several strains of mice (4, 18). Also of note in ascertaining its presence in mouse colonies is the organism's ability to efficiently colonize the lower gastrointestinal tract of mouse strains susceptible to hepatic infection as well as mouse strains resistant to hepatic disease. It therefore becomes important in developing *H. hepaticus* eradication modalities to ascertain absence of the organism not only in the target organ, the liver, but also in the intestine, the natural niche of the organisms. A/JCr mice naturally infected with *H. hepaticus* were used to evaluate eradication regimens. Previous studies indicated nearly 100% colonization of cecum and colon with the organism and development of *H. hepaticus*-associated hepatitis (4). No other murine helicobacters have been detected in mice from this source; however, the use of selective filtration (0.45-μm-pore-size filter) and plating inocula onto agar containing antibiotics greatly facilitates identification of *H. hepaticus* from other murine helicobacters as well as minimizing overgrowth by other intestinal bacteria. Gram staining helps to distinguish the smaller *H. hepaticus* from the larger *H. bilis*, *H. rappini*, and *H. muridarum*. The latter are 0.35 to 0.6 μm in diameter and have periplasmic fibers. *H. hepaticus* is 0.2 to 0.3 μm in diameter and has no periplasmic fibers.

The triple antimicrobial treatment regimens, AMB and TMB which have been effective in eradicating *H. pylori* in humans, *H. mustelae* in ferrets, and *H. felis* in mice also proved to be 100% effective in eradicating *H. hepaticus* in naturally infected A/JCr mice (1, 2, 12, 15). Failure to isolate *H. hepaticus* at 2 weeks posttreatment in mice receiving only tetracycline or metronidazole monotherapy reflects suppression of *H. hepaticus* but not eradication. Similar results are commonly encountered in unsuccessful antimicrobial strategies for *H. pylori* eradication. Also, when the tetracycline in the TMB protocol was reduced 10-fold (i.e., 0.15 mg) and administered to another group of mice, *H. hepaticus* was recovered from all TMB-treated mice (unpublished data), thus emphasizing the importance of proper concentrations of antibiotics. Our definition of eradication is based on clinical data used to describe elimination of *H. pylori* which stipulate a negative gastric culture for *H. pylori* four weeks after completion of the antibiotic treatment (1, 12). The inability to isolate *H. hepaticus* from ceca, colons, and livers of all the mice 4 weeks after administration of the triple therapy containing amoxicillin or tetracycline and the lack of *H. hepaticus*-specific PCR product support the definition of 100% eradication. High concentration of *H. hepaticus* in the colonic and cecal crypts may make eradication more difficult than eliminating *H. felis*, which colonizes the superficial gastric mucus and, to a lesser extent, gastric glands (2, 8). Complete eradication of *H. hepaticus* from each mouse is necessary if the goal is elimination of the murine pathogen from a given mouse colony. Persistence of *H. hepaticus*, even in a small number of mice, would be unacceptable because of the apparent ease of fecal-oral transmission and efficient colonization of *H. hepaticus* in the lower gastrointestinal tract.

We recognize that multiple daily doses of three antimicrobial agents administered by gavage are not practical for treatment of large numbers of *H. hepaticus*-infected mice. Now that we have ascertained that *H. hepaticus* can be eradicated from inbred mice by extensive antimicrobial dosing regimens, our laboratory will test efficacy of selected antibiotics in drinking water or feed in an attempt to develop eradication strategies to eliminate *H. hepaticus* from large mouse colonies.

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