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Helicobacter marmotae sp. nov. Isolated from Livers of Woodchucks and Intestines of Cats

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Woodchucks (*Marmota monax*) have a high incidence of hepatocellular carcinoma (HCC) associated with chronic infection with woodchuck hepatitis virus (WHV) and serve as a model of hepatitis B virus-associated HCC in humans. *Helicobacter hepaticus*, an enterohepatic helicobacter in mice, is known to cause hepatocellular adenomas and carcinomas in susceptible mouse strains. In long-term chemical bioassays conducted with B6C3F$_1$ mice, *H. hepaticus* has been regarded as a confounding factor because of its tumor-promoting activity. In order to determine if woodchucks harbor a *Helicobacter* sp. that might play a role in potentiating hepatic inflammation or neoplasia, a study was undertaken to determine whether woodchucks' livers were infected with a *Helicobacter* sp. Frozen liver samples from 20 (17 WHV-infected and 3 noninfected) woodchucks, 10 with WHV-associated hepatic tumors and 10 without tumors, were cultured by microaerobic techniques and analyzed by using genus- and species-specific helicobacter PCR primers. A 1,200-bp *Helicobacter* sp.-specific sequence was amplified from 14 liver samples. Southern hybridization confirmed the specific identity of the PCR products. Nine of the 10 livers with tumors had positive *Helicobacter* sp. identified by PCR, whereas 5 of the 10 livers without tumors were positive. By use of 16S rRNA species-specific primers for *H. marmotae*, two additional liver samples from the nontumor group had positive PCR amplicons confirmed by Southern hybridization. A urease-, catalase-, and oxidase-positive bacterium was isolated from one liver sample from a liver tumor-positive woodchuck. By 16S rRNA analysis and biochemical and phenotypic characteristics, the organism was classified as a novel *Helicobacter* sp. Subsequently, four additional bacterial strains isolated from feces of cats and characterized by biochemical, phenotypic, and 16S rRNA analysis were determined to be identical to the woodchuck isolate. We propose the name *Helicobacter marmotae* sp. nov. for these organisms. Further studies are required to ascertain if this novel *Helicobacter* sp. plays a tumor promotion role in hepadnavirus-associated tumors in woodchucks or causes enterohepatic disease in cats.

The genus *Helicobacter* is a rapidly expanding group of gram-negative, microaerophilic, spiral organisms that persistently colonize a variety of mammalian hosts and in some cases are responsible for significant clinical diseases (6). The type species, *H. pylori*, causes a persistent gastritis in the human stomach and is directly linked to peptic ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma (15). Although gastric helicobacters have been the focus of experimental studies because of the importance of *H. pylori*-associated gastric disease in humans, the discovery of enterohepatic helicobacters in animals and humans has sparked an interest in exploring the pathogenic potential of these organisms as well.

Several years ago, two novel helicobacters, *H. hepaticus* and *H. bilis*, were isolated from livers of mice with hepatitis (8, 17). *H. hepaticus* infection is also associated with hepatic neoplasms in A/JCr and B6C3F$_1$ mice, and this bacterium causes hepatitis and typhlitis in A/JCr inbred mice and AXB recombinant inbred mice (22, 38). Indeed, *H. hepaticus* infections in B6C3F$_1$ mice used for carcinogenesis bioassays are considered a confounding factor in mouse liver tumorigenesis studies because

of the bacteria's tumor-promoting ability (9, 14, 21). Other *Helicobacter* species, originally called "*Flexispira rappini*," have also been isolated from the inflamed livers of aborted sheep fetuses, as well as livers of guinea pigs experimentally infected with "*F. rappini*" (2, 24). Additionally, novel helicobacters have been associated with enterohepatic disease in domesticated species. *H. pullorum* has been cultured from diseased chicken livers and from the feces of diarrheic humans (33). *H. canis* has been isolated from feces of diarrheic dogs, cats, and humans and the liver of a puppy with hepatitis (5, 34). *H. cinaedi* isolated from bacteremic humans has recently been isolated from the liver of a rhesus monkey with hepatitis and idiopathic colitis (10, 36).

In 1978, woodchuck hepatitis virus (WHV) was discovered in woodchucks, a large rodent native to North America. Chronic infection with WHV causes hepatitis and is associated with hepatocellular carcinoma (HCC) (35). This virus has been found in wild woodchuck populations in several eastern states of the United States. More than 90% of both wild-caught WHV-infected and colony-born experimentally infected woodchucks die of HCC within 2 to 3 years. These animals have chronic hepatitis with significant components of inflammation and regeneration, but cirrhosis does not occur (26). HCC also developed in up to 20% of woodchucks that had recovered from WHV infections (surface-antigen-negative, anti-HBc/anti-HBs-positive animals) but did not develop in any uninfected

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animals. These data support the view that WHV infection is necessary for HCC but may not be sufficient by itself to induce tumors. The possibility of cofactors that could contribute to tumor formation has been examined. Dietary aflatoxin B$_1$ exposure has a synergistic effect with chronic WHV infection on hepatic tumorigenesis (1, 20). The presence of hepatic inflammation and biliary proliferation (both lesions are also noted in *H. hepaticus*-infected mouse livers) has been previously reported for WHV-seronegative woodchucks and suggested to be a response to chronic infection (27, 28). Since *Helicobacter* species can establish chronic infection and serve as a cocarcinogen in other rodents, and woodchucks without WHV infection have a background incidence of hepatitis, it is conceivable that a helicobacter might contribute to woodchuck HCC. However, a bacterial infection has not been previously entertained as a possible cofactor (26).

This study describes the isolation and characterization by morphology, biochemistry, and 16S RNA analysis of a novel *Helicobacter* sp., *H. marmotae*, isolated from the liver of a woodchuck infected with WHV as well as the feces of commercially reared cats. This finding raises a testable hypothesis that novel *Helicobacter* spp. may be responsible for hepatitis in other hosts and that the presence of a persistent bacterial infection may be a cofactor in virally induced hepatic neoplasms.

MATERIALS AND METHODS

Animals. Liver specimens from 20 wild-caught adult woodchucks maintained in captivity were evaluated. Their livers were collected aseptically at necropsy and stored at −70°C, until submitted for culture and PCR analysis.

Four cats (three received in one shipment in 1998 and one cat in a shipment received in 1999 from a specific-pathogen-free animal commercial source certified to have cats free of feline leukemia virus, feline immunodeficiency virus, and feline coronavirus) were part of a previous survey of enteric *Helicobacter* spp. and *Campylobacter* spp. (31). At the time of fecal culture, all cats were clinically healthy.

Slot blot analysis of serum WHV DNA. For analysis of WHV DNA, whole blood was collected and stored at −70°C until analysis. WHV DNA was extracted from 200 μl of serum and eluted into 200 μl of Tris buffer (10 mM, pH 8.5) with a QIAMP blood kit (Qiagen, Valencia, Calif.). The probe was an *Eco*RI-digested WHV2 genome (3.32 kb) labeled with digoxigenin-II-dUTP (DIG High Prime DNA labeling detection starter kit II; Boehringer Mannheim, Indianapolis, Ind.). The WHV DNA standard used as a control was whole WHV2 genomic DNA linearized with *Eco*RI. Ten microliters of the extracted DNA solution was denatured with 30 μl of denaturing buffer at 65°C for 40 min and was loaded on each slot. Denatured DNA was fixed to a nylon filter (Nytran; Schleicher & Schuell, Keene, N.H.) by a UV cross-linking method. The filter and probe were incubated together, and after washing, the membranes were immediately scanned with the Lumi-Imager (Boehringer Mannheim) to generate a numerical value for the strength of the hybridization signal. Extracted negative and positive sera and several dilutions of standard DNA (0.01, 0.03, 0.06, and 0.1 ng of DNA) were included.

Bacterial isolation and biochemical characterization. Samples from each liver were homogenized in 1.0 ml of phosphate-buffered saline. Each slurry was gently passed through a 0.45-μm-pore-size syringe filter, and the homogenate was streaked onto a Trypticase soy agar (with 5% sheep blood) plate (Remel Laboratories, Lenexa, Kans.) containing TVP (trimethoprim, vancomycin, and polymyxin) or CVA (cefoperzone, vancomycin, and amphotericin B) (Remel). In addition, a selective antibiotic medium was prepared as follows: blood agar base (Oxoid; Remel), 5% horse blood (Remel), 50 μg of amphotericin B/ml, 100 μg of vancomycin/ml, 3.3 μg of polymyxin B/ml, 200 μg of bacitracin/ml, and 10.7 μg of nalidixic acid/ml (all from Sigma Chemical Co., St. Louis, Mo.). Feces from the cats were directly streaked onto CVA medium. The cultures were then incubated at 37°C under microaerobic conditions in vented jars containing N$_2$, H$_2$, and CO$_2$ (90:5:5). A detailed biochemical characterization analysis was performed on five isolates as previously described by Fox et al. (17).

Electron microscopy. The isolate was examined by electron microscopy. Cells grown on blood agar for 48 h were gently suspended in 10 mM Tris-HCl buffer (pH 7.4) at a concentration of about 10^8 cells per ml. Samples were negatively stained with 1% (wt/vol) phosphotungstic acid (pH 6.5) for 20 to 30 s. Specimens were examined with a JEOL model JEM-1200EX transmission electron microscope operating at 100 kV.

Genomic DNA extraction for 16S rRNA sequencing. Bacteria isolated from the liver of one woodchuck were cultured on blood agar plates, and cells were harvested and washed once with 1 ml of phosphate-buffered saline. The High Pure PCR template preparation kit was used for DNA extraction (Roche Molecular Biochemicals, Indianapolis, Ind.).

16S rRNA gene sequencing. The sequences of the 16S rRNA gene of three isolates of *H. marmotae* MIT 98-6070, MIT 98-1705-1, and MIT 99-4759 were determined. For amplification of 16S rRNA cistrons, 16S rRNA gene sequencing, and 16S rRNA data analysis we used the methods described by Fox et al. (7). Briefly, primers C70 and B37 (7) were used to amplify the 16S rRNA genes. The amplicons were purified and directly sequenced by using a TAQuence cycle sequencing kit (U.S. Biochemicals, Cleveland, Ohio). The 16S rRNA gene sequences were entered into RNA, a program for analysis of 16S rRNA data in Microsoft Quickbasic for use with IBM PC-compatible computers, and were aligned as described previously (25). The database used contains approximately 100 *Helicobacter*, *Wolinella*, *Arcobacter*, and *Campylobacter* sequences and more than 900 sequences for other bacteria. Similarity matrices were constructed from the aligned sequences by using only those base positions for which 90% of the strains had data and were corrected for multiple base changes by the method of Jukes and Cantor (23). Phylogenetic trees were constructed by the neighbor-joining method (29).

DNA extraction for PCR analysis. DNA was extracted from the liver samples and the cultured organisms with the High Pure PCR template preparation kit (Roche Molecular Biochemicals) according to the manufacturer's directions. Briefly, the samples were lysed and incubated with 40 μl of proteinase K for 1 h at 55°C. Two hundred microliters of binding buffer was added to each sample and allowed to incubate for 10 min at 72°C before 100 μl of isopropanol was added. The samples were placed in a filter tube and centrifuged at 6,000 × *g* for 1 min. The flowthrough was discarded, and 500 μl of wash buffer was added to the samples and centrifuged as before. This washing step was repeated twice. Prewarmed (70°C) elution buffer (200 μl) was used to elute DNA from the filter.

PCR amplification of bacterial DNA. One set of primer sequences chosen for PCR amplification recognize a region of the 16S rRNA gene specific for members of the *Helicobacter* genus. This set of primers produces an amplified product of 1.2 kb. PCR amplification was achieved by the method previously described (7). Briefly, 20 μl of the DNA preparation was added to 100 μl of a reaction mixture containing 1× *Taq* polymerase buffer (supplied by the manufacturer but supplemented with 1 M MgCl$_2$ to a final concentration of 2.25 mM), 0.5 μM (each) primer, 200 μM (each) deoxynucleotide and 200 μg of bovine serum albumin per ml. Samples were heated at 94°C for 4 min, briefly centrifuged, and cooled to 58°C. At this time, 2.5 U of *Taq* polymerase (Roche Molecular Biochemicals) and 1.0 U of polymerase enhancer (Perfect Match; Stratagene, La Jolla, Calif.) were added, and then the samples were overlaid with 100 μl of mineral oil. The following conditions were used for amplification of the 462-bp fragment: 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 2 min, and elongation at 72°C for 2 min, followed by an elongation step of 8 min at 72°C. For amplification of the 1.2-kb fragment the following conditions were used: 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 2 min, and elongation at 72°C for 3 min, followed by an elongation step of 8 min at 72°C. Fifteen microliters of the sample was then electrophoresed through a 1% agarose gel followed by ethidium bromide staining and visualized by UV illumination.

Subsequent to obtaining the full sequences for one strain of the novel *Helicobacter* sp., PCR primers (forward primer G70, 5′-GCG GGT AAT TAA GTC AGA TG; reverse primer G69, 5′-TGT TTT CAA GCT CCC CAA AG) were designed for identification of *H. marmotae* in woodchuck liver tissues.

Southern blot analysis. To confirm that the amplicons were of bacterial 16S rRNA rather than nonspecific mammalian genomic origin, Southern blot analysis was performed with a 16S ribosomal DNA helicobacter probe. Fifteen microliters of amplicons was electrophoresed through a 1% agarose gel and transferred onto a Hybond N nylon membrane as outlined by the manufacturer (Amersham, Arlington Heights, Ill.). DNA was then UV cross-linked. The fixed DNA was then hybridized with a helicobacter probe generated by PCR amplification of *H. pylori* with primers C97 and C05 or the PCR product amplified from the woodchuck isolate with *H. marmotae*-specific primers. The probe was labeled with horseradish peroxidase, hybridized overnight to the nylon membrane at 42°C, and exposed in the presence of Luminol to Hyperfilm-ECL as outlined by the manufacturer (Amersham).

Histopathology. Ten formalin-fixed liver samples were processed by standard methods and embedded in paraffin. Six of the 10 samples were from woodchucks with liver tumors. Five-micrometer-thick sections were stained with either hematoxylin and eosin or Warthin-Starry silver stain. These sections were examined by light microscopy for evidence of inflammatory and degenerative lesions and for the presence of a bacterium with morphology consistent with members of the genus *Helicobacter*. Archival histologic sections of liver tissue from two WHV-negative woodchucks were also used for comparison. Frozen liver samples from these two archival woodchucks were not available for culture or PCR. Tissues were not available for the four cats.

Nucleotide sequence accession numbers. The nucleotide sequence accession numbers for each strain studied are in Fig. 4, the phylogenetic tree. The accession no. for *H. marmotae* type strain MIT 98-6070 is AF333341.

RESULTS

WHV status. Based on serum WHV DNA analysis, 17 of the woodchucks used in this study were naturally infected with WHV. At the time of necropsy, 10 of these woodchucks had WHV-associated hepatic tumors and 10 (3 of which were negative for WHV) had no grossly observable hepatic tumors.

***Helicobacter* sp. characterization.** Despite culturing all 20 livers, we were able to isolate a pure culture of the novel *Helicobacter* species from only one frozen liver from a tumor-positive woodchuck. In addition, we isolated pure cultures of the novel helicobacter from the feces of four cats. The bacteria were gram negative and had spiral morphology. The bacteria grew under microaerobic conditions as a spreading film on blood agar at 37 and 42°C but not at 25°C. The isolate was oxidase, catalase, urease, and alkaline phosphatase positive but did not hydrolyze indoxyl acetate and was γ-glutamyl transpeptidase negative. The bacterium did not reduce nitrate to nitrite; however, the bacterium grew in 1% glycine and was resistant to nalidixic acid and cephalothin (Table 1).

Ultrastructure. Bacterial cells had a spiral appearance and measured 0.2 by 1.5 μm. The cells had bipolar sheathed flagella typical of most enterohepatic helicobacters (Fig. 1).

PCR identification in liver tissue. Nine of the 10 livers from tumor-positive woodchucks had positive *Helicobacter* spp. identified by PCR (with *Helicobacter* genus-specific primers) and confirmed by Southern blot hybridization (Fig. 2A), whereas 5 of 10 livers from tumor-negative woodchucks (3 of which were WHV negative) were *Helicobacter* spp. positive with the genus-specific PCR primers (Fig. 2B). By use of 16S rRNA species-specific primers for *H. marmotae*, two additional liver samples from the nontumor group had positive PCR amplicons confirmed by Southern hybridization (Fig. 3).

Based on the cumulative PCR data with both sets of primers, 9 of 10 livers from tumor-positive woodchucks and 7 of 10 livers from tumor-negative woodchucks were helicobacter positive, for an overall prevalence of 80%. Because of the high prevalence of both helicobacter infection and tumors in the study group, no statistical association between the helicobacter PCR status and the presence of tumors could be determined.

Phylogenetic analysis. Full 16S rRNA sequences (approximately 1,500 bases) were determined for three isolates (MIT 98-6070, MIT 98-1705-1, and MIT 99-4759). Comparison of these sequences with over 100 *Helicobacter* sequences in our database indicated that the isolates represent a new *Helicobacter* species, for which we propose the name *H. marmotae*. The sequence for *H. marmotae* is most closely related to sequences from helicobacters isolated from rhesus monkeys (AF333339

TABLE 1. Characteristics which differentiate *H. marmotae* from other nongastric *Helicobacter* species[a]

Taxon	Catalase production	Nitrate reduction	Alkaline phosphatase hydrolysis	Urease	Indoxyl acetate hydrolysis	γ-Glutamyl transpeptidase	Growth At 42°C	Growth With 1% glycine	Nalidixic acid (30-μg disk)	Cephalothin (30-μg disk)	Periplasmic fibers	No. of flagella	Distribution of flagella	G+C content (mol%)
H. marmotae	+ (5/5)	− (5/5)	+ (5/5)	+ (5/5)	− (5/5)	− (5/5)	+ (5/5)	+ (5/5)	R (5/5)	R (5/5)	−	2	Bipolar	ND
H. canadensis	+	−	−	−	+	−	−	+	R	R	−	2	Bipolar	ND
H. rodentium	+	+	−	−	+	−	+	+	R	R	−	2	Bipolar	ND
H. pullorum	+	+	−	−	−	ND	+	+	R	R	−	1	Monopolar	34–35
H. fennelliae	+	−	+	−	+	−	−	+	S	S	−	2	Bipolar	35
H. trogontum	+	+	−	+	−	+	ND	ND	R	S	+	5–7	Bipolar	ND
H. muridarum	+	−	−	+	+	+	+	+	R	R	+	10–14	Bipolar	34
H. hepaticus	+	+	−	+	+	+	+	−	R	R	+	2	Bipolar	ND
H. canis	−	−	+	−	+	+	+	+	I	R	−	2	Bipolar	48
H. bilis	+	+	+	+	+	+	+	+	R	R	+	3–4	Bipolar	ND
"*H. rappini*"	+	+	−	+	ND	ND	+	+	R	R	+	10–20	Bipolar	34
H. cinaedi	+	+	−	−	−	−	−	+	I	S	+	1–2	Bipolar	37–38
H. pametensis	+	+	+	−	+	+	+	+	S	S	−	2	Bipolar	38
H. winghamensis	−	−	−	−	−	−	ND	ND	S	S	−	1–2	Bipolar	ND
H. mesocricetorum	+	+	+	−	+	+	+	+	S	R	ND	2	Bipolar	ND
H. aurati	+	+/−	+	+	ND	ND	+	−	R	R	+	7–10	Bipolar	ND
H. typhlonius	+	+	+	+	−	+	−	ND	R	R	−	1	Bipolar	ND
H. cholecystus	+	+	−	−	−	−	−	+	I	R	−	2	Bipolar	ND

[a] Data were obtained from references 12, 18, and 37. Symbols and abbreviations: +, positive reaction; −, negative reaction; S, susceptible; R, resistant; I, intermediate; ND, not determined.
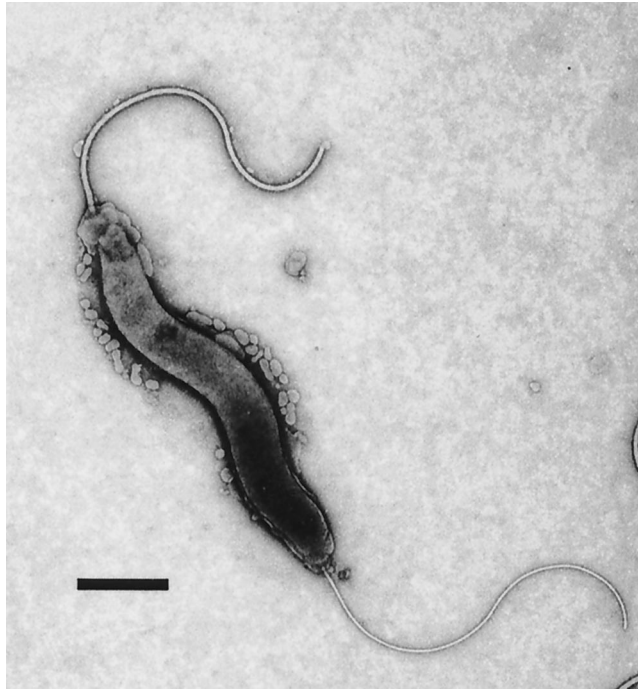
FIG. 1. Novel *Helicobacter* sp. has a curved to spiral appearance. The bacterium measures 0.2 by 1.5 to 5.0 μm and has bipolar sheathed flagella. Bar = 0.3 μm.

and AF333340 [11]) and cotton-top tamarins (AF107494 [30]) and *H. fennelliae*. The *H. marmotae* sequence differs from these sequences by 4 to 5% (Fig. 4).

Histopathology. Liver samples from woodchucks with HCC and positive for *Helicobacter* spp. by PCR were characterized by portal tracts with moderate to marked degrees of oval cell or bile duct hyperplasia with a portal bridging pattern and periportal inflammation comprised primarily of a lymphoplasmacytic infiltrate. The parenchyma was characterized by multifocal necrosis of hepatocytes accompanied by phagocytic infiltration and lymphoplasmacytic inflammation. The liver
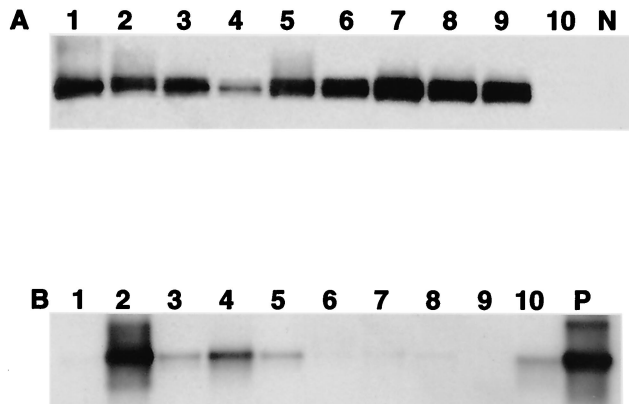

FIG. 2. Southern blot hybridization of the 1,200-bp PCR products with genus-specific *Helicobacter* spp. primers. (A) Tumor-positive group. (B) Tumor-negative group. Lanes 1 to 10, liver tissue samples. P, *Helicobacter* DNA as positive control; N, reagent control.
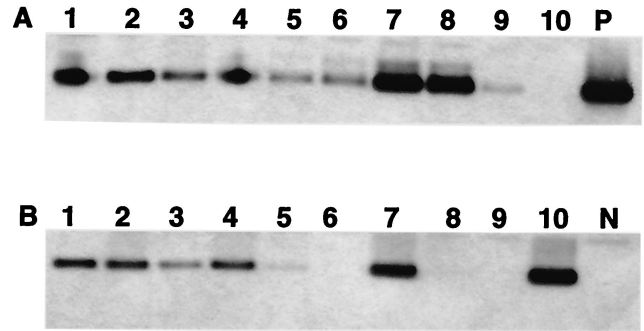

FIG. 3. Southern blot hybridization of PCR products with *Helicobacter* species-specific primers for the novel *Helicobacter* sp. (A) Tumor-positive group. (B) Tumor-negative group. Lanes 1 to 10, Liver tissue samples. P, *Helicobacter* DNA as positive control; N, reagent control.

samples from WHV-positive woodchucks without tumors, including the liver from which *H. marmotae* was isolated, had mild to moderate, chronic hepatitis centered on the portal areas. The hepatic parenchyma often contained small foci of necrotic hepatocytes accompanied by mild phagocytic and lymphoplasmacytic infiltrates. Mild peribiliary fibrosis and bile duct or oval cell proliferation were present in some specimens. Of interest, comparable histopathologic changes were observed in the liver samples from the three woodchucks that were negative for WHV but positive for *Helicobacter* spp. by PCR, consisting of chronic multifocal periportal hepatitis and biliary proliferation. Inflammation in the portal areas and parenchyma was comprised of lymphoplasmacytic infiltrates predominantly, with lesser numbers of macrophages and granulocytes (Fig. 5). Bacteria were not identified in Warthin-Starry-stained liver tissue.

DISCUSSION

The identification of a novel *Helicobacter* sp., *H. marmotae*, from woodchuck liver by culture and *H. marmotae*-specific PCR and the feces of cats raises the number of enterohepatic *Helicobacter* spp. isolated from livers to eight. The others include *H. bilis* and *H. hepaticus* from mice, *H. cholecystus* in hamsters, *H. pullorum* from chickens, *H. canis* from dogs, *H. cinaedi* from monkeys (11), and *Helicobacter* sp. Flexispira taxa 2 and 5 from sheep (6). DNA of *H. bilis* and *H. pullorum* and *Helicobacter* sp. Flexispira taxon 8 has also been identified in Chilean patients with chronic cholecystitis (7).

Whether the *H. marmotae* isolated from a woodchuck is responsible for the hepatitis noted in these animals and other woodchucks not infected with the woodchuck hepadnavirus is at present unknown and will require further studies. The isolation of *H. marmotae* from only one liver despite the presence of helicobacters in DNA from a large percentage of the woodchuck livers can be partially explained by the fact that *Helicobacter* spp. often do not survive freezing unless samples are stored in glycerol broth medium (37). Though similar in morphology to *H. canis*, which is also isolated from cats, *H. marmotae* can be differentiated from *H. canis* biochemically by being urease positive and indoxyl acetate hydrolysis and γ-glutamyl transpeptidase negative (5, 31). We have previously an-

(% Difference)

Helicobacter pylori ATCC 43504ᵀ {M88157} Human
Helicobacter nemestrinae ATCC 49396ᵀ {X67854} Monkey
Helicobacter acinonychis ATCC 51101ᵀ {M88148} Cheetah
Helicobacter sp. MIT 99-5656 {AF292378} Dolphin
Helicobacter sp. MIT 99-5657 {AF292377} Dolphin
"Gastrospirillum hominis" 1 Solnick G1A1 {L10080} Human
"Gastrospirillum hominis" 2 Solnick G2A9 {L10079} Human
Helicobacter felis ATCC 49179ᵀ {M57398} Cat
Helicobacter salomonis CCUG 37845ᵀ {U89351} Cat
Helicobacter bizzozeronii ATCC 70030ᵀ {Y09404} Dog
Helicobacter sp. Flexispira Taxon 1 ATCC 43968 {U96300} Pig
Helicobacter mesocricetorum ATCC 700932ᵀ {AF072471} Hamster
Helicobacter sp. Eaton 94-536 {U96299} Mouse
Helicobacter rodentium ATCC 700285ᵀ {U96296} Rat
Helicobacter rodentium-like MIT 95-2011 {U96298} Mouse
Helicobacter canadensis NLEP 16143ᵀ {AF262037} Human
Helicobacter pullorum NCTC 12824ᵀ {L36141} Chicken
Helicobacter sp. Flexispira Taxon 2 ATCC 49314 {AF225546} Sheep
Helicobacter sp. Flexispira Taxon 3 ATCC 49320 {AF225547} Pig
Helicobacter sp. CLO-3 CCUG 14564 {M88151} Human
Helicobacter sp. MIT 99-5507 {A333340} Rhesus Monkey 2
Helicobacter fennelliae CCUG 18820ᵀ {M88154} Human
Helicobacter sp. MIT 97-6194-5 {AF107494} Cotton-Top Tamarin
Helicobacter marmotae MIT 98-6070ᵀ {AF333341} Woodchuck ⟵
Helicobacter sp. MIT 99-5504 {AF333339} Rhesus Monkey 1
Helicobacter aurati MIT 97-5075ᵀ {AF297868} Hamster
Helicobacter sp. MIT 94-022 {AF225550} Mouse
Helicobacter trogontum ATCC 700114ᵀ {U65103} Rat
Helicobacter sp. Flexispira Taxon 4 ATCC 49310 {AF225548} Sheep
Helicobacter sp. Flexispira Taxon 5 ATCC 43966 {M88138} Sheep
Helicobacter hepaticus ATCC 51448ᵀ {U07574} Mouse
Helicobacter muridarum ATCC 49282ᵀ {M80205} Mouse
Helicobacter sp. Flexispira Taxon 7 Eaton 1302 {U51874} Dog
Helicobacter canis NCTC 12739ᵀ {L13464} Dog
Helicobacter bilis ATCC 51630ᵀ {U18766} Mouse
Helicobacter sp. Flexispira Taxon 8 ATCC 49317 Dog
Helicobacter cinaedi CCUG 18818ᵀ {M88150} Human
Helicobacter westmeadii {U44756} Human
Helicobacter sp. MIT 91-266-11 {M88152} Ferret
Helicobacter pametensis ATCC 51478ᵀ {M88147} Tern
Helicobacter cholecystus ATCC 700242ᵀ {U46129} Hamster
Helicobacter sp. Bird C ATCC 51482T {M88144} House sparrow
Helicobacter sp. Bird B ATCC 51480T {M88139} Tern
Helicobacter mustelae ATCC 43772ᵀ {M35048} Ferret
Helicobacter suncus Kaz-2 {AB006148} Shrew
Wolinella succinogenes ATCC 29543ᵀ {M88159} Cow
Campylobacter rectus ATCC 33238ᵀ {L04317} Human
Campylobacter concisus ATCC 33237ᵀ {L04322} Human
Campylobacter upsaliensis CCUG 14913ᵀ {L14628} Dog
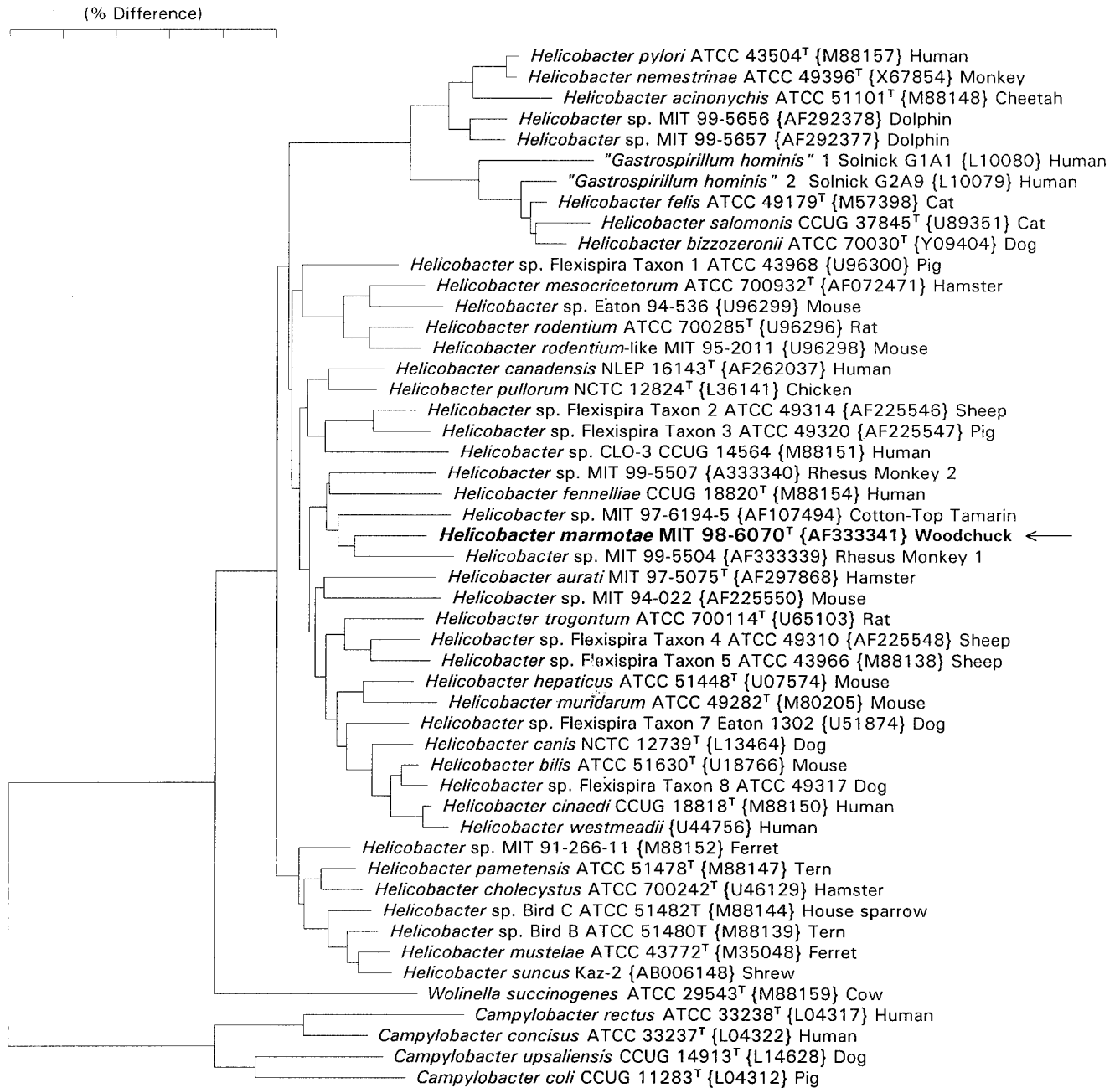*Campylobacter coli* CCUG 11283ᵀ {L04312} Pig

FIG. 4. Phylogenetic tree comparing *H. marmotae* with other closely related helicobacters.

alyzed the 1.2-kb products from *Helicobacter* genus-specific PCR analysis of 19 cat helicobacter isolates by *Alu*I enzyme digestion (31). Five patterns were observed. Three patterns grouped taxonomically with *H. bilis* or *Helicobacter* ("Flexispira") taxon 8, one was similar to *H. canis*, and the remaining pattern matched that of the novel *H. marmotae* isolated from woodchucks (31). Thus, restriction fragment length polymorphism analysis clearly supports our data that *H. marmotae* can be taxonomically distinguished from other feline enteric helicobacters.

Interestingly, *H. marmotae* experimentally inoculated into

A/JCr mice produces chronic hepatitis and typhlocolitis (J. G. Fox, unpublished observations). Given that cats are often diagnosed with chronic cholangiohepatitis of unknown etiology, it will be interesting to ascertain whether this novel helicobacter causes liver disease in cats as well as other mammals (19).

A body of evidence is accumulating which supports the conjecture that enterohepatic helicobacters may act as cofactors in carcinogenesis. We have previously shown that *H. mustelae*, a natural gastric pathogen in ferrets, strongly promotes gastric adenocarcinoma initiated by the gastric carcinogen *N*-methyl-
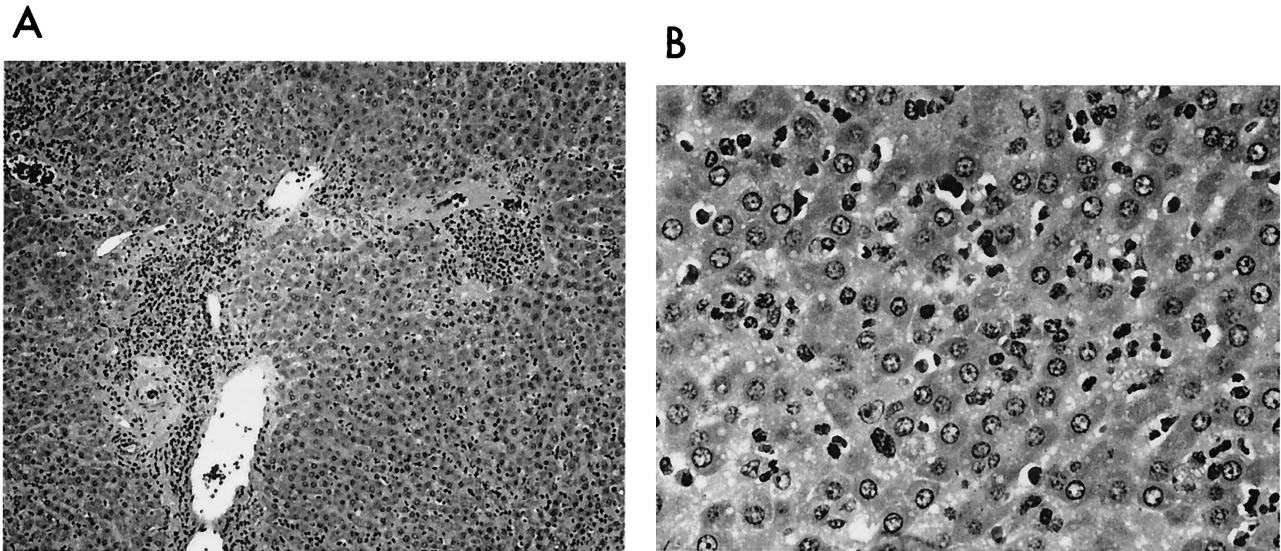
A

B

FIG. 5. (A) The portal areas are moderately infiltrated by a mixed inflammatory cell population, comprised chiefly of lymphocytes, plasma cells, and some macrophages. Inflammation abuts all portal structures, but a tendency to peribiliary condensation is present. Bile duct proliferation is present and extends into the adjacent parchyma. Hematoxylin and eosin stain. (B) An unstructured, mixed inflammatory infiltrate is present in the hepatic parenchyma, comprised chiefly of plasma cells, macrophages, and lymphocytes. Hematoxylin and eosin stain.

N'-nitrso-N-nitrosoguanidine (16). Experimentally, nitrosamine-induced gastric adenocarcinoma is promoted by *H. pylori* infection in gerbils (15). Others have demonstrated that bile duct cancer is strongly promoted by liver fluke infection in hamsters (4). It is interesting to speculate as to whether these hamsters were also infected with *H. cholecystus*, a novel helicobacter known to colonize the bile of hamsters (18). Also, several recent studies of A/J and B6C3F$_1$ mice infected with *H. hepaticus* suggest that the bacterium is a tumor promoter in the mouse liver (3, 13, 14, 21, 32, 38). Additional studies are required to ascertain whether *H. marmotae* acts as a tumor promoter in WHV-infected woodchucks and whether *H. marmotae* colonizes the lower bowel of woodchucks, the site of colonization of other enterohepatic helicobacters.

In summary, the finding of a novel *Helicobacter* sp., *H. marmotae*, in an animal model used to study virus-induced liver cancer raises intriguing questions as to whether similar *Helicobacter* spp. may be present in humans infected with hepatitis virus as well as cats with chronic cholangiohepatitis (19). If present, it will be important to determine whether these persistent helicobacters play a role in the development of hepatocellular cancers.

Description of *H. marmotae* sp. nov. (mar′mo.tae. F *marmotte*, fusion of OF *marmotte*, a monkey, and Romansch *murmont*, a marmot [L *mus*, *muris*, a mouse, plus *montis*, a mountain], relating to a short-tailed burrowing rodent species found in North America.) Cells are slender, curved to spiral rods (0.2 by 1.5 to 5 μm), which have one to three spirals. The bacterium is gram negative and nonsporulating; it is motile by means of sheathed, single unipolar or bipolar flagella. Cultures grown on solid agar media appear as spreading layers. Cells exhibit microaerobic but not aerobic or anaerobic growth. Growth occurs at 37 and 42°C. The bacteria are oxidase, catalase, urease, and alkaline phosphatase positive but γ-glutamyl transpeptidase negative. The organism does not hydrolyze indoxyl acetate nor

reduce nitrate to nitrite. Cells are resistant to nalidixic acid and cephalothin. Bacteria have been isolated from the liver of woodchucks and feces of clinically normal cats. The type strain is MIT 98-6070 (ATCC BAA-546). The 16S rRNA sequence accession no. for the type strain is AF333341.

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