Helicobacter marmotae and novel Helicobacter and Campylobacter species isolated from the livers and intestines of prairie dogs

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Abstract
Prairie dogs (*Cynomys ludovicianus*) are used to study the etiology and prevention of gallstones because of the similarities of prairie dog and human bile gallstone composition. Epidemiologic and experimental studies have suggested a connection between *Helicobacter* spp. infection and cholesterol cholelithiasis, cholecystis and gallbladder cancer. Ten of thirty four prairie dogs in our study had positive *Helicobacter* spp. identified by PCR with *Helicobacter* genus-specific primers (C97, CO5). Fourteen of thirty four prairie dogs had positive *Campylobacter* spp. identified in the intestine by PCR with *Campylobacter* genus-specific primers (C98 and C99). Six *Helicobacter* sp. isolates and 3 *Campylobacter* isolates were taxonomically identified by 16S rRNA analysis. The prairie dog helicobacters fell into three clusters adjacent to *H. marmotae*. On the basis of 16S rRNA sequence analysis, three strains in two adjacent clusters are included in the species *H. marmotae*. Three strains are only 97.1% similar to the sequence of *H. marmotae*, and are considered a novel species with the provisional designation *Helicobacter* sp. Prairie Dog 3. The prairie dog campylobacters formed a single novel cluster and represent a novel *Campylobacter* species with the provisional designation *Campylobacter* sp. Prairie Dog. They branched with *C. cuniculorum* at 96.3% similarity and had the greatest sequence similarity to *C. helveticus* at 97.1% similarity. Whether *H. marmotae* or the novel *Helicobacter* sp. and *Campylobacter* sp. identified in prairie dogs play a role in cholesterol gallstones or hepatobiliary disease requires further studies.
Introduction

Bacteria belonging to the genus *Helicobacter* are now recognized as a common inhabitant of the hepatobiliary and gastrointestinal tract of mammals. These gram-negative enterohpatic bacteria have become a subject of intense research because of their association with gastrointestinal diseases (Fox, 2002; Kobayashi *et al.*, 2005; Pellicano *et al.*, 2008). *Helicobacter* species have been isolated and identified from the gastrointestinal tract of humans, dogs, cats, ferrets, pigs, cattle, monkeys, dolphins, seals and cheetahs; and are associated with variable degrees of pathology in their respective hosts (Whary & Fox, 2004). In humans, *H. pylori* causes peptic ulcers and because of its association with gastric adenocarcinoma is classified a Class I carcinogen by WHO (Fox & Wang, 2007). In recent years enterohpatic *Helicobacter* spp. have also been isolated from wild and laboratory rodents (Comunian *et al.*, 2006; Fox *et al.*, 2010a; Fox *et al.*, 2002; Franklin *et al.*, 1996; Goto *et al.*, 2004; Won *et al.*, 2002; Zenner, 1999).

Epidemiologic and experimental studies have suggested a connection between *Helicobacter* spp. infection and cholesterol cholelithiasis, cholecystitis, and gallbladder cancer (Fox *et al.*, 1998; Maurer *et al.*, 2005). Prairie dogs are used to study gallstones etiology and prevention (Davis *et al.*, 2003; Li *et al.*, 1994; Narins *et al.*, 2005; Strichartz *et al.*, 1989) and electrogenic bicarbonate secretion in the gallbladder (Moser *et al.*, 2007).

We hypothesized that prairie dogs, like other wild rodents, harbor *Helicobacter* spp. and that these microaerophilic bacteria might be associated with overt or subclinical disease and effect research studies. The goal of this study was to determine if *Helicobacter* spp. could be isolated from the gastrointestinal tract and/or liver of prairie dogs used as models to study gallbladder physiology. It is also recognized that *Helicobacter* spp. co-colonize with a variety of *Campylobacter* spp. in the intestines of humans, dogs and cats (Fox *et al.*, 1995; Lastovica & le Roux, 2000; Rossi *et al.*, 2008; Shen *et al.*, 2001). We therefore asked whether *Campylobacter* spp. also could be identified in the gastrointestinal tract of prairie dogs.

Materials and Methods

Animals. Adult, black-tailed male and female prairie dogs (*Cynomys ludovicianus*), trapped in the wild were purchased from Flyers Speciality Pets (Lubbock, TX). The prairie dogs were caged individually in a 23°C thermoregulated room at the Drexel University College of Medicine, Philadelphia. These animals were used on studies of cholesterol gallstone pathophysiology. From 2004-2008, selected samples of liver, cecum, and fecal specimens from 34 adult prairie dogs, of mixed sex were collected during necropsy. In 13 animals, liver and ceca from the same prairie dog were available for culture. In another 8 prairie dogs, feces only were cultured; two prairie dogs had gallbladder and liver cultures, and 11 prairie dogs livers only were cultured. The specimens were shipped to MIT on dry ice and immediately stored at -70°C, until evaluated by culture and PCR analysis. All experiments were approved by the institutional animal care and use committee.

Microaerobic culture and biochemical characterization. The liver and cecal tissue were rinsed with sterile physiological saline and placed in a vial with 1.5 ml of 20% glycerol in brucella broth (Remel, Lenexa, KA). The vials were maintained at -70°C prior to culture. Samples were plated on TSA (Trypsic Soy Agar, BBL-), CVA (cefoperazone, vancomycin, and amphotericin B, Remel), and Blood agar base (Oxoid, Cambridge, UK) media containing amphotericin, vancomycin, polymyxin, bacitracin, and naladixid acid (Sigma Chemical Company, St. Louis, MO)(44). A small amount of tissue or feces was homogenized in 1ml of brucella broth.
containing 5% fetal calf serum (ATLAS, Biolabs.) in a disposable plastic tissue-grinder. Approximately 100 µl of sample was applied and streaked directly onto the three selective media. Half of the remaining sample was filtered through a 0.45 µm pore-size filter onto a blood agar plate. The plates were incubated at 37 °C under microaerobic conditions as described previously (Fox et al., 2006). A detailed biochemical characterization analysis was performed on selected isolates of Campylobacter spp. and Helicobacter spp. as previously described (Shen et al., 2001).

**DNA extraction for PCR analysis.** The High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, IN) was used for extraction of DNA from the bacterial isolates and the tissue samples according to the manufacturer’s direction. QIAamp DNA Stool Mini Kit was used to extract DNA from fecal samples (Qiagen Valencia, CA).

**PCR amplification of bacterial DNA.** Helicobacter genus-specific primers C97 and C05 and Campylobacter genus-specific primers C98 and C99 were used to amplify a 1.2 kb and a 420 bp PCR fragment respectively as described previously (Fox et al., 1998; Shen et al., 2001).

**Nested PCR.** The 1.2 kb PCR product from Helicobacter-genus specific primers C97 and C05 amplified from DNA of 21 liver specimens, were subjected to nested PCR using Helicobacter genus- specific primers C98F (5’-TGG TGT AGG GGT AAA ATC C), the reverse complement of primer C98 (Fox et al., 1998) and H3A20 (5’ GCC GTG CAC CTG TTT C) (Bohr et al., 2002) to generate 386 bp fragments within the 1.2 kb PCR products. Briefly, 5 µl of PCR product using C97/CO5 primers was used for another PCR amplification using primers C98F and H3A20 under the same reaction and cycling conditions (Fox et al., 1998). Fifteen microliters of the sample was then electrophoresed through a 2% agarose gel followed by ethidium bromide staining and visualized by UV illumination.

**Restriction fragment length polymorphism (RFLP) analysis.** The PCR-amplified 1.2-kb fragment of the 16S rRNA gene (20 µl) was digested with 10 U of restriction endonuclease Alul and Hhal (New England BioLabs, Beverly, MA) in the appropriate buffer recommended by the manufacturer at 37°C for 3 hours (Shen et al., 2000). Restriction patterns were compared after the digested PCR products were separated on a 6% Visigel separation matrix (Stratagene, Cedar Cree, TX).

**Amplification of 16S cistron by PCR and purification of PCR product from cultured bacteria.** The rRNA cistron from the Helicobacter spp. isolates (accession numbers MIT 04-8588, 07-6167, MIT 07-5168, 07-5155, and 07-5158) and Campylobacter spp. (MIT 07-5158, MIT 07-5158, and MIT 07-5167) were amplified with universal bacterial primers F24 and F25 for 16S rRNA (Fox et al., 1998). Hot-start PCR was performed in thin-walled tubes with a Perkin-Elmer 9700 thermocycler and 1 µl of the DNA template was added to a reaction mixture (50 µl volume) containing 20 pmol of each primer, 40 nmol of deoxynucleoside triphosphates, and 1 U of Taq 2000 polymerase (Stratagene) in buffer containing Taqstart antibody (Sigma, St. Louis). The amplification was achieved by previously described methods (Marini et al., 2010).

**16S rRNA gene sequencing and data analysis.** Three purified DNA samples from 2004 and 3 DNA samples from 2007 were sequenced with an ABI prism cycle-sequencing kit (BigDye Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase FS; Perkin-Elmer). The primers used were as previously described by Fox et al. (Fox et al., 1998). Quarterdye chemistry was used with 80 µM primers and 1.5 µl of PCR product in a final volume of 20 µl. Cycle sequencing was performed with an ABI GeneAmp PCR System 9700 with 25 cycles of
denaturation at 96°C for 10 s and annealing and extension at 60°C for 4 min. Sequencing reactions were run on an ABI 3100 DNA instrument. Sequence data were entered into RNA, a program set for data entry, editing, sequence alignment, secondary structure comparison, similarity matrix generation, and dendrogram construction for 16S rRNA (Dewhirst et al., 1999). Our database contains over 1000 sequences obtained in our laboratory and over 500 retrieved from GenBank for 16S rRNA. Dendrograms were constructed by the neighbor-joining method (Saitou & Nei, 1987).

Histopathology. Select specimens of liver from 5 prairie dogs were shipped to MIT in vials containing neutral-buffered 10% formalin; tissues were processed by standard histologic methods, embedded in paraffin, and 5 µm sections were stained with hematoxylin and eosin and evaluated by a comparative pathologist.

RESULTS

Microaerobic culture and biochemical characterization of H. marmotae, novel Helicobacter spp. and Campylobacter spp.

After incubation under microaerobic conditions, bacterial growth was visible as a thin, spreading film. Helicobacter spp. were confirmed using catalase, oxidase, and urease activity as well as cell morphology, lack of growth aerobically, and PCR using Helicobacter species specific PCR. Helicobacter was isolated from the feces or cecal tissue of 5 of 34 prairie dogs, and from the liver of one animal. In the 13 prairie dogs in which tissue samples from both the liver and ceca were available, pure cultures of Helicobacter spp. were isolated from the ceca of two animals (07-5165 & 07-5167), and the liver of a third (07-85-89). In the 8 prairie dogs that had feces cultured Helicobacter spp. were isolated from three animals. The bacteria were gram negative and grew under microaerobic conditions at 37°C and 42°C, but not at 25°C. All isolates were oxidase positive and urease negative. The bacteria did not hydrolyze indoxyl acetate and alkaline phosphatase or reduce nitrate to nitrite, and the isolates did not have γ-glutamyl transpeptidase activity. They did not grow in 1% glycine and were resistant to cephalothin except 04-8584 but sensitive or intermediate to nalidixic acid (Table 1).

Despite culturing all specimens of 34 prairie dogs for Campylobacter spp., a pure culture of a novel Campylobacter species was isolated from only three animals; from the cecum of two animals and from the liver of a third. The bacteria were gram negative and grew under microaerobic conditions at 42°C, but not at 37°C. All isolates were oxidase and catalase positive but urease negative. The bacteria did hydrolyze indoxyl acetate but not alkaline phosphatase and did not reduce nitrate to nitrite. The isolates did not have γ-glutamyl transpeptidase activity. They did not grow in 1% glycine and were resistant to cephalothin but sensitive to nalidixic acid (Table 2).

PCR identification of Helicobacter spp. Ten of thirty four prairie dogs were positive for Helicobacter spp. by PCR with Helicobacter genus-specific primers (C97, CO5). Six of the ten were amplified from pure culture. In addition, two of the prairie dog liver-samples that were negative using PCR were positive for Helicobacter spp. using nested PCR. Thus, Helicobacter spp. were detected in 9 of 20 males and 3 of 14 females.

PCR identification of Campylobacter spp. In 3 of 20 males and 7 of 14 females Campylobacter spp. were identified by PCR with Campylobacter genus-specific primers (C98 and C99).
**RFLP analysis.** By RFLP analysis of the PCR 1.2 kb products (Fig. 1), three different RFLP patterns were noticed by use of restriction enzyme AluI: Strains in line 1-3 share a common pattern and correspond to 16S rRNA prairie dog 3 cluster (see below). The prairie dog isolate in lane 4 is identical to the woodchuck isolate in lane 5 and correspond to 16S rRNA prairie dog 2 cluster. The prairie dog isolates in lanes 6-7 are identical to the woodchuck *H. marmotae* type strain in lane 8, and correspond to 16S rRNA prairie dog 1 cluster. Two patterns were observed by use of restriction enzyme HhaI: the isolates corresponding to 16S rRNA prairie dog cluster 3 had one pattern (lanes 1-3); while the remaining isolates and *H. marmotae* had a second pattern (lanes 4-8).

**16S rRNA Sequence Analysis.** As noted in Fig. 2, the prairie dog helicobacters fell into three clusters adjacent to *H. marmotae*. Prairie dog 1 cluster contained strains MIT 04-8584 and MIT 04-8589 were 99.0% similar to the sequence of *H. marmotae* and contain a 295 base intervening sequence (IVS) in the 198-219 helix (E. coli numbering), which has not been noted in other *H. marmotae* strains. This IVS is about 80% similar to the IVS in *H. muricola*. Prairie dog 2 cluster contained strain MIT 07-5165 was 98.6% similar to *H. marmotae* and also contains a 295 base IVS essentially identical to the of the Prairie Dog 1 strains. On the basis of 16S rRNA sequence and phenotypic properties we propose that the three strains in prairie dog clusters 1 & 2 be included in the species *H. marmotae*. The three prairie dog 3 cluster strains, MIT 04-8588, MIT 07-5167, and MIT 07-5168 are only 97.1% similar to the sequence of *H. marmotae*, and should be considered a novel species.

The three prairie dog campylobacters formed a single identifiable cluster (Fig. 3). They branched with *C. cuniculorum* at 96.3% similarity and had a sequence similarity to *C. helveticus* at 97.1% similarity. These strains based on 16S rRNA analysis represent a novel *Campylobacter* species.

**Histopathology.** In all five animals the most consistent findings noted in livers included mild portal lymphocytic inflammation, mild bile duct and arteriolar proliferation, and mild centrilobular fatty change in their respective livers. Interestingly, the livers of 3 of the prairie dogs also had mild accumulations of neutrophils and myeloid precursor cells along the capsular border. Although the precursor cells indicate extramedullary hematopoiesis in the liver, the mature neutrophils suggest a degree of peritonitis, although the cause of this was not identified histologically. The most significant lesion was the hepatocellular carcinoma seen in the liver of animal 08-9090 (Figure 4). This tumor was comprised of a variably well-demarcated proliferation of neoplastic cells with hepatocellular differentiation. Cells formed trabeculae and nests (often composed of more than 10 cell layers) separated by a fine connective tissue stroma and were round to polygonal with abundant eosinophilic cytoplasm and a single, round nucleus with a prominent nucleolus. Nuclear and cellular pleomorphism were mild, and mitoses were 0-5 per ten x40 objective fields. Occasional attempts at acinar formation were seen. Extensive intratumoral hemorrhage, necrosis and intracellular fatty change were also seen.

**Discussion**

Prairie dogs are used mostly in gallbladder studies particularly in experiments involving cholesterol gallstones (Abedin et al., 2001; Davis et al., 2003; Narins et al., 2005; Strichartz et al., 1989). The advantages of utilizing the prairie dog gallstone model are the similarity of the prairie dog and human bile and gallstone composition and also the reliability of sufficient quantities of bile, cholesterol stones and tissue for analysis (Brenneman et al., 1972). The isolation of *H. winghamensis*-like organisms from the liver of a prairie dog being used to study
cholesterol gallstone formation raises the number of Helicobacter spp. isolated from the livers of mammals and birds to ten. The other H. spp. isolated from animal livers include H. bilis and H. hepaticus cultured from mice (Fox et al., 1994; Fox et al., 1996c; Fox et al., 1995), H. bilis from hamsters (Fox et al., 2009), H. cholecystus from hamsters (Franklin et al., 1996), H. canis from dogs (Fox et al., 1996a), H. cinaedi from monkeys (Fox et al., 2001), Helicobacter sp. Flexispira taxa 2 and 5 from sheep (Kirkbride et al., 1985), H. marmotae has been cultured from the liver of woodchucks and feces of cats (Fox et al., 2002) and H. pullorum from chicken livers and mouse intestines (Boutin et al., 2010; Stanley et al., 1994).

Recent experimental results and epidemiological studies suggest a possible association between enteroheliotic Helicobacter spp. and cholesterol cholelithiasis, chronic cholecystitis, and gallbladder cancer. Our laboratory prospectively investigated the effect of Helicobacter spp. infection in cholesterol gallstone pathogenesis in the highly susceptible C57L/J mouse model (Maurer et al., 2005). Helicobacter spp.-free adult male C57L mice were infected with several different enteroheliotic helicobacters or left uninfected and fed either a lithogenic diet or standard mouse chow for 8 and 18 weeks. At the conclusion of the study, bile was examined microscopically and diagnostic culture and PCR were performed. Mice infected with H. bilis or coinfected with H. hepaticus and H. rodentium and fed a lithogenic diet developed cholesterol gallstones at 80% prevalence by 8 weeks compared with approximately 10% in uninfected controls. Monoinfections with H. hepaticus, Helicobacter cinaedi, and H. rodentium gave a cholesterol gallstone prevalence of 40%, 30%, and 20%, respectively; the latter 2 groups did not differ statistically from controls. Neither infected nor uninfected mice fed a standard chow diet developed cholesterol gallstones. These findings suggest that Helicobacter spp. may play a role in the pathophysiology of cholesterol gallstone formation in mice (Maurer et al., 2005). We have also previously documented Helicobacter spp. in Chilean females with chronic cholecystitis and cholesterol gallstones (Fox et al., 1998). Others have also documented Helicobacter spp. in a variety of hepatobiliary diseases in humans (see review by (Pellicano et al., 2008)).

In this study, H. marmotae was isolated from ceca of two prairie dogs and was identified by PCR and sequencing of the 700 basepair PCR product in the liver of two additional prairie dogs. Helicobacter spp. have been detected in various other wild rodents (Comunian et al., 2006; Fox et al., 2002; Parker et al., 2009; Won et al., 2002). H. hepaticus, H. apodemus, H. canadensis, and H. winghamensis identified by partial 16S rRNA sequence analysis suggests that enteric helicobacter infections may be widespread in wild rodents in China (Goto et al., 2004). H. muricola was isolated and characterized in Korean wild mice (Won et al., 2002). Comunian et al. (Comunian et al., 2006) also described DNA of Helicobacter by PCR in feces of wild rodents in Brazil. Species tentatively identified included H. marmotae, H. cinaedi and H. rodentium (Comunian et al., 2006).

Five out of 12 helicobacter positive prairie dogs were coinfected with Campylobacter spp. Three of the Campylobacter strains whose 16S rRNA were sequenced are considered a novel species, related to C. cuniculorum and C. helveticus. This finding of coinfection is not surprising given that enteric helicobacters are often found in hosts co infected with Campylobacter spp. For example, 64 of 227 commercially reared cats had microaerobic bacteria isolated from their feces and were initially identified as Campylobacter-like organisms based on biochemical and phenotypic characteristics (Shen et al., 2001). DNA extractions were subjected to PCR using primers specific for Helicobacter spp. and Campylobacter spp. Of the isolates, 92% (47 of 51) were positive for Campylobacter spp., 41% (21 of 51 isolates) were positive for Helicobacter spp., and 33% (17 of 51 isolates) were positive for both genera. Co-infection with Campylobacter
spp., enteric *Helicobacter* spp. and *Anaerobiospirillum* spp. 52 healthy and 138 diarrheic dogs and 63 diarrheic and 21 healthy cats has been reported (Rossi *et al.*, 2008); about 10% of both dogs and cats were found to be infected with *Campylobacter* spp. and *Helicobacter* spp. However, no statistically significant correlation between isolation of single or mixed infections and the presence of diarrhea was observed (Rossi *et al.*, 2008).

Investigators in South Africa have published results for a protocol that allows primary isolation of multiple *Campylobacter* spp. and *Helicobacter* spp. from the diarrheic specimens of individual children. Filtrates are plated onto antibiotic-free blood agar plates and incubated in an H2-enriched atmosphere (Lastovica & le Roux, 2000). The authors have reported a 16% prevalence of multiple *Campylobacter* spp. and *Helicobacter* spp. based on primary isolation, biochemical characterization, and serologic confirmation. They frequently recovered between two and five species of campylobacter-and helicobacter-from one stool sample, with *C. jejuni*, *C. coli*, *C. upsaliensis*, *Helicobacter fennelliae*, and *H. cinaedi* being commonly isolated (Lastovica & le Roux, 2000).

Recently we have reported that *H. marmotae* can successfully colonize mice and cause enterohepatic disease (Patterson *et al.*, 2010). Inflammatory lesions present in the livers of 5 prairie dogs and the hepatocellular carcinoma present in one animal may be related to helicobacter infection as noted in *H. marmotae* infected mice or mice infected with *H. hepaticus* (Fox *et al.*, 2010b; Fox *et al.*, 1996b; Garcia *et al.*, 2008; Patterson *et al.*, 2010). Isolation and identification of a novel campylobacter from the intestine and liver of prairie dogs is also interesting. Experimentally *C. jejuni* can induce chronic hepatitis in mice (Kita *et al.*, 1986; Kita *et al.*, 1992; Kita *et al.*, 1990). It is also interesting to note the gender differences in the presence of *Helicobacter* spp. and *Campylobacter* spp. in the prairie dog. *Helicobacter* spp. were detected in 9 of 20 males and 3 of 14 females while *Campylobacter* spp. were detected in 3 of 20 males and 7 of 14 females. It is not known whether female gender bias of *Campylobacter* spp. is associated with the female gender bias of gallstone and gallbladder cancer incidence in humans. Our results suggest that further studies examining the role of *H. marmotae*, other *Helicobacter* spp. and *Campylobacter* spp. in the pathogenesis of cholesterol gallstones and chronic inflammation in the liver and intestine in this prairie dog model should be conducted.
References


Fox, J. G., Yan, L. L., Dewhirst, F. E., Paster, B. J., Shames, B., Murphy, J. C., Hayward, A., Belcher, J. C. & Mendes, E. N. (1995). *Helicobacter bilis* sp. nov., a novel Helicobacter species isolated from bile, livers, and intestines of aged, inbred mice. *J Clin Microbiol* 33, 445-454.


### Table 1 Biochemical characterization of *Helicobacter* sp.

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* Include in *H. marmotae*, **Novel taxa Helicobacter sp. Prairie Dog 3

### Table 2 Biochemical characterization of Campylobacter sp. Nov.

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+ NA – nalidixic acid (30mg)
++ CE – cephalothin (30mg)
Figure Legends

Figure 1
Products (1.2 kb) of PCR using Helicobacter genus-specific primers were digested by Alu I and Hha I and analyzed by electrophoresis on 6% Visigel matrix. Lane 1- 4 prairie dog isolates: (MIT 07-5168; MIT 07-5167; MIT 04-8588; ; MIT 07-5165; lane 5: woodchuck fecal isolate (MIT 02-6901); lane 6-7: prairie dog isolates (MIT 04-8589; MIT 04-8584) and lane 8: H. marmotae; (MIT 98-6070) isolated from woodchuck liver; M. 100 bp DNA ladder.

Figure 2
Neighbor-joining tree for prairie dog helicobacter isolates based on comparison of 16S rRNA sequences. The marker bar shows a 3% difference in nucleotide sequence. GenBank accession numbers are given in brackets. The sequences in boldface are those described in this study.

Figure 3
Neighbor-joining tree for prairie dog campylobacter isolates based on comparison of 16S rRNA sequences. The marker bar shows a 5% difference in nucleotide sequence. GenBank accession numbers are given in brackets. The sequences in boldface are those described in this study.

Figure 4
(1) Normal liver parenchyma (N) is expanded by a tumor (T). An arrow marks the zone of demarcation between normal liver parenchyma and the hepatocellular carcinoma. (Bar = 500 µm). (2) A fine connective tissue stroma (arrow) delineates nests of well-differentiated, neoplastic hepatocytes. Lipid vacuoles (*) scattered throughout characterize the fatty change within the tumor. (Bar = 50 µm). (3) A zone of liquefactive necrosis within the tumor, with characteristic loss of cell detail and admixed, large amounts of neutrophilic cell debris (arrow). Fatty change is again evident by the presence of lipid vacuoles (*). (Bar = 50 µm). (4) A portal area within the non-tumorous region of liver is expanded by proliferations of bile ducts (white arrows) and arterioles (black arrows). Increased numbers of lymphocytes also surround the bile ducts. (Bar = 20 µm).
Figure 3

Figure 4