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

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Prairie dogs (Cynomys ludovicianus) are used to study the aetiology and prevention of gallstones because of the similarities of prairie dog and human bile gallstone composition. Epidemiological and experimental studies have suggested a connection between infection with Helicobacter species and cholesterol cholelithiasis, cholecystis and gallbladder cancer. Ten of the 34 prairie dogs in this study had positive Helicobacter species identified by PCR using Helicobacter genus-specific primers. Ten of 34 prairie dogs had positive Campylobacter species identified in the intestine by PCR with Campylobacter genus-specific primers. Six Helicobacter sp. isolates and three Campylobacter sp. isolates were identified taxonomically by 16S rRNA gene analysis. The prairie dog helicobacters fell into three clusters adjacent to Helicobacter marmotae. On the basis of 16S rRNA gene sequence analysis, three strains in two adjacent clusters were included in the species H. marmotae. Three strains were only 97.1 % similar to the sequence of H. marmotae and can be 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 sp. with the provisional designation Campylobacter sp. Prairie Dog. They branched with Campylobacter cuniculorum at 96.3 % similarity and had the greatest sequence similarity to Campylobacter 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 enterohepatic bacteria have become the 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, Helicobacter pylori causes peptic ulcers and, because of its association with gastric adenocarcinoma, is classified as a class I carcinogen by the World Health Organization (Fox & Wang, 2007). In recent years, enterohepatic Helicobacter species have also been isolated from wild and laboratory rodents (Comunian et al., 2006; Fox et al., 2002, 2010; Franklin et al., 1996; Goto et al., 2004; Won et al., 2002; Zenner, 1999).

Epidemiological and experimental studies have suggested a connection between infection with Helicobacter species and cholesterol cholelithiasis, cholecystitis, and gallbladder cancer (Fox et al., 1998; Maurer et al., 2005). Prairie dogs

are used to study gallstone aetiopathogenesis 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, harbour *Helicobacter* species, and that these microaerophilic bacteria might be associated with overt or subclinical disease and might affect research studies. The goal of this study was to determine whether *Helicobacter* species could be isolated from the gastrointestinal tract and/or liver of prairie dogs, which are used as models to study gallbladder physiology because of the similarities of prairie dog and human bile gallstone composition. It is also recognized that *Helicobacter* species co-colonize with a variety of *Campylobacter* species 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* species could also be identified in the gastrointestinal tract of prairie dogs.

METHODS

Animals. Adult, black-tailed male and female prairie dogs (*Cynomys ludovicianus*), trapped in the wild, were purchased from Flyers Speciality Pets (Lubbock, TX, USA). The prairie dogs were caged individually in a 23 °C thermoregulated room at the Drexel University College of Medicine, PA, USA. These animals were used for studies of cholesterol gallstone pathophysiology. From 2004 to 2008, selected samples of liver, caecum and faecal specimens from 34 adult prairie dogs, of both sexes, were collected during necropsy. In 13 animals, liver and caeca from the same prairie dog were available for culture. In another eight prairie dogs, faeces only were cultured; from two prairie dogs, gallbladder and liver cultures were grown, and from 11 prairie dogs only livers were cultured. The specimens were shipped to the Massachusetts Institute of Technology (MIT; MA, USA) on dry ice and stored immediately at −70 °C until evaluated by culture and PCR analysis. All experiments were approved by the Drexel Institutional Animal Care and Use Committee.

Microaerobic culture and biochemical characterization. Liver and caecal tissues were rinsed with sterile physiological saline and placed in a vial with 1.5 ml 20 % glycerol in *Brucella* broth (Remel). Vials were maintained at −70 °C prior to culture. Samples were plated on tryptic soy agar (BBL), CVA medium (containing cefoperazone, vancomycin and amphotericin B; Remel) and blood agar base (Oxoid) media containing amphotericin, vancomycin, polymyxin, bacitracin and nalidixic acid (Sigma Chemical Company). A small amount of tissue or faeces was homogenized in 1 ml *Brucella* broth containing 5 % fetal calf serum (ATLAS Biolabs) in a disposable plastic tissue grinder. Approximately 100 μl 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* species and *Helicobacter* species as described previously (Shen *et al.*, 2001).

DNA extraction for PCR analysis. A High Pure PCR Template Preparation kit (Roche Molecular Biochemicals) was used for extraction of DNA from the bacterial isolates and the tissue samples

according to the manufacturer's direction. A QAIamp DNA Stool Mini kit (Qiagen) was used to extract DNA from faecal samples.

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 the DNA of 21 liver specimens was subjected to nested PCR using *Helicobacter* genus-specific primer C98F (5′-TGGTGTAGGGGTAAAATCC-3′), which is the reverse complement of primer C98 (Fox *et al.*, 1998), and H3A20 (5′-GCCGTGCAGCACCTGTTTC-3′) (Bohr *et al.*, 2002) to generate 386 bp fragments. Briefly, 5 μl PCR product using primers C97 and C05 was used for another PCR amplification using primers C98F and H3A20 under the same reaction and cycling conditions (Fox *et al.*, 1998). Fifteen microlitres of the sample was then electrophoresed through a 2 % agarose gel followed by ethidium bromide staining, and visualized by UV illumination.

RFLP analysis. The PCR-amplified 1.2 kb fragment of the 16S rRNA gene (20 μl) was digested with 10 U *Alu*I and *Hha*I (New England BioLabs) in appropriate buffer, as recommended by the manufacturer, at 37 °C for 3 h (Shen *et al.*, 2000). Restriction patterns were compared after the digested PCR products had been separated on a 6 % Visigel separation matrix (Stratagene).

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

16S rRNA gene sequencing and data analysis. Three purified DNA samples from 2004 and three 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 have been described previously by Fox *et al.* (1998). Quarterdye chemistry was used with 80 μM primers and 1.5 μl 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 neighbour-joining method (Saitou & Nei, 1987).

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

RESULTS

Microaerobic culture and biochemical characterization of *Helicobacter marmotae* and novel *Helicobacter* and *Campylobacter* species

After incubation under microaerobic conditions, bacterial growth was visible as a thin, spreading film. *Helicobacter* species were confirmed using catalase, oxidase and urease activity, as well as cell morphology, lack of growth aerobically and PCR using *Helicobacter* species-specific primers. *Helicobacter* was isolated from the faeces or caecal tissue of five of the 34 prairie dogs and from the liver of one animal. In the 13 prairie dogs in which tissue samples from both the liver and caeca were available, pure cultures of *Helicobacter* species were isolated from the caeca of two animals (MIT 07-5165 and 07-5168) and the liver of a third (MIT 07-5167). In the eight prairie dogs from which faeces were cultured, *Helicobacter* species were isolated from three animals (MIT 04-8584, 04-8589 and 04-8588). The bacteria were Gram-negative and grew under microaerobic conditions at 37 and 42 °C and 25 °C. All isolates were oxidase positive and urease negative. The bacteria did not hydrolyse indoxyl acetate or show alkaline phosphatase activity, and did not reduce nitrate to nitrite; they did not have γ-glutamyl transpeptidase activity. They did not grow in 1 % glycine and were resistant to cephalothin, except for 04-8584, but were sensitive or showed intermediate resistance to nalidixic acid (Table 1).

Despite culturing all specimens from the 34 prairie dogs for *Campylobacter* species, a pure culture of a novel *Campylobacter* sp. was isolated from only three animals: from the caecum of two animals and from the liver of a third. The bacteria were Gram-negative and grew under microaerobic conditions at 42 °C and 37 °C. All isolates were oxidase and catalase positive but urease negative. The bacteria hydrolysed indoxyl acetate but did not show alkaline phosphatase activity, and did not reduce nitrate to nitrite; they 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* species

Ten of the 34 prairie dogs were positive for *Helicobacter* species by PCR with *Helicobacter* genus-specific primers (C97 and CO5). Six of these ten were amplified from pure culture. In addition, two of the five prairie dog liver samples also analysed by histopathology that were negative using conventional PCR were positive for *Helicobacter* species using nested PCR. Overall, *Helicobacter* species were detected in nine out of 20 males and three out of 14 females.

PCR identification of *Campylobacter* species

Campylobacter species were identified by PCR using *Camyplobacter* genus-specific primers (C98 and C99) in three out of 20 males and seven out of 14 females.

Table 1. Biochemical characterization of some of the *Helicobacter* species isolated in this study

+, Positive reaction; −, negative reaction; S, sensitive; I, intermediate; R, resistant; NA, nalidixic acid (30 mg); CE, cephalothin (30 mg).

MIT strain (sample)	16S rRNA cluster	Urease activity	Oxidase activity	Catalase production	Nitrate reduction	Alkaline phosphatase hydrolysis	Indoxyl acetate hydrolysis	γ-Glutamyl transpeptidase activity	Growth at 42 °C	Growth with 1 % glycine	Susceptibility to NA	Susceptibility to CE
04-8584 (faeces)*	1	−	+	+	−	−	−	−	+	−	S/I	S
04-8589 (faeces)*	1	−	+	+	−	−	−	−	+	−	S/I	R
07-5165 (caecum)*	2	−	+	+	−	−	−	−	+	−	S/I	R
04-8588 (faeces)†	3	−	+	−	−	−	−	−	+	−	S	R
07-5167 (liver)†	3	−	+	−	−	−	−	−	+	−	S/I	R
07-5168 (caecum)†	3	−	+	−	−	−	−	−	+	−	S/I	R/I

*Included within the species *H. marmotae*.
†Novel taxa *Helicobacter* sp. Prairie Dog 3.

Table 2. Biochemical characterization of the *Campylobacter* sp. nov. isolated in this study

+, Positive reaction; −, negative reaction; S, sensitive; NA, nalidixic acid (30 mg); CE, cephalothin (30 mg).

MIT strain (sample)	Urease activity	Oxidase activity	Catalase production	Nitrate reduction	Alkaline phosphatase hydrolysis	Indoxyl acetate hydrolysis	γ-Glutamyl transpeptidase activity	Growth at 42 °C	Growth with 1% glycine	Susceptibility to	
										NA	CE
07-5167 (liver)	−	+	+	−	−	+	−	+	−	S	R
07-5155 (caecum)	−	+	+	−	−	+	−	+	−	S	R
07-5158 (caecum)	−	+	+	−	−	+	−	+	−	S	R

RFLP analysis

By RFLP analysis of the 1.2 kb PCR products (Fig. 1), three different RFLP patterns were noted using the restriction enzyme *Alu*I: strains in Fig. 1(a) lanes 1–3 shared a common pattern and corresponded to 16S rRNA prairie dog 3 cluster (see below). The profile of the prairie dog isolate in Fig. 1, lane 4, was identical to that of the woodchuck isolate in lane 5 and corresponded to 16S rRNA prairie dog 2 cluster. The profiles of the prairie dog isolates in Fig. 1(a), lanes 6 and 7, were identical to that of the woodchuck *H. marmotae* type strain in lane 8 and corresponded to 16S rRNA prairie dog 1 cluster. Two patterns were observed using restriction enzyme *Hha*I: the
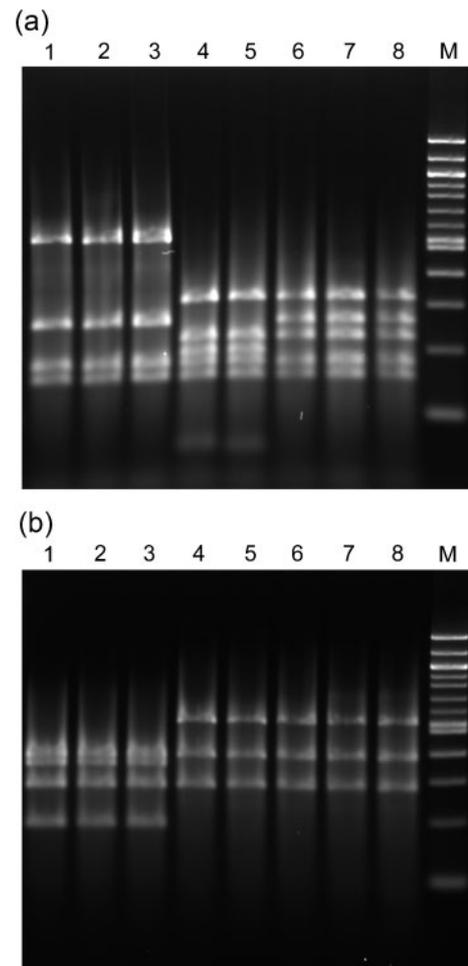
Fig. 1. PCR products of 1.2 kb produced using *Helicobacter* genus-specific primers were digested by *Alu*I (a) or *Hha*I (b) and analysed by electrophoresis on a 6 % Visigel matrix. Lanes 1–4, prairie dog isolates MIT 07-5168, MIT 07-5167, MIT 04-8588 and MIT 07-5165; lane 5, woodchuck faecal isolate MIT 02-6901; lanes 6 and 7, prairie dog isolates MIT 04-8589 and MIT 04-8584; lane 8, *H. marmotae* MIT 98-6070 isolated from woodchuck liver; M, 100 bp DNA ladder.

isolates corresponding to 16S rRNA prairie dog cluster 3 had one pattern (Fig. 1b, lanes 1–3), whilst the remaining isolates and *H. marmotae* showed a second pattern (Fig. 1b, lanes 4–8).

16S rRNA gene sequence analysis

As shown 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, which were 99.0 % similar to the sequence of *H. marmotae* and contained a 295 bp intervening sequence (IVS) in the helix at nt 198–219 (*Escherichia coli* numbering), which has not been noted in other *H. marmotae* strains. This IVS was about 80 % similar to the IVS in *Helicobacter muricola*. Prairie dog 2 cluster contained strain MIT 07-5165 and was 98.6 % similar to *H. marmotae* and also contained a 295 bp IVS essentially identical to that of the prairie dog 1 strains. On the basis of 16S rRNA gene sequence and phenotypic properties, we propose that the three strains in prairie dog clusters 1 and 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 were 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* with 96.3 % similarity and had a sequence similarity to *C. helveticus* of 97.1 %. Based on 16S rRNA analysis, these strains 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 changes. Interestingly, the livers of three of the prairie dogs, two of which were positive for *Helicobacter* species by nested PCR, also had mild accumulations of neutrophils and myeloid precursor cells along the capsular border. Although the precursor cells indicated extramedullary haematopoiesis in the liver, the mature neutrophils suggested 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, which was one of the prairie dogs whose liver was positive for *Helicobacter* species by nested PCR (Fig. 4). This tumour comprised a variably well-demarcated proliferation of neoplastic cells with hepatocellular differentiation. Cells formed trabeculae and nests (often composed of more than ten 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 pleomorphisms were mild, and the number of mitoses was between zero and five per ten objective fields (×40). Occasional attempts at acinar
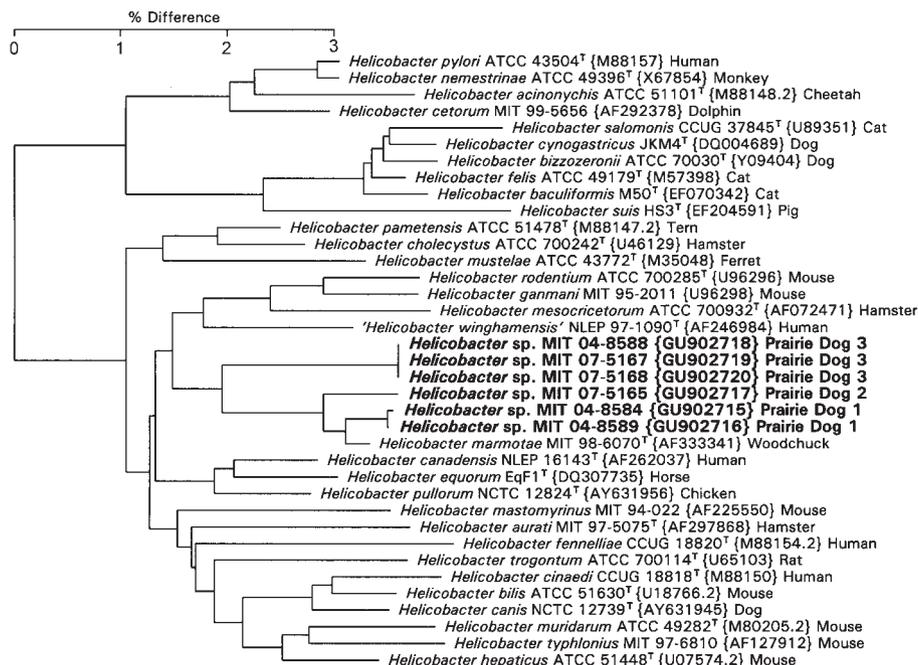
Fig. 2. Neighbour-joining tree for prairie dog *Helicobacter* isolates based on comparison of 16S rRNA gene sequences. Bar, percentage difference in nucleotide sequence. GenBank accession numbers are shown in brackets and host species is indicated. Numbers in bold are isolates described in this study.
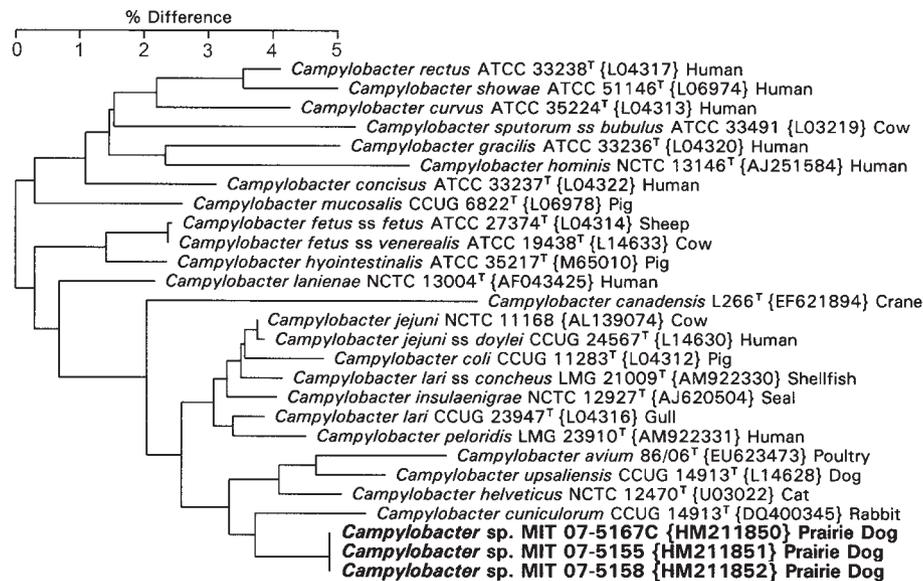
Fig. 3. Neighbour-joining tree for prairie dog *Campylobacter* isolates based on comparison of 16S rRNA gene sequences. Bar, percentage difference in nucleotide sequence. GenBank accession numbers are shown in brackets and host species is indicated. Numbers in bold are isolates described in this study.

formation were seen. Extensive intratumoral haemorrhage, necrosis and intracellular fatty changes 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 the reliability of sufficient quantities of bile, cholesterol stones and tissue for analysis (Brenneman *et al.*, 1972). The isolation of *Helicobacter marmotae*-like organisms from the liver of a prairie dog being used to study cholesterol gallstone formation raises the number of *Helicobacter* species isolated from the livers of mammals and birds to ten. The other *Helicobacter* species isolated from animal livers include *Helicobacter bilis* and *Helicobacter hepaticus* cultured from mice (Fox *et al.*, 1994, 1995, 1996c), *H. bilis* from hamsters (Fox *et al.*, 2009), *Helicobacter cholecystus* from hamsters (Franklin *et al.*, 1996), *Helicobacter canis* from dogs (Fox *et al.*, 1996a), *Helicobacter cinaedi* from monkeys (Fox *et al.*, 2001) and *Helicobacter* sp. flexispira taxa 2 and 5 from sheep (Kirkbride *et al.*, 1985). *H. marmotae* has been cultured from the liver of woodchucks and the faeces of cats (Fox *et al.*, 2002) and *Helicobacter 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 enterohepatic

Helicobacter species and cholesterol cholelithiasis, chronic cholecystitis and gallbladder cancer. Our laboratory prospectively investigated the effect of infection by *Helicobacter* species in cholesterol gallstone pathogenesis in the highly susceptible C57L/J mouse model (Maurer *et al.*, 2005). *Helicobacter*-free adult male C57L mice were infected with several different enterohepatic 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 co-infected with *H. hepaticus* and *Helicobacter 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*, *H. cinaedi* and *H. rodentium* gave a cholesterol gallstone prevalence of 40, 30 and 20 %, respectively; the latter two 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* species may play a role in the pathophysiology of cholesterol gallstone formation in mice (Maurer *et al.*, 2005). We have also previously documented *Helicobacter* species in Chilean females with chronic cholecystitis and cholesterol gallstones (Fox *et al.*, 1998). Others have documented *Helicobacter* species in a variety of hepatobiliary diseases in humans (reviewed by Pellicano *et al.*, 2008).

In this study, *H. marmotae* was isolated from caeca of two prairie dogs and identified by PCR and sequencing of a 700 bp PCR product in the liver of two additional prairie dogs. *Helicobacter* species have been detected in various other wild rodents (Comunian *et al.*, 2006; Fox *et al.*, 2002;
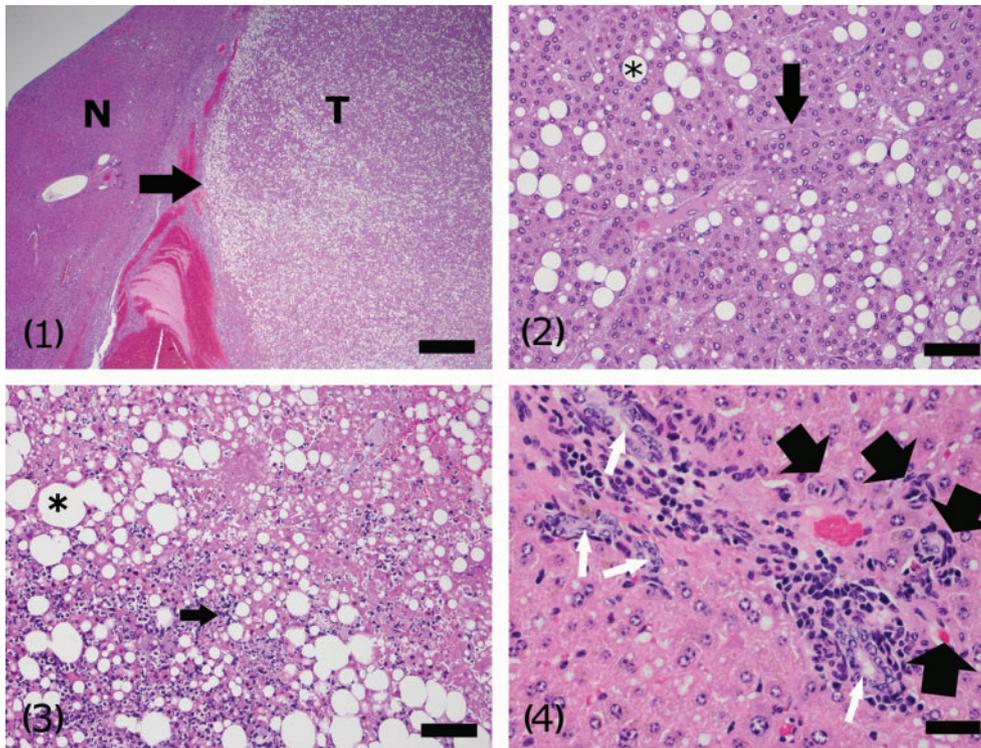
Fig. 4. Hepatocellular carcinoma seen in the liver of prairie dog 08-9090, whose liver was positive for *Helicobacter* species by nested PCR. (1) Normal liver parenchyma (N) is expanded by a tumour (T). An arrow marks the zone of demarcation between normal liver parenchyma and the hepatocellular carcinoma. Bar, 500 μm. (2) A fine connective tissue stroma (arrow) delineates nests of well-differentiated, neoplastic hepatocytes. Lipid vacuoles (*) scattered throughout characterize the fatty changes within the tumour. Bar, 50 μm. (3) A zone of liquefactive necrosis within the tumour, with characteristic loss of cell detail and mixed, large amounts of neutrophilic cell debris (arrow). Fatty changes are again evident by the presence of lipid vacuoles (*). Bar, 50 μm. (4) A portal area within the non-tumorous region of the 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.

Parker *et al.*, 2009; Won *et al.*, 2002). *H. hepaticus*, *Helicobacter apodemus*, *Helicobacter canadensis* and *H. winghamensis* were identified by partial 16S rRNA gene sequence analysis, suggesting 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.* (2006) also detected *Helicobacter* DNA by PCR in faeces 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 co-infected with *Campylobacter* species Three of the *Campylobacter* strains whose 16S rRNA gene was sequenced were considered a novel species, related to *C. cuniculorum* and *C. helveticus*. This finding of co-infection is not surprising given that enteric helicobacters are often found in hosts co-infected with *Campylobacter* species. For example, 64/227 commercially reared cats had microaerophilic bacteria isolated from their faeces that 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* species and *Campylobacter* species. Of the isolates, 92 % (47/51) were positive for *Campylobacter* species, 41 % (21/51 isolates) were positive for *Helicobacter* species and 33 % (17/51 isolates) were positive for both genera. Co-infection with *Campylobacter* species, enteric *Helicobacter* species and *Anaerobiospirillum* species in 52 healthy and 138 diarrhoeic dogs and in 63 diarrhoeic and 21 healthy cats has been reported (Rossi *et al.*, 2008); about 10 % of both dogs and cats were found to be co-infected with *Campylobacter* species and *Helicobacter* species. However, no statistically significant correlation between isolation of single or mixed infections and the presence of diarrhoea was observed (Rossi *et al.*, 2008).

Investigators in South Africa have published results for a protocol that allows primary isolation of multiple *Campylobacter* species and *Helicobacter* species from the diarrhoeic specimens of individual children. Filtrates are plated onto antibiotic-free blood agar plates and incubated in an H$_2$-enriched atmosphere (Lastovica & le Roux, 2000). The authors reported a 16% prevalence of multiple *Campylobacter*

species and *Helicobacter* species based on primary isolation and biochemical characterization. They frequently recovered between two and five species of *Campylobacter* and *Helicobacter* from single stool samples, with *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter upsaliensis*, *Helicobacter fennelliae* and *H. cinaedi* being commonly isolated (Lastovica & le Roux, 2000).

Recently, we reported that *H. marmotae* can successfully colonize mice and cause enterohepatic disease (Patterson *et al.*, 2010). Inflammatory lesions present in the livers of five prairie dogs and a hepatocellular carcinoma in a prairie dog whose liver was positive for *Helicobacter* species by PCR may be related to *Helicobacter* infection, as noted in *H. marmotae*-infected mice or mice infected with *H. hepaticus* (Fox *et al.*, 1996b, 2011; García *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, 1990, 1992). It is also interesting to note the gender differences in the presence of *Helicobacter* species and *Campylobacter* species in the prairie dog. *Helicobacter* species were detected in nine out of 20 males and three out of 14 females, whilst *Campylobacter* species were detected in three out of 20 males and seven out of 14 females. It is not known whether a female gender bias of *Campylobacter* species 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* species and *Campylobacter* species in the pathogenesis of cholesterol gallstones and chronic inflammation in the liver and intestine in this prairie dog model should be conducted.

ACKNOWLEDGEMENTS

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