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Helicobacter canis Isolated from a Dog Liver with Multifocal Necrotizing Hepatitis

J. G. FOX,[1]* R. DROLET,[2] R. HIGGINS,[2] S. MESSIER,[2] L. YAN,[1]
B. E. COLEMAN,[3] B. J. PASTER,[3] AND F. E. DEWHIRST[3]

Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge,[1] and Department of Molecular Genetics, Forsyth Dental Center, Boston,[3] Massachusetts, and School of Veterinary Medicine, University of Montreal, Montreal, Quebec, Canada[2]

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On the basis of biochemical, phenotypic, and 16S rRNA analysis, a novel gram-negative bacterium, isolated from normal and diarrheic dogs as well as humans with gastroenteritis, has been recently named *Helicobacter canis*. A 2-month-old female crossbred puppy was submitted to necropsy with a history of weakness and vomiting for several hours prior to death. The liver had multiple and slightly irregular yellowish foci up to 1.5 cm in diameter. Histologically, the liver parenchyma contained randomly distributed, occasionally coalescing hepatocellular necrosis, often accompanied by large numbers of mononuclear cells and neutrophils. Sections of liver stained by the Warthin-Starry silver impregnation technique revealed spiral- to curve-shaped bacteria predominantly located in bile canaliculi and occasionally in bile ducts. Aerobic culture of liver was negative, whereas small colonies were noted on *Campylobacter* selective media after 5 days of microaerobic incubation. The bacteria were gram negative and oxidase positive but catalase, urease, and indoxyl acetate negative; nitrate was not reduced to nitrite, and the organism did not hydrolyze hippurate. The bacteria were also resistant to 1.5% bile. Electron microscopy revealed spiral-shaped bacteria with bipolar sheathed flagella. By 16S rRNA analysis, the organism was determined to be *H. canis*. This is the first observation of *H. canis* in active hepatitis in a dog and correlates with recent findings of *Helicobacter hepaticus*- and *Helicobacter bilis*-related hepatic disease in mice. Further studies are clearly warranted to ascertain whether *H. canis*-associated hepatitis is more widespread in canines as well as a cause of previously classified idiopathic liver disease in humans.

A *Campylobacter*-like organism similar to *Helicobacter fennelliae* was isolated from the feces of a child suffering from gastroenteritis (4, 7, 26). The organism was distinguished from *H. fennelliae* by its ability to grow at 42°C, its failure to produce catalase, and its marked tolerance to bile. This bacterium more recently has been isolated from feces of normal and diarrheic dogs and has been classified, on the basis of 16S rRNA sequencing, as a novel helicobacter and named *H. canis* (24). Morphologically, the bipolar sheathed flagella of *H. canis* are similar to those in *Helicobacter cinaedi* and *H. fennelliae*, and they are useful in characterizing the organism.

We recently isolated, characterized, and named two novel helicobacters, *H. hepaticus* and *H. bilis*, from livers of mice with hepatitis (11, 13). *H. hepaticus* is also associated with hepatic neoplasms in A/JCr mice, and experimentally this bacterium causes hepatitis in A/JCr inbred mice (29). Additionally, of interest is the isolation of another helicobacter, *H. pullorum*, from diseased chicken livers and from the feces of diarrheic humans (23). The purpose of this report is to characterize, by morphology, biochemistry, and 16S rRNA sequence analysis, bacteria isolated from a diseased liver of a puppy as *H. canis*. This novel finding raises the distinct possibility that other *Helicobacter* spp. induce hepatitis in a variety of mammals, including humans, and thus could be responsible for some cases of what is described in the literature as idiopathic hepatitis.

* Corresponding author. Mailing address: MIT-Division of Comparative Medicine, 77 Massachusetts Ave., 45-106, Cambridge, MA 02139. Phone: (617) 253-1757. Fax: (617) 258-5708.

CASE REPORT

A 2-month old, female mixed-breed puppy was submitted for necropsy with a history of weakness and vomiting for several hours prior to death. At necropsy, the 1-kg puppy was thin and had pale mucous membranes suggestive of anemia. The lungs were diffusely edematous and slightly congested. In the liver there were multiple and slightly irregular yellowish foci up to 1.5 cm in diameter. The pancreatic parenchyma was pale and firmer than normal. The stomach was empty, and the small intestine contained large numbers of ascarid nematodes. Feces appeared normal.

Significant histological changes were restricted to the liver and pancreas. The liver parenchyma contained multiple and irregularly shaped foci of hepatocellular coagulative necrosis usually infiltrated by large numbers of mononuclear cells and neutrophils (Fig. 1). These foci of necrotizing hepatitis were randomly distributed and occasionally coalescing. A prominent sinusoidal leukocytosis was generally found in the areas surrounding these foci. Sections of liver stained by the Warthin-Starry silver impregnation technique revealed numerous spiral- to curve-shaped bacteria predominantly located in the hepatic parenchyma at the periphery of the necrotic lesions (Fig. 2). These organisms were often concentrated between adjacent hepatocytes in the bile canaliculi. A few morphologically similar bacteria were found occasionally in the lumen of the bile ducts. Lesions observed in the pancreas were those of a subacute to chronic active interstitial and necrotizing pancreatitis in which no organisms could be seen.

MATERIALS AND METHODS

Bacterial isolation and biochemical characterization. A piece of liver was submitted for bacteriological analysis. The sample was inoculated on Trypticase soy agar (Difco Laboratories, Detroit, Mich.) with 5% bovine blood and on

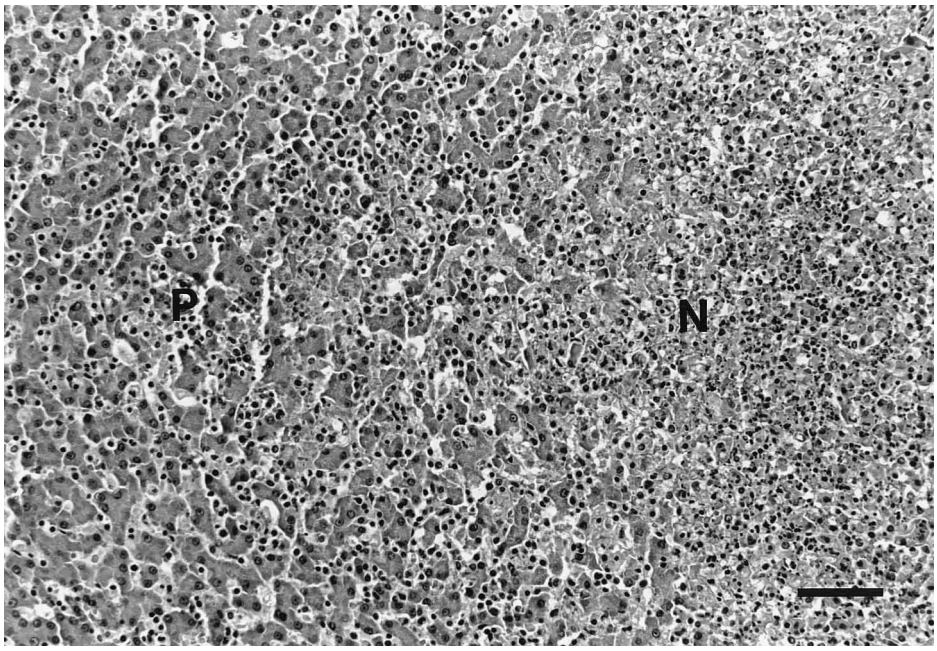

FIG. 1. Dog liver tissue treated with hematoxylin and eosin stain. Focal hepatic necrosis (N) and mixed inflammatory cell infiltration with extension into the parenchyma (P) are present. Bar = 65.6 μm.

Columbia agar (Oxoid, Basingstoke, England) with 10% bovine blood and *Campylobacter* selective supplement SR98 (Blaser-Wang, Oxoid). The blood agar plate was incubated at 37°C in an atmosphere of 5% CO_2 for 2 days. The Columbia agar plate was incubated in a microaerophilic atmosphere (10% CO_2, 4% O_2, ~85% N_2) for 5 days.

Biochemical tests and phenotypic characterization employed were based on previously described media and methods employed by us and others when identifying, characterizing, and naming other *Helicobacter* spp. (11, 13). Colony morphology and Gram staining of bacteria were determined by using organisms obtained after 72 h of incubation of blood agar under microaerobic conditions. Bile tolerance was determined by growth of organisms on 1.5% bile media (1.5% desiccated ox bile [Oxoid] in 5% blood agar).

Electron microscopy. Cells (10^8 per ml) grown on Trypticase soy blood agar plates were suspended in 10 mM Tris buffer (pH 7.4). The bacteria were negatively stained with 1% (wt/vol) phosphotungstic acid (pH 6.5) for 15 to 20 s. Specimens were examined with a JEOL model JEM-1200EX transmission electron microscope operating at 100 kV (13).

16S rRNA sequence analysis. DNA was isolated from cultured cells, the genes coding for 16S rRNA were amplified by PCR, and the amplicon was cycle sequenced as previously described for *H. bilis* isolates (13).

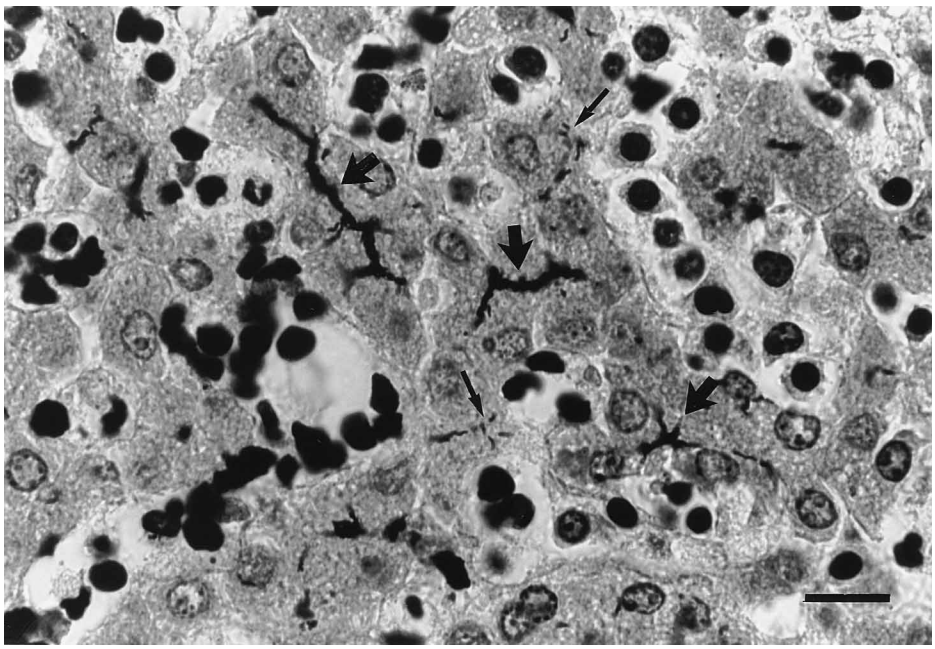

FIG. 2. Dog liver tissue treated with Warthin-Starry stain. Warthin-Starry stain reveals clustered (thick arrows) and individual (thin arrows) spiral-shaped organisms compatible with *H. canis* within the bile canaliculi. Bar = 13.1 μm.

Nucleotide sequence accession numbers. *H. canis* MIT 95-5359 has accession number U65102, and strain CCUG 29176 has accession number L14634.

RESULTS

No growth was detected on the plate incubated under aerobic conditions, whereas small colonies were visible after 3 days on the plate incubated under microaerophilic conditions. Direct examination of colonies revealed the presence of curved gram-negative rods. On the basis of ultrastructural analysis the bacteria were spiral shaped, had bipolar flagella that were sheathed, and measured 3 to 4 μm in length and 0.25 to 0.5 μm in width.

The isolate grew at 37 and 42°C but not at 25°C. It was oxidase positive and catalase, urease, and indoxyl acetate negative; it did not reduce nitrate to nitrite; and it did not hydrolyze hippurate, but it could grow in the presence of 1.5% bile. It was resistant to cephalothin (30 μg) but sensitive to nalidixic acid (30 μg). With the exception of the indoxyl acetate test, which had been reported to be positive for four of five strains, and of the resistance to cephalothin, which was reported for two of five strains (24), this bacterium fulfilled the characteristics of *H. canis*.

The sequence of the puppy isolate MIT 95-5359 differs from the type strain of *H. canis* (L13464) by about 8 bases. The sequence appears to be nearly identical to that for *H. canis* A804-92 (GenBank accession number U04344) as described previously (17). However, the actual sequence in GenBank as of April 1996 is corrupted, particularly in the region from base 760 to base 1010 (*Escherichia coli* numbering). There is an intervening sequence (IVS) in the 16S rRNA gene in about one-third of all *H. canis* strains (17). The puppy isolate, MIT 95-5359, contained an IVS essentially identical to that of *H. canis* A804-92. Prior to the description of *H. canis*, we determined the 16S rRNA sequence of an unusual helicobacter isolate, CCUG 29176, which was isolated from a bacteremic patient with agammaglobulinemia (6a). The sequence for strain CCUG 29176 is almost identical to those for strains MIT 95-5359 and A804-92, but it has a distinctly different IVS, which is 17 bases shorter than those of other *H. canis* strains with IVSs. Whether the strains with IVSs represent one or more distinct subspecies has not been determined.

DISCUSSION

To date, the genus *Helicobacter* includes 13 named species as well as other formally unnamed closely related organisms (6, 10, 11, 13, 16, 18, 23, 24, 27). Since the discovery that *Helicobacter pylori* is a cause of gastritis and peptic ulcer disease and that it has a possible relationship to gastric cancer, other gastric helicobacters have been linked to gastritis in a variety of mammalian hosts (9, 30). More recently, however, an increasing number of *Helicobacter* spp. have been isolated from the lower intestinal tracts of mammals and birds (11, 13, 22–24). Some of these, e.g., *H. cinaedi* and *H. fennelliae*, have been linked to proctitis and colitis in immunocompromised humans (7, 25). In addition, *H. hepaticus* and *H. bilis* have been isolated from livers of mice with hepatitis as well as from intestines of asymptomatic mice (11, 13).

In recent epidemiologic studies regarding the incidence of *Campylobacter*-like organisms in 1,000 dogs, 4% of the animals had an organism, later determined to be *H. canis*, isolated from their feces (2, 24). These bacteria were previously given the name "*H. canis* group" because they evidenced weak DNA homology to *H. cinaedi* and *H. fennelliae* but were distinguishable from these organisms because they were resistant to polymixin B and were unable to reduce nitrate. The organism was also isolated from the feces of a child with gastroenteritis during a similar survey of children to determine the prevalence of *Campylobacter*-like organisms (4). Like that of *H. hepaticus*, *H. bilis*, and *H. pullorum*, its marked resistance to bile probably enables the organism to colonize the liver (11, 13, 23, 24). Unlike the two murine helicobacters, which are strongly urease positive and also colonize livers, *H. canis* and *H. pullorum* are urease negative; this would argue against this phenotype being important for hepatic colonization.

The best-characterized liver lesion caused by a *Helicobacter* sp. is *H. hepaticus*-associated hepatitis in A/JCr mice. In infected mice, the organism causes a multifocal hepatic lesion with cholangitis and vasculitis which progresses in severity to include bile duct hyperplasia, hepatomegaly, oval-cell hyperplasia, hepatocellular proliferation, and in aged A/JCr mice hepatoma or hepatocellular carcinoma (11, 12, 29). The *H. canis*-associated liver lesion noted in the young dog consisted of an acute multifocal necrotizing hepatitis. Interestingly, *Helicobacter*-like organisms were present at the periphery of the hepatic lesion and appeared to be located in bile canaliculi. This pattern of colonization is also noted in *H. hepaticus*-infected livers (11, 12, 29). Determination of whether the lesion persists in the dog, as *H. hepaticus* persists in A/JCr mice, will require further studies (12).

Other types of bacterial hepatitis documented infrequently for dogs have been caused by *Yersinia pseudotuberculosis*, *Salmonella* spp., and rarely *Clostridium* (*Bacillus*) *piliforme*, the causative agent of Tyzzer's disease. Gram-negative enteric bacteria are the most commonly cited microorganisms isolated from dogs with cholecystitis (5). More recently *Campylobacter jejuni* has been isolated from two dogs with bacteremia and cholecystitis (19). Presenting signs included anorexia, fever, and icterus. By ultrasonography, a fluid-filled abnormally thickened gallbladder wall was observed in both dogs. In human cholecystitis cases, *C. jejuni* as well as *Campylobacter fetus* is also recovered infrequently from the bile (27, 28). However, in the future with the recognition that *Helicobacter* spp. are present in liver tissue and bile of various hosts, detailed biochemical and phenotypic descriptions will be necessary to fully characterize the microaerophilic organisms and determine whether they are *Campylobacter* spp. or *Helicobacter* spp.

The mechanism whereby certain species of helicobacters, whose normal ecological niche is the lower intestine, colonize the liver is unknown. Like another intestinal enteropathogen, *Salmonella typhi*, the organism may gain access to the liver by initial M-cell uptake, with spread to the liver via the portal circulation and finally discharge of the bacteria from the liver into the biliary tract (14). Alternatively, there may be direct translocation through enterocytes or migration of the helicobacters from the lumen of the gut into the bile duct. Whether the presence of ascarids in the puppy in the present study facilitated the ability of *H. canis* to colonize the liver and to cause hepatitis is unknown. Organisms compatible with *H. bilis* morphology have been noted previously in bile canaliculi of rats experimentally infected with *Fasciola hepaticus* (8). The author speculated that the liver fluke infection may have promoted the organism's colonization of the liver. Also, "*Helicobacter* (*Flexispira*) *rappini*" has been associated with abortion in sheep, necrotic hepatitis in the aborted fetuses, and intestinal disease in animals and humans (1, 15, 21). It is conceivable but certainly not proven that the puppy had been infected in utero. The cause of the pancreatic lesions in this puppy is unknown; unfortunately, the pancreas was not cultured, but the absence of organisms in pancreatic tissue as determined with special stains may indicate that the pancreatitis was unrelated to *H. canis*.

Although *H. canis* and *H. pullorum* have been isolated from diarrheic children and adults with gastroenteritis, at this juncture evidence that either organism can cause hepatitis in humans is indirect. However, the fact that *H. canis* has been isolated from the blood of humans increases the likelihood that liver infection with *H. canis* also occurs (6a, 18). Also, a patient with *H. pullorum*-associated diarrhea had persistent increases in levels of three liver enzymes as well as hepatomegaly as determined by abdominal ultrasonography (3). Thus, we predict that with the use of appropriate diagnostic media and microaerobic culture conditions, various other *Helicobacter* spp. will also be isolated in cases of hepatitis in humans and companion pet animals.

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REFERENCES

1. **Archer, J. R., S. Romero, A. E. Ritchie, M. E. Hamacher, B. M. Steiner, J. H. Bryner, and R. F. Schell.** 1988. Characterization of an unclassified microaerophilic bacterium associated with gastroenteritis. J. Clin. Microbiol. **26:**101–105.
2. **Burnens, A. P., B. Angeloz-Wick, and J. Nicolet.** 1992. Comparison of *Campylobacter* carriage rates in diarrheic and healthy pet animals. Zentralbl. Veterinaermed. **39:**175–180.
3. **Burnens, A. P., J. Stanley, R. Morgenstern, and J. Nicolet.** 1994. Gastroenteritis associated with *Helicobacter pullorum*. Lancet **344:**1569–1570.
4. **Burnens, A. P., J. Stanley, U. B. Schaad, and J. Nicolet.** 1993. Novel *Campylobacter*-like organism resembling *Helicobacter fennelliae* isolated from a boy with gastroenteritis and from dogs. J. Clin. Microbiol. **31:**1916–1917.
5. **Center, S. A.** 1990. Hepatobiliary infection, p. 150–151. *In* C. E. Greene (ed.), Infectious diseases of the dog and cat. W. B. Saunders, Philadelphia.
6. **Dewhirst, F. E., C. Seymour, C. J. Fraser, B. J. Paster, and J. G. Fox.** 1994. Phylogeny of *Helicobacter* isolates from bird and swine feces and description of *Helicobacter pametensis* sp. nov. Int. J. Syst. Bacteriol. **44:**553–560.

6a. **Domellöf, L.** Personal communication.

7. **Fennell, C. L., P. A. Totten, T. C. Quinn, D. L. Patton, K. K. Holmes, and W. E. Stamm.** 1984. Characterization of *Campylobacter*-like organisms isolated from homosexual men. J. Infect. Dis. **149:**58–66.
8. **Foster, J. R.** 1984. Bacterial infection of the common bile duct in chronic fascioliasis in the rat. J. Comp. Pathol. **94:**175–181.
9. **Fox, J. G.** 1994. *In vivo* models of gastric *Helicobacter* infections, p. 3–27. *In* R. H. Hunt and G. N. J. Tytgat (ed.), *Helicobacter pylori*: basic mechanisms to clinical care. Kluwer Academic Publishers, Boston.
10. **Fox, J. G., T. Chilvers, C. S. Goodwin, N. S. Taylor, P. Edmonds, L. I. Sly, and D. J. Brenner.** 1989. *Campylobacter mustelae*, a new species resulting from the elevation of *Campylobacter pylori* subsp. *mustelae* to species status. Int. J. Syst. Bacteriol. **39:**301–303.
11. **Fox, J. G., F. E. Dewhirst, J. G. Tully, B. J. Paster, L. Yan, N. S. Taylor, M. J. Collins, P. L. Gorelick, and J. M. Ward.** 1994. *Helicobacter hepaticus* sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. J. Clin. Microbiol. **32:**1238–1245.
12. **Fox, J. G., X. Li, L. Yan, R. J. Cahill, R. Hurley, R. Lewis, and J. C. Murphy.** 1996. Chronic proliferative hepatitis in A/JCr mice associated with persistent *Helicobacter hepaticus* infection: a model of helicobacter-induced carcinogenesis. Infect. Immun. **64:**1548–1558.
13. **Fox, J. G., L. Yan, F. E. Dewhirst, B. J. Paster, B. Shames, J. C. Murphy, A. Hayward, J. C. Belcher, and E. N. Mendes.** 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.
14. **Hornick, R. B.** 1995. Enteric fever, p. 325–332. *In* M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg, and R. L. Guerrant (ed.), Infections of the gastrointestinal tract. Raven Press, Ltd., New York.
15. **Kirkbride, C. A., C. E. Gastes, J. E. Collins, and M. S. Ritchie.** 1985. Ovine abortion associated with an anaerobic bacterium. J. Am. Vet. Med. Assoc. **186:**789–791.
16. **Lee, A., M. W. Phillips, J. O'Rourke, B. Paster, F. E. Dewhirst, G. J. Fraser, J. G. Fox, L. I. Sly, P. J. Romaniuk, T. J. Trust, and S. Kouprach.** 1992. *Helicobacter muridarum* sp. nov., a microaerophilic spiral bacterium with a novel ultrastructure isolated from the intestinal mucosa of rodents. Int. J. Syst. Bacteriol. **42:**27–36.
17. **Linton, D., J. P. Clewley, A. Burnens, R. J. Owen, and J. Stanley.** 1994. An intervening sequence (IVS) in the 16S rRNA gene of the eubacterium *Helicobacter canis*. Nucleic Acids Res. **22:**1954–1958.
18. **On, S. L. W., and B. Holmes.** 1995. Classification and identification of campylobacters, helicobacters and allied taxa by numerical analysis of phenotypic characters. Syst. Appl. Microbiol. **18:**374–390.
19. **Oswald, G. P., D. C. Twedt, and P. Steyn.** 1994. *Campylobacter jejuni* bacteremia and acute cholecystitis in two dogs. J. Am. Anim. Hosp. Assoc. **30:** 165–169.
20. **Paster, B. J., A. Lee, J. G. Fox, F. E. Dewhirst, L. A. Tordoff, G. J. Fraser, J. L. O'Rourke, N. S. Taylor, and R. Ferrero.** 1991. The phylogeny of *Helicobacter felis* sp. nov., *Helicobacter mustelae*, and related bacteria. Int. J. Syst. Bacteriol. **41:**31–38.
21. **Romero, S., J. R. Archer, M. E. Hamacher, S. M. Bologna, and R. F. Schell.** 1988. Case report of an unclassified microaerophilic bacterium associated with gastroenteritis. J. Clin. Microbiol. **26:**142–143.
22. **Seymour, C., R. J. Lewis, M. Kim, D. F. Gagnon, J. G. Fox, F. E. Dewhirst, and B. J. Paster.** 1994. Isolation of *Helicobacter* strains from wild bird and swine feces. Appl. Environ. Microbiol. **60:**1025–1028.
23. **Stanley, J., D. Linton, A. P. Burnens, F. E. Dewhirst, S. L. W. On, A. Porter, R. J. Owen, and M. Costas.** 1994. *Helicobacter pullorum* sp. nov.—genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. Microbiology (Reading) **140:**3441–3449.
24. **Stanley, J., D. Linton, A. P. Burnens, F. E. Dewhirst, R. J. Owen, A. Porter, S. L. W. On, and M. Costas.** 1993. *Helicobacter canis* sp. nov., a new species from dogs: an integrated study of phenotype and genotype. J. Gen. Microbiol. **139:**2495–2504.
25. **Totten, P. A., C. L. Fennel, F. C. Tenover, J. M. Wezenberg, P. L. Perine, W. E. Stamm, and K. K. Holmes.** 1985. *Campylobacter cinaedi* (sp. nov.) and *Campylobacter fennelliae* (sp. nov.): two new *Campylobacter* species associated with enteric disease in homosexual men. J. Infect. Dis. **151:**131–139.
26. **Vandamme, P., E. Falsen, R. Rossau, B. Hoste, P. Segers, R. Tytgat, and J. De Ley.** 1991. Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Acrobacter* gen. nov. Int. J. Syst. Bacteriol. **41:**88–103.
27. **Vanderhoop, A. G., and E. M. Veringa.** 1993. Cholecystitis caused by *Campylobacter jejuni*. Clin. Infect. Dis. **17:**133.
28. **Verbruggen, P., U. Creve, A. Hubens, and J. Verhaegen.** 1986. *Campylobacter fetus* as a cause of acute cholecystitis. Br. J. Surg. **73:**46.
29. **Ward, J. M., J. G. Fox, M. R. Anver, D. C. Haines, C. V. George, M. J. Collins, Jr., P. L. Gorelick, K. Nagashima, M. A. Gonda, R. V. Giden, J. G. Tully, R. J. Russell, R. E. Beneviste, B. J. Paster, F. E. Dewhirst, J. C. Donovan, L. M. Anderson, and J. M. Rice.** 1994. Chronic active hepatitis and associated liver tumors in mice caused by a persistent bacterial infection with a novel *Helicobacter* species. J. Natl. Cancer Inst. **86:**1222–1227.
30. **Warren, R. J., and B. J. Marshall.** 1983. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet **i:**1273–1275.