Helicobacter canadensis sp. nov. Isolated from Humans with Diarrhea as an Example of an Emerging Pathogen

JAMES G. FOX,1* CHIH CHING CHIEN,1 FLOYD E. DEWHIRST,2 BRUCE J. PASTER,2 ZELI SHEN,1 PASQUALE L. MELITO,3 DAVID L. WOODWARD,3 AND FRANK G. RODGERS3

Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge,1 and Forsyth Institute, Boston,2 Massachusetts, and National Laboratory for Enteric Pathogens, Winnipeg, Canada3

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We recently analyzed 11 helicobacter isolates cultured from diarrhea patients in Canada. These isolates had been characterized biochemically by restriction fragment length polymorphism (RFLP; AluI, HhaI) analysis and by fatty-acid analysis as Helicobacter pullorum. However, four of the isolates differed biochemically from H. pullorum by their inability to hydrolyze indoxyl acetate and their resistance to nalidixic acid. Using complete 16S rRNA analysis, we determined that these four strains clustered near H. pullorum but had a sequence difference of 2% and therefore represent a novel helicobacter, Helicobacter canadensis. This novel helicobacter could also be distinguished from H. pullorum by RFLP analysis using ApaLI. The number of novel Helicobacter spp. associated with gastrointestinal disease in humans and animals is rapidly increasing. There are now six Helicobacter spp. isolated from diarrheic humans, the other five being H. pullorum, H. canis, “H. r rappini,” H. fennellae, and H. cinerea. This finding highlights the importance of careful molecular analysis in addition to standard biochemical tests in identifying the increasing number of Helicobacter spp. isolated from humans and animals.

Enterohepatic Helicobacter spp. are increasingly recognized as microbial pathogens in humans and animals (6). Helicobacter pullorum was initially isolated from the feces and diseased livers of chickens (14). H. pullorum is now known to colonize many chicken flocks and is commonly isolated from the cecal contents and carcasses of slaughtered chickens (1, 2).

This microaerobe has been linked to a number of cases of gastrointestinal disease in humans (7, 14, 15; A. P. Burnens, J. Stanley, and J. Nicolet, Letter, Lancet 344:1569–1570, 1994). One case in particular was of interest because the organism was isolated from a male with chronic diarrhea who was also suspected of having liver disease based on elevated liver enzymes and an abdominal ultrasound examination (Burnens et al., letter). Reports subsequent to the original description of H. pullorum have cited the difficulty in correctly distinguishing H. pullorum from other enteric helicobacters as well as campylobacters (14, 15). H. pullorum, though clearly identifiable as a helicobacter by 16S rRNA analysis and biochemical features, differs from most other Helicobacter spp. in lacking sheathed flagella. H. pullorum is inert in most biochemical assays, and like most other enteric helicobacters isolated from humans, with the exception of “H. rappini,” H. pullorum is urease negative. However, this biochemical feature does not distinguish it from Campylobacter coli, so the inability of H. pullorum to hydrolyze indoxyl acetate was relied upon to differentiate between these two microaerophiles. Furthermore, H. pullorum had the same biochemical features as Campylobacter lari except its intolerance to 2% NaCl and its sensitivity to nalidixic acid.

It was of interest to us that several strains of H. pullorum previously confirmed using purported H. pullorum 16S rRNA-specific primers, fatty acid analysis, and restriction enzyme analysis with AluI and HhaI (7, 14) were indoxyl acetate positive, a biochemical feature not previously noted in H. pullorum (7). The purpose of this report is to describe indoxyl acetate-positive “H. pullorum” strains isolated from diarrheic humans based on 16S RNA analysis and biochemical profiling as a novel Helicobacter species, Helicobacter canadensis.

MATERIALS AND METHODS

Case histories. Four strains of indoxyl acetate-positive H. pullorum were isolated from diarrheic humans from 1994 to 1999. Unfortunately, any clinical information other than the patients’ residency in Canada is not available. Two of the diarrheic individuals were 31-year-old females. The two males were 17 and 27 years old.

Biological and phenotypic characterization. The four strains were collected and initially characterized by the National Laboratory for Enteric Pathogens, Laboratory Center for Disease Control (LCDC), Health Canada, as H. pullorum based on morphology, biochemical characteristics, and H. pullorum-specific PCR analysis (7, 9). The four indoxyl acetate-positive “H. pullorum” isolates (NLEP-16143, NLEP-16767, NLEP-17813, and NLEP-99-3017) shipped to the Massachusetts Institute of Technology were subjected to a detailed biochemical characterization as previously described by Shen et al. (13). The isolates were examined for catalase, oxidase, and urease activities. With the RapID NH System (Innovative Diagnostic Systems Inc., North Canton, Ohio), the isolates were examined for the presence of alkaline phosphatase hydrolysis and γ-glutamyl transpeptidase and for the hydrolysis of urea. Indoxyl acetate hydrolysis was determined by using indoxyl acetate discs (Remel, Lenexa, Kan.). The isolates were also tested for their ability to reduce nitrate by using nitrate broth (GIBCO Laboratories, Grand Island, N.Y.) (5). Growth at 37 and 42°C under microaerobic conditions was examined at 3- to 4-day intervals for up to 2 weeks. Susceptibility to cephalothin (30 µg/disc) and nalidixic acid (30 µg/disc) was determined by cultivating the organisms in the presence of discs impregnated with the antibiotic in question (Difco Laboratories). The bacteria were also Gram stained and examined for motility in sterile phosphate-buffered saline by phase-contrast microscopy.

Electron microscopy. Isolate NLEP-16143 was examined by electron microscopy. Cells grown on blood agar plates were centrifuged and gently suspended in 10 mM Tris-HCl buffer (pH 7.4) at a concentration of about 107 cells per ml. Samples were negatively stained with 1% (wt/vol) phosphotungstic acid (pH 6.5) for 20 to 30 s. The specimens were examined with a JEOL model JEM-1200EX transmission electron microscope.

DNA extraction. The High Pure PCR template preparation kit (Roche Molecular Biochemicals, Indianapolis, Ind.) was used to extract DNA from bacterial pellets as outlined by the manufacturer and previously described (12).

Helicobacter-specific PCR amplification. A 16S rRNA-based primer set that is genus specific for all Helicobacter spp. was used for PCR amplification. Primer C97 (5′ GCT ATG AGC GGT ATC C) and primer C95 (5′ ACT TCA CCC CAG
The 1.2-kb Helicobacter-specific PCR products of the 16S rRNA gene were subjected to digestion by *Alul* I, *Hha*I, and *Apa*LI. *H. pullorum* and *H. canadensis* had similar RFLP patterns when digested by *Alul* I, *Hha*I, and *Apa*LI, whereas *H. pullorum* does not; *H. canadensis* was therefore digested into two fragments, one of 250 bp and the other of 950 bp (Fig. 2).

**RESULTS**

**Biochemical characterization.** All four strains grew as a spreading film on blood agar at 37 and 42°C and were oxidative and catalase positive and urease, alkaline phosphatase, and \( \gamma \)-glutamyl transpeptidase negative. Unlike other reported *H. pullorum* strains, the organisms were indoxyl acetate positive and naldixic acid (30 mg) and cephalothin (30 mg) resistant. Nitrate reduction was variable (two of four) (Table 1). Phylogenetic trees were constructed by the neighbor-joining method using only those base positions for which data were available for 90% of the bacteria. Similarity matrices were constructed from the aligned sequences by similarity matrix generation, and dendrogram construction and is written in Microsoft QuickBasic for use with PCs (11).

**Restriction fragment length polymorphism (RFLP) of the 16S rRNA gene.** PCR-amplified DNA (20 \( \mu \)g) was digested with 10 U of enzyme in the appropriate buffer recommended by the enzyme manufacturer at 37°C for 3 h. Restriction patterns were compared after the digested PCR products were separated on a 6% Visigel separation matrix. Restriction enzymes *Hha*I, *Apa*I, and *Apa*LI were used for digestion.

**Nucleotide sequence accession number.** The 16S rRNA sequence for NLEP-16143 has been deposited in GenBank under accession no. AF262037.

**TABLE 1. Characteristics which differentiate *H. canadensis* strains from other *Helicobacter* species**

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Catalase production</th>
<th>Nitrate reduction</th>
<th>Alkaline phosphatase activity</th>
<th>Urease activity</th>
<th>( \gamma )-glutamyl transpeptidase activity</th>
<th>Growth with indoxyl acetate</th>
<th>Growth with naldixic acid</th>
<th>Growth with cephalothin</th>
<th>Streaking on TCBS</th>
<th>Polymorphic flagella</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. canadensis</em></td>
<td>ND</td>
<td>R</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>H. pullorum</em></td>
<td>ND</td>
<td>R</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>H. rodentium</em></td>
<td>ND</td>
<td>R</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>H. hepaticus</em></td>
<td>ND</td>
<td>R</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>2</td>
</tr>
<tr>
<td><em>H. pylori</em></td>
<td>ND</td>
<td>R</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>2</td>
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<tr>
<td><em>H. felis</em></td>
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<td>R</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>H. acinonychis</em></td>
<td>ND</td>
<td>R</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>2</td>
</tr>
<tr>
<td><em>H. nemestrinae</em></td>
<td>ND</td>
<td>R</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tbody>
</table>

**TABLE 2. Biochemical characteristics of strains used for comparison of RFLP patterns**

<table>
<thead>
<tr>
<th>Species</th>
<th>Catalase</th>
<th>Indoxyl acetate</th>
<th>Naldixic acid</th>
<th>Cephalothin</th>
<th>Polymorphic flagella</th>
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</thead>
<tbody>
<tr>
<td><em>H. canadensis</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
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<tr>
<td><em>H. pullorum</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td><em>H. rodentium</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td><em>H. hepaticus</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td><em>H. pylori</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td><em>H. felis</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td><em>H. acinonychis</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td><em>H. nemestrinae</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
</tr>
</tbody>
</table>

**Figure 1.** RFLP patterns of the 16S rRNA gene of Helicobacter sp. strain NLEP-16143. (A) Lane 1: *H. canadensis* NLEP-16767, lane 2: *H. canadensis* NCTC 12826, lane 3: *H. canadensis* NLEP-16143. (B) Lane 1: *H. canadensis* NLEP-16767, lane 2: *H. canadensis* NCTC 12826, lane 3: *H. canadensis* NLEP-16143.
DISCUSSION

In this study we identified, based on 16S rRNA analysis and biochemical traits, a novel helicobacter, *H. canadensis*, previously isolated from the feces of diarrheic humans and classified as *H. pullorum*. *H. canadensis* strains are indoxyl acetate-positive helicobacters first described as *H. pullorum* based on 16S rRNA primers designed to be specific for *H. pullorum* (14), as well as on other phenotypic and biochemical features which characterize the organism. Subsequent analysis has revealed that errors in printed sequence and base positions occurred in the *H. pullorum*-specific primers described by Stanley et al. (14). The primers should be as follows: forward, positions 819 to 839, 5′ ATG AAT GCT AGT TGT TGT GAG 3′ (E. coli numbering). Under the conditions used, these primers amplify *H. pullorum* and *H. canadensis* sequences. The reverse primer, with a one-base mismatch near the 3′ end, is evidently not sufficient to prevent PCR amplification. Thus, primers developed for the early description of *Helicobacter* may not be specific and may misidentify newly described *Helicobacter* spp.

In support of our findings, Gibson et al. recently described genetic diversity in *H. pullorum* strains isolated from humans and chickens (7). Two of these strains, NLEP-16143 and NLEP-16767, which we have now characterized as *H. canadensis*, were included in their study (7). Interestingly, these two strains, when analyzed by an amplified RFLP technique, were 73% similar to each other but showed only 33% similarity to 18 other *H. pullorum* strains, all of which grouped at the 70% similarity level (7). The authors concluded that these two Canadian strains were distinct from the other Canadian *H. pullorum* isolates as well as from other strains isolated in other countries (7). These two strains also differed from other *H. pullorum* strains in their *Sac* II pulsed-field gel electrophoresis profiles by not being digested by *Sma* I (7).

We have recently identified cytotoxic distending toxin (CDT) in several enterohelical helicobacters, including *H. pullorum* (3, 16, 17). *H. pullorum* from both human and avian sources has DNA sequence homology and cytotoxic activity which position it as a member of the CDT family of bacterial toxins. Interestingly, *H. canadensis* strains NLEP-16143 and -16767 were tested for the presence of the *cdtB* gene by PCR as well as for the production of the CDT cytotoxic effect and cell cycle arrest and were negative, thus providing further evidence that these novel strains are distinct from *H. pullorum* (17).

In summary, we have identified and named a novel *Helicobacter* sp., *H. canadensis*, associated with diarrhea in humans. Our study reemphasizes that enteric helicobacters cannot always be reliably identified by biochemical reactions or PCR. Proper classification of these novel helicobacters will allow the more accurate description of their pathogenic potential as well.
as elucidation of key features of their epidemiology. For example, since its original isolation and description, *H. pullorum* has been isolated from diarrheic humans in North America and Europe (1, 7, 14; Burnens et al., letter). Because of its association with chicken feces and carcasses, studies have suggested that, as in the case of *Campylobacter jejuni*, a zoonotic link to chicken consumption may exist with *H. pullorum* infection in people as well (7, 14; Burnens et al., letter). Whether *H. canadensis* has similar reservoir hosts and zoonotic potential requires further study.

**Description of *H. canadensis* nov. *H. canadensis* relating to the country of original isolation.** Cells are slender, curved to spiral rods (0.3 by 1.5 to 4 μm), which have one to three spirals. The bacterium is gram negative and nonsporulating; it is motile by means of nonsheathed, 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 urease, alkaline phosphatase, and γ-glutamyl transpeptidase negative but catalase and oxidase positive. The organism hydrolyzes indoxyl acetate, and some strains reduce nitrate to nitrite. Cells are resistant to nalidixic acid and cephalothin. Bacteria have been isolated from the feces of diarrheic humans. The type strain is NLEP-16143 (MIT 98-5491) (= ATCC 700968).

**ACKNOWLEDGMENTS**

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**REFERENCES**