Atypical *Helicobacter canadensis* Strains Associated with Swine†

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Received 2 December 2005/Accepted 4 April 2006

Forty-two *Helicobacter* isolates were isolated from swine feces in The Netherlands and Denmark. All 12 isolates sequenced (16S rRNA gene) formed a robust clade with *Helicobacter canadensis* (~99% similarity). Species-specific PCR indicated that all of the isolates were *H. canadensis* isolates. Although the appearance of the porcine isolates was similar to the appearance of *H. canadensis*, only one of these isolates was able to hydrolyze indoxyl acetate, a cardinal characteristic of this taxon. Examination of the 23S rRNA and *hsd*60 genes revealed high levels of similarity between the porcine isolates and *H. canadensis*. However, amplified fragment length polymorphism genomic typing showed that isolates recovered from swine feces were genetically distinct from *H. canadensis* strains obtained from humans and geese.

Although its pathogenicity has not been fully resolved, *Helicobacter canadensis* is a possible emerging pathogen of humans (7, 23). This taxon is closely related to *H. pullorum* (7, 23, 24), which is found primarily in poultry and retail chicken products (2, 22). *H. canadensis* has been isolated from wild Barnacle and Canada Geese in Europe (24), and Goto et al. (9) detected *H. canadensis* DNA in rodent feces in China. Despite these observations, the significance of avian, rodent, or other animals as reservoirs of *H. canadensis* has yet to be resolved. To our knowledge, *H. canadensis* has never been isolated from domesticated animals.

During a study to assess the antimicrobial susceptibilities of zoonotic and commensal bacteria obtained from food-producing animals in Europe (3), a number of colonies typical of fastidious *Campylobacter* species were isolated from porcine feces after 48 h of incubation on modified charcoal cefoperazone desoxycholate agar containing a supplement of cefoperazone (2). This characteristic is found primarily in poultry and retail chicken products (7, 23). This taxon is closely related to *H. pullorum* (7, 23, 24, 26), and as predicted (by using NCBI GenBank nonredundant nucleotide database using BLASTN). Subsequently, the nucleotide sequences of the isolates recovered from swine were analyzed using programs in the PHYLIP phylogenetic software. Phylogenetic estimates were obtained based on the neighbor-joining distance method. BLASTN analysis indicated that there was a very high degree of sequence similarity with *H. canadensis*, and all 12 isolates formed a robust clade with *H. canadensis* strains isolated from humans and geese (Fig. 1). For 11 of the isolates, the 16S rRNA gene sequence similarity with the type strain of *H. canadensis* (strain ATCC 70968) was ~99% (one to three base substitutions). This level of similarity was higher than that observed by Tee et al. (23) for a strain of *H. canadensis* recovered from an Australian man suffering from bacteremia (isolate W. Tee-Cro) (26 base substitutions). Interestingly, L222 recovered from a Danish pig was found to form a clade with W. Tee-Cro (75% bootstrap support), but there were only 12 base differences between it and the type strain of *H. canadensis* (Fig. 1). A level of similarity of less than 97% in the 16S rRNA gene sequence is typically used to underpin most proposals for new species (5). The high levels of similarity for the 16S rRNA gene between *H. canadensis* and the *Helicobacter* strains from swine that we observed indicated that the porcine isolates are *H. canadensis*. Furthermore, the 16S rRNA gene sequence analysis indicated that isolates obtained from porcine feces were clearly not members of species of *Helicobacter* previously recovered from swine (e.g., *H. pametensis*, *H. rappini*, *H. trogontum*, and “Candidatus H. suis”).

Isolates were then subjected to taxon-specific PCR using the *Helicobacter* (8) and *H. pullorum* (22) primer sets and PCR conditions (Table 1). All porcine isolates were positive for the genus *Helicobacter* (Table 2), and as predicted (by using NCBI Search for short, nearly exact matches), the *H. pullorum* primers also provided an amplicon for all *H. canadensis* reference strains, as well as the isolates obtained from swine (Table 2).

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Therefore, an *H. canadensis*-specific primer set targeting the 16S rRNA gene was designed (Table 1). The PCR conditions used were 15 min at 95°C, followed by 30 cycles of 30 s at 94°C, 60 s at 55°C, and 75 s at 72°C and then 10 min at 72°C. The positive template controls consisted of reference strains of *H.
canadensis (ATCC 700968, ATCC BAA-439, Gotland 93, Gotland 44, ISL 793/92, and ISL 790/92), H. pullorum (ATCC 51801), and H. pylori (SS1, LC20, and LC11). The negative template control consisted of optima water alone. The H. canadensis-specific primer set that we designed resulted in an amplicon with all H. canadensis isolates and all Helicobacter isolates recovered from swine, but not with the H. pullorum or H. pylori reference strains (Table 2).

To confirm that the 42 isolates were H. canadensis, physiological characteristics of all isolates were determined using standard methods (4), with the following modifications: reduction of nitrate was determined using the nitrate disk method (Remel Inc., Lenexa, KS); the ability to grow at 42°C and on media containing 1% glycine was assessed on Karmali agar and Mueller-Hinton agar; hydrolysis of indoxyl acetate was assessed using two commercial methods (the disk method marketed by Remel Inc. and the strip method sold by Fluka); and the sensitivities to nalidixic acid and cephalothin were assessed using the disk impregnation method described by Mills and Gherna (15). The MICs of ciprofloxacin were determined using the agar dilution method described by the Clinical and Laboratory Standards Institute. Sensitivity to sodium fluoride (0.5%) also was assessed (24). Most physiological tests were conducted at least two times; the exception was hydrolysis of indoxyl acetate, which was determined three times with the Remel Inc. disk method, two times with the Fluka strip method, and two or three times with the disk impregnation method.

All porcine isolates formed shiny, gray, translucent to opaque, smooth colonies that were 0.3 to 0.8 mm in diameter.
 TABLE 1. Primer information

<table>
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<th>Target</th>
<th>Primer</th>
<th>Melting temp (°C)</th>
<th>Sequence (5' to 3')</th>
<th>Size (bp)</th>
<th>Reference</th>
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a The primers are degenerate primers in which changes in previously published primer sequences are indicated by boldface type, where K = G or T, R = A or G, and Y = C or T.

b The primers are degenerate primers, where D = A, G, or T; N = A, C, G, or T; R = A or G; and Y = C or T.

on Karmali agar and charcoal cefoperazone desoxycholate agar at 40°C after 48 h. Growth on the former medium was superior. The growth on 5% sheep blood agar was similar to the growth on Karmali agar. None of the isolates were hemolytic, able to grow in the presence of 2.0% or 3.5% (wt/vol) NaCl at 40°C, or produced acid, gas, or H2S in triple sugar iron agar. For many of the isolates, the growth on Columbia agar (50%) and Mueller-Hinton agar (93%) without blood was reduced compared with the growth on Karmali agar. About 15 to 25% of the colonies on Karmali agar swarmed. All isolates were microaerophilic and thermophilic and able to grow well at 40°C and 42°C. Under microaerophilic conditions, no isolates grew at 25°C, restricted growth of some isolates (45%) occurred at 30°C, and all isolates grew at 35°C. All isolates also grew under anaerobic conditions, and detectible growth was observed for some isolates (33%) under aerobic conditions at 40°C on Karmali agar (first transfer). The results of physiological tests for Helicobacter species (4) are shown in Table 2. With the exception of indoxyl acetate hydrolysis, all of the results were consistent with the original description of the four H. canadensis strains described by Fox et al. (7). Hydrolysis of indoxyl acetate is considered a cardinal physiological characteristic of H. canadensis (7, 24). However, the inability of the vast majority (98%) of the Helicobacter isolates from swine to utilize indoxyl acetate raised questions concerning their identity as H. canadensis isolates and/or concerning the robustness of this characteristic to distinguish H. canadensis from H. pullorum; only four and seven isolates of H. canadensis were examined by Fox et al. (7) and Waldenström et al. (24), respectively. Interestingly, we observed that one of the porcine isolates was able to hydrolyze indoxyl acetate. Although weak reactions do occur with some strains belonging to positive taxa,

TABLE 2. Sources of porcine Helicobacter isolates and Helicobacter reference strains, and results of physiological tests and PCR

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Strain(s)</th>
<th>Host</th>
<th>Location(s)</th>
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<th>Ure</th>
<th>IA</th>
<th>42°C</th>
<th>Gly</th>
<th>Cip</th>
<th>NA</th>
<th>Cep</th>
<th>NaF</th>
<th>Alk</th>
<th>Glu</th>
<th>Gen</th>
<th>Hpu</th>
<th>Hcan</th>
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<td>2</td>
<td>100</td>
<td>98</td>
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<td>88</td>
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<td>2</td>
<td>100</td>
<td>98</td>
<td>0</td>
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<td>Gotland, Sweden</td>
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<td>100</td>
<td>98</td>
<td>0</td>
<td>88</td>
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<td>United Kingdom</td>
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</table>

a Cat, catalase; Nit, nitrate reduction; Ure, urease production; IA, indoxyl acetate hydrolysis; 42°C, growth at 42°C; Gly, growth with 1% glycine; Cip, resistance to ciprofloxacin; NA, resistance to nalidixic acid; Cep, resistance to cephalothin; NaF, resistance to sodium fluoride; Alk, alkaline phosphatase hydrolysis; Glu, γ-glutamyl transpeptidase production; Gen, Helicobacter genus-specific primers (16S rRNA gene); Hpu, H. pullorum-specific primers (16 rRNA gene); Hcan, H. canadensis-specific primers (16S rRNA gene); S, sensitive (distinct clearing zone); I, intermediate resistance (reduced number of colonies in clearing zone); R, resistant (no visible clearing zone); +, positive; −, negative; 0, variable; ND, not determined.

b Forty-two strains of H. canadensis isolated from porcine feces at various locations in The Netherlands and Denmark were tested.

c Percentage of positive strains.

d Percentage of resistant strains as determined using the agar dilution method (breakpoint, 4 μg ml−1).

e Percentage of resistant or intermediate resistant strains as determined using the disk diffusion method.

f T = type strain.

g Exact location unknown.
indoxyl acetate utilization is considered to be a constant characteristic of many *Campylobacter* and *Helicobacter* species (15, 18). However, On et al. (16) noted that a small number of isolates of *Campylobacter lari*, an indoxyl acetate-negative taxon, were positive for indoxyl acetate hydrolysis. Consistent with the original description of *H. canadensis* strains isolated from humans (7), none of the porcine isolates of *Helicobacter* were able to hydrolyze alkaline phosphatase. This contrasted with the strains of *H. canadensis* isolated from geese in Sweden and the United Kingdom (24). Furthermore, we observed that all 42 *Helicobacter* isolates recovered from porcine feces were urease negative. Gastric *Helicobacter* species are typically urease positive (21), suggesting that the intestines are the primary niche occupied by the isolates of *Helicobacter* recovered from porcine feces.

Given the anomalous indoxyl acetate results for the porcine *Helicobacter* isolates, representative isolates were examined further morphologically and genetically (targeting the 23S rRNA and *hsp60* genes). Cells of isolates recovered from swine (L172, L178, L179, L184, and L222), *H. canadensis* ATCC BAA-439, and *H. pullorum* ATCC 51801 were examined with a transmission electron microscope. Cells that were 48 h old were fixed in 2% glutaraldehyde and examined with an Hitachi H-7100 microscope operated at 30 kV. *Helicobacter* cells 2.14 to 3.18 μm long and 0.36 to 0.59 μm wide. The morphology of *Helicobacter* isolates from swine were spiral, and all taxa produced cells that possessed bipolar flagella, although some cells were unflagellar (Fig. 2). The flagella were sheathed, and no periplasmic fibers were observed. Cells were 2.14 to 3.18 μm long and 0.36 to 0.59 μm wide. The morphology and dimensions of *Helicobacter* isolates from swine were similar to the morphology and dimensions of the *H. canadensis* reference strain examined and different from the morphology and dimensions of the *H. pullorum* reference strain since this taxon produces unflagellar cells exclusively (22).

To sequence the 23S rRNA gene, the basic primers and PCR conditions described by Sallen et al. (20) for *Listeria* species were used. Based on sequence alignments with *Helicobacter* 23S rRNA sequences, slight modifications were made to primers 8f, 9f, and 16f (Table 1). Contigs of the partial 23S rRNA gene were obtained for L179, L184, L229, and L231; sequences were compared directly with the NCBI GenBank nonredundant nucleotide database; and a majority-rule consensus tree was generated. The four porcine isolates along with the *H. canadensis* strains formed a robust clade compared to other *Helicobacter* species (Fig. 3), with levels of similarity of >99%. Although the taxa were closely related (based on branch length), there was bootstrap support (93%) which suggested that the two *H. canadensis* isolates from humans were distinct from the *Helicobacter* isolates recovered from swine.

To further resolve the phylogenetic relationship with *H. canadensis*, the *hsp60* gene was targeted. This gene has been demonstrated to provide better resolution for *Campylobacter* species than the 16S rRNA gene (12), and Mikkonen et al. (14) utilized this gene to resolve species relationships of 15 *Helicobacter* species, but not *H. canadensis*. Unfortunately, we were unable to obtain an amplification product using primers described by Rusanganwa et al. (19). Therefore, we designed new degenerate primers with the CODEHOP program (http://bioinformatics.weizmann.ac.il/blocks/help/EODEHOP); the primers that we designed (Table 1) were based on the Hsp60 amino acid sequence of nine *Helicobacter* species accessioned in the NCBI database. The PCR mixture consisted of 2 μl of 10× buffer, 0.4 μl of a solution containing each deoxynucleoside triphosphate at a concentration of 10 mM, 1 μl of a solution containing 10 μM HSP60AF and 10 μM HSP60DR, 0.1 μl of a 5-U/μl solution of QIAGEN HotStar Taq, 64.5 μl of optima water, and 2 μl of DNA (0.2 to 1.0 ng). We used a touchdown protocol that consisted of one cycle of 95°C for 15 min, one cycle of 94°C for 30 s, 63°C for 30 s, and 72°C for 1 min, a decrease in the annealing temperature by 1°C per cycle until 53°C, 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min, and a final extension cycle of 72°C for 10 min. Sequences were obtained for strains L178, L184, L222, and L231 and for *H. canadensis* reference strains ATCC BAA-439,
Gotland 93, and ISL 793/92. Sequences were compared directly with the NCBI GenBank nonredundant nucleotide database, and a majority-rule consensus tree was generated. The Helicobacter isolates from porcine feces formed a clade with H. canadensis isolates from humans and geese (Fig. 4). However, the porcine isolates grouped separately from H. canadensis strains from humans and geese, and there was strong bootstrap support for this clade; there were 17 to 20 base substitutions in the hsp60 gene for the isolates recovered from porcine feces compared to the hsp60 gene of the type strain (isolate ATCC 700968), and there were only 1 to 7 base substitutions for the goose and human strains compared to the type strain. For the taxa for which there were hsp60 sequence data for multiple strains (H. bizzozeronii, H. canadensis, H. felis, H. pylori, H. salomonis, and H. trogontum), similar levels of divergence from H. canadensis isolates were observed for H. bizzozeronii and, to a lesser extent, for H. felis (data not shown). This level of within-taxon divergence is consistent with that observed by Mikkonen et al. (14) and Jalava et al. (11).

To address genetic variability, isolates were subsequently subjected to a genomic typing analysis using amplified fragment length polymorphism (AFLP) analysis as described by Owen et al. (17) for H. pylori. Amplified DNA products were separated by electrophoresis on 1.5% agarose gels (Ultrapure electrophoresis grade) at 100 V for 6 h, stained with ethidium bromide, and scanned using a Typhoon variable-mode imager (Amersham Biosciences), and the sizes of amplicons were determined using Fragment Analysis, version 1.1 (Amersham Biosciences). Electrophoretic patterns were converted into binary matrices for all three primers, and the data were combined for analysis. All but one of the porcine isolates, along with reference strains of H. canadensis, H. pullorum, and H. pylori, were analyzed. The DNADIST program of PHYLIP was used to generate a distance matrix by applying the Jukes-Cantor correction. The genetic distance matrix was then analyzed with the NEIGHBOR program of PHYLIP using the unweighted-pair group method using average linkages for clustering. The AFLP patterns indicated that Helicobacter isolates from swine were distinct from H. canadensis isolates from geese and humans and from H. pullorum and H. pylori (Fig. 5).

FIG. 3. Dendrogram based on a majority-rule consensus tree obtained by analyzing the partial 23S rRNA gene of selected porcine Helicobacter isolates (designations beginning with L). The outgroup used in the analysis was Wolinella succinogenes. All four of the porcine isolates of Helicobacter included in the analysis were recovered in The Netherlands (NLD). Isolate L184 was indoxyl acetate hydrolysis positive. All other helicobacters are indicated by their taxonomic names, NCBI accession numbers (in parentheses), and isolate designations (in brackets). Type strains are indicated by “T.” The bar indicates 0.01 nucleotide substitution per base, and the numbers at nodes indicate the support for the internal branches of the tree obtained by bootstrap analysis (percentages of 1,000 bootstraps).
For the 41 porcine isolates of Helicobacter examined, considerable genetic diversity was observed. Only four isolates (isolates L229 and L233 and isolates L226 and L224) were deemed to be potentially members of the same genotype (≥90% similar). The four isolates recovered from Denmark formed a clade with an isolate (L235) from The Netherlands. For other isolates, there were no clear trends for genetic similarity and location of isolation. However, in some instances, isolates that were from the same location and were isolated at similar times formed clades (e.g., isolates L185 and L186 and isolates L229 and L230).

Here we describe a bacterium associated with swine in Europe that is closely related to H. canadensis, including strains isolated from humans suffering from gastroenteritis. Evidence indicated that the 16S rRNA and 23S rRNA gene sequences of the porcine isolates exhibited ~99% similarity to the 16S rRNA and 23S rRNA gene sequences of H. canadensis, indicating the bacteria from swine were H. canadensis sensu stricto. However, only 1 of the 42 porcine isolates was able to hydrolyze indoxyl acetate, a cardinal physiological characteristic that distinguishes H. canadensis from H. pullorum. Furthermore, typing of the isolates indicated that they were genetically distinct from H. canadensis strains obtained from humans and geese, raising questions about the taxonomic status of the porcine H. canadensis isolates. A detailed examination of the taxonomy of H. pullorum and H. canadensis is warranted, and in particular, whether the porcine H. canadensis isolates represent a distinct taxonomic entity, such as a subspecies or genomospecies (1), needs to be resolved. The fact that swine in both Denmark and The Netherlands (various locations) were positive for H. canadensis suggests that this bacterium is widespread in northern Europe, and studies that examine its association with swine and the prevalence at which cells are shed in porcine feces and subsequently contaminate pork also are war-
ranted. Furthermore, the high density of swine production throughout the world, including Europe, coupled with the emerging pathogen status of *H. canadensis*, emphasizes the need to determine whether porcine strains of this bacterium are a health concern for humans.

**Nucleotide sequence accession numbers.** The 16S rRNA gene sequences of isolates L166, L172, L178, L179, L180, L184, L219, L222, L223, L227, L229, and L231 have been deposited in the GenBank database under accession numbers DQ438112, DQ438113, DQ438114, DQ438115, DQ438116, DQ438117, DQ438118, DQ438119, DQ438120, DQ438121, DQ438122, and DQ438123, respectively. Contigs of a partial 23S rRNA gene of L179, L184, L229, and L231 have been deposited in the GenBank database under accession numbers DQ438108, DQ438109, DQ438110, and DQ438111, respectively. *hsp60* sequences of strains L178, L184, L222, and L231 and of *H. canadensis* reference strains ATCC BAA-439, Gotland 93, and ISL 793/92 have been deposited in the GenBank database under accession numbers DQ438101, DQ438102, DQ438106, DQ438107, DQ438103, DQ438104, and DQ438105, respectively.

This research could not have been completed without the excellent work of a number of individuals at the Agriculture and Agri-Food Canada Research Center at Lethbridge. We thank Jenny Gusse for conducting the AFLP genotyping and sequencing of the 23S rRNA and *hsp60* genes; Kathaleen House for carrying out the physiological tests, the taxon-specific PCR, and sequencing of the 16S rRNA gene; and Byron Lee for conducting the transmission electron microscopy.

![FIG. 5. Unweighted-pair group method using average linkages dendrogram of AFLP fingerprints from 41 Helicobacter isolates recovered from swine (designations beginning with L), five reference strains of *H. canadensis*, and one reference strain each of *H. pullorum* and *H. pylori*. Isolate designations not preceded by a taxon name are isolates obtained from swine in The Netherlands or Denmark. Isolates indicated by an asterisk were collected in Denmark. The bar indicates percentages of genetic similarity. The vertical dotted line represents isolates that are ≥90% similar in terms of banding patterns and thus are similar or closely related.](image)
We also thank the European Animal Health Study Center (CEESA) for providing the strains, Georg Hausner (University of Manitoba) for providing advice on analysis of the AFLP data, Stephen On (Danish Veterinary Institute, Copenhagen, Denmark) for providing reference strains of H. canadensis isolated from geese, Andre Buret (University of Calgary) for providing the reference strains of H. pylori, and Ron Teather (Agriculture and Agri-Food Canada, Lethbridge) for his comments on the manuscript.

REFERENCES
6. Reference deleted.
13. Reference deleted.