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High Prevalence of *Helicobacter* Species Detected in Laboratory Mouse Strains by Multiplex PCR-Denaturing Gradient Gel Electrophoresis and Pyrosequencing

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Rodent models have been developed to study the pathogenesis of diseases caused by *Helicobacter pylori*, as well as by other gastric and intestinal *Helicobacter* spp., but some murine enteric *Helicobacter* spp. cause hepatobiliary and intestinal tract diseases in specific inbred strains of laboratory mice. To identify these murine *Helicobacter* spp., we developed an assay based on PCR-denaturing gradient gel electrophoresis and pyrosequencing. Nine strains of mice, maintained in four conventional laboratory animal houses, were assessed for *Helicobacter* sp. carriage. Tissue samples from the liver, stomach, and small intestine, as well as feces and blood, were collected; and all specimens (n = 210) were screened by a *Helicobacter* genus-specific PCR. Positive samples were identified to the species level by multiplex denaturing gradient gel electrophoresis, pyrosequencing, and a *H. ganmani*-specific PCR assay. Histologic examination of 30 tissue samples from 18 animals was performed. All mice of eight of the nine strains tested were *Helicobacter* genus positive; *H. bilis*, *H. hepaticus*, *H. typhlonius*, *H. ganmani*, *H. rodentium*, and a *Helicobacter* sp. flexispira-like organism were identified. *Helicobacter* DNA was common in fecal (86%) and gastric tissue (55%) specimens, whereas samples of liver tissue (21%), small intestine tissue (17%), and blood (14%) were less commonly positive. Several mouse strains were colonized with more than one *Helicobacter* spp. Most tissue specimens analyzed showed no signs of inflammation; however, in one strain of mice, hepatitis was diagnosed in livers positive for *H. hepaticus*, and in another strain, gastric colonization by *H. typhlonius* was associated with gastritis. The diagnostic setup developed was efficient at identifying most murine *Helicobacter* spp.

Mouse models are widely used in biomedical research because of the physiological and genetic similarities of mice with humans, low maintenance costs, and the availability of immunological reagents and a large number of inbred as well as transgenic and knockout strains (20). Moreover, the complete mouse genome has recently been published (http://www.informatics.jax.org/) (21).

The genus *Helicobacter* comprises 24 formally named species and is a group of microaerophilic, gram-negative, spiral to curve-shaped bacteria isolated from the stomachs and intestines of humans and various animal species (8). After the first isolation of *Helicobacter muridanum* from the intestinal mucosa of rats and mice (16), other *Helicobacter* spp., such as *H. hepaticus*, *H. bilis*, *H. rodentium*, *H. typhlonius*, *H. trogontum*, and *H. ganmani*, were isolated from laboratory mice (23, 31). *H. hepaticus* infects the liver and intestinal tract and causes enterocolitis, typhlitis, and hepatitis in germfree mice (7). Furthermore, in susceptible strains (e.g., A/JCr mice), *H. hepaticus* causes chronic hepatitis and hepatocellular carcinoma (5, 29). *H. bilis* colonizes the liver and intestinal tract of mice, has been associated with multifocal chronic hepatitis, and in particular, induces inflammatory bowel disease in interleukin-10-deficient (IL-10−/−) mice (3, 6). *H. typhlonius* causes colitis and typhlitis in severe combined immunodeficient (SCID) and IL-10−/− mice (11). *H. rodentium* and *H. ganmani* have been isolated from the mouse intestine, but the pathogenic potentials of these species are unclear (23, 26), although *H. rodentium* has been isolated from a colony of SCID mice with diarrhea coinfected with *H. bilis* (27).

Various methods for the diagnosis of *H. pylori* infections in humans have been developed and evaluated, such as culture, microscopy, urease activity tests, PCR assays, and serology (2). For other helicobacters, detection depends on culture, and PCR assays were developed for some species (12, 24). Conventional culturing for the detection of *Helicobacter* spp. is time-consuming, up to 3 weeks of culture may be necessary for biochemical and phenotypic characterization of some species, and many enteric species are difficult to culture (24). In addition, the presence of a growing numbers of *Helicobacter* spp. in several animal hosts as well as humans precludes species-specific PCR assays for detection.

The 16S ribosomal DNA (rDNA) consists of highly conserved and highly variable regions (4). Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA has previously been used to identify total microbial populations or groups of bacteria in activated sludge and soil, to analyze seasonal changes of marine bacterial communities and the bacterial compositions of different biofilms, and to study the affiliations of the predominant bacteria in human feces (19). In our laboratory, PCR-DGGE was previously optimized to detect and identify various *Helicobacter* spp. (1). Recently, pyrosequencing successfully identified *Helicobacter* spp. and other bacteria,
based on sequencing of short segments of the 16S rDNA (15, 18).

The aim of this study was to further increase the efficiency of PCR-DGGE by analyzing two regions of the Helicobacter 16S rDNA (the V3 and V6-7 regions), followed by pyrosequencing of the V3 region. The efficiency of the optimized method was evaluated with gastric, intestinal, and hepatic murine tissue specimens in order to study the distribution of Helicobacter spp. and their association with disease, as determined by histopathological analysis of tissue samples.

MATERIALS AND METHODS

Bacterial strains. The murine reference Helicobacter strains, obtained from the Culture Collection of the University of Gothenburg (CCUG), Gothenburg, Sweden, included in this study were H. pylori (CCUG 17878), H. bilis (CCUG 38995, CCUG 41387), H. gastri (CCUG 43527), H. hepaticus (CCUG 44777, CCUG 36367), H. muridarum (CCUG 29262), and Helicobacter sp. flexipara taxon 8 (CCUG 23433). Additional strains were H. rodentium 1707, 2060, and 2178; H. typhlonius MIT 955,369,9136; and H. muridarum ST2. The strains were cultured on brucella blood agar supplemented with 0.1% activated charcoal, as described previously (28), in an atmosphere-generating Anoxomat WS 9000 apparatus (Marr Microbiology, Lichtenvoorde, The Netherlands) at 37°C under microaerobic conditions for all strains except H. gastri, which was cultured anaerobically.

Animals. The mouse strains used in this study were obtained from four different laboratory animal houses (animal houses AH-4, C57BL/6 IL-10 pathogen-free (SPF) and SCID mice, B6sJ1 mice (three females). All animals were housed in a separate trough, where 70% (vol/vol) ethanol was aspirated through the filter probes. The Prep Tool of the workstation was then placed in a trough of 0.5 M sodium hydroxide to denature and release the single-stranded DNA while 5' biotinylated strands remained immobilized on the beads. Next, the beads were washed (10 mM Tris-acetate buffer [pH 7.6]) and transferred to a 96-well pyrosequencing plate containing 1 μl annealing buffer (20 mM Tris-acetate, 2 mM magnesium acetate tetrahydrate [pH 7.6]) and sequencing primer (GCCGAAGAACCTTACC). With the vacuum pressure switched off, a gentle shake of the Prep Tool released the beads into the pyrosequencing plate, which was heated (80°C, 5 min) and left to cool at room temperature to allow annealing of the sequencing primer. The pyrosequencing plate was placed into the process chamber of a PSQ 96 (Pyrosequencing AB) instrument, enzymes, substrates, and nucleotides from the PSQ 96 SQA reagent kit (Pyrosequencing AB) were dispensed. The nucleotide dispensing order was TCAGCTGACATGATGAGAGATCTCTAGATGAGTCGCCCGGCTCGTTGC-3′ with a GC clamp (5′-modi/H11003 of sterile deionized water solution contained 7 M urea and 40% [vol/vol] formamide). DGGE analysis of the V6-7 region (15 μl) was performed as described previously (1). Electrophoresis was performed at 60°C in 0.5 X TAE buffer (20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA [pH 8.3]) at 200 V for 4 h with a 0.6 cm magnesium acetate (Bio-Rad). The gels were stained with etidium bromide (0.2 μg/ml in 0.5 X TAE buffer) for 15 min and visualized with a GelFotostation (Techtm Lab, Umsel, Sweden).

Pyrosequencing of the V3 region. The separated DNA fragments were excised from DGGE gels with a scalpel and transferred to microcentrifuge tubes containing 160 μl of sterile deionized water filtered through a Millipore filter. To separate the DNA from the polyclaramide gel, the tubes were briefly centrifuged (6,000 × g, 10 s) and subjected to two freeze-thaw cycles (−80°C for 1 h, room temperature for 1 h, and −80°C for 1 h). Subsequently, the supernatants were thawed at 4°C for 2 h and 2.0 μl was used as a template in a PCR mixture containing biotinylated primers BSFI97 and BSR114 under the conditions described above. Helicobacter genus-specific PCR products, extracted from the agarose gels with Ultrafree DA centrifuge tubes (Millipore). Single-stranded DNA was obtained with a Vacuum Prep workstation (Pyrosequencing AB, Uppsala, Sweden). Streptavidin-coated Sepharose beads (Amersham Biosciences) were added to the PCR plate containing the biotinylated PCR products, and the mixture was agitated (10 min, room temperature). A vacuum was applied, and the beads with immobilized PCR products were moved to a separate trough, where 70% (vol/vol) ethanol was aspirated through the filter. The Prep Tool of the workstation was then placed in a trough of 0.5 M sodium hydroxide to denature and release the single-stranded DNA while 5' biotinylated strands remained immobilized on the beads. Next, the beads were washed (10 mM Tris-acetate buffer [pH 7.6]) and transferred to a 96-well pyrosequencing plate containing 1 μl annealing buffer (20 mM Tris-acetate, 2 mM magnesium acetate tetrahydrate [pH 7.6]) and sequencing primer (GCCGAAGAACCTTACC). With the vacuum pressure switched off, a gentle shake of the Prep Tool released the beads into the pyrosequencing plate, which was heated (80°C, 5 min) and left to cool at room temperature to allow annealing of the sequencing primer. The pyrosequencing plate was placed into the process chamber of a PSQ 96 (Pyrosequencing AB) instrument, enzymes, substrates, and nucleotides from the PSQ 96 SQA reagent kit (Pyrosequencing AB) were dispensed. The nucleotide dispensing order was TCAGCTGACATGATGAGAGATCTCTAGATGAGTCGCCCGGCTCGTTGC-3′ with a GC clamp (5′-modi/H11003 of sterile deionized water solution contained 7 M urea and 40% [vol/vol] formamide). DGGE analysis of the V6-7 region (15 μl) was performed as described previously (1). Electrophoresis was performed at 60°C in 0.5 X TAE buffer (20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA [pH 8.3]) at 200 V for 4 h with a 0.6 cm magnesium acetate (Bio-Rad). The gels were stained with etidium bromide (0.2 μg/ml in 0.5 X TAE buffer) for 15 min and visualized with a GelFotostation (Techtm Lab, Umsel, Sweden).

Histologic examination. H. hepaticus and H. typhlonius have been previously shown to cause pathological changes in specific strains of deficient mice (7, 11). On the basis of the identification results obtained in this study, 30 tissue biopsy specimens from 18 animals, comprising mainly liver tissue (n = 15), stomach tissue (n = 3), and lungs (n = 4) and some intestinal tissue (n = 12), and some samples that generated genus-specific PCR products, were extracted from the V3 region (15 μl) DNA was added to the reaction mixture, whereas 5 μl of sterile deionized water filtered through a Millipore filter (Bedford, Mass., filter was used as a negative control. Detection of the amplified PCR products was done by agarose gel electrophoresis (1)).

DGGE. The 16S rDNA sequences of different Helicobacter spp. were analyzed with WinMelt software (Bio-Rad, Hercules, Calif.) to assist with primer selection and DGGE analysis. DGGE analysis of the V3 region was performed on 9% polyacrylamide (acrylamide-bisacrylamide [37:5:1]) gels containing a urea and formamide gradient from 20 to 40% (100% denaturing solution contained 7 M urea and 40% [vol/vol] formamide). DGGE analysis of the V6-7 region (15 μl) was performed as described previously (1). Electrophoresis was performed at 60°C in 0.5 X TAE buffer (20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA [pH 8.3]) at 200 V for 4 h with a 0.6 cm magnesium acetate (Bio-Rad). The gels were stained with etidium bromide (0.2 μg/ml in 0.5 X TAE buffer) for 15 min and visualized with a GelFotostation (Techtm Lab, Umsel, Sweden).
TABLE 1. Prevalence of Helicobacter spp. in conventional mice determined by Helicobacter genus-specific PCR analysis

<table>
<thead>
<tr>
<th>Animal house</th>
<th>Mouse strain</th>
<th>Feces</th>
<th>Stomach</th>
<th>Liver</th>
<th>Small intestine</th>
<th>Blood</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH-1</td>
<td>C57BL/6</td>
<td>6/6</td>
<td>3/6</td>
<td>0/6</td>
<td>1/6</td>
<td>0/6</td>
<td>10/30</td>
</tr>
<tr>
<td>AH-2</td>
<td>SPF-SCID</td>
<td>5/5</td>
<td>3/5</td>
<td>4/5</td>
<td>1/5</td>
<td>3/5</td>
<td>16/25</td>
</tr>
<tr>
<td>AH-2</td>
<td>B6sJl</td>
<td>3/3</td>
<td>2/3</td>
<td>1/3</td>
<td>0/3</td>
<td>0/3</td>
<td>6/15</td>
</tr>
<tr>
<td>AH-2</td>
<td>SCID</td>
<td>2/2</td>
<td>1/2</td>
<td>1/2</td>
<td>2/1</td>
<td>2/2</td>
<td>6/10</td>
</tr>
<tr>
<td>AH-3</td>
<td>BALB/cA</td>
<td>5/5</td>
<td>4/5</td>
<td>3/5</td>
<td>2/5</td>
<td>1/5</td>
<td>15/25</td>
</tr>
<tr>
<td>AH-3</td>
<td>C57BL/6</td>
<td>6/6</td>
<td>3/6</td>
<td>0/6</td>
<td>1/6</td>
<td>0/6</td>
<td>10/30</td>
</tr>
<tr>
<td>AH-3</td>
<td>C3H/HeJ</td>
<td>4/4</td>
<td>3/4</td>
<td>0/4</td>
<td>1/4</td>
<td>0/4</td>
<td>8/20</td>
</tr>
<tr>
<td>AH-3</td>
<td>ApoE−/−</td>
<td>5/5</td>
<td>4/5</td>
<td>1/5</td>
<td>0/5</td>
<td>0/5</td>
<td>10/25</td>
</tr>
<tr>
<td>AH-4</td>
<td>IL-10−/−</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/30</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>36/42</td>
<td>23/42</td>
<td>9/42</td>
<td>7/42</td>
<td>6/42</td>
<td>81/210</td>
</tr>
</tbody>
</table>

PCR detection of Helicobacter spp. in mice. The distribution of Helicobacter-positive samples among the different mouse strains is shown in Table 1. The Helicobacter genus-specific PCR assay detected Helicobacter DNA in at least one of the five specimens sampled from each mouse, i.e., in 81 of 210 (38.6%) of the specimens examined and in 36 of 42 (85.7%) of the mice analyzed. All animals except for the six IL-10−/− mice from AH-4 were PCR positive for Helicobacter in feces; all samples from the IL-10−/− mice tested were Helicobacter negative. Gastric tissue samples from 23 of 42 (54.8%) animals were positive. The lowest prevalence of helicobacter was observed in blood samples (6 of 42 [14.3%]), followed by those from the small intestine (7 of 42 [16.7%]) and then those from the liver (9 of 42 [21.4%]). Helicobacter DNA was equally distributed in gastric and intestinal tissue specimens, regardless of the mouse strain and source (i.e., animal house). However, a strain-dependent Helicobacter prevalence was observed in liver tissue and blood samples (Table 1). For liver tissue samples, 80% (4 of 5) of the SPF-SCID mice from AH-2 and 60% (3 of 5) of the BALB/cA mice from AH-3 were Helicobacter positive, whereas liver samples from only 2 of the remaining 32 animals were Helicobacter positive. Helicobacter DNA was detected in the blood of five of seven (71%) SCID mice (conventional SCID mice plus SPF-SCID mice), whereas the blood of only one additional mouse was positive. The prevalence of Helicobacter sp. DNA among the different mouse strains was highest in the SPF-SCID strain of mice (16 of 25 mice [64%]), followed by conventional SCID mice (6 of 10 [60%]) and BALB/cA mice (15 of 25 [60%]). The rates of detection of Helicobacter sp. in specimens of the remaining mouse strains ranged from 0 to 40%. A similar prevalence was observed in C57BL/6 mice, which were collected from two animal houses (AH-1 and AH-3) (Table 1).

PCR-DGGE and pyrosequencing analysis. The principle of the diagnostic assay developed in this study is shown in Fig. 1. PCR-DGGE analysis of the V3 and V6-7 regions was efficient at identifying murine Helicobacter spp. by defining a specific mobility pattern of the PCR product for each species except H. ganmani and H. rodentium, whose amplicons could not be differentiated on the DGGE gels (Fig. 2 and Table 2). Therefore, an H. ganmani-specific PCR assay was applied to specimens with PCR products that migrated similar to those of H. ganmani and H. rodentium in the DGGE analysis (Table 2). DGGE detected 94 PCR products from 81 PCR-positive specimens, due to the presence of more than one PCR product in some samples with different DGGE profiles. DGGE of the V6-7 region of the PCR products amplified from specimens from mice in AH-2 and AH-3 showed migration patterns different from those of the other murine Helicobacter type strains tested (Fig. 2), whereas DGGE of the V3 region showed a migration pattern similar to that for H. muridarum for 38 of 84 (45%) of Helicobacter-positive specimens. BLAST analysis of
FIG. 2. Migration patterns of V3 and V6-7 regions of 16S rDNA by DGGE analysis of murine Helicobacter spp.

distributions of the different Helicobacter spp. in the different mouse specimens were highly variable. H. hepaticus and H. typhlonius were detected in all tissue types, whereas H. bilis was detected in fecal samples only. H. ganmani and the Helicobacter sp. flexispira-like helicobacter were found in fecal and gastric samples (one blood sample was also positive for the Helicobacter sp. flexispira-like helicobacter), and H. rodentium was identified in of stomach and small intestine tissue specimens.

Histologic examination. Histological changes were observed in 9 of 30 (30%) of the tissue specimens examined, of which 7 were helicobacter positive (Table 4). Except for the liver and the stomach tissue of one IL-10−/− mouse that was helicobacter negative and that showed few abscesses, no changes were observed in tissues negative for Helicobacter spp. However, changes were seen in 11 Helicobacter-positive specimens. Hepatitis was diagnosed in all the livers of three BALB/cA mice and one ApoE−/− mouse that were positive for a Helicobacter sp. identified as H. hepaticus. Helicobacter-negative livers from the same mouse strains were normal (Table 4). The hepatic inflammation in the affected animals consisted of a few foci of infiltrating polymorphonuclear leukocytes and lymphocytes in various areas of the lobules. The hepatocytes in the foci had increased cytoplasmic basophilia, with a loss of nuclei in some of the cells (Fig. 4A). Kupffer cells appeared to be prominent in one liver, and slight hepatic steatosis was detected in two of the livers. Chronic gastritis and duodenitis were observed in all three C57BL/6 mice cocolonized with H. typhlonius and H. rodentium or H. ganmani (Table 4). The gastritis was characterized by heavy infiltration of polymorphonuclear leukocytes in the basal part of the lamina propria, the submucosa of the corpus, and, to some extent, the adjacent muscular layers (Fig. 4B).

DISCUSSION

An increasing number of Helicobacter spp. are being isolated from a large number of animal species as well as from humans; and some Helicobacter spp., such as H. pullorum and H. cinaedi, infect multiple hosts (8). Hosts, such as rodents and humans, can be colonized with a range of helicobacters, some of which cause or are associated with gastrointestinal disorders. Diagnostic methods such as culture, microscopy, PCR assays, PCR-restriction fragment length polymorphism analysis, and serology have been developed for the detection of murine helicobacters (12, 22, 25, 30). Mahler et al. (17) reported that differentiation of murine Helicobacter spp. by colony morphological or histologic features was not possible. Moreover, bacteriological culturing for the detection of Helicobacter spp. may require weeks, and isolation can be compromised by the overgrowth of other bacteria. Helicobacter sp.-specific PCR assays are limited by the close relatedness of the 16S rDNA and by the large and continuously increasing number of species. PCR-restriction fragment length polymorphism analysis is efficient when it is used with genomic DNA from cultured organisms, but its value is uncertain for the direct detection of Helicobacter in animal tissues, in which colonization with more than one Helicobacter spp. can occur (13, 27). Consequently, culture-independent methods for the direct detection and identification of species of this genus in biological samples are important.
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</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>2 SPF-SCID</td>
<td>17% (10) 100% H. rodentium</td>
<td>33% H. hepaticus</td>
<td>39% H. muridarum</td>
<td>20% H. bilis</td>
<td>83% H. trogontum</td>
<td>56% H. typhlonius</td>
<td>17% H. ganmani</td>
</tr>
<tr>
<td>SCID</td>
<td>2 B6sJl</td>
<td>17% (10) 100% H. rodentium</td>
<td>33% H. hepaticus</td>
<td>39% H. muridarum</td>
<td>20% H. bilis</td>
<td>83% H. trogontum</td>
<td>56% H. typhlonius</td>
<td>17% H. ganmani</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>3 SPF</td>
<td>17% (10) 100% H. rodentium</td>
<td>33% H. hepaticus</td>
<td>39% H. muridarum</td>
<td>20% H. bilis</td>
<td>83% H. trogontum</td>
<td>56% H. typhlonius</td>
<td>17% H. ganmani</td>
</tr>
</tbody>
</table>

**TABLE 2. Identification of Helicobacter spp. in Helicobacter genus-positive mouse specimens by multiplex PCR-DGGE.**

- Helicobacter spp. were identified in 100% of the mice from C57BL/6, 9/10 (90%) from SCID, and 7/8 (87.5%) from SPF mice.
- H. rodentium was identified in 100% of the C57BL/6, 90% of the SCID, and 75% of the SPF mice.
- H. hepaticus was identified in 33% of the C57BL/6, 10% of the SCID, and 20% of the SPF mice.
- H. muridarum was identified in 39% of the C57BL/6, 10% of the SCID, and 10% of the SPF mice.
- H. bilis was identified in 20% of the C57BL/6, 10% of the SCID, and 10% of the SPF mice.
- H. trogontum was identified in 83% of the C57BL/6, 10% of the SCID, and 10% of the SPF mice.
- H. typhlonius was identified in 56% of the C57BL/6, 10% of the SCID, and 10% of the SPF mice.
- H. ganmani was identified in 17% of the C57BL/6, 10% of the SCID, and 10% of the SPF mice.
Previously, a PCR-DGGE assay targeting the V6-7 region of 16S rDNA was developed for the identification of most Helicobacter spp. in the guts of zoo animals, which was based on an optimized Helicobacter genus-specific PCR assay with a detection sensitivity of 500 CFU of H. pylori/g of spiked feces (1). As this may represent a limiting step, we also included a new region of the 16S rDNA (the V3 region) for the PCR-DGGE analysis used in the present study. The close relatedness of the 16S rDNA among certain species of the genus Helicobacter may lead to similar migration profiles by PCR-DGGE; this was observed for reference strains of H. bilis, H. ganmani, and H. rodentium in the analysis of the V6-7 region and in the analysis of the V3 region for H. ganmani and H. rodentium, as well as for H. typhlonius and H. muridarum. By contrast, using another set of 16S rDNA-specific primers, Grehan et al. (13) obtained different DGGE profiles for the PCR products, whose sequences were homologous to the H. ganmani sequence by DNA sequence analysis. In this study, only one DGGE profile for the PCR products of the H. ganmani strains was detected. Optimally, the primers used for PCR-DGGE should be selected so that they amplify conserved regions of different strains within a species but, simultaneously, retain variability to consistently differentiate organisms at the species level, thereby avoiding interpretation errors leading to misidentification.

PCR-DGGE analysis of both the V3 and the V6-7 regions combined with pyrosequencing allowed identification of most helicobacters, but not H. ganmani and H. rodentium, as well as a Helicobacter sp. flexispira-like helicobacter which possessed a V6-7 mobility pattern different from those of any of the PCR products of the reference strains but similar to that of H. trogontum in the V3 analysis. Given the high level of strain-to-strain variation in the 16S rDNA reported for H. trogontum and the closely related Helicobacter sp. flexispira-like helicobacter strains (14), a definitive identification would not be possible. Distinction of H. ganmani and H. rodentium required an H. ganmani-specific PCR assay. The close relationship of H. ganmani and H. rodentium has been thoroughly examined previously, and a published PCR assay targeting the 16S rDNA of H. rodentium was shown to amplify both species (23). For this reason, we developed a PCR assay for H. ganmani that targeted the 16S-23S rDNA internal spacer region but that did not amplify genomic DNA of H. rodentium (Tolia et al., submitted).

Pyrosequencing has been used for the identification of Helicobacter spp. in different specimens of naturally infected mouse strains from three animal houses (AH-1 to AH-3). Mouse strains shown are C57BL/6 (AH-1); SPF-SCID, B6sJl, and SCID (AH-2); and BALB/cA, C57BL/6, C3H/HeJ, and ApoE−/− (AH-3). The specimens examined were feces (F), stomach tissue (S), liver tissue (L), small intestine tissue (SI), and blood (B).
icobacter spp. and other bacteria. Pyrosequencing is done by sequencing short segments (25 to 30 nucleotides) of variable 16S rDNA, creating a 16S rDNA signature (15, 18). In the present study, we obtained up to 73 nucleotides (average length, 51 bases; 29 to 73 bases in total) by pyrosequencing of the V3 region of the 16S rDNA. Pyrosequencing alone could not separate closely related murine Helicobacter spp., but analyses of the pyrosequences with the BLAST algorithm confirmed the results of the multiplex DGGE analysis.

The isolation of several novel murine helicobacters may call into question the suitability of some laboratory mouse strains as experimental animal models of *H. pylori* infection, especially those involving potential vaccine candidates and other immunological aspects. In addition, past results obtained with a number of experimental murine models in other areas of research should be interpreted with caution. Importantly, exposure to *Helicobacter* spp. antigens prior to experimental infection may influence the host immune response and, potentially, the results obtained. Furthermore, enteric helicobacters such as *H. hepaticus* and *H. typhlonius* have been shown to cause chronic liver and enteric diseases in susceptible strains of mice (11, 29). Moreover, *H. hepaticus* infection has been shown to be commonly associated with chronic active hepatitis (10) and is widespread among the mice offered by commercial mouse breeders in the United States (24). In the present study, liver specimens of male BALB/cA mice that were positive for *H. hepaticus* by PCR-DGGE were affected by hepatitis (Fig. 4A), whereas BALB/cA livers negative for *Helicobacter* spp. appeared normal. Our findings support previous observations of investigators in the United States (29) and further demonstrate the widespread pathogenic potential of *H. hepaticus* for mice. In addition, *H. typhlonius* has been shown to cause enteric lesions in immunodeficient mice (11), and we observed an acute gastritis associated with *H. typhlonius* colonization in C57BL/6 mice. Although it is possible to deduce that *Helicobacter* spp. are involved in the gastric and hepatic disease development observed in the present study, a disease association does not prove a causal effect, and thus, further studies are required.

Some mice, such as the ApoE−/− strain, were colonized with a single *Helicobacter* sp., whereas all other mouse strains in the same animal house tested were colonized with at least three *Helicobacter* spp., suggesting that host factors influence the susceptibility to colonization with *Helicobacter* spp. Moreover, various degrees of immune deficiency may influence colonization with *Helicobacter* spp., as observed with SCID mice, which displayed the highest prevalence of *Helicobacter* sp. infections. On the other hand, it cannot be excluded that environmental factors, such as food composition, water, and/or fecal contamination, also contribute to differences in *Helicobacter* sp. colonization among the various mouse colonies.

In conclusion, the broad-range diagnostic setup developed in this study was efficient at detecting and identifying most *Helicobacter* spp. colonizing the different parts of the gastrointestinal tracts of laboratory mice. Moreover, we have shown
an intriguing pattern of murine Helicobacter sp. colonization and the association of Helicobacter spp. with gastric and hepatic diseases not previously demonstrated in European rodent colonies. The PCR-DGGE approach efficiently detected coloni- zation of a single specimen with multiple species and could prove favorable for the study of this widespread genus and the zoonotic potentials of some helicobacters. It seems likely that other colonies of laboratory rodents also host Helicobacter spp. and that more studies should be undertaken to reveal possible routes of transmission, with the ultimate aim of formulating practical guidelines for the creation and maintenance of Helico- bacter-free rodent colonies.

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