Development of a Noninvasive Method for Detecting and Monitoring the Time Course of Helicobacter pylori Infection

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Received 26 August 2003/Returned for modification 30 November 2003/Accepted 1 June 2004

Helicobacter pylori infection status following experimental inoculation of mice presently requires euthanasia. The purpose of this study was to develop a method for following the time course of H. pylori infection in live experimental animals. Twenty-six C57BL/6, Helicobacter-free female mice were inoculated with H. pylori Sydney strain 1, and 16 mice were sham inoculated. The mice were repeatedly tested during a period of about 1 year with an H. pylori species-specific primer-based PCR analysis of DNA extracted from fecal pellets of mice. The mice were euthanized at 6 months (n = 15) and 10 months (n = 15) to determine their infection status by histology, culture, and PCR of gastric specimens. H. pylori-inoculated mice were tested via the PCR method at 6 and 10 months prior to necropsy. Nine of 13 (69%) and 10 of 13 (77%) mice tested at 6 and 10 months, respectively, were positive. All sham-inoculated mice were negative. These two PCR results suggested a specificity of 100% with a sensitivity range between 69 and 77%. In contrast, sensitivity and specificity rose to 90 and 100% if groups of mice were tested once daily for 4 days. Seventy-seven to 85% of the experimental mice were also positive for H. pylori by culture. The histopathology demonstrated mild to severe gastritis. These findings demonstrate that the persistence or transience of H. pylori infection in live mice can be repeatedly evaluated over time. This method could allow the determination of the time course of infection and the efficacy of medications and/or vaccine without necropsy.

Helicobacter pylori is a spiral gram-negative bacterium that colonizes the stomachs of over half of the world population (23). It consistently causes chronic active gastritis and represents a major risk factor for peptic ulcer disease (3, 24), adenocarcinoma of the stomach, and mucosa-associated lymphoid tissue lymphoma (11, 19, 30, 31, 37). The World Health Organization has classified H. pylori as a class I carcinogen (2). Therefore, it is important to improve our understanding of the mechanism of H. pylori pathogenicity and to develop novel diagnostic and therapeutic approaches.

Only a few animal species can be persistently colonized following experimental inoculation with H. pylori. This has been demonstrated by the development and validation of useful models (9, 16, 21, 22, 23). The mouse model has been widely used, as it combines a number of advantages. Mice are relatively inexpensive, and there are several inbred strains as well as transgenic and knockout mice with resulting pathologies similar to those of H. pylori-infected humans (22, 23). The use of transgenic and knockout mice to study Helicobacter infection is growing (12, 29). However, the evaluation of H. pylori persistence following experimental inoculation of mice usually requires euthanasia of the animals and subsequent histology and culture of the gastric specimen. As a result, multifaceted experiments requiring the repetitive demonstration of H. pylori in individual mice over time have not been performed. For example, an inoculated mouse cannot be tested for bacterial colonization prior to antimicrobial treatment, and the time course of the infection cannot be studied in the same animal once euthanized.

Noninvasive methods that can detect H. pylori infection in humans can be considered for use in mice. For example, immunoglobulin G serology (32) would demonstrate the presence of anti-H. pylori antibodies. However, this test does not allow detection of the early stages of infection and it remains positive for over 6 months after eradication of H. pylori or after resolution of gastritis (30). The 13C-urea breath test (4) and the more recently developed 14C-urea blood test (8) are sensitive, but these tests may lack specificity in mice that often carry urease-positive bacteria in the stomach. Detection of H. pylori by culture of human feces has been successful in only a few studies (20). Agha-Amiri et al. (1) recently reported a novel assay for the detection of H. pylori antigens in human stool specimens with monoclonal antibodies. A theoretically more sensitive and specific approach is the use of PCR, which can detect small amounts of H. pylori nucleic acid in gastric biopsy specimens (7, 15).

The goal of the present study was to evaluate the sensitivity, specificity, and applicability of a PCR-based detection method for H. pylori in fecal pellets of mice, thus eliminating the requirement for euthanasia.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The H. pylori strain used in this study was Sydney strain 1 (SS1), kindly provided by A. Lee and Jani O’Rourke, University of New South Wales, Sydney, Australia (22). The strain was grown for 16 to 18 h at 37°C in a microaerophilic atmosphere and in bisulphite-less Brucella broth (BLBB) (17) containing 10% fetal bovine serum (HyClone, Logan, Utah). For solid medium, 1.5% agar was added. Cultures were tested for urease, catalase, and oxidase activities and monitored microscopically (including a Gram stain) (27, 33). Helicobacter bilis (ATCC 51632), Helicobacter hepticus (ATCC
Mass.) were used in compliance with guidelines and protocol approved by the National Institutes of Health (NIH) animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (Rockville, Md.). The mice were housed in a specific-pathogen-free animal care holding room and were confirmed to be free of the following microorganisms: cilium-associated respiratory bacillus, crotalaria, mouse rotavirus, mouse euecholomenylitis virus, lymphocytic choriomeningitis virus, murine cytomegalovirus, mouse hepatitis virus, mouse adenovirus, minute virus of mice, Mycoplasma pulmonis, paraviruses, polyomavirus, pneumonia virus of mice, reovirus 3, and Sendai virus.

Mice were housed in 7.5- by 11.5- by 5-in. sterilized ventilated Thoren cages (Thoren Caging System, Inc., Hazleton, Pa.) on Tek Fresh bedding (Harlan Teklad, Madison, Wis.). Cages were changed weekly. The animal holding room was maintained under environmental conditions of 20°C, 40 to 70% relative humidity, 15 air changes/h and a 12-h light-dark cycle. Mice were fed an autoclaved pelleted rodent diet (rodent NIH-31 autoclavable NA; Zeigler Brothers, Gardners, Pa.) ad libitum and provided sterilized individual water bottles for ad libitum water source. Upon arrival, the mice were acclimated for a minimum of 7 days prior to being used in the experiments. Mice were identified by numerical stainless steel rodent ear tags (National Band and Tag Co., New-Hampshire, Inc., Gardners, Pa.) and Accreditation of Laboratory Animal Care International (Rockville, Md.).

Animal housing and diet. The mice were maintained in a National Institutes of Health (NIH) animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (Rockville, Md.). The mice were housed in a specific-pathogen-free animal care holding room and were confirmed to be free of the following microorganisms: cilium-associated respiratory bacillus, crotalaria, mouse rotavirus, mouse euecholomenylitis virus, lymphocytic choriomeningitis virus, murine cytomegalovirus, mouse hepatitis virus, mouse adenovirus, minute virus of mice, Mycoplasma pulmonis, paraviruses, polyomavirus, pneumonia virus of mice, reovirus 3, and Sendai virus.

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Animal inoculations and follow-up. Forty-two 6- to 8-week-old Helicobacter- and pathogen-free female C57BL/6 mice (n = 42) (Charles River, Wilmington, Mass.) were used in compliance with guidelines and protocol approved by the Animal Care and Use Committee of the NIH. Using a 20-µg ballpoint metal feeding tube (Harvard Apparatus, Inc., Holliston, Mass.), 26 mice were inoculated intragastrically with 0.1 ml of H. pylori SS1 cell suspension (10^6 CFU/ml) from three separate overnight cultures on three alternate days. Sixteen control mice were inoculated with BLBB containing 10% fetal bovine serum. The mice were maintained in a specific-pathogen-free animal care holding room and were confirmed to be free of the following microorganisms: cilium-associated respiratory bacillus, crotalaria, mouse rotavirus, mouse euecholomenylitis virus, lymphocytic choriomeningitis virus, murine cytomegalovirus, mouse hepatitis virus, mouse adenovirus, minute virus of mice, Mycoplasma pulmonis, paraviruses, polyomavirus, pneumonia virus of mice, reovirus 3, and Sendai virus.

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Isolation of DNA from fecal pellets. Fecal pellets were collected by holding a mouse above a sterile microcentrifuge tube and gently stroking the lower left side of the abdomen. The tubes were placed on ice and processed immediately. DNA was isolated from fecal pellets (2 pellets per mouse) by using the DNAeasy kit or DNA isolation kit (Qiagen, Valencia, Calif.), 26 mice were inoculated intragastrically with 0.1 ml of H. pylori SS1 cell suspension (10^6 CFU/ml) from three separate overnight cultures on three alternate days. Sixteen control mice were inoculated with BLBB containing 10% fetal bovine serum. The mice were repeatedly tested for the presence of H. pylori by PCR analysis of DNA extracted from fecal pellets. During necropsy at 6 and 10 months, stools were again tested by PCR and stomachs were harvested for H. pylori recovery, histology, and PCR.

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DNA stool kit (QIAGEN, Valencia, Calif.). These methods are based on selective removal of debris, the binding of extracted DNA to commercial spin columns, and elution with water. The DNA was quantitated by using a spectrophotometric method or by using the QIAGEN fluorescence dye binding method. DNA isolation and PCR experiments were performed on coded Genta-Robason-stained (14) slides viewed at a magnification of ×100. The amplicons were analyzed by electrophoresis on a 2% NuSieve agarose gel. All amplification reactions were performed on an automated thermocycler (GeneAmp PCR system 2400; Perkin-Elmer, Foster City, Calif.).

PCR detection of H. pylori in fecal pellets. To noninvasively determine the H. pylori status of mice, fecal pellets of animals terminated at 6 months (n = 15) were tested by PCR at 4 and 5 months postinoculation, and animals terminated at 10 months (n = 15) were tested at 9 months postinoculation. In addition, fecal pellets were collected immediately before euthanasia and tested by PCR. Small groups of mice (usually 2 control mice and 3 inoculated mice) were tested for H. pylori status daily for four consecutive days. Fecal pellets were taken for each animal on days 1, 3, 5, and were tested for H. pylori infection. This experiment was repeated four times.

H. pylori status at time of euthanasia. To determine the H. pylori status at the time of euthanasia, the animals were subjected to fasting for 14 h and euthanized by CO2 asphyxiation and cervical dislocation. The stomach was harvested and dissected along the greater curvature.

(iii) Histology. One half of the entire stomach was placed with the mucosal side up in a Tissue-Tek Uni-Cassette (Sakura, Allegheny Healthcare Corp., McGraw Park, Ill.), immersed in neutral 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Gastritis was evaluated on coded slides viewed at a magnification of ×100. H. pylori colonization was assessed on coded Genta-Robason-stained (14) slides viewed at a magnification of ×100 to ×1,000.

(iv) Culture of H. pylori. Gastric tissue specimens (0.11 to 0.12 g) were taken from the antral and corpus regions, immediately immersed in 50 μl of BLBB containing 30% glycerol and 10% fetal calf serum, placed on dry ice, and stored at −80°C. Harvested corpus and antral samples were homogenized with a sterile motorized pestle (Daigger, and 100 g of 1,000-fold dilutions of the homogenate were streaked on Campy blood agar plates (Remel, Lenexa, Kan.) and BLBB-Glaxo selective supplement A (GSSA) plates containing 5 μg of amphotericin B/ml, 20 μg of bacitracin/ml, 1.07 μg of nalidixic acid/ml, 0.33 μg of polymixin B/ml, and 10 μg of vancomycin/ml (GSSA) (25). The plates were incubated under microaerophilic conditions at 37°C and examined 3 to 4 days later. Pure colonies of H. pylori were obtained by subculturing the small transparent colonies, on the BLBB-Glaxo medium. These colonies were identified by a modified Gram stain (i.e., employing carbol-fuchsin as a counterstain), examined microscopically, and tested for urease.
ase, catalase, and oxidase activities (28, 33). Finally, DNA extracted from cultures of the colonies was subjected to PCR analysis to test for \textit{H. pylori}-specific 16S rRNA (with Hp1-Hp2 primers) and for their RAPD fingerprint profiles.

(iii) PCR analysis. DNA samples were prepared from stored gastric tissue specimens by using the QIAGEN DNeasy kit protocol as described above. These DNA samples were tested for the presence of \textit{H. pylori} by PCR with the Hp1-Hp2 primer set, and PCR conditions were as described above (18).

RESULTS

Specificity of PCR assay. A 109-bp PCR product was observed only with \textit{H. pylori} DNA and not with \textit{H. hepaticus}, \textit{H. bilis}, or \textit{H. muridarum} when the Hp1-Hp2 species-specific primer set was used (Fig. 1A). In contrast, \textit{H. pylori}, \textit{H. hepaticus}, \textit{H. bilis}, and \textit{H. muridarum} demonstrated amplification of 375-bp products with the 16S1-16S2 genus-specific primer set (Fig. 1B), thus suggesting the generality of the 16S1-16S2 primer set for \textit{Helicobacter} as opposed to the specificity of the Hp1-Hp2 primer set for \textit{H. pylori}.

Specificity and selectivity of PCR assay in stool containing genomic DNA from other \textit{Helicobacter} species. To demonstrate the specificity and selectivity of the PCR-based \textit{H. pylori} assay in stool contaminated with genomic DNA from other \textit{Helicobacter} species, serially diluted genomic DNA mixtures contami-nated with fecal material were amplified with genus- and species-specific primers. The results shown in Fig. 1C and D indicate that, in stool containing genomic DNA from three other \textit{Helicobacter} species (lanes 2 to 4), the specificity and selectivity of the PCR-based \textit{H. pylori} assay are unaltered. Lanes 5 and 11 show the results of the amplification of a DNA mixture that does not contain \textit{H. pylori} SS1 by using species- and genus-specific primers, respectively.

FIG. 1. (A) Specificity of the species-specific primer pair (Hp1-Hp2). Only \textit{H. pylori} SS1 DNA is PCR amplified, producing a 109-bp fragment (arrows). Lane 1, \textit{H. pylori} DNA; lane 2, \textit{H. bilis} DNA; lane 3, \textit{H. hepaticus} DNA; lane 4, \textit{H. muridarum} DNA; lane 5, 100-bp DNA marker. (B) PCR detection of \textit{Helicobacter} species with genus-specific primer pair (16S1-16S2) and electrophoretic analysis of 375-bp PCR products. Lane 6, PCR reagent control; lane 7, \textit{H. pylori} SS1; lane 8, \textit{H. bilis} DNA; lane 9, \textit{H. hepaticus} DNA; lane 10, \textit{H. muridarum} DNA. (C) Specificity and selectivity of the species-specific primer pair (Hp1-Hp2) in the presence of genomic DNA of three other \textit{Helicobacter} species and fecal material. Only \textit{H. pylori} SS1 DNA was PCR amplified, producing a 109-bp fragment in the presence of other \textit{Helicobacter} species genomic DNAs and fecal material (arrow, lanes 1 to 4). Lane 1, 100-bp ladder; lane 2, 10× dilution of a DNA mixture containing 9 ng of genomic DNA from four \textit{Helicobacter} species (\textit{H. pylori} SS1, \textit{H. bilis}, \textit{H. hepaticus}, and \textit{H. muridarum}); lane 3, 100× dilution of the same DNA mixture; lane 4, 1,000× dilution of the same DNA mixture; lane 5, 10× dilution of a DNA mixture (9 ng of genomic DNA per organism/µl) containing only \textit{H. bilis}, \textit{H. hepaticus}, and \textit{H. muridarum}; lane 6, PCR reagent control. (D) PCR amplification of four \textit{Helicobacter} species genomic DNAs with the genus-specific primer pair (16S1-16S2) in the presence of fecal material. Lane 8, 10× dilution of the DNA mixture described above; lane 9, 100× dilution; lane 10, 1,000× dilution; lane 11, 10× dilution of a DNA mixture without \textit{H. pylori} SS1 genomic DNA; lane 12, PCR reagent control.
product in lane 5 versus lane 11 is consistent with the specificity and selectivity of the \( H. pylori \) species-specific primers, Hp1-Hp2.

**Sensitivity of PCR assay in the absence of fecal pellet.** To determine the detection limit of the assay, nucleic acid extracted from the serial dilutions (10\(^5\) to 10\(^0\) CFU/ml) of \( H. pylori \) SS1 culture was amplified with primers Hp1-Hp2. The results demonstrated that the sensitivity of the assay is 1 to 10 CFU/ml in aqueous solution (Fig. 2A).

**Sensitivity of PCR assay in the presence of fecal pellet.** To examine whether feces interfered with the assay, 100 \( \mu l \) of a 10-fold serial dilution (10\(^5\) to 10\(^0\) CFU/ml) of \( H. pylori \) SS1 culture was centrifuged, the supernatant was discarded, and one fecal pellet of a noninoculated mouse was added to each tube. After suspension, homogenization, and DNA extraction, the samples were analyzed by PCR. \( H. pylori \) DNA was detected in serially diluted samples (Fig. 2B) as great as 10\(^8\) (i.e., 10\(^2\) bacterial CFU/ml). These results demonstrated that in the presence of fecal components the sensitivity of the assay was reduced 10-fold. Thus, the sensitivity of this stool assay is estimated at 10 to 100 bacterial cells per fecal pellet. The average weight of 20 fecal pellets from C57BL/6 was 0.032 g, indicating that the sensitivity of the assay in the presence of stool is 10 to 100 bacterial cells/0.032 g of stool. It should be noted that we have detected \( H. pylori \) infection in 0.025 g of human stool (data not shown).

**Detection of \( H. pylori \) in fecal pellets.** DNA (unquantitated) extracted from the supernatant fractions of fecal pellets of infected and noninfected mice was subjected to PCR amplification with primer pair Hp1-Hp2. The reaction produced 109-bp PCR fragments in most of the infected mice, detecting infection in 9 of 13 mice at 6 months and 10 of 13 mice at 10 months and detecting no infection in the 4 negative control mice (Table 1; Fig. 3).

**DNA fingerprints of recovered bacteria.** To determine whether the \( H. pylori \) strain recovered from cultures of gastric tissue of infected mice was the same as the \( H. pylori \) SS1 strain used for inoculation, the arbitrarily primed PCR DNA fingerprinting method was used. The DNA samples were tested independently with the 10-nucleotide primers 1281 and 1290. The DNA of bacteria recovered from mouse gastric specimens

### Table 1. Percentage of \( H. pylori \) positivity by histology, culture, and PCR at time of necropsy

<table>
<thead>
<tr>
<th>Assay</th>
<th>6 mo Inoculation(^a)</th>
<th>6 mo Sham inoculation(^a)</th>
<th>10 mo Inoculation(^a)</th>
<th>10 mo Sham inoculation(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology of gastric tissue</td>
<td>13/13 (100)</td>
<td>0/2 (0)</td>
<td>12/13 (92)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>Culture of gastric tissue</td>
<td>10/13 (77)</td>
<td>0/2 (0)</td>
<td>11/13 (85)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastric tissue</td>
<td>9/13 (69)</td>
<td>0/2 (0)</td>
<td>10/12 (83)(^b)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>Fecal pellets</td>
<td>9/13 (69)</td>
<td>0/2 (0)</td>
<td>10/13 (77)</td>
<td>0/2 (0)</td>
</tr>
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\(^a\) All mice were positive by at least two of the three tests.

\(^b\) No tissue was available for one of the mice.

\(^c\) Seven additional control animals remained negative for up to 21 months.
produced a DNA fragment array pattern identical to that of DNA isolated from the *H. pylori* SS1 strain used for inoculation (Fig. 4).

**Detection of *H. pylori* at the time of necropsy.** (i) **Histopathologic evaluation of gastric tissue.** Gastritis was mild at 6 months (Fig. 5B), and it became severe at 10 months (Fig. 5C). The presence of *H. pylori* was detected in Genta-stained slides of inoculated mice (13 of 13 at 6 months and 12 of 13 at 10 months) (Fig. 5B and C, insets; Table 1) and in 0 of 4 sham-inoculated animals (Fig. 5A).

(ii) **Culture of *H. pylori* from gastric tissue.** Culture of gastric specimens on Campy blood agar plates and BLBB-GSSA plates resulted in the growth of small transparent colonies consistent with *H. pylori* in the majority of inoculated animals (10 of 13, or 77%, at 6 months; 11 of 13, or 85%, at 10 months) but in none of the 4 sham-inoculated mice (Table 1). Microscopy of positive cultures revealed motile, curved, and spiral bacteria that tested positive for urease, catalase, and oxidase and were gram negative (28, 33). Counterstaining with carbolfuchsin instead of safranin provided visualization of pink, curved organisms morphologically consistent with *H. pylori*. In addition, DNA extracted from these cultures and amplified with species-specific primers (Hp1-Hp2) produced a 109-bp PCR fragment, and RAPD fingerprinting of the same DNA with primers 1281 and 1290 demonstrated that the resulting profile was identical to that of the *H. pylori* SS1 strain used for inoculation (Fig. 4). No growth was detected on BLBB-GSSA plates for the 4 negative control mice.

(iii) **PCR of gastric tissue.** Inoculated mice were positive by PCR (9 of 13 at 6 months and 10 of 12 at 10 months), whereas none of four sham-inoculated mice tested positive (Table 1).

(iv) **PCR of fecal pellets.** At 6 months, fecal pellets were positive for 9 of 13 (69%) inoculated mice and for 0 of 2 sham-inoculated control mice. At 10 months, 10 of 13 (77%) were positive (Fig. 3; Table 1). Importantly, 26 of 26 inoculated

![FIG. 4. *H. pylori* SS1 genomic fingerprinting by the RAPD method. The test performed with 10-nucleotide primers 1281 (lanes 1 and 2) and 1290 (lanes 4 and 5) confirmed that the RAPD profile identity of the bacterial strain recovered from the mice matched that of the strain used for inoculation. Lanes 1 and 4, *H. pylori* SS1 recovered from infected mice; lane 3, 1-kb ladder; lanes 2 and 5, *H. pylori* SS1-positive control.](http://iai.asm.org/)

**FIG. 5. Mouse gastric tissue (corpus, Genta and H&E stains; original magnification, ×100 [main picture] and ×1,000 [inset]).** (A) Sham-inoculated mouse (Genta stain). (B) Mouse 6 months after inoculation with *H. pylori* strain SS1 (H&E stain). Note the minimal infiltration (arrow). The inset shows *H. pylori* near the epithelial surface. (C) Mouse 10 months after inoculation with strain SS1 (Genta stain). Note the marked infiltration by inflammatory cells (arrow). The inset shows bacteria near the epithelial surface of the gland (original magnification, ×1,000).
mice were positive by at least two of the three invasive tests performed directly on the stomach, and 4 of 4 sham-inoculated mice were negative by all three tests. Therefore, the sensitivity of the stool PCR can be estimated at 77%, and the specificity can be estimated at 100%.

Noninvasive detection of *H. pylori* in feces prior to euthanasia. All 13 experimental mice that were terminated at 6 months tested positive at least once: 9 of 13 were positive at 3 months (69%), and 9 of 13 were positive at 6 months (1 to 3 days before euthanasia), including three of the four mice that tested negative at 3 months. The fecal pellets collected on the day of euthanasia from the fourth mouse (which had tested negative at 3 months) also tested positive. Similarly, the 13 animals euthanized at 10 months were tested three times by PCR of the stools (at 9, 9.5, and 10 months). All 13 animals tested positive at least once (8 were positive 3 of 3 times, 3 were positive 2 of 3 times, and 2 were positive once). The fecal pellets collected at the time of euthanasia from one of the mice that previously tested positive only once also tested positive while those from the other were negative. In addition, we tested the fecal pellets of a group of 5 mice (2 controls and 3 *H. pylori* infected) once daily for 4 days: 3 of 3 (100%) of the infected mice were positive 4 of 4 times tested and the two uninfected controls were negative. Three additional repeats of this 4-day testing procedure gave similar results with a range of 90 to 100% detection of *H. pylori* infection.

**DISCUSSION**

The present study illustrates that PCR analysis of fecal pellets permits documentation of the *H. pylori* status of C57BL/6 mice for up to 10 months following inoculation. Prior to necropsy at 6 and 10 months, groups of 13 mice were tested. The specificities at both test intervals were 100%, but the sensitivities were 69 and 77%, respectively. In contrast, the sensitivity and specificity of the stool PCR increased to 90 and 100% when four groups of mice were tested daily for 4 days. This approach allows repeated determination of the *H. pylori* status of each of the mice over time without requiring euthanasia of the animal and could be applied to determine the time course of persistent versus transient infection in mice. Thus, it provides information similar to that obtained for humans by using the 13C- or 14C-urea breath or blood test (4, 8).

The method was first validated in vitro by demonstrating that it was specific and sensitive for the detection of *H. pylori* in aqueous solutions and also in fecal pellets. In a second step, in vivo validation was achieved by demonstrating that PCR of the fecal pellets was positive at least one of four times in animals that tested positive by histology, culture, and/or PCR of gastric mucosal samples at the time of euthanasia. Moreover, PCR of the fecal pellets was negative in all sham-inoculated animals. Interestingly, four of the inoculated animals that were positive by PCR of the fecal pellets, histology, and PCR of gastric specimens were negative by culture, suggesting that this latter assay is the least sensitive of the three tests. A similar experience has been reported by others (P. Ghiara, personal communication). However, one animal was positive by culture and stool PCR but negative by histology. Hence, negative culture in five inoculated animals could be due to a loss of bacterial culturability (coccoid form) (6) or to the absence of colonization of the portion of the gastric specimen that was cultured. Also, seven additional control mice remained negative for up to 21 months. A number of PCR assays for the detection of *H. pylori* have been described. When the mouse model was used, all of the assays available to date required the euthanasia of experimental animals. In the study by Ho et al. (18), the Hp1-Hp2 primers were designed to amplify a region of the 16S rRNA, thus allowing maximum differentiation between *H. pylori* and closely related *Helicobacter* species, and *H. pylori* was then detected in gastric biopsy specimens by PCR (19). These results were reproduced with gastric biopsy specimens and gastric aspirates (16, 36). In the present study, fecal matter of live experimental mice served as the substrate for the direct, sensitive, and specific detection of *H. pylori* by PCR. However, multiple products, including complex lipopolysaccharides (26), that are inhibitory to PCR are known to be present in the feces, and attempts to eliminate them represent one of the most difficult challenges to PCR amplification of fecal DNA extracts. To overcome this problem, two steps were introduced in the fecal DNA preparation: (i) the use of a silica-based column which binds the nucleic acid, thus allowing for an extensive wash of the columns to remove most, if not all, inhibitors, and (ii) the dilution of fecal DNA samples to be used as the template. This approach helped to reduce the effect of inhibitory substances present in the DNA extracted from fecal matter, thereby improving the detection of *H. pylori* in fecal samples. However, it is important to note that the negative results in inoculated mice do not necessarily support the absence of infection because the variability of fecal samples (i.e., content water and polysaccharides, etc.). DNA loss during the extraction process, failure to uniformly reduce PCR-inhibitory substances, animal variations in *H. pylori* infection, or the variability of *H. pylori* titer in the fecal samples may interfere with assay sensitivity.

The selection of primers for any PCR is critical to the success of the test, and the use of primers that target specific organisms is important. Mice can be colonized by different species of *Helicobacter*, and bacteria that are closely related to *Helicobacter* species can infect mice (13). This can produce confounding results if *H. pylori* is the target organism. Therefore, distinction of *H. pylori* from other phylogenetically related organisms by detecting its nucleic acid requires specific and sensitive methods of DNA amplification. The published genus-specific primer set (16S1-16S2) (34) generally detected all *Helicobacter* tested in our investigation, whereas the species-specific primer set (Hp1-Hp2) specifically detected only *H. pylori* (Fig. 1). This approach eliminated the need for species identification by restriction and sequence analysis or hybridization (35) and provided results that were in agreement with those obtained from direct analysis of gastric samples. Also, analysis of the PCR products showed that DNA from approximately 10 cells of *H. pylori* (Fig. 2A) is detectable in aqueous solution. Thus, this study demonstrated a sensitive and specific method for the detection of *H. pylori*.

The present study also provided a histological demonstration of the colonization of the gastric corpus at 6 and 10 months. Figure 5B (inset) shows minimal colonization of the surface epithelium while Fig. 5C (inset) shows significant bacterial colonization. Infiltration by lymphocytes and some neutrophilic granulocytes tended to increase with time. There was
mild to severe gastritis, but no ulcers were observed (Fig. 5B and C). These latter observations are similar to those reported by Eaton et al. (10).

In conclusion, PCR analysis of fecal pellets can facilitate studies of the time course of H. pylori infection in small rodents, thus allowing studies of novel therapeutic approaches. Moreover, this method may also represent an alternative to the 13C- or 14C-urea breath test in the clinical setting to assess H. pylori eradication in infected patients and to detect the relapse of infection after antimicrobial treatment. Although additional technical improvement may be needed before this diagnostic tool can be relied upon as the sole diagnostic test, the present report has demonstrated the sensitivity and specificity of the method as well as its usefulness.

ACKNOWLEDGMENTS

This work was partially supported by the National Center on Minority Health and Health Disparities, NIH, and by a grant from the NIH (grant CA82312). The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Health Education and Welfare, the NIH, the Department of Defense, or the Uniformed Services University of the Health Sciences.

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Editor: J. F. Urban, Jr.