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Fecal PCR Assay for Diagnosis of *Helicobacter* Infection in Laboratory Rodents

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A fecal PCR assay for detection of *Helicobacter* infections in laboratory rodents was developed. DNA was isolated from murine fecal pellets, and a region of the 16S rRNA gene conserved among murine *Helicobacter* species was amplified. The fecal PCR was sensitive and specific. This assay does not require euthanasia of rodents, which is especially important for valuable rodents, such as transgenic mice.

Members of the bacterial genus *Helicobacter* are recognized as important infectious agents of many host species (4, 9, 13). The best known of these organisms, *Helicobacter pylori*, is the leading cause of gastritis and peptic ulcers in people (2, 11, 12). Two species of *Helicobacter* are associated with disease in laboratory mice. *Helicobacter hepaticus* causes hepatitis and hepatocellular neoplasia and has recently been associated with inflammatory bowel disease in immunocompromised mice (5, 18–20). *Helicobacter bilis* is associated with hepatitis in aged mice (7). In addition, *Helicobacter muridarum* may be associated with gastritis in mice (10, 14). For each of these species, the intestinal tract is the principal site of colonization.

Helicobacter infections cause no overt signs of illness in immunocompetent rodents. However, these organisms interfere with research. In fact, *H. hepaticus* was first identified as a pathogen when it was discovered to be the cause of chronic hepatitis and hepatocellular carcinoma in mice in a long-term toxicology study (5, 18, 19). In addition to histological lesions, preliminary studies have shown altered serum enzyme and bile acid profiles in mice infected with *H. hepaticus* (6, 18). The full scope of complications in research that are associated with helicobacters in laboratory rodents is unknown, but there is growing evidence suggesting that effects may be far reaching and, in some cases, so severe that entire studies are invalidated by the use of *Helicobacter*-infected rodents.

Because of the potential of *Helicobacter* spp. to confound research, it is important to identify *Helicobacter*-infected animals. Previous reports have determined that there is widespread infection among mice and rats in research institutions and commercial production colonies (16, 17). PCR is an excellent diagnostic test for *Helicobacter* infections because it is specific, sensitive, and rapid. Our laboratory recently developed a PCR assay that uses DNA isolated from cecal tissue for diagnosis of murine helicobacters. That assay amplifies the 16S rRNA gene of all *Helicobacter* species known to infect laboratory mice and rats and further identifies the organisms to the species level by restriction enzyme analysis. The PCR assay developed in this study uses feces as the test sample. Since feces can be collected from live animals, the advantage of our fecal PCR assay is that it does not require euthanasia of the animals being tested. The ability to test live animals is especially important for monitoring valuable and rare rodents, such as transgenic and knockout mice for *Helicobacter* infections. The fecal PCR assay amplifies the same region of the *Helicobacter* 16S rRNA gene amplified in the cecal tissue assay and can be followed by restriction enzyme analysis to determine the species of the organisms. Detection of multiple *Helicobacter* organisms with identification to the species level is important, since tests for single species fail to detect other helicobacters that may also interfere with research.

Bacterial strains. *H. hepaticus* was isolated from the liver of a naturally infected A/JNCr mouse as previously described (19). *H. bilis* (ATCC 51630) and *H. muridarum* (ATCC 51212) were purchased from the American Type Culture Collection (Rockville, Md.).

Isolation of DNA. DNAs were isolated from cultured organisms and cecal tissue with a QIAamp Tissue Kit (Qiagen Inc., Chatsworth, Calif.) as previously described (16). DNA from mouse feces was isolated by an adaptation of the QIAamp Tissue Kit. Briefly, a single mouse fecal pellet was suspended in 1.7 ml of phosphate-buffered saline (PBS), pH 7.4. The suspension was centrifuged at 700 × *g* for 5 min. A 100-μl volume of supernatant was diluted 1:2 with PBS and processed according to the QIAamp Tissue Kit protocol for blood.

PCR. *Helicobacter* genus-specific primers 5′-TATGACGGG TATCCGGC-3′ and 5′-ATTCCACCTACCTCTCCCA-3′, designed from regions of the 16S rRNA gene conserved among members of the *Helicobacter* genus (16), were synthesized at the DNA Core Facility, University of Missouri—Columbia. PCRs were prepared with 1 μM each primer, 200 μM each dNTP (dATP, dCTP, dGTP, and dTTP), PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl [pH 8.3]), template DNA, 1.25 U of *Taq* polymerase, and distilled water in a total volume of 50 μl. For PCR of feces, 5 μl of unquantitated fecal DNA was used as the template for all samples. For PCR of cecal tissue, 1.25 μg of cecal DNA was used as the template. PCR mixtures were heated to 94°C for 30 s once, followed by 45 cycles of denaturation at 94°C for 2 s, primer annealing at 53°C for 2 s, and extension at 72° for 30 s in a Perkin-Elmer model 2400 thermocycler. A 16-μl volume of each PCR product was subjected to electrophoresis on an agarose gel containing 0.2 μg of ethidium bromide per ml. The following controls were included with each PCR run: a no-DNA template control, a positive control DNA prepared from cecal tissue of an *H. hepaticus*-infected mouse, and a negative control DNA prepared from cecal tissue of a *Helicobacter*-free mouse. Positive samples contained a 375-bp band that was visualized under UV light. The size of the product was confirmed with DNA molecular weight standards.

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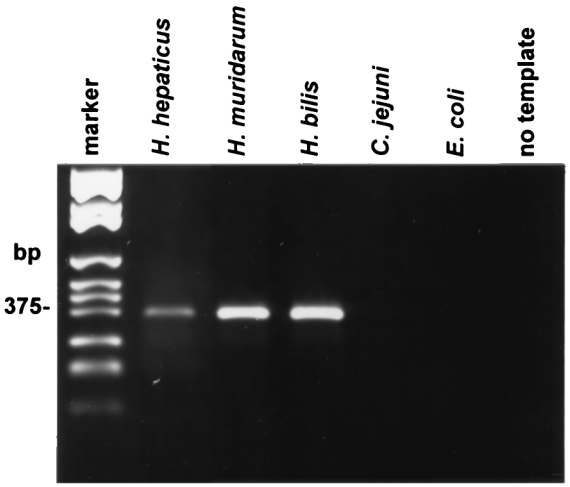

FIG. 1. Ethidium bromide-stained gel showing PCR amplification products from DNAs of various bacterial species amplified in the presence of fecal DNAs from uninfected mice. Ten picograms each of *H. hepaticus*, *H. muridarum*, and *H. bilis* DNA, 30 ng of *Campylobacter jejuni* DNA, and 100 ng of *E. coli* DNA were added to fecal DNAs from uninfected mice.

Specificity. The *Helicobacter* genus-specific primers amplified the 375-bp fragment from *H. hepaticus*, *H. bilis*, *H. muridarum*, *H. pylori*, and the related species "*Flexispira rappini*" (16). The expected fragment was not amplified from *Enterococcus faecalis*, *Escherichia coli*, *Proteus mirabilis*, and *Bacillus subtilis* (16). To verify primer specificity under conditions of the fecal assay, DNAs from *E. coli* and *Campylobacter jejuni* were added to separate PCRs containing pooled negative fecal DNA because these two organisms were believed to have the greatest potential to interfere with the assay; *E. coli* was included because it is commonly present in normal rodent feces, and *C. jejuni* was included because members of the *Campylobacter* and *Helicobacter* genera have a high degree of homology in their 16S rRNA genes. PCRs with *E. coli* and *C. jejuni* DNAs were negative based on the absence of bands after PCR and electrophoresis (Fig. 1). As another indicator of specificity, we examined fecal pellets from BALB/c mice free of *Helicobacter* infections; these reactions produced no bands.

PCR products amplified from mouse feces were sequenced by the dideoxy chain termination method with the ABI Prism Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems Division, Foster City, Calif.) to confirm the specificity of the PCR assay. Sequencing reactions utilized the *Helicobacter* genus-specific primers. DNA sequences were analyzed and compared with sequences in GenBank with the Genetics Computer Group, Inc. (Madison, Wis.) software package. There was greater than 99% identity in the 375-bp overlap between the sequences of PCR products amplified from feces of *H. bilis*-, *H. hepaticus*-, and *H. muridarum*-infected mice and GenBank sequences of the 16S rRNA genes of these *Helicobacter* species.

Sensitivity. The sensitivity of the fecal PCR assay for *H. hepaticus*, *H. muridarum*, and *H. bilis* was determined by adding 10-fold serial dilutions of DNAs from cultured bacteria to separate PCR mixtures in the presence of 5 μl of fecal DNAs from *Helicobacter*-free mice. PCR mixtures containing 10-fold serial dilutions of *Helicobacter* DNA without fecal DNA were also prepared. The lower limit of detection of *H. hepaticus*, *H. bilis*, and *H. muridarum* in the presence of fecal DNA was 100 fg (Fig. 2). The lower limit of detection of *Helicobacter* DNA in the absence of fecal DNA was 10 fg (data not shown).

Comparison of fecal and cecal tissue PCRs. The fecal PCR assay was compared with the previously described PCR assay of cecal tissue for *Helicobacter* diagnosis (16) which utilizes the same primer set. Ten A/JNCr mice and 10 BALB/c mice were tested from colonies enzootically infected with *H. hepaticus* and *H. bilis*, respectively. Mice were euthanized after feces collection, ceca were collected aseptically, and specimens were stored at −20°C until they were processed for DNA isolation and PCR. Ten of 10 fecal samples and 10 of 10 cecal tissue samples from the *H. hepaticus*-infected A/JNCr mice yielded the 375-bp product after PCR and electrophoresis. For the *H. bilis*-infected BALB/c mice, 10 of 10 fecal samples and 9 of 10 cecal tissue samples yielded the 375-bp product.

Stability of *Helicobacter* DNA in mouse feces. The stability of *Helicobacter* DNA in mouse feces was also evaluated. Fecal pellets were collected from three *H. hepaticus*-infected A/JNCr mice housed individually in solid-bottom cages without bedding. Fecal pellets from each mouse were stored for 0, 1, 2, 3, and 7 days at room temperature and then frozen at −20°C until they were processed. Two pellets per mouse per time period were processed. All fecal pellets from *H. hepaticus*-infected mice yielded the expected fragment after PCR (Fig. 3). As expected, no products were amplified from fecal pellets

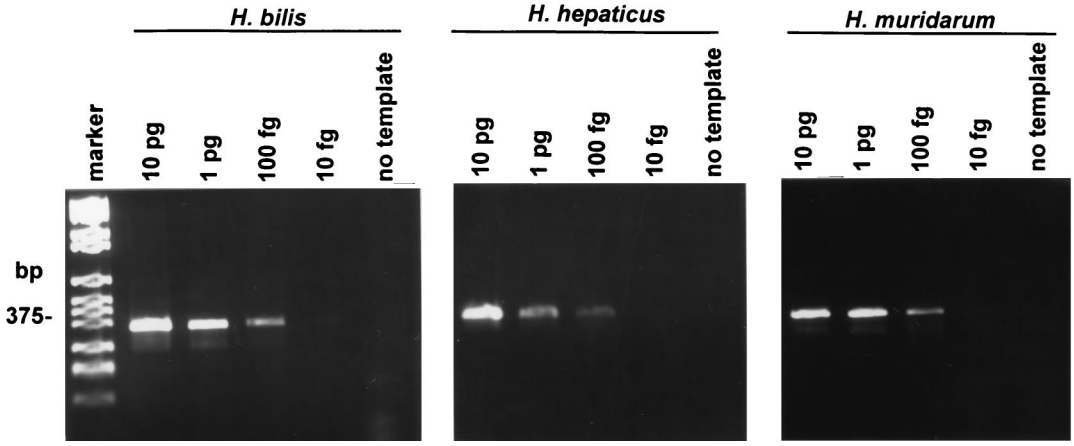

FIG. 2. Ethidium bromide-stained gel showing PCR products resulting from amplification of serial dilutions of *H. hepaticus*, *H. bilis*, and *H. muridarum* DNAs in the presence of fecal DNAs from uninfected mice.

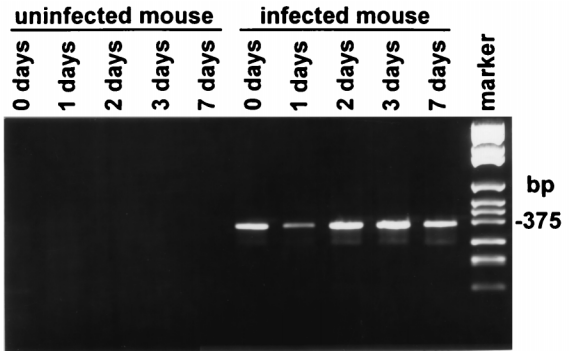

FIG. 3. Ethidium bromide-stained gel showing PCR amplification products from mouse feces stored for various times. Products in lanes 1 to 5 were from PCR of feces from an uninfected mouse. Products in lanes 6 to 10 were from feces of an *H. hepaticus*-infected mouse. Feces were stored at room temperature for the indicated number of days before processing.

that were obtained from *Helicobacter*-free BALB/c mice and that were collected and stored in the same manner.

When large numbers of mice are to be tested, it is cumbersome and inefficient to prepare specialized caging solely for the purpose of feces collection. For this reason, we completed an additional test to determine if *Helicobacter* DNA remains stable in mouse feces in contact with bedding soiled with urine and feces. Ten fecal pellets were collected 5 days after the last bedding change directly from the paper bedding (Canbrands International Ltd., Moncton, New Brunswick, Canada) of a cage that housed three male, *H. hepaticus*-infected A/JNCr mice. The pellets were stored at room temperature an additional 3 days before being processed to simulate the time that may be required for shipping samples to a diagnostic laboratory. All 10 pellets yielded a fragment of the expected size after PCR amplification.

Discussion. Several diagnostic PCR assays for *Helicobacter* diagnosis have recently been described (1, 16, 17). The PCR assay described in this study is unique in offering advantages over previously described PCR assays for *Helicobacter* detection as follows. (i) The use of feces as the test substrate allows the testing of live rodents, whereas tissue-based assays require euthanasia of animals. Antemortem detection is especially important for *Helicobacter* diagnosis in valuable or rare rodents, such as transgenic mice. (ii) The PCR primers amplify a region of the 16S rRNA gene highly conserved among rodent helicobacters to provide genus-specific diagnosis. Species identification as described by Riley et al. (16) can be accomplished by digestion of the amplified product with various restriction endonucleases in separate reactions followed by agarose gel electrophoresis. Amplified DNA from each rodent *Helicobacter* species yields a different restriction pattern based on the unique sequence of its 16S rRNA gene, thus allowing identification of multiple *Helicobacter* organisms to the species level. This advantage over tests which detect only *H. hepaticus* may become increasingly important, since we have identified additional *Helicobacter* species that have the potential to interfere with research with laboratory rodents (8, 15). (iii) The 100-fg sensitivity of this PCR assay for feces is comparable to those of previously described tissue-based assays for detection of *Helicobacter* DNA and exceeds those of other *Helicobacter* fecal PCR assays. Battles and colleagues (1) recently described a PCR-liquid hybridization assay. The sensitivity of their assay was assessed by amplifying cloned copies of the *H. hepaticus* 16S rRNA gene in the absence of fecal DNA and is estimated to be approximately three *H. hepaticus* organisms, assuming three copies of the 16S rRNA gene per cell. The 10-fg sensitivity of our PCR assay with *Helicobacter* DNAs from cultured bacteria in the absence of fecal DNA corresponds to between 5 and 10 organisms, when the 1,700-kb genome size of *H. pylori* (3) is used as an approximation of the genome sizes of rodent helicobacters. Thus, the sensitivity of our assay appears comparable to that of the assay described by Battle et al. However, the high sensitivity of Battle et al.'s assay requires hybridization of the PCR product with a ^{32}P-labeled oligonucleotide probe, a step which increases sample manipulation and exposes personnel to a hazardous radioisotope. The PCR assay developed in this study offers high sensitivity without these disadvantages.

Shames et al. (17) recently reported development of a fecal PCR assay with a sensitivity of 0.5 to 0.1 ng of *H. hepaticus* DNA. The lower sensitivity of that assay in comparison to the 100-fg sensitivity of our assay may be due to the method of DNA purification. Initial studies in our laboratory demonstrated that purity of the DNA was crucial to assay sensitivity with fecal specimens. Fecal PCR assays are sometimes problematic to develop, because factors present in feces inhibit *Taq* polymerase. Two methods were used to overcome inhibition in this fecal assay: (i) fecal samples were diluted with PBS, which resulted in large-scale dilution of both feces and inhibitors prior to DNA isolation and (ii) DNA in the diluted fecal sample bound the silica matrix column in the QIAamp Tissue Kit and was washed extensively to remove impurities, including inhibitors of *Taq* polymerase. In the presence of fecal DNA, the PCR assay detected 100 fg of *Helicobacter* DNA, and in the absence of fecal DNA, the assay detected 10 fg of *Helicobacter* DNA. The loss of only 10-fold sensitivity in the presence of fecal DNA indicated that the purified fecal samples were substantially free of polymerase inhibitors.

The determination that *Helicobacter* DNA is stable in mouse feces is important, because it facilitates collection and screening of fecal samples from large numbers of rodents. Our results indicate that *Helicobacter* DNA is stable in mouse feces collected immediately or within 5 days of a bedding change. Thus, feces collection is convenient, even if large numbers of rodents are to be tested, because it can be taken directly from the bedding in the cages. In addition, submission of samples to the laboratory is simplified, because feces can be shipped at ambient temperature if they will be received within 3 days.

In preliminary experiments we have also used fecal PCR to detect *Helicobacter* infections in rats and hamsters. Fecal and cecum samples from rats have been directly compared in this manner with a perfect correlation of results. On the basis of these results, it is likely that this fecal PCR assay can be adapted for use with other animal species.

Detection of *Helicobacter* infections in laboratory rodents is necessary in order to provide research animals free of confounding variables. This fecal PCR assay is a sensitive, specific, and convenient method of antemortem diagnosis of *Helicobacter* infection in rodents.

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