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Simple Duplex Fecal PCR Assay That Allows Identification of False-Negative Results in *Helicobacter* sp.-Infected Mice

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We designed a simple and sensitive duplex polymerase chain reaction (PCR) assay for detection of false-negative results during routine *Helicobacter* sp. feces analysis. We took advantage of the various *Lactobacillus* species that form part of the normal intestinal flora of laboratory rodents to improve our PCR diagnostic assays. Using this one-step PCR assay, we were able to rule out false-negative results without the need of adding internal standard molecules. This is an important quality control for PCR diagnostic tests, since the presence of inhibitors in feces can prevent detection of *Helicobacter* infections using PCR analysis. Use of this *Lactobacillus* group-specific PCR assay can be extended to other feces tests used in mouse quality-control programs.

Helicobacter species are gram-negative, microaerophilic, spiral bacteria that commonly colonize the gastrointestinal tract of mammals and other animals. In the laboratory mouse, the currently identified *Helicobacter* species are *H. bilis*, *H. hepaticus*, *H. muridarum*, "*H. rappini,*" *H. rodentium, H. typhlonius*, and *H. ganmani* (8, 10, 12, 32). Some of these species are associated with chronic gastrointestinal disorders (6), and hepatitis (e.g., *H. hepaticus* and *H. bilis*) in susceptible strains of mice (most immunocompetent strains used in research, though readily infected, are resistant to hepatitis) (9, 29). *Helicobacter hepaticus* also is associated with development of hepatocellular adenomas and carcinomas in A/J mice (predominantly males) (29, 30), and inflammatory bowel disease in SCID ($Prkdc^{scid}$/$Prkdc^{scid}$), nude ($Foxn1^{nu}$/$Foxn1^{nu}$), and other immunocompromised mice (2, 13, 18, 25, 28). *Helicobacter* species principally colonize the distal portion of the gastrointestinal tract, but some species, like *H. hepaticus* and *H. bilis*, also have localized in the liver (8, 10). *Helicobacter* organisms are thought to be transmitted by the fecal-oral route (29).

Although cesarean re-derivation, embryo re-derivation, and neonatal transfer (26) are potentially effective methods for eliminating these spiral organisms from mouse colonies, the prevalence of *Helicobacter* infections in animal facilities is still high. Although *H. hepaticus*, *H. bilis*, and *H. typhlonius* are the recognized principal pathogenic *Helicobacter* sp. in the mouse, the potential pathogenicity of the other *Helicobacter* species, as well as the research impact of these organisms, needs to be fully determined (14, 16, 19).

The presence of *Helicobacter* sp. in mouse colonies was initially detected by use of microaerobic bacteriologic culture of specimens from the large intestine, liver, or feces (10, 23), or histologic evaluation of liver using Steiner's silver stain (28, 29). During the past decade, routine *Helicobacter* testing procedures have incorporated use of PCR analysis, which is specific and non-expensive, compared with culture screening, which is more skill based and more costly, and to silver staining, which is less sensitive (1, 5, 20).

The PCR assays for detection of murine *Helicobacter* sp. are generally based on the amplification of bacterial 16S ribosomal RNA (rRNA) gene sequences (16S rDNA), and are carried out on DNA obtained from feces or tissues (e.g., cecum), using specifically designed oligonucleotide primers. Species- as well as genus-specific PCR assays, including use of fluorogenic nuclease PCR (also known as "real time" PCR [4, 15]) analysis, have been developed (20, 22-24, 31). This new technology eliminates the post-PCR analytic, electrophoretic steps, enhances specificity, and provides quantitative data. Due to its great sensitivity, PCR analysis currently is the method of choice for determination of the *Helicobacter* carrier status of mouse colonies (3, 17). In addition, fecal PCR analytic methods can be used as a noninvasive means of rapidly screening large numbers of mice (20).

However, one of the main concerns of all fecal tests is the potential of obtaining false-negative results due to PCR inhibitors in the reaction (i.e., complex polysaccharides possibly originating from vegetable material in the diet) (21). To deal with this limitation, we took advantage of the various *Lactobacillus* species that form the normal intestinal flora of the laboratory mouse (also included in the "altered Schaedler Flora" or "cocktail" used to colonize germ-free rodents). We designed a one-step, duplex, PCR assay that is based on use of *Lactobacillus* group-specific primers (27), along with *Helicobacter* group-specific primers (7, 11).

Using this simple PCR assay, we were able to detect false-negative results without the need of adding internal standard molecules or any other extra manipulation. We tested the duplex PCR assay at two independent laboratories (Institut Pasteur and M.D. Anderson Cancer Center), and performed the

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amplifications using slightly dissimilar PCR conditions, as well as different thermocyclers. Even under these alternate conditions, we obtained 100% coincident results in a series of blind experiments performed using fecal specimens obtained from our mouse facilities.

Materials and Methods

Animals. Mice of various strains were housed in suspended polycarbonate cages or individually ventilated cages (Lab Products Inc., Maywood, N.J.) on autoclaved hardwood bedding (PJ Murphy Forest Products Corp., Montville, N.J.) in an AAALAC-accredited facility (M.D. Anderson Cancer Center, Science-Park Research Division, Smithville, Tex.). Room conditions included controlled temperature (20 to 22°C), humidity (60 to 70%), and light (14/10-h light/darkness period). Commercial rodent pelleted food (Harlan Teklad, Madison, Wis.) and autoclaved reverse osmosis (R.O.) water were available ad libitum. All procedures were in compliance with the Public Health Service's *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996).

Bacterial DNA. The DNA samples from *H. hepaticus* (ATCC 51448), *H. bilis* (ATCC 51630 and CIP 204753T), and *H. muridarum* (ATCC 49282) were a generous gift from Dr. Richard Ferrero (Unité de Pathogénie Bactérienne des Muqueuses, Institut Pasteur, Paris, France). The DNA from *Lactobacillus* species was extracted using standard protocols from cultures of *L. rhamnosus* (CIP A158) and *L. plantarum* (CIP A159) obtained from the Collection de l'Institut Pasteur (CIP).

Fecal DNA extraction. Using the Qiagen Stool Kit (Qiagen, Inc., Valencia, Calif.), DNA was extracted from fecal pellets (6 to 8/cage) obtained from intramural mouse colonies as per the manufacturer's instructions for isolation of DNA from feces for pathogen detection. The DNA was eluted in 200 µl of sterile water, and DNA concentration and purity were determined spectrophotometrically by measuring the A_{260}-to-A_{280} optical density (OD) ratio.

Primers. Oligonucleotide primers were purchased from Eurogentec SA (Liege, Belgium) and Invitrogen (Carlsbad, Calif.). Primers Lac1 (5'-AGC AGT AGG GAA TCT TCC A-3') and Lac2 (5'-ATT YCA CCG CTA CAC ATG-3') were previously derived for the amplification of 340 bp of the 16S rRNA gene (16S rDNA) of lactobacilli (where Y is a degenerated nucleotide, standing for C or T). Use of in silico primer specificity analysis indicated that these primers would not bind exclusively to the 16S rDNA of lactobacilli, but would also anneal to that of *Pediococcus*, *Leuconostoc*, and *Weissella* spp. (27). The primers for *Helicobacter* sp. detection, C97 (5'-GCT ATG ACG GGT ATC C-3') and CO5 (5'-ACT TCA CCC CAG TCG CTG-3'), were reported to generate a 16S rDNA amplicon of 1,220 bp from the 16S rRNA gene of all known *Helicobacter* sp. (7). The species-specific primers, o008 (5'-TAGCTTGCTAGAAGTGGATT-3') and o009 (5'-ACCCTCTCAG-GCCGGATACC-3'), were previously reported to yield a 210-bp band for *H. muridarum, H. hepaticus*, and "*H. rappini*" and a 390-bp band for *H. bilis* (20).

Duplex PCR assay. Amplifications were carried out by use of *Lactobacillus* group-specific primers Lac1/Lac2 (27) and *Helicobacter* group-specific primers C97/C05 (7), using different protocols in the two laboratories.

Protocol 1 (Institut Pasteur). All PCR analyses were performed using 15 ng of DNA in a final volume of 25 µl with an automated BioRad iCycler (BioRad, Marnes-la-Coquette, France). Oligonucleotide primers were set at a final concentration of 1 µ*M* for primers Lac1 and Lac2 and 2.5 µ*M* for primers C97 and C05 (duplex PCR). The final concentration of $MgCl_2$ was 1.5 m*M*. We used a "touchdown" PCR protocol composed of four steps. The first step was initial denaturation at 94°C for 4 min. The second step consisted of 15 cycles of denaturation for 1 min at 94°C, annealing for 2 min at a temperature decreasing from 64°C to 57°C at a rate of 0.5°C/cycle, and extension at 72°C for 15 sec. The third step consisted of 15 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 58°C, and extension at 72°C for 15 sec. The last step was a final extension at 72°C for 7 min. The PCR products were separated by use of electrophoresis in 2% NuSieve agarose gels (FMC BioProducts, Rockland, Maine), stained with ethidium bromide, and visualized under UV light.

Protocol 2 (M.D. Anderson Cancer Center). All amplifications were made using the puReTaq Ready-To-Go PCR Beads kit (Amersham Biosciences, Inc., Piscataway, N.J.), designed for performing standard PCR analysis (containing approx. 2.5 U of puReTaq DNA polymerase, 10 m*M* Tris-HCl, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 µ*M* each dNTP, stabilizers, and bovine serum albumin) in a 96-well format, where the only additional reagents are water, template DNA, and primers. In this instance, we added 15 µl of sterile water, 3 µl of sample DNA (approx. 20 ng/µl), 1.0 µl of each of the two *Lactobacillus* primers (Lac1/Lac2), and 2.5 µl each of the two *Helicobacter* primers (C05/C97). The PCR cycling conditions were: initial denaturation for 1 min at 94°C; 35 cycles of 1 min at 94°C, 2 min at 58°C, and 3 min at 72°C; and final extension of 8 min at 72°C. All analyses were performed using a RoboCycler 96 Temperature Cycler (Stratagene, La Jolla, Calif.). The PCR products were resolved by use of electrophoresis through a 2% agarose gel (Sigma, St. Louis, Mo.) in Tris-Borate-EDTA (TBE) buffer, and bands were detected by use of ethidium bromide staining. For the partially species-specific duplex PCR assay, we combined primers o008/o009 (20) with primers Lac1/Lac2, using the same conditions described for protocol 2.

Results

Evaluation of the *Helicobacter*/*Lactobacillus* duplex PCR assay. The specificity of the duplex PCR assay was determined by amplifying fecal specimens obtained from mice in animal rooms (M.D. Anderson Cancer Center) that historically tested positive by use of routine PCR analysis quality control testing (performed by Charles River Laboratory, Wilmington, Mass., or Research Animal Diagnostic Laboratory, University of Missouri, Columbia, Mo.), and from *Helicobacter*-free mice housed under specific-pathogen-free (SPF) conditions. Excluded agents in SPF rooms were: mouse hepatitis virus, Sendai virus, minute virus of mice, mouse parvovirus, mouse encephalomyelitis virus, reovirus type 3, rotavirus, pneumonia virus of mice, ectromelia virus, lymphocytic choriomeningitis virus, polyoma virus, mouse adenovirus, Hantaan virus, *Mycoplasma pulmonis*, *Staphylococcus aureus, Citrobacter rodentium*, *Clostridium piliforme*, *Helicobacter* sp., *Salmonella* sp., *Pasteurella* sp., and endoparasites and ectoparasites.

The PCR amplifications using the combination of primers Lac1/Lac2 and C97/C05 generated two bands of the expected size (1,220 bp for *Helicobacter* sp. and 340 bp for *Lactobacillus* sp.) in all the fecal specimens originating from test-positive mice in the aforementioned rooms and the respective control bands

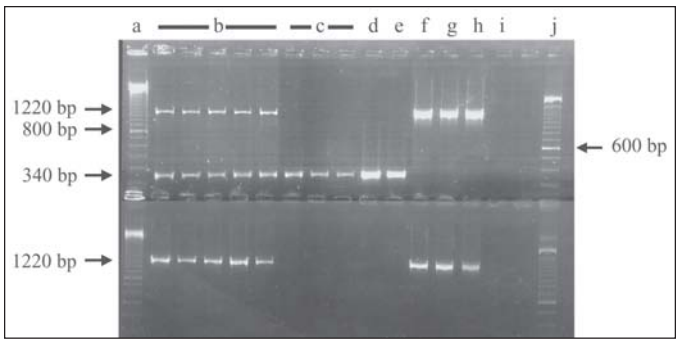

Figure 1. Ethidium bromide-stained agarose gel electrophoresis of polymerase chain reaction (PCR) products (protocol 1). Top: amplification using C05 + C97 + Lac1 + Lac2 primers (duplex PCR assay). Bottom: amplification using C05 + C97 primers alone. (a) A 100-bp ladder molecular marker (Pharmacia Bio-Tec, Uppsala, Sweden) with a strong band at 800 bp. (b) DNA isolated from feces of five *Helicobacter*-infected mice. (c) DNA isolated from feces of three *Helicobacter*-free mice. (d) *Lactobacillus plantarum* DNA. (e) *Lactobacillus rhamnosus* DNA. (f) *Helicobacter bilis* DNA. (g) *Helicobacter muridarum* DNA. (h) *Helicobacter hepaticus* DNA. (i) Control with no DNA. (j) A 100-bp ladder molecular marker (Invitrogen), with a strong band at 600 bp. The *Lactobacillus* group-specific band is amplified from the feces of all mice, acting as an internal quality control for PCR analysis. *Lactobacillus* sp. = 340 bp; *Helicobacter* sp. = 1,220 bp.

for the purified bacterial DNA (extracts of *H. hepaticus*, *H. muridarum*, *H. bilis*, *L. plantarum*, and *L. rhamnosus*). Amplifications using primers C97/C05 alone yielded the same results for *Helicobacter* sp. On the other hand, the fecal specimens obtained from mice in SPF rooms (consistently negative results of the *Helicobacter* PCR assay) yielded only one band of 340 bp, the expected size for *Lactobacillus* sp. (Fig. 1).

To analyze the overall sensitivity of our duplex PCR assay, we derived a standard curve using DNA isolated from feces of a *Helicobacter*-infected mouse at various concentrations. To evaluate whether the amplification of *Lactobacillus* sp. (shorter PCR product) competes for the PCR reagents more efficiently than does that of *Helicobacter* sp., serial dilutions of DNA isolated from feces of a *Helicobacter*-infected mouse were tested. The dilutions were made using DNA isolated from feces of a *Helicobacter*-free mouse to keep *Lactobacillus* DNA concentration as constant as possible. Using primers C97/C05 alone, an amplification product of the expected size for *Helicobacter* sp. was detected in ethidium bromide-stained gels, when as little as 0.185 ng of DNA was used as template. On the other hand, using the combination of primers Lac1/Lac2 and C97/C05 (duplex PCR), *Helicobacter* group-specific product was detected when as little as 0.55 ng of DNA was used as template (Fig. 2).

Comparison of group-specific with species-specific duplex PCR analysis. We re-amplified the fecal DNA from the five *Helicobacter*-infected mice and the three uninfected mice that was analyzed by use of the group-specific duplex PCR assay (Fig. 1) with species-specific primers. The amplifications performed with primers Lac1/Lac2 in combination with primers o008/o009 generated two bands of the expected size (210 bp for *Helicobacter* sp., and 340 bp for *Lactobacillus* sp.) in three of the samples, and three bands (210 bp, 340 bp, and 390 bp for *H. bilis*) in the other two samples from infected mice, indicating that some samples had positive results for more than one *Helicobacter* species. Fecal DNA from the three uninfected mice generated only the expected 340-bp band for *Lactobacillus* sp. (Fig. 3). These results

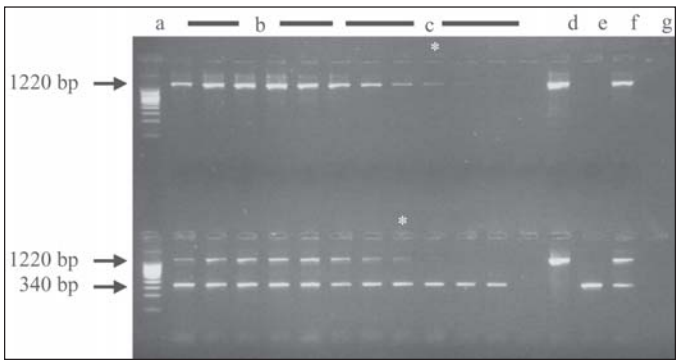

Figure 2. Ethidium bromide-stained agarose gel electrophoresis of PCR products (protocol 1). Top: amplification using C05 + C97 primers alone. Bottom: amplification using C05 + C97 + Lac1 + Lac2 primers (duplex PCR assay). (a) SmartLadder SF molecular marker, with bands ranging from 100 to 1,000 bp (Eurogentec, Seraing, Belgium). (b) To evaluate the overall sensitivity of PCR amplification, DNA isolated from feces of a *Helicobacter*-infected mouse was tested at various concentrations, from left to right: 78, 52, 39, 26, and 15 ng/reaction. (c) To evaluate loss of sensitivity in *Helicobacter* detection due to competition by *Lactobacillus* amplification, serial dilutions of DNA isolated from feces of a *Helicobacter*-infected mouse were tested. Dilutions were made with DNA isolated from feces of a *Helicobacter*-free mouse to keep *Lactobacillus* DNA concentration as constant as possible (at least equal to 15 ng in all reactions). Amount of DNA isolated from feces of the *Helicobacter*-infected mouse (from left to right): 5, 1.66, 0.55, 0.185, 0.061, and 0.0203 ng. White stars indicate the last well with clear amplification products. (d) *Helicobacter hepaticus* DNA (15 ng). (e) *Lactobacillus plantarum* DNA (15 ng). (f) DNA isolated from feces of another *Helicobacter*-infected mouse (15 ng). (g) Control with no DNA. *Lactobacillus* sp. = 340 bp; *Helicobacter* sp. = 1,220 bp.

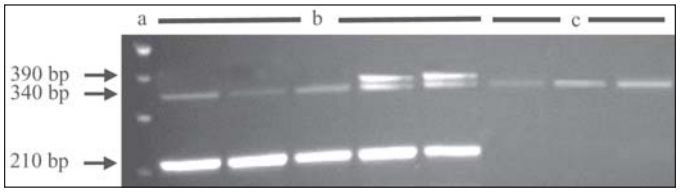

Figure 3. Ethidium bromide-stained agarose gel electrophoresis of PCR products (protocol 2). Amplification using o008 + o009 + Lac1 + Lac2 primers (duplex PCR). The species-specific primers o008 and o009 were previously reported to generate bands of approximately 210 bp for *H. muridarum, H. hepaticus*, and "*H. rappini*," and 390 bp for *H. bilis*. (a) A 100-bp DNA ladder, with bands ranging from 200 to 500 bp (Promega, Madison, Wis.). (b) DNA isolated from feces of five *Helicobacter*-infected mice (*see* Fig. 1). (c) DNA isolated from feces of three *Helicobacter*-free mice (*see* Fig. 1). *Lactobacillus* sp. = 340 bp.

indicate that it is possible to adapt the proposed duplex PCR assay using species-specific primers that yield PCR products closer to the size of the *Lactobacillus* sp. product.

Rate of false-negatives results. Using the developed duplex PCR assay, we examined 202 fecal DNA samples from the Animal Facilities at M. D. Anderson Cancer Center Science-Park Research Division during a 9-month period in 2003-2004. During the development of this duplex PCR assay, we occasionally observed samples that did not develop bands in the agarose gel (4/202). These DNA samples were re-purified, the DNA concentration was determined by OD_{260} and OD_{280} measurements, and the PCR assay was repeated. After using the new PCR assay, we could amplify the *Lactobacillus*-specific band with primers Lac1/Lac2, as well as *Helicobacter*-specific bands with primers C97/C05, indicating that these specimens were actually *Helicobacter* positive. Even

though the false-negative rate was low (< 2%), results for these samples would have been designated negative without the inclusion of the *Lactobacillus* sp. primers as an internal quality control.

Inter-laboratory repeatability of the duplex PCR assay. Identical results were obtained for each fecal DNA sample evaluated at each of the two independent laboratories, even though the amplifications were performed using non-identical PCR conditions and different thermocyclers (data not shown). In addition, use of the puRe*Taq* Ready-To-Go PCR Beads kit proved to be an efficient way to avoid contamination during specimen handling and processing, thus reducing the risk of false-positive results.

Discussion

The risk of obtaining false-negative results during fecal PCR assays routinely used to screen for *Helicobacter* sp. infections has always been a concern in face of the impact of obtaining a wrong diagnosis. In this study, we addressed this point by designing a simple quality control for false-negative results that makes use of the *Lactobacillus* species present in the normal intestinal flora of the laboratory mouse.

The procedure is simple and does not require use of internal DNA (mimic) controls or any other manipulation, only the addition of the *Lactobacillus* primers to the duplex PCR assay. The proposed duplex PCR method of *Helicobacter* detection was evaluated independently at two laboratories. We did not observe discrepant results in a series of blind tests using samples from our intramural mouse colonies. Using Lac1/Lac2 primers in combination with *Helicobacter*-specific primers in the duplex PCR assay, we consistently obtained a specific band (340 bp) for *Lactobacillus* sp. for the DNA purified from fecal specimens, although for some of them, we needed to repeat the extraction and adjust the concentration. Without this internal quality control, these specimens could have represented a false-negative result for *Helicobacter* diagnosis. In addition, using the PCR bead method greatly reduced the risk of contamination with previously amplified DNA. Compared with those of standard PCR analysis, these "ready-to-go" reagents are more expensive, but the benefits of preventing DNA contamination (false-positive results) clearly outweigh the higher costs.

In conclusion, we have developed a simple duplex PCR assay that is sensitive and specific for detecting false-negative results during routine *Helicobacter* feces tests. This is an important quality control for PCR diagnostic tests because of the potential presence of inhibitors in feces, which can diminish detection of *Helicobacter* infections. This *Lactobacillus* group-specific PCR assay could also potentially be useful for application to fecal PCR assays used to detect other pathogens in mouse quality-control programs.

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