Fluorogenic PCR-Based Quantitative Detection of a Murine Pathogen, Helicobacter hepaticus

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Helicobacter hepaticus infection in mice is being used as an animal model for elucidating the pathogenesis of gastrointestinal and biliary diseases in humans. H. hepaticus, which forms a spreading film on selective agar, is not amenable to routine quantitative counts of organisms in tissues using a CFU method. In this study, a fluorogenic PCR-based assay was developed to quantitatively detect H. hepaticus in mouse ceca and feces using the ABI Prism 7700 sequence detection system. A pair of primers and a probe for this assay were generated from the H. hepaticus cdTB gene (encoding subunit B of the H. hepaticus cytolethal distending toxin). Using this assay, the sensitivity for detection of H. hepaticus chromosomal DNA prepared from pure culture was 20 fg, which is equivalent to approximately 14 copies of the H. hepaticus genome based on an estimated genome size of ∼1.3 Mb determined by pulsed-field gel electrophoresis. H. hepaticus present in feces and cecal samples from H. hepaticus-infected mice was readily quantified. The selected PCR primers and probe did not generate fluorescent signals from eight other helicobacters (H. canis, H. cinedai, H. felis, H. mustelae, H. nemestrinae, H. pullorum, H. pylori, and H. rodentium). A fluorescent signal was detected from 20 ng of genomic DNA but with much lower sensitivity (10^9-fold) than from H. hepaticus DNA. Therefore, this assay can be used with high sensitivity and specificity to quantify H. hepaticus in experimentally infected mouse models as well as in naturally infected mice.

Helicobacter hepaticus, an enterohelpeic member of the genus Helicobacter, colonizes the lower gastrointestinal tract, including the cecum, colon, and hepato-biliary system of mice (4, 5). H. hepaticus infection can cause chronic active hepatitis and typhlitis in immunocompetent mice (6, 7, 22, 23) and can also lead to liver carcinoma in male mice of susceptible strains (4, 6, 9, 22, 23). Natural and experimental infection with H. hepaticus in certain immunodeficient mice can induce inflammatory bowel disease (1, 3, 10, 21). These studies have prompted the increased use of murine models with H. hepaticus infection to begin elucidating the possible roles of helicobacters in the development of gastrointestinal diseases in humans, particularly liver carcinogenesis and inflammatory bowel disease.

Progress in studying the pathogenic role of H. hepaticus in gastrointestinal diseases has been hampered by the difficulty in quantifying this pathogen in naturally and experimentally infected mice. The most commonly used technique is quantitative culture on selective agar plates, yielding CFU per unit (often gram) of sample weight. This technique is insensitive and inaccurate in quantifying CFU of H. hepaticus because of the organism’s fastidious growth requirements, and more importantly, H. hepaticus forms a spreading film on agar plates, making it impossible to count individual colonies (4). Recently, a fluorogenic TaqMan assay has been developed (12). It has been reported that this technique can detect approximately two copies of the Yersinia enterocolitica genome in blood samples (17). In this report, a sensitive and specific fluorogenic PCR assay for quantifying H. hepaticus in murine cecum and feces is described.

MATERIALS AND METHODS

Bacterial strains. Ten members of the genus Helicobacter, i.e., H. hepaticus type strain 3B1 (ATCC 51448), H. bilis ATCC 51630, H. canis ATCC 43772, H. cinedai ATCC 35683, H. felis ATCC 49179, H. mustelae ATCC 43772, H. nemestrinae ATCC 49396, H. pylori NCTC 11639, H. pullorum ATCC 12825, and H. rodentium ATCC 700285, were used in this study. These bacteria were grown under microaerobic conditions (5% O2, 10% H2, and 85% N2) on blood agar plates (Remel, Lenexa, Kans.) at 37°C for 3 to 5 days.

Experimental infection of mice with H. hepaticus. Viral antibody-free and helicobacter-free female A/JCr mice were purchased from the National Cancer Institute (Frederick, Md.). Mice were housed under environmental conditions of 22°C, 40 to 70% humidity, 15 air changes/h, and a 12 h–12 h light-dark cycle. Eight-week-old female A/JCr mice (n = 2) received 0.2 ml of fresh inoculum of H. hepaticus type strain ATCC 51448 by oral gavage every other day for a total of three doses. At 6 months postinfection, feces of the helicobacter-free (n = 2) and experimentally infected mice were collected, and these mice were then necropsied. Cecal and feces from these mice were stored at −20°C until use.

Preparation of DNA. Chromosomal DNA from bacterial cultures and total DNA from mouse cecum were prepared using a High Pure PCR kit according to the instructions of the supplier (Roche Molecular Biochemicals, Indianapolis, Ind.). For the isolation of DNA from mouse feces, five fecal pellets were suspended in 1 ml of phosphate-buffered saline, followed by brief centrifugation in a microcentrifuge. DNA was isolated from 200 μl of the supernatant using a QiAampDNA minikit according to the protocol of the supplier (Qiagen, Valencia, Calif.). The concentration of DNA was determined using Genequant (Amersham Pharmacia Biotech, Piscataway, N.J.).

Design of primers and probe. Two primers and a probe for fluorogenic PCR assays in the ABI Prism TaqMan 7700 sequence detection system (PE Biosystems, Foster City, Calif.) were derived from the H. hepaticus cdTB gene (encoding subunit B of bacterial cytolethal distending toxin) with the aid of the software Primer Express (PE Biosystems) (2, 25). The nucleotide sequences of the forward primer (cdTBF), reverse primer (cdTBR), and probe (cdTBP) are given in Fig. 1. This pair of PCR primers produces an 81-bp PCR DNA fragment. The probe was labeled with FAM (6-carboxyfluorescein, a fluorescent reporter) and with TAMARA (6-carboxytetramethylrhodamine, a fluorescent quencher) at its 3′ end. These sequences were compared with the corresponding regions of the
**RESULTS**

**Sensitivity of quantitative PCR using TaqMan.** To determine the detection limit of this assay, chromosomal DNA from pure *H. hepaticus* cultures was used as the PCR template. The concentrations of the primers and probe for optimal amplification were 400 and 100 nM, respectively. As shown in Fig. 4, 20 fg of DNA could be detected, whereas there was no significant difference in fluorescent signal intensity between 2 fg of this DNA and the no-template controls. The genome size of *H. hepaticus* was previously estimated as 1.3 Mb using pulsed-

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**FIG. 1.** Sequence comparison between the *H. hepaticus* primers and probe and the corresponding regions of the *cdtB* genes in selected bacteria. *Hh*, *H. hepaticus*; *Cj*, *C. jejuni*; *Hb*, *H. bilis*; *Hc*, *H. canis*. Numbers indicate the positions of the respective primers and probe in the nucleotide sequence determined by Young et al. (25). The nucleotides different from those present in the *H. hepaticus* *cdtB* gene are indicated in boldface. The sequence of primer *cdtBR* is reverse and complementary to the sequence shown here. For *H. bilis* and *H. canis*, only the partial sequence of the *cdtB* gene is available (2).

**FIG. 2.** Detection limitation of the TaqMan PCR assay for *H. hepaticus* genomic DNA. Amplification plots 1 to 6 were generated from 2 × 10⁷, 2 × 10⁶, 2 × 10⁵, 200, 20, and 2 fg, respectively. The *r*² value from the linear regression in this assay is >0.99.
field gel electrophoresis (16). Since the molecular weight for 1 bp is 650, the 1.3-Mb Fig. 1. Evaluation of the specificity of the primers and probe for H. hepaticus. Amplification plots 1 and 2 were generated using $2 \times 10^7$ and $2 \times 10^6$ fg of H. hepaticus chromosomal DNA, respectively, whereas plots 3 to 11 were produced using $2 \times 10^7$ fg of chromosomal DNA from H. bilis, H. canis, H. cineadi, H. felis, H. mustelae, H. nemestrinae, H. pullorum, H. pylori, and H. rodentium, respectively.

H. hepaticus genome is equal to approximately $8.45 \times 10^8$ g/mol, which contains $6.0 \times 10^{23}$ molecules/mol (Avogadro’s number). Thus, H. hepaticus genomic DNA contains $7.1 \times 10^{14}$ molecules and 20 fg is equivalent to $\approx 14$ copies. We demonstrate that 14 or more copies of the H. hepaticus genome in a sample can be detected in this assay.

Specificity of primer and probe. To investigate the specificity of the primers and the probe used in the TaqMan PCR assay, genomic DNA samples from nine species of helicobacters, i.e., H. bilis, H. canis, H. cineadi, H. felis, H. mustelae, H. nemestrinae, H. pylori, H. pullorum, and H. rodentium, were assayed using these primers and probe. The enterohepatic helicobacters H. bilis, H. canis, and H. pullorum have been shown to have cytolethal distending toxin activity that causes progressive cell enlargement and eventual death (2, 24, 25). There were two reasons for selecting the H. hepaticus cdtB gene as a PCR target in this assay. First, the designed primers and probe will be used to investigate the role of cytolethal distending toxin in the pathogenesis of H. hepaticus. Second, the partial sequences of the cdtB gene in H. bilis and H. canis, which are close relatives of H. hepaticus, have been determined, so they were used for sequence comparison with that of H. hepaticus (2).

These limited nucleotide sequences of the cdtB gene in H. bilis and H. canis encompass only the forward primer cdtBF and part of the cdtBP sequence. Sequence comparison revealed that cdtBF and cdtBP display little sequence similarity to the H. canis cdtB gene but have significant sequence identity to the H. bilis cdtB gene (Fig. 1). In addition, these primers and probe did not display significant sequence similarity to the cdtB gene in C. jejuni (Fig. 1). In the TaqMan PCR assay, Ct values for 20 and 2 ng of the H. hepaticus genomic DNA are 16 and 20, respectively (Fig. 3). In contrast, there was no fluorescent signal detected from 20 ng of eight selected non-H. hepaticus helicobacterial DNA templates. A weak signal (Ct $\approx 36.4$), which is approximately equivalent to 20 fg of H. hepaticus DNA, was detected from 20 ng of H. bilis DNA. The results demonstrate that these primers and probe can be used to detect H. hepaticus with high specificity.

Detection of the H. hepaticus genome present in mouse cecum and feces in TaqMan PCR assay. To test the applicability of this assay in samples from experimentally infected mice, chromosomal DNA was prepared from ceca and feces of two A/JCr mice experimentally inoculated with H. hepaticus and two control (helicobacter-free) mice. Subsequently, the H. hepaticus genomic DNA (20 ng and 0.2 pg) was spiked into 20 ng of either cecal or fecal DNA prepared from the helicobacter-free mouse and then analyzed using the TaqMan PCR assay for investigating if the cecal and fecal DNA preparations interfere with the PCR amplification of H. hepaticus DNA. The spiked H. hepaticus DNA produced Ct values (16.4 at 20 ng and 36.5 at 2 ng) similar to those for the pure standard DNA (Fig. 2, plots 1 and 2), demonstrating that there is no inhibitory effect on the PCR amplification in the presence of murine chromosomal DNA. Twenty nanograms of each DNA template in parallel with the H. hepaticus DNA standards was evaluated using the TaqMan PCR assay with the primer pair cdtBF-cdtBR and probe cdtBP. The quantity of H. hepaticus DNA in each sample was calculated using the standard curve (Fig. 4) and then converted into the number of copies of the H. hepaticus genome (Table 1). Using the same quantity of the
initial DNA templates from the ceca and feces, different copy numbers of the H. hepaticus genome were detected: 13.6 × 10^4 and 9.1 × 10^3 in the cecum and feces, respectively, of one mouse (mouse I) and 6.2 × 10^4 and 7.8 × 10^3 in the cecum and feces, respectively, of a second mouse (mouse II). In the two uninfected control mice, no H. hepaticus DNA was detected in any of the samples. These data demonstrate that H. hepaticus present in murine ceca and feces was specifically and sensitively quantified using this assay.

**DISCUSSION**

Quantitative analysis of a microbial pathogen in its host provides information useful in elucidating the mechanisms utilized by the pathogen to elicit disease and to evade immune defenses. This analysis is also useful in evaluating the efficacy of vaccines and new drugs for eradicating specific pathogens. Quantitative culture has been widely used to quantify bacterial pathogens which form single colonies on agar plates. In the case of H. hepaticus, which does not form single colonies (4), an alternative technique is needed for its quantification. In this study, a rapid, sensitive, and reproducible fluorogenic PCR assay in the ABI Prism 7700 sequence detection system was developed to quantify H. hepaticus in samples from experimentally infected mice. PCR primers producing an 81-bp amplicon and a probe were generated from the H. hepaticus cdtB gene. This technique is readily applicable to DNA templates prepared from ceca and feces of mice and will be a powerful tool in determining the pathogenic role of H. hepaticus in the induction of murine gastrointestinal and biliary diseases as an animal model for understanding the development of similar human diseases. More recently, the copy numbers of the H. hepaticus genome in ceca from experimentally infected A/JCr (n = 23) and C57BL/6 (n = 20) mice were determined by this technique; the numbers of H. hepaticus in A/JCr and C57BL/6 mice were significantly different (P < 0.003; 7.14 × 10^5 and 2.27 × 10^7 bacteria/ng of mouse DNA, respectively) (M. T. Whary, Z. Ge, and J. G. Fox, unpublished data).

The designed primers and probe did not generate PCR amplification signals from eight helicobacters, including H. pylori, H. felis, H. mustelae, H. pullorum, H. cinaedi, H. rodentium, H. nemestrinae, and H. canis, or from mouse DNA. PCR amplification from H. bilis DNA was detected (Fig. 3); however, this amplification was much lower in sensitivity (Ct = ∼36.4 for H. bilis at 20 ng) than that for H. hepaticus (Ct = ∼16.3 at 20 ng). This cross-amplification between H. hepaticus and H. bilis could be due to the fact that primer cdtBF and probe cdtBP share significant sequence similarity to the corresponding regions of the H. bilis cdtB gene (Fig. 1). In addition, the reverse primer cdtBR should be expected to have a similar degree of sequence identity to the corresponding region of the H. bilis cdtB gene, since there is 69.6% nucleotide sequence identity in the 702-bp region of the cdtB gene between H. bilis and H. hepaticus (2). It has been reported that the mismatched

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**TABLE 1. Detection of H. hepaticus present in mouse tissues and feces**

| Sample (20 ng) | Ct | Quantity (fg) of H. hepaticus DNA | Copy no. |
|---------------|----|----------------------------------|----------|
| Mouse I       |    |                                   |          |
| Cecum (C1)    | 23.81 | 1.95 × 10^5                                                      | 136,500  |
| Feces (F1)    | 27.68 | 1.3 × 10^4                                                      | 9,100    |
| Mouse II      |    |                                   |          |
| Cecum (C2)    | 25.46 | 6.2 × 10^4                                                      | 43,400   |
| Feces (F2)    | 28.45 | 7.8 × 10^3                                                      | 5,460    |

*a* The Ct values for the samples from the control mice were greater than 39.

*b* The copy numbers were calculated by converting 1 fg into 0.7 copy of the H. hepaticus genome.
3’ end of a primer discriminated point mutations in the *H. pylori* genome (8, 19) and in plasmid DNA (13, 14, 20). However, the discriminatory efficiency of such a primer depends on PCR conditions (annealing temperature and concentrations of deoxynucleoside triphosphates and Mg\(^{2+}\)) and the nature of a mismatch (such as A:A, A:G, G:G, T:T, or T:C, etc.) (11, 13, 14). In this assay, cdtBF contains a thymidine mismatched with a cytosine of the *H. bilis* cdtB gene and the PCR conditions, including reagents and thermocycling parameters, were used as recommended by the manufacturer; this likely contributed to the low-efficiency amplification from the *H. bilis* chromosomal DNA. It is worth noting that the detection limit for *H. bilis* with the primer pair we used is 10\(^6\)-fold more insensitive than that for *H. hepaticus*, since the Ct value for 20 ng of *H. bilis* DNA is approximately equivalent to that for 20 fg of *H. hepaticus* DNA. Although the theoretical limit should have no or little impact on the final copy number of *H. hepaticus*, in addition, the presence of the *H. bilis* DNA in a sample can be easily discriminated using the *H. bilis*-specific primers targeting the 16S rRNA gene (18). Furthermore, additional primers and probes with higher specificity for *H. hepaticus* can be designed when more information on the *H. hepaticus* genome is available.

In theory, a single copy of a target nucleotide sequence can be detected in a sample under optimal conditions using the ABI Prism 7700 sequence detection system. However, many factors, such as the purity and complexity of a PCR template and concentrations of Mg\(^{2+}\), primers, and the probe, could significantly influence this sensitivity. In our assay, the detection limit is 20 fg, which is approximately equivalent to 14 copies of the *H. hepaticus* genome. Although the theoretical detection limit could not be achieved, this sensitivity is much higher than those of other available techniques and should be sufficient for quantifying *H. hepaticus* in experimental animal models and probably in natural infections as well. Further investigations on the dynamic relationship between the colonization site or number of *H. hepaticus* organisms and the inflammatory state in infected mice during chronic infection will increase our understanding of the mechanism of helicobacter-induced gastrointestinal and liver diseases.

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