Comparative Medicine
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Vol 52, No 4
August 2002
Pages 347-353

Detection of Rodent *Helicobacter* spp. by Use of Fluorogenic Nuclease Polymerase Chain Reaction Assays

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Polymerase chain reaction (PCR) analysis is the standard method for detection of *Helicobacter* spp. infections in laboratory rodents, with *H. hepaticus*, *H. bilis*, and *H. typhlonius* considered primary pathogens. Fluorogenic nuclease PCR assays that detect all known rodent *Helicobacter* spp., or that specifically detect *H. hepaticus*, *H. bilis*, or *H. typhlonius* were developed to eliminate post-PCR processing, enhance specificity, and provide quantitative data on starting template concentration. Each fluorogenic PCR assay detected a minimum of 10 copies of target template, had comparable or greater sensitivity when compared directly with corollary gel detection PCR assays, and detected only targeted species when numerous *Helicobacter* spp. and other enteric bacteria were analyzed. Fluorogenic nuclease PCR analysis of fecal DNA samples obtained from numerous laboratory mice sources detected all samples with positive results by use of *Helicobacter* spp., *H. hepaticus*, *H. bilis*, and/or *H. typhlonius* gel detection PCR analysis, except for one sample that had positive results by *H. typhlonius* gel detection PCR but negative results by *H. typhlonius* fluorogenic nuclease PCR analysis. Among fecal DNA samples that were *Helicobacter* spp. negative by use of all gel detection PCR assays, the fluorogenic nuclease PCR assays detected target template in only one sample that was positive by use of the *Helicobacter* spp. and the *H. bilis* fluorogenic nuclease PCR assays. In conclusion, fluorogenic nuclease PCR assays provide sensitive, specific, and high-throughput diagnostic assays for detection of *Helicobacter* spp., *H. hepaticus*, *H. bilis*, and *H. typhlonius* in laboratory rodents, and the quantitative data generated by these assays make them potentially useful for bacterial load determination.

The genus *Helicobacter* is composed of gram-negative, microaerophilic, spiral to curve-shaped bacteria that commonly inhabit the gastrointestinal system of humans and animals. During the past decade, numerous *Helicobacter* species have been isolated from laboratory rodents, of which *H. hepaticus*, *H. bilis* and *H. typhlonius* are considered primary pathogens. *Helicobacter hepaticus* has been associated with hepatitis, proliferative typhlitis, colitis, and proctitis in multiple mouse strains (1-7), and with increased liver tumor incidence in A/JCr mice (3, 8). *Helicobacter bilis* is known to cause hepatitis in CBA/CA, DBA/2, and C56BL/6 mice (9), proliferative typhlitis in *scid* mice (10, 11), and proliferative and ulcerative typhlitis, colitis, and proctitis in athymic nude rats (12, 13). *Helicobacter typhlonius* has been associated with proliferative typhlocolitis in *scid* and interleukin 10-deficient mice (14-16). Therefore, accurate identification of rodents infected with *Helicobacter* spp., and specifically those infected with *H. hepaticus*, *H. bilis*, and *H. typhlonius*, is essential due to the deleterious effects these bacteria may have on animal-based research. Although histologic examination, culture, and serologic testing have been used to detect rodent *Helicobacter* spp., polymerase chain reaction (PCR) analysis, due to its enhanced sensitivity, specificity, and availability, is currently the favored diagnostic tool for evaluating the *Helicobacter* status of laboratory rodents (11, 15, 17-21). However, conventional PCR analysis requires gel electrophoresis for amplicon detection, which increases labor, reagent costs, and the potential for carry-over contamination (22). Results obtained from different laboratories can also vary tremendously, indicating a general lack of sensitivity and/or specificity among existing *Helicobacter* PCR assays as performed at these laboratories (23, 24). Therefore, PCR-based diagnostic assays that are sensitive and specific, and have potential for high throughput are needed for detection of rodent *Helicobacter* species.

A recently developed technique, fluorogenic nuclease PCR (TaqMan) (25), has several advantages over gel-detection PCR. Fluorogenic nuclease PCR eliminates the need for post-PCR processing, which decreases labor, turn-around time, and expense, qualities that allow application of this technology to development of high-throughput diagnostic assays. In addition, use of an internal fluorogenic probe potentially confers greater specificity than does gel detection PCR. A major advantage of fluorogenic nuclease PCR is the ability to generate quantitative data, which can be used to determine bacterial load (26, 27). Because *Helicobacter* spp. grow as a thin spreading film on agar plates, determination of bacterial load by use of standard plate counting methods is extremely difficult, and this difficulty has slowed progress of pathogenesis studies of these bacteria. As *H. hepaticus*, and to a lesser extent, *H. bilis* and *H. typhlonius*, are used in the murine host as a model for human inflammatory bowel disease, a method to accurately

Received: 2/25/02. Revision requested: 3/14/02. Accepted: 5/17/02.
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quantify bacterial load for each of these bacterial species is critically needed. Therefore, the goals of the study reported here were to develop quantitative fluorogenic nuclease PCR assays that could be used to diagnose rodents naturally infected with *Helicobacter* spp. and to quantify *Helicobacter* spp. load in naturally and experimentally infected rodents. To this end, a generic fluorogenic nuclease PCR assay that detects all known rodent *Helicobacter* spp. and three specific quantitative fluorogenic nuclease PCR assays that detect *H. hepaticus*, *H. bilis*, or *H. typhlonius,* respectively, were developed.

Materials and Methods

Bacteria. All *Helicobacter* isolates and purified DNA from *H. typhlonius* (strain MU 96-1) and *H. rodentium* (strain MIT 95-1707) were obtained from the University of Missouri Research Animal Diagnostic Laboratory (Columbia, Mo.). *Helicobacter hepaticus* (strain MU 94-1), *H. bilis* (strain MU-96-1), *H. muridarum* (ATCC 49282), *H. cholecystus* (strain MU Hkb- 1), and *H. mesocricetorum* (strain MU 97-1514) isolates were grown in *Brucella* broth at 37°C with shaking under microaerophilic conditions (90% N_2, 5% H_2, and 5% CO_2) for one to three days. *Campylobacter* isolates, *Bacillus subtilis*, and *Clostridium perfringens* were kindly provided by Drs. Lynn Joens, Wayne Nicholson, and Stephen Billington, respectively (University of Arizona, Tucson, Ariz.). *Citrobacter amalonaticus*, *Proteus mirabilis*, *Escherichia coli*, *Klebsiella oxytoca*, and *Pseudomonas aeruginosa* isolates were obtained from aerobic enteric cultures of rodents maintained in intramural animal facilities.

Fluorogenic Nuclease PCR assays. Available rodent *Helicobacter* spp. 16S rRNA sequences were obtained from GenBank and were aligned with the ClustalW and Pretty software programs (Genetics Computer Group, Madison, Wis.). Oligonucleotide primers and probe sequences were selected from regions conserved among *Helicobacter* spp., or unique to *H. bilis*, and *H. typhlonius*, using the Primer Express software program (PE Applied Biosystems, Foster City, Calif.). *Helicobacter hepaticus* primers and probe were selected from a region of the *H. hepaticus* urease gene (28). The specificity of the oligonucleotide primer/probe sequences for each assay was verified by use of the BLAST database search program (29). Oligonucleotide primers and fluorogenic probes were synthesized by Sigma-Genosys (Woodlands, Tex.) and PE Applied Biosystems, respectively. All fluorogenic nuclease PCR reactions were performed in a PE Applied Biosystems GeneAmp 5700 Sequence Detection System, and products were analyzed by use of the accompanying software. Each 25-µl reaction contained 2.5 µl of template, 1X TaqMan Buffer A (50 m*M* KCl, 10 µ*M* EDTA, 10 m*M* Tris-HCl [pH 8.3], and 60 n*M* Passive Reference), 5.5 m*M* $MgCl_2$, 200 µ*M* (each) dATP, dCTP, and dGTP, 400 µ*M* dUTP, 0.05% gelatin, 0.01% Tween-20, 100 n*M* probe, 300 n*M* primers, 0.25 U of AmpErase uracil-N-glycosylase (UNG), and 0.625 U of AmpliTaq Gold Polymerase (PE Applied Biosystems).

Thermal cycling conditions consisted of 50°C for 2 min (UNG incubation), 95°C for 10 min (polymerase activation), and 45 cycles at 95°C for 15 sec, followed by 60°C for 1 min. Samples used for evaluating sensitivity were considered test positive if they had mean fluorescence (Rn) > 0.05 and cycle threshold (Ct) < 45. Diagnostic and fecal samples were considered test positive if they had mean fluorescence (Rn) > 0.05 and Ct < 40. The baseline Rn value was selected so as to intersect the amplification curve in the middle of the linear amplification phase (as recommended by the manufacturer). The slightly lower Ct limit used for evaluation of the diagnostic samples was selected on the basis of our experience with this technique to minimize incidence of false-positive results.

Amplicon cloning. Amplicon DNA from each fluorogenic nuclease PCR assay was generated in a PE Applied Biosystems GeneAmp 2400 with the aforementioned reagents and thermocycling times; however, 200 µ*M* dTTP was used instead of dUTP and UNG was omitted. Amplicon DNA was resolved by agarose gel electrophoresis, and the appropriately sized target bands were cut from the gel and eluted, using a QIAquick Spin kit (Qiagen, Inc., Valencia, Calif.) according to the manufacturer's instructions. The 208-basepair (bp) *Helicobacter* spp. product, 99-bp *H. hepaticus* product, 92-bp *H. bilis* product, and 115-bp *H. typhlonius* product were each cloned separately into PT7Blue-2 vectors, using the Perfectly Blunt Cloning Kit, and were amplified in Tuner (DE3) pLac1-competent cells (Novagen, Madison, Wis.). The plasmid was purified, using the Qiagen Plasmid Purification kit, and the vector insert was sequenced by the University of Arizona's Molecular Core Facility, using the BigDye Terminator cycle sequencing kit (PE Applied Biosystems) to verify presence of the complete amplicon. Plasmid DNA concentration was determined by use of an MBA 2000 UV-vis spectrophotometer (PE Applied Biosystems), then was expressed as template copy number concentration through use of the calculated molecular weight of the plasmid with the amplicon insert.

Specificity. The specificity of each fluorogenic nuclease PCR assay was determined by evaluation of DNA extracts of *H. hepaticus*, *H. bilis*, *H. typhlonius*, *H. rodentium*, *H. muridarum*, *H. cholecystus*, *H. mesocricetorum, Bacillus subtilis*, *Campylobacter jejuni*, *C. coli*, *Citrobacter amalonaticus*, *Clostridium perfringens, Escherichia coli*, *Klebsiella oxytoca*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. The DNA was extracted from each bacterium, using a QIAamp DNA kit (Qiagen) according to manufacturer's instructions.

Sensitivity. The absolute sensitivity of each fluorogenic nuclease PCR assay was determined by evaluation of 10-fold serial dilutions of cloned amplicon DNA, from an estimated 10^7 to10^0 copies of template, with and without 2.5 µl of fecal DNA extract. The fecal DNA was prepared as subsequently described for the Qiagen Stool kit. Relative sensitivity of gel detection PCR assays for *Helicobacter* spp. and *H. bilis* (20), *H. hepaticus*, and *H. typhlonius* (15, 16) and of the corollary fluorogenic nuclease PCR assays were determined by evaluation of serially diluted genomic DNA extracted from *H. hepaticus*, *H. bilis*, and *H. typhlonius*. The PCR reactions were performed in a PE Applied Biosystems GeneAmp 2400, as described (20). Briefly, each PCR reaction (50-µl total volume) contained 5 µl of template DNA, 10X assay buffer (50 m*M* KCl, 10 m*M* Tris-HCl [pH 9.0], and 0.1% Triton X-100), 3 m*M* $MgCl_2$, 200 µ*M* (each) dATP, dGTP, dCTP, and dTTP, 1 µ*M* each primer, and 1 U of *Taq* polymerase. Thermal cycling conditions consisted of 94°C for 5 min, then 45 cycles at 94°C for 15 sec, 53°C for 15 sec, and 72°C for 1 min, followed by a final extension at 72°C for 12 min. The PCR products were separated on an ethidium bromide-stained 3% agarose gel, and viewed under UV light.

Diagnostic sample evaluation. Fecal DNA extracts from multiple laboratory mice sources that had been previously evaluated by PCR analysis were used to evaluate the specificity

of the fluorogenic nuclease PCR assays. Fecal DNA was extracted, using an adaptation of the QIAamp Tissue Kit (Qiagen), as described (18), and was evaluated by use of *Helicobacter* spp., *H. hepaticus*, *H. bilis*, *H. typhlonius*, and *H. rodentium* gel detection PCR assays in a multiplex format (30). Aliquots of these fecal DNA samples were then evaluated by use of the *Helicobacter* spp., *H. hepaticus*, *H. bilis*, and *H. typhlonius* fluorogenic nuclease PCR assays. Amplicon size was determined for all discrepant samples by use of agarose gel electrophoresis, and at least three discrepant samples for each fluorogenic nuclease PCR assay were sequenced by the University of Arizona Molecular Core Facility after gel extraction, using a QIAquick Spin kit (Qiagen).

Fecal DNA extraction method comparison. The DNA was extracted from fecal pellets obtained from intramural mouse colonies, using the Qiagen Stool Kit and the hot sodium hydroxide solution with Tris buffer (HotSHOT) method (31). Approximately 20 fecal pellets were collected per mouse pan, of which 180 to 200 mg of feces (approx. 10 fecal pellets) were used for the Qiagen kit, and one fecal pellet was used for the HotSHOT method. Fecal DNA was extracted, using the Qiagen Stool Kit as per the manufacturer's instructions, and was eluted in 200 µl of water. The HotSHOT method was performed as described (31). Briefly, 3 ml of alkaline lysis reagent (25 m*M* NaOH and 0.2 m*M* EDTA, pH 12) were added to a single mouse fecal pellet, vortexed for one minute or until the pellet was dispersed, incubated in a 95°C water bath for 10 min, and centrifuged for 1 min at 200 ×*g*. Equal volumes (500 µl) of supernatant and 40 m*M* Tris HCl (pH 5.0) were then combined and vortexed. The DNA extracted by use of both methods and HotSHOT DNA diluted 1:10 in water were each evaluated by use of the *Helicobacter* spp. fluorogenic nuclease PCR assay.

Results

Selection and evaluation of primer and probe sequences. Sequence alignment of the rodent *Helicobacter* 16S rRNA genes revealed an area conserved among all known rodent *Helicobacter* spp., but heterologous to other bacterial genera. In addition, intervening sequences unique to *H. bilis* and *H. typhlonius* were identified in the 16S rRNA gene sequences of these species. These three regions of the 16S rRNA gene were used to design the primers and probes for the *Helicobacter* spp., *H. bilis*, and *H. typhlonius* assays, respectively (Table 1). Regions in the 16S rRNA gene sequence unique to *H. hepaticus* that fulfilled the parameters necessary for fluorogenic nuclease PCR primer/probe design could not be identified; therefore, the *H. hepaticus* urease gene was used for the *H. hepaticus*-specific fluorogenic nuclease PCR assay (Table 1). All primers were optimized at a concentration of 300 n*M* through use of checkerboard titrations of 50, 300, and 900 n*M* of each primer with a 125 n*M* probe. Similarly, the probes were optimized at 100 n*M* through evaluation of 25, 50, 75, 100, 125, 150, 175, 200, and 225 n*M* concentrations of probe with 300 n*M* primer concentration.

Specificity. The specificity of each fluorogenic nuclease PCR assay was determined by evaluation of DNA extracts of *H. hepaticus*, *H. bilis*, *H. typhlonius*, *H. rodentium*, *H. muridarum*, *H. cholecystus*, *H. mesocricetorum, Bacillus subtilis*, *Campylobacter jejuni*, *C. coli*, *Citrobacter amalonaticus*, *Clostridium perfringens, Escherichia coli*, *Klebsiella oxytoca*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. The *Helicobacter* spp. assay detected all rodent *Helicobacter* spp., and the *H. hepaticus*, *H. bilis*, and *H. typhlonius* assays detected only *H. hepaticus*, *H. bilis*, and *H. typhlonius*, respectively. *Bacillus subtilis*, *Campylobacter jejuni*, *C. coli*, *Citrobacter amalonaticus*, *Clostridium perfringens, Escherichia coli*, *Klebsiella oxytoca*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* were not detected by use of any of the fluorogenic nuclease assays.

Sensitivity. The absolute sensitivity of each fluorogenic nuclease PCR assay was determined by evaluation of serial dilutions of cloned amplicon DNA. The *H. hepaticus* and *H. bilis* assays detected an estimated one copy of target template, whereas the *Helicobacter* spp. and *H. typhlonius* assays de-

Table 1. Primer and probe sequences for *Helicobacter* spp., *H. hepaticus*, *H. bilis*, and *H. typhlonius* fluorogenic nuclease polymerase chain reaction (PCR) assays

Assay	Sequence (5'–3')	Position (5'–3')[1]
Helicobacter spp.		
Forward primer	GATCAGCCTATGTCCTATCAGCTTG	384–408
Reverse primer	AGTTTACAATCCTAAAACCTTCATCCTC	591–564
Probe	TCCGATCACCCTCTCAGGCCGGATAC	466–441
H. hepaticus		
Forward primer	GAGATTAAATTTGGCGGAGGAA	814–835
Reverse primer	CATTGCATTTGTGATGACTGCA	912–891
Probe	AGTGCTTGCGCTTTGTGCCATACCA	873–849
H. bilis		
Forward primer	TGGCACAAAATTCTAGTATTTGGAATG	232–258
Reverse primer	AATTTGCTTGTGCGACAGACACTA	323–300
Probe	CCGCACAAATTGCTTCACAACATCAAT	289–263
H. typhlonius		
Forward primer	GGACTCTTAAATATGCTCCTAGAGTATTTTTAA	165–197
Reverse primer	CGTGTTTGAATGCGTCAAATTG	279–258
Probe	CTTCTACAATTTTCCTTTTCACTCACGCGACTTCTT	236–201

[1]Nucleotide positions for *Helicobacter* spp., *H. hepaticus*, *H. bilis*, and *H. typhlonius* obtained from GenBank accession numbers u18766, u75749, u18766, and af061104, respectively.

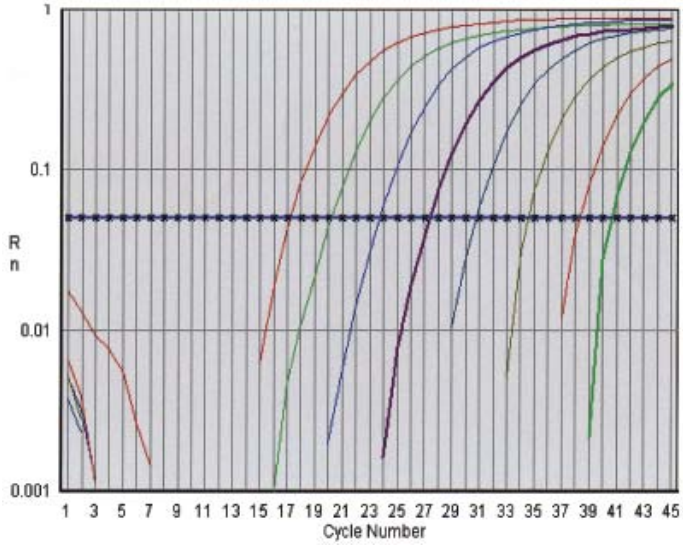

Figure 1. Absolute sensitivity of the *Helicobacter hepaticus* fluorogenic nuclease polymerase chain reaction (PCR) assay as documented by an amplification plot of 10-fold serially diluted cloned amplicon DNA. Target template concentrations from left to right on the amplification plot range from 10^7 to 10^0 copies (labeled 1–8, respectively). All values with mean fluorescence (Rn) > 0.05 and cycle threshold (Ct) < 45 are considered positive results.

Table 2. Evaluation of fecal DNA extracts by use of the fluorogenic nuclease PCR and multiplex gel detection PCR assays

	Fluorogenic Nuclease PCR			
Helicobacter infection status[1]	*H. hepaticus* pos.	*H. bilis* pos.	*H. typhlonius* pos.	*Helicobacter spp.* pos
H. hepaticus pos.	25/25[2]	2/25	4/25	25/25
H. bilis pos.	3/23	23/23	1/23	23/23
H. typhlonius pos.	4/15	3/15	14/15	15/15
H. rodentium pos.	2/7	0/7	0/7	7/7
H. hepaticus/H. bilis	8/8	8/8	0/8	8/8
H. hepaticus/H. typhlonius	4/4	1/4	4/4	4/4
H. hepaticus/H. rodentium	3/3	1/3	1/3	3/3
H. bilis/H. typhlonius	1/1	1/1	1/1	1/1
H. hepaticus/H. bilis/H. typhlonius	2/2	2/2	2/2	2/2
Helicobacter spp. neg.	0/46	1/46	0/46	1/46

[1]As defined by multiplex gel detection PCR results.
[2]No. of animals positive/No. of animals tested.
pos. = positive; neg.= negative

tected an estimated 10 copies of target template (Fig. 1). When identical dilutions of cloned amplicon DNA were evaluated in the presence of fecal DNA extract, the absolute sensitivity of each assay was reduced approximately 10-fold. The relative sensitivities also were determined for each fluorogenic nuclease PCR assay, compared with previously reported gel detection PCR assays for *Helicobacter* spp., *H. hepaticus*, *H. bilis*, and *H. typhlonius*. Tenfold serial dilutions of *H. bilis* genomic DNA were used for the *Helicobacter* spp. and *H. bilis* assays, 10-fold serial dilutions of *H. hepaticus* genomic DNA were used for the *H. hepaticus* assays, and 10-fold serial dilutions of *H. typhlonius* genomic DNA were used for the *H. typhlonius* assays. The *Helicobacter* spp., *H. hepaticus*, and *H. bilis* fluorogenic nuclease PCR assays were approximately 10,000-fold, 10-fold, and 100-fold more sensitive than their respective gel detection PCR assays, whereas the *H. typhlonius* fluorogenic nuclease PCR assay was 10-fold less sensitive than the corresponding gel detection PCR assay (data not shown).

Diagnostic sample evaluation. Fecal DNA extracts were obtained from multiple laboratory mice sources and were evaluated by each fluorogenic nuclease PCR assay and a recently developed multiplex gel detection PCR assay (Table 2). All samples that were PCR-positive for one or more *Helicobacter* spp. also were positive by use of the *Helicobacter* spp. fluorogenic nuclease PCR assay. All *Helicobacter* spp. PCR-negative samples were negative by use of the *Helicobacter* spp. fluorogenic nuclease PCR assay, with the exception of one PCR-negative sample that was positive by use of the *Helicobacter* spp. and *H. bilis* fluorogenic nuclease PCR assays. Amplicon DNA generated by each of the two fluorogenic nuclease PCR assays from this PCR-negative sample were sequenced, and each amplicon sequence was 100% homologous to the target sequence for the *Helicobacter* spp. and *H. bilis* fluorogenic nuclease PCR assays, respectively. Use of the *H. hepaticus* fluorogenic nuclease PCR assay detected all samples that were *H. hepaticus* PCR-positive, and did not detect any of the *Helicobacter* spp. PCR-negative samples. Use of the *H. bilis* fluorogenic nuclease PCR assay detected all *H. bilis* PCR-positive samples and only one of the *Helicobacter* spp. PCR-negative samples, the aforementioned sample that was also test positive by use of the *Helicobacter* spp. fluorogenic nuclease assay. Use of the *H. typhlonius* fluorogenic nuclease PCR assay detected all but one of the samples that were *H. typhlonius* PCR positive, and did not detect any of the *Helicobacter* spp. PCR-negative samples.

Interestingly, the *H. hepaticus* fluorogenic nuclease PCR assay detected target template in several samples that were PCR negative for *H. hepaticus* but PCR positive for *H. bilis*, *H. typhlonius* and/or *H. rodentium*. Similarly, the *H. bilis* and *H. typhlonius* fluorogenic nuclease PCR assays detected target template in samples that were PCR negative for *H. bilis* or *H. typhlonius* respectively, but were PCR positive for at least one other *Helicobacter* species. Amplicon DNA generated by the fluorogenic nuclease PCR assay for each discrepant sample was the expected size, as determined by ethidium bromide-stained agarose gel electrophoresis. In addition, amplicon DNA from at least three of these discrepant samples for each fluorogenic nuclease PCR assay were sequenced and had complete homology with the genomic sequence targeted by each assay.

Fecal DNA extraction method comparison. Because DNA quality may impact PCR assay performance, two methods of DNA extraction were evaluated for the fluorogenic PCR assays. Fecal pellets were obtained from multiple intramural mouse colonies. The DNA was then extracted from the fecal pellets by use of the Qiagen Stool Kit and the HotSHOT method and was evaluated by use of the *Helicobacter* spp. fluorogenic nuclease PCR assay. *Helicobacter* DNA was detected in 19 out of 47 samples by use of at least one of the methods. Of these 19 samples, 18 were detected by use of the Qiagen Stool Kit, and 12 were detected by use of the HotSHOT method, either from undiluted DNA or DNA diluted 1:10 in water (Table 3). Of the 12 samples detected by use of the HotSHOT method, seven samples were positive with use of undiluted and diluted DNA, three were positive only with use of undiluted DNA, and two were positive only with use of diluted DNA.

Discussion

Fluorogenic nuclease PCR assays that detect all rodent *Helicobacter* spp., or specifically detect *H. hepaticus*, *H. bilis*, and *H. typhlonius* were developed. All assays proved to be specific for their respective target when other rodent *Helicobacter* spp. and enteric bacteria were evaluated, and had excellent sensitivity, detecting an estimated 10 or fewer copies of target template. The relative sensitivities of the fluorogenic nuclease PCR assays, compared with those of previously published gel-detection PCR assays, were then evaluated. The fluorogenic nuclease assays were as much as five logarithms more sensitive than were the gel-detection PCR assays, except for the *H. typhlonius* fluorogenic nuclease PCR assay, which was approximately 10-fold less sensitive than the corresponding gel-detection PCR assay. The improved sensitivity of the *Helicobacter* spp., *H. hepaticus*,

Table 3. Comparison of Qiagen and HotSHOT fecal extraction methods as evaluated by use of the *Helicobacter* spp. fluorogenic nuclease PCR assay

Sample No.	Qiagen	HotSHOT	HotSHOT 1:10[1]
1	28.8	-[2]	38.8
2	29.3	-	-
3	25.6	23.7	27.1
4	-	-	38.1
5	24.4	28.1	29.9
6	32.7	-	-
7	29.7	-	-
8	30.3	39.7	-
9	37.1	28.8	29.4
10	29.4	-	-
11	33.1	37.8	-
12	28.3	25.5	26.9
13	27.3	-	-
14	27.6	-	-
15	24.3	31.3	29.2
16	39.2	37.7	-
17	26.2	23.5	26.7
18	26.1	-	-
19	30.3	38.8	39.7

[1]DNA diluted 1:10 in water.
[2]Ct value > 40.
Numerical values reflect the Ct value for each sample.

and *H. bilis* fluorogenic nuclease PCR assays may reflect the shorter amplicons generated by the fluorogenic nuclease assays, use of a hot-start *Taq* polymerase, which reduces non-specific primer binding, or possibly, improved sensitivity of fluorogenic detection versus gel detection. In other experiments with identical PCR products, we have determined that detection by use of a fluorogenic probe can improve sensitivity 10- to 100-fold over detection by use of ethidium bromide-stained agarose gels (data not shown). Whether this improved sensitivity makes a difference in the ability to qualitatively detect *Helicobacter* spp. DNA is unclear, although it could play a role in detecting DNA in fecal samples, which can have low amounts of total DNA.

Fecal DNA extracts obtained from multiple laboratory mice sources were evaluated by use of the fluorogenic nuclease PCR assays and recently developed multiplex gel detection PCR assays. Overall, the results obtained from the fluorogenic nuclease PCR assays and the corresponding gel-detection PCR assays compared well, in contrast to previous reports of markedly different PCR results when identical samples were evaluated by different assays at different laboratories (23, 24). The fluorogenic nuclease PCR assays detected target template in all samples that were PCR-positive for the targeted *Helicobacter* spp., with the exception of one sample that was *H. typhlonius* PCR-positive but was negative by the *H. typhlonius* fluorogenic nuclease PCR assay. This finding may be explained by the slightly greater sensitivity of the *H. typhlonius* gel detection PCR assay, compared with that of the corresponding fluorogenic nuclease PCR assay, or could possibly be a PCR false-positive result. In addition, each fluorogenic nuclease PCR assay did not detect target template in any of the *Helicobacter* spp. PCR-negative samples, with the exception of one sample that was fluorogenic nuclease PCR-positive by use of the *Helicobacter* spp. and the *H. bilis* assays. The presence of *Helicobacter* spp., or more specifically, *H. bilis* DNA, was verified by sequencing the amplicon DNA. This discrepancy may be due to the enhanced sensitivity of the fluorogenic nuclease PCR assays, although contamination of the sample with *H. bilis* DNA prior to evaluation by use of the fluorogenic nuclease PCR assays also is a possibility. The *H. hepaticus*, *H. bilis*, and *H. typhlonius* fluorogenic nuclease PCR assays also detected target template in multiple samples that were PCR-negative for that respective species, but were positive for other pathogenic *Helicobacter* spp. Most likely, this is the result of performing the fluorogenic nuclease PCR assays as singleplex reactions, whereas the gel detection PCR assays were run in a multiplex format, in which reagent competition and depletion could result when multiple *Helicobacter* species are present. Alternatively, the potentially enhanced relative sensitivity of the *H. hepaticus* and *H. bilis* fluorogenic nuclease assays, compared with that of gel detection assays, could have resulted in detection of additional isolates, although direct comparison of the fluorogenic nuclease PCR assays with the multiplex gel detection PCR assay was not performed.

Finally, the potential of these findings to reflect false-positive fluorogenic nuclease PCR results cannot be ruled out, although this is considered unlikely since the no-template (negative control) reactions were uniformly test negative when diagnostic samples were evaluated by use of the fluorogenic nuclease PCR assay. The importance of detecting additional pathogenic *Helicobacter* species, when one is known to be present, is debatable, although this information could be useful for epidemiologic studies or monitoring the *Helicobacter*-infection status of mouse and rat colonies over time. Regardless, the overall correlation of results obtained by the fluorogenic nuclease PCR assays and the multiplex gel detection PCR assay was excellent, with the few differences observed unlikely to change the clinical management of any of the rodent colonies from which samples were collected.

Two methods of DNA extraction from fecal pellets were evaluated to determine whether an alkaline lysis/neutralization (HotSHOT) method would yield amplifiable DNA equivalent to fecal DNA extracted by use of a commercial kit. A previous report indicated the HotSHOT method provided DNA of sufficient quality for PCR detection of *Helicobacter* spp. in rodents, with the band intensity of PCR product amplified from HotSHOT DNA equivalent to or more intense than DNA prepared, using a QIAamp DNA Mini Kit (Qiagen) (31). When DNA extracted by use of the Qiagen Stool kit and the HotSHOT method were evaluated by use of the *Helicobacter* spp. fluorogenic nuclease PCR assay, however, the Qiagen kit detected multiple samples that were test negative by the HotSHOT method, and there were several discrepant samples between undiluted and diluted HotSHOT DNA. The Qiagen Stool kit may have detected more *Helicobacter*-positive samples than did the HotSHOT method since the former extracts DNA from 10 fecal pellets, compared with the HotSHOT method that extracts DNA from only one pellet. The discrepant samples obtained by use of the HotSHOT method had high Ct values, indicating low template copy number in these samples. Therefore, sample dilution may have reduced the amount of template DNA to undetectable amounts (resulting in loss of signal in diluted samples), or may have reduced the concentration of PCR inhibitors that are common in feces (resulting in gain of signal in diluted samples) (32, 33). Although the HotSHOT method is more cost and time effective, this procedure did not appear to consistently produce amplifiable fecal DNA, compared with that of the Qiagen stool kit, for detection of *Helicobacter* spp. by use of the fluorogenic nuclease PCR assay.

In contrast to how well the fluorogenic nuclease PCR assays correlated to gel detection PCR assays in the study reported

here, previous reports have indicated that testing of identical samples for rodent *Helicobacter* spp. by several commercial laboratories generated discrepant results (23, 24). Although the reasons for these findings were not determined in those studies, several factors encountered during our studies may have played a role (e.g., use of multiplex PCR assays, which may not detect the presence of multiple species due to reagent depletion, presence of fecal inhibitors in HotSHOT extracted DNA, or intermittent shedding of *Helicobacter* spp. in the feces). In addition, false-positive results may be obtained secondary to use of primers that are not specific for the targeted sequence or to sample contamination during DNA extraction and PCR assay set-up. The fluorogenic nuclease PCR assays offer a definitive advantage with respect to the latter, as the closed tube fluorogenic detection system eliminates handling of PCR product, which is considered the most common source of contaminating DNA in PCR reactions.

In conclusion, each of the fluorogenic nuclease PCR assays is sensitive and specific at detecting the targeted bacterial DNA. The *Helicobacter* spp. fluorogenic nuclease PCR assay should provide an accurate screening test to detect presence of any rodent *Helicobacter* spp., followed by speciation, using the *H. hepaticus*, *H. bilis*, and *H. typhlonius* fluorogenic nuclease PCR assays to determine whether pathogenic species are present. Compared with gel detection PCR analysis, reagent costs are similar, and the replacement of gel detection with fluorogenic detection reduces labor costs, although the initial cost of a real-time thermocycler is substantially higher than that of a traditional thermocycler. Although, in the past, this high equipment cost may have limited this technique to high-volume laboratories, the quick expansion of fluorogenic nuclease technology has made real-time thermocyclers more accessible, especially at larger research institutions. In addition, these assays are amenable to automation and, therefore, provide a high-throughput method for detection of rodent helicobacters. Finally, the ability of the fluorogenic nuclease PCR assays to generate quantitative results could prove useful in rodent *Helicobacter* research for determination of bacterial loads in intestine or feces. Recently, a fluorogenic nuclease PCR assay that targets the cytolethal distending toxin subunit B gene sequence of *H. hepaticus* has been developed for this application (26). Although this assay has limited specificity for use as a diagnostic assay since it detects *H. hepaticus* and *H. bilis*, the report indicated that the quantitative results generated by use of the fluorogenic nuclease PCR assay correlated precisely to the bacterial load in mouse cecum and feces. The assay was recently used to document a significant difference in cecal bacterial load between A/J mice and C57BL/6 mice, mouse strains that are susceptible and resistant, respectively, to *H. hepaticus*-induced hepatitis, thus showing the potential usefulness of this method for investigating rodent *Helicobacter* spp. pathogenesis (27). Although evaluation of the fluorogenic nuclease PCR assays developed in our laboratories focused on their application as diagnostic assays for detection of rodent *Helicobacter* species, the sensitivity, specificity, and quantitative accuracy documented for each assay suggest they would also perform well for cecal or fecal bacterial load determination.

Acknowledgments

We thank April Wagner, Jessie Loganbill, Beth Livingston, and Allen Maddy for technical assistance, and Lynn Joens, Stephen Billington, and Wayne Nicholson for providing various isolates used in specificity studies. This work was supported by Public Health Service grant R01 RR14072 from the National Center for Research Resources.

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