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Detection of *Helicobacter* Colonization of the Murine Lower Bowel by Genus-Specific PCR-Denaturing Gradient Gel Electrophoresis

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Helicobacter **genus-specific PCR and denaturing gradient gel electrophoresis can detect and speciate the helicobacters that colonize the lower bowel of laboratory mice. The method's sensitivity is comparable to that of species-specific PCR and may detect unnamed *Helicobacter* species. This approach should prove useful for commercial and research murine facilities.**

A number of the *Helicobacter* species that colonize the murine lower bowel may confound experimental data because of their association with typhlocolitis, hepatitis, and hepatic neoplasia in susceptible murine strains (3, 6–11, 13–15, 24, 25, 27). Screening of laboratory mice for lower bowel colonization is particularly desirable, because *Helicobacter* species are transmitted by the fecal-oral route (16, 28) and are prevalent in commercial and research animal facilities (9, 18, 22, 28). Testing can be performed using culture or PCR; however, the former is labor-intensive and the latter requires multiple PCRs for species identification. As denaturing gradient gel electrophoresis (DGGE) of PCR products (PCR-DGGE) generated with genus-specific primers has been used successfully to detect and speciate the bacteria of a targeted genus (12, 21), we sought to develop this method for the identification of *Helicobacter* species in the lower bowel of laboratory mice.

Primer 1067R that targets the 16S ribosomal DNA (rDNA) of the *Helicobacter* genus was designed by comparing the 16S rDNA sequences of 73 lower bowel *Helicobacter* species and 25 other colonic bacterial species (2, 26). This primer was used in combination with a reversed and GC-clamped version of primer H676 (18) (Table 1). Hot-start PCR using this primer pair was performed on a PCR Sprint thermal cycler (Hybaid, Ashford, Middlesex, United Kingdom), using a 50-μl reaction mixture containing 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.01 mg of gelatin, 2.5 mM $MgCl_2$, 200 nM concentrations of each nucleotide triphosphate, 20 pmol of each primer, 1.1 U of *Taq* DNA polymerase (Biotech International, Belmont, Western Australia, Australia), and 10 to 30 ng of template DNA. Thermal cycling consisted of 94°C for 5 min, 30 cycles of 94°C for 10 s, 62°C for 10 s, 72°C for 30 s, and 72°C for 2 min. DNA template was obtained from bacterial cultures using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn.) according to the manufacturer's instructions. The specificity of the PCR was confirmed by the amplification of template DNA from *Helicobacter hepaticus* (ATCC 51448), *Helicobacter rodentium* (ATCC 700285), *Helicobacter muridarum* (ATCC 49282), *Helicobacter bilis* (ATCC 51630), *Helicobacter trogontum* (ATCC 700114), and two laboratory strains of *Helicobacter ganmani*, but not 13 other colonic bacteria, including *Campylobacter fetus* and *Campylobacter coli*.

DGGE of the *Helicobacter* genus-specific PCR product on a 6% polyacrylamide gel (acrylamide-bisacrylamide, 37.5:1) containing a 41-to-48% gradient of urea and formamide (100% is 7 M urea and 40% deionized formamide) was performed for 16 h at 75 V and 60°C (Bio-Rad, Hercules, Calif.). Bands were visualized with ethidium bromide staining. PCR products were directly sequenced using the ABI PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. The results of PCR-DGGE with American Type Culture Collection (ATCC) and laboratory helicobacter strains are shown in Fig. 1. Band positions were generally species specific; however, those of *H. rodentium* and a laboratory strain of *H. ganmani* isolated from wild-type C57BL/6 mice were practically indistinguishable due to the very high degree of sequence homology in the amplified region. It is noteworthy that laboratory strains of *H. ganmani* isolated from interleukin-10-deficient (IL-$10^{-/-}$) and wild-type C57BL/6 mice had differing gel positions as a result of a 2-base difference in their 16S rDNA sequence (T versus G at position 971 and A versus G at position 1045; *Escherichia coli* 16S rDNA numbering).

The sensitivity of *Helicobacter* genus-specific PCR-DGGE was determined by assessing the detection of *Helicobacter* species in spiked murine fecal samples and by direct comparison with species-specific PCR. Equal portions of a murine fecal sample were spiked with serial dilutions of cultured *H. hepaticus*, and DNA was extracted according to the animal tissue protocol of the Puregene DNA isolation kit (Gentra). The limit of detection of PCR-DGGE was 10^7 *H. hepaticus* organisms per g of feces. The sensitivity of *Helicobacter* genus-specific PCR-DGGE was also directly compared with PCRs specific for individual *Helicobacter* species by using fresh fecal samples from 13 12-week-old C57BL/6 cagemates obtained from the same supplier. Primer sequences and references for these PCRs are given in Table 1. PCR for *H. muridarum* used a 2.5 mM $MgCl_2$ concentration, and thermal cycling consisted of 94°C for 4 min, 35 cycles of 94°C for 10 s, 58°C for 10 s, 72°C for 30 s, and finally 72°C for 2 min. Combining the results, all of the mice were colonized with *H. bilis* and *H. ganmani* but not

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TABLE 1. Primer sequences used in *Helicobacter* genus-specific and species-specific PCRs

Target	Primer	Sequence (5′–3′)	Reference
Helicobacter genus	GC658F	CGC CCC CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC TGG GAG AGG TAG GTG GAA T	Riley et al. (18)[a]
	1067R	GCC GTG CAG CAC CTG TTT TCA	This paper
H. rodentium and *H. ganmani*[b]	D86	GTC CTT AGT TGC TAA CTA TT	Shen et al. (23)
	D87	AGA TTT GCT CCA TTT CAC AA	Shen et al. (23)
H. bilis	H276f	CTA TGA CGG GTA TCC GGC	Riley et al. (18)
	Hbr	TCT CCC ATA CTC TAG AAA AGT	Riley et al. (18)
H. hepaticus	B38	GCA TTT GAA ACT GTT ACT CTG	Shames et al. (22)
	B39	CTG TTT TCA AGC TCC CC	Shames et al. (22)
H. muridarum[c]	Hmur	ACA GAA GTG GCA CTC CCA	This paper

[a] GC658F is a GC-clamped and reversed version of H676r.
[b] Primer set will amplify 16S rDNA from both species (19).
[c] Primer used in combination with H276f.

the other *Helicobacter* species. PCR-DGGE detected *H. ganmani* in 92% and *H. bilis* in 100% of mice, while species-specific PCRs were 92% sensitive for the same organisms, suggesting that the sensitivities of both methods were comparable and consistent with previous reports (1, 17, 22).

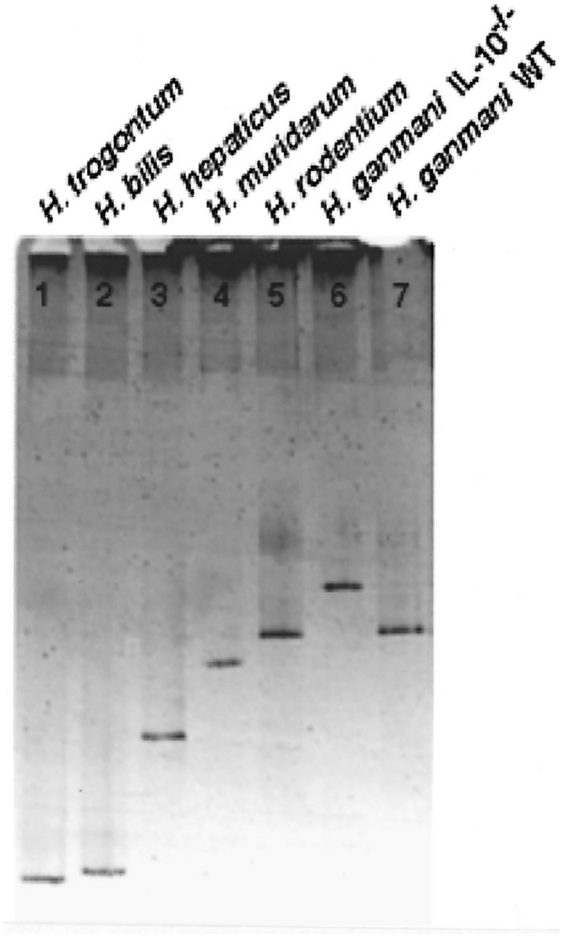

FIG. 1. Results of *Helicobacter* genus-specific PCR-DGGE for ATCC and laboratory strains of *Helicobacter* species. Lane 1, *H. trogontum*, lane 2, *H. bilis*; lane 3, *H. hepaticus*; lane 4, *H. muridarum*; lane 5, *H. rodentium*; lane 6, *H. ganmani* isolated from IL-10$^{-/-}$ C57BL/6 mice; lane 7, *H. ganmani* isolated from wild-type C57BL/6 mice.

In order to examine the utility of *Helicobacter* genus-specific PCR-DGGE for the identification of colonizing *Helicobacter* species, the method was applied to eight mice housed in different cages in four rooms of our animal facility. These mice were between 2 and 12 months of age and had originated from three Australian suppliers. The resulting DGGE gel is shown in Fig. 2. Bands were excised from the gel and DNA obtained using the "crush and soak" method (20) was amplified and sequenced. The results of comparing these sequences with the BLAST database (2) are shown in Table 2. For six of the mice, the bands matched the gel position and sequence of the ATCC and laboratory strains of *Helicobacter* species. As noted previously, bands representing one strain of *H. ganmani* had an identical gel position to *H. rodentium*. One mouse did not have detectable helicobacter colonization. Interestingly, two bands derived from the remaining mouse (cage A) did not match the gel position of known standards; sequencing showed that one

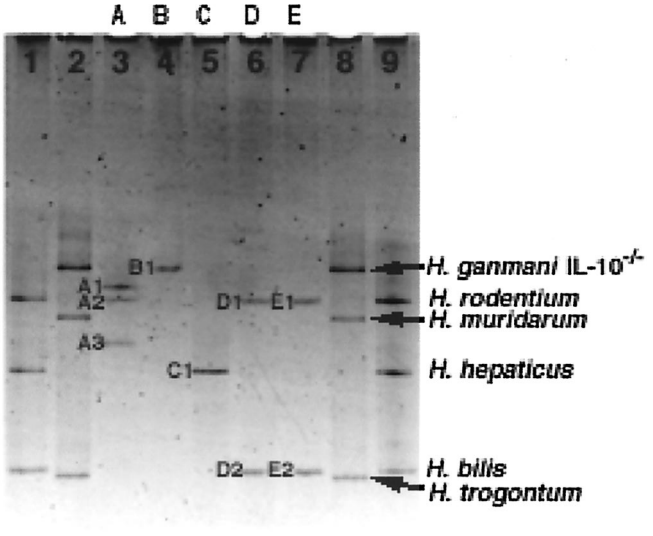

FIG. 2. *Helicobacter* genus-specific PCR-DGGE results for Animal Facility mice. Lanes 1 and 9, marker containing *H. rodentium*, *H. hepaticus*, and *H. bilis*; lanes 2 and 8, marker containing *H. ganmani* of IL-10$^{-/-}$ mice, *H. muridarum*, and *H. trogontum*; lane 3, cage A mouse; lane 4, cage B mouse; lane 5, cage C mouse; lane 6, cage D mouse; lane 7, cage E mouse. (Results for cages F, G, and H are not shown.)

TABLE 2. Combined results of *Helicobacter* genus-specific PCR-DGGE and sequencing

Cage	Strain	Age (mos)	*Helicobacter* species
A	BALB/c	9	HSU96299(23) *H. ganmani*[a] HSU51874(5)
B	C57BL/6 IL10$^{-/-}$	8	*H. ganmani*[b]
C	BALB/c	12	*H. hepaticus*
D	C57BL/6	2	*H. ganmani*[a] *H. bilis*
E	BALB/c	5	*H. ganmani*[a] *H. bilis*
F	BALB/c	12	*H. hepaticus*
G	BALB/c	11	Negative
H	BALB/c	12	*H. hepaticus*

[a] Laboratory strain of *H. ganmani* with 971G/1045G (*E. coli* numbering).
[b] Laboratory strain of *H. ganmani* with 971T/1045A (*E. coli* numbering).

(band A1) was closely related to *H. rodentium* (23) and the second (band A3) was 98.3% homologous to 16S rDNA from a helicobacter previously isolated from dog stomach (5). The presence of unnamed *Helicobacter* species in the colony is not surprising, as a significant number of murine helicobacters have not yet been named (4).

To accurately apply this method to the screening of laboratory mice, PCR standards representing the range of *Helicobacter* species and the strains present in a given colony must first be developed. Once established, however, *Helicobacter* spp. may be identified in a single PCR and the presence of a novel species may be detected. Murine fecal samples may be stored at room temperature for up to a week without affecting the outcome of PCR for *Helicobacter* species (1). In addition, recent studies of the prevalence of *Helicobacter* species in animal facilities and their rates of transmission to helicobacter-free sentinels suggest that the results obtained from just a few mice are likely to reflect the colonization status of their cagemates (16, 28). With appropriate standards, *Helicobacter* genus-specific PCR-DGGE could also be adapted for the screening of other laboratory animals, e.g., gerbils, ferrets, and rats.

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