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Use of Pulsed-Field Gel Electrophoresis To Determine Genomic Diversity in Strains of *Helicobacter hepaticus* from Geographically Distant Locations

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In 1992 a helical microorganism associated with chronic active hepatitis and a high incidence of hepatocellular tumors was identified in the hepatic parenchyma of A/JCr mice. By using biochemical tests, phenotypic characterization, and 16S rRNA gene sequence analysis, the organism was classified as a novel *Helicobacter* species and named *Helicobacter hepaticus*. Recent surveys completed in our laboratory indicate that *H. hepaticus* is widespread in academic and commercial mouse colonies. The aim of this study was to examine the *H. hepaticus* genome by pulsed-field gel electrophoresis (PFGE) to determine the degree of genomic variation and genomic size. This technique has been used to identify significant genomic diversity among strains of *Helicobacter pylori* and to demonstrate only slight genomic diversity among strains of *Helicobacter mustelae*. Genomic DNAs from 11 isolates of *H. hepaticus* from the United States, Germany, France, and The Netherlands were subjected to PFGE after digestion with *Sma*I. Isolates from three independent sources within the United States had very similar PFGE patterns, suggesting that the genomic DNAs of these isolates are conserved. Genomic DNA isolated from a fourth source within the United States had a PFGE pattern different from those of the other U.S. isolates. Isolates obtained from Germany, France, and The Netherlands had PFGE patterns that differed markedly from those of the U.S. isolates and from one another. The use of DNA fingerprinting may be useful in subsequent epidemiological studies of *H. hepaticus* when the source and method of spread of this murine pathogen need to be ascertained. By PFGE, the genomic size of *H. hepaticus* is estimated to be roughly 1.3 Mb, which compares to 1.67 Mb for *H. pylori* and 1.7 Mb for *H. mustelae*.

Members of the genus *Helicobacter* are gram-negative, curved bacteria that infect humans and a wide variety of animals (9). In humans and several animals, including the ferret, they have been isolated from the gastric mucosa (9, 13). Several other species of *Helicobacter* colonize the livers and intestinal tracts of mice, and an additional species has been cultured from the intestines of birds (12, 17, 24, 32).

It is now known that different *Helicobacter* species can cause various degrees of gastritis, hepatitis, and intestinal disease in susceptible humans and animals (9, 10, 16, 18, 23, 25, 32, 38–40) and several are associated with an increased risk for developing certain forms of neoplastic disease (8, 12, 15, 20, 28, 29, 41, 42, 45).

The type species of this genus, *Helicobacter pylori*, can be isolated from the gastric mucosae of humans and is associated with gastritis and duodenal ulcers (18, 23, 25). It is also considered a risk factor for the development of gastric adenocarcinoma and lymphoma of the mucosa-associated lymphoid tissue (11, 28, 29, 42). A number of epidemiological studies have shown that *H. pylori* is distributed globally and may be the most prevalent chronic infection of humans (34).

Following the discovery of *H. pylori*, *Helicobacter mustelae* was isolated from pyloric ulcers in ferrets (13). Like *H. pylori*, *H. mustelae* is associated with chronic gastritis, ulcers, and gastric adenocarcinoma (9, 10, 15, 45). Natural infection of ferrets with *H. mustelae* has been used to study natural, persistent *Helicobacter* infections. The organism has a worldwide distribution, having been isolated from ferrets in the United States, England, Canada, and Australia (9).

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In 1992 *Helicobacter hepaticus* was identified in mice (12). This microorganism was associated with chronic active hepatitis and a high incidence of hepatocellular tumors (41). The lesions associated with infection in the liver include chronic inflammation; oval cell, Kupffer cell, and Ito cell hyperplasia; hepatomegaly; and bile duct proliferation (14, 38). A recent finding also indicates that germfree outbred mice infected with *H. hepaticus* can develop panenteritis or colitis (16). Like infection with *H. pylori* in humans, which is associated with increased risk for developing gastric adenocarcinoma, infection with *H. hepaticus* is associated with an increased risk of hepatic cancer in selected strains of mice and is therefore being used as an important model for bacterium-induced carcinogenesis (16, 38, 41).

Recent studies have shown that infection with *H. hepaticus* is common in commercial and academic mouse colonies and can approach 100% prevalence in affected groups (31). The endemic nature of this organism and its deleterious effects on the livers and intestines of some strains of mice have the potential to seriously confound many research projects. As a result of these findings, several commercial vendors have eradicated *H. hepaticus* from their colonies, making uninfected animals commercially available.

As *H. hepaticus* is a newly recognized murine bacterial pathogen, information about its genetic makeup and epidemiology of infection is currently lacking. *H. pylori* and *H. mustelae* have been extensively characterized by a variety of analytical techniques, including pulsed-field gel electrophoresis (PFGE), and the degree of genomic diversity and genomic size have been determined (35, 36).

The purpose of this study was to evaluate mice from several sources within the United States as well as from overseas locations to determine if they were infected with *H. hepaticus*,

to characterize the genomic diversity of these isolates by PFGE, and to estimate the genomic size of the type strain of *H. hepaticus.*

MATERIALS AND METHODS

Bacterial isolation techniques. Bacterial isolates used in this study were obtained from intestinal scrapings collected aseptically as previously described or by culturing a suspension of feces in phosphate-buffered saline that had been passed through a 0.45-μm-pore-size filter (12, 31, 33).

Bacterial isolates and growth media. The organisms were cultured on blood agar plates (Remel, Lenexa, Kans.) or in brucella broth (Difco Laboratories, Detroit, Mich.) containing 5% bovine calf serum (Summit Biotechnologies, Fort Collins, Colo.) under microaerobic conditions (5% H_2, 5% CO_2, and 84% N_2) at 37°C for 4 to 7 days.

Biochemical and morphological characterization. All isolates were tested for urease, catalase, and oxidase activities; Gram stain reaction; and motility and morphology under phase-contrast microscopy as described previously (12).

DNA extraction for PCR and restriction enzyme analysis. An InstaGene Matrix kit (Bio-Rad, Hercules, Calif.) was used according to the manufacturer's instructions. Briefly, bacterial pellets were washed twice with double-distilled H_2O. One hundred microliters of InstaGene Matrix was added to the pellet, and this mixture was incubated at 56°C for 30 min and then vortexed at high speed for 10 s. The samples were placed in a boiling-water bath for 10 min, vortexed again, and centrifuged at 11,000 × *g* for 5 min.

PCR amplification of bacterial DNA. The primer sequences chosen for PCR amplification recognized a region of the 16S rRNA specific for *H. hepaticus* (12, 31). These two oligonucleotides, 5′ GCA TTT GAA ACT GTT ACT CTG 3′ and 5′ CTG TTT TCA AGC TCC CC 3′, produced an amplified product of 417 bp. Twenty microliters of the DNA preparation was added to 100 μl (final volume) of a reaction mixture containing 1× *Taq* polymerase buffer (supplied by the manufacturer but supplemented with 1 M $MgCl_2$ to a final concentration of 2.25 mM), 0.5 μM each primer, 200 μM each deoxynucleotide, and 200 μg of bovine serum albumin per ml. Samples were heated at 94°C for 4 min, briefly centrifuged, and cooled to 61°C. At this time, 2.5 U of *Taq* polymerase (Pharmacia, Uppsala, Sweden) and 1.0 U of polymerase enhancer (Perfect Match; Stratagene, La Jolla, Calif.) were added, and then 100 μl of mineral oil was laid over the samples. The following conditions were used for amplification: 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 2 min, and elongation at 72°C for 2 min, followed by an elongation step of 7 min at 72°C. A 15-μl aliquot of the sample was then electrophoresed through a 6% Visigel separation matrix (Stratagene); this procedure was followed by ethidium bromide staining and viewing by UV illumination.

DNA preparation and restriction endonuclease digestion for PFG analysis. After 4 to 7 days of growth, the organisms were harvested and suspended in TES buffer (10 mM Tris-HCl [pH 7.2], 20 mM NaCl, 100 mM EDTA). The resuspended cells were then embedded in 1% low-melting-point (LMP) agarose with plug molds (Bio-Rad) and lysed by soaking the plugs in 1 mg of lysozyme per ml in a solution containing 10 mM Tris-HCl (pH 7.2), 50 mM NaCl, 100 mM EDTA, 0.2% sodium deoxycholate, and 0.5% sodium *N*-lauryl-sarcosine at 37°C for 2 h. The plugs were treated with 1 mg of proteinase K per ml in 100 mM EDTA, 0.2% sodium deoxycholate, and 1% sodium *N*-lauryl-sarcosine at 42°C as described previously for *H. pylori* (3). The plugs were incubated at 4°C for 1 h in 1 mg of phenylmethylsulfonyl fluoride solution per ml and then washed three times with TE buffer (20 mM Tris [pH 8.0], 50 mM EDTA). The DNA plugs were washed two times with 300 μl of the appropriate restriction buffer (New England Biolabs, Beverly, Mass.) before digestion. DNA digestion was accomplished with the addition of 40 to 400 U of restriction enzyme to 300 μl of fresh restriction buffer and incubation overnight at the appropriate temperature, and then the same amount of restriction enzyme was added prior to PFGE.

PFGE. The endonuclease-digested genomic DNA of *H. hepaticus* contained within the LMP agarose plugs was separated by PFGE by the contour-clamped homogeneous electric field method of electrophoresis. The LMP agarose plugs were placed in the wells of a 1% agarose gel in 1× Tris-borate-EDTA, and separation of the DNA fragments was achieved with the Bio-Rad CHEF-DR III system. The PFGE was run for 16 to 24 h at 14°C with an initial switching time of 5 s and a final switching time of 10 s at 6 V/cm. Concatamers of DNA isolated from bacteriophage λ (New England Biolabs) were run next to the DNA fragments for size comparison. At completion of the electrophoresis, the gel was stained with ethidium bromide and photographed with a Polaroid MP4 camera with Polaroid 667 film.

RESULTS

Bacterial isolation. *H. hepaticus* isolates used in this study are listed in Table 1. In addition to isolates of *H. hepaticus* previously cultured in our laboratory, three isolates were cultured from mice obtained from another source within the United States. Mice from five European sources were also examined for the presence of *H. hepaticus*. Of these five sources, we were able to isolate *H. hepaticus* from three. These mice came from academic institutions in The Netherlands, Germany, and France.

TABLE 1. Isolates of *H. hepaticus* used in this study

Strain	Geographic origin	Supplier (source)	Reference
3B1 (ATCC 51448)	United States	1	12
95-659	United States	2	31
94-223[a]	United States	3	31
94-224	United States	3	31
94-225	United States	3	31
B200	United States	4	This study
B203	United States	4	This study
B230	United States	4	This study
96-1809[a]	The Netherlands	5	This study
96-284	Germany	6	This study
96-1313[a]	France	7	This study

[a] This isolate does not appear on the gel in Fig. 1.

Biochemical and morphological characteristics. All 11 isolates of *H. hepaticus* were subjected to a number of biochemical tests and examined for motility and culture purity by phase-contrast microscopy. All isolates were urease, catalase, and oxidase positive, gram negative, curved, and motile, which is consistent with the properties of *H. hepaticus.*

Verification of *H. hepaticus* by PCR. The *Helicobacter* isolates were confirmed to be *H. hepaticus* by PCR with *H. hepaticus*-specific primers, which amplify a portion of the 16S rRNA gene (12, 31). PCR was performed as described in Materials and Methods, and the amplified fragments were visualized on an analytical gel. The *H. hepaticus*-specific primers amplify a 417-bp fragment. All isolates in this study were confirmed to be *H. hepaticus* by PCR analysis.

Identification of restriction endonucleases useful for digestion of *H. hepaticus* DNA. A series of restriction enzymes were tested to determine one that would be suitable for PFGE analysis. Chromosomal DNA from *H. hepaticus* was digested with *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, *Not*I, *Nru*I, *Sac*II, *Sal*I, and *Sma*I. The majority of the enzymes were not suitable for PFGE, as they either failed to cut the DNA or cut it in too few places. *Sma*I (restriction sequence, 5′ CCCGGG 3′), however, gave approximately 15 fragments that could be resolved by PFGE and that ranged in size from 23 to 194 kbp. One sample of the type strain of *H. hepaticus* DNA (ATCC 51448) analyzed by PFGE without restriction endonuclease digestion revealed a single band at 294 kbp, indicating that *H. hepaticus* may contain a plasmid (data not shown).

Comparison of different *H. hepaticus* isolates by PFGE. Digestion of chromosomal DNAs by *Sma*I gave restriction patterns that were very similar among five of the bacterial isolates from the United States. These five isolates, 3B1, 95-659, 94-223, 94-224, and 94-225, represented three independent sources. Three additional isolates from another source within the United States, B200, B203, and B230, showed patterns that were very similar to one another but that differed from the patterns of the other five U.S. isolates. Genomic DNAs of *H. hepaticus* that were isolated from The Netherlands, Germany, and France (96-1809, 96-284, and 96-1313) gave restriction patterns that differed widely from those of the U.S. isolates and from each another (Fig. 1).

Genomic size of *H. hepaticus.* The genomic size of the type strain of *H. hepaticus* (ATCC 51448) was estimated by PFGE. By determining the size of the genomic DNA fragments ob-

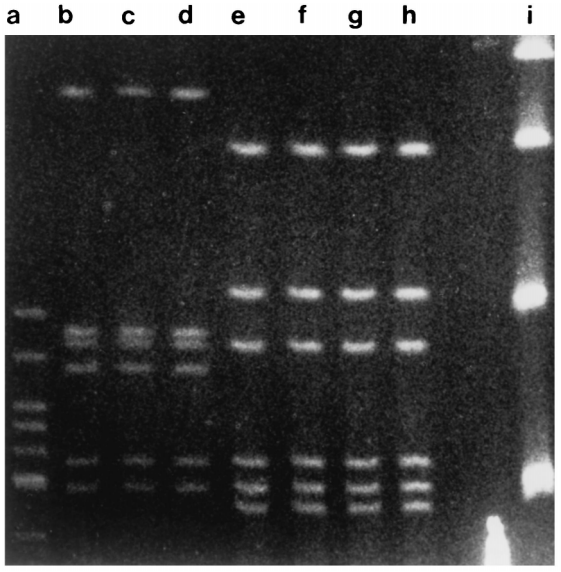

FIG. 1. PFGE of various isolates of *H. hepaticus* DNA (Table 1) digested with *Sma*I and electrophoresed for 24 h. *H. hepaticus* isolates were as follows: 96-284 (lane a), B230 (lane b), B203 (lane c), B200 (lane d), 94-225 (lane e), 94-224 (lane f), 95-659 (lane g), and 3B1 (lane h). Phage λ DNA concatamers were used as size markers (lane i).

tained after digestion with *Sma*I, the genomic size was found to be approximately 1.3 Mb (Table 2 and Fig. 2).

DISCUSSION

H. hepaticus is a newly recognized bacterial pathogen that colonizes the livers, ceca, and colons of mice and is associated with various degrees of hepatitis, enteritis, and hepatocellular tumors (12, 16, 41). The pathogenic potential of this organism makes its presence in research colonies extremely undesirable.

Examination of mice from several commercial and academic sources within the United States has revealed that infection with *H. hepaticus* is widespread; however, information regarding the presence of this organism in colonies overseas was unavailable (31). This study recognizes the presence of *H. hepaticus* in mouse colonies outside of the United States. We isolated *H. hepaticus* from sources within The Netherlands, Germany, and France as well as additional sources from within the United States.

TABLE 2. *Sma*I restriction digests of *H. hepaticus* (ATCC 51448) DNA

H. hepaticus DNA fragment	Fragment size (kb)[a]
1	267 ± 29
2	186 ± 33
3	162 ± 26
4	102 ± 18
5	92 ± 16
6	83 ± 14
7	61 ± 11
8	57 ± 12
9	54 ± 11
10	47 ± 12
11	41 ± 9
12	37 ± 10
13	30 ± 15
14	29 ± 15
15	28 ± 17
Total	1,276 ± 248

[a] Sizes were determined by PFGE as described in Materials and Methods from results with at least three different gels (means ± standard deviations).

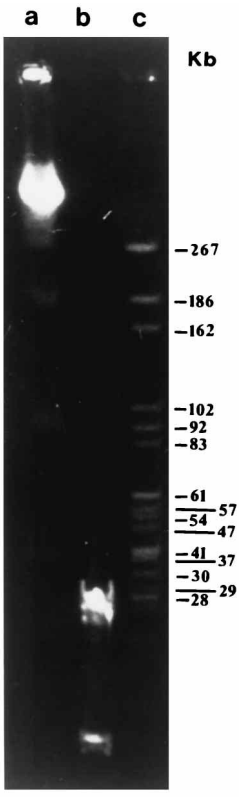

FIG. 2. PFGE of *H. hepaticus* DNA from the type strain, 3B1 (lane c). Phage λ DNA concatamers were used as size markers (lanes a and b).

Traditional bacterial typing techniques have relied on phenotypic characterization, such as serotyping and antibiotic sensitivity, to discriminate among related bacterial isolates and to provide information about the epidemiology of infection. Several studies have shown that these methods are not as sensitive as molecular characterization in identifying subtypes of bacteria responsible for disease outbreaks. Genomic DNA fingerprinting by PFGE has been used successfully to study the molecular epidemiology of several bacterial species, including *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter fetus*, *Mycobacterium tuberculosis*, *Vibrio cholerae* O1, and *Pseudomonas* species (4, 19, 30, 44, 46). Recently, PFGE was used to identify a subtype of *Escherichia coli* O157:H7 that was involved in a multistate food-borne outbreak of disease associated with the consumption of undercooked hamburgers (1). Public health laboratories currently use PFGE to subtype isolates of *E. coli* O157:H7 due to the reproducibility and discriminatory ability of this technique.

In this study, we determined that polymorphism exists among PFGE profiles of *H. hepaticus* isolates from several sources within the United States, The Netherlands, Germany, and France and that homogeneity exists among PFGE profiles from three separate sources within the United States. To better understand the significance of genomic diversity in *H. hepaticus*, we can compare the PFGE profiles of other members

of the *Helicobacter* genus. *H. pylori*, like *H. hepaticus*, has a worldwide distribution. The *H. pylori* genome has also been analyzed by PFGE. *H. pylori* strains isolated from people who are unrelated and who reside within the same geographic location or from distant geographic locations as well as members of the same family show a wide variation in PFGE patterns when genomic DNAs are examined. This difference indicates a very diverse genome for this species. This technique also determined the size of the genome to be 1.6 to 1.73 Mb (36). The range of sizes can be attributed to the large number of strains present in this species. The genomic size of the type strain of *H. hepaticus* was roughly estimated to be 1.3 Mb, which is comparable to the finding of 1.6 to 1.73 Mb for *H. pylori* and 1.7 Mb for *H. mustelae* (35, 36).

H. mustelae also has a worldwide distribution (9). In contrast to *H. pylori*, when the *H. mustelae* genome was analyzed by PFGE, it showed only a very slight diversity of the genome, indicating that all isolates were closely related to one another. This slight diversity was present whether the organism was isolated from ferrets or mink or whether the animals were bred in the United Kingdom or the United States.

After examining the *H. hepaticus* genomic DNAs isolated from the United States and from international sources, it is apparent that the level of genomic diversity of *H. hepaticus* lies between those of *H. pylori* and *H. mustelae*. The levels of conservation of the genomes of several isolates from the United States, although obtained from different sources, parallel the level reported for *H. mustelae* (35). The genomic diversity present among *H. hepaticus* isolates from around the world is similar to the genomic diversity present among *H. pylori* isolates (36). The significance of this finding is unclear, but studies are under way in our laboratory to further characterize these strains of *H. hepaticus.*

Our study indicates that *H. hepaticus* can be found in commercial and academic mouse colonies throughout Europe and the United States. This supports the results of an earlier report that suggested that *H. hepaticus* may be present in a high percentage of mouse colonies (31). Because infection with *H. hepaticus* is associated with hepatitis, enterocolitis, and hepatocellular tumors, the presence of this pathogen in research colonies can have disastrous effects.

It is known that in *H. pylori* strains, genome differences result in different levels of pathogenicity (43). Specifically, there exists in some strains a pathogenicity island which is a large segment of chromosomal DNA carrying a series of genes (5, 6, 22). One of the genes present on this island, *cagA*, encodes a protein that is a marker for enhanced virulence. *H. pylori* strains possessing the *cagA* gene are more likely to be isolated from patients with peptic ulcer disease and gastric carcinoma (2, 7, 21, 37). We have evidence that homologs of two of the genes present on this pathogenicity island, *cagA* and *picB*, are also present in the type strain of *H. hepaticus* (27). The *cagA* gene is not, however, present in all isolates of *H. hepaticus*, suggesting that the bacterium's genomic diversity, along with host factors, may be related to pathogenic differences (26). Further characterization of the *H. hepaticus* isolates is under way.

The PFGE technique we developed demonstrating the genomic diversity of *H. hepaticus* will also be an invaluable tool when the molecular epidemiology of this murine pathogen is investigated.

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