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Genetic Similarity of Intestinal Spirochetes from Humans and Various Animal Species

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The chromosomal DNA of spirochetes isolated from human, swine, dog, mouse, rat, and chicken intestine or feces was subjected to restriction enzyme analysis and hybridization with three different DNA probes, derived from a flagellin gene, a hemolysin gene, and the 16S rDNA sequence of the pathogenic swine intestinal spirochete *Serpulina hyodysenteriae*. This genetic analysis showed that intestinal spirochetes represent a heterogenous but related population of bacteria. In general, unique genotypes were distinguished among isolates from the same host species; they were not present among isolates from other host species. This suggests the host specificity of some strains. An exception to this are isolates from humans and dogs suffering from gastrointestinal disorders; these isolates showed highly similar or even identical genotypes. None of them resembled any of the genotypes of isolates found in other host species without apparent disease.

The spirochete *Serpulina hyodysenteriae* (formerly called *Treponema hyodysenteriae*) is the causative agent of swine dysentery, a mucohemorrhagic enteritis of the colon of swine (20, 38, 39). Similar organisms have been found in the intestine of humans (26) and several animal species, including mice (24), dogs (21, 31, 37, 42), rats (6, 10), and chickens (11). An immunological (14) and genetic (7, 15) relationship between *S. hyodysenteriae* and spirochetes isolated from humans with gastrointestinal disorders has been reported, and it was suggested that domestic animals may be sources of spirochetes pathogenic for humans (7).

Currently, the epidemiology of intestinal spirochetes is poorly understood, and their role in gastrointestinal disease is obscure. A major reason for this is the difficulty in typing these organisms by conventional techniques. Typing techniques that are commonly used to identify and classify *S. hyodysenteriae* isolates, such as serological assays (based on lipopolysaccharide) (3, 28, 30), have been used. However, even *S. hyodysenteriae* isolates do not always fit into the typing scheme, and the outcome of the test may be difficult to interpret because certain isolates possess multiple lipopolysaccharide antigen specificities and therefore react with more than one typing serum (19). Nonserological typing methods have also been used to type swine intestinal spirochetes. These methods are mainly based on enzymatic profiles of the organisms. Although these methods are of some use in discriminating between *S. hyodysenteriae* and the apathogenic swine spirochete *Serpulina innocens* (1, 5, 23, 29), often these tests are not sensitive enough to discriminate among individual isolates of *S. hyodysenteriae*, much less to distinguish them from other intestinal spirochetes. The outcome of these tests can also be influenced by environmental and culture conditions. Thus, there is an obvious need for a method that can be used for the classification and identification of new isolates of intestinal spirochetes. DNA-based typing methods may be suitable for this purpose (8, 9).

To investigate the relatedness between intestinal spirochetes of human and animal origin, we used restriction endonuclease analysis of chromosomal DNA combined with hybridization to three different DNA probes, derived from a 16S rDNA sequence, a flagellar gene (27), and a hemolysin gene (33) of *S. hyodysenteriae*. rDNA typing with a 16S rDNA probe has been used successfully in the characterization of a variety of bacterial species (2, 12, 16–18, 32). Analysis of aligned 16S rRNA sequences has been used to investigate the relationship between representatives of a number of spirochetal species (34). It showed that strains of *S. hyodysenteriae* and *S. innocens* form a very tight cluster (e.g., 16S rRNA sequence identity of more than 99%) which is distantly related to the other spirochetes. Probes derived from the flagellin gene or hemolysin gene of *S. hyodysenteriae* may be useful in discriminating between pathogenic and apathogenic intestinal spirochetes, since these genes may be involved in the virulence of *S. hyodysenteriae*. The hemolysin probe has been used to discriminate between *S. hyodysenteriae* and *S. innocens* (33), whereas with the flagellin probe, 10 different genotypes could be distinguished among 11 individual isolates of *S. innocens* (40).

The spirochetes used in this study are listed in Table 1. The human intestinal spirochetes were independent clinical isolates from human immunodeficiency virus-positive men with intestinal disorders (25a, 26) and a stool isolate from a woman with gastrointestinal complaints (strain number 16). Other intestinal spirochetes originated from the intestines of dogs suffering from diarrhea or from mice (*Mus musculus*) or rats (*Rattus norvegicus*) that showed no pathological lesions in the gut (4). Mouse and rat isolates designated DM and DR, respectively, originated from a farm where swine dysentery was a problem, whereas isolates designated FM and FR, respectively, were cultivated from rodents captured on a farm with no history of swine dysentery. The chicken intestinal spirochete was isolated from an animal with diar-

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TABLE 1. Description of strains used

No.	Strain	Source	Hemolysis[a]	Remarks	Origin or reference
1	*S. hyodysenteriae* C5	Swine	S	Pathogenic	41
2	FM2	Mouse	W	From farm without history of SD[b]	Amtsberg and Beckman
3	DM12	Mouse	W	From farm with SD	Amtsberg and Beckman
4	DM18	Mouse	W	From farm with SD	Amtsberg and Beckman
5	DM23	Mouse	W	From farm with SD	Amtsberg and Beckman
6	DR5	Rat	W	From farm with SD	Amtsberg and Beckman
7	DR7	Rat	W	From farm with SD	Amtsberg and Beckman
8	DR10	Rat	W	From farm with SD	Amtsberg and Beckman
9	FR11	Rat	W	From farm without history of SD	Amtsberg and Beckman
10	A3036	Dog	W	Dog had diarrhea	Amtsberg and Beckman
11	A3888	Dog	W	Dog had diarrhea	Amtsberg and Beckman
12	A5660	Dog	W	Dog had diarrhea	Amtsberg and Beckman
13	A3077	Dog	W	Dog had diarrhea	Amtsberg and Beckman
14	A5687	Dog	W	Dog had diarrhea	Amtsberg and Beckman
15	D126	Human	W	Gastrointestinal complaints	Käsbohrer et al.
16	D65	Human	W	Gastrointestinal complaints	Käsbohrer et al.
17	D71	Human	W	Gastrointestinal complaints	Käsbohrer et al.
18	D160	Human	W	Gastrointestinal complaints	Käsbohrer et al.
19	D26	Human	W	Gastrointestinal complaints	Käsbohrer et al.
20	D14	Human	W	Gastrointestinal complaints	Käsbohrer et al.
21	D117	Human	W	Gastrointestinal complaints	Käsbohrer et al.
22	S16	Human	W	Gastrointestinal complaints	Käsbohrer et al.
23	D51	Human	W	Gastrointestinal complaints	Käsbohrer et al.
24	D200	Human	W	Gastrointestinal complaints	Käsbohrer et al.
25	*Brachyspyra aalborgi*	Human	W	NCTC 11492	22
26	*Borrelia burgdorferi* B31	Human	A	ATCC 35210	25
27	4742	Chicken	I	Chicken had diarrhea	H. F. Smit
28	*S. innocens* B256	Swine	W	Nonpathogenic	3
29	*S. hyodysenteriae* B204	Swine	S	Pathogenic	3

[a] Hemolysis was tested on blood agar plates. S, strong; I, intermediate; W, weak; A, absent.
[b] SD, swine dysentery.

rhea (kindly provided by H. F. Smit, Doorn, The Netherlands). *S. hyodysenteriae* C5 was isolated from the intestine of a swine severely affected by swine dysentery (41) and is the origin strain for the flagellar gene probe and 16S rDNA probe used in the present study. *S. hyodysenteriae* B204 (3) is the origin strain for the hemolysin gene probe. *S. innocens* B256, *Borrelia burgdorferi* B31, and *Brachyspyra aalborgi* have been described elsewhere (3, 22, 25). Human, mouse, rat, dog, swine, and chicken intestinal spirochetes and the spirochete *Brachyspyra aalborgi* were cultured anaerobically at 37°C on tryptic soy agar (Becton Dickinson, Cockeysville, Md.) plates supplemented with 10% sheep blood, 400 μg of spectinomycin per ml, and 0.06% yeast extract (Oxoid, Basingstoke, England). On these plates, *S. hyodysenteriae* is strongly hemolytic, the chicken isolate shows intermediate hemolysis, and all other intestinal spirochetes examined in this study are weakly hemolytic. *Borrelia burgdorferi* was cultured in modified Barbour-Stoenner-Kelly medium as described before (36).

Chromosomal DNA was isolated from approximately 10^9 spirochetes. Spirochetes were collected from blood agar plates or from broth cultures in phosphate-buffered saline containing 5 mM $MgCl_2$ (pH 7.2) and centrifuged at 10,000 × *g*. The bacterial pellet was washed once with TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and resuspended in 2 ml of buffer containing 100 mM Tris-HCl (pH 8.0), 100 mM EDTA, 150 mM NaCl, and 10 mg of lysozyme per ml. Following a 1-h incubation at 37°C, bovine pancreatic RNase A was added to a final concentration of 20 μg/ml, and incubation was continued for an additional 20 min at 70°C. Cells were lysed by the addition of 0.2 ml of 30% Sarkosyl and gentle mixing, and the cells were incubated at 65°C for 20 min and then at 37°C for 1 h. Proteinase K was added to a final concentration of 500 μg/ml, and incubation was continued for 4 h at 37°C. The lysate was dialyzed overnight at 4°C in TE and then extracted once with an equal volume of TE-saturated phenol-chloroform (1:1) and once with an equal volume of chloroform-isoamyl alcohol (24:1). The chromosomal DNA was subsequently precipitated with isopropanol and dissolved in 0.5 ml of TE.

Chromosomal DNA was digested with the restriction endonuclease *Eco*RV (Pharmacia, Woerden, The Netherlands) according to the specifications of the manufacturer. Restriction fragments were separated on a 0.6% agarose gel, stained with ethidium bromide, illuminated with UV light, and photographed. A large number of restriction fragments were generated throughout the length of the gel (Fig. 1). A wide range of different patterns was observed, but some similarities were obvious as well. For instance, three of four mouse isolates showed related or identical restriction patterns, and the human isolates clearly have several bands in common. However, because of the large number of bands, the differences and similarities among the isolates are sometimes difficult to interpret.

For more detailed analysis, *Eco*RV-digested chromosomal DNA was therefore Southern blotted and hybridized to a hemolysin gene probe, a flagellin gene probe, and a 16S rDNA probe. The digested DNA was transferred from the agarose gel to a Hybond-N membrane (Amersham, Buckinghamshire, England) by standard procedures (35). After blotting, DNA was fixed to the filters by exposure to UV light. Filters were prehybridized in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–5× Denhardt's reagent–0.5% sodium dodecyl sulfate (SDS)–100

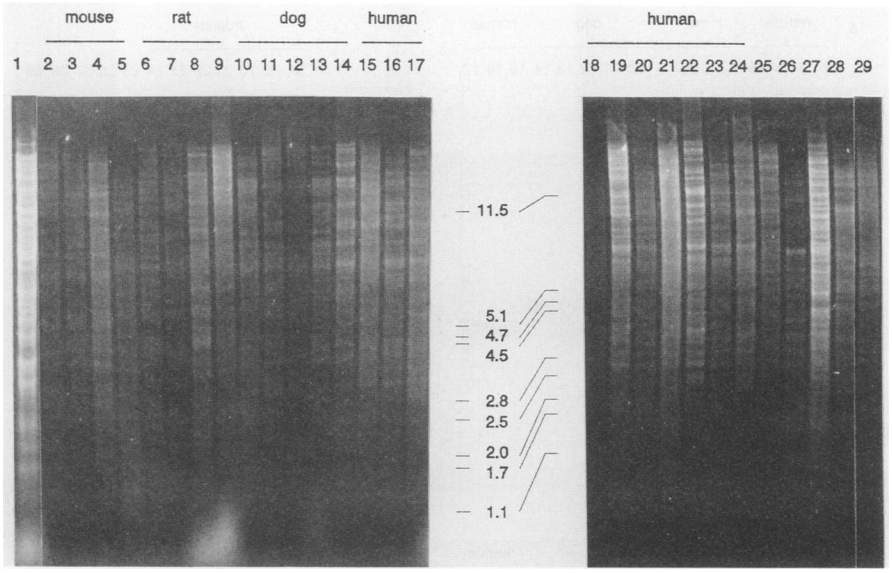

FIG. 1. Restriction fragment length polymorphisms in spirochetes of human and animal origin. Chromosomal DNA was digested with restriction endonuclease *Eco*RV, separated on a 0.6% agarose gel, stained with ethidium bromide, illuminated with UV light, and photographed. Numbers above the lanes correspond to the strain numbers listed in Table 1. Positions of molecular size markers are indicated (in kilobases).

μg of heat-denatured salmon sperm DNA per ml for 6 h at 45°C. Radiolabeled flagellin or hemolysin probe was added, and hybridization was performed at 45°C for 16 h. With the 16S rDNA probe, prehybridization and hybridization were performed at a higher temperature (65°C) because 16S rDNA sequences are strongly conserved among bacteria. After hybridization, the filters were washed with 2× SSPE–0.1% SDS at 68°C. Autoradiography was performed with intensifying screens at −70°C with Fuji XR film (Fuji Photo Film Co., Ltd., Tokyo, Japan).

The flagellin probe was derived from phage λfla6, containing a flagellar sheath gene of *S. hyodysenteriae* C5 (27). The hemolysin probe was derived from plasmid pJBA, containing the *tly* hemolysin gene of *S. hyodysenteriae* B204 (33). In order to be able to obtain a DNA probe containing the entire open reading frame of the genes without adjacent noncoding sequences, the polymerase chain reaction (PCR) was used. Phage λfla6 DNA was used in the PCR with oligodeoxynucleotides (Pharmacia) 5′-TTAACAAACTCAACTTTG-3′ and 5′-TTATTGAGCTTGTTCTT-3′ (nucleotides 160 to 177 and nucleotides 1065 to 1049, respectively [27]) to amplify the flagellar sheath gene. Plasmid pJBA was used in the PCR with oligonucleotides 5′-ATGCGATTAGATGAATATGT-3′ and 5′-TCGTGATAATAATAGAAGCG-3′ (nucleotides 471 to 490 and nucleotides 1449 to 1430, respectively [33]) to amplify the hemolysin gene. The 16S rDNA probe was obtained by performing PCR amplification of the chromosomal DNA of *S. hyodysenteriae* C5 with oligonucleotides fD1 and rD1. This oligonucleotide pair, described by Weisburg et al. (43), is capable of amplifying the 16S rDNA of most bacteria and produces an rDNA fragment of approximately 1,500 bp. PCR was performed (35 cycles) on 10 ng of DNA with the following cycle parameters: 1 min at 95°C, 1 min at 48°C, and 2 min at 72°C. All of these PCR-amplified DNA probes were purified from agarose gels with GeneClean (Bio 101, La Jolla, Calif.) and labeled with [α-^{32}P] dATP (Amersham) with a random-primer labeling kit (Boehringer, Mannheim, Germany).

The results of the Southern blot hybridization experiments are shown in Fig. 2. The photographs are composites of autoradiographs exposed for different periods because the probes hybridized more strongly to *S. hyodysenteriae* chromosomal DNA than to DNA from the other spirochetes. Sequences homologous to the hemolysin gene (Fig. 2A) and flagellin gene (Fig. 2B) of *S. hyodysenteriae* were present in all the intestinal spirochetes tested (human, dog, rat, mouse, and chicken origin) but not in *Brachyspyra aalborgi* or *Borrelia burgdorferi* (this study) or in *Treponema pallidum*, *Treponema phagedenis*, or *Spirochaeta aurantia* (unpublished observations).

With the hemolysin probe and the flagellin probe, three related hybridization patterns were distinguished among the 10 human intestinal spirochetes and 5 dog isolates tested. These patterns did not occur among isolates from other host species. Likewise, three of four mouse isolates had an identical and unique hybridization pattern that was not found among the isolates from other host species. The banding patterns of isolates from rats were unique to this species. Two rat isolates had a number of bands in common. Apart from *S. hyodysenteriae*, the chicken isolate hybridized most strongly to the probes. The hybridization patterns obtained with the 16S rDNA probe confirmed these findings: isolates that showed similar or identical banding patterns with the flagellin or hemolysin probe gave similar or identical patterns with the 16S rDNA probe as well (Fig. 2C). The appearance of two hybridizing fragments in *S. hyodysenteriae* C5 with the flagellin probe is explained by the presence of an *Eco*RV restriction site in the probe. Apparently, the flagellin gene of *S. hyodysenteriae* B204 is different; this bacterium shows a different pattern of hybridization with the flagellin gene probe. Recently, we showed that two distinct patterns occur among 43 isolates of *S. hyodysenteriae* with the flagellin probe (40). When weakly hemolytic swine spirochetes were examined, the flagellin probe appeared to be more discriminatory; 10 different patterns occurred among 11 isolates (40).

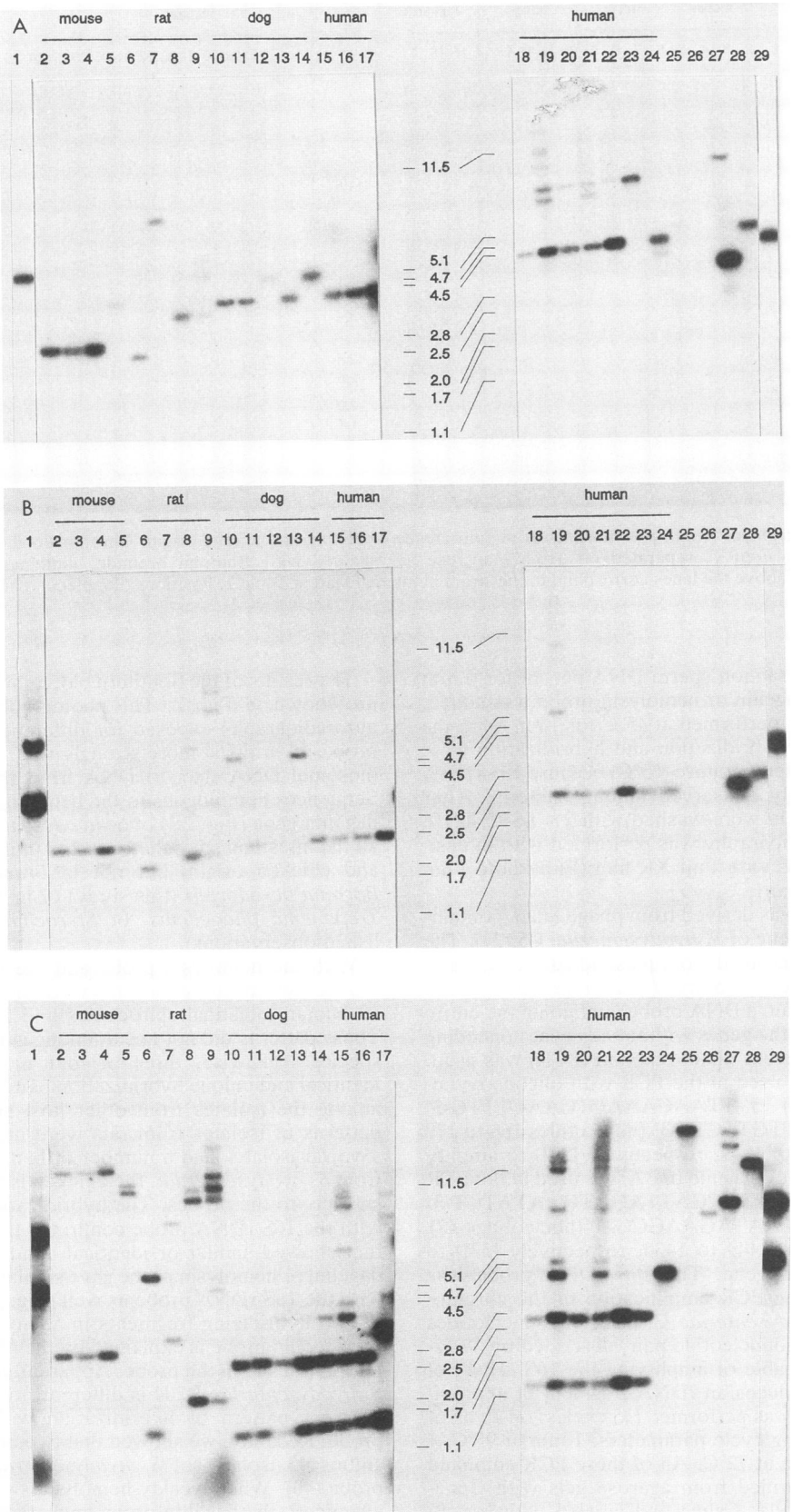

FIG. 2. Autoradiographs of the gel shown in Fig. 1 probed with the hemolysin gene (A), the flagellar sheath gene (B), and a 16S rDNA sequence (C) of *S. hyodysenteriae*. Lanes and size markers are the same as in Fig. 1.

The results of the present study indicate that the intestinal spirochetes of humans, dogs, rats, mice, and chickens are related to *S. hyodysenteriae*. However, their precise taxonomic positions are unclear, since their restriction endonuclease patterns and hybridization patterns differ considerably from those of type strains of *S. hyodysenteriae*. In addition, they do not resemble the patterns of *S. innocens* or any of the patterns observed at our laboratory for a large collection of swine spirochetes (40). Perhaps the intestinal spirochetes from humans and various animal species represent different populations of bacteria or even different species.

Although the number of isolates from each host species examined in this study is small, our findings suggest that some intestinal spirochetes are host restricted. For instance, identical genotypes were observed among isolates from mice that had been captured on different farms, whereas these genotypes did not occur among intestinal spirochetes from other hosts, not even among isolates from rats captured at the same farm. Also, the hybridization pattern of the single chicken isolate differed from that of all other isolates tested.

In contrast, identical genotypes were found among independent isolates originating from humans and dogs with gastrointestinal disorders. These genotypes were not observed among isolates from other host species without apparent disease, indicating that intestinal spirochetes may be transmitted between human and dog. The reduced genetic diversity observed among isolates from humans and dogs suffering from gastrointestinal disorders also suggests a role of these organisms in disease. This hypothesis is supported by the recent findings of Dettori et al. (13). Using four randomly cloned genomic DNA fragments from a human intestinal spirochete as a probe, they found essentially two types of hybridization patterns among nine human intestinal spirochetes that were isolated from the feces of patients suffering from various intestinal disorders. Both our findings and those of Dettori et al. (13) show that the hybridization patterns of human intestinal spirochetes are different from those observed among *S. hyodysenteriae* isolates. Hence, it seems unlikely that pigs are a reservoir of intestinal spirochetes that are pathogenic for humans. It could be that pathogenic intestinal spirochetes are transmitted between dog and human, but at the moment this is not proven. Considering the wide genetic diversity observed among intestinal spirochetes, it could be speculated that both pathogenic and commensal strains exist. The typing methods used in the present study will enable more extensive epidemiological studies to elucidate the role of intestinal spirochetes in clinical diseases and to investigate transmission between different host species.

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