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Certain Canine Weakly β-Hemolytic Intestinal Spirochetes Are Phenotypically and Genotypically Related to Spirochetes Associated with Human and Porcine Intestinal Spirochetosis†

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Four canine weakly β-hemolytic intestinal spirochetes associated with intestinal spirochetosis (IS-associated WBHIS) were compared with IS-associated human and porcine WBHIS and the type species for *Serpulina hyodysenteriae* and *S. innocens* by using phenotypic and genotypic parameters. The IS-associated canine, human, and porcine WBHIS belonged to a phyletic group distinct from but related to previously described *Serpulina* type species.

Well-characterized hemolytic intestinal spirochetes include *Serpulina* (*Treponema*) *hyodysenteriae*, the cause of swine dysentery, and *S. innocens*, a nonpathogenic spirochete of the swine colon (3, 12, 17, 32, 43). *S. hyodysenteriae* is strongly β-hemolytic and produces a zone of enhanced hemolysis or ring phenomenon when grown near an area where the agar has been stabbed, while *S. innocens* is weakly β-hemolytic and does not produce a ring phenomenon (3). As the name implies, *S. innocens* is assumed to be harmless; however, this might not be the case for all weakly β-hemolytic intestinal spirochetes (WBHIS). Some porcine and human WBHIS are different from *S. innocens*, on the basis of the number and arrangement of periplasmic flagella (PF), biochemical profiles, multilocus enzyme electrophoretic patterns, sequence of the 16S rRNA gene, and DNA hybridization using whole-genome and gene-specific probes (7, 18, 22–24, 28). Some of these WBHIS have been associated with colonization of the lower intestine by a mechanism involving intimate attachment to the brush border of enterocytes (1, 2, 9, 10, 34–36, 38, 39), a condition known as intestinal spirochetosis (IS). Feeding of IS-associated porcine and human WBHIS to susceptible hosts has produced lesions consistent with IS (1, 4, 35).

Leach and coworkers (21) identified spirochetes by light microscopic and ultrastructural examination of colonic crypts of dogs with normal stools and postulated that spirochetes are passively shed during diarrhea from other causes. Others have reported a causal relationship between profuse diarrhea and the presence of spirochetes in the stools and have proposed a role for spirochetes as either primary or secondary pathogens in enteric diseases of dogs (26). Because of variations in the pattern and intensity of hemolysis, spirochetes isolated from feces of dogs have been classified as either *S. hyodysenteriae* or *S. innocens* (17, 31, 41). However, Turek and Meyer (38) isolated a WBHIS with six PF, isolate K9-12, from the feces of a dog with ultrastructural evidence of spirochetal attachment to the colonic mucosa. More recently, we described spirochetal attachment accompanied by invasion of the colonic mucosa in a young dog with diarrhea and giardiasis, a lesion similar to IS in human beings, nonhuman primates, and swine (2). Ultrastructural examination of PF (37), together with DNA-based typing methods (18), suggests that a subset of canine WBHIS, different from *S. innocens*, may be responsible for canine IS. This study compares the phenotypic and genotypic characteristics of four canine WBHIS, obtained from dogs with clinical signs or lesions of IS, with those of IS-associated human and porcine WBHIS, and the type species for *S. hyodysenteriae* and *S. innocens*.

The reference isolates, *S. hyodysenteriae* isolate B78 (ATCC 27164 [17]), and *S. innocens* isolate B256 (ATCC 29796 [17]) were obtained from J. M. Kinyon, College of Veterinary Medicine, Iowa State University, Ames, and the American Type Culture Collection, Rockville, Md., respectively. The WBHIS isolate 16 (ATCC 49776 [15]), obtained from a human immunodeficiency virus-positive individual with IS, was provided by R. M. Smibert, Virginia Polytechnic Institute, Blacksburg. The IS-associated porcine WBHIS isolate P43/6/78 (35) was provided by T. B. Stanton, National Animal Disease Center, Ames, Iowa. The canine WBHIS associated with IS, isolates K9-12 (37) and 16242-94 (2), were obtained from M. J. Wannemuehler, Veterinary Medical Research Institute, College of Veterinary Medicine, Iowa State University, Ames, and the authors' laboratory at the University of Nebraska, respectively. The canine WBHIS, isolates 24072-93 A and B, were isolated from the feces of two 4-month-old poodle littermate pups (A and B) with a chronic mucoid diarrhea containing flecks of blood suggestive of IS. The diarrhea had been unresponsive to oral sulfadimethoxine, but the clinical signs in both pups rapidly subsided after oral administration of metronidazole, a response similar to that of human patients with IS (36). Pure cultures of spirochetes were propagated either in trypticase soy agar plates with 5% citrated sheep blood (TSA) incubated at 42°C in the GasPak Anaerobic System (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.), as previously described (3), or in prereduced anaerobically sterilized trypticase soy broth, as previously described (19).

Because of a complete growth inhibition of certain canine WBHIS in selective agar media for primary isolation of intes-

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TABLE 1. Summary of phenotypic characteristics of intestinal spirochetes

Source and isolate	No. of PF	Indole production	Hippurate hydrolysis	Growth[a] on medium			
				CVS	BJ	Spiramycin	Rifampin
Porcine							
S. hyodysenteriae B78	7–14	+	−	+++	+++	ND	ND
S. innocens B256	9–14	−	−	−	−	ND	ND
P43/6/78	5–7	−	+	+++	+++	ND	ND
Human (16)	4–6	−	+	+++	++	ND	ND
Canine							
K9-12	6	−	+	+++	++	+	+++
16242-94	5–7	−	+	+++	+++	+++	+++
24072-93A	5–7	−	+	+++	−	−	+++
24072-93B	12–14	−	−	+++	−	−	+++

[a] Growth was scored subjectively by culture on selective agar medium incubated at 42°C in the GasPak Anaerobic System (BBL) for 5 days as follows: −, no growth; +, minimal growth; ++, moderate growth; +++, marked growth. ND, not determined.

tinal spirochetes, the growth of each isolate was assessed subjectively by using the following media: (i) a modification of the CVS medium (11) consisting of TSA with 100 U of colistin per ml, 20 μg of vancomycin per ml, and 400 μg of spectinomycin per ml; (ii) TSA with 5% pig fecal extract and 6.25 μg each of colistin and vancomycin per ml, 200 μg of spectinomycin per ml, 25 μg of spiramycin per ml, and 12.5 μg of rifampin per ml (BJ medium [20]); (iii) TSA with 25 μg of spiramycin per ml; and (iv) TSA with 12.5 μg of rifampin per ml. Each spirochete culture was streaked on selective agar media, incubated at 42°C in the GasPak Anaerobic System (BBL), and scored subjectively for growth after 5 days (−, no growth; +, minimal growth; ++, moderate growth; +++, marked growth). Growth inhibition of the canine WBHIS on selective BJ medium was attributable to sensitivity to the macrolide antibiotic spiramycin (Table 1). Sensitivity to the antibiotic spectinomycin accounts for the growth inhibition of *S. innocens* isolate B256 on the CVS and BJ selective media, as previously reported (25). Although Kunkle and Kinyon (20) did not observe a complete growth inhibition of *S. innocens* isolate B256 in BJ medium, a greater than 10^5 depression of viable cell numbers, from 3×10^8 to $< 1 \times 10^3$ CFU/ml, was reported with the BJ medium compared with the TSA medium. Discrepancies in the sensitivity results between laboratories may be attributable to variations in strains of isolate B256. It is of interest that isolate B256 was first isolated by filtration of intestinal scrapings and culture on artificial media without antibiotics (8, 16). We speculate that sensitivity to either spectinomycin or spiramycin might have been responsible for growth inhibition of more than one-third of field isolates of porcine WBHIS when cultured in the BJ medium compared with TSA (3). Consequently, we concluded that selective media without spiramycin may be more appropriate for primary isolation of IS-associated WBHIS from diagnostic specimens.

The number of PF of each intestinal spirochete was determined by transmission electron microscopy, as previously described (28). While the IS-associated canine, human, and porcine WBHIS had four to seven PF, *S. hyodysenteriae* isolate B78 and *S. innocens* isolate B256 and the canine WBHIS isolate 24072-93B had greater than seven PF (Table 1). On the basis of indole production (28) and hippurate hydrolysis (29), the IS-associated canine, human, and porcine WBHIS with four to seven PF were similar (Table 1). By contrast, *S. innocens* isolate B256 and the canine WBHIS isolate 24072-93B did not form indole or hydrolyzed hippurate. *Serpulina hyodysenteriae* isolate B78 was indole positive and hippurate negative.

A search for a *S. hyodysenteriae*-specific DNA sequence in purified DNA (28) from the canine, human, and porcine intestinal spirochetes, using a *S. hyodysenteriae*-specific PCR assay (5), detected a 1.55-kb amplified DNA fragment only in *S. hyodysenteriae*. As expected, the *S. hyodysenteriae* amplified DNA fragment hybridized with a *S. hyodysenteriae*-specific internal oligonucleotide probe (data not shown). Purified DNA from the canine WBHIS (28) was compared with that of related porcine and human intestinal spirochetes, using arbitrarily primed (AP)-PCR (27) with the M13 reverse-sequencing primer (5′-GGAAACAGCTATCACCATGA-3′). The amplified products were resolved on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels and stained with silver nitrate (Fig. 1), as previously described (30). A total of 7 to 16 discrete DNA bands were identified for each isolate and were used as data to compare banding patterns among isolates, using the bootstrap analysis method of the phylogenetic inference package PAUP (27, 33, 42). In this analysis, the tree length corresponds to the sum of the weight of each character for each isolate, with a character defined as presence or absence of an individual DNA band in each isolate compared with all other isolates. The branch length is defined as the relative minimum phylogenetic difference needed to explain the banding pattern observed between isolates. For example, canine WBHIS isolate 24072-93A had 13 discrete DNA bands or characters, and all of them were shared with canine WBHIS isolate K9-12 (Fig.

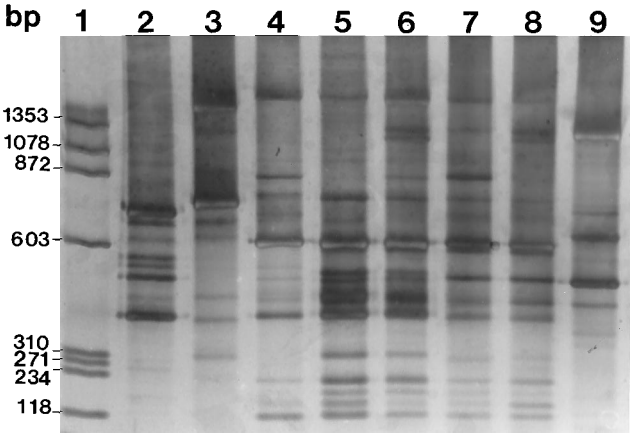

FIG. 1. Silver-stained SDS-polyacrylamide gel containing molecular weight markers (lane 1) and AP-PCR-amplified products of *S. hyodysenteriae* isolate B78 (lane 2), *S. innocens* isolate B256 (lane 3), swine WBHIS isolate P43/6/78 (lane 4), human WBHIS isolate 16 (lane 5), and canine WBHIS isolates K9-12 (lane 6), 16242-94 (lane 7), 24072-93A (lane 8), and 24072-93B (lane 9).

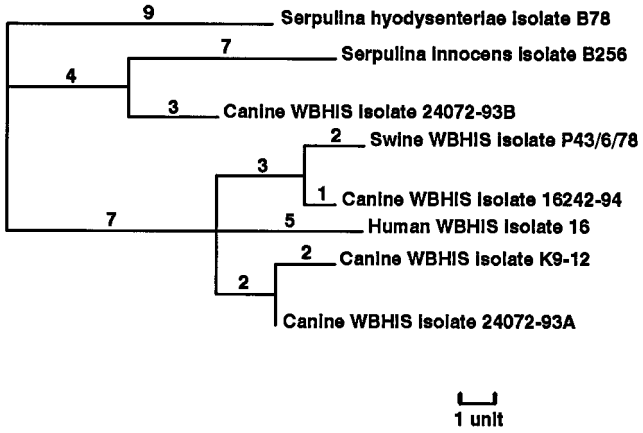

FIG. 2. Phylogenetic relationships of intestinal spirochetes on the basis of parsimony analyses of DNA fingerprints generated by AP-PCR.

1); thus the branch length for isolate 24072-93A compared with isolate K9-12 was determined to be zero (Fig. 2). Conversely, isolate K9-12 had two discrete DNA bands in addition to those present in isolate 24072-93A; thus, isolate K9-12 was placed on a separate branch 2 units further down from 24072-93A. Overall, the phylogenetic analyses revealed that the IS-associated, indole-negative, hippurate-positive canine, human, and porcine WBHIS with four to seven PF formed a closely related group, phylogenetically distinct from *S. hyodysenteriae* and *S. innocens* (Fig. 2). The canine WBHIS isolate 24072-93B was phylogenetically related to *S. innocens* isolate B256 (Fig. 2).

DNA was extracted from late-logarithmic-phase broth cultures of canine, human, and porcine intestinal spirochetes, using a modification of a previously described method (14) except that the lysing solution did not contain proteinase K and the lysate was extracted once instead of twice with phenol chloroform. Also, the DNA spooling and precipitation steps were done using 100% ethanol, and the DNA was dissolved in TE buffer instead of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The levels of DNA sequence homology between each spirochete were estimated from the results of DNA-DNA reassociation using the S1 nuclease method (13). Reassociation studies indicated that there was greater than 95% DNA sequence homology between the IS-associated canine, human, and porcine WBHIS (Table 2). In contrast, there was less than 32% DNA sequence homology between the IS-associated WBHIS isolates and *S. hyodysenteriae* and *S. innocens* (Table 2). The AP-PCR results were comparable to genetic distances determined by DNA-DNA reassociation.

Results from this study indicated that of the four canine WBHIS obtained from dogs with clinical signs or lesions of IS, isolate 24072-93B was similar to the nonpathogenic WBHIS of swine, *S. innocens* isolate B256, while the other three canine WBHIS isolates had phenotypic and genotypic characteristics similar to a human and a porcine WBHIS associated with IS (6, 7, 9, 22–24, 28). Genomic fingerprint analyses, using AP-PCR, and DNA-DNA reassociation, using the S1 nuclease method, correlated with the structural and biochemical analyses and those of Lee and Hampson (22) and further demonstrated that canine, human, and porcine hemolytic intestinal spirochetes were members of the genus *Serpulina* but belonged to three separate phyletic groups, with *S. hyodysenteriae* and *S. innocens* in groups I and II, respectively, and the IS-associated indole-negative, hippurate hydrolysis-positive WBHIS with four to seven PF in group III. These results were in agreement with those of Koopman and coworkers (18), indicating that WBHIS isolated from individuals affected with the human immunodeficiency virus and dogs with IS have genotypes that are similar to but distinct from those of *S. hyodysenteriae* and *S. innocens*. These results also extend the observation made by Fellström and Gunnarsson (6) with porcine WBHIS, indicating that hippurate hydrolysis may be the simplest method for rapid differentiation of the nonpathogenic WBHIS *S. innocens* from IS-associated canine, human, and porcine WBHIS.

TABLE 2. Relative percentages of DNA-DNA reassociation between intestinal spirochetes

Unlabeled DNA	% DNA-DNA reassociation with labeled DNA		
	S. hyodysenteriae B78	*S. innocens* B256	WBHIS 16242-94
S. hyodysenteriae B78	100	38	24
S. innocens B256	46	100	32
WBHIS			
P43/6/78	24	32	95
16	26	32	100
16242-94	ND[a]	ND	100

[a] ND, not determined.

Although the canine WBHIS, isolate 24072-93B, was obtained from a diarrheic fecal specimen, the phenotypic and genotypic characteristics of this isolate were similar to those of *S. innocens* isolate B256. This is consistent with previous reports indicating heterogeneity among canine WBHIS with some isolates more closely related to *S. innocens* (41). It is interesting that the original description of *S. innocens* (17) included an indole-negative and hippurate-negative WBHIS with four to seven PF, obtained from a young dog with catarrhal diarrhea. The isolate, designated "Puppy," was no longer viable and could not be included in our study (40). However, on the basis of ultrastructural characteristics, the "Puppy" isolate may be similar to the IS-associated WBHIS rather than *S. innocens*.

Turek and Meyer (37) recovered WBHIS with six PF from 12 of 18 dogs with normal stools and, using scanning electron microscopy, demonstrated spirochetal attachment to the colonic mucosa of some of the dogs (38). Presence of adherent spirochetes in the colons of dogs with normal stools suggests that IS-associated WBHIS might not be a primary cause of gastrointestinal disease. Alternatively, in mature dogs, infection with IS-associated WBHIS might be subclinical, and some unknown factors may contribute to development of spirochetal diarrhea. The latter possibility appears more likely for several reasons: (i) the canine WBHIS isolate 16242-94 was obtained from a young dog with chronic diarrhea associated with giardiasis together with IS; (ii) in immunocompetent adult individuals, spirochetal attachment is seen as an incidental finding in appendices (34, 36), whereas immunocompromised individuals affected with the human immunodeficiency virus exhibit massive spirochetal attachment along the superficial epithelium of the lower intestines and chronic diarrhea (15, 36); and (iii) a diarrheal syndrome associated with infection by WBHIS different from *S. innocens* is seen in children in developing countries (22, 24).

Although the phylogenetic classification of IS-associated WBHIS in humans and animals is incomplete, several names have been proposed to describe these organisms, including "*Brachyspira aalborgi*" (8), "*Serpulina joneseae*" (22, 28), "*Anguillina coli*" (22–24), and "*Serpulina coli*" (3). "*Brachyspira aalborgi*" has not been widely studied; however, the taxonomic classification of this isolate appears to be distantly related to the genus *Serpulina*, as determined by multilocus enzyme elec-

trophoresis (22). Lee and coworkers (23, 24) identified three groups of human and porcine intestinal spirochetes on the basis of multilocus enzyme electrophoresis, biochemical analyses, and PF number and arrangement. They proposed the name "*Anguillina coli*" for indole-negative WBHIS with four to six PF associated with human and porcine IS. Interestingly, a WBHIS, obtained from the feces of a dog with diarrhea and classified as "*Anguillina coli*," had an electrophoretic type similar to that of WBHIS isolated from the feces of (i) Australian Aboriginal children with diarrhea living in the same community; (ii) adult individuals with diarrhea living in distant communities, including isolate 16; and (iii) swine with spirochetal diarrhea in Australia, the United States, and the United Kingdom, including isolate P43/6/78 (22).

On the basis of currently available data, it is reasonable to propose that WBHIS inhabiting the intestinal tract of humans and animals form a diverse group with some isolates capable of attaching to the colonic mucosa, particularly in young individuals and immunocompromised adult hosts. Because of the close phenotypic, genotypic, and pathogenetic relationships between canine, human, and porcine WBHIS associated with IS, these spirochetes might cause a zoonotic infection with public health significance.

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