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Identification and Characterization of *Serpulina pilosicoli* Isolates Recovered from the Blood of Critically Ill Patients

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The phenotypic and genetic characteristics of spirochetes isolated from the blood of one U.S. and six French patients with severe clinical disease or impaired immunity were examined. All spirochetes were anaerobic, weakly β-hemolytic, positive for hippurate hydrolysis, and negative for β-glucosidase activity. Cell lengths ranged from 4 to 8 μm, and each isolate had between 8 and 12 periplasmic flagella per cell. These features were consistent with the spirochetes' being *Serpulina pilosicoli*, the agent of intestinal spirochetosis. All isolates were positive in a PCR assay amplifying a portion of the *S. pilosicoli* 16S rRNA gene, and they all grouped with fecal isolates of *S. pilosicoli* in multilocus enzyme electrophoresis (MLEE). The blood isolates could be differentiated from each other by MLEE, although the U.S. and two French isolates were closely related. Apparently *S. pilosicoli* may translocate from the large intestine to establish spirochetemia. The clinical significance of this finding remains uncertain and requires further investigation.

Intestinal spirochetes of the genus *Serpulina* inhabit the large intestines of humans and animals (15). A number of reports have documented the isolation of morphologically similar spirochetes in other organ systems of humans. Cholestatic hepatitis due to invasion of the liver with spirochetes has been reported for an AIDS patient (11), and spirochetes were isolated from the stools and blood of a French patient with nonobstructive cardiomyopathy who went into shock following acute hemorrhagic diarrhea and later died from cardiac insufficiency (13). A more recent report documented the isolation of spirochetes from the blood of six severely ill French patients with a variety of clinical problems, including vascular disease, malignancy, intoxication with ethylene glycol, and cecal necrosis (5). Four of the patients subsequently died. These spirochetes were morphologically similar to those in the genus *Serpulina*, and they were provisionally identified as *Serpulina hyodysenteriae*, the agent of swine dysentery in pigs.

In the present study we examined the six isolates from France, together with a spirochete isolated from the blood of a patient in the United States, in order to establish their identities and relationships to each other. We examined some key phenotypic traits of spirochetes in the genus *Serpulina* and then performed a PCR test that is specific for *Serpulina pilosicoli*. We then undertook multilocus enzyme electrophoresis (MLEE) analysis to compare the strains with other known intestinal isolates of *S. pilosicoli* from humans, a dog, and a pig. *S. pilosicoli* is the etiological agent of porcine intestinal spirochetosis, a diarrheal disease of growing pigs resulting in the passage of watery to mucoid feces (23, 25). A pathognomonic feature of the infection is the attachment of large numbers of spirochetes by one cell end to the cecal or colonic epithelium. *S. pilosicoli* also has been isolated from the feces of humans with clinical signs and lesions suggestive of intestinal spirochetosis, most notably in individuals in developing communities and in AIDS patients and homosexual males in Western societies (14, 15). The organism also naturally colonizes dogs and birds, which may be reservoirs of human pathogens (2, 3).

Primary culture of the seven isolates from the bloodstream was performed in anaerobic blood culture medium, with growth observed by dark-field microscopy. The first six strains were recovered from unrelated French patients (5). Strains PE90 and BR 81/80 were isolated from patients who died following a stroke; strain 28/94 was isolated from an alcoholic who died from shock associated with complete jejunal necrosis following ingestion of 1 liter of ethylene glycol. Strain RA/87 was recovered from a patient who died from severe arteriopathy. The other two strains were obtained from patients with severe clinical illness who later recovered following surgery and/or therapy. Strain HJ 128/90 was isolated from a patient with peritonitis (following necrosis of the cecum), and strain 382/91 was isolated from an alcoholic with myeloma. Primary isolation was performed in Hemoline blood culture bottles (bioMerieux, Lyon, France) or, for strain 382/91, in BioArgos Sanofi diagnostic anaerobic medium (Pasteur Institute, Paris, France). The seventh strain, H1, was isolated from a Californian AIDS patient receiving chemotherapy for Kaposi's sarcoma. The patient presented with thrombophlebitis in the arm used to infuse the chemotherapy. No growth signal was obtained in the ESP automated blood culture system (Difco, Detroit, Mich.), but the spirochete was observed by microscopy and subsequently isolated from a subculture. No attempt to isolate spirochetes from the feces of the patients was made, although strains BR 81/80 and 382/91 were isolated from the blood of patients with diarrhea.

In the identification process, the human blood strains were compared with the type strains of each of the three recognized species of *Serpulina*: *S. hyodysenteriae* B78, *Serpulina innocens* B256, and *S. pilosicoli* P43/6/78 (25). Additional strains for comparison included *S. pilosicoli* WesB, isolated from an Ab-

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original child with diarrhea (14), and porcine strains from two proposed species: "*Serpulina intermedius*" PWS/A (ATCC 51140) (16) and "*Serpulina murdochii*" 56/150 (ATCC 51284) (15). For MLEE, the results for the blood strains were compared with previously published results obtained for 68 *S. pilosicoli* strains isolated from human feces in various areas of the world, 1 *S. pilosicoli* strain isolated from the feces of a dog, and *S. pilosicoli* type strain P43/6/78 (15). For identification, each isolate was grown on Trypticase soy agar (Becton Dickinson, Cockeysville, Md.), supplemented with 5% defibrinated ovine blood, under an atmosphere of 94% H_2 and 6% CO_2. After growth for 3 to 5 days at 37°C, the strength of β-hemolysis around individual colonies was compared with that for type strains B78 (strong hemolysis) and B256 (weak hemolysis). Indole production was assessed by extracting 2 ml of each active 72-h culture grown in Kunkle's medium (12) with 1 ml of xylene and adding 4 drops of Kovács reagent (16). The biochemical activities of cells harvested from agar plates were assessed with API ZYM (API, Montalieu-Vercieu, France), and profiles were recorded as recommended for intestinal spirochetes (10, 16). Hippurate hydrolysis was assessed by the ninhydrin method, as described for intestinal spirochetes (4). The lengths and number of periplasmic flagella per cell harvested from agar were recorded with a Philips 301 transmission electron microscope, as previously described for porcine spirochetes (16). A specific PCR then was applied to all strains, using *S. pilosicoli*-specific primers designed to amplify a 1,330-bp portion of the 16S rRNA gene (18). Whole cells were washed and boiled for 10 min prior to PCR amplification. The product was detected by ethidium bromide staining following electrophoresis in 1.5% agarose. The electrophoretic mobilities of 15 constitutive enzymes were determined for the seven blood spirochete strains by MLEE (16). Details of electrophoretic running conditions, buffers, and enzyme assays have been given previously (15, 16). Allele profiles generated for the seven strains were grouped into electrophoretic types (ETs) which were compared with ETs generated for the other 70 strains previously examined (15). Genetic distance between ETs was calculated as the proportion of fixed loci at which dissimilar alleles occurred. A phenogram illustrating genetic distance between ETs was constructed as described previously (Fig. 1) (16).

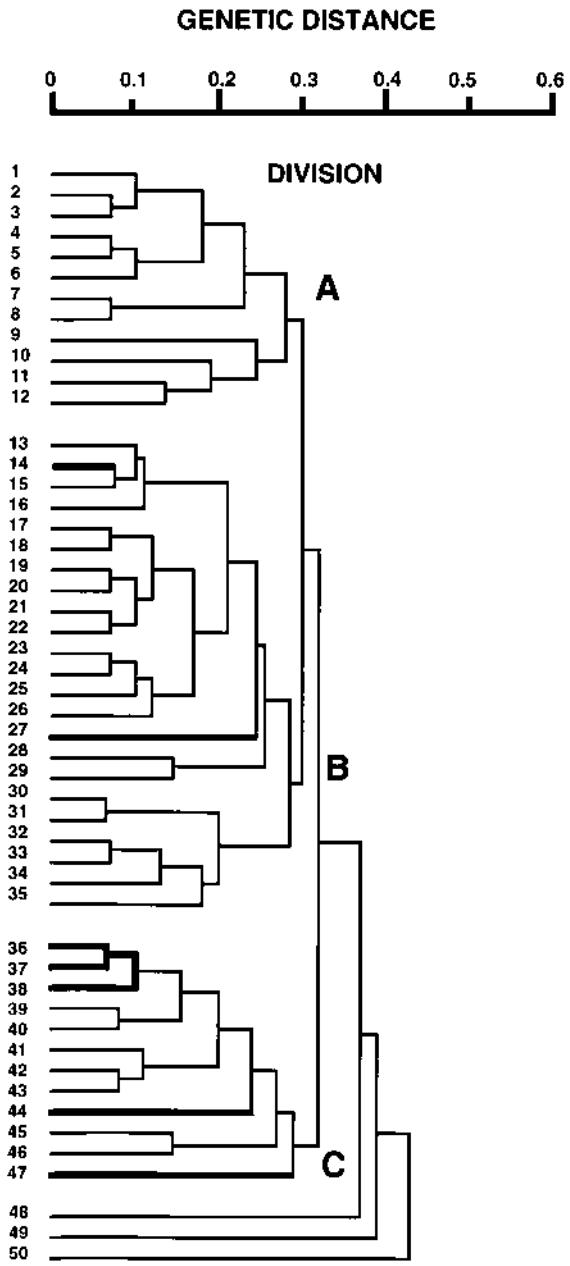

FIG. 1. Phenogram illustrating genetic relationships among 50 ETs of *S. pilosicoli*, including the porcine type strain of ET 2, a canine fecal strain of ET 26, 68 human fecal strains, and 7 strains isolated from human blood, indicated by thick lines and listed in Table 1.

The results of the phenotypic tests are summarized in Table 1. The seven blood strains were all weakly hemolytic, positive for hippurate hydrolysis, and negative for β-glucosidase activity in the API ZYM system. They were from 4 to 8 μm long and had between 8 and 12 periplasmic flagella per cell (4 to 6 at each cell end). These phenotypic characteristics were shared by *S. pilosicoli* WesB and P43/6/78^T. Three of the strains (RA/87, HJ 128/90, and H1) were positive for indole production. A PCR product of 1,330 bp was generated for each of the human blood spirochetes and for *S. pilosicoli* P43/6/78^T and WesB but not for any of the type strains of the other species of *Serpulina*. In the MLEE analysis the 77 strains were divided into 50 ETs. Three major divisions of ETs separated by a genetic distance of 0.32 were created. Each blood strain had a unique allele profile (ETs 14, 27, 36, 37, 38, 44, and 47 [depicted in Fig. 1 and listed in Table 1]). Two of the blood strains were located in division B, and five were located in division C. Strain H1 from the United States (ET 36) and French strains BR 81/80 and 28/94 (ETs 37 and 38) were closely related, each being differentiated from the others by single alleles.

The seven human blood spirochetes were identified as strains of *S. pilosicoli* on the basis of their distinguishing phenotypic characteristics and their positive reaction in a specific PCR assay. Hippurate hydrolysis, lack of β-glucosidase activity, and number of periplasmic flagella per cell (8 to 12) have been used to identify strains of *S. pilosicoli* (24, 25). Interestingly, three of the blood spirochete strains gave positive reactions for indole production, a trait not previously reported for *S. pilosicoli* and usually possessed only by strains of *S. hyodysenteriae* and the proposed species of weakly β-hemolytic spirochete "*Serpulina intermedius*" (16). The results of MLEE also helped confirm the identities of the blood spirochetes. The two human blood strains located in division B of the phenogram and the other five strains in division C were separated from the type strain of *S. pilosicoli*, P43/6/78 (assigned to ET 2 of division A), by genetic distances of 0.30 and 0.32, respectively. In previous

TABLE 1. Phenotypic characteristics of spirochetes isolated from the blood of seriously ill patients, human intestinal spirochete *S. pilosicoli* WesB, and the type strains of *S. pilosicoli*, *S. hyodysenteriae*, *S. innocens*, "*S. intermedius*," and "*S. murdochii*"

Isolate (geographic origin)	ET[a]	Hemolysis	Indole production	Hippurate hydrolysis	PFN[b]	API ZYM profile[c]
RA/87 (France)	14	Weak	+	+	8–12	14.0.12.3.0
382/91 (France)	27	Weak	−	+	8–12	14.0.4.3.0
H1 (United States)	36	Weak	+	+	8–12	14.0.12.3.0
BR 81/80 (France)	37	Weak	−	+	8–12	14.0.12.11.0
28/94 (France)	38	Weak	−	+	8–12	14.0.12.3.0
HJ 128/90 (France)	44	Weak	+	+	8–12	14.0.12.2.0
PE90 (France)	47	Weak	−	+	8–12	14.0.12.3.0
S. pilosicoli WesB	19	Weak	−	+	8–12	14.0.4.3.0
S. pilosicoli P43/6/78^T	2	Weak	−	+	8–12	14.0.4.3.0
S. hyodysenteriae B78^T	NT	Strong	+	−	16–24	14.0.4.10.1
S. innocens B256^T	NT	Weak	−	−	20–28	14.0.4.3.1
"*S. intermedius*" PWS/A	NT	Weak	+	−	16–22	14.0.4.10.1
"*S. murdochii*" 155/20	NT	Weak	−	−	16–24	14.0.4.2.1

[a] ET in MLEE (Fig. 1); NT, not tested.
[b] PFN, number of periplasmic flagella per cell.
[c] According to Hunter and Wood (10). A zero in the final position indicates a lack of β-glucosidase activity.

studies another canine strain of *S. pilosicoli* had 95% DNA homology with strain P43/6/78^T and 100% homology with strain ATCC 49776 (3). In the present study, strain ATCC 49776, assigned here to ET 50, was separated from P43/6/78^T by a genetic distance of 0.43. These close DNA-DNA reassociation values between isolates with relatively large MLEE genetic distances between them help to confirm that all strains examined belonged to *S. pilosicoli*. The MLEE study also demonstrated that while generally the blood isolates of *S. pilosicoli* were genetically heterogeneous, the U.S. and two French isolates were closely related and could be considered to represent a clonal group. All the blood isolates were closely related to fecal isolates which previously had been recovered in several studies in different parts of the world (15); this suggests that the blood isolates were likely to have originated in the intestinal tract. Translocation of bacteria or endotoxin from the intestine commonly occurs in debilitated patients as a result of overgrowth of gram-negative bacteria, impaired host immune defenses, or injury to the gut mucosa resulting in increased mucosal permeability (6). It has been suggested that the predominant biotype or serotype in the gut will be the bacteria most likely to translocate to other organ systems (6). Although fecal culture for *S. pilosicoli* was not undertaken in any of the cases described here, two patients were reported to have diarrhea. Previously, simultaneous culture of spirochetes resembling *S.* (then *Treponema*) *hyodysenteriae* from the blood and feces of a patient was recorded for a debilitated patient (13), although unfortunately this organism is not available for further characterization. The factors initiating translocation of *S. pilosicoli* and the clinical significance of this bacteremia remain uncertain.

In most previous studies involving intestinal spirochetes in humans, spirochetal cells were reported to be attached to the epithelial brush border in biopsy sections, but no attempts to culture the organisms to establish their identity were made (19). Generally, these spirochetes have been assumed to be *Brachyspira aalborgi* (9) and have been seen attached to host epithelial cells, with little inflammation or penetration beyond the host cell membrane. In some cases, however, spirochetes have been observed within rectal epithelial cells, subepithelial macrophages, and Schwann cells (1, 7), with an associated increase in the number of immunoglobulin E-secreting plasma cells in the lamina propria (7). They have also been observed in colonic epithelial cells, goblet cells, macrophages, and Schwann cells in immunocompetent heterosexual patients with diarrhea (17). Two recent case study reports of intestinal spirochetes in AIDS patients have demonstrated more severe inflammatory reactions. In one study, spirochetes were reported to be present in intestinal crypts and beyond the mucosa (8). In the second, invasion of spirochetal cells beyond the brush border epithelium was associated with crypt abscesses and epithelial ulceration and necrosis (11). Spirochetal invasion of the bile canaliculi, with associated inflammation, also has been observed in an AIDS patient (11). Again, it was assumed that the spirochetes colonizing these patients belonged to *B. aalborgi*, although this should now be reconsidered in light of the present findings. Interestingly, crypt abscessation and penetration of *S. pilosicoli* cells through tight junctions has been observed in the colons of pigs naturally or experimentally infected with *S. pilosicoli* (21, 23).

Apart from AIDS patients and homosexual males, the prevalence of *S. pilosicoli* in Western communities is low (14, 20). To determine the significance of *S. pilosicoli* bacteremia, future investigations should simultaneously examine fecal and blood cultures from AIDS and other immunocompromised patients in Western societies as well as from individuals in developing communities, where both intestinal carriage and immune deficiencies are common. To date, the organism's low growth rate, fastidious growth requirements, and failure to provide growth signals in certain automated blood culture systems may have prevented it from being isolated more frequently. Further work is required to improve isolation methods. The animal models developed to demonstrate the enteropathogenicity of *S. pilosicoli* strains (22, 23) also could be used to evaluate their enteroinvasive capability and their capacity to survive in the blood and initiate disease in other organ systems. The strains described here are available for further study.

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