Isolation of *Serpulina pilosicoli* from Rectal Biopsy Specimens Showing Evidence of Intestinal Spirochetosis

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Histologic evidence of intestinal spirochetosis (IS) was found in 22 of 41 (53.7%) rectal biopsy specimens from homosexual men attending a sexually transmitted diseases clinic. *Serpulina pilosicoli* was cultured from 11 of the IS-positive biopsy specimens (50%) and from 2 specimens (10.5%) in which spirochetes were not observed. The association between seeing spirochetes in biopsy specimens and isolating *S. pilosicoli* was statistically significant, clearly indicating that this spirochete is the agent of IS.

The term intestinal spirochetosis (IS) was coined in 1967 for an infection of the large bowel in which uncharacterized spirochetes were found attached by one end to the colonic epithelium to form a dense "false brush border" (11). Subsequently, there has been considerable controversy over the pathologic and epidemiologic significance of this colonization. Some investigators have reported symptoms such as rectal bleeding and/or diarrhea in patients with IS (6, 7, 9, 20, 30, 42), and in some cases clinical improvement has occurred after treatment with antibiotics which eliminated the spirochetes (7, 14, 29). Other investigators have been unable to relate the presence of intestinal spirochetes to gastrointestinal symptoms (3, 26, 31, 32).

Most studies of IS have involved histologic examination of biopsy material, without concurrent bacterial culture (17, 21, 24, 31, 42); a smaller number of studies have involved only fecal culture for spirochetes (3, 13, 18, 30, 33). One exception was a study by Hovind-Hougen et al. (12) in which the newly described spirochete *Brachyspira aalborgi* was isolated from the feces of one of five patients showing histologic evidence of IS in rectal biopsy specimens. Since then, when IS has been diagnosed on the basis of histologic examination, it has usually been assumed that the organism involved was *B. aalborgi* (10, 15, 26, 27, 29). This is despite the fact that in another study in which spirochetes were seen in rectal biopsy specimens from five of eight homosexual men, a different, incompletely characterized spirochete was isolated (5).

Histologic studies have suggested that spirochetes are present in 2.5 to 9% of rectal biopsy specimens from unselected Europeans (11, 17, 20, 26), but are more common (9 of 14; 64%) in biopsy specimens from individuals in southern India (22) and are present in 30 to 39% of biopsy specimens from homosexual men in western societies (16, 24). Studies involving selective culture for spirochetes have established that they can be isolated from the feces of only 1.2 to 1.5% of unselected individuals in the United Kingdom and Australia (18, 33), while they were isolated from 12 of 27 (44%) samples from untreated human immunodeficiency virus (HIV)-positive

homosexual males in Germany (14). Rates of colonization in adult Omani Arabs (3), Australian Aboriginal children (18), and villagers of all ages in the Highlands of Papua New Guinea (38) have also been high, ranging from 11.4 to 32.6%. These highly variable colonization rates may be influenced by a variety of factors, including immune function, sexual practices, diet, sanitation, and community structure (4, 19, 33, 38).

Representative isolates from the studies described above were recently analyzed by multilocus enzyme electrophoresis (MLEE). None of these isolates was *B. aalborgi*; instead, all were shown to be *Serpulina pilosicoli* (formerly "*Anguillina coli*") (19, 36), the recently described agent of porcine intestinal spirochetosis (37). Infection of pigs with *S. pilosicoli* is widespread and is associated with poor growth rates, colitis, and diarrhea. *S. pilosicoli* also infects dogs and birds and again is associated with diarrhea (8, 23). Experimentally, infection of pigs with a human isolate of *S. pilosicoli* caused colitis with crypt abscessation (35). In experimentally infected chicks, human isolates have caused watery diarrhea and reduced growth rates, and they also attached by one end to the cecal epithelium (25, 34). These findings strongly suggest that human strains of *S. pilosicoli* have pathogenic potential.

To date systemic disease associated with *S. pilosicoli* has not been reported in animals, but *S. pilosicoli* has recently been isolated from the bloodstreams of critically ill human patients, some of whom had a history of intestinal disease (39). In humans, spirochetes have occasionally been seen in colonic or rectal epithelial cells, goblet cells, macrophages, and Schwann cells in both immunocompetent and immunocompromised patients (1, 10, 15, 27). In some cases severe inflammatory reactions have been recorded, including crypt abscessation and epithelial ulceration and necrosis (15). In all these cases it was assumed that the spirochetes were *B. aalborgi*. The purpose of the present study was to isolate and identify spirochetes from rectal biopsy specimens taken from a group of high-risk individuals with histologic evidence of IS. This would then answer the question of whether spirochetes that can be seen in biopsy specimens from patients with IS are *B. aalborgi* or the more recently described spirochete *S. pilosicoli*.

The subjects of the study were a group of 40 homosexual men attending a sexual health clinic in Sydney, Australia. Approximately half the men were HIV antibody positive, but none was suffering from AIDS. Most reported having minor

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nonspecific gastrointestinal symptoms. Informed consent was obtained for participation in the study. For one individual a second biopsy specimen was taken 6 months after the first biopsy specimen was obtained. Proctoscopy was performed with disposable sigmoidoscopes. Rectal biopsy specimens were taken 10 to 15 cm from the anal verge by using flexible colonoscopy biopsy forceps as described previously (16). Half of the biopsy specimen was placed in neutral buffered formalin for histologic examination, and the other half was streaked directly onto selective Trypticase soy agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) supplemented with 5% defibrinated horse blood and 400 μg of spectinomycin (Sigma-Aldrich Pty Ltd., Castle Hill, Australia) per ml (33). Plates were incubated at 37°C in an environment of 85% N_2– 10% H_2–5% CO_2 and were examined daily for up to 14 days for the presence of spirochetes. Suspected spirochete colonies were examined by dark-field microscopy to determine cell morphology. Isolated spirochetes were maintained on antibiotic-free Columbia agar plates (Columbia Agar base; Oxoid Unipath Ltd., Basingstoke, United Kingdom) containing 5% defibrinated horse blood.

Isolates were subjected to a *S. pilosicoli*-specific PCR designed to amplify a 1.33-kb portion of the *S. pilosicoli* 16S rRNA gene (28). Whole cells were harvested from plates in phosphate-buffered saline, washed, and then boiled for 10 min prior to amplification. The product was detected by ethidium bromide staining following electrophoresis in 1.5% agarose. For nine isolates, including the two isolates recovered from biopsy specimens collected from the same individual 6 months apart, the electrophoretic mobilities of 15 constitutive enzymes were determined by MLEE. The same electrophoretic running conditions, buffers, and enzyme assays reported previously were used (19). The allele profiles generated were grouped into electrophoretic types (ETs) and were compared with the ETs generated for 70 human fecal strains of *S. pilosicoli* which we had examined previously (19, 39). Genetic distances between ETs were calculated as the proportion of fixed loci at which dissimilar alleles occurred. A phenogram illustrating genetic distances between ETs was constructed as described previously (19).

Formalin-fixed biopsy tissues were dehydrated through alcohol, paraffin embedded, cut to a thickness of 5 μm, stained with hematoxylin and eosin, and examined by light microscopy. A total of 22 of the 41 biopsy specimens (53.7%) exhibited a blue-stained haze of spirochetes on the surface of the epithelium, but there was no evidence of spirochete penetration or inflammation. The positive samples were examined further by transmission electron microscopy. These biopsy specimens were washed and postfixed in 2% osmium tetroxide (buffered in 0.1 M cacodylate) for 4 h. Samples were dehydrated through alcohol and prepared for embedding by absorption in a 1:1 mixture of acetone and low-viscosity epoxy resin. The tissue was embedded in 100% low-viscosity epoxy resin and polymerized at 70°C for a minimum of 8 h. Semithin (0.5 μm) and ultrathin (70 to 90 nm) sections were cut from the biopsy specimen by ultramicrotomy. These were stained with 2% uranyl acetate containing 50% ethanol for 15 min and then washed and restained with lead citrate for 4 min. The preparations were examined with a Phillips CM10 transmission electron microscope.

A 20-μm-thick layer of spirochetes was observed attached end on to the mucosal layer of the rectal epithelial cells of the 22 positive biopsy specimens. These spirochetes were situated between and parallel to the microvilli, with little obvious loss of microvilli (Fig. 1). At the point of attachment between the ends of the spirochetes and the mucosal cell membrane was seen a
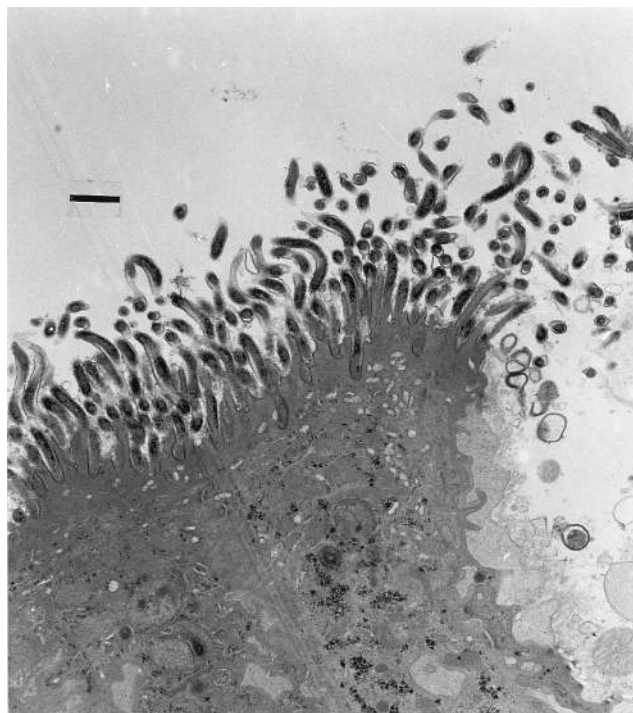

FIG. 1. Transmission electron microscopic view of sectioned rectal biopsy specimen showing a "false brush border" of intestinal spirochetes attached by one end to the mucosa, with minimal disruption of the microvilli. Bar, 3 μm.

depression or pocket, forming a small electron-lucent pit, and some exhibited an electron-dense cap at this point (Fig. 2). The epithelial cell membranes remained intact, with the spirochetes confined to the cell surface.

Spirochetes were isolated from 11 of the 22 (50%) biopsy specimens showing histologic evidence of IS and from 2 of the 19 specimens (10.5%) not showing histologic evidence of colonization. The association between histologic evidence of spirochete colonization and isolation was examined by a chi-squared test and was shown to be significant ($\chi^2 = 7.34$; $P < 0.01$).

Two different weakly β-hemolytic colony types were observed on blood agar plates, and both were visible only after 6 days of incubation. The first colony type was <1 mm in diameter, convex, grey, and translucent, and the other was 1 to 1.5 mm in diameter, crenated, mucoid, grey, and translucent. Bacteria from the two colony types were indistinguishable by both light and electron microscopy; they were 6 to 10 μm long, with a tapered end, and had four to six periplasmic flagella at each cell end (Fig. 3). All gave positive DNA amplifications in the *S. pilosicoli*-specific PCR, and the 9 that were subjected to MLEE analysis all grouped with well-characterized fecal isolates of *S. pilosicoli* (Fig. 4). Each of the nine isolates belonged to a different ET. One, in ET 62, was relatively distantly related to the others. Pairs of isolates in ETs 16 and 17 and ETs 28 and 29 differed by only a single allele. The two isolates cultured from the same individual 6 months apart were different, being located in ETs 28 and 32, respectively.

This study has shown a clear and significant relationship between the presence of intestinal spirochetes attached as a "false brush border" in histologic sections from the rectum—a condition consistent with previous reports of IS—and isolation of *S. pilosicoli* from the biopsy specimens. It is therefore highly likely that in other studies in which IS has been diagnosed on
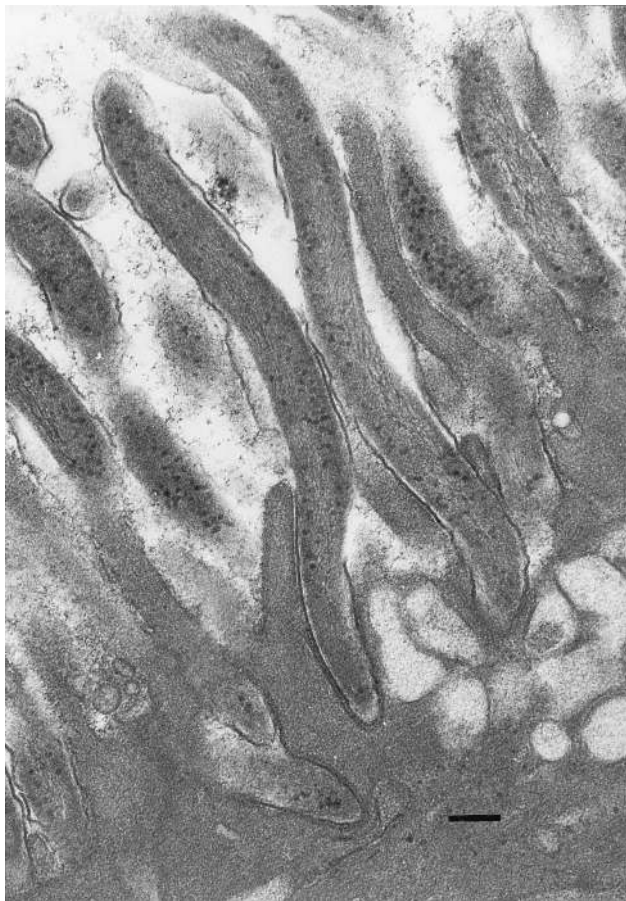
FIG. 2. Transmission electron micrograph of sectioned rectal biopsy tissue showing spirochetes on the mucosal layer of rectal epithelial cells, invaginated into the terminal web of the cells. Bar, 0.5 μm.

FIG. 3. Transmission electron micrograph of a transversely sectioned spirochete showing five periplasmic flagella. Bar, 0.1 μm.

the basis of histologic examination alone, these spirochetes also were *S. pilosicoli*. In no case was *B. aalborgi* isolated in this study, even though the medium used supports its growth, and plates were incubated for 2 weeks, as described when the organism was originally isolated (12). Since that original report, no other isolations of this organism have been made. Unlike *S. pilosicoli*, *B. aalborgi* recently failed to colonize experimentally infected chicks (41). Clearly, over the years, the significance of *B. aalborgi* appears to have been greatly overestimated.

The high prevalence of rectal biopsy specimens showing evidence of IS (53.7%) among the group of individuals examined in the present study was similar to that in previous reports of studies with homosexual males with or without HIV infection (5, 14, 16). The relatively low rate of isolation of *S. pilosicoli* from the biopsy specimens showing evidence of IS (50%) was interesting and suggests that studies based on culture alone, including fecal culture, may underestimate the true prevalence of infection. In the two cases in which *S. pilosicoli* was isolated from biopsy specimens without histologic evidence of IS, these organisms may have been present in the intestinal lumen, having originated from the mucosa in more proximal colonic sites.

S. pilosicoli is a known pathogen of animals, and its capacity to cause disease in humans now requires further assessment. In the current study no attempt was made to associate histologic evidence of IS and disease. All the subjects described here
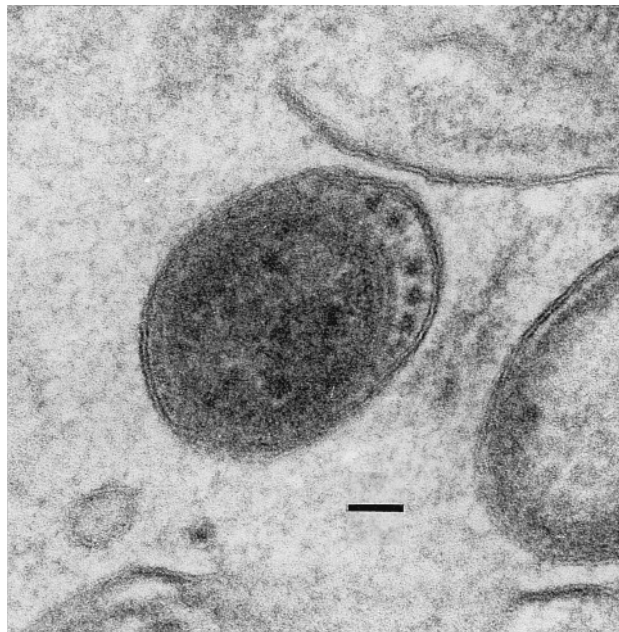
reported only mild nonspecific gastrointestinal disorders. Rectal spirochetosis has been reported in patients with proctitis (5, 7, 42), but this was not present in any of the current subjects, and indeed, no evidence of inflammation was found at the histologic level. Because samples were taken only from the rectum, it was not known whether colonization extended beyond this site. This is significant, since diarrheal disease in pigs is associated with extensive colonization of the cecum and colon (37). Similarly, in humans it seems likely that extensive colonization at other sites is more likely than rectal colonization to be problematic, but it is not known how frequently rectal colonization and colonic colonization coexist. Lindboe et al. (20) reported finding spirochetes more commonly in the rectum (2.5%) than at other large intestinal sites (1.2 to 1.9%) in patients undergoing biopsy, while Lo et al. (21) reported finding spirochetes in the proximal colon but not the distal colon or rectum of one of their patients. When spirochetes are isolated from the feces, their major site of colonization in the intestine remains unknown. This may help explain some of the difficulties in linking the presence of *S. pilosicoli* in the feces or in rectal biopsy specimens to the presence or absence of specific intestinal symptoms.

The large genetic diversity found among the strains isolated here was consistent with previous findings for strains recovered from humans and animals (2, 19). The isolation of different strains of *S. pilosicoli* from biopsy specimens from the same patient taken 6 months apart was also consistent with findings for some individuals in an Australian Aboriginal community (18, 19) and in villages in the Highlands of Papua New Guinea (38, 40). Nevertheless, in the former study some other subjects were found to be colonized with the same strain of *S. pilosicoli* over a 12-month period, while in the latter studies the average duration of colonization was calculated to be approximately 4 months. It is also possible that individuals can be colonized concurrently with different strains of the spirochete, and this could have important implications for the development of protective immunity and for antimicrobial drug therapy if one or
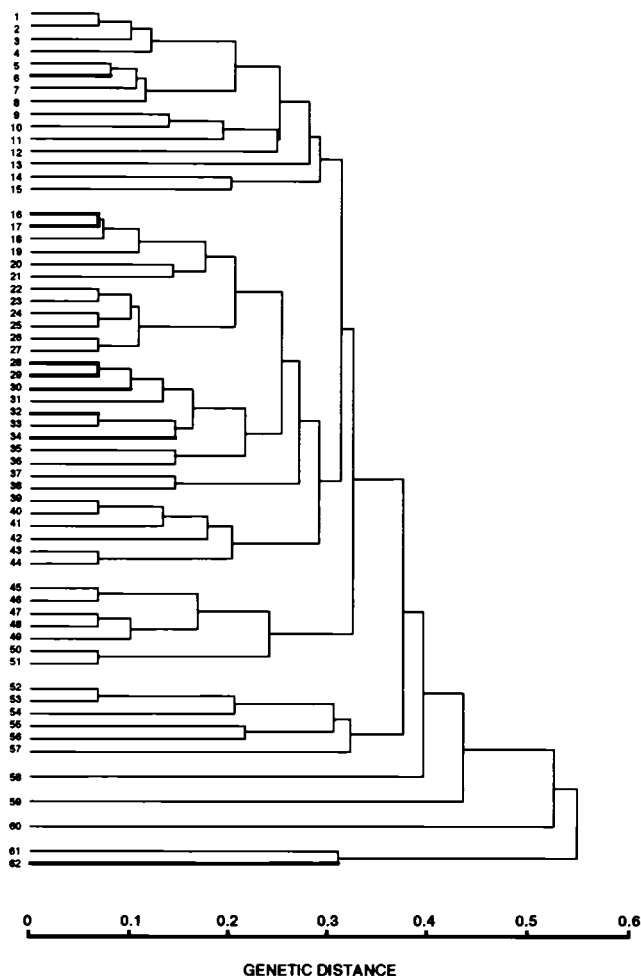
FIG. 4. Phenogram illustrating relationships of nine isolates of *S. pilosicoli* cultured from rectal biopsy specimens, indicated by bold lines, and 70 fecal isolates from previous studies located in another 53 ETs.

more resistant strains were also present. This possibility requires further investigation, for example, by undertaking strain typing with multiple isolates obtained from a range of sites sampled along the length of the large intestine.

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