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Comparative Prevalences of *Brachyspira aalborgi* and *Brachyspira* (*Serpulina*) *pilosicoli* as Etiologic Agents of Histologically Identified Intestinal Spirochetosis in Australia

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DNA from gastrointestinal biopsy specimens from 28 Australian patients with histologic evidence of intestinal spirochetosis (IS) was subjected to PCRs to amplify segments of the 16S rRNA and NADH oxidase genes of *Brachyspira aalborgi* and *Brachyspira (Serpulina) pilosicoli*. *B. aalborgi* was identified in specimens from 24 (85.7%) patients and *B. pilosicoli* in those from 4 (14.3%) patients (2 of whom were also positive for *B. aalborgi*). For two patients, no product was amplified. This study demonstrates that *B. aalborgi* is much more commonly involved in histologically identified IS in Australian patients than is *B. pilosicoli*. This is the first report of amplification of *B. pilosicoli* DNA from humans with IS.

Since the first histologic description of intestinal spirochetosis (IS) in 1967 (3), diagnosis of the condition has been problematic. Clinical microbiologists have had difficulty culturing the causal spirochetes; consequently, IS has been predominantly diagnosed on the basis of histologic findings in biopsy material. The characteristic diagnostic feature is the presence of a thin, carpet-like layer of spirochetes attached by one cell end to the colorectal surface epithelium. The colonization is often not associated with significant changes in the underlying mucosa, and unless the specimen is examined at a 1,000-fold magnification, the spirochetes may go unnoticed. Since colonoscopy with or without biopsy is relatively routine only in affluent western societies, the histologic identification of IS has been predominantly in developed countries.

Two spirochete species, *Brachyspira aalborgi* (4) and *Brachyspira (Serpulina) pilosicoli* (12, 14), have been implicated as causal agents of IS in humans. The relative involvement of the two species in IS in different groups of patients is unclear, and it is not known whether both species have similar pathogenic potential. Both are slow-growing anaerobes requiring specialized media and prolonged incubation, but *B. pilosicoli* is much easier to isolate than *B. aalborgi*. Perhaps because of this difference, *B. pilosicoli* is currently recognized as a pathogen of the large intestine in various animal species (14), while *B. aalborgi* has only been demonstrated by PCR in macaques and humans (2, 11). By using specialized culturing techniques on human feces, rather than histologic examination, *B. pilosicoli* has been shown to commonly colonize (>30% prevalence) Australian Aboriginal children (8), Gulf Arabs (1), villagers in Papua New Guinea (13), and homosexual males and human immunodeficiency virus (HIV)-positive patients in Western societies (9, 12). However, it is not clear whether these individuals also have histologic IS caused by *B. pilosicoli*. There is only one report where concurrent histologic and microbiologic investigation has demonstrated the involvement of *B. pilosicoli*. In this study, involving Australian homosexual males, *B. pilosicoli* was isolated from 50% of rectal biopsy specimens that showed histologic attachment of spirochetes to the mucosa (12).

Currently there are only two reports, both from Scandinavia, of *B. aalborgi* having been cultured from biopsy samples from patients with IS (4, 7). Consequently, the relative importance of *B. aalborgi* as an etiologic agent of IS has been uncertain. However, in a recent study, PCRs were carried out on DNA extracted from human colorectal biopsy tissue obtained from 16 patients, mainly from Norway, who had histologic evidence of IS (11). *B. aalborgi* was detected in 10 of the patients (63%), while *B. pilosicoli* was not detected in any individual. This finding suggests that *B. aalborgi* may be the main agent of histologic IS in nonhomosexual Caucasian populations, particularly in Scandinavian countries.

In the Australian context, we have used selective culture to examine specimens from relatively narrow population groups, and we concluded that colonization with *B. pilosicoli* was common in rural Aboriginal children, and in homosexual males in Sydney, but was virtually absent from children in the cities of Perth and Darwin (8, 12). The purpose of the present study was to broaden our understanding of the relative importance of the two spirochete species in Australia by using the PCR technique on retrospective archival biopsy material from a relatively large number of nonselected, mainly urban adult patients with both histologic IS and gastrointestinal symptoms.

Permission for this study was obtained from the Murdoch University Human Ethics Committee and the ethics committees of the hospitals involved. Sixty-nine formalin-fixed routine biopsy samples from 28 Australian patients with histologic diagnoses of IS were examined (Table 1). The majority of the patients resided in cities: 14 in Perth, Western Australia (WA), 7 in Darwin in the Northern Territory, and 1 in Melbourne, Victoria. The other six patients lived in relatively remote small rural towns and communities throughout WA. Two of the patients from Perth were HIV positive. The number of samples

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TABLE 1. Sources of the intestinal samples from 28 Australian patients diagnosed with IS, and subsequent PCR results using primers specific for *B. aalborgi* and *B. pilosicoli* 16S rRNA and *nox* genes

Source[a]	Sex, age[b]	Symptoms and/or endoscopic findings	Biopsy location	Histologic findings and/or clinical histology[c]	Results for 16S rRNA and *nox* PCRs	
					B. aalborgi	*B. pilosicoli*
Perth, WA	F, 35	12-yr ulcerative-colitis disease history; inactive at time of biopsy	Cecum	IS	+	−
			Transverse colon	Minor changes	+	−
			Ascending colon	IS	+	−
			Hepatic flexure	IS	+	−
			Splenic flexure	IS	+	−
			Sigmoid	IS	+	−
			Rectum	IS	+	−
Perth, WA	F, 41	Diarrhea; O/E ulcers	Colon at 130 cm	IS; inflammation	+	−
			Colon at 80 cm	IS; active inflammation	+	−
			Rectum	IS; active inflammation	+	−
Perth, WA	F, 49	Diarrhea	Duodenum	Negative	−	−
			Random colon	IS; lymphocyte aggregates	+	−
Perth, WA	F, 53	Polyps	Cecum	IS	+	−
			Transverse colon	IS	+	−
			Descending colon	IS	+	−
			Hepatic flexure	IS	+	−
			Sigmoid colon	IS	+	−
			Rectum	IS	+	−
Perth, WA	F, 56	Altered bowel habit	Rectum	IS; lymphocyte aggregates	+	−
Perth, WA	M, 25	Epigastric pain; lower abdominal cramps	Gastric antrum	Negative	−	−
			Gastroesophageal junction	Inflammation	−	−
			Colon at 80 cm	IS; minor changes	+	−
			Colon at 35 cm	IS; inflammation	+	−
			Rectum	IS; minor lymphocyte aggregates	+	−
Perth, WA	M, 29	Right iliac fossa pain	Appendix	IS	+	−
			Appendix	IS	+	−
Perth, WA	M, 35	Anemia	Gastric antrum	Negative	−	−
			Duodenum	Negative	−	−
			Terminal ileum	Negative	−	−
			Right colon	IS; lymphocyte aggregates	+	−
			Left colon	IS; lymphocyte aggregates	+	−
			Rectum	Minor changes	−	−
			Rectal polyp	Adenoma	−	−
Perth, WA	M, 36	HIV^+; homosexual	Liver	Negative	−	−
			Sigmoid	IS; minor changes; lymphocyte aggregates	+	−
			Anal canal	Inflammation; fibrosis; no dysplasia	−	−
Perth, WA	M, 43	Not recorded	Rectum	IS; active inflammation; CMV	+	−
Perth, WA	M, 81	Gastritis	Gastric antrum	Gastritis	−	−
			Cecum	IS; minor changes	+	−
			Ascending colon	IS; minor changes	+	−
			Transverse colon	IS	+	−
			Splenic flexure	IS	+	−
			Descending colon	IS	+	−
			Sigmoid colon	IS	+	−
			Rectum	IS	+	−
Denmark, WA	F, 52	Polyp	Transverse colon	IS	+	−
Bridgetown, WA	M, 27	Rectal bleeding; pain; proctitis	Ileum	Normal	−	−
			Descending colon	IS	+	−
			Right colon	IS	+	−
			Rectum	Proctitis	−	−
Port Headland, WA	M, 27	Rectal bleeding	Rectum	IS; minor changes	+	−
			Rectum	IS	+	−
Geraldton, WA	M, 35	Ulcerative proctitis	Rectum	IS; lymphocyte aggregates	+	−
Kalgoorlie, WA	M, 36	Diarrhea; congestion	Rectum	IS; minor changes; lymphocyte aggregates	+	−
Darwin, NT	F, 25	Rectal bleeding	Sigmoid colon	IS; inflammation; tubular adenoma formation; mild epithelial atypia	+	−
Darwin, NT	F, 47	1-yr history of intermittent and mild right iliac fossa pain	Colon	IS; metaplastic polyps	+	−
Darwin, NT	F, 47	Irritable bowel	Cecum	IS; associated mild increase in eosinophils within lamina propria	+	−
Darwin, NT	M, 35	Bloody diarrhea; diverticulitis?	Colon	IS	+	−
Darwin, NT	M, 45	Rectal bleeding	Rectum	IS	+	−
Melbourne, Vic	F, 51	Not recorded	Colon	IS	+	−
Wyndham, WA	M, 36	Trauma-ischemia	Appendix	IS	+	+
			Transverse colon	IS	+	−
Darwin, NT	M, 47	Not recorded	Mid-transverse colon	IS; hyperplastic polyps	+	+
Perth, WA	M, 30	HIV^+; diarrhea	Sigmoid colon	IS; mild nonspecific inflammatory changes	−	+
Darwin, NT	F, 16	Appendicitis	Appendix	IS; acute appendicitis	−	+
Perth, WA	F, 67	Diarrhea	Ascending colon	Minor changes	−	−
			Transverse colon	Possibly IS; minor changes	−	−
			Sigmoid colon	Adenoma	−	−
Perth, WA	M, 38	Acute appendicitis?	Appendix	IS	−	−

[a] NT, Northern Territory; Vic, Victoria.
[b] F, female; M, male. Ages are given in years.
[c] CMV, cytomegalovirus.

from any particular patient varied from one to seven and, for comparative purposes, included samples from the liver, stomach, and small intestine for six patients. Samples from the large intestine were taken at various sites from the appendix through to the anus. The patients were all suffering from a range of gastrointestinal symptoms, which prompted endoscopy with biopsy to aid diagnosis (Table 1).

DNA from paraffin-embedded tissue (PET) samples was extracted using a modification of a method previously described (11). Several sections approximately 10 μm thick were cut from the PET samples. Each sample was dewaxed with 400 μl of xylene and then an equivalent volume of 100% ethanol. Samples were centrifuged at 10,000 × *g* for 10 min, and the supernatant was discarded. If residual paraffin was present, the process was repeated. The samples were dried at 50°C in an oven for at least 30 min. Twenty micrograms of proteinase K in 200 μl of 50 mM Tris-HCl, pH 8.3, was added and incubated for 1 to 2 h at 55°C, with occasional gentle mixing. The samples then were boiled for 8 min, and 2 μl of each resultant extract was used as template DNA for PCR analysis. The negative controls, comprising biopsy specimens from patients without histologic IS ($n = 14$), were the same as those used in the previous PCR study (11).

Specific PCR procedures amplifying portions of the 16S rRNA and NADH oxidase (*nox*) genes of *B. aalborgi* and *B. pilosicoli* were applied to the extracted DNA, as previously described (11). The only exception was the use of a different reverse primer (5′-CCCCTACAATATCCAAGACT-3′) for the 16S rRNA PCR specific for *B. pilosicoli*. This produced a larger, 439-bp product compared to the previous 196-bp product. Amplification mixtures were as previously described (11), except that dimethyl sulfoxide was not added. The specificities of the PCRs were confirmed as previously described (11), by amplification of DNA extracted from a set of test organisms, and by direct sequencing of eight selected amplicons from PET giving amplification in either *B. pilosicoli* or *B. aalborgi* 16S rDNA or *nox* PCRs. In each case the product produced had a sequence identical to or very closely related to the target sequence.

DNA from all 14 negative-control large-intestinal biopsy specimens, and from all biopsy specimens taken from gastric or small-intestinal tissue, failed to amplify in any of the PCRs. Furthermore, there was no histologic evidence of IS in any of these samples. Results for the samples from the 28 IS patients are presented in Table 1. Where amplification occurred, there was perfect agreement between the results of 16S rRNA and *nox* PCRs for both organisms. Two patients were positive for both *B. pilosicoli* and *B. aalborgi* (7.1%). For one of these patients, the appendix sample was positive for both organisms but a concurrent transverse colon biopsy specimen was positive for *B. aalborgi* only. For the other patient, a biopsy specimen taken from the midpoint of the transverse colon was positive for both species. One patient (3.6%), who was HIV positive, was positive for *B. pilosicoli* alone in both PCRs. Histologically it was not possible to distinguish *B. pilosicoli* from *B. aalborgi* in the biopsy sections. Forty-eight specimens from 22 patients (78.6%) were positive for *B. aalborgi* alone in both PCRs. These positive samples came from various sites along the large intestine from the appendix and cecum through to the rectum. For eight patients multiple samples were taken along the large intestine, including the rectum, and for five of these DNA was amplified from *B. aalborgi* at all sites sampled, while three (37.5%) patients had positive amplifications from the colon samples and negative PCR results from the rectum samples. Rectal biopsy specimens, therefore, do not necessarily reflect the occurrence of IS in more proximal parts of the large intestine, even where there is otherwise extensive colonization along the tract.

Samples from two patients (7.1%) from Perth failed to amplify when any of the PCR protocols were applied. For one of these patients, however, for whom multiple biopsy specimens were tested, the histologic diagnosis of IS was uncertain (Table 1). Failure of amplification for the other patient may have been due to technical problems associated with obtaining appropriate high-quality DNA, or it could have been the result of the patient having been colonized by a different species of spirochete.

This is the first report of direct detection of *B. pilosicoli* in human colonic biopsy tissue using PCR. It is also the first time that both *B. aalborgi* and *B. pilosicoli* have been found concurrently colonizing humans with IS. Previously, concurrent colonization has been detected in macaques (2). It is possible that other uncharacterized spirochete species were also present in some patients; to investigate this possibility, it would be necessary to use less species-specific primers and to sequence the PCR product. Overall, *B. aalborgi* was the predominant species detected in Australian patients with IS; it was present in 24 (85.7%) of the patients. This finding is in contrast to the findings of previous studies in Australia, using selective culture on different population groups, where *B. pilosicoli* was commonly isolated both from Aboriginal children and male homosexuals. Such contrasting results from one country clearly indicate that *B. aalborgi* and *B. pilosicoli* have very distinctive epidemiologic patterns and infect different population groups. It is still not known whether the two species have different pathogenic potentials, and it will be necessary to obtain more biopsy samples from patients colonized with *B. pilosicoli* before such comparisons can be made. It was also of interest that in this study *B. pilosicoli* was identified in samples from the colon and appendix, but not in the rectal biopsy specimens of any of the patients, whereas in the previous study on homosexual males it was commonly isolated from rectal biopsy specimens (12).

The patients in the present study were not known to have any increased risk factors for IS, apart from two who were known to be HIV positive. One of the HIV-positive patients was one of the four colonized by *B. pilosicoli*. Investigators in Germany have reported recovering spirochetes resembling *B. pilosicoli* from the feces of about half of a series of HIV patients (6). The other HIV patient in this study, who was a homosexual and a chronic hepatitis C virus carrier, was positive for *B. aalborgi*. This organism was previously identified by the same PCR protocols in biopsy tissue from two HIV-positive patients with histologic IS from Washington, D.C. (11). Hence HIV-positive patients may be colonized by either species of spirochete.

Interestingly, the two patients who were colonized by both *B. pilosicoli* and *B. aalborgi* came from Wyndham, a rural settlement in the north of WA, and Darwin in the Northern Territory, respectively. Many Aboriginal people reside in these areas, but unfortunately ethical considerations prevented us from obtaining specific information regarding the patients' eth-

nic identities. If these two patients were Aboriginal, the presence of *B. pilosicoli* would fit with the known high (>30%) prevalence of fecal carriage of *B. pilosicoli* by Aborigines in the north of Australia (8). To date it has been difficult to obtain appropriate specimens to determine whether people with fecal carriage of *B. pilosicoli* also show histologic evidence of IS. Certainly a study from southern India, where 64% of normal villagers showed attachment of spirochetes to the rectal mucosa, suggests that people in developing countries (where *B. pilosicoli* is commonly found in human feces) do often have histologic IS (10). It is also worth noting that many pigs with chronic diarrhea associated with *B. pilosicoli* infection do not show end-on attachment of the spirochetes to the colonic mucosa (5). A similar situation may occur in humans, in which case chronic diarrhea and failure to thrive caused by *B. pilosicoli* (and perhaps *B. aalborgi*) would go undiagnosed because the organism would not be detectable by histology or by the inappropriate culture techniques currently routinely used in medical diagnostic laboratories.

In summary, in this study *B. aalborgi* was shown to be a common agent of histologic IS in Australian patients who had undergone biopsy because of the presence of various relatively nonspecific gastrointestinal symptoms. *B. pilosicoli* was identified as an infrequent cause of IS in this group of patients, even though this species has previously been shown to be a common cause of histologic IS in Australian homosexuals (12). Similarly, *B. pilosicoli* has been shown to commonly colonize Australian Aboriginal children, but it is not known whether these individuals also suffer from histologic IS. Further studies, perhaps including techniques such as direct PCR on feces, are required on these and other population groups to determine the relative carriage rates and disease potentials of the two spirochete species.

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