Brachyspira Species and Gastroenteritis in Humans

L. J. Westerman,[a] R. F. de Boer,[b] J. H. Roelfsema,[c] I. H. M. Friesema,[c] L. M. Kortbeek,[c] J. A. Wagenaar,[d,e] M. J. M. Bonten,[a] J. G. Kusters[a]

Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, the Netherlands[a]; Laboratory for Infectious Diseases, Department of Research and Development, Groningen, the Netherlands[b]; National Institute for Public Health, Center for Infectious Disease Control, Bilthoven, the Netherlands[c]; Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands[d]; Central Veterinary Institute of Wageningen University and Research Center, Lelystad, the Netherlands[e]

***Brachyspira* species have been implicated as a potential cause of gastroenteritis in humans; this is, however, controversial. In 733 gastroenteritis cases and 464 controls, we found 29 samples positive for *Brachyspira* species (2.3% of cases and 2.6% of controls; $P = 0.77$). *Brachyspira* species were not associated with gastroenteritis in humans.**

uman intestinal spirochetosis (HIS) is caused by spirochetes from the genus *Brachyspira* (1) and is histologically characterized by the apical attachment of these bacteria to the mucosa of the colon (2). Three *Brachyspira* species have been reported to colonize humans: *Brachyspira aalborgi*, *Brachyspira pilosicoli*, and the provisionally named "*Brachyspira hominis*" (3–7). Although it is unclear whether HIS is associated with clinical symptoms, there is evidence suggesting an association between *B. pilosicoli* and diarrhea in humans (3, 8–11). However, due to the obligate anaerobic culture requirements for these bacteria, most studies on HIS are based on histopathology without species differentiation, as histopathology requires additional techniques to differentiate between species (3, 12). As a result, the clinical significance of HIS has remained controversial for decades (1, 13–16). Several studies have investigated the prevalence of *Brachyspira* species in patients with diarrhea, and various incidences have been reported, depending on the investigated population (high- versus low-income countries) and techniques used (culture versus histopathology). In this study, we investigated the prevalence of *Brachyspira* species in Dutch primary care patients with and those without symptoms of gastroenteritis. For this purpose, we used fecal samples stored from a case-control study reported in 2001 (17). DNA was extracted as previously described (18), directly from the fecal samples without selenite enrichment. Amplification was performed by using a previously described real-time PCR amplifying a 142-bp region of the 16S rRNA of all *Brachyspira* species (3), with some minor modifications. The modifications consisted of three species-specific probes targeting *B. pilosicoli*, *B. aalborgi*, and "*B. hominis*" and another reverse primer. These probes were validated against a known set of 66 *Brachyspira*-positive samples (cultured isolates and histopathologically positive samples) and 26 *Brachyspira*-negative samples (3). The PCR protocol and primer and probe sequences are supplied in Table 1. PCR inhibition was defined as a PhHV cycle threshold value greater than the average of all PCRs plus 2 times the standard deviation. Samples exceeding this value and all *Brachyspira*-positive samples were reextracted from the original fecal samples. Samples were excluded from analysis if the internal control exceeded the cutoff again. Statistical analyses were performed by using IBM SPSS Statistics version 20 (release 20.0.0) for Macintosh OS X.

Samples from 736 cases and 465 controls were available. Four samples (three cases and one control) had to be excluded due to inhibition of the PCR. Twenty-nine samples were positive for

TABLE 1 Primers and probes[a]

Primer or probe	Sequence (5′–3′)
PhHV Fw	GGGCGAATCACAGATTGAATC
PhHV Rev	GCGGTTCCAAACGTACCAA
PhHV Probe	LC640-TTTTTATGTGTCCGCCACCATCTGGATC-BBQ
Bspp Fw	AACATGGACTAATACCGCATATAC
Bspp Rev	CTCAGGTCGGCTACCTATC
Bspp Probe	LC610-TGAGCCTGCGGCCTATTAGCC-BBQ
Bpilo Probe	R6G-AAGTAGAGTAGAGGAAAGTTTTTTCGCTTC-BBQ
Bhomi Probe	6FAM-TCTTGACACATAAGTGTAGTAGAGGAAAGG-BBQ
Baalb Probe	Cyan500-TGACGCTAAAGCGTAGTAGAGGAAAG-BBQ

[a] The PCR protocol consisted of 95°C for 10 min (preincubation) and 45 cycles of 95°C for 10 s (denaturation) followed by 60°C for 60 s (amplification). PCR runs included a negative control (high-performance liquid chromatography-grade water) and positive controls (*B. aalborgi* [513A^T], *B. pilosicoli* [P43/6/78^T], and a biopsy sample positive for *B. hominis*). Primers and probes were synthesized by TIB Molbiol GmbH, Berlin, Germany. PhHV, phocine herpesvirus. Reporter dyes: LC640, LightCycler Red 640; LC610, LightCycler Red 610; R6G, rhodamine 6G; 6FAM, 6-carboxyfluorescein; BBQ, BlackBerry quencher.

Brachyspira species: 17 cases (2.3%) and 12 controls (2.6%) ($P = 0.77$, determined by a chi-square test). All PCR-positive samples were confirmed after reextraction.

B. aalborgi was detected in 18 samples (11 cases and 7 controls), and "*B. hominis*" was detected in 7 samples (4 cases and 3 controls). Four samples were positive for both *B. aalborgi* and "*B. hominis*" (two cases and two controls). *B. pilosicoli* was not detected in any of the included samples, although this species was responsible for 15.4% of HIS cases in a previous study in the Netherlands using similar methods (3). No statistically significant association ($P < 0.05$) was found regarding the presence of *Brachyspira* species in cases and controls or regarding age, gender, age, coinfections, urbanization, seasons, year of study, or duration of symptoms.

Other studies investigating the presence of *Brachyspira* species in Western societies used microscopy of colon biopsy specimens taken for various reasons and reported a prevalence between 0.0% and 2.5% (8, 19–22), but those studies did not include a control group or used samples taken for other suspected pathologies as controls. In a culture-based study, 1,018 fecal samples from patients with diarrhea and 509 samples taken for screening purposes were analyzed, and spirochetes resembling *B. pilosicoli* were detected in 8 (0.8%) patients and in 15 (2.9%) of the screening samples (23). However, all positive samples were from either persons of Asian descent (*n* = 16; 12 of whom had recently been to the Indian subcontinent) or homosexuals (*n* = 7), whereas HIS is reported to be more frequent in HIV-infected patients or in men who have sex with men (23–26) and is known to be more prevalent in low-income countries (8, 21, 22, 27, 28). In the present study, one patient was HIV positive (*B. aalborgi* and "*B. hominis*" coinfection), all but one *Brachyspira*-positive patients were native Dutch inhabitants (one was born in Africa but immigrated to the Netherlands 19 years before the original study), and three had been abroad in the week before sampling (eastern Europe, Central/South Africa, and North Africa).

Infections with *Brachyspira* species have been associated with male gender (8, 14, 22), and in the present study there was a non-significant trend toward a higher prevalence of *Brachyspira* species in fecal samples of men (3.5% versus 1.8%; *P* = 0.075, determined by a chi-square test).

The storage of feces in peptone-glycerol at −80°C for more than 10 years is a potential limitation, as this may have had a negative impact on DNA quality. However, PCR-based retesting (18, 29) of 12 fecal samples that were positive in 1997 did not suggest a loss of DNA quality (see Table S1 in the supplemental material). Moreover, if quality had decreased, this would have affected samples from both cohorts equally. One sample was found to be negative for *Dientamoeba fragilis* by PCR, whereas it was microscopically positive in the original study. This is likely due to the fact that *D. fragilis* excretion is known to be highly variable, and routinely, three fecal samples are collected for microscopy on three different days, while only a single fecal sample was stored and available for PCR analysis. Furthermore, seven additional positive results were obtained by PCR, illustrating the higher sensitivity of PCR than conventional diagnostic procedures. In conclusion, these results were indicative of correct preservation of the DNA.

Two important limitations of this study are to be taken into consideration: as our study was performed in general practices in the Netherlands, our findings may not be generalizable to countries with a different prevalence of *Brachyspira* species among humans or to different patient populations, and since no *B. pilosicoli* isolates were identified in this population, this does not allow for conclusions on its pathogenic potential but strongly suggests that it is not relevant as a pathogen in general practice.

This is the first study of the association of *Brachyspira* species and gastroenteritis in humans using prospectively collected fecal samples from patients with and those without symptoms of gastroenteritis. Based on our results, we conclude that *Brachyspira* species are not associated with gastroenteritis in humans in general practice.

REFERENCES

1. Hovind-Hougen K, Birch-Andersen A, Henrik-Nielsen R, Orholm M, Pedersen JO, Teglbjaerg PS, Thaysen EH. 1982. Intestinal spirochetosis: morphological characterization and cultivation of the spirochete *Brachyspira aalborgi* gen. nov., sp. nov. J. Clin. Microbiol. 16:1127–1136.
2. Harland WA, Lee FD. 1967. Intestinal spirochaetosis. Br. Med. J. iii:718–719.
3. Westerman LJ, Stel HV, Schipper MEI, Bakker LJ, Neefjes-Borst EA, van den Brande JHM, Boel CHE, Seldenrijk CA, Siersema PD, Bonten MJM, Kusters JG. 2012. Development of a real-time PCR for identification of *Brachyspira* species in human colonic biopsies. PLoS One 7:e52281. doi:10.1371/journal.pone.0052281.
4. Mikosza AS, Munshi MA, Hampson DJ. 2004. Analysis of genetic variation in *Brachyspira aalborgi* and related spirochaetes determined by partial sequencing of the 16S rRNA and NADH oxidase genes. J. Med. Microbiol. 53:333–339.
5. Pettersson B, Wang M, Fellstrom C, Uhlen M, Molin G, Jeppsson B, Ahrne S. 2000. Phylogenetic evidence for novel and genetically different intestinal spirochetes resembling *Brachyspira aalborgi* in the mucosa of the human colon as revealed by 16S rDNA analysis. Syst. Appl. Microbiol. 23:355–363.
6. Sato H, Nakamura S, Habano W, Wakabayashi G, Adachi Y. 2010. Human intestinal spirochaetosis in northern Japan. J. Med. Microbiol. 59:791–796.
7. Mikosza AS, Hampson DJ. 2001. Human intestinal spirochetosis: *Brachyspira aalborgi* and/or *Brachyspira pilosicoli*? Anim. Health Res. Rev. 2:101–110.
8. Calderaro A, Bommezzadri S, Gorrini C, Piccolo G, Peruzzi S, Villanacci V, Zambelli C, Dettori G, Chezzi C. 2007. Infective colitis associated with human intestinal spirochetosis. J. Gastroenterol. Hepatol. 22:1772–1779.
9. Peghini PL, Guccion JG, Sharma A. 2000. Improvement of chronic diarrhea after treatment for intestinal spirochetosis. Dig. Dis. Sci. 45:1006–1010.
10. Gad A, Willen R, Furugard K, Fors B, Hradsky M. 1977. Intestinal spirochaetosis as a cause of longstanding diarrhoea. Ups. J. Med. Sci. 82:49–54.
11. Naresh R, Song Y, Hampson DJ. 2009. The intestinal spirochete *Brachyspira pilosicoli* attaches to cultured Caco-2 cells and induces pathological changes. PLoS One 4:e8352. doi:10.1371/journal.pone.0008352.
12. Jensen TK, Teglbjaerg PS, Lindboe CF, Boye M. 2004. Demonstration of *Brachyspira aalborgi* lineages 2 and 3 in human colonic biopsies with intestinal spirochaetosis by specific fluorescent in situ hybridization. J. Med. Microbiol. 53:341–343.
13. Kraaz W, Pettersson B, Thunberg U, Engstrand L, Fellstrom C. 2000. *Brachyspira aalborgi* infection diagnosed by culture and 16S ribosomal DNA sequencing using human colonic biopsy specimens. J. Clin. Microbiol. 38:3555–3560.
14. Weisheit B, Bethke B, Stolte M. 2007. Human intestinal spirochetosis: analysis of the symptoms of 209 patients. Scand. J. Gastroenterol. 42:1422–1427.
15. Van Mook WNKA, Koek GH, Van Der Ven AJAM, Ceelen TL, Bos RP. 2004. Human intestinal spirochaetosis: any clinical significance? Eur. J. Gastroenterol. Hepatol. 16:83–87.
16. Körner M, Gebbers JO. 2003. Clinical significance of human intestinal spirochetosis—a morphologic approach. Infection 31:341–349.
17. de Wit MA, Koopmans MP, Kortbeek LM, van Leeuwen NJ, Bartelds AI, van Duynhoven YT. 2001. Gastroenteritis in sentinel general practices, The Netherlands. Emerg. Infect. Dis. 7:82–91.
18. de Boer RF, Ott A, Kesztyus B, Kooistra-Smid AM. 2010. Improved detection of five major gastrointestinal pathogens by use of a molecular screening approach. J. Clin. Microbiol. 48:4140–4146.
19. Henrik-Nielsen R, Orholm M, Pedersen JO, Hovind-Hougen K, Tegl-

bjaerg PS, Thaysen EH. 1983. Colorectal spirochetosis: clinical significance of the infestation. Gastroenterology **85**:62–67.

20. **Lindboe CF, Tostrup NE, Nersund R, Rekkavik G.** 1993. Human intestinal spirochaetosis in mid-Norway. A retrospective histopathological study with clinical correlations. APMIS **101**:858–864.

21. **Esteve M, Salas A, Fernandez-Banares F, Lloreta J, Marine M, Gonzalez CI, Forne M, Casalots J, Santaolalla R, Espinos JC, Munshi MA, Hampson DJ, Viver JM.** 2006. Intestinal spirochetosis and chronic watery diarrhea: clinical and histological response to treatment and long-term follow up. J. Gastroenterol. Hepatol. **21**:1326–1333.

22. **Tanahashi J, Daa T, Gamachi A, Kashima K, Kondoh Y, Yada N, Yokoyama S.** 2008. Human intestinal spirochetosis in Japan; its incidence, clinicopathologic features, and genotypic identification. Mod. Pathol. **21**:76–84.

23. **Tompkins DS, Foulkes SJ, Godwin PG, West AP.** 1986. Isolation and characterisation of intestinal spirochaetes. J. Clin. Pathol. **39**:535–541.

24. **Law CL, Grierson JM, Stevens SM.** 1994. Rectal spirochaetosis in homosexual men: the association with sexual practices, HIV infection and enteric flora. Genitourin. Med. **70**:26–29.

25. **Surawicz CM, Roberts PL, Rompalo A, Quinn TC, Holmes KK, Stamm WE.** 1987. Intestinal spirochetosis in homosexual men. Am. J. Med. **82**:587–592.

26. **McMillan A, Lee FD.** 1981. Sigmoidoscopic and microscopic appearance of the rectal mucosa in homosexual men. Gut **22**:1035–1041.

27. **Margawani KR, Robertson ID, Brooke CJ, Hampson DJ.** 2004. Prevalence, risk factors and molecular epidemiology of *Brachyspira pilosicoli* in humans on the island of Bali, Indonesia. J. Med. Microbiol. **53**:325–332.

28. **Munshi MA, Traub RJ, Robertson ID, Mikosza AS, Hampson DJ.** 2004. Colonization and risk factors for *Brachyspira aalborgi* and *Brachyspira pilosicoli* in humans and dogs on tea estates in Assam, India. Epidemiol. Infect. **132**:137–144.

29. **Friesema IH, de Boer RF, Duizer E, Kortbeek LM, Notermans DW, Norbruis OF, Bezemer DD, van Heerbeek H, van Andel RN, van Enk JG, Fraaij PL, Koopmans MP, Kooistra-Smid AM, van Duynhoven YT.** 2012. Etiology of acute gastroenteritis in children requiring hospitalization in the Netherlands. Eur. J. Clin. Microbiol. Infect. Dis. **31**:405–415.