Colonic Spirochetal Infections in Nonhuman Primates That Were Associated with \textit{Brachyspira aalborgi}, \textit{Serpulina pilosicoli}, and Unclassified Flagellated Bacteria. \textit{G. E. Duhamel, R. O. Elder, N. Munappa, M. R. Mathiesen, V. J. Wong, and R. P. Tarara.} From the Department of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, Nebraska; and the California Regional Primate Research Center, University of California at Davis, Davis, California

Colonic spirochetosis (CS) in humans and nonhuman primates is characterized by attachment of poorly characterized spirochetes, alone or together with flagellated bacteria, to the brush border of the colonic epithelium \cite{1}. CS in colony-raised rhesus monkeys (\textit{Macaca mulatta}) and some wild-caught baboons (\textit{Papio} species) has been documented by using light and electron microscopy, but the spirochetes have not been characterized \cite{1}. Our group \cite{2, 3} has reported that certain human, canine, and porcine intestinal spirochetes associated with CS belong to the new phyletic group \textit{Serpulina pilosicoli}. However, it has been reported that a phenotypically different spirochete designated \textit{Brachyspira aalborgi} causes CS in humans \cite{4}. To ascertain the role of \textit{Brachyspira aalborgi} and \textit{S. pilosicoli} in colonic spirochetal infection in macaques, a PCR amplification method specific for the 16S ribosomal RNA genes from each spirochete was applied to total DNA extracted from either pure cultures of intestinal spirochetes or formalin-fixed paraffin-embedded colonic tissue specimens obtained from 10 colony-born rhesus monkeys (MMU) and two crab-eating monkeys (\textit{Macaca fascicularis}; MCY) with CS or colitis.

The macaques were selected based on histopathologic changes consistent with CS or colitis (table 1). Light microscopic examination of hematoxylin-eosin-stained sections of colon specimens from macaques with CS revealed a diffuse, densely basophilic 4-\micro meter layer covering the brush border of the superficial epithelium that extended a short distance into the neck of the crypts. The basophilic layer could not be demonstrated by gram staining but was dark brown to black by Warthin-Starry staining. With the exception of colon tissue specimens from MMU20571 and MMU26501, a mild multifocal infiltrate of mixed mononuclear cells also was present in the lamina propria.

Electron microscopic examination revealed large numbers of spirochetes, often associated with flagellated bacteria, attached perpendicularly by one pole to the brush border of the surface epithelium that caused deep indentations into the apical cell membrane and effacement of microvilli (table 1). In some specimens, there were spirochetes between epithelial cells or free in the lamina propria. Spirochetes also were present in the cytoplasm of degenerative cells in necrotic foci of the surface epithelium in some specimens from macaques with CS. The ages of these animals ranged from 8 months to 11 years and 8 months. Seven macaques were killed for reasons unrelated to intestinal disorders, whereas MMU19755 and MMU25659 died of simian immunodeficiency virus–related pneumonia.

Light microscopic examination of hematoxylin-eosin-stained colon specimens from macaques with colitis revealed marked inflammatory cell infiltrates in the lamina propria consisting of varying numbers of lymphocytes, plasma cells, histiocytes, and neutrophils with multifocal crypt abscesses and multifocal to extensive erosion of the surface epithelium. By contrast, moderate to large numbers of argyrophilic spirochetes in the crypt lumina, but not on the mucosal surface, were found in these specimens. These animals, whose ages ranged from 9 to 22 months, were killed (MMU26717) or died (MMU26986 and MMU27669) because of chronic diarrhea, dehydration, and cachexia. Concurrent enteric infections with \textit{Shigella flexneri} and \textit{Yersinia pseudotuberculosis} were present in MMU26717 and MMU27669. Although \textit{S. flexneri} had been isolated from a rectal swab taken 8 months before death, no enteric bacterial pathogens were isolated from MMU26986 at necropsy.

Colonic mucosal swabs were taken from some of the macaques at necropsy; these swabs were placed in transport medium, frozen at –70°C, and shipped to the University of Nebraska-Lincoln for spirochete isolation. After thawing, the swabs were placed on a selective agar medium for growth of intestinal spirochetes \cite{2} and incubated in the Gas Pak Anaerobic System (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD) at 42°C for 10 days. Pure cultures of spirochetes were propagated anaerobically at 42°C on trypticase soy agar with 5% citrated sheep blood and in repressed anaerobically sterilized trypticase soy broth incubated at 37°C \cite{2}. The macaque intestinal spirochetes grew as a thin film that gave a ground glass appearance to the surface of the agar and produced discrete weak β-hemolysis without a zone of enhanced hemolysis or ring phenomenon \cite{2}. Transmission electron microscopy of negatively stained spirochete isolates from MMU26717 and MMU27669 revealed five periplasmic flagella inserted in one row at each end of the cytoplasmic membrane, a characteristic of \textit{S. pilosicoli} \cite{1–3}.

Total DNA was extracted from formalin-fixed paraffin-embedded colonic tissues. The colonic tissues were excised from the paraffin blocks by using sterile razor blades and placed in xylene for 30 minutes at room temperature. After centrifugation at 12,000g for 5 minutes, the supernatant was discarded, and the samples were extracted once more with xylene and twice with 100% ethanol. The final pellet was placed in acetonitrile, dried at 50°C, and subjected to purple m M Tris (pH, 8.5), 1 mM EDTA, 0.5% N-lauroyl sarcosine, and 200 μg of protease K/mL for 3 hours at 55°C or overnight at 37°C.

The PCR assay was conducted with use of a 21-base forward oligonucleotide primer designed to be complementary to a region of a 16S ribosomal RNA gene from the reference \textit{S. pilosicoli} isolate P43/6/78 (GenBank Data Library accession no. U14927) recovered from a pig with clinical signs and lesions of CS \cite{3} that extended from base position 165 and consisted of nucleotides 5′-AGAGGAAAGTTTTTTCGCTTC-3′ in combination with a 20-
substituted for template DNA was used with each PCR assay.

Amplification of DNA was done by using a 20-base reverse oligonucleotide primer designed to be complementary to a unique sequence of a 16S ribosomal RNA gene from Serpulina pilosicoli. Amplification of DNA was done by using a previously described PCR method except that initial denaturing was for 4 minutes at 94°C, followed by 40 cycles of 30 seconds at 55°C, 30 seconds at 72°C, and 30 seconds at 94°C [5]. The amplified products were visualized in 1.7% agarose gels after ethidium bromide staining. A negative control consisting of reaction buffer, deoxyribonucleoside triphosphates, and distilled water substituted for template DNA was used with each PCR assay.

PCR amplification of DNA extracted from the reference S. pilosicoli isolate P43/6/78 (ATCC [American Type Culture Collection] 51139) and the macaque intestinal spirochetes yielded S. pilosicoli-specific products (table 1). PCR amplification of DNA extracted from the reference B. aalborgi isolate (GenBank Data Library accession no. Z22781) recovered from a human with CS [4] that consisted of nucleotides 5' -GCCTGTTGGTAGATAAAAG-3' was used in combination with the 20-base reverse oligonucleotide primer from Serpulina species. Amplification of DNA was done by using a previously described PCR method except that initial denaturing was for 4 minutes at 94°C, followed by 40 cycles of 30 seconds at 55°C, 30 seconds at 72°C, and 30 seconds at 94°C [5]. The amplified products were visualized in 1.7% agarose gels after ethidium bromide staining. A negative control consisting of reaction buffer, deoxyribonucleoside triphosphates, and distilled water substituted for template DNA was used with each PCR assay.

NOTE. NBG = no bacterial growth; ND = not determined; TEM = transmission electron microscopic; 1 = colitis; 2 = diffuse basophilic layer covering the brush border of the superficial colonic epithelium; + = present; - = absent.

* Spirochetes or flagellated bacteria either attached to the epithelial brush border in colon specimens from macaques with colonic spirochetosis or were free in the crypt lumina in colon specimens from macaques with colitis.

1 After anaerobic incubation of colonic swabs on selective agar medium for spirochetes at 42°C for 10 days and S. pilosicoli 16S ribosomal RNA-specific PCR.

1 PCR amplification of purified DNA from formalin-fixed paraffin-embedded colon tissues by using a 16S ribosomal RNA gene from S. pilosicoli or a 16S ribosomal RNA gene from B. aalborgi.

Figure 1. Ethidium bromide-stained 1.7% agarose gel of PCR-amplified products of a 314-bp 16S ribosomal RNA gene from Brachyspira aalborgi and a 361-bp 16S ribosomal RNA gene from Serpulina pilosicoli that were extracted from formalin-fixed paraffin-embedded colonic tissue specimens from macaques with colonic spirochetosis or colitis. Lanes 1 and 13, molecular size standards (1-kilobase DNA ladder; GIBCO BRL, Gaithersburg, MD); lane 2, MMU26717 and PCR-amplified S. pilosicoli; lane 3, MMU27669 and PCR-amplified B. aalborgi; lane 4, MMU24748 and PCR-amplified S. pilosicoli; lane 5, MMU27314 and PCR-amplified B. aalborgi; lane 6, MMU24951 and PCR-amplified B. aalborgi; lane 7, MMU24748 and PCR-amplified S. pilosicoli; lane 8, MMU24951 and PCR-amplified B. aalborgi; lane 9, MMU27314 and PCR-amplified S. pilosicoli; lane 10, negative control; lane 11, reference porcine S. pilosicoli isolate P43/6/78 and PCR-amplified S. pilosicoli; lane 12, reference human B. aalborgi isolate and PCR-amplified B. aalborgi.
adherent spirochetes had *S. pilosicoli* infection alone. These results are consistent with previous transmission electron microscopic studies that demonstrated spirochetes with phenotypic characteristics suggestive of *B. aalborgi* and *S. pilosicoli* in colonic tissue specimens from humans and rhesus monkeys with CS [1, 4]. The role of *S. flexneri* and *Y. pseudotuberculosis* in concurrent enteric infection with *S. pilosicoli* in two of our macaques with colitis is unknown. It is possible that intestinal infection by *S. flexneri* and *Y. pseudotuberculosis* provided an environment suitable for concurrent spirochetal infection. However, MMU26986 had colitis and *S. pilosicoli* infection, but enteric bacterial pathogens were not found at necropsy, thus suggesting that *S. pilosicoli* was the only bacterial agent accounting for colitis.

Because colitis in macaques with *S. pilosicoli* infection was strikingly similar to ulcerative colitis in humans, it may represent a more advanced stage of CS in which colonic invasion and persistence of *S. pilosicoli* are involved. Conversely, *B. aalborgi* may lack invasive attributes or might not persist beyond the brush border interface. Further studies with in vivo and in vitro models of surface attachment and invasion are needed to determine the significance of each spirochete in CS and colitis. These findings have important implications for the diagnosis, prognosis, and treatment of inflammatory bowel diseases (including CS in humans and nonhuman primates) and provide evidence to support the use of a nonhuman primate animal model in the study of the pathogenesis of CS and colitis.

References


