Experimental Infection of C3H mice with Avian, Porcine, or Human Isolates of *Serpulina pilosicoli*

RANDY E. SACCO,[1] DARRELL W. TRAMPEL,[2] AND MICHAEL J. WANNEMUEHLER[1]*

Veterinary Medical Research Institute[1] and Veterinary Extension,[2] College of Veterinary Medicine, Iowa State University, Ames, Iowa 50011

C3H/HeJ (lps^d/lps^d) and C3H/HeOuJ (lps^n/lps^n) mice were infected via gastric intubation with avian, porcine, or human isolates of weakly hemolytic spirochetes classified as *Serpulina pilosicoli*. Upon histopathological examination of cecal tissue from mice infected with avian or porcine isolates, colonization of spirochetes attached end-on to the apical surface of enterocytes was observed. There were no apparent differences in severity of cecal lesions between the lipopolysaccharide (LPS)-responsive (C3H/HeOuJ) and LPS-hyporesponsive (C3H/HeJ) mouse strains infected with these isolates. Transmission electron microscopy showed spirochetes invaginated into the host cell membrane with resultant effacement of microvilli and loss of the glycocalyx. End-on attachment of the human isolate *S. jonesii* was not observed in the present studies, although weakly hemolytic spirochetes were reisolated from mice infected with *S. jonesii*. Moreover, results of Western immunoblot experiments showed mice developed serum antibody responses to the *S. pilosicoli* isolates examined. Thus, the present results indicate that specific isolates of *S. pilosicoli* can colonize mice and exhibit end-on attachment to cecal enterocytes.

The genus *Serpulina* is comprised of intestinal spirochetes which have been classified on the basis of hemolysis. *S. hyodysenteriae*, a strongly β-hemolytic gram-negative anaerobic spirochete, is the etiological agent of swine dysentery, a severe mucohemorrhagic diarrheal disease of growing pigs. Weakly β-hemolytic spirochetes, known to be present in the normal microflora of several animal species, including humans, were originally thought to be nonpathogenic. However, Taylor et al. provided the first evidence that a weakly hemolytic spirochete (designated P43/6/78) could produce diarrhea in experimentally infected pigs (12). Colitis was observed in several of the inoculated pigs, with excess mucus and punctate hemorrhages seen in the colonic mucosa. Upon histological examination, extensive colonization of enterocytes by end-on attaching spirochetes was observed. Intestinal spirochetosis or spirochetal diarrhea has been the term used to describe the condition where numerous spirochetes have been observed attached end-on to the epithelial surface of the intestinal mucosa.

The clinical significance of intestinal spirochetes in humans is at present a matter of debate. Spirochetes attached to the intestinal mucosa have been observed in clinically normal individuals and have been regarded as members of normal intestinal microflora (6, 11). However, in some human patients, enteric spirochetes have been associated with clinical symptoms such as diarrhea, mucus discharge, and rectal bleeding (2, 3). Indeed, intestinal spirochetes are commonly isolated from indigenous people including Persian Gulf Arabs and Australian Aborigines (1, 7). In Western societies, intestinal spirochetes have been most frequently isolated from patients with AIDS and from homosexual males (5, 13). The weakly hemolytic spirochetes isolated from humans are closely related to the weakly hemolytic spirochetes which are associated with intestinal spirochetosis in pigs. These weakly hemolytic spirochetes, previously referred to as "*Anguillina coli*," are known to

be genetically distinct from other members of the genus *Serpulina* (10) and have recently been given the name *S. pilosicoli*.

The specific-pathogen-free chick model has been utilized as an experimental animal model to study the pathogenesis of intestinal spirochetosis. In this model, human, canine, and porcine intestinal spirochetes have been shown to colonize the mucosal surface of cecal enterocytes, exhibiting an end-on attachment, and to produce a mucoid diarrhea (8, 15). The development of a murine model would be highly desirable for studies of intestinal spirochetosis given the availability of immunological reagents and genetically defined mouse strains, as well as transgenic mice and mice with targeted disruption of specific genes. However, there have been no reports of mice being used as an experimental animal model of intestinal spirochetosis. In addition to their usefulness in experimental studies of intestinal spirochetosis, mice represent a potential reservoir of intestinal spirochetes in livestock production systems. Therefore, the objectives of the present study were to determine whether *S. pilosicoli* could colonize the mucosal surface of the colonic epithelium of mice and whether gross and/or histological cecal lesions would be observed in mice following infection.

The avian isolate utilized in the present study was a weakly β-hemolytic spirochete that was isolated from a commercial laying flock exhibiting diarrhea and a 5% decrease in egg production (designated 42167) (14). Isolate 42167 has been determined to belong to the *S. pilosicoli* species by PCR amplification of 16S rDNA which includes a nucleotide sequence specific for this species (4a). The porcine isolate examined in the present study was the well-characterized strain P43/6/78 (12). The weakly hemolytic spirochete with the originally proposed name "*S. jonesii*" (ATCC 49776), isolated from a homosexual male with diarrhea, was the human strain utilized in the present study (5). Spirochetes were grown in brain heart infusion broth supplemented with 6% fetal calf serum (BHIS) under anaerobic conditions at 37°C. These isolates were indole negative as determined by the addition of Kovac's reagent to 48-h cultures. Ninety-seven male and female C3H/HeJ (lps^d/lps^d) and C3H/HeOuJ (lps^n/lps^n) mice (8 to 16 weeks of age)

* Corresponding author. Mailing address: Veterinary Medical Research Institute, 1802 Elwood Dr., Iowa State University, Ames, IA 50011. Phone: (515) 294-3270. Fax: (515) 294-1401. E-mail: mjwannem @iastate.edu.

from breeding colonies at Iowa State University (originally obtained from Jackson Laboratory, Bar Harbor, Maine) were utilized in these experiments. Mice were fed Teklad 85420 (Harlan Teklad, Madison, Wis.), a nutritionally complete defined diet (9), for 48 h prior to infection followed by a 4-h fast. Mice were inoculated twice within a 4-h period with 10^8 highly motile spirochetes via gastric intubation.

Mice were euthanized at 4, 8, 16, 20, and 30 days postinfection. Blood was collected from individual animals via cardiac puncture following CO_2 asphyxiation. At necropsy, antibiotic-containing blood agar plates (9) were inoculated with cecal contents from each mouse and incubated at 37°C in anaerobic jars (BBL Microbiology Systems, Cockeysville, Md.) with an atmosphere of 10% H_2, 10% CO_2, and 80% N_2. Following subsequent in vitro passage on blood agar plates without antibiotics, isolated spirochetes were grown in BHIS broth. The production of indole was determined by the addition of Kovac's reagent to 48-h broth cultures.

Cecal tissue was fixed in Bouin's fluid (70% saturated picric acid, 9% formaldehyde, 5% glacial acetic acid) for histological examination by light microscopy. Tissue samples for transmission electron microscopy (TEM) were placed in chilled half-strength Karnovsky's fixative (1.5% gluteraldehyde, 0.8% paraformaldehyde in 0.1 M phosphate buffer [pH 7.3]). Samples for TEM were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at room temperature, dehydrated in acetone, and embedded in plastic. Ultrathin sections were cut and mounted on carbon-coated grids. Grids were stained with freshly prepared 2% uranyl acetate in 50% methanol and with lead citrate. Sections were examined on a Hitachi 500 transmission electron microscope at 75 kV.

For Western immunoblot analyses, serpulinal cultures were diluted to an optical density of 1.0 at 595 nm and 1 ml of each culture was used to prepare lysates. An *S. hyodysenteriae* B204 culture was included in this series of experiments. Bacterial lysates were prepared by the addition of 50 µl of lysis buffer to bacterial pellets (4). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using 12% separating gels with 4% stacking gels. Ten microliters of each sample was added per well. Samples were electrophoresed at 75 V for approximately 2 h and transferred to polyvinylidene difluoride membranes (Micron Separations, Inc., Westboro, Mass.) at 60 V for 2 h. Membranes were incubated with pooled serum samples (diluted 1:100) from mice infected with avian isolate 42167 or human isolate *S. jonesii* overnight at 4°C. Membranes were incubated with a 1:500 dilution of goat anti-mouse immunoglobulin G (heavy plus light chains) conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) for 1.5 h at 37°C. The substrate/chromogen was napthol-fast red (Sigma Chemical Co., St. Louis, Mo.), and development was for 15 min.

Results of bacteriological cultures from mice at 4 to 20 days following infection with avian, porcine, or human spirochete isolates showed weakly β-hemolytic growth on five-way antibiotic-containing blood agar plates after 48 to 96 h of incubation (all 76 infected mice were culture positive). Moreover, culture-positive results were obtained for mice infected for more than 30 days with the avian, porcine, or human isolates (all 21 infected mice were culture positive). Spirochetes reisolated from mice were weakly hemolytic or nonhemolytic and indole negative, as these spirochetes were prior to infection.

Ceca of mice were examined visually for gross lesions. Excess intraluminal mucus was periodically observed in the ceca of infected mice and could be seen as early as day 4 postinfection. However, cecal atophy was not seen in either C3H/HeOuJ (*n* = 45) or C3H/HeJ (*n* = 52) mice infected with the
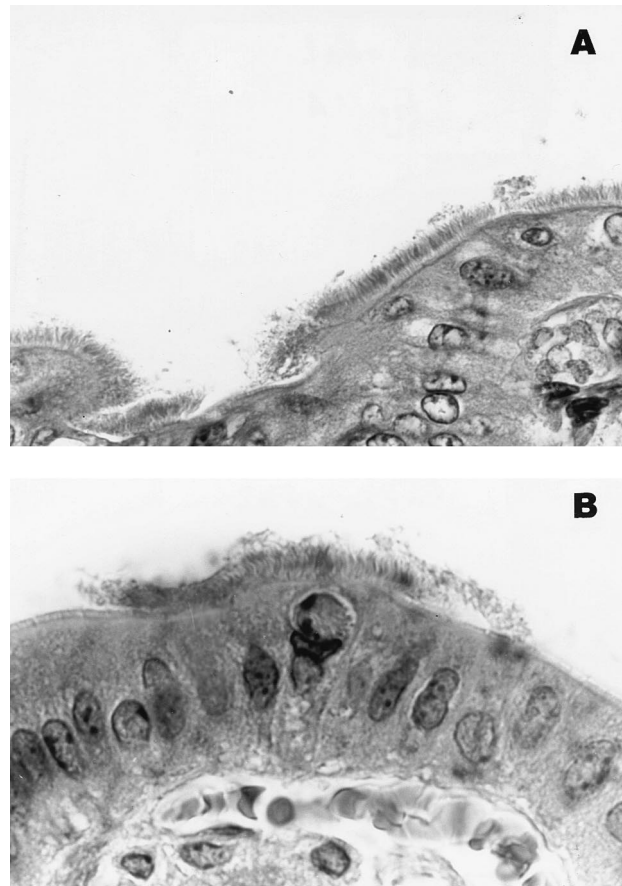

FIG. 1. Photomicrographs of ceca from mice experimentally infected with *S. pilosicoli*. (A) Cecum from a C3H/HeJ mouse infected with chicken isolate 42167. A dense, uniform population of spirochetes can be observed attached to the mucosal surface. Stain, hematoxylin and eosin; original magnification, ×61. (B) Cecum from a C3H/HeOuJ mouse infected with porcine isolate P43/6/78. Note focal attachment of spirochetes to the apical surface of enterocytes. Stain, hematoxylin and eosin; original magnification, ×154.

isolates of *S. pilosicoli* described in the present study, nor were differences in severity of lesions observed between mouse strains. In contrast, cecal atophy is consistently observed in C3H mice on day 3 following infection with *S. hyodysenteriae* B204 (9).

Upon light microscopic examination of histological sections of ceca from mice infected with the avian isolate, a dense, uniform layer of spirochetes was observed attached end-on to the apical surface of cecal enterocytes (Fig. 1A). In contrast, the porcine isolate was observed to exhibit a focal attachment to cecal enterocytes (Fig. 1B). Spirochetes were readily observed when stained with hematoxylin and eosin, although the Warthin-Starry silver stain was more sensitive. Spirochetes were observed to be aligned parallel to each other and perpendicular to the epithelial surface. However, as previously reported for *S. pilosicoli* (8, 14, 15), spirochetal attachment was not observed to extend into the cecal crypts. Furthermore, in mice infected with the *S. pilosicoli* isolates examined in the present studies, cecal tissues generally exhibited little or no edema and there was no evidence of mucosal inflammation. End-on attachment of the human isolate *S. jonesii* was not observed in either strain of mice in the present studies, although weakly hemolytic spirochetes were reisolated through-
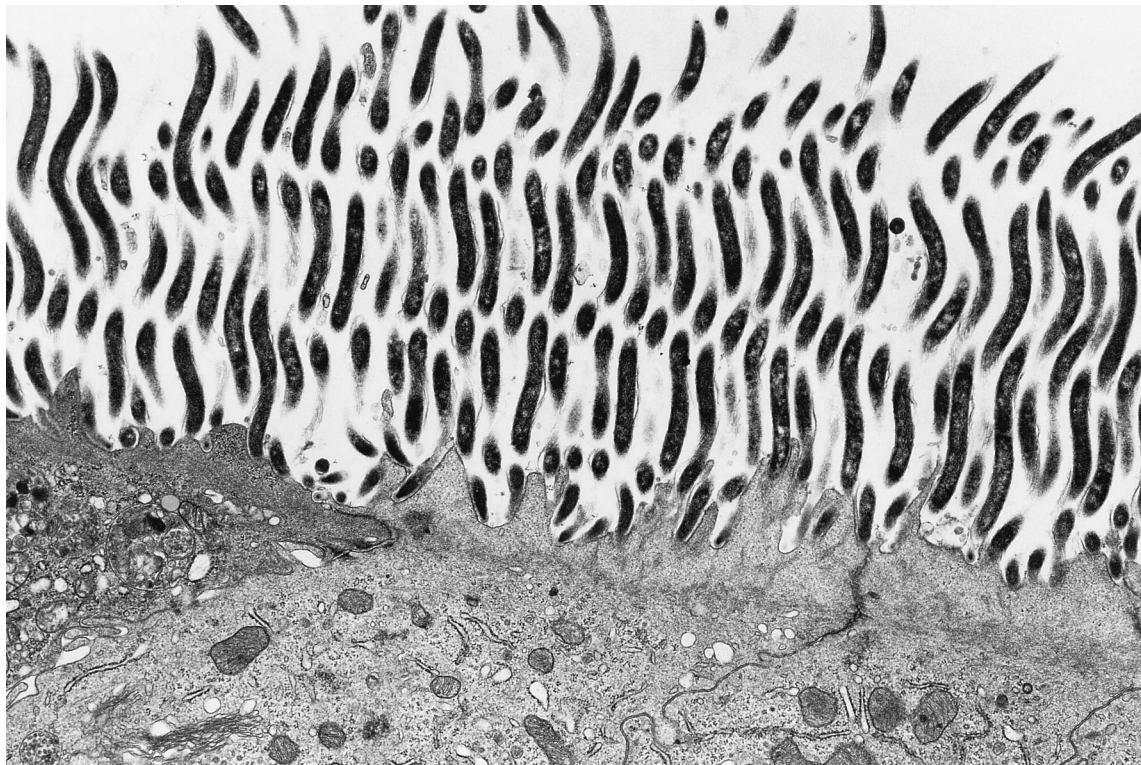
FIG. 2. Transmission electron micrograph of cecal tissue from a mouse infected with chicken isolate 42167. Spirochetes are invaginated into the cellular membrane of the host enterocyte. Stain, uranyl acetate and lead citrate; original magnification, ×13,983.

out the test period. No differences in microscopic lesions were noted between C3H/HeJ and C3H/HeOuJ mouse strains.

As can be observed in the transmission electron micrograph of a thin section of cecal tissue of a mouse infected with avian isolate 42167 (Fig. 2), spirochetes are invaginated into the cellular membrane of the host enterocytes. In such cases where numerous spirochetes are attached, normal brush border microvilli are displaced by the spirochetes and the glycocalyx is absent. We observed an increased vacuolization of the apical cytoplasm of the host cells and mitochondrial swelling but did not observe a lateral separation between columnar epithelial cells, as has been described following colonization of the ceca of specific-pathogen-free chicks with the WesB isolate of *S. pilosicoli* (15). No spirochetes were observed intracellularly within epithelial cells. As previously observed for cecal enterocytes in the chick model (8), spirochetal attachment resulted in a cap-like structure over the terminal web of infected cells, as shown in Fig. 3. Note that the micovilli of an adjacent host enterocyte without spirochetal attachment appear intact but are blunted in comparison to microvilli of control mice.

Higher-magnification TEM shows invaginations into the terminal web cytoplasm of the epithelial cells at the sites of spirochetal attachment (Fig. 4). The spirochetes are readily characterized by the presence of periplasmic flagella. Electron-lucent pits can be seen between host cell membranes and the spirochetes, with bristle-like structures at the tip of the spirochetes. Moreover, a thin zone of electron-dense material beneath the cell membrane was visible at the point of contact between the cell and the spirochete.

Figure 5 presents results of Western immunoblot analyses of bacterial lysates of *S. hyodysenteriae* (lane 1), *S. pilosicoli* isolates 42167 (lane 2) and P43/6/78 (lane 3), and *S. jonesii* (lane 4) developed with pooled antisera of mice infected with either avian isolate 42167 (Fig. 5A) or human isolate *S. jonesii* (Fig. 5B). Results indicate that infected mice developed serum antibody responses to *S. pilosicoli*. However, there was little or no reactivity to *S. hyodysenteriae* lysates. Sera of mice infected with 42167 recognized a 16- to 24-kDa band from 42167 lysates which is similar in size to the lipopolysaccharide band typically recognized for *S. hyodysenteriae* (Fig. 5A). Similar results were obtained for *S. jonesii* lysates probed with antisera from *S. jonesii*-infected mice (Fig. 5B).

In the present study, avian and porcine isolates of *S. pilosicoli* were observed attached end-on to the mucosal surface of cecal enterocytes. At the time of necropsy, excess intraluminal mucus in the ceca of infected mice was the only gross lesion observed. Histological examination of cecal tissue showed a layer of spirochetes attached end-on to the apical surface of cecal enterocytes. TEM showed spirochetes invaginated into the host cell membrane concurrent with effacement of microvilli and absence of the glycocalyx. The light and electron microscopic observations for mice were similar to those for the 1-day-old chick model. There were no apparent differences in severity of cecal lesions between the lipopolysaccharide-responsive (C3H/HeOuJ) and -hyporesponsive (C3H/HeJ) mouse strains infected with the isolates described in the present studies. This result suggests that spirochetal lipopolysaccharide may not be a critical factor in the pathogenesis of intestinal spirochetosis. Moreover, end-on attachment of the human isolate *S. jonesii* was not observed in either strain of mice in the present studies even at 30 days postinfection. Nevertheless, weakly hemolytic spirochetes were reisolated from these mice and serum antibody responses to *S. jonesii* lysates were detected.
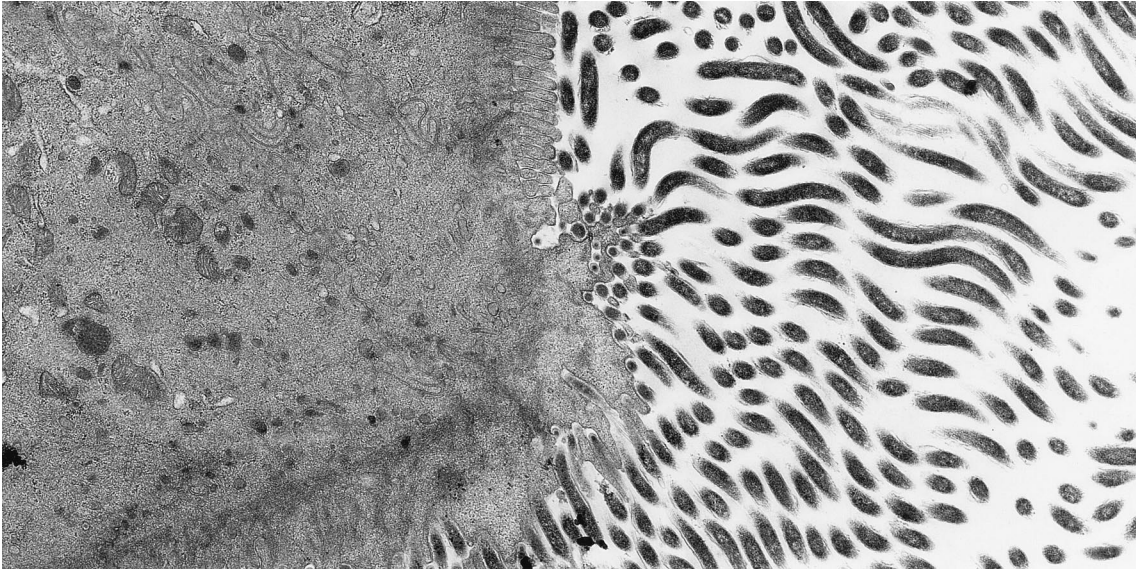
FIG. 3. Transmission electron micrograph of cecal tissue from a mouse infected with chicken isolate 42167. The microvilli of a host enterocyte without spirochetal attachment appear intact but are blunted in comparison to microvilli of enterocytes in noninfected mice. Stain, uranyl acetate and lead citrate; original magnification, ×9,654.

The observations that the porcine isolate exhibited focal attachment and the human isolate did not exhibit end-on attachment may be due to their long-term passage in vitro or to strain variation. The avian isolate utilized in the present experiments which produced dense end-on attachment was re-

cently isolated and passaged short-term in vitro prior to infection of mice. Thus, further experimental examination of additional avian, porcine, and human isolates of *S. pilosicoli* is warranted to determine whether isolates differ in their ability to colonize mice and induce intestinal lesions. However, the
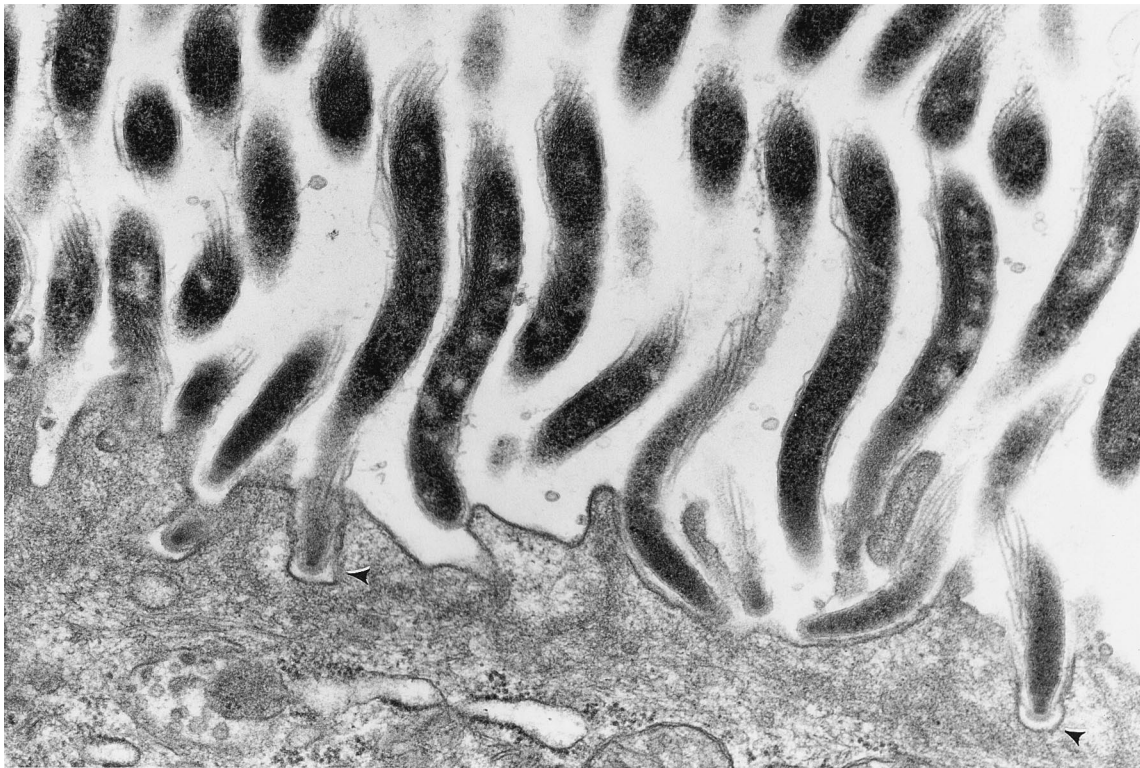

FIG. 4. Transmission electron micrograph of cecal tissue from a mouse infected with chicken isolate 42167. An electron-lucent pit can be seen between the spirochete and the host cell membrane (arrowheads). In addition, note the thin zone of electron-dense material beneath the host cell membrane visible at the point of contact between the host cell and the spirochete. Stain, uranyl acetate and lead citrate; original magnification, ×41,483.
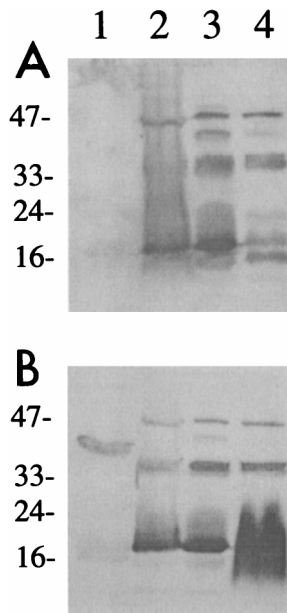
FIG. 5. Western immunoblots of sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated serpulinal lysates probed with pooled antisera from mice infected with avian isolate 42167 (day 45 postinfection) (A) or with human isolate *S. jonesii* (day 30 postinfection) (B). Lane 1, *S. hyodysenteriae* B204; lane 2, avian isolate 42167; lane 3, porcine isolate P43/6/78; lane 4, human isolate *S. jonesii.*

present studies indicate that mice can be infected with *S. pilosicoli* and may represent a potential reservoir for intestinal spirochetosis.

Editor: P. E. Orndorff

REFERENCES

1. **Barrett, S. P.** 1990. Intestinal spirochaetes in a Gulf Arab population. Epidemiol. Infect. **104:**261–266.
2. **Douglas, J. G., and V. Crucioli.** 1981. Spirochaetosis: a remediable cause of diarrhoea and rectal bleeding? Br. Med. J. **283:**1362–1363.
3. **Harland, W. A., and F. D. Lee.** 1967. Intestinal spirochaetosis. Br. Med. J. **3:**718–719.
4. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
4a. **Jensen, N. S.** Personal communication.
5. **Jones, M. J., J. N. Miller, and W. L. George.** 1986. Microbiological and biochemical characterization of spirochetes isolated from feces of homosexual males. J. Clin. Microbiol. **24:**1071–1074.
6. **Lee, F. D., A. Kraszewski, J. Gordon, and J. G. R. Howie.** 1971. Intestinal spirochaetosis. Gut **12:**126–133.
7. **Lee, J. I., and D. J. Hampson.** 1992. Intestinal spirochaetes colonising Aborigines in the remote North of Western Australia. Epidemiol. Infect. **109:**133–141.
8. **Muniappa, N., G. E. Duhamel, M. R. Mathiesen, and T. W. Bargar.** 1996. Light microscopic and ultrastructural changes in the ceca of chicks inoculated with human and canine *Serpulina pilosicoli.* Vet. Pathol. **33:**542–550.
9. **Nibbelink, S. K., and M. J. Wannemuehler.** 1992. An enhanced murine model for studies of *Serpulina* (*Treponema*) *hyodysenteriae* pathogenesis. Infect. Immun. **60:**3433–3436.
10. **Park, N. Y., C. Y. Chung, A. J. McLaren, R. F. Atyeo, and D. J. Hampson.** 1995. Polymerase chain reaction for identification of human and porcine spirochaetes recovered from cases of intestinal spirochaetosis. FEMS Microbiol. Lett. **125:**225–230.
11. **Takeuchi, A., H. R. Jervis, H. Nakazawa, and D. M. Robinson.** 1974. Spiral-shaped organisms on the surface colonic epithelium of the monkey and man. Am. J. Clin. Nutr. **27:**1287–1296.
12. **Taylor, D. J., J. R. Simmons, and H. M. Laird.** 1980. Production of diarrhoea and dysentery in pigs by feeding pure cultures of a spirochaete differing from *Treponema hyodysenteriae.* Vet. Rec. **106:**326–332.
13. **Tompkins, D. S., M. A. Waugh, and E. M. Cooke.** 1981. Isolation of intestinal spirochaetes from homosexuals. J. Clin. Pathol. **39:**535–541.
14. **Trampel, D. W., N. S. Jensen, and L. J. Hoffman.** 1994. Cecal spirochetosis in commercial laying hens. Avian Dis. **38:**895–898.
15. **Trott, D. J., A. J. McLaren, and D. J. Hampson.** 1995. Pathogenicity of human and porcine intestinal spirochetes in one-day-old specific-pathogen-free chicks: an animal model of intestinal spirochetosis. Infect. Immun. **63:**3705–3710.