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Pathogenicity of Human and Porcine Intestinal Spirochetes in One-Day-Old Specific-Pathogen-Free Chicks: an Animal Model of Intestinal Spirochetosis

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One-day-old chicks were infected orally with two strains of weakly hemolytic spirochetes isolated from a human and a pig with intestinal spirochetosis. These spirochetes both colonized birds, attached end-on to their cecal enterocytes, induced watery diarrhea, and significantly depressed growth rates. Cultures of *Serpulina innocens* failed to colonize the chicks.

In 1971, a strongly hemolytic intestinal spirochete called *Treponema* (now *Serpulina*) *hyodysenteriae* was shown to be the etiologic agent of swine dysentery, a severe mucohemorrhagic colitis of pigs (13, 32, 37). Weakly hemolytic spirochetes, collectively termed *Treponema* (*Serpulina*) *innocens*, also were found to colonize the intestinal tract of pigs, but these were thought to be nonpathogenic (18, 32). In 1980, Taylor and coworkers isolated a weakly hemolytic spirochete (P43/6/78) from a pig with diarrhea and subsequently used pure cultures of the organism to reproduce mucoid diarrhea in experimental pigs (38). This condition was termed porcine intestinal spirochetosis or spirochetal diarrhea and was characterized by end-on attachment of spirochetes to the colonic epithelium. Subsequently, other researchers have shown an association between colonization of the porcine intestinal tract with certain weakly hemolytic spirochetes and the occurrence of diarrhea (1, 6, 14, 27, 30).

By using multilocus enzyme electrophoresis (MEE), the weakly hemolytic porcine intestinal spirochetes, previously known as *Serpulina innocens*, now have been differentiated into at least four distinct genetic groups. The name "*Anguillina coli*" was suggested as a new genus and species name for those isolates which were genetically and phenotypically similar to P43/6/78 and to other isolates recovered from cases of porcine intestinal spirochetosis (23). More recent analysis of 16S ribosomal DNA (genes encoding 16S rRNA) sequence data obtained for these pathogenic organisms suggests that they probably belong to a separate species in the genus *Serpulina* (9, 33).

Spirochetes also colonize the intestinal tract of humans, and they are commonly cultured from members of a number of indigenous groups, including Gulf Arabs (2), Africans (25), Indians of the subcontinent (26), and Australian Aborigines (20). By contrast, infection rarely occurs among westernized populations and is confined mainly to AIDS patients and homosexual males (16, 17, 40). Where large numbers of spirochetes are found attached end-on to the colonic epithelium, the condition again is referred to as intestinal spirochetosis and has been associated with a number of gastrointestinal disturbances including chronic diarrhea and rectal bleeding (3–5, 10–12, 16, 20, 29). On the basis of the results of DNA-DNA hybridization and MEE, isolates from these patients have been shown to belong to the same genetic group of spirochetes as those responsible for porcine intestinal spirochetosis (21, 24). This finding supports the contention that they may act as enteric pathogens in humans. Recently, human intestinal spirochetes that were closely related to the porcine pathogenic strain P43/6/78 were used to infect day-old chicks (8). Whilst no clinical signs developed during the 22-day test period, some strains were shown to attach to the cecal wall and to produce focal erosions. This experiment highlighted the usefulness of this animal model in the study of intestinal spirochetosis. Day-old chicks previously have been used to demonstrate the pathogenicity of both the porcine pathogen *Serpulina hyodysenteriae* (34) and a number of uncharacterized spirochetes associated with wet litter and dirty eggshells in commercial poultry (7, 36).

The purpose of the present study was to use the day-old specific-pathogen-free (SPF) chick model to examine the pathogenicity of weakly hemolytic spirochetes that had previously been isolated from cases of intestinal spirochetosis in humans and pigs. The strains used to infect chicks were WesB, which is a well-characterized weakly hemolytic spirochete recovered from an Australian Aboriginal child with chronic diarrhea (20, 24); weakly hemolytic strain 1648, isolated from an 8-week-old Australian pig with intestinal spirochetosis; weakly hemolytic *S. innocens* 155-5, which was recovered from a normal pig; and *S. hyodysenteriae* WA15 (22). The two weakly hemolytic non-*S. hyodysenteriae* strains that were recovered from individuals with diarrhea were confirmed to belong to the same genetic group as P43/6/78 and other isolates with four to six periplasmic flagella on the basis of their electrophoretic type in MEE (24), their reactivity in a PCR assay that is specific for this group of spirochetes (28), and their characteristic morphology and number of periplasmic flagella. Strain 1648 also previously has been used to induce watery mucoid diarrhea, without blood, in newly weaned pigs fed a highly fermentable diet (41). Isolate 155-5 was confirmed as belonging to the same genetic group as *S. innocens* B256 and 4/71 on the basis of its biochemical profile in the API-ZYM system and its electrophoretic type in MEE (23).

Each strain was propagated in prereduced anaerobic medium consisting of Trypticase soy broth supplemented with 2% fetal bovine serum and 1% ethanolic cholesterol solution (19) and was incubated at 37°C on a rocking platform until early-

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TABLE 1. Body weight and isolation of spirochetes from cloacal swabs taken from experimentally infected day-old SPF chicks

Group	Spirochetal strain[a]	No.	Mean wt (± SD) at days p.i.[b]		No. (%) of chicks infected with spirochetes at days p.i.			
			0	21	0	7	14	21
1A	Control 1	17	39.7 ± 2.7	139.1 ± 22.2	0 (0)	0 (0)	0 (0)	0 (0)
1B	WesB	16	40.1 ± 2.9	118.5 ± 22.5	0 (0)	0 (0)	10 (62)	16 (100)
1C	1648	16	40.0 ± 2.5	123.9 ± 19.3	0 (0)	8 (50)	13 (81)	16 (100)
1D	WA15	14	40.4 ± 2.9	120.0 ± 18.2	0 (0)	0 (0)	11 (79)	14 (100)
2A[c]	Control 2	10	43.2 ± 3.4	160.7 ± 20.3	0 (0)	0 (0)	0 (0)	0 (0)
2B	155-5	10	41.5 ± 3.0	155.1 ± 16.3	0 (0)	0 (0)	0 (0)	0 (0)

[a] Control 1, chicks sham inoculated with broth; WesB, chicks inoculated with intestinal spirochete WesB from a human with diarrhea; 1648, chicks inoculated with intestinal spirochete 1648 from a pig with diarrhea; WA15, chicks inoculated with *Serpulina hyodysenteriae* WA15; Control 2, chicks sham inoculated with broth; 155-5, chicks inoculated with *Serpulina innocens* 155-5.

[b] In one-way analysis of variance, weights of chicks in groups 2 to 4 at 21 days p.i. were significantly less ($P < 0.05$) than those of sham-inoculated control chicks in group 1 at 21 days p.i.

[c] Group 2 experiments were conducted separately from group 1 experiments.

log-phase growth was achieved. Samples were examined for contamination daily with a phase-contrast microscope.

In the first experiment, 65 SPF day-old Commonwealth Scientific and Industrial Research Organisation hybrid White Leghorn chicks were obtained from the Western Australian Animal Resource Centre. The chicks were weighed and then divided randomly into three groups of 17 birds (groups 1A, 1B, and 1C) and one group of 14 birds (1D). Birds in group 1A acted as uninoculated controls, those in group 1B were dosed orally with strain WesB, those in group 1C were dosed with strain 1648, and those in group 1D were dosed with *S. hyodysenteriae* WA15. In the second experiment, 20 SPF day-old chicks were divided into two groups of 10 birds (groups 2A and 2B). Birds in group 2A acted as uninoculated controls, and birds in group 2B were dosed with *S. innocens* 155.5. A total inoculum of 10^8 spirochetes in early-log-phase culture was given by crop tube to each chick in groups 1B, 1C, 1D, and 2B, three times over 24 h. The control groups were treated simultaneously with an equivalent volume of sterile culture medium. All chicks were housed in sterile Zeitz-filtered boxes and fed sterile chick mash and water ad libitum for the duration of the experiment. Cloacal swabs were taken weekly and cultured for the presence of spirochetes. The chicks were inspected daily, and the presence of wet litter was noted. A final weight was obtained 3 weeks after inoculation.

Twenty-one days postinfection (p.i.), prior to postmortem examination, chicks were anesthetized with 0.1 mg of diazepam administered intramuscularly, followed 10 min later by a bolus injection of 20 mg of xylazine kg^{-1} and 100 mg of ketamine kg^{-1} intramuscularly (35). Samples for histology and scanning and transmission electron microscopy were removed within 30 s from proximal, middle, and distal sections of one cecum. An intestinal wall scraping was taken from the second cecum for culturing. The chicks then were killed by cardiac puncture.

Tissue for histological examination was placed in Bouins fixative for 4 h before being washed three times in 50% ethanol and transferred into 70% ethanol. Sections (4 μm) were made from paraffin-embedded tissue and were stained with hematoxylin and eosin and with Warthin-Starry silver stain. Tissue samples for scanning and transmission electron microscopy were placed in chilled half-strength Karnovsky's fixative (1.5% gluteraldehyde–0.8% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3) for 24 h and then postfixed in 1% aqueous osmium tetroxide for 1 h at 4°C. After fixation, the samples for transmission electron microscopy were dehydrated in an ethanol series and then in propylene oxide and were embedded in Epon 812 (Taab Laboratories, Reading, England). Ultrathin sections were cut on a Reichert Ultracut E ultramicrotome and mounted on carbon-coated grids. Grids were stained with freshly prepared uranyl acetate and lead citrate and examined under a Philips 301 transmission electron microscope at 80 kV. Sections for scanning electron microscopy were dehydrated in ethanol and amyl acetate, critical point dried in a Balzers Union critical point dryer with carbon dioxide as the exchange medium, and mounted on stubs. Stubs were sputter coated with gold to a thickness of 90 nm in a Balzers sputter coater and viewed under a Philips XL 20 scanning electron microscope.

Cloacal swabs taken weekly and cecal swabs taken at postmortem were directly inoculated onto Trypticase soy agar (BBL) plates supplemented with 5% defibrinated bovine blood, 400 μg of spectinomycin ml^{-1}, and 25 μg each of vancomycin and colistin ml^{-1}. After streaking out, the plates were incubated at 37°C in anaerobic jars in an atmosphere of 94% N_2 and 6% CO_2 for 5 days and then examined for the presence of spirochetes. Identification of spirochetes was based on morphological appearance under a phase-contrast microscope. In addition, single isolates obtained at postmortem from chicks infected either with WesB, 1648, or WA15 were typed by MEE, as previously described (23).

The one-tailed *t* test was used to determine the significance of differences among groups 1A to 1D and between 2A and 2B at day 1 and again at day 21 of the experiment. One of the chicks in group 1C (1648) died during the first week, and a single chick in group 1B (WesB) was culture negative for the duration of the experiment, so data from both these chicks were not included in the statistical analysis.

The mean and standard deviations of body weights at days 1 and 21 of the experiment and weekly swab results of each treatment group are shown in Table 1. No significant differences in weight were recorded between any treatment groups at day 1 of the experiment, but at day 21, the three groups infected with spirochetes in experiment 1 were significantly lighter than the uninfected birds in group 1A. No significant difference was demonstrated between groups 2A and 2B. Differences in weights among the three infected groups in which weight gain was depressed were not significant. The greatest difference in weight from the uninfected group 1A was demonstrated for chicks infected with WesB ($t = 2.649$; $P = 0.013$), followed by chicks infected with WA15 ($t = 2.577$; $P = 0.015$) and 1648 ($t = 2.096$; $P = 0.044$).

At 7 days p.i., only those chicks infected with 1648 had begun to shed spirochetes (eight chicks, 50%). By 14 days p.i., the number of positive swabs had increased to 11 (79%) for chicks infected with WesB (group 1B), to 13 (81%) for those infected with 1648 (group 1C), and to 10 (62%) for those infected with

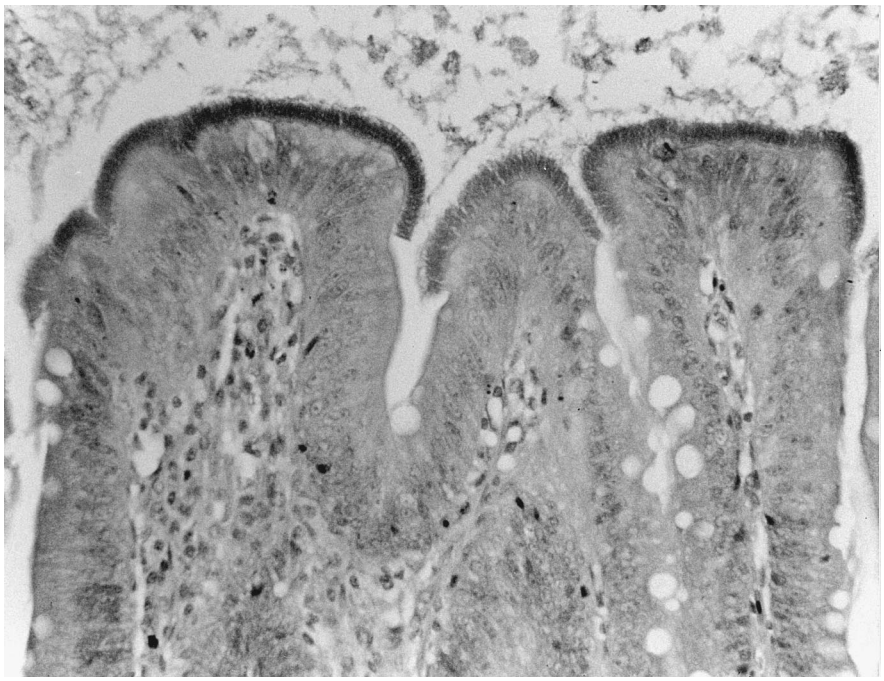

FIG. 1. Hematoxylin-eosin-stained section of cecal tissue from an SPF chick experimentally infected with human intestinal spirochete WesB. The spirochetes appear on the villous tips as a dark fringe covering vacuolated enterocytes at the mucosal surface. No inflammatory reaction is evident (magnification, ×400).

WA15 (group 1D). At postmortem examination, spirochetes were isolated from cecal swabs taken from all birds infected with either WesB, 1648, or WA15. Spirochetes were not cultured from any chicks inoculated with 155-5 (group 2B) or in either of the control groups throughout the trial period.

After day 7 p.i., the droppings of birds infected with *S. hyodysenteriae* WA15 became pasty and mucoid. The droppings of chicks infected with WesB and 1648 initially were indistinguishable from those of the uninfected chicks; however, after day 14 p.i. they became progressively more watery, and wet litter was noted in the isolator boxes. Cecal and normal droppings of some birds were poorly formed and contained sloppy fecal material and urates surrounded by a clear gelatinous fluid. This was not observed in birds infected with 155-5 or in either of the control groups.

At postmortem examination, the ceca of chicks infected with WesB, 1648, and 155-5 were indistinguishable from the ceca of uninfected chicks in size, shape, and surface appearance, but the contents of those infected with WesB and 1648 were more fluid. The ceca of chicks infected with *S. hyodysenteriae* were much smaller than those of uninfected chicks and contained very little contents. The luminal surface appeared thickened, and some portions of the ceca were filled with mucus.

Isolates recovered from swabs taken from chicks infected with WesB and 1648 were weakly hemolytic, whereas those obtained from chicks infected with WA15 were strongly hemolytic. At postmortem examination, in each case the isolates obtained from the ceca of infected chicks had the same allelic profile in MEE as the original isolate that was used to infect the chick.

On histological examination, no differences were apparent in the thickness of the mucosa, the number of inflammatory cells present in the lamina propria, or the number of active goblet cells at the mucosal barrier in sections from uninfected chicks compared with sections from the chicks inoculated with 155-5 or infected with WesB or 1648. Necrotic cells were occasionally present in the lamina propria of infected birds in the latter two groups. A total of 5 (29.4%) of the sections from chicks infected with WesB and 11 (68.7%) of the sections from chicks infected with 1648 were characterized by the presence of a dense carpet of spirochetes attached end-on to the epithelial surface in proximal, middle, and distal cecal sections (Fig. 1). Colonization of the epithelial surface was greater in sections from chicks infected with WesB than in those from chicks infected with 1648. Vacuolation and protein deposition were a common feature in the apical cytoplasm of luminal enterocytes colonized by WesB but were seen less frequently in tissue sections from ceca infected with 1648. Spirochetal attachment extended throughout the section but was limited to the villous tips of the luminal epithelium and did not extend into the intestinal crypts. There was no invasion of spirochetes beyond the enterocyte brush border. Compared with control sections, tissue sections from ceca infected with *S. hyodysenteriae* exhibited thickening in the mucosal layer and a large increase in the number of goblet cells. Many spirochetes were present in the intestinal crypts, and occasional penetration of the mucosal barrier was apparent. No spirochetes or histological abnormalities were detected in birds infected with strain 155-5.

Scanning electron micrographs of mounted sections from chicks infected with WesB and 1648 demonstrated extensive colonization of the epithelium by spirochetes. The mucosal surface was completely obscured, with the majority of spirochetes aligned longitudinally in parallel sequence. Detached spirochetes were seen lying perpendicular to the underlying cluster. In comparison, dense mats of spirochetes combined with debris and mucin were seen deep within intestinal crypts in sections from chicks infected with *S. hyodysenteriae*. Individual spirochetes were attached to the luminal surface, and blood cells were occasionally present. Again, no spirochetes were observed in chicks inoculated with 155-5.

Under the transmission electron microscope, sections from chicks infected with 1648 and WesB showed the bacteria at-

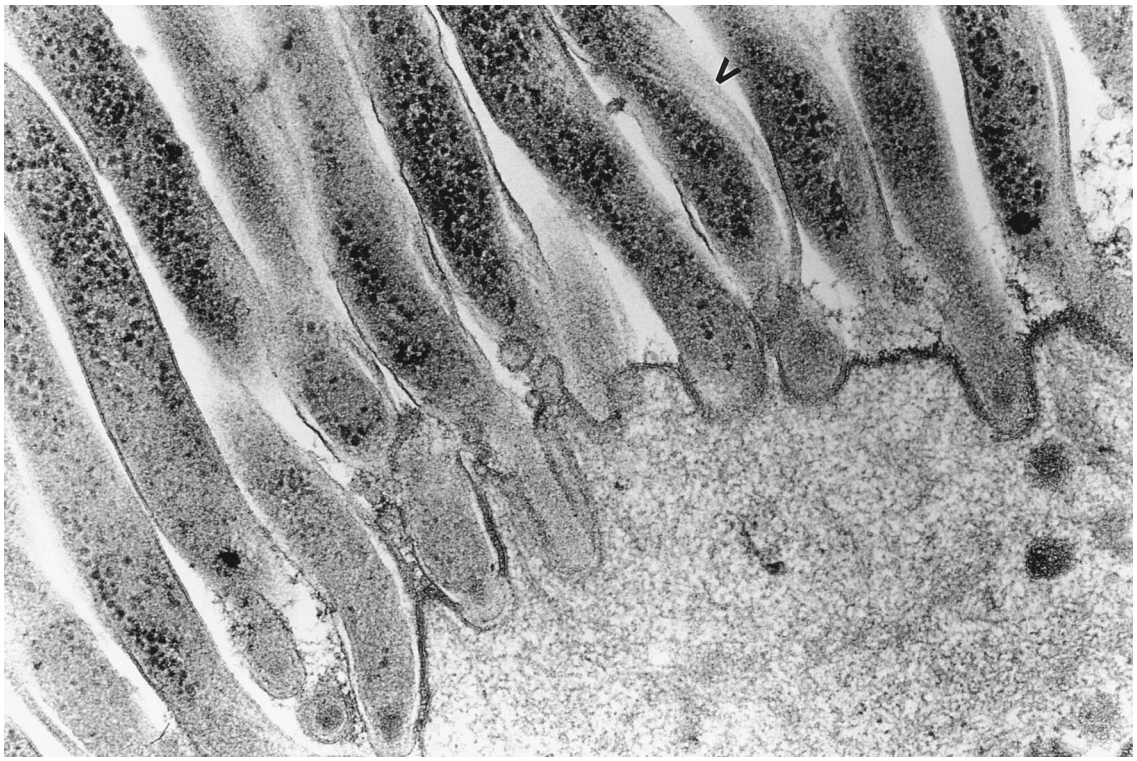

FIG. 2. High-power transmission electron micrograph of cecal tissue from a chick infected with porcine intestinal spirochete 1648. Spirochetes, characterized by the presence of periplasmic flagella (arrowhead), are invaginated into the host cell membrane (magnification, ×41,917).

tached directly to the columnar epithelial cells of the villous tips, located between adjacent microvilli. Where heavy colonization occurred, the microvilli were completely obscured, damaged, or obliterated by large numbers of bacteria. At higher power, individual spirochetes were seen to be invaginated into the cellular membrane of the host, which was indented into the terminal web cytoplasm (Fig. 2). No direct penetration into the cytoplasm was evident; however, the spirochetes were intimately associated with the host cell plasmalemma. Compared with sections from uninfected tissue, sections infected with WesB were characterized by large numbers of vacuoles in the apical cytoplasm and by lateral separation between adjacent columnar epithelial cells (Fig. 3). Changes were less noticeable in the cytoplasm of cells colonized by strain 1648.

This study clearly demonstrated that weakly hemolytic spirochetes isolated from pigs and humans with diarrhea can colonize and induce disease in young chicks. In contrast, a strain of the nonpathogenic spirochetal species *S. innocens*, recovered from a healthy pig, failed to colonize and cause disease. The experimental condition produced by WesB and 1648 closely resembled the findings in natural cases of intestinal spirochetosis in humans and pigs. The clinical signs and pathological changes induced were more severe in the chicks infected with the human isolate WesB than in those infected with the porcine isolate 1648, but chicks in both test groups developed an identical syndrome of watery diarrhea and significant reduction in growth rate. The same type of attachment to enterocytes in the large intestine that previously has been recorded in both human and porcine intestinal spirochetosis was reproduced. A striking feature was the extent of spirochetal attachment to enterocytes over the villous tips, with their complete absence from cells in the intestinal crypts. The reason for this distribution was unclear, but it could have been related either to factors in the local intestinal environment or to the expression and availability of cell surface receptors for the spirochetes by more mature apical enterocytes. Spirochetal attachment to some of these cells appeared to cause significant intracellular changes. The microvilli of infected enterocytes were distorted, and they could not be seen in areas where heavy colonization had occurred. Cytoplasmic vacuolation in the apical portion of some cells also was evident. The lateral separation of colonized epithelial cells, which previously has not been reported in descriptions of intestinal spirochetosis, may have arisen from the activity of the spirochetes. Similar changes have been observed in chicks infected with *S. hyodysenteriae* (34).

The mechanism by which diarrhea was induced in the chicks was not studied, but as suggested by Spitz et al. (31), the physical presence of large numbers of bacteria at the level of the brush border and covering the entire surface of the ceca may have altered absorption or affected paracellular pathways. Either a physical malabsorption-type syndrome or a secretory mechanism induced by the spirochetes in the cecum and/or colon could result in the production of diarrhea. Generally, this is the major clinical sign seen in intestinal spirochetosis of both humans and pigs.

In the only other study in which intestinal spirochetes from humans were inoculated into day-old chicks, diarrhea or weight depression did not occur during the 22-day trial period, despite the fact that attachment and focal epithelial erosion were apparent in some chicks at postmortem examination (8). The three human isolates that were used in the experiment were from Europeans and were located in the same genetic group as P43/6/78 (21). Although the current experiment was conducted over a similar time period (21 days), in this case infected chicks developed watery diarrhea and significant

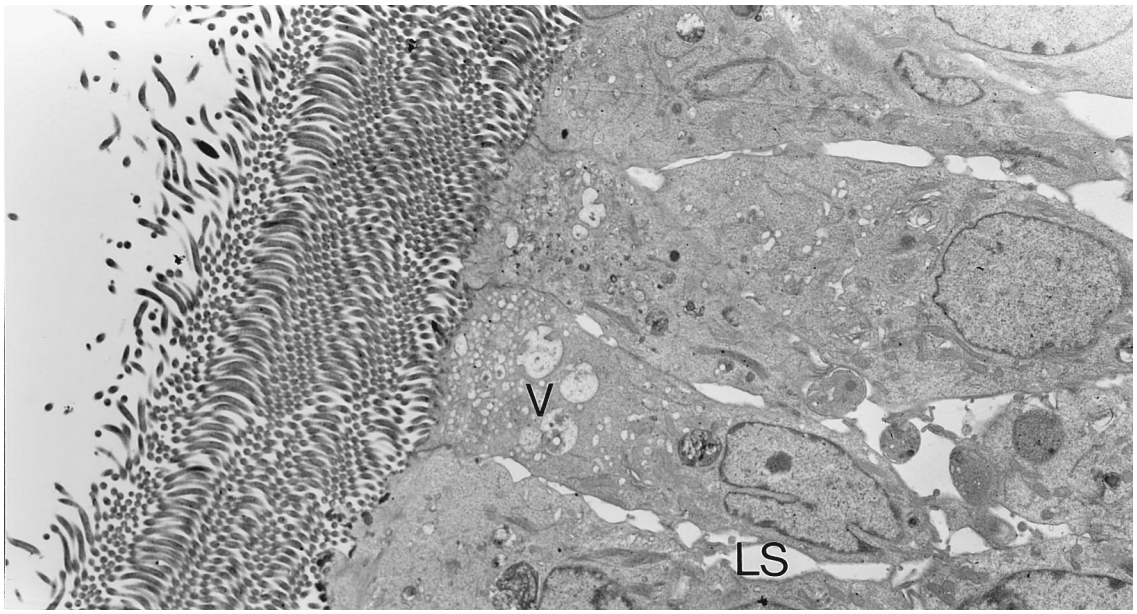

FIG. 3. Transmission electron micrograph of the cecum from a chick experimentally infected with human intestinal spirochete WesB. Large numbers of spirochetes are attached to individual enterocytes. Vacuolation (V) is present in the apical cytoplasm, and lateral separation (LS) is evident between adjacent columnar cells (magnification, ×3,679).

weight depression. Besides strain variation, other factors which may have influenced the different outcomes in the two experiments include the number of previous subcultures to which each spirochete was subjected and the type of medium in which the spirochetes were propagated. WesB and 1648 were subcultured fewer than five times on solid medium, and the chicks were inoculated with cells grown in broth culture. In the previous work, the isolates were subcultured 10 times on solid medium and the chicks were inoculated with spirochetes harvested from plates and suspended in 0.9% saline and egg yolk. Extended subculturing may reduce the virulence of pathogenic intestinal spirochetes, as has occurred for B78, the type strain of *S. hyodysenteriae* (15).

This study has demonstrated that two genetically similar intestinal spirochetes derived from a human and a pig have pathogenicity in young chicks and produce a syndrome that closely resembles natural cases of intestinal spirochetosis. The young chick is a useful model for the study of intestinal spirochetosis and could be utilized in pathophysiological studies to demonstrate the presence and nature of any functional impairment in large intestinal epithelium that is colonized by intestinal spirochetes.

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