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Experimental *Campylobacter* Diarrhea in Chickens

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An animal model for *Campylobacter fetus* subsp. *jejuni* enteritis was developed in 3-day-old chickens. Diarrhea was induced in 88% (22 of 25) of chickens inoculated with 9×10^7 bacteria given orally. The mean incubation time was 45 h (range, 24 to 72 h). Considerable weight loss was observed in the experimental group compared with the control group. Ninety bacteria was the minimal infective dose capable of inducing diarrhea in 90% of the chickens. Overall mortality was 32% (8 of 25). Light microscopy, immunofluorescence, and electron microscopy of the gastrointestinal tract of serially sacrificed chickens were performed in control and experimental groups. A moderate infiltration of mononuclear cells was observed in ileum and cecum in the experimental group, with no disruption of intestinal mucosa. By immunofluorescence and electron microscopy, campylobacter was located within the epithelial cells and phagocytosed to a greater degree by mononuclear cells of the lamina propria.

Campylobacter fetus subsp. *jejuni* has been recognized as a common agent of human enteritis. Several studies reported a rate of 3 to 30% (1, 5, 7, 10, 11, 13, 14, 16, 22, 23). At the Instituto Nacional de la Nutrición in Mexico City, it was isolated in 5% of gastroenteritis cases (N. Torres, thesis, Instituto Politécnico Nacional, Mexico, 1980).

Neither the exact mechanism of diarrhea production nor the immune mechanisms induced in the host are known. Mucus and blood in the stool (1, 10, 23), sigmoidoscopic observation of congestion and edema of the mucosa (15), and the presence of intestinal congestion and hemorrhage at necropsy of a patient who died from campylobacter infection (14) suggest that *C. fetus* subsp. *jejuni* is an invasive organism. Enterotoxins produced by campylobacter have been looked for recently (11), but few strains have been found that produce temperature-stable toxins (8).

Attempts to induce diarrhea in animals have not been fully successful (8, 21), and therefore a suitable animal model is needed to study the pathogenesis of campylobacter enteritis. Jones et al. (12) were probably the first to transmit the disease experimentally in cattle, implicating microaerophilic vibrios, which they called *Vibrio jejuni*, as the cause of winter dysentery. By oral inoculation of a pure culture in healthy animals, they reproduced a disease which simulated human campylobacter enteritis. No further studies to confirm or extend these results have been made, but winter scourse in cattle is no longer attributed to campylobacter infection (9).

Campylobacter has been isolated from the liver, small intestine, bile, and cecum of turkeys with bluecomb enteritis (24). The disease has been reproduced experimentally in these birds, but the attack rate and histological findings are not described (27). King (14) reported association of infectious hepatitis with the isolation of microaerophilic related vibrios (probable *C. fetus* subsp. *jejuni*) from liver and feces in chickens. This, plus the fact that avian species (3, 24), and chickens in particular (6, 22, 25), are common carriers of this organism, suggested newborn chickens as a suitable animal model for the study of campylobacter enteritis.

The purpose of this study was to develop an experimental model for *C. fetus* subsp. *jejuni* enteritis whereby diarrhea could be consistently induced through oral inoculation of a human strain.

MATERIALS AND METHODS

Characterization of strain. Strain INN-1-79 of *C. fetus* subsp. *jejuni* was isolated from an adult patient with severe dysenteric diarrhea at the Instituto Nacional de la Nutrición. Media used for isolation were Campy-BAP and Campy-THIO (28), incubated in an atmosphere of 10% CO_2, 5% oxygen, and 85% nitrogen for 48 h at different temperatures. Characteristic S-shaped, curved, and spiral forms were seen in Gram stain and phase-contrast microscopy. Complete characterization of the strain was based on its ability to grow at 37 and 42°C, in glycine and 1% bile, but not with 3.5% NaCl nor at 25°C. Microorganisms were motile and produced catalase and oxidase; they produced H_2S in lead acetate strips but not in iron-containing media.

Animals. One-day-old *Gallus gallus domesticus* obtained from a local poultry farm were caged in groups of 10 at a constant temperature of 37°C. To ensure the absence of diarrhea and rule out fecal shedding of campylobacter, daily stool cultures were made for 2 days before experimental infection. Only 1 of 86 chickens tested had a positive culture upon arrival from the farm.

Preparation of inoculum. A pure culture of *C. fetus* subsp. *jejuni* strain INN-1-79 was grown in 10% sheep blood brucella agar for 48 h. A suspension containing 9×10^8 colony-forming units (CFU) per ml was standardized with phosphate-buffered saline, pH 7.2, using a McFarland nephelometer.

Preparation of antiserum. New Zealand rabbits were inoculated intravenously with 0.5 ml of formaldehyde-inactivated bacteria for 2 consecutive days, followed by weekly schedules of a 1-ml suspension given intravenously for 2 days during 1 month. In addition, on the 2nd day of the 3rd and 4th weeks, a 2-ml mixture of incomplete Freund adjuvant and heat-inactivated suspension was given intramuscularly in several sites. One month after the last dose, 0.5 ml of the inactivated bacterial suspension was applied subcutaneously in multiple sites on the back in addition to 2 ml of the mixture with Freund incomplete adjuvant given intramuscularly. Rabbits were bled 8 days later, and sera had titers of 1:1,600 by tube and slide agglutination.

Infection of chickens. Chicken weights were recorded daily, and rectal swabs and stool samples were collected for culture on 2 consecutive days. On the 3rd day, 0.1 ml of a suspension containing 9×10^8 CFU of campylobacter per ml in phosphate-buffered saline, pH 7.2, was administered orally, using a 1-ml syringe connected to an Intracat 21 gauge. To determine the minimal infective dose, the initial suspension was serially diluted, and the number of colonies was verified by plating the suspension in 10% sheep blood brucella agar.

After inoculation, chickens were observed for appearance of diarrhea and weighed daily until death or cessation of diarrhea. Chickens were considered to have diarrhea if (i) their feces were liquid or semiliquid or contained mucus and (ii) their perinea were wet and soiled. Campylobacter shedding after inoculation was followed by daily culture of rectal swabs and stool samples for 7 days, and survivors were cultured monthly. Food was cultured to keep it free of campylobacter. Heart blood samples were obtained from dead chickens for culture.

Transmission studies. Five-day-old healthy chickens, not shedding campylobacter in their stool, were transferred to a cage with sick chickens on the 3rd day after oral inoculation. Weight, presence of diarrhea, and cultures were observed as in the infected group.

Bacterial isolation. Stool, rectal swabs, and blood samples were inoculated in Campy-THIO, whereas feces were also plated on Campy-BAP. All samples were incubated in a 10% CO_2–5% O_2–85% N atmosphere for 48 h at 37 and 42°C. Nonhemolytic, flat, gray, mucoid colonies, measuring 1 to 2 mm, were examined by Gram stain for typical curved and S-shaped morphology. Suspicious colonies were confirmed by catalase reaction, slide agglutination, and immunofluorescence, using antiserum against strain INN-1-79.

Indirect immunofluorescence. Slides of acetone-fixed campylobacter isolates or chicken intestine sections were incubated with a dilution of antiserum against strain INN-1-79. Fluorescein-labeled goat anti-rabbit immunoglobulin was used as a second antibody. Preparations were observed with a Zeiss epifluorescent ultraviolet microscope. Control slides containing *Escherichia coli*, *Klebsiella*, and intestinal tissues from uninfected chickens showed no specific immunofluorescence.

Histological examination. Sections of intestine were fixed with 10% Formalin in phosphate-buffered saline, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Electron microscopy. Sections of intestine were fixed with 2.5% glutaraldehyde, embedded in Epon, and stained with uranyl acetate. Grids were observed under a Hitachi electron microscope.

RESULTS

Clinical findings. In three separate trials, 25 chickens inoculated with 9×10^7 CFU of campylobacter and 20 control chickens were followed up simultaneously (Fig. 1). Diarrhea developed in 22 (88%) infected chickens, with a mean onset of 45 h and a range of 24 to 72 h.

The mean duration of diarrhea was 8 days, with a range of 7 to 10 days. Diarrhea was not present in the control group. Chickens inoculated with the strain INN-1-79 had considerable weight loss compared with the control group. Thirty-two percent of the infected chickens died between days 4 and 7 after infection. Campylobacter was isolated from heart blood cultures of all dead infected chickens. The cause of one

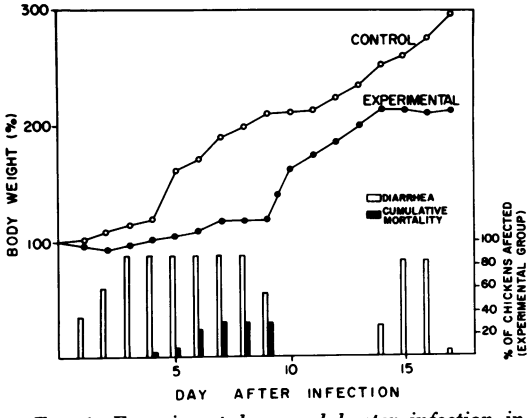

FIG. 1. *Experimental campylobacter infection in 3-day-old chickens induced orally with 9×10^7 CFU of C. fetus subsp. jejuni. The weight of the chickens on day zero was considered 100%. Empty bars represent percentage of chickens with diarrhea on a specific day; solid bars represent cumulative mortality.*

death in the control group could not be determined, but campylobacter was not isolated from stool or blood. Shedding of campylobacter in the experimental group started at 24 h and persisted for up to 3 months after challenge. A short episode of diarrhea recurred 5 days after the first episode in 80% of the survivors, and no further deaths were observed.

Minimal infective dose. Three groups of 10 chickens each were inoculated with 9,000, 90, and 9 CFU, respectively. Diarrhea developed in 90% (9 of 10) of the chickens inoculated with either 9,000 or 90 CFU, but only in 20% (2 of 10) of the chickens infected with 9 CFU. Campylobacter shedding started 24 h after challenge in all chickens from the first two groups and only in two of those receiving 9 CFU. There were no deaths in any of the three groups until day 6, when chickens were sacrificed.

Transmission studies. Ten healthy chickens were transferred to a cage containing eight chickens previously infected with 9×10^7 CFU of campylobacter that were shedding the organism and that had had diarrhea for 2 days. The transferred group displayed a disease pattern similar to that found in the previously infected animals, although the incubation period was longer in the former (72 ± 12 h) and the mortality rate was lower (20% compared with 32% in the inoculated group) (Fig. 2).

Microscopic findings. Histological sections of bowel were made from chickens sacrificed 6, 9, 12, 24, and 48 h after infection. Segments of duodenum, jejunum, ileum, cecum, and colon were separated and processed for light microscopy, immunofluorescence, and electron microscopy.

(i) Light microscopy. Moderate edema and infiltration of mononuclear cells were observed in the lamina propria of the last portion of the small bowel and the first portion of the colon including cecum, with no disruption of the mucosa.

(ii) Immunofluorescence. Campylobacter was observed penetrating epithelial cells as early as 12 h after inoculation. By 24 h, specific fluorescence was seen inside phagocytic cells in the lamina propria of the last portion of jejunum, the ileum, and the first portion of the colon. Control animals showed no specific fluorescence in any portion of the intestine (Fig. 3).

(iii) Electron microscopy. The characteristic S shape of campylobacter without flagella and other cells in various stages of replication were seen in the lumen of the jejunum, ileum, and cecum (Fig. 4a). They were also seen penetrating epithelial cells with disruption of the cell membrane (Fig. 4b).

DISCUSSION

Three-day-old chickens seem to provide a useful animal model to study *C. fetus* subsp. *jejuni* enteritis, since diarrhea was easily induced, even with a small oral inoculum. The diarrhea attack rate was very high, with watery diarrhea prevalent. Our transmission studies, designed to simulate the natural spread of infection, confirm the high susceptibility of this species to the disease.

Shortly after we began our trials in newborn chickens, Butzler and Skirrow (8) reported failure to induce diarrhea in older chickens, although they observed campylobacter in epithelial cells and recovered the organism from stools of all inoculated animals and from the blood of half of the chickens. In earlier experiments, we

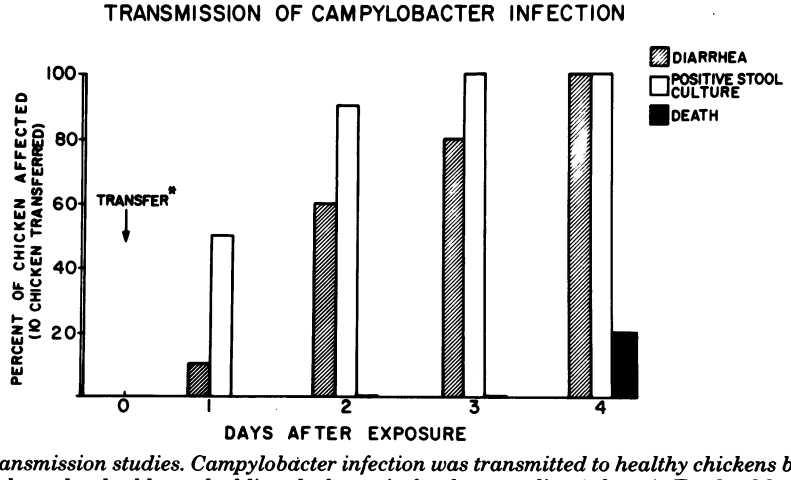

FIG. 2. *Transmission studies. Campylobacter infection was transmitted to healthy chickens by caging them with sick chickens that had been shedding the bacteria for the preceding 3 days. *, Ten healthy chickens were transferred on day zero.*

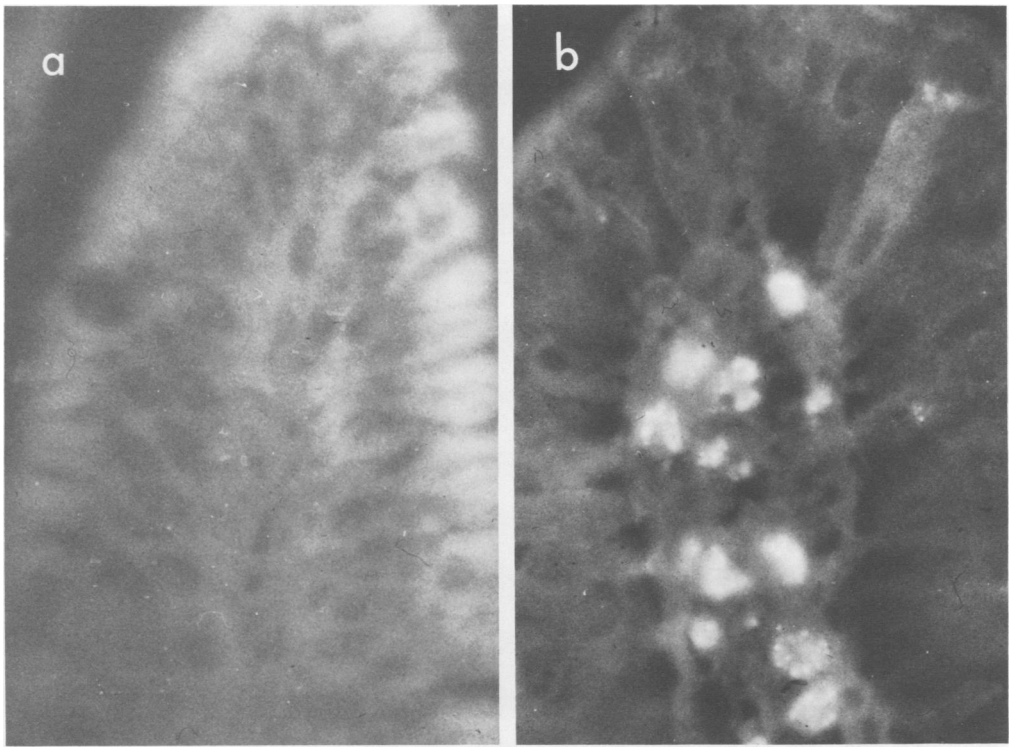

FIG. 3. *Indirect immunofluorescence of tissue sections of ileum reacted with hyperimmune antiserum made of heat-stable antigens of C. fetus subsp. jejuni strain INN-1-79. (a) Villi of a noninfected control chicken; (b) specific immunofluorescence observed intracellulary in the lamina propria of the villi of an infected animal.*

observed that 2-week-old chickens were more resistant to diarrhea. In a group of 10, it was induced in only 3 (30%). Butzler and Skirrow's failure could have been due to the use of older chickens, which seem more resistant. On the other hand, other strains isolated from carriers, patients with diarrhea, and animals need to be studied with this model to establish differences in virulence.

The jejunum and ileum were the bowel segments most affected in this animal model. Even though human data are scarce, one case was reported by King (14), who described the anatomical findings in a poultry farmer who died with campylobacter diarrhea and whose jejunum and ileum showed evidence of congestion and hemorrhage.

Colitis in chickens caused by strain INN-1-79 was not as severe as in humans, where bloody diarrhea appears in 20% of the cases (1, 10, 23). Bacteremia was common, even though blood cultures were obtained only from dead animals. The frequency of bacteremias associated with intestinal infection due to *C. fetus* subsp. *jejuni* in humans has not been established, but Blaser et al. (4) reported its isolation in two of seven patients with diarrhea.

Invasiveness was confirmed by electron microscopy and immunofluorescence. The initial process of penetration into the mucosa is by disruption of the cell membrane into the epithelial cells. The mechanism by which *C. fetus* subsp. *jejuni* destroyed the cell membrane is unknown, but it could possible be due to cytotoxic enzymes.

After campylobacter is given orally, it replicates in the small intestine; 12 h later it penetrates the mucosa; and in 24 h, as shown by immunofluorescence, it has been phagocytosed. The infiltration of mononuclear cells observed by light microscopy may implicate macrophages as the major phagocytic cells. Phagocytosis studies in vitro may help to elucidate the interaction between this organism and phagocytic cells.

Attempts to reproduce the disease in rhesus monkeys have been made by several authors, with inconsistent results (8). R. L. Kaplan, using marmosets, has been able to induce diarrhea with better success (personal communication), but high costs and difficulties in obtaining these animals are important limitations in their use. Although campylobacter enteritis has been associated with canine infection (2), and there are reports of fatal infections in dogs (20), induction

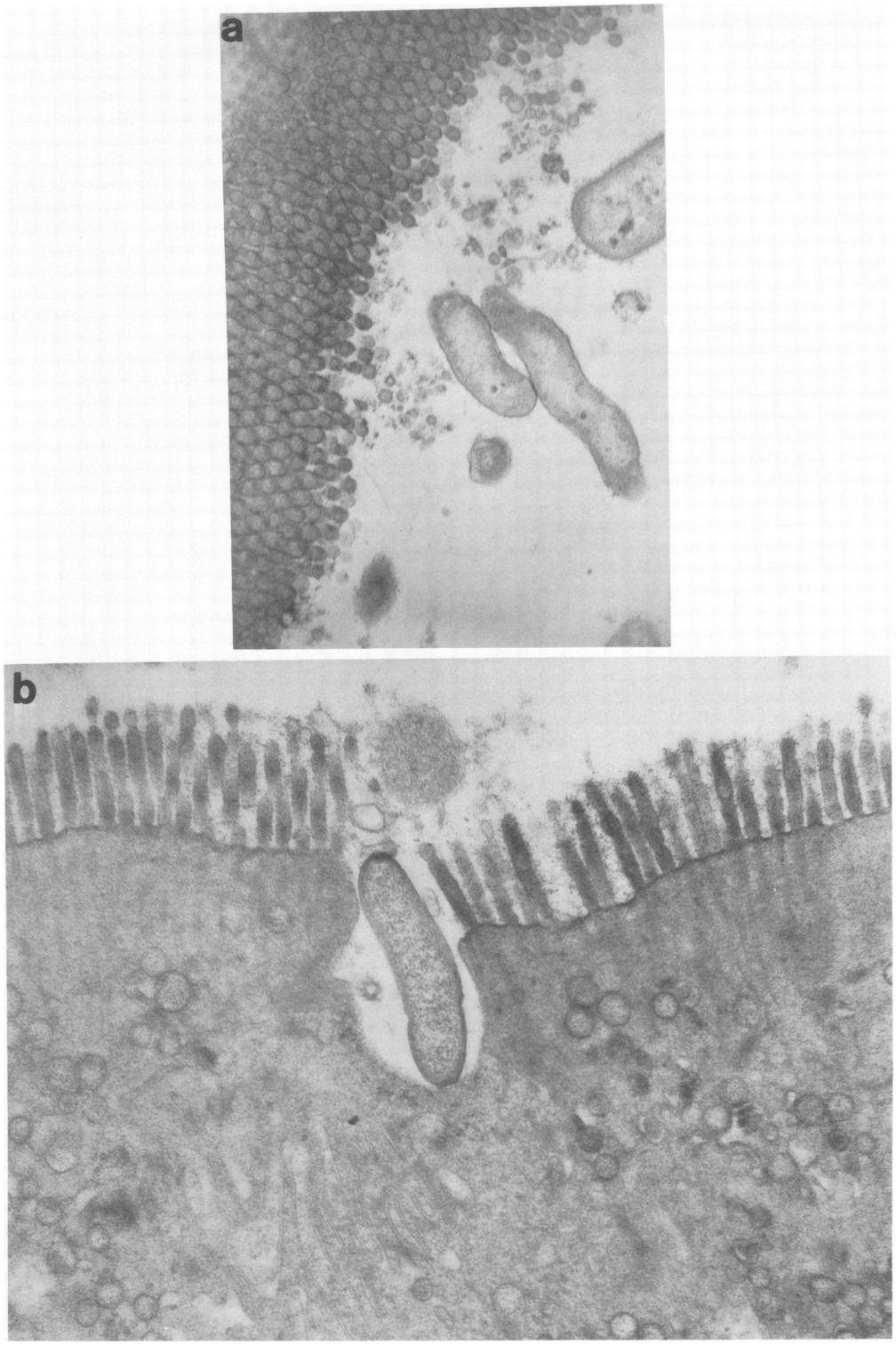

FIG. 4. *Electron micrographs of intestine of infected chickens. (a) Characteristic S shape of campylobacter found in the lumen of jejunum; (b) penetrating epithelial cells with disruption of the membrane.*

of diarrhea with *C. fetus* subsp. *jejuni* in puppies has been unsuccessful (21).

The animal model we propose fills most of the requirements for an ideal experimental model: (i) disease reproduction similar to its occurrence in humans; (ii) route of inoculation equivalent to natural infection; (iii) low inoculum needed; (iv) reproducibility of experiments; and (v) low costs of animals and maintainance.

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