Campylobacter jejuni Diarrhea Model in Infant Chickens

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To study the pathogenic mechanisms of *Campylobacter jejuni* infection, 36- to 72-h-old chickens were fed 10^3 to 10^6 live cells, using strains isolated from 40 patients with watery diarrhea and 6 with bloody mucoid diarrhea from whom no other known enteropathogen was detected. Chickens of Starbro strain were more likely to develop *C. jejuni*-induced diarrhea than were White Leghorn chickens. Diarrhea was defined on the basis of amounts of gut fluid in 288 chicks fed with live *C. jejuni* versus 183 saline-fed control as an accumulation ≥0.4 ml of fluid in the guts (excluding ceca) of chickens. Twenty-five percent of the chickens developed diarrhea on day 2, 49% on day 4, and 81% on day 5. The intestines, including ceca, were distended with watery fluid. The majority of the strains, irrespective of whether they were isolated from watery or bloody mucoid enteritis patients, caused watery diarrhea in chickens, and a few caused mucoid diarrhea. No correlation was observed between the source of a strain and the outcome in the experimental model. Bloody diarrhea was never observed in chickens. The peak incidence of diarrhea on day 5 coincided with the mean of maximum fluid accumulation. The organisms multiplied by 3 to 4 logs in all parts of the intestine, with a steady increase in number until day 5. Systemic invasion occurred frequently: *C. jejuni* could be recovered from the spleen in 47% of the chickens on day 5, in 25% from the liver on day 6, and in 11% from heart blood on day 4. Histopathological examination of gut tissue of the chickens having watery diarrhea did not reveal any abnormality except slight submucosal edema. However, in chickens with mucoid diarrhea, the organisms were found to adhere to brush borders and penetrate into the epithelial cells with formation of a breach in continuity of the brush border lining. The electrolyte composition of the intestinal fluid from chickens infected with *C. jejuni* and from saline-fed controls did not show significant differences, except for depletion of K^+ in the test group. The results obtained in this highly reproducible chicken diarrhea model indicate that (i) most chickens develop nonexudative watery diarrhea 2 to 5 days after oral feeding of 10^3 to 10^6 live cells of *C. jejuni*; (ii) the organism multiples in all parts of a chicken intestine, (iii) systemic invasion is common, and (iv) local invasion is sometimes observed.

Campylobacter jejuni has been isolated from cases of human enteritis in many parts of the world (2, 4). The existence of differences in the epidemiology and clinical consequences of *C. jejuni* infection in developed and less-developed countries has been suggested (7). The organism was isolated from healthy individuals and diarrheal patients in almost equal frequency in selected groups of population in Bangladesh (3). *C. jejuni* has been implicated in the etiology of diarrhea in the industrialized world on the basis of thorough clinical and epidemiological studies, although little work has been done on its pathogenic mechanisms. Hypotheses that the organism can produce enterotoxin or is invasive have been proposed (2, 8, 9). Several potential animal models such as calf, lamb, dog, and chicken, and invasion in HeLa cell culture, have also been described (1, 6, 8, 10, 11), but the reports are preliminary.

The purpose of this study was to develop a simple, sensitive, and reproducible animal model to mimic *C. jejuni* diarrhea with strains isolated from human cases of enteritis caused by this organism and to elucidate its role in the pathogenesis of diarrhea in Bangladesh.

MATERIALS AND METHODS

Organisms. Strains of *C. jejuni* isolated from 40 patients with watery diarrhea and 6 with bloody, mucoid diarrhea from whom no other known enteropathogen was detected were included in the study. The presence of parasites in stool

specimens was established by microscopy of direct and iodine-stained smears. These specimens were also cultured and tested for different enteropathogenic species of *Vibrio*, *Shigella*, *Salmonella*, enterotoxigenic *Escherichia coli*, *Aeromonas*, and *Plesiomonas* by following standard procedures (5). *C. jejuni* strains that were isolated together with any of these organisms were not included in this study. *Yersinia enterocolitica*, being uncommon in Bangladesh (12), was not investigated. Examination of all of the fecal specimens for rotavirus was done by the method of Yolken et al. (13), with negative results. The *C. jejuni* strains were isolated in Campy-BAP medium by the method of Blaser et al. (2) and identified by characteristic colonial morphology, Gram strain, motility and catalase production, no growth at 25°C in brucella broth and in 3.5% sodium chloride, H_2S production with the lead acetate strip method, hippurate hydrolysis in 1% aqueous sodium hippurate, and sensitivity to nalidixic acid at a concentration of 30 µg per disk. The strains were preserved in brucella broth (BBL Microbiology Systems) containing 15% glycerol and 0.12% Bacto Agar at −70°C and did not undergo more than two subcultures before use in this study.

Experimental model. Chickens of White Leghorn and Starbro strains that hatched overnight were brought from a local pilot poultry farm and kept in a cage at ca. 37 to 39°C in an isolated room. Cloacal swabs were cultured for *C. jejuni* before experimental infection. Of 500 chickens tested, none carried the organism. Death before infection was rare.

Inoculum. Freshly isolated *C. jejuni* strains were grown in fluid thioglycolate medium (BBL) at 37°C in a candle jar for 48 h. Ten-fold dilutions were made in phosphate-buffered

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saline (PBS), pH 7.2, with growths at the upper part of the medium to make suspensions of ca. 10^4 to 10^7 CFU/ml, 0.05-ml amounts of which were used as inocula. In some experiments, 0.05 ml of undiluted growth served as inoculum. Viable counts of the bacteria were done on Campy-BAP medium by the spread plate method in duplicate, and the actual dose fed to each chicken was calculated for every strain.

Experimental procedure. The chickens were divided into groups of either 10 or 5 each and kept in separate wooden cages in a room at approximately 37°C. Every chicken in each group was given an identification mark with ink in different regions of the body. A chart was made for each experiment wherein identification of all the chicks was noted individually and against which daily observations such as death, frequency and consistency of stools, perineal soiling, and measurement of volume of intestinal fluid were entered. The chicks were allowed their usual food and water throughout the experiments.

In a set of experiments, one group of chicks received PBS and the remaining groups were fed with live cells of different strains of C. jejuni. Feeding was done with a tuberculin syringe without the needle. The mouth of each chick was opened with a pair of plain forceps, the beaks were held apart, and 0.05 ml of the live cell suspension (as described above) was dropped onto the tongue. Each strain was fed to a group of 10 or 5 chicks. Experimental infection was usually done when the chicks were 36 to 72 h old. One or two of the premarked chicks were sacrificed every day with an excess dose of anesthetic ether from day 1 to day 7 after feeding to avoid any statistical bias. On observation that fluid accumulation did not occur before 48 h of experimental infection, the birds were sacrificed from day 3 to day 7. Before sacrificing a chick, we noted its general appearance and any soiling in the perineal region. After sacrifice, the abdomen and any distension of the gut with fluid or gas were noted, and the cloacal and gizzard ends were clamped with artery forceps and removed with scissors. The jejunum, ileum, and large gut were separated in different petri dishes and cut open through the entire lengths, and the fluid in each part— excluding solid fecal material, if any—was measured with sterile pipettes. Mucoid fluid, when present, was milked out gently with fingers in tubes and measured. Cecal fluid was not measured. Campy-BAP, MacConkey, salmonella-shigella (Difco), and gelatin agar media were inoculated with cecal and large gut fluids of each test and control chick.

Tests for reproducibility. Fourteen of the 46 strains were randomly selected for this experiment. Each strain was fed to only two chickens. A control group of two birds fed with PBS was included in each set of experiments. The chickens were sacrificed on day 5, and the fluid accumulations were noted and cultured on Campy-BAP and different standard media for isolation of C. jejuni and other known pathogens, if any. This set of experiments was repeated three times.

Multiplication of C. jejuni in the guts of chickens. Fluids in 0.05-ml amounts from the jejunum, ileum, and large gut were collected aseptically on autopsy, immediate serial 10-fold dilutions were made in PBS, and 0.1-ml amounts were plated in duplicate by the spread method in Campy-BAP medium and incubated in a candle jar at 42°C. Counts of viable bacteria were done after 48 h. When there was measurable fluid in any part of the gut, that segment was washed three times with 1 ml of PBS, using a sterile syringe, and plated accordingly. Counts of viable bacteria were done after 48 h.

Systemic invasion. Cultures of heart blood in 0.05-ml quantities and smears from sections of liver and spleen for C.

jejuni were done on Campy-BAP medium, taking aseptic precautions immediately on autopsy of almost all of the experimentally infected chickens. Smears of spleen were cultured for isolation of C. jejuni from 30 chickens every day starting from 24 h. A total of 210 chickens were examined for this purpose over a period of 7 days. Daily cultures from heart blood ($n = 33$) and liver ($n = 33$) were done similarly. In the case of liver, one extra chicken each was used on days 5 and 6.

Histopathology of the guts of chickens fed with live cells of C. jejuni. Portions of jejunum, ileum, and large gut of chickens developing watery or mucoid diarrhea were taken into separate sterile petri dishes, cut open through the lengths, cultured on different media as described earlier, and preserved in neutral buffered Formol saline in MacCartney bottles. Pieces of these segments of intestine from PBS-fed control birds were also preserved at that time from the same set of experiments. Paraffin sections of these gut tissues stained with hematoxylin and eosin and by Gram's method were examined microscopically for any change in histology and bacterial invasion of the mucosa.

Electrolyte contents of experimental diarrheal fluid. Diarrhea was induced in chickens by feeding live cells of C. jejuni strains, and the intestinal fluids were collected in sterile tubes for estimation of Na^+, K^+, and Cl^- in millimoles per liter. Fluids were similarly collected from PBS-fed chickens at the same time from the same sets of experiments.

Microscopy of the experimental diarrheal fluids. Diarrheal fluids of 20 experimentally infected and 10 saline-fed chickens were examined by direct microscopy for pus cells and erythrocytes immediately after autopsy.

RESULTS

Diarrhea could not be induced regularly by oral feeding of live cells of C. jejuni in White Leghorn chickens. We therefore changed over to 36- to 72-h-old chicks of Starbro strain, in which reproducible diarrhea was observed after experimental infection. Diarrhea, as defined by increased volume of stool output, was observed in tested chickens. For example, massive soiling of the animal in its excreta (see Fig. 2B) was a feature of many animals after experimental infection but was never seen in saline-fed birds. However, the presence of cloaca and the difficulty of collecting excreta in chickens made the quantitation of diarrhea difficult. Therefore, a more convenient indirect measurement of transient intestinal fluid accumulation was attempted which yielded a reproducible indicator of diarrhea in this model. In the group of saline-fed controls, 178 of 183 chickens had an amount of ≤ 0.2 ml of fluid in the gut, excluding solid materials. We therefore defined diarrhea in C. jejuni-fed chicks as ≥ 0.4 ml of fluid accumulation in the gut, excluding ceca. Solid material, if there was any, was not included in the measurement. Of 288 chickens fed with live suspensions of 46 strains of C. jejuni, 25% developed diarrhea on day 2, 49% on day 4, and a maximum of 81% on day 5 (Fig. 1). The incidence at 144 h (day 6) was almost similar (80%) and then dropped steadily. No variations in time of onset, maximal fluid accumulation, and duration of diarrheal response were noted within the dosage range of ca. 10^3 to 10^6 live bacteria. An inoculum of 10^7 CFU also did not result in any differences in these measurements. However, a dose of 100 bacteria induced diarrhea only occasionally. Five of the 183 chickens fed with PBS developed diarrhea, but no pathogen could be detected. Two and 7 chickens of 290 and 190 in the test and control groups, respectively, died for unknown
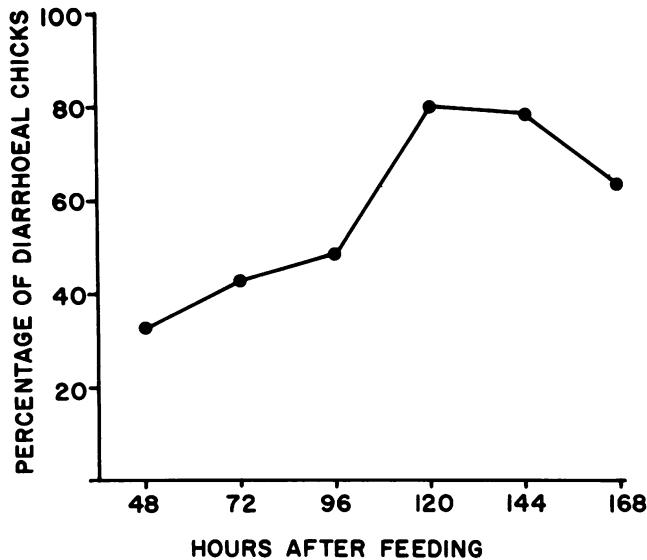
FIG. 1. Occurrence of diarrhea in chickens (n = 288) fed with live cells of *C. jejuni* isolated from diarrheal patients.

reasons during the course of the experiments, and they were not included in the data shown. Some of the infected chickens developed severe diarrhea leading to cachectic condition (Fig. 2B) as compared with saline-fed controls at 120 h (Fig. 2A). The intestines, including ceca, were distended with watery fluid (Fig. 3B), whereas the guts of control chickens were normal, usually with shrunken ceca (Fig. 3A). The majority of the strains caused watery diarrhea irrespective of whether they were isolated from patients with watery

or bloody mucoid diarrhea. On the other hand, some of the strains isolated from watery or dysenteric diarrhea cases induced mucoid diarrhoea in experimentally infected birds. Bloody diarrhea was never observed in chickens. *C. jejuni* was isolated from all of the diarrheal chickens infected experimentally, but not from controls.

The mean fluid accumulations in test and control chicken guts with standard errors at different time intervals after feeding (Fig. 4) indicated that maximum fluid accumulation in infected birds occurred on day 5 (n = 39), declining steadily thereafter. The rise from 96 (n = 53) to 120 h was very sharp. Little fluid was found in the guts of control chickens at any given time.

Twelve of the 14 strains, when fed thrice, caused diarrhea in chickens every time on day 5. One of the remaining two strains caused borderline fluid accumulation only once.

The mean number of CFU/ml of fluid in jejunum, ileum, and large gut of experimentally infected chickens reached a maximum on day 5 in all three parts of the gut (Fig. 5). The counts in jejunum were low throughout the course of the experiment and never exceeded 10^6 CFU/ml. The counts in ileum and colon increased rapidly after day 2, reaching the highest value on day 5 with more than 10^7 CFU/ml. From day 2 to day 5, the mean number of CFU/ml of fluid was higher in the colon than in the ileum; after day 5 the counts were higher in the ileum.

C. jejuni organisms were isolated from the spleen, liver, and heart blood of experimentally infected chickens (Fig. 6). Maximum isolation rates were from spleen (47%) on day 5, followed by liver (25%) and heart blood (11%) on days 6 and 4, respectively.

No histopathological changes, except for slight submucosal edema, were observed in the jejunum, ileum, or colon of chickens with watery diarrhea as compared with controls. However, examination of the Gram-stained sections of the

FIG. 2. Severe diarrhea leading to cachetic condition on day 5 in experimentally infected chickens (B) as compared with saline-fed control (A).
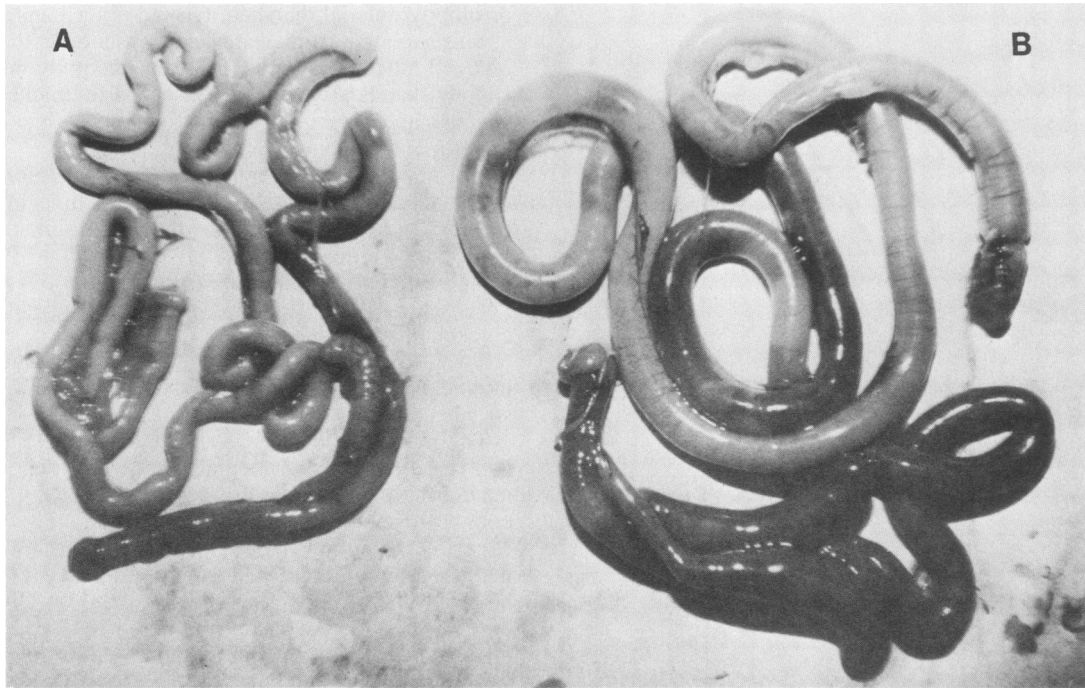
FIG. 3. Distended intestine, including ceca with watery fluid of experimentally infected chickens (B) as compared with that of saline-fed control (A).

small and large intestinal mucosa of chickens with mucoid diarrhea revealed gram-negative curved rods adherent to the brush borders, penetrating through them and within the epithelial layers. In the same specimens, *C. jejuni* could be isolated in the absence of any other organism. As we lack the facilities for immunofluorescence or electron microscopy, the identities of the bacteria cannot be finally proven.

The electrolyte composition of intestinal fluid from test and control birds did not show significant differences except for depletion of K^+ ($P < 0.05$) in the former group in a small series of measurements (Table 1).

Direct microscopy of the experimental diarrheal fluid showed the presence of a few pus cells per high-power field but no erythrocytes.
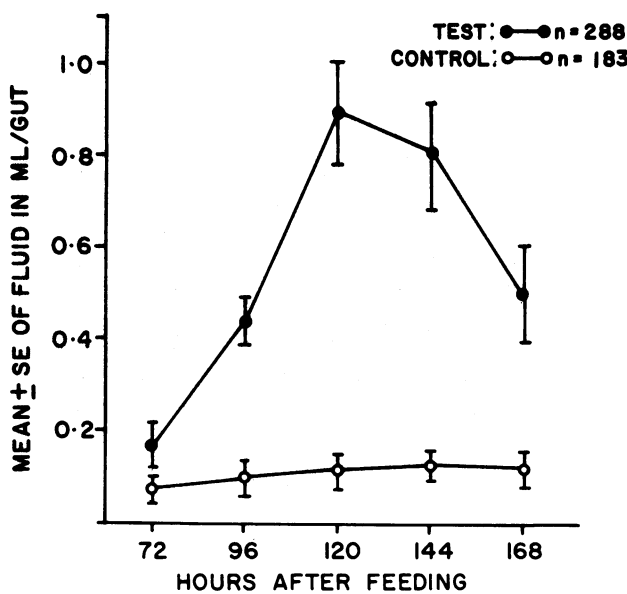

FIG. 4. Fluid accumulations in test and control chicken guts at different time intervals after feeding of live *C. jejuni* isolated from diarrheal patients.
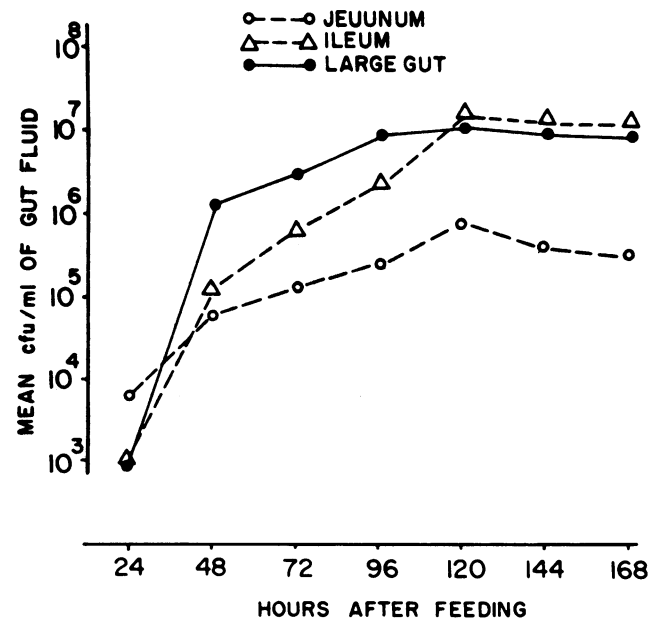

FIG. 5. Multiplication of *C. jejuni* in different parts of the guts of experimentally infected chickens at different time intervals after feeding (inoculum = $6 \times 10^4 \pm 9.0$; $n = 15$).
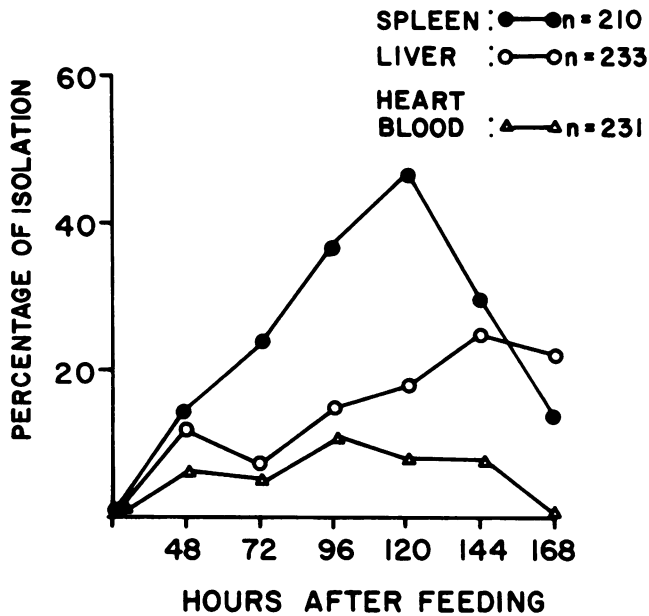
SPLEEN : ●—● n = 210
LIVER : ○—○ n = 233
HEART
BLOOD : ▲—▲ n = 231

FIG. 6. Systemic invasion after feeding of live *C. jejuni* organisms in chickens.

DISCUSSION

Eight-day-old chickens, in which Butzler and Skirrow (4) observed occasional invasion caused by *C. jejuni*, were tested with little success for inducing diarrhea by this organism in the early stages of this study initiated in 1981. Later, following the age-old conception that younger animals are more susceptible to enteric pathogens, chickens of all ages less than 8 days were tried with variable success until the 36- to 72-h-old birds of Starbro strain gave reproducible results. The White Leghorn chickens were less susceptible to *C. jejuni* diarrhea and were not pursued further. The other factor that influenced the study was the delayed onset of diarrhea in this model. Unlike other enteric pathogens causing diarrhea in different animals, usually within a period of 24 h, *C. jejuni* requires a longer time to produce detectable diarrhea. The period of observation after experimental infection had to be extended for up to 1 week to obtain a plateau and downward slope.

The present study indicates that strains of *C. jejuni* isolated from enteritis patients in Bangladesh can produce diarrhea in 36- to 72-h-old chickens. Since birds possess cloaca and pass stools mixed with urine, assessment of diarrhea in chickens based on consistency or frequency of their excreta as done in an earlier study (11) is not useful. Unless they develop severe diarrhea, their perinea also are not wet or soiled.

Measurable fluid in the guts of control chickens rarely reached 0.2 ml; therefore, accumulation of ≥ 0.4 ml was abnormal and quite apart from what was seen in the guts of normal chickens. Following this definition, diarrhea was observed in 81% of the chickens on day 5, which is comparable to other biological models. That all the chickens did not have diarrhea might be due to the following reasons: (i) some of the experimentally fed chickens were not susceptible to the organisms, (ii) some of the strains were not pathogenic, or (iii) diarrhea as defined might not have been detected in all

cases. Almost an equal number of chicks continued to have diarrhea on day 6. This allowed a wider range for the period of observation. The incidence of diarrhea was very low before day 4 and after day 7 or later. This period could not be reduced significantly with an inoculum as high as 10^7 CFU. The optimal inoculum was found to be 10^3 to 10^6 CFU, although occasional diarrhea could be induced with only approximately 100 bacteria. This indicates the sensitivity of the model.

The time (120 h) for the maximum incidence of diarrhea in chickens coincided with the peak of mean maximum fluid accumulation. This is indicative of the reproducibility of the model and the observed time of highest diarrheal incidence. The two curves of percentage of diarrheal chickens and mean of fluid accumulation plotted against time were almost similar, confirming each other.

The results of three repeat experiments in which the same 12 strains of 14 tested caused diarrhea consistently and the other 2 did not indicate that the model is highly reproducible.

C. jejuni multiplied in the intestines of chickens, as evinced by the isolation of several logs more bacteria per milliliter of gut fluid. A steady increase in the number of bacteria in jejunum and ileum over a period of 120 h might be indicative of their multiplication in these regions. However, a similar although not identical trend of the curve of mean bacterial counts per milliliter in colon might be suggestive of its involvement in the disease process as well. The consistently low counts of bacteria in jejunal fluid might be due to their constant downward propulsion by peristaltic movements. The ceca of infected chickens always carried the organism, indicating its possible role in pathogenesis, especially in prolongation of the excretion period and repeat attacks of diarrhea that occur in some birds as observed by some workers (10). The peak multiplication of luminal *C. jejuni* in day 5 coincided with the maximal incidence of observable diarrhea and intestinal fluid accumulation. This coherence further validates the specificity of the chicken diarrhea model.

C. jejuni organisms were isolated from spleens and other organs of a large number of chickens, indicating its capability to cause systemic invasion. Maximum isolation was made from spleens on the day when diarrhea also was at its peak. This may indicate some relationship between these two events. Fewer blood cultures were positive, probably due to the clearing activity of the macrophages, which were particularly effective in the spleen. The relative density of macrophages exposed by the tissue section may account for the lower isolation from liver by the smear technique.

It may be recalled that strains of *C. jejuni* were isolated from 40 patients with watery diarrhea and 6 patients with bloody mucoid diarrhea. Most of the strains of both groups caused watery and some mucoid diarrhea in chickens. No relationship was observed between the type of experimental diarrhea and source of strain from either watery or bloody mucoid diarrhea patients, indicating that there is no correla-

TABLE 1. Mean and standard deviations of electrolyte contents of the intestinal fluids of diarrheal and control chickens

Group	Concn (mmol/liter) of the following electrolyte:		
	Na+	K+	Cl−
Test (n = 11)	114.8 ± 15.19	20.04 ± 5.87[a]	125.7 ± 13.47
Control (n = 6)	118.3 ± 19.15	14.45 ± 2.88[a]	129.5 ± 7.77

[a] $P < 0.05$, using Student's t test (two-tailed).

tion between the source of strain and the outcome of experimental diarrhea in this model.

Histopathological examination of gut tissue was performed in chickens with watery and mucoid diarrhea. In the first group, no abnormality could be found. However, in a group of chickens with mucoid diarrhea, we observed adherence of bacteria to the brush borders and penetration of epithelial cells, resulting in damage and destruction of the superficial luminal part of the gut mucosa. The bacteria were gram-negative curved rods, and only *C. jejuni* organisms were isolated from the same specimens. These observations indicate that experimental infection in chickens with *C. jejuni* strains isolated from patients can cause occasional local invasion. Most of the chickens, however, developed watery diarrhea and had no histopathological lesions in the gut mucosa. The elaboration of enterotoxic substances by *C. jejuni*, therefore, cannot be excluded.

Not much difference was observed in Na^+ and Cl^- contents of gut fluid between experimentally induced diarrheal and control chickens; this is indicative of watery diarrhea. Significant depletion of K^+ in diarrheal chickens as compared with controls might indicate involvement of the colon in the disease process, as colonic mucosa is rich in potassium. However, since the fluids were collected from the entire guts, not from jejunal, ileal, or colonic segments, this may not reflect any significant change in the electrolyte movements through the mucosal surface.

The present study thus describes the development of a reproducible *Campylobacter* diarrhea model in 36- to 72-h-old chickens. The organism multiplies in the gut, causing frequent systemic and rare intestinal invasion. From the observations of the watery, nonexudative nature of the diarrheal fluid and the usual absence of any damage to the intestinal mucosa, involvement of some enterotoxic substance(s) in the experimental diseases cannot be excluded. This study, however, does not establish that all *C. jejuni* strains isolated in Bangladesh are enteropathogenic.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Al-Mashat, R., and D. J. Taylor. 1980. Production of diarrhoea and dysentery in experimental calves feeding pure culture of *C. fetus* ssp. *jejuni*. Vet. Rec. 107:459–464.
2. Blaser, M. J., I. D. Berkowitz, and M. LaForce. 1979. *Campylobacter* enteritis: clinical and epidemiologic features. Ann. Intern. Med. 91:179–185.
3. Blaser, M. J., R. I. Glass, M. I. Huq, B. J. Stoll, G. M. Kibriya, and A. R. M. A. Alim. 1980. Isolation of *Campylobacter fetus* subsp. *jejuni* from Bangladeshi children. J. Clin. Microbiol. 12:744–747.
4. Butzler, J. P., and M. B. Skirrow. 1979. *Campylobacter* enteritis. Clin. Gastroenterol. 8:737–765.
5. Cowan, S. T. 1974. Cowan and Steel's manual for the identification of medical bacteria, 2nd ed. Cambridge University Press, Cambridge.
6. Firehammer, B. D., and L. L. Myers. 1981. *Campylobacter fetus* subsp. *jejuni*: its possible significance in enteric diseases of calves and lambs. Am. J. Vet. Res. 42:918–922.
7. Glass, R. I., M. I. Huq, B. J. Stoll, A. K. M. G. Kibriya, and M. J. Blaser. 1982. Epidemiologic features of *Campylobacter* enteritis in Bangladesh, p. 28–29. In D. G. Newell (ed.), *Campylobacter* epidemiology, pathogenesis and biochemistry. MTP Press Ltd., Lancaster, England.
8. Gubina, M., J. Zajc-Satler, A. Z. Dragas, Z. Seleznik, and J. Mehle. 1982. Enterotoxin activity of *Campylobacter fetus*, p. 188–195. In D. G. Newell (ed.), *Campylobacter* epidemiology, pathogenesis and biochemistry. MTP Press Ltd., Lancaster.
9. Manninen, K. I., J. F. Prescott, and I. R. Dohoo. 1982. Pathogenicity of *Campylobacter jejuni* isolates form animals and humans. Infect. Immun. 38:46–52.
10. Prescott, J. F., I. K. Barker, K. I. Manninen, and O. P. Miniats. 1981. *Campylobacter jejuni* colitis in gnotobiotic dogs. Can. J. Comp. Med. 45:377–383.
11. Ruiz-Palacios, G. M., E. Escamilla, and N. Torres. 1981. Experimental *Campylobacter* diarrhea in chickens. Infect. Immun. 34:250–255.
12. Samadi, A. R., K. Wachsmuth, M. I. Huq, M. Mahbub, and D. E. Agbonlahor. 1982. An attempt to detect *Yersinia enterocolitica* infection in Dhaka, Bangladesh. Trop. Geogr. Med. 34:151–154.
13. Yolken, R. H., H. W. Kim, T. Clem, R. G. Wyatt, A. R. Kaliea, R. M. Chanock, and A. Z. Kapikian. 1977. Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis. Lancet ii:263–267.