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# Survival of *Campylobacter fetus* subsp. *jejuni* in Biological Milieus

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To provide new information on the epidemiology and pathophysiology of human infection with *Campylobacter fetus* subsp. *jejuni*, we studied its survival in several milieus. Standard inocula of organisms were placed in hydrochloric acid, human bile and urine, bovine milk, or stream water and kept at 4, 25, or 37°C; viable organisms were then counted. Stools from humans infected with *Campylobacter* were similarly tested. Survival in acid was pH and time dependent, with 7-log kill within 5 min in solutions at pH 2.3. Organisms multiplied in bile at 37°C and survived for 2 months. Organisms survived better in feces, milk, water, and urine kept at 4°C than they did in these milieus at 25°C. Maximal viabilities of *Campylobacter* organisms kept at 4°C were 3 weeks in feces, 3 weeks in milk, 4 weeks in water, and 5 weeks in urine. Study results suggest that when these milieus are contaminated with *C. fetus* subsp. *jejuni*, they may be significant environmental reservoirs.

*Campylobacter fetus* subsp. *jejuni* is now known to be a significant enteric pathogen for humans (1, 7, 11, 19). Studies from six continents demonstrate that *Campylobacter* is distributed worldwide and is as commonly associated with diarrhea as is *Salmonella* or *Shigella*. Despite these recent advances, the epidemiology and pathophysiology of *Campylobacter* enteritis are not fully defined (12).

We therefore performed in vitro experiments to study the behavior of this bacterium in several environments and physiological milieus. To understand the role of gastric pH in *Campylobacter* infection, we tested the survival of *C. fetus* subsp. *jejuni* in hydrochloric acid. Survival in bile and urine was examined to better delineate the localization of infection, and the organisms in stools were counted to determine the bacterial load during infection. Since infected feces (3) and milk (2, 18) and contaminated water (24) have been implicated in the transmission of *Campylobacter* infection, we also tested the level at which the organisms survived in these vehicles.

## MATERIALS AND METHODS

**Bacterial strains and media.** In this study we used 11 strains of *C. fetus* subsp. *jejuni* isolated from humans with diarrhea (1 per patient) (1); all the isolates selected were characteristic *C. fetus* subsp. *jejuni* (21). Organisms were either used within two subcultures after isolation from patients or frozen at −70°C. Frozen organisms were then thawed and used by the second subculture. Subcultures were done on blood agar plates, were incubated at 42°C in an atmosphere consisting of 5% oxygen, 10% carbon dioxide, and 85% nitrogen for 48 h (1), and then were kept at 4°C for up to 72 h before the next subculture. No organism used was passaged more than four generations on artificial media.

The media used for growing *C. fetus* subsp. *jejuni* included 5% sheep blood agar, *Brucella* agar (BBL Microbiology Systems, Cockeysville, Md.) with 10% sheep erythrocytes, *Brucella* broth, Campy-BAP (*Brucella* agar with 10% erythrocytes and the following antimicrobial concentrations per liter: vancomycin, 10 mg; trimethoprim, 5 mg; polymyxin B, 2,500 IU; amphotericin B, 2 mg; cephalothin, 15 mg), and Campy-thio (thioglycolate broth with 0.16% agar and the above antimicrobial agents) (1). The antibiotic-containing media were used for cultures of feces.

**Milieus tested. (i) Hydrochloric acid.** Dilutions of 0.1 N hydrochloric acid (4.5 ml) at pH 1.7 to 4.0 and sterile distilled water (pH 7.0) were kept at 37°C and adjusted to $10^7$ to $10^9$ colony-forming units (CFU) per ml with 0.5-ml samples of suspensions of *C. fetus* subsp. *jejuni*. The samples were removed at 0, 1, 5, 10, 20, and 30 min to prepare cultures used for quantitation.

The same initial concentrations of *C. fetus* subsp. *jejuni* were introduced to each milieu in the following experiments except that an additional $10^3$ CFU/ml were added to bile, and no organisms were added to the stools from infected humans.

**(ii) Bile.** Bile was obtained at autopsy from patients who had not received antimicrobial agents, was tested for sterility, and was frozen at −70°C until used. Multiple samples were inoculated with bacterial suspensions at time zero and then kept at 4, 25, and 37°C.

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Initial counts were made and repeated daily for the first week; intervals were then lengthened according to the slope of the survival curve. This method for determining sampling intervals was used for all milieus tested. If contamination of a sample was observed, we then continued our counts with an unopened sample which had also been inoculated at time zero and kept at the same temperature.

**(iii) Urine.** Urine from two human volunteers was passed through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) and maintained at 4, 25, and 37°C before bacterial suspensions were added to multiple samples.

**(iv) Feces.** Stool specimens were obtained from patients with *Campylobacter* enteritis and kept at 4 and 25°C. The specimens at 25°C were exposed to air to simulate natural conditions.

**(v) Milk.** Pasteurized commercial bovine milk was autoclaved for 15 min and maintained at 4 and 25°C before standard inocula were added.

**(vi) Water.** Surface water was obtained from four streams in Colorado (elevations, 5,000, 7,500, 9,000, and 11,000 feet [ca. 1,524, 2,285, 2,743, and 3,352 m, respectively]), autoclaved for 15 min and maintained at 4 and 25°C before standard inocula were added.

**Method of counting.** For determining initial and subsequent counts (CFU/ml), samples were withdrawn from the test milieus. For each test milieu at each time, 0.1 ml was withdrawn and diluted in normal saline (0.1 N sodium bicarbonate solution [pH 8.5] for the hydrochloric acid experiment). Serial 10-fold dilutions were done as necessary to obtain an optimal range for counting colonies per plate. Dilutions were inoculated on duplicate blood agar plates or Campy-BAP (for feces) according to the Miles-Misra method (17).

For determining endpoints, except for feces, 0.5 ml of the test material was inoculated into *Brucella* broth. The plates and *Brucella* broths were then incubated at 42°C in 5% oxygen–10% carbon dioxide–85% nitrogen. For determining endpoints for fecal specimens, samples were added to Campy-thio broths. Campy-thio can be used for recovery of small numbers of *C. fetus* subsp. *jejuni* from stools (1). Earlier experiments showed better recovery of *C. fetus* subsp. *jejuni* from Campy-thio kept at 4°C than from that kept at 25°C (W.-L. L. Wang and M. J. Blaser, unpublished data). In this experiment, Campy-thio broths were refrigerated for 8 h and then subcultured to Campy-BAP and incubated as above.

After 48 h of incubation, *Campylobacter* growth was examined on each of the primary plates (blood agar or Campy-BAP). The broths were inoculated in parallel; if no growth was detected on the plates, the broths were subcultured to the plate media for the determination of endpoints.

## RESULTS

The survival of three strains of *C. fetus* subsp. *jejuni* in hydrochloric acid was both time and pH dependent. The results for the three strains were quite similar, and only the mean values are shown in Fig. 1. Water and a solution at pH ≥3.6 had virtually no effect on viability, but in a solution at pH 3.0, there was more than a 2-log drop by 30 min. However, in a solution at pH 2.5, there was 4-log kill within 1 min, and in a solution at pH 2.3, there was 7-log kill within 5 min.

*C. fetus* subsp. *jejuni* survived well in bile (Fig. 2), with the briefest survival being 1 to 3 weeks in preparations held at 25°C. At 4°C, organisms survived for 1 to 2 months, and at 37°C, they survived for 2 to 3 months. At 37°C, organisms multiplied to reach levels of $10^8$ CFU/ml within 1 week and remained viable at levels of $>10^6$ CFU/ml for up to 80 days. Changes in pH did not affect viability, since all pH values remained constant, in the range of 6.5 to 6.9, throughout the study.

Blood and polymorphonuclear leukocytes were observed in the stools of five and eight

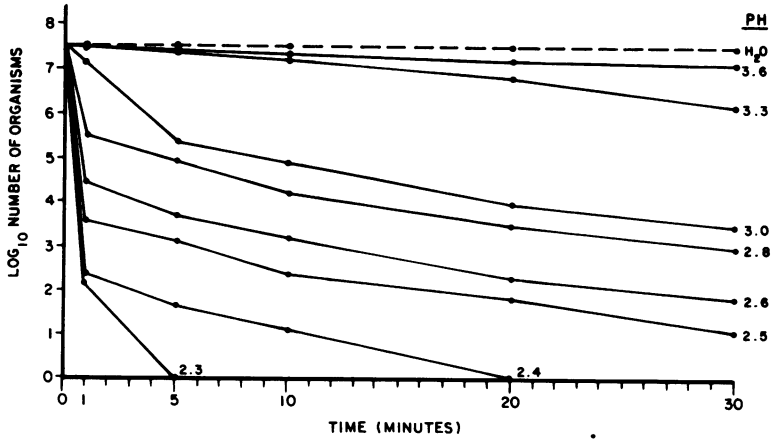


FIG. 1. *Survival of three strains of C. fetus subsp. jejuni in hydrochloric acid (solid line; pH 3.6 to 2.3) and water (broken line) at 37°C. Each point represents the mean number of organisms for the strains tested.*

patients, respectively, with *Campylobacter* enteritis (1). Stools from the eight patients with polymorphonuclear leukocytes contained from $6 \times 10^6$ to $1 \times 10^9$ CFU of *C. fetus* subsp. *jejuni* per g (median, $2.8 \times 10^7$ CFU/g). Five of these specimens then were stored at 4°C, and *Campylobacter* was recovered for up to 3 weeks (Fig. 3). Portions from three of the same specimens kept at 25°C and exposed to air dried within 96 h, however, and no viable organisms could be recovered after 1 week.

Two strains of *C. fetus* subsp. *jejuni* placed in specimens of autoclaved stream water survived for various periods (Fig. 4). Organisms in samples kept at 25°C died within 4 days, whereas those in samples kept at 4°C survived for 1 to >4 weeks. There was no correlation between the length of survival and the altitude of the source or the known mineral content of the water.

Five strains of *C. fetus* subsp. *jejuni* survived better in pasteurized milk at 4°C than at 25°C. At 4°C, organisms remained viable up to 22 days, but at 25°C, no viable organisms could be detected after 3 days. Viability of four strains in urine was also temperature dependent. Orga-

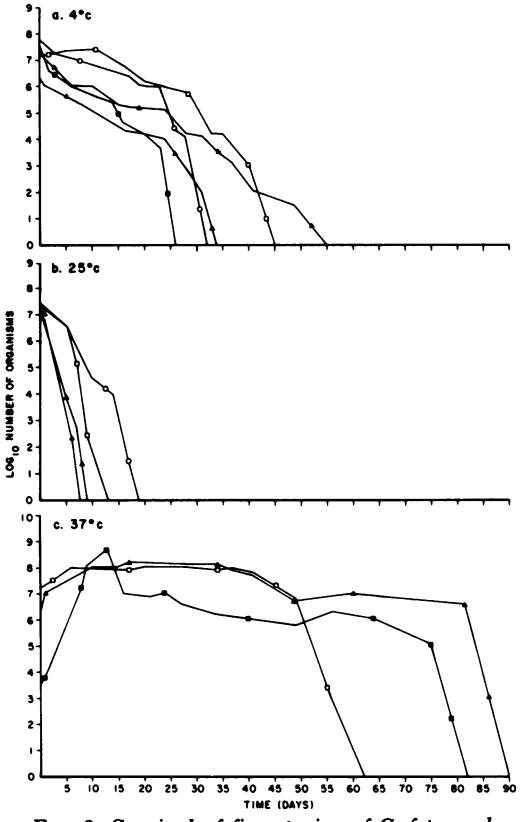


FIG. 2. *Survival of five strains of C. fetus subsp. jejuni in human bile kept at 4, 25, and 37°C.*

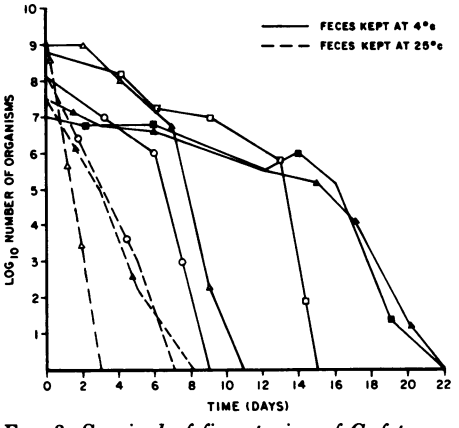


FIG. 3. *Survival of five strains of C. fetus subsp. jejuni in human feces kept at 4 and 25°C.*

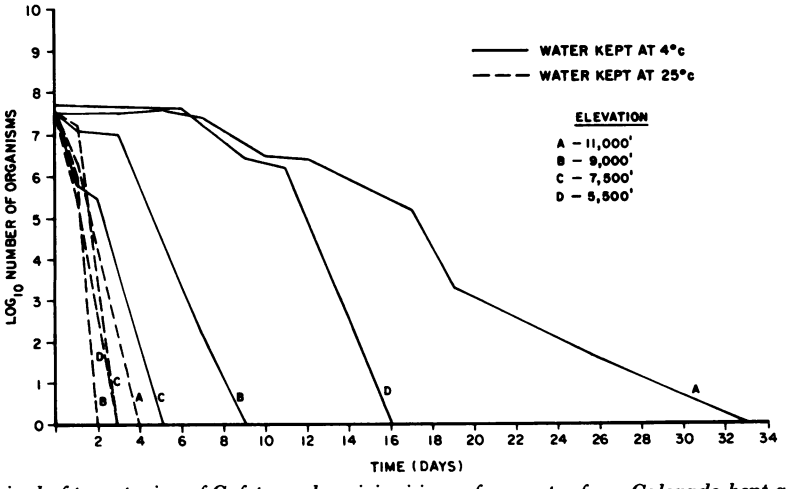


FIG. 4. *Survival of two strains of C. fetus subsp. jejuni in surface water from Colorado kept at 4 and 25°C.*

nisms survived less than 48 h in urine kept at 37°C, but they survived for as long as 5 weeks in urine kept at 4°C.

## DISCUSSION

In these experiments we used laboratory strains of *C. fetus* subsp. *jejuni* to evaluate survival of this organism in several environmental and physiological milieus. Although these strains were minimally passaged, adaptation to artificial media may have affected the results we obtained, and these results may not be identical to those seen in nature with wild-type strains. Only the experiments with stools used "naturally" infected material and unpassaged strains.

Gastric acid is a potent barrier against ingested pathogens and is an important determinant of infectious dose (10). The effect is primarily a pH-hydrochloric acid-dependent mechanism; survival characteristics of *Salmonella typhimurium* are nearly identical in gastric acid and in HCl (9). In our study we found that HCl solutions with pH values of <3.0 adversely affected the survival of *C. fetus* subsp. *jejuni* to a significant degree, although solutions with pH values of >3.6 had virtually no effect on survival.

These data parallel the pattern for *S. typhimurium*, and we might speculate from this marked acid sensitivity that *Campylobacter* infection may result from being exposed to large numbers of organisms, from ingesting them in buffers, or from having rapid washthrough. Our data could help to explain the reports of *Campylobacter* infection associated with consuming milk (an excellent buffer) (2, 18) and water (rapid washthrough) (24) and are consistent with the few reports of person-to-person spread which generally involve smaller inocula. However, the importance of this acid sensitivity in vivo may be circumvented by hypochlorhydria in exposed individuals or complex stoichiometric interactions between bacterium and food.

*C. fetus* subsp. *jejuni* has been isolated from the biliary tracts of infected sheep (6), cattle (6), fowl (20) and rodents (M. J. Blaser, F. M. LaForce, and W.-L. L. Wang, unpublished data). Recently, a case of acute cholecystitis presumably caused by *C. fetus* subsp. *jejuni* was reported (16), and tissue from patients with *Campylobacter* enteritis has shown involvement of the small intestine (5, 13). Our data show that *C. fetus* subsp. *jejuni* can multiply and survive in bile for long periods at 37°C, which is consistent with the observed localization of the organism in the biliary tract and in the bile-rich upper small bowel during human infection. These data also suggest that adding bile to primary media might improve isolation of *C. fetus* subsp. *jejuni* from clinical specimens.

*C. fetus* organisms have been isolated from the genitourinary tracts of several mammalian species (22), and a recent report described a urinary tract infection in a human caused by *C. fetus* subsp. *jejuni* (8). In our study, survival in urine was brief at 37°C, which may correlate with the rarity of isolation of *Campylobacter* in human urinary tract infections. Survival of organisms in urine for several weeks at 4°C suggests that infected urine from animals might be a potential source of environmental contamination.

Our studies showed that patients with *Campylobacter* enteritis (1) shed *C. fetus* subsp. *jejuni* organisms in large numbers, similar to those associated with *Salmonella* and *Shigella* infections (23). In contrast, mice which are chronically colonized with *Campylobacter* shed $10^4$ to $10^6$ CFU/g of stool (M. J. Blaser, F. M. LaForce, and W.-L. Wang, unpublished data), and asymptomatic humans shed few if any (4).

Organisms in stools kept at environmental temperatures survived better at 4°C than at 25°C, although the difference may have been due to drying of specimens at 25°C. At 4°C, these bacteria survive for up to 3 weeks, which is consistent with an earlier report that *C. fetus* organisms survived for 20 days in sheep manure kept at 6°C (15).

Recently an outbreak of *Campylobacter* enteritis involving up to 2,000 persons was reported. Consumption of contaminated water was highly associated with illness, although *C. fetus* subsp. *jejuni* was not isolated from the implicated vehicle (24). In another study, *C. fetus* subsp. *jejuni* was isolated from several water sources in the environment (14). Raw water is a common vehicle for transmitting enteric infection, and our data suggest that *C. fetus* subsp. *jejuni* may live for up to 4 weeks in groundwater. The organisms survived longer at 4°C than at 25°C, suggesting that cold water may be a more effective vehicle when there has been a point source of contamination.

Cattle frequently carry *C. fetus* subsp. *jejuni* in their intestinal flora (22), and recently several outbreaks of *Campylobacter* enteritis due to consuming unpasteurized milk have been reported from the United States (2) and England (18). Our data suggest that *C. fetus* subsp. *jejuni* may remain viable in refrigerated milk for several weeks. Since milk is such an excellent buffer of gastric acidity, milk containing even a few organisms may be sufficient to initiate human infection.

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