Isolation of *Campylobacter jejuni* from Raw Milk

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Campylobacter jejuni was isolated from raw milk by a method that can routinely detect ≤1 organism per ml. This procedure was used in a survey of 195 separate farms and showed a 1.5% incidence of *C. jejuni* in milk from bulk tanks.

The first reported instance of human *Campylobacter* enteritis (12) implicated raw milk as the vehicle for the infective agent. Recently, raw milk has been frequently described as the vehicle for foodborne enteritis caused by *Campylobacter fetus* subsp. *jejuni* (*Campylobacter jejuni*) (2, 19), but the organism has been isolated only once from incriminated milk (14). Perhaps the difficulty is due to low concentrations of the organism in raw milk. Even low concentrations represent a health hazard (16) and are likely to survive in raw milk long enough to reach consumers (1, 3, 4, 6, 8). Pasteurization destroys this pathogen (4, 6).

Attempts at isolating *C. jejuni* from milk suspected as the vehicle of an enteritis outbreak have employed direct plating on selective agar (1) or a combination of selective enrichment followed by plating on selective agars (5, 14, 17). The single confirmation of a milkborne outbreak (14) employed an enrichment broth and agar made selective by the addition of antibiotics after the formulation of Skirrow (18) and incubation in a microaerobic atmosphere.

Recently, two methods were reported for isolating *C. jejuni* from raw milk (7; C. E. Park and Z. K. Stankiewicz, Abstr. 95th Annu. Meet. Assoc. Offic. Agric. Chem., 1981, abstr. no. 212) with recovery limits of <1.0 organisms per ml of milk. One of these methods (7) was used in a survey for *C. jejuni* in milk from nine farms of a university dairy herd. The organism was isolated from 0.9% of the farm bulk milk tank samples over a 3-month period and from 64% of fecal swabs from the same herd (8).

Our purpose in this study was twofold: (i) to develop a method capable of isolating *C. jejuni* present in low concentrations in raw milk, and (ii) to survey the incidence of *C. jejuni* in the raw milk supply of the area.

MATERIALS AND METHODS

Bacterial strains. Both animal- and human-derived strains of *C. jejuni* were used in developing and testing the method used for isolating *C. jejuni* from milk. The strains designated CH were from stool specimens of patients with diarrhea in Children's Hospital, Cincinnati, Ohio. Bovine strains, designated B, and one ovine strain, designated L, were supplied by B. D. Firehammer, Montana State University, Bozeman, Mont. Strains designated J were supplied by D. M. Jones, Withington Hospital, Manchester, England, and were identified as human strains obtained during investigations of milkborne outbreaks of *C. jejuni* enteritis. Human blood strains, designated HB, were supplied by Sabine Lauwers, Vrige University, Brussels, Belgium. In addition, one human strain was from the collection of C. E. Park, Department of Health and Welfare, Ottawa, Canada; one bovine strain was from Norman Stern, U.S. Department of Agriculture, Beltsville, Md.; one porcine strain was from Peter Turnbull, Central Public Health Laboratory, London, England; and a single chicken isolate was from our own collection. Contributed strains were passed once in our laboratory for purity and an identity check, grown up in brucella broth, and frozen at −70°C in 15% dimethyl sulfoxide.

***C. jejuni* recovery experiments.** Strains selected for use in recovery experiments were grown for 24 h in brucella broth at 42°C in 5% O_2–10% CO_2–85% N. Dilutions to produce the proper inoculum levels were made in portions of the milk to be inoculated. The actual concentration of *C. jejuni* in the inoculum was determined by diluting the original brucella broth culture in 0.1% peptone and plating on brucella agar containing ferrous sulfate sodium metabisulfite, and pyruvate, each at a concentration of 0.25 g/liter (FBP) (9). The concentration of indigenous microorganisms in raw milk was determined by a standard aerobic plate count (13). Uninoculated milk and enrichment broth (EB) culture of inoculated strains served as controls.

A 40-g amount of raw milk inoculated with a test strain of *C. jejuni* was weighed into a centrifuge tube and spun at 34,000 × *g* for 20 min at 4°C. The fat and aqueous layers were discarded. The remaining pellet was suspended in 100 ml of brucella broth supplemented with FBP, 15 mg of vancomycin per liter, 7.5 mg of trimethoprim per liter, and 5,000 U of polymyxin B per liter in a 250-ml Erlenmeyer flask. This EB culture was incubated at 42°C in the previously described gas mixture by delivering the gas through Teflon tubing (outside diameter, ⅛ in. [3.2 mm]) into the flask below the surface of the EB. The plug closure of the flask was inserted after the tube was in place, and the flask was further sealed with parafilm. The gas flow rate was adjusted to approximately 10 ml/min with a meter-

ing valve by observing the bubbling rate. Between runs, the Teflon tubing was stored in 70% ethanol and boiled in distilled water for 1 h before reuse.

After 24 h of incubation, 5 ml of the EB culture was removed and filtered through a 0.65-μm membrane. Both filtered and unfiltered EB cultures were streaked onto Skirrow selective agar (18) supplemented with FBP. Plates were incubated 24 h at 42°C in the previously described gas mixture provided by evacuating and refilling an anaerobic jar containing the plates three times.

After incubation, typical suspect colonies were picked for presumptive identification by phase microscopy of wet mounts. Presumptively identified colonies were streaked onto nonselective blood agar for purification. Colonies picked from nonselective agar were confirmed by growth and biochemical reactions previously described (15), with the addition of hippurate hydrolysis (11).

To test the inhibitory properties of our EB, 23 human-derived strains and 13 animal-derived strains of *C. jejuni* were inoculated into EB and incubated for 24 h at 42°C in the microaerobic atmosphere. Portions of each culture were diluted in 0.1% peptone, and 0.1 ml was spread on the surface of brucella agar supplemented with FBP. Counts were made after 48 h at 42°C in the microaerobic atmosphere. The original inoculum level was approximately 100 organisms per ml. A strain was judged to be inhibited if it failed to show a concentration increase of 2 logs in 24 h.

Farm survey. Raw milk samples were supplied by Milk Marketing, Inc., Cincinnati, Ohio, which serves several hundred farms in northern and central Kentucky, southwest Ohio, and southeast Indiana. Farm herd sizes varied from approximately 200 to as few as 10, averaging about 45 cows per herd.

The farm portion of this work was divided into two segments. From 16 February to 30 March 1982, 105 individual farm bulk milk tank samples were received and analyzed on the day of receipt by the schema

previously described. These raw milk samples were collected on the day before delivery to our laboratory and held at refrigeration temperature until analysis, usually within 36 h after collection. Milk Marketing was instructed not to duplicate farm samples, and this was monitored by checking code numbers that indicated route and farm.

From 17 May to 6 July 1982, an additional 105 individual farm samples were received and analyzed. Although the Milk Marketing staff was not instructed to prevent duplication of first-period samples during the second sampling, only 15 duplicates were in fact analyzed. Thus, overall, 195 separate farm bulk milk tanks were sampled during the survey. The same analysis procedure was used with one exception. After the second sampling period began, Milk Marketing changed the sample size, making only 25 to 30 ml available.

RESULTS AND DISCUSSION

The starting point in our effort to produce an enrichment medium for *C. jejuni* was the formulation used by Park et al. to isolate the organism from poultry (15). That formulation prevented the growth of many strains in our collection. We varied the components to arrive at the antibiotic concentration that would successfully inhibit competitors but minimally affect *C. jejuni*. Polymyxin proved to be the responsible inhibitory ingredient.

During this work, *C. jejuni* was never isolated from unfiltered EB when it was not also isolated from filtered EB. Plates streaked with filtered EB sometimes appeared as pure cultures of *C. jejuni*. In other instances, reduced concentrations of competing organisms on filtrate-inoculated plates made recognition and picking of *C.*

TABLE 1. Growth of *C. jejuni* strains in EB

Strain	Inoculum (cells per ml)	Final concn (cells per ml)	Strain	Inoculum (cells per ml)	Final concn (cells per ml)
HB1	120	1.5×10^8	CH3	290	1.3×10^7
HB2	42	1.7×10^8	CH4	24	ND
HB4	79	5.6×10^7	CH5	2	4.4×10^7
HB7	14	1.3×10^8	CH6	48	1.3×10^8
HB8	43	1.6×10^8	CH7	98	1.1×10^8
HB9	60	1.8×10^8	B1	270	1.6×10^8
HB11	36	6.3×10^2	B2	180	1.3×10^5
HB12	80	2.3×10^8	B3	42	6.5×10^7
HB14	4	4.0×10^1	B4	150	1.8×10^7
HB15	1	6.9×10^7	B5	6	ND
Park 6	200	5.0×10^7	B6	38	1.2×10^5
J1	18	1.1×10^8	B7	44	5.0×10^7
J2	32	8.4×10^6	B8	33	3.3×10^8
J3	32	ND[a]	B9	64	1.4×10^8
J4	8	8.9×10^7	B10 (Stern)	50	4.6×10^6
J5	120	1.0×10^4	L3	82	1.1×10^9
CH1	39	1.3×10^8	Porcine	96	ND
CH2	470	1.3×10^7	Chicken	84	9.8×10^7

[a] ND, Not detected.

TABLE 2. Recovery of *C. jejuni* added to raw milk

Strain	Detection level (cells per ml)	Standard plate count per ml of milk	Strain	Detection level (cells per ml)	Standard plate count per ml of milk
CH1	0.6	58,000	B1	1.0	48,000
	0.6	46,000		1.0	1,600,000
	0.6	140,000		10.0[a]	140,000
CH2	3.5	140,000	B2	0.7	150,000
	3.5	150,000		0.7	1,100,000
	3.5	64,000		0.7	48,000
CH3	2.4	380,000	B3	1.1	48,000
	2.4	150,000		1.1	1,600,000
	2.4	1,150,000		1.1	140,000
CH5	0.1	67,000	B4	1.7	150,000
	0.1	1,300,000		1.7	1,100,000
	1.2[a]	100,000		1.7	48,000
CH6	0.2	67,000	J1	0.7	41,000
	2.4[a]	1,300,000		0.7	6,200,000
	2.4[a]	100,000		0.7	13,000,000
CH7	0.9	380,000	J4	0.3	41,000
	8.9[a]	150,000		0.3	6,200,000
	0.9	1,500,000		0.3	13,000,000

[a] Lower concentration was negative. All other concentrations were lowest levels inoculated.

jejuni considerably easier. However, aware that others have shown a 1- to 2-log reduction in *C. jejuni* concentration from filtration (10), we recommend streaking both filtered and unfiltered EB.

In our examination of market poultry for *C. jejuni* (15), we grew accustomed to seeing the organism on blood agar as spreading growth that was barely discernable from surface moisture. This colony type could easily be missed by those inexperienced in *C. jejuni* isolation. The addition of FBP to the agar formulation of Skirrow eliminated that colony type. On this agar, the organism appeared as a clear to white or tannish convex colony with an irregular edge that sometimes spread along streak lines.

A total of 10 *C. jejuni* strains isolated from human blood, 13 strains isolated from human feces, and 13 animal-derived strains were tested for their ability to grow in EB. The results are shown in Table 1. Two blood-derived strains failed to increase 2 logs in 24 h, although some growth was noted in both strains. The overall inhibition rate for EB was 17%. Only the blood strains had no previous antibiotic exposure, so the inhibition rate for EB could be as high as 20%. Balancing this view is the observation that natural strains demonstrate less inhibition by polymyxin than do laboratory-passed strains (C. E. Park, personal communication).

The recovery of *C. jejuni* added to raw milk is shown in Table 2. When the inoculum was ≤1 cell per ml, the organism was recovered in 19 of 24 trials (83%). The best performance was the recovery of 0.1 cell per ml in the presence of 1.3 × 10⁶ competitors.

The survey of milk from bulk tanks recovered *C. jejuni* from 3 of 210 samples (1.4%), i.e., from 2 of the first segment of 105 samples and from 1 of the second 105 samples. Since 15 of the second 105 samples duplicated those previously sampled, we conclude that 195 separate farms were sampled, yielding three positive samples for a farm incidence rate of 1.5%.

We can only speculate on the effect of the reduced size of the last 75 samples. Whereas the small size undoubtedly reduced the recovery capability, it reduced the overall percentages insignificantly.

Our findings support those of Doyle and Roman (8), who reported a *C. jejuni* incidence of 0.9% from repeated samplings of nine farms in a university dairy herd. Our survey of 195 farms yielded a farm incidence rate of 1.5%. These studies, using two separate but sensitive methods, indicate that the incidence of *C. jejuni* in farm bulk milk is at the level of 1 to 2%.

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