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Pathogenesis of *Campylobacter fetus* Infections: Serum Resistance Associated with High-Molecular-Weight Surface Proteins

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Campylobacter fetus subspecies *fetus* causes both systemic and diarrheal illnesses. We studied 38 strains of *C. fetus* isolated from 34 patients; underlying illness was present in eight (89%) of nine patients with only systemic isolates compared with three (20%) of 15 patients with only fecal isolates ($P = .002$). In a standardized assay of susceptibility to normal human serum, 27 (71%) strains were resistant, six (16%) had intermediate susceptibility, and five (13%) were serum sensitive. Major protein bands migrating at 100 kDa or 125 kDa on polyacrylamide gels were present in all of the 25 serum-resistant strains tested but in only four of seven serum-sensitive isolates of *C. fetus* from humans and animals ($P = .007$). The presence of these bands was associated with type A lipopolysaccharide. A low-passaged strain, 82-40, was serum resistant and contained the 100-kDa protein; however, a spontaneous mutant of this strain lacked this band and was serum sensitive. The 100-kDa and 125-kDa proteins of three strains of *C. fetus* were antigenically cross reactive or identical and were exposed on the surface of the *C. fetus* cell. Serum resistance is inherent to most *C. fetus* isolates from humans and is associated with the presence of cross-reactive surface proteins

Campylobacter fetus subspecies *fetus* has been increasingly recognized in humans as an opportunistic pathogen causing extraintestinal infection [1-4], in contrast to the more-common *Campylobacter jejuni* that chiefly causes diarrheal illnesses [2]. Although most *C. fetus* infections reported in the literature have involved systemic sites in compromised hosts [1, 3, 4], recent reports suggest that *C. fetus* also can cause an acute diarrheal illness [5, 6]. Virulence mechanisms of *C. fetus* strains have been only partially explored [1, 7, 8], and there have not been studies comparing fecal and systemic isolates of *C. fetus*. *C. fetus* strains may be serotyped on the ba-

sis of heat-stable lipopolysaccharide (LPS) antigens [9, 10], but no serotype-specific differences in virulence have been found. McCoy et al. [8] had reported that strain 23D was not agglutinable in serogroup A antiserum unless boiled, but that a spontaneous mutant, strain 23B, was agglutinable without boiling. Subsequently, strain 23D was found to possess a protein microcapsule that was absent in strain 23B [11] and that enabled strain 23D to resist phagocytosis by bovine neutrophils.

For a variety of gram-negative pathogens, susceptibility of strains to the complement-mediated bactericidal activity in normal mammalian serum is associated with infection or colonization confined to mucosal surfaces, whereas serum resistance is associated with systemic spread [12, 13]. In previous studies [14], we found that, as anticipated by this model, most fecal isolates of *C. jejuni* were serum sensitive, whereas four isolates of *C. fetus* from systemic sites were serum resistant [14]. Because *C. fetus* can produce infection restricted to the intestinal tract, we now question whether fecal isolates are serum resistant. An alternative hypothesis is that differences in clinical outcome of *C. fetus* infections may be due to differences in host characteristics. The

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goals of this study were to (1) determine the relation of host characteristics to the site of *C. fetus* infection, (2) determine whether fecal isolates of *C. fetus* are serum resistant, and (3) assess which bacterial characteristics are associated with serum resistance.

Materials and Methods

Bacterial strains. We reviewed the records of the Denver *Campylobacter* laboratory culture collection to identify all isolates of *C. fetus* subsp. *fetus* from humans. Isolates were from patients in Colorado or had been received from clinical laboratories between 1979 and 1985. We examined all isolates from sporadic cases of *C. fetus* infection, four isolates from an outbreak of gastroenteritis in Wisconsin [15], and three isolates from an outbreak of gastroenteritis and meningitis in New York [16]. For comparative purposes, we examined nine isolates of nonhuman origin, including bovine strains 23D and 23B [8] supplied by Dr. Alex Winter (Cornell University); strains 81-170, 84-90, and 84-04 that had been described in previous studies [8, 10, 14, 17]; and reptile isolates 85-387, 85-388, and 85-389 supplied by Dr. Sidney Harvey (Irvine Diagnostic). All isolates were identified as *C. fetus* subsp. *fetus* according to standard criteria including cell morphology, growth at 25 C, susceptibility to naladixic acid, resistance to cephalothin, and inability to hydrolyze hippurate [18]. Strains were passaged four or fewer times on artificial media before use in these studies; bacterial suspensions were prepared as previously described [14]. For comparative studies, we also used *C. jejuni* strain 79-193, a serum-sensitive fecal isolate from a patient with acute enteritis [14]. All *C. fetus* strains were serotyped on the basis of their heat-stable (LPS) antigens, as previously described [10].

Clinical information. Medical records were reviewed for the 34 patients from whom *C. fetus* was isolated to determine demographic features, predispositions to *C. fetus* infection, and nature of illness produced; information was incomplete for seven patients.

Serum susceptibility. The susceptibility of the test strains of *C. fetus* to the bactericidal activity present in pooled serum from normal adult men was assessed in a standardized assay, as previously described [14]. *C. fetus* strains resistant to killing by 10% normal human serum when incubated for 60 min were incubated in 67% serum for 240 min. Because normal human serum does not contain spe-

cific antibodies to *C. fetus* [19], we incubated *C. fetus* strain 82-40 with hyperimmune rabbit sera to live, whole *C. fetus* strain 82-40 and 81-170 cells, as previously described [10]. To visually assess bacteria-serum interactions, we incubated *C. jejuni* strain 79-193 and *C. fetus* strain 82-40 for 60 min at 37 C, with 50% pooled or heat-inactivated (56 C for 30 min) normal human serum in Medium 199 (GIBCO, Grand Island, NY). Electron microscopy was performed according to the method of Harriman et al. [20].

Production of antisera. Adult New Zealand white rabbits were inoculated sc with formalin-killed bacterial suspensions (10^8 cells/ml) from 24-hr plate cultures, as previously described [21]. Preimmune sera were used as control sera; postimmunization sera showed titers $>1:320$ by an indirect immunofluorescence assay or showed significant reactivity on western blots against homologous whole cell preparations [21]. Some antisera were adsorbed with homologous or heterologous whole cell suspensions as follows. Growth from one plate was suspended in sterile distilled water and centrifuged (12,000 g for 15 min); the pellet was mixed with 1.0 ml of rabbit serum and incubated (37 C for 1 hr). Antibodies to the bacterial suspension were removed by recentrifugation, and the supernatant was reabsorbed five times.

PAGE. To assess the relation between protein profiles and serum susceptibility, we prepared solubilized, whole *C. fetus* cells for SDS-PAGE, as previously described [22]. Because a *C. fetus* strain (23D) isolated from an infected cow was reported to have an acid-extractable microcapsular surface protein, we followed the method of McCoy et al. [8, 11] for treating cells of other *C. fetus* strains to extract this material. The extracts were then subjected to SDS-PAGE, as described [22]. After electrophoresis at 35 mA for 2 hr, gels were fixed and proteins resolved by silver stain.

Immunoblot procedure. The methods of Towbin et al. [23], as modified by Burnette [24], formed the basis for the western blot procedure we used, as previously described [21, 25]. In brief, SDS-PAGE-separated components were transferred from the slab gel to nitrocellulose paper soaked in electrode buffer by electroblotting for 12 hr at 100 mA. After non-specific binding was blocked with bovine serum albumin (Sigma Chemical, St. Louis)-borate buffer, the nitrocellulose paper was incubated (25 C for 4 hr) in a 1:100 or 1:200 dilution of the test serum sample in bovine serum albumin-borate buffer. Af-

ter washing, the nitrocellulose paper was incubated (25 C for 2 hr) with a 1:1,000 dilution of horseradish peroxidase-conjugated *Staphylococcus aureus* protein A (Amersham, Arlington Heights, Ill). After being washed, the nitrocellulose paper was developed in diaminobenzidine solution.

Radioiodination of intact *C. fetus* cells. *C. fetus* cells were extrinsically radiolabeled, as previously described [22], with the following modifications. In brief, cells from 24-hr cultures grown confluent on trypticase-soy sheep blood agar plates (PASCO, Wheatridge, Colo) were added to Dulbecco's PBS to make a suspension of standard OD. Cells from this suspension were pelleted, and Dulbecco's PBS and 1 mM potassium iodide were added. The suspension was transferred to an Iodogen®-coated (Pierce Chemical, Rockford, Ill) tube, incubated with 500 µCi of ¹²⁵I for 10 min at 25 C, then washed in Dulbecco's PBS, and resuspended in Tris (pH 7.4).

Radioimmunoprecipitation. The radioimmunoprecipitation procedure used to identify surface-exposed antigens of *C. fetus* was similar to a previously described method [25]. Extrinsically ¹²⁵I-radiolabeled whole *C. fetus* cells were washed and then incubated (4 C for 18 hr) with immune rabbit serum. After centrifugation, the resultant cell pellet was suspended in solubilization buffer for 1 hr and recentrifuged; the supernatant was then drawn off. At least 60% of the initial radioactivity added to the system was recovered in this supernatant that was then incubated with formaldehyde-treated, protein A-containing *S. aureus* cells. The resultant complexes containing *S. aureus*, antibody, and membrane were pelleted and washed five times with W buffer [25]. The *S. aureus*-adsorbed complexes containing antibody and membrane were recovered by suspending the washed pellet in D buffer [25], heating this suspension at 100 C for 4 min, and pelleting out the *S. aureus* cells. In all cases, >90% of the radioactivity originally bound to *S. aureus* was recovered in the supernatant fluid. The supernatant material was then boiled in sample buffer containing 2-mercaptoethanol and SDS, and the radioiodinated antigens present were resolved by SDS-PAGE. Gels were silver stained [21], dried, and processed for autoradiography, as previously described [25].

Hydrophobicity assay. The method used to measure adherence of bacterial cells to hexadecane droplets has been described [26]. In brief, *C. fetus* cells from 48-hr cultures on blood agar were harvested in phosphate-urea-magnesium (PUM) buffer

Table 1. Serum susceptibility of *C. fetus* isolates from humans, by isolation site.

Site	No. of isolates examined	Completely resistant (<.10*)	Intermediate (0.10-0.99)	Sensitive (≥1.0)
Bloodstream	15	9	2	4
Other				
systemic	5	4	1	0
Feces	18	14	3	1
Total	38	27	6	5

* Log₁₀ kill in standardized bactericidal assay with pooled normal human serum, as determined by using previous definitions of serum susceptibility [14].

[26], washed, and resuspended in PUM to an OD of 1.0 at 400 nm. To 5 ml of the aqueous phase, we vigorously mixed 2.5 ml of hexadecane, and after 30-min incubation at room temperature, we compared the OD of the aqueous phase with that of a control suspension not mixed with hexadecane. Each assay was done six times.

Results

Characteristics of patients from whom *C. fetus* strains were isolated. We obtained clinical information pertinent to 38 isolates of *C. fetus* from humans; 17 isolates were from feces, 16 were from the bloodstream, and 5 were from other sites. Twenty-five (76%) isolates had serotype A LPS, whereas eight (24%) had non-A serotypes (six were B and two were AB). Fourteen isolates were from Switzerland, and 24 isolates were from various areas in the United States; isolates were from 34 patients including two each from four patients. The ages of the patients ranged from three days to 87 years (median, 35 years), and 16 of the patients were women. Among the nine patients with only systemic isolates and for whom information could be obtained, eight (89%) had a known underlying disease including malignancies, renal failure, and alcoholism. In contrast, among 15 patients with only fecal isolates, three (20%) had an underlying illness, a significantly ($P = .002$, Fisher's exact test) lower rate. Among the total of 17 patients who had fecal isolates, two had diarrhea lasting two weeks or longer, and 15 others had an acute diarrheal illness (three with bloody diarrhea). Among the total of 16 patients from whom systemic isolates were made, two (13%) had diarrheal illnesses. Two patients had thrombophlebitis, and two patients



Figure 1. Electron photomicrographs of *Campylobacter* cells after incubation with 50% human serum for 60 min at 37 C. After incubation, cells were pelleted, washed in veronal-buffered saline, set on formvar carbon-coated grids, air dried, and stained with uranyl formate. Bar = 1 μ m. *Left*, *C. jejuni* 79-193 with heat-inactivated normal human serum. *Middle*, *C. jejuni* 79-193 with normal human serum. *Right*, *C. fetus* 82-40 with normal human serum.

died. Two of the patients with fecal isolates received erythromycin, to which their strains were susceptible, and recovered rapidly.

Serum susceptibility of the *C. fetus* isolates. In the standardized bactericidal assay used, 5 (13%) of the 38 human isolates tested were serum sensitive ($\geq 1.0 \log_{10}$ killing), six (16%) isolates had intermediate serum susceptibility (0.1–0.99 \log_{10} killing), and 27 (71%) were serum resistant ($< 0.1 \log_{10}$ killing; table 1). All isolates from both clusters of infection were serum resistant; eliminating all but the index cases from analysis [15, 16], 22 (67%) of the remaining 33 strains of *C. fetus* studied were serum resistant. Two isolates each from four patients were examined. In three pairs, both isolates were serum resistant, and from the fourth patient, the fecal isolate had intermediate susceptibility, whereas the isolate from the bloodstream was serum resistant. We confirmed the absence of damage to our prototypic, serum-resistant *C. fetus* strain 82-40 by using electron microscopy. Incubating a serum-sensitive strain,

C. jejuni 79-193, with heat-inactivated serum showed intact rod and curved forms (figure 1, left), but incubation with normal serum resulted in a variety of conformational changes including swelling, poor staining, and loss of rodlike structure with both irregular and rounded forms (figure 1, middle). In contrast, *C. fetus* 82-40 incubated with normal serum showed fully intact, well-defined cells (figure 1, right).

Susceptibility under more-stringent conditions. To further assess serum susceptibility, we incubated eight strains of *C. fetus* with 66% pooled normal human serum for 240 min. For three strains, in which the standard assay showed $< 0.01 \log_{10}$ killing, incubation under the more-stringent conditions showed no killing (data not shown). For five strains for which susceptibility in the standard bactericidal assay ranged from less than 0.01 to 0.73 \log_{10} killing, increasing the serum concentration to 66% produced from 0.07 to 2.16 \log_{10} killing, and after 240-min incubation in 66% serum, \log_{10} killing ranged from

Table 2. Susceptibility of a human-serum-susceptible strain of *C. jejuni* and a human-serum-resistant strain of *C. fetus* to normal and immune rabbit sera.

Serum source	Log ₁₀ killing in 60 min	
	<i>C. jejuni</i> (79-193)	<i>C. fetus</i> (82-40)
Medium 199 control	<.01	<.01
Normal rabbit serum	4.14	<.01
Immune serum to <i>C. fetus</i>		
82-40 (homologous)	4.35	<.01
Immune serum to <i>C. fetus</i>		
(heterologous)	1.25	<.01

1.09 to 3.55; the two most serum-resistant strains in the standard assay were least affected. We further characterized the susceptibility of a completely resistant strain, *C. fetus* 82-40, to hyperimmune rabbit serum. As expected, both normal rabbit serum and the two immune rabbit sera produced >1.0 log₁₀ killing of a known serum-sensitive strain of *C. jejuni* (table 2). The *C. fetus* strain was, however, completely resistant to both the homologous and heterologous immune sera. Addition of serum with full complement activity (from a hypogammaglobulinemic patient) to heat-inactivated immune serum also did not permit killing of this organism (data not shown).

Outer membrane proteins of *C. fetus* strains. We first examined cellular proteins of 10 strains of *C. fetus* by using SDS-PAGE. All six serum-resistant strains and one strain with intermediate susceptibility, but none of three sensitive strains, showed a major band migrating at ~100 kDa or 125 kDa. These high-molecular-mass bands, shown in figure 2 (strains 79-22 [100 kDa], 80-109 [125 kDa], 82-40 low-passage [100 kDa], and 23D [100kDa]), also were present in acid-extractable protein preparations from these strains, usually in higher concentrations, but were not present in the preparations from the serum-susceptible strains. All *C. fetus* subsp. *fetus* strains showed similar profiles in the region below 70 kDa, with the major outer membrane proteins migrating at 45 kDa and 47 kDa and the flagellar proteins migrating at 63 kDa [22] (figure 2). Pronase or proteinase K treatment of whole cell lysates [17] of five strains removed the 100-kDa and/or 125-kDa band from each.

Relation of outer membrane protein, LPS type, and serum susceptibility. In subsequent studies, we examined 33 isolates of *C. fetus* from humans and added seven animal isolates (table 3). Twenty-eight

(70%) isolates had type A LPS, and 12 (30%) had non-A serotypes (nine were serotype B and three were AB; table 4). None of the type A strains were serum susceptible versus 58% of the non-type A strains ($P = .00004$, by Fisher's exact test). Conversely, 79% of type A strains but only 25% of type B strains ($P = .002$, by Fisher's exact test) were fully serum

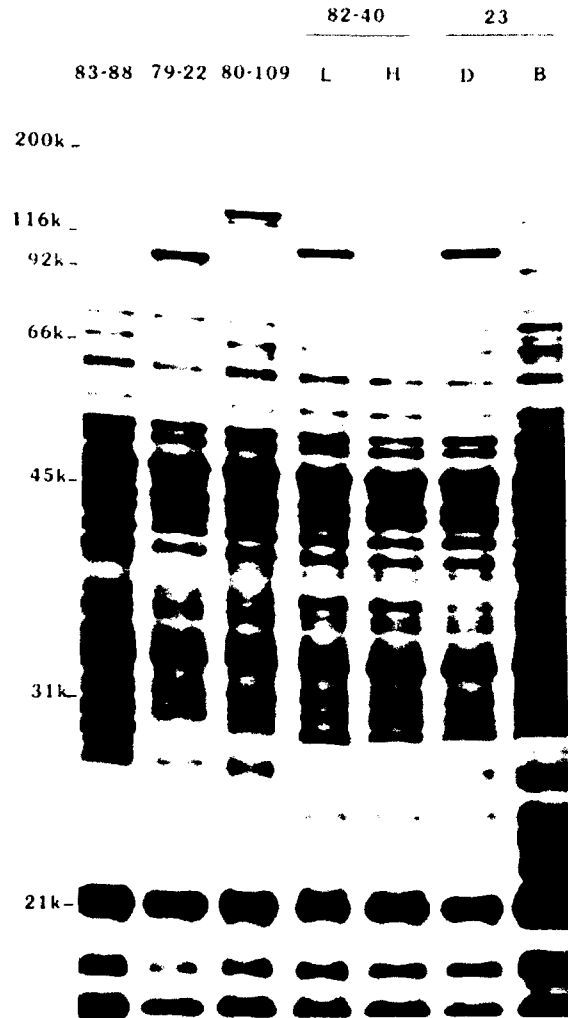


Figure 2. SDS-PAGE in 10% acrylamide of whole cell preparations from human and animal isolates of *C. fetus*. Materials were prepared as described [22], and ~1.0 µg of protein was loaded per lane; after electrophoresis, gels were silver stained. Molecular masses of marker proteins (in kilodaltons) are shown at the left [22]. Preparations from *C. fetus* strains (and serum susceptibility of the strain) are as follows: 83-88 (susceptible), 79-22 (intermediate), 80-109 (resistant), 82-40 low passage (L; resistant), 82-40 high-passage (H; susceptible), 23D (resistant), 23B (sensitive).

Table 3. Characteristics of *C. fetus* strains of nonhuman origin.

Strain no.	Source	Isolation site	LPS serotype	Presence of high-molecular-weight band	Log ₁₀ kill in bactericidal assay
81-170	Bovine	Fetus	B	No	2.50
84-32 (23D)	Bovine	Fetus	A	Yes	<.01
84-90	Bovine	Fetus	B	No	3.93
84-104	Monkey	Blood	AB	Yes	<.01
85-387	Reptile	Feces	B	Yes	.13
85-388	Reptile	Feces	A	Yes	.03
85-389	Reptile	Feces	A	Yes	.10
84-54 (23B)	Reference [8]		A	No	2.10
82-40 HP	This study†		A	No	1.02

* Laboratory mutant of strain 23D.

† Laboratory mutant of strain 82-40 LP.

resistant. In a separate analysis, high-molecular-weight bands were present on SDS-PAGE in all 25 serum-resistant strains, 88% of the intermediate strains, and 57% of the serum-sensitive strains. The difference in proportion among the resistant and sensitive strains is statistically significant ($P = .007$, by Fisher's exact test). All four strains with high-molecular-weight proteins that were serum sensitive were not type A.

Analysis of spontaneously mutated strains. In pursuing this analysis, we noted the absence of the 100-kDa band in SDS-PAGE of whole cell and acid-extractable preparations of a high-passage (HP) strain of *C. fetus* 82-40, whereas preparations from earlier passages had shown a 100-kDa band. This high-passage strain had been cultured many times on artificial medium and now was serum sensitive (1.02 log₁₀ kill). In contrast, the original frozen stock culture of 82-40 (low passage strain [LP]) was serum resistant (<.01 log₁₀ kill) and had the 100-kDa band (figure 2). The only differences between the two

strains by SDS-PAGE were the presence on the low-passage strain of the 100-kDa band and a minor 28-kDa band. LPS profiles [17] of the two strains were identical (data not shown), and on the basis of heat-stable (LPS) antigens [10], both strains were serotype A. Using the hexadecane adherence assay [26], we found that the aqueous-phase retention of strain 82-40 HP was 72.6% ± 1.0%, whereas retention of strain 82-40 LP was 26.4% ± 2.7% ($P < .001$, Student's *t* test).

We found that McCoy's microcapsule-containing strain 23D [8, 11] was resistant to 66% serum for 240 min, whereas strain 23B that lacked the capsule was killed within 1 hr by 10% normal human serum (figure 3). By SDS-PAGE, strain 23D possesses a band migrating at 100 kDa, whereas this is absent in strain 23B (figure 2); a minor 28-kDa band is also present on strain 23D. For strain 23B, there is enhancement of the 45-kDa band.

Western blotting of *C. fetus* strains. The antigenicity of cells of three strains of *C. fetus* containing

Table 4. Relation of serum susceptibility of *C. fetus* strains, LPS type, and presence of high-molecular-weight, acid-extractable surface proteins.

LPS type	LPS type and presence		No. of strains by serum susceptibility			Total
	High-molecular-weight band present	Resistant (< 0.1)*	Intermediate (0.1-0.99)	Sensitive (≥1.0)		
A	Yes	22	5	0	27	
	No	0	1	0	1	
Non-A†	Yes	3	2	4	9	
	No	0	0	3	3	
Total	Yes	25	7	4‡	36	
	No	0	1	3‡	4	

* Log₁₀ kill in standardized assay [14].

† Types B and AB that are unrelated to type A but strongly cross reactive with one another [10].

‡ Compared with resistant strains, $P = .007$, Fisher's exact test.

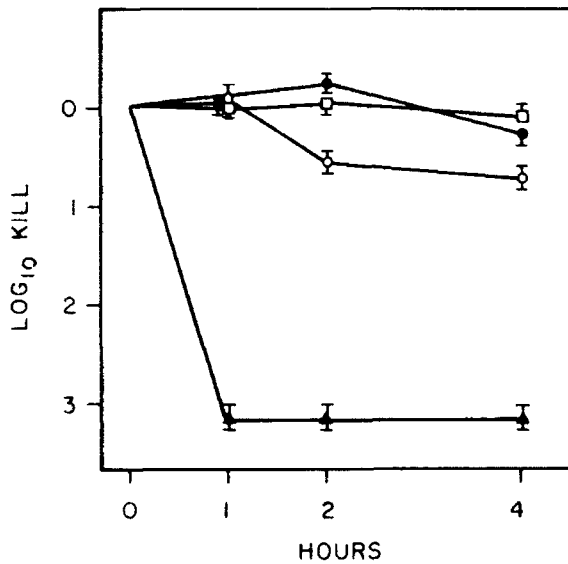


Figure 3. Susceptibility of *C. fetus* strains 23D and 23B to normal human serum. Overnight cultures of strains 23D and 23B were incubated in Medium 199 alone or with 10% or 66% pooled serum from healthy uninfected men. Bacterial counts were determined by serial dilutions and replicate plating just before adding serum and after 1-hr, 2-hr, and 4-hr incubations at 37 C, as described [14]. Points represent mean \log_{10} killing, and bars indicated SE for (triplicate determinations). ○, 23B control; □, 23D control; ▲, 23B 10% serum; ●, 23D 66% serum.

either the 100-kDa (23D, 82-40 LP) or 125-kDa (81-200) band was assessed by using western blotting (figure 4). We used antisera to strains 23D and 82-40 LP that were studied unadsorbed (lanes a and g) or after adsorption with five *C. fetus* strains. Unadsorbed antisera recognized the 100-kDa or 125-kDa bands for each of the three strains, whereas adsorption with cells of any of these three strains essentially abolished reactivity to this band and most other bands (lanes b, d, f, h, j, l). In contrast, adsorption of the antisera with the mutant strains lacking the 100-kDa band (strains 23B and 82-40 HP) had little effect on the reactivity to any band (lanes c, e, i, k). In studies not shown, western blotting of strains 23B and 82-40 HP with the same sera used in figure 3 showed that no immunoreactive band was present at 100 kDa, a result confirming the SDS-PAGE results shown in figure 2.

Surface exposure of the 100-kDa bands. To determine if the high-molecular-weight bands were surface exposed on intact *C. fetus* cells, we extrinsically labeled these cells with ^{125}I . One hypothesis tested by this study was whether the 100-kDa bands seen in the two serum-sensitive strains, 84-91 and 84-107, were indeed surface exposed. We found, however, that the 100-kDa bands seen by silver staining were always labeled by ^{125}I , a phenomenon indicating their

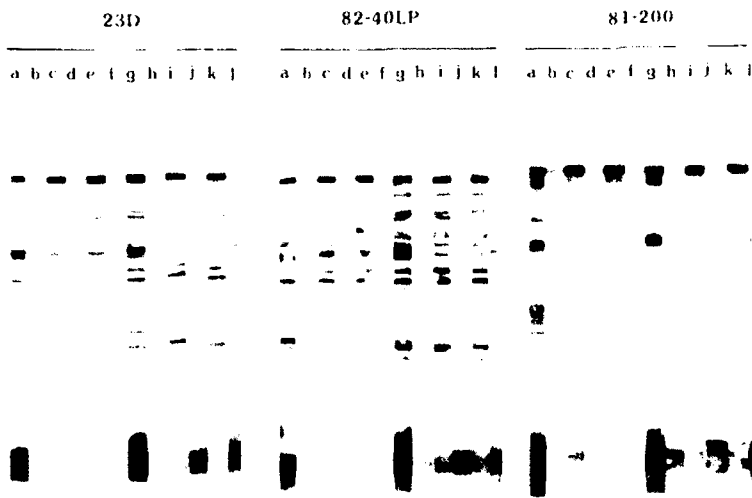


Figure 4. Immunoblot of *C. fetus* whole cells with unadsorbed and adsorbed antisera to *C. fetus*. Cells are from *C. fetus* strains 23D, 82-40 LP, and 81-200. SDS-PAGE was done in 10% acrylamide, and cellular components were electrotransferred to nitrocellulose paper. Antisera are to *C. fetus* 23D (lanes a-f) and 82-40 LP (lanes g-l). Antisera are unadsorbed (lanes a and g) or adsorbed with 23D cells (lanes b and h), 23B cells (lanes c and i), 82-40 LP cells (lanes d and j), 82-40 HP cells (lanes e and k), or 81-200 cells (lanes f and l). Sera were diluted 1:100, and the conjugate used was horseradish peroxidase-conjugated staphylococcal protein A. Adsorbing sera with 23B or 82-40 HP cells did not remove reactivity to either the 100-kDa band in strains 23D or 82-40 LP or the 125-kDa band in strain 81-200. However, adsorbing the sera with 23D, 82-40 LP, or 81-200 cells removed essentially all reactivity to these bands.

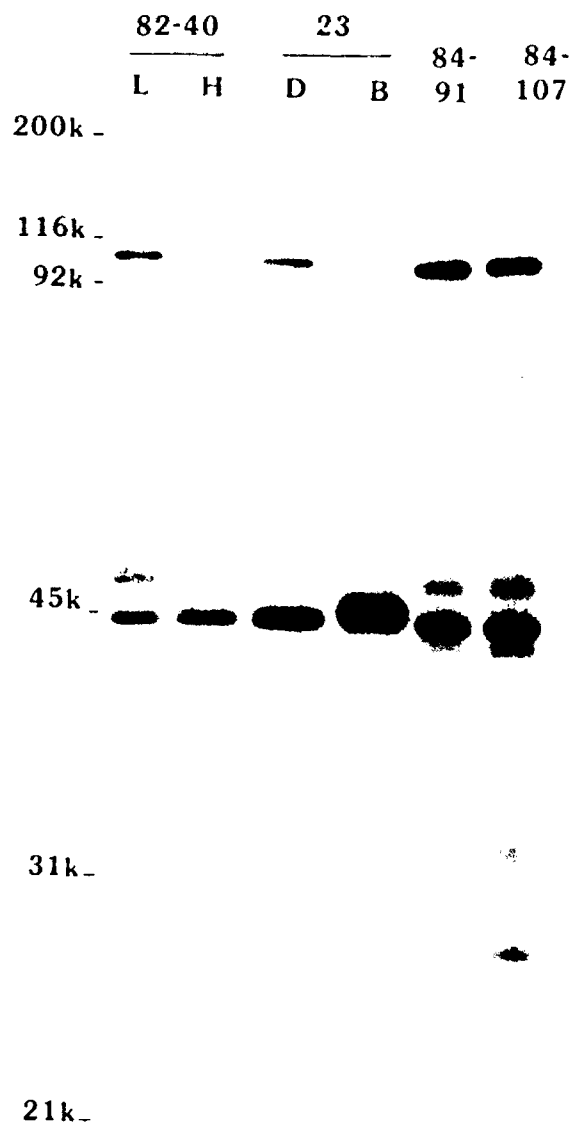


Figure 5. Autoradiograph of ^{125}I -labeled strains of *C. fetus*. Intact whole cells were extrinsically labeled with ^{125}I ; the cells were then solubilized and electrophoresed in 10% acrylamide SDS-PAGE. Cells were from *C. fetus* strains 82-40 low passage (L), 82-40 high-passage (H), 23D, 23B, and the two serum-sensitive, non-group A strains 84-91 and 84-107. Each strain that had a 100-kDa band present on silver staining also had a 100-kDa band labeled with ^{125}I .

surface exposure (figure 5). As expected, no significant labeling was seen in the 100-kDa region for strains 82-40 HP and 23B. In gels not shown, the 125-kDa band of strain 81-200 was also labeled.

Radioimmunoprecipitation. By performing radioimmunoprecipitation, we then attempted to de-

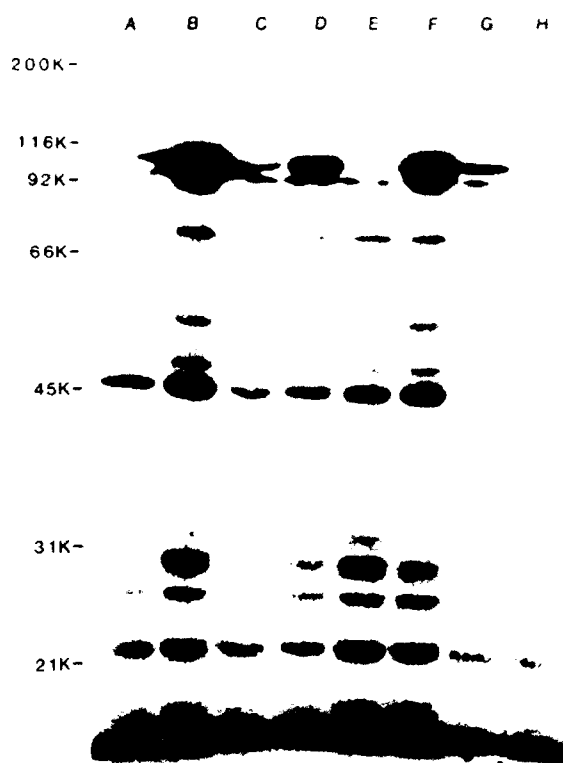


Figure 6. Radioimmunoprecipitation of ^{125}I -labeled cells of *C. fetus* 23D with immune and control rabbit serum. Serum samples were mixed with the radioiodinated cells, and the mixture was solubilized. Antigen-antibody complexes were isolated by adsorption to protein A-containing *S. aureus* cells and processed by SDS-PAGE followed by autoradiography to detect radiolabeled protein antigens, as described in Materials and Methods. Sera (diluted 1:100) were from rabbits immunized with *C. fetus* strains 23B (lane A), 23D (lane B), 80-109 (lane D), 81-170 (lane E), 82-40 LP (lane F), *C. jejuni* strain 79-193 (lane C), normal rabbit serum (lane G), and a saline control without serum (lane H).

termine if these ^{125}I -labeled, surface-exposed proteins were immunologically cross-reactive. Radiolabeled cells of strain 23D were incubated with a variety of immune and control sera, cellular constituents were solubilized, and those with reactive antibodies were precipitated by protein A-containing *S. aureus*, as previously described [25]. By SDS-PAGE and autoradiography (figure 6), it is clear that the surface-exposed, radiolabeled 100-kDa band of 23D is recognized by immune serum to strains 23D (lane B), 82-40 LP (lane F), and 80-109 (lane D). Immune serum to *C. fetus* strains 23B and 81-170 that were lacking the 100-kDa or 125-kDa band and control se-

rum showed minor reactivity, which may have represented spillover from adjacent lanes. In other gels, normal rabbit serum showed no reactivity to *C. fetus* proteins. In studies not shown, as expected there was no reactivity in the 100-kDa region for strain 23B with antisera to any organism. These studies confirm the western blot results and specifically show that surface-exposed 100-kDa bands are antigenically cross reactive. That antiserum to the 125-kDa-containing strain 80-109 recognized the 100-kDa band on strain 23D further suggests an antigenic similarity between these two proteins.

In our next studies, we asked whether the surface-exposed, 100-kDa bands of serum-sensitive strains 84-91 and 84-107 could also be precipitated. We found that these bands were precipitated by immune serum to strain 23D, but not 23B, and were not precipitated by normal rabbit serum (data not shown). This study further illustrates that the 100-kDa bands for these two serum-sensitive strains were extremely similar or identical to the 100-kDa bands on the serum-resistant strains.

Discussion

Most systemic *C. fetus* infections that we report occurred in compromised hosts, whereas, similar to *C. jejuni*, enteric *C. fetus* infections occurred most often in normal hosts. In a summary of surveillance of *Campylobacter* infections in the United States [27], 63% of *C. fetus* isolates were from the bloodstream. Twelve (75%) of 16 isolates from blood but only one (8%) of 13 stool isolates were from persons 55 years of age or older. In a review of 50 patients with bloodstream infections due to *C. fetus*, 31 (62%) had a predisposing condition [1], and 13 (26%) had diarrhea, findings similar to ours. Host characteristics thus appear to be important determinants of whether systemic *C. fetus* infections will occur.

We also found that resistance to bactericidal activity present in normal human serum was a common property of *C. fetus* strains, whether they were isolated from systemic sites or from feces. The strains studied were from symptomatic patients; we have no data on strains from asymptomatic *C. fetus* infections [6]. That the resistance of some *C. fetus* strains to mammalian serum is high grade is evidenced by failure of hyperimmune rabbit or human [14] serum to kill these strains. Several strains were completely serum resistant in the stringent assay, whereas for

others the resistance could be overcome by prolonging the incubation period and increasing the serum concentration. Therefore, as with *C. jejuni* isolates from humans, a range of serum susceptibilities was observed for *C. fetus*, although as a group the *C. fetus* isolates were more serum resistant than were *C. jejuni* isolates [14, 28]. Because *C. jejuni* isolates are largely serum sensitive [14], the infrequency of extraintestinal infection is understandable; however, the serum resistance found in fecal isolates of *C. fetus* leads to the question of why these have not caused disseminated infections. One hypothesis is that the serum resistance of *C. fetus* is not sufficient to cause systemic infections. Host defects, most notable those involving impaired phagocytosis by the reticuloendothelial system, T cell dysfunction, or a site of sequestration for the organism, are common antecedents to systemic *C. fetus* infection [1-4] and may be the necessary cofactors. Three of the four patients with isolates from the bloodstream that were serum-sensitive strains of *C. fetus* had malignant conditions. An association of bloodstream infection due to serum-sensitive strains and host compromise has been previously noted for *C. jejuni* [28].

Because the electron microscopic studies illustrated the complement-mediated damage to *C. jejuni* and the absence of damage to *C. fetus*, we then studied characteristics that might permit *C. fetus* strains to resist complement-mediated killing. For other gram-negative organisms, two factors associated with serum resistance are smooth-type LPS with long polysaccharide side chains [29] and outer membrane capsular-type proteins [30, 31]—either of which may hinder complement binding to vital cell-surface targets [32]. Both type A and non-type A strains of *C. fetus* have smooth-type LPS with long polysaccharide side chains [10]; however, in this study we found a correlation between serum resistance and type A LPS, whereas the majority of non-type A strains were serum sensitive. We also found an association between serum resistance among *C. fetus* strains and the presence of high-molecular-weight, acid-extractable surface proteins. The central question is whether LPS structure or presence of high-molecular-weight surface protein, or a combination of these factors is essential for serum resistance. The most-convincing evidence came from the accidents of nature in which during the course of serial in vitro passage, strains 82-40 and 23D lost their 100-kDa bands without any other significant change in protein or type A lipopolysaccharide structure and be-

came serum sensitive. That the low-passaged, serum-resistant strain containing the 100-kDa protein has a hydrophobic surface, whereas the high-passaged, serum-sensitive strain lacking the 100-kDa protein has a hydrophilic surface [26] further confirms the superficial localization and biologic significance of this protein.

The immunoblotting studies clearly showed the antigenic similarities or identity of the 100-kDa and 125-kDa bands from three serum-resistant strains of *C. fetus*. In contrast, because adsorption with the isogenic, serum-sensitive mates of two of the strains did not remove the reactivity of the antisera to the 100-kDa or 125-kDa bands, it is unlikely that these or cross-reacting antigens are present on their cell surfaces. The radioimmunoprecipitation studies support this observation because immune serum to several serum-resistant strains containing bands precipitated the 100-kDa protein from strain 23D but not 23B.

McCoy et al. [8, 11] described a capsular glycoprotein that enabled *C. fetus* to resist phagocytosis by bovine neutrophils; this capsule was not present on strain 23B that was fully susceptible to phagocytosis. Our study has shown that the only difference between strains 23D and 23B by SDS-PAGE is the 100-kDa band, which presumably represents the capsular protein and which is present on nearly every strain of *C. fetus* isolated from humans. However, despite the association between serum resistance and the presence of high-molecular-weight surface proteins for *C. fetus*, the presence of these proteins on serum-sensitive strains is not easily explained. These proteins on the serum-sensitive strains migrate at 100-kDa, are surface exposed, and are antigenically cross reactive with the proteins of the serum resistant strains. One possibility is that despite these similarities, the proteins themselves are intrinsically different from those on the serum-resistant strains. Attempts at purifying these proteins, which are ongoing in our laboratory, should be able to answer this question. A second explanation is that the proteins of the serum-sensitive and serum-resistant strains are identical but that their concentration, localization, or relation with deeper outer membrane structures is different. Supporting this hypothesis is that the serum-sensitive strains have LPS types (B and AB) that are not commonly found on *C. fetus* isolates from humans and that are usually associated with serum sensitivity [10]. It is possible that for a *C. fetus* strain to be fully serum resistant, both the typi-

cal type A LPS and one of the high-molecular-weight proteins must be present.

References

- Guerrant RL, Lahita RG, Winn WC Jr, Roberts RB. Campylobacteriosis in man: pathogenic mechanisms and review of 91 bloodstream infections. *Am J Med* 1978; **65**:584-92.
- Blaser MJ, Reller LB. *Campylobacter* enteritis. *N Engl J Med* 1981; **305**:1444-52.
- Bokkenheuser V. *Vibrio fetus* infection in man. I. Ten new cases and some epidemiologic observations. *Am J Epidemiol* 1970; **91**:400-9.
- Francioli P, Herzstein J, Grob J-P, Vailotton J-J, Mombelli G, Glauser MP. *Campylobacter fetus* subspecies *fetus* bacteremia. *Arch Intern Med* 1985; **145**:289-92.
- Harvey SM, Greenwood JR. Probable *Campylobacter fetus* subsp. *fetus* gastroenteritis. *J Clin Microbiol* 1983; **18**:1278-9.
- Quinn TC, Goodell SE, Fennell C, Wang S-P, Schuffler MD, Holmes KK, Stamm WE. Infections with *Campylobacter jejuni* and *Campylobacter*-like organisms in homosexual men. *Ann Intern Med* 1984; **101**:187-92.
- Corbeil LB, Corbeil RR, Winter AJ. Bovine venereal vibriosis: activity of inflammatory cells in protective immunity. *Am J Vet Res* 1975; **36**:403-6.
- McCoy EC, Doyle D, Burda K, Corbeil LB, Winter AJ. Surface antigens of *Campylobacter (Vibrio) fetus*: characterization of an antiphagocytic component. *Infect Immun* 1975; **11**:517-25.
- Berg RL, Jutila JW, Firehammer BD. A revised classification of *Vibrio fetus*. *Am J Vet Res* 1971; **32**:11-22.
- Perez-Perez GI, Blaser MJ, Bryner JH. Lipopolysaccharide structures of *Campylobacter fetus* are related to heat-stable serogroups. *Infect Immun* 1986; **51**:209-12.
- Winter AJ, McCoy EC, Fullmer CS, Burda K, Bier PJ. Microcapsule of *Campylobacter fetus*: chemical and physical characterization. *Infect Immun* 1978; **22**:963-71.
- Roantree RJ, Pappas NC. The survival of strains of enteric bacilli in the blood stream as related to their sensitivity to the bactericidal effect of serum. *J Clin Invest* 1960; **39**:82-8.
- Schoolnik GK, Buchanan TM, Holmes KK. Gonococci causing disseminated gonococcal infection are resistant to the bactericidal action of normal human sera. *J Clin Invest* 1976; **58**:1163-73.
- Blaser MJ, Smith PF, Kohler PF. Susceptibility of *Campylobacter* isolates to the bactericidal activity of human serum. *J Infect Dis* 1985; **151**:227-35.
- Klein BS, Vergeront JM, Blaser MJ, Edmonds P, Brenner DJ, Janssen D, Davis JP. *Campylobacter* infection associated with raw milk. An outbreak of gastroenteritis due to *Campylobacter jejuni* and thermotolerant *Campylobacter fetus* subsp. *fetus*. *JAMA* 1986; **255**:361-4.
- Lee MM, Welliver RC, La Scole J J Jr. *Campylobacter* meningitis in childhood. *Ped Infect Dis* 1985; **4**:544-7.
- Perez GI, Blaser MJ. Lipopolysaccharide characteristics of pathogenic campylobacters. *Infect Immun* 1985; **47**:353-9.
- Smibert RM. *Campylobacter*. In: Krieg NR, Holt JG, eds.

- Bergey's manual of systematic bacteriology. Vol 1. Baltimore: Williams and Wilkins, 1984:111-8
19. Bokkenheuser V. *Vibrio fetus* infection in man: a serological test. *Infect Immun* 1972;**5**:222-6
 20. Harriman GR, Podack ER, Braude AI, Corbeil LC, Esser AF, Curd JG. Activation of complement by serum-resistant *Neisseria gonorrhoeae*. Assembly of the membrane attack complex without subsequent cell death. *J Exp Med* 1982;**156**:1235-49
 21. Perez GI, Hopkins JA, Blaser MJ. Antigenic heterogeneity of lipopolysaccharides from *Campylobacter jejuni* and *Campylobacter fetus*. *Infect Immun* 1985;**48**:528-33
 22. Blaser MJ, Hopkins JA, Berka RM, Vasil ML, Wang W-L.L. Identification and characterization of *Campylobacter jejuni* outer membrane proteins. *Infect Immun* 1983;**42**:276-84
 23. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;**76**:4350-4
 24. Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 1981;**112**:195-203
 25. Blaser MJ, Hopkins JA, Vasil ML. *Campylobacter jejuni* outer membrane proteins are antigenic for humans. *Infect Immun* 1984;**43**:986-93
 26. Rosenberg M, Gutnick D, Rosenberg E. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* 1980;**9**:29-32
 27. Riley LW, Finch MJ. Results of the first year of national surveillance of *Campylobacter* infections in the United States. *J Infect Dis* 1985;**151**:956-9
 28. Blaser MJ, Perez GP, Smith PE, Patton C, Tenover FC, Lastovica AJ, Wang W-L.L. Extraintestinal *Campylobacter jejuni* and *Campylobacter coli* infections: host factors and strain characteristics. *J Infect Dis* 1986;**153**:552-9
 29. Joiner KA, Hammer CH, Brown EJ, Cole RJ, Frank MM. Studies on the mechanism of bacterial resistance to complement-mediated killing. I. Terminal complement components are deposited and released from *Salmonella minnesota* S218 without causing bacterial death. *J Exp Med* 1982;**155**:797-808
 30. Moll A, Manning PA, Timmis KN. Plasmid-determined resistance to serum bactericidal activity: a major outer membrane protein, the *traT* gene product, is responsible for plasmid-specified serum resistance in *Escherichia coli*. *Infect Immun* 1980;**28**:359-67
 31. Lambden PR, Heckels JE, James LT, Watt PJ. Variations in surface protein composition associated with virulence properties in opacity types of *Neisseria gonorrhoeae*. *J Gen Microbiol* 1979;**114**:305-12
 32. Joiner KA, Hammer CH, Brown EJ, Frank MM. Studies on the mechanism of bacterial resistance to complement-mediated killing. II. C8 and C9 release C5b67 from the surface of *Salmonella minnesota* S218 because the terminal complex does not insert into the bacterial outer membrane. *J Exp Med* 1982;**155**:809-19