JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1979, p. 227-232
0095-1137/79/02-0227/06$02.00/0

Vol. 9, No. 2

Detection of Enteric Campylobacteriosis in Children

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Received for publication 8 November 1978

Campylobacter fetus subsp. *jejuni* was recovered as the sole bacterial pathogen from 31% of 0- to 8-month-old children with acute gastroenteritis and from 5% of asymptomatic children ($P < 0.05$). In children 8 to 24 months old, the respective recovery rates were 38 and 40%. With the exception of one case of simultaneous bacteremia, the clinical course of the symptomatic infection was benign and rarely lasted more than a week. The isolates were sensitive to most commonly used antibiotics. Several isolates shared antigenic determinants with *C. fetus* subsp. *intestinalis*. A bacteriophage specific for *C. fetus* subsp. *jejuni* lysed 73% of the strains. Several phage-resistant isolates carried a phage that lysed the stock strain of *C. fetus* subsp. *jejuni* (NADC 917).

At least 134 cases of disseminated campylobacteriosis have been cited in the literature (2; an updated list of references is available upon request) since the first case of human *Campylobacter fetus* bacteremia was reported more than 30 years ago. Although the infection is a recognized zoonosis, there is no evidence that exposure to *C. fetus* in its natural habitat, i.e., the intestinal tract of sheep, swine, goat, dog, antelope, chicken, and turkey (12, 13, 19) immediately precedes disseminated campylobacteriosis in humans (2). Modes of transmission currently remain unexplained.

It has been observed that cases of disseminated *C. fetus* subsp. *jejuni* infection often are associated with diarrhea (19, 21). Investigators using specialized techniques (6, 8, 20) recovered *C. fetus* subsp. *jejuni* from 4 to 15% of children with diarrhea and from 0 to 1.5% of asymptomatic children and adults. Furthermore, five cases have been reported in which both hemocultures and coprocultures yielded *C. fetus* subsp. *jejuni* (7, 11). These findings indicate that colonization of the human gut is an important aspect of the disseminated *C. fetus* subsp. *jejuni* infection. Whether or not that is true also for *C. fetus* subsp. *intestinalis*, the predominant subspecies causing disseminated infection of adults (2, 11) is unknown. It has not been isolated from human feces.

A recent serological survey revealed that antibody titers to *C. fetus* subsp. *intestinalis* were four times higher in preparations of commercial human gamma globulin obtained from South Africa than in batches from other parts of the world (3). This, together with the fact that many cases of infantile gastroenteritis in South Africa are still of unknown etiology (14), prompted us to search for *C. fetus* in the feces of children with and without diarrhea.

MATERIALS AND METHODS

Selection of children. The investigation was conducted in the gastroenteritis ward of the Pediatric Division of the Baragwanath Hospital during the summer of 1977. The hospital serves the residents of Soweto at the outskirts of Johannesburg. Stool specimens were obtained from 78 black children, 0 to 2 years old, with acute gastroenteritis. Specimens from nondiarrhea cases were collected from diapers of children of the same age attending the clinics. According to their mothers, they had not had diarrhea for at least 2 weeks and had not received antibiotics recently.

The children were examined clinically, and notes were made of their nutritional status (1) and of the degree of dehydration (mild: sunken eyes or impaired turgor; moderate: sunken eyes and fontanelle and poor turgor; severe: features of moderate dehydration associated with shock and/or acidosis).

Fecal specimens. Within 2 h of arrival in the hospital, fecal specimens were obtained from the children. In some cases, mechnical stimulation of the rectum was required. The specimens were transported to Johannesburg at the prevailing temperature and processed within 4 h of collection.

Clot cultures. Blood samples were taken from all patients with gastroenteritis. After the serum had been removed, the clot was incubated at 37°C overnight in 10 ml of streptokinase broth (meat infusion broth, 1 liter; sodium taurocholate, 5 g; streptokinase [Lederle

Laboratories, Pearl River, N.Y.], 100,000 units) in a 10% CO_2 atmosphere. The mixture was then transferred to Todd-Hewitt broth (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated. Subcultures to 10% horse blood agar were made after 3 and 8 to 10 days.

Isolation of *Salmonella* and *Shigella* from stool specimens. *Salmonella* and *Shigella* were isolated, identified to species, and serotyped with conventional techniques. Enteropathogenic *Escherichia coli* were detected by analysis of the K and O antigens of *E. coli* strains.

Isolation and identification of *C. fetus*. (i) Media. Three media were used routinely: (a) 45 g of tryptose agar (Difco Laboratories, Detroit, Mich.), 100 ml of citrated horse blood, and distilled water to 1 liter (BTA); (b) 51 g of cystine heart agar (Difco Laboratories), 100 ml of citrated horse blood, 5 mg of novobiocin (Albamycin; Upjohn, Kalamazoo, Mich.), 1 ml of 0.1% brilliant green, and distilled water to 1 liter (BCHA); (c) 39 g of Columbia agar base (Oxoid Ltd.), 10% citrated horse blood, and distilled water to 1 liter (BCA).

(ii) Cultural technique. To obtain a consistency suitable for filtration, 1 to 2 g of soft or 4 ml of liquid feces was diluted in about 10 ml of buffered saline (pH 7.2) to form a thin slurry. After vigorous shaking, the suspension was centrifuged at 1,000 × *g* for 10 min. From 3 to 4 ml of supernatant fluid was transferred to a syringe for membrane filtration (0.65 μm; Millipore Corp., Bedford, Mass.). The first few drops were discarded; then 0.2 ml of filtrate was spread over the surface of BTA and BCHA, which were then incubated at 37°C in an atmosphere of 10% CO_2 and 90% air. The plates were examined daily for 5 days. Colonies morphologically resembling *C. fetus* were selected and subcultured for further examination.

(iii) Morphology. In the Gram stain, 0.2% carbol fuchsin was employed as the counterstain. Due to the low refractory index of the organisms, motility was examined by phase-contrast or dark-field microscopy.

(iv) Air tolerance. Duplicate BCA plates were seeded and incubated for 3 days, one under aerobic conditions and the other in a 10% CO_2 atmosphere.

(v) Biochemical identification procedures. The strains were identified to subspecies by conventional methods (18). Briefly, Albimi broth (Pfizer Diagnostics, New York) was supplemented with 0.15% agar and one of the following: 1% glucose, 1% glycine, 3.5% NaCl, 8% glucose, or (to detect H_2S production) 0.02% or 0.05% cystine. BCA plates supplemented with cephalothin (3 μg/ml), nalidixic acid (3 μg/ml), or triphenyltetrazolium chloride (0.4 mg/ml) were inoculated for differential diagnostic purposes (9).

(vi) Temperature tolerance. Replicate plates of BCA were seeded and incubated under microaerophilic conditions (7% O_2, 81% N_2, 6% CO_2, and 6% H_2) at 25, 37, or 42°C for 2 days.

(vii) Fluorescence technique. The direct test was performed with fluorescein isothiocyanate-conjugated rabbit antisera prepared against one strain of *C. fetus* subsp. *fetus*, six strains of *C. fetus* subsp. *intestinalis* representing both antigenic types, one strain of *Campylobacter sputorum* subsp. *bubulus*, and three strains of *C. fetus* subsp. *jejuni* (one of animal and two of human origin). Air-dried smears of bacterial suspensions were fixed for 10 min in 95% ethanol, rinsed in distilled water, and dried. The slides, flooded with conjugate, were incubated in moist chambers for 30 min, rinsed, and dried. Cover slips were mounted with phosphate-buffered glycerol (pH 8.6).

Indirect fluorescence determinations were performed with rabbit antiserum and fluorescein-conjugated goat anti-rabbit gamma globulin (10, 16, 17). Rabbit antisera were prepared against *C. fetus* subsp. *fetus* (A-1), *C. fetus* subsp. *intestinalis* (A-2 and B), and *C. fetus* subsp. *jejuni* (C). The slides were prepared and stained by conventional techniques.

Control slides, incorporated with all batches, revealed good staining with homologous bacterial strains; heterologous strains did not stain.

(viii) Agglutination of isolates. The strains were grown on Albimi agar (Pfizer Diagnostics) and harvested in buffered saline (pH 6.8) containing 0.3% formaldehyde. One drop of a suspension, adjusted to 10 times 0.4 optical density unit at 550 nm, was mixed on a glass plate with 50 μl of hyperimmune rabbit serum. After gentle rotation of the plate for 3 min, the test was read in a Bang's Box (a lighted box with a glass top). The strains were tested for autoagglutinability in a mixture of normal serum and saline.

(ix) Phage typing. Phages I to IV, originally recovered from *C. fetus* subsp. *fetus*, were propagated in *C. fetus* subsp. *intestinalis*. These phages lysed strains of both subspecies but not *C. fetus* subsp. *jejuni*. Phage C, obtained from and propagated in *C. fetus* subsp. *jejuni*, lysed strains of the latter subspecies only. The test was performed as previously described (4).

Antibiotic susceptibility testing. Minimal inhibitory concentration determinations were performed on plates containing graded concentrations of the different antimicrobial agents. Sulfonamide antagonistic-free agar (Mast Laboratories, Ltd., Liverpool, England) was used throughout and was supplemented with 0.5% lysed horse blood for sulfonamide and cotrimoxazole minimum inhibitory concentration estimations. The plates inoculated with 0.01 ml of a suspension containing approximately 10^6 bacteria per ml were incubated at 37°C in a 10% CO_2 atmosphere for 2 days.

Antibiotic susceptibility was also tested by a disk diffusion method on 5% horse blood agar plates (Oxoid Columbia base) with an inoculum obtained from 48-h cultures in Todd-Hewitt broth (Oxoid) adjusted to a density corresponding to 0.5 McFarland no. 1 barium sulfate standard. For sulfonamide and cotrimoxazole susceptibility testing, a lighter inoculum containing about 10^3 bacteria per ml (15) was used on sulfonamide antagonistic-free plates containing 0.5% lysed horse blood. Plates were inoculated with a sterile cotton swab on a wooden applicator and incubated as described above. The disk contents of the different antimicrobial agents are given in Table 8. Inhibition zones were measured in millimeters.

RESULTS

Isolates from stool specimens. *C. fetus* was recovered from 35% of children with diarrhea and from 16% of asymptomatic children (Table 1). The incidence was related neither to age nor

TABLE 1. *Isolates from stool specimens*

Clinical status	Age (mo.)	No. of children	% Positive with			
			C. fetus	*Salmonella*	*Shigella*	EPEC[a]
Diarrhea	0–8	47	32	0	2	38
	9–24	31	39	16	0	23
	Total	78	35	6	1	32
No diarrhea	0–8	45	4	2	0	13
	9–24	18	44	22	0	28
	Total	63	16	8	0	17

[a] Enteropathogenic *E. coli*.

to sex. The difference was most pronounced in the 0- to 8-month age group (32% positive for *C. fetus* in the group with diarrhea versus 4% of the nondiarrheal). By the time the children were 9 to 24 months of age, this difference no longer existed (39 and 44% in diarrhea and nondiarrheal groups, respectively). The fecal samples, in addition, yielded growth of one shigella, some salmonellae, and 49 serologically diagnosed enteropathogenic *E. coli*.

Subjects yielding *C. fetus* only. *C. fetus* was the sole bacterial pathogen in the feces of 34% of cases with diarrhea (Table 2). The recovery rate was similar in younger and older children. This compared with a prevalence of *C. fetus* in nondiarrheal children of 13%, increasing from 5% in the youngest to 40% in the oldest group. The younger children are of particular interest. In this group *C. fetus* was isolated from 31% of those with diarrhea as against 5% from the asymptomatic children, a highly significant difference ($\chi^2 = 7.92$, $P < 0.05$).

In one child with diarrhea (V54), identical strains of *C. fetus* were recovered from the stool and blood clot. For many children the present illness was their first experience with gastroenteritis. The stools usually were very soft to watery and often were bile stained and contained flakes of mucus. Nearly all children vomited. In 2/3 of the cases, the temperature ranged from normal to 38°C. Hyperpyrexia was not observed. Mild dehydration was common; severe dehydration and/or malnutrition occurred in 10% of the children.

There was no clear correlation between diet and infection. It is noteworthy, however, that *C. fetus* was recovered from the stools of twins who were breast fed only.

C. fetus was isolated from 17% of the children with diarrhea on the first day of illness and from 67% within the first week.

Treatment. On admission, electrolyte imbalance of dehydrated children was immediately corrected. Antibiotics were withheld in uncomplicated cases. Penicillin G or ampicillin was given to 17 of 28 children who were infected with *C. fetus* and showed evidence of respiratory tract complications. Candidiasis was treated with nystatin.

TABLE 2. *Children with C. fetus as the sole pathogen*

Clinical status	Age (mo.)	No. of children	No. positive (%)
Diarrhea	0–8	29	9 (31)[a]
	9–24	21	8 (38)
	Total	50	17 (34)
No diarrhea	0–8	38	2 (5)[a]
	9–24	10	4 (40)
	Total	48	6 (13)

[a] $\chi^2 = 7.92$; $P < 0.05$.

All infected patients, including the child with bacteremia, made an uneventful recovery, in most cases within 3 to 6 days.

Identification of *C. fetus*. Because of their similarity, isolates from diarrheal and nondiarrheal cases are considered together.

(i) Biochemical and tolerance tests. All isolates were H_2S positive on cystine media, tolerant to 1% glycine, and capable of growing at 42°C but not at 25°C (Table 3). The majority of strains were catalase positive, nitrate reducers, and resistant to cephalothin (3 μg/ml) and nalidixic acid (3 μg/ml). They were inhibited by 3.5% NaCl, 8% glucose, and triphenyltetrazolium (0.4 mg/ml).

(ii) Agglutination. Most of the isolates were agglutinated by antiserum to *C. fetus* subsp. *jejuni*, strain 958 (Table 4). A few strains also reacted weakly with antisera to *C. fetus* subsp. *fetus* and *C. fetus* subsp. *intestinalis*. Strain K35 agglutinated strongly with the two latter antisera but not at all with antiserum to *C. fetus* subsp. *jejuni*. Four strains were not agglutinated by any of the antisera.

(iii) Fluorescence technique. Cellular staining of the isolates was noted, but flagellar staining, which occurred with many antisera, was ignored. Fluorescein-conjugated antisera to hu-

man isolates of *C. fetus* subsp. *jejuni*, PB 1/175 and PB 1/68, reacted with 39 and 32 of 39 isolates, respectively (Table 5). A conjugate of antiserum to *C. fetus* subsp. *jejuni* (AF/58/3) of porcine origin reacted with 37 of 39 strains. Five isolates were stained with one of the six *C. fetus* subsp. *intestinalis* conjugates, but no two strains reacted with the same conjugate; conjugates to *C. fetus* subsp. *fetus* and *C. sputorum* subsp. *bubulus* did not stain any of the strains. K35 was one of the two isolates that failed to react with *C. fetus* subsp. *jejuni* (AF/58/3) conjugate.

The results from the direct fluorescence test were essentially confirmed by the indirect method (Table 6). However, reactions were weaker and more scattered.

(iv) Phage typing. Phage C, lytic for many strains of *C. fetus* subsp. *jejuni*, but not for *C. fetus* subsp. *fetus* and *C. fetus* subsp. *intestinalis*, lysed 30 of 41 isolates (Table 7). A few isolates were also lysed by phages I to IV, considered to be specific for strains of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *intestinalis*. Spontaneous plaque formation occurred with many of the phage-resistant isolates. Phages recovered from these plaques lysed *C. fetus* subsp. *jejuni* NADC 917.

TABLE 3. *Biochemical characterization of 39 C. fetus strains recovered from diarrheal and nondiarrheal children*

Test	No. of strains positive
Catalase	38
H_2S in cystine 0.02%	35
H_2S in cystine 0.05%	39
NO_3 reduction	36
Growth in 1% glycine	39
Growth in 3.5% NaCl	0
Growth on tetrazolium agar	3
Growth in 8% glucose	0
Growth in thiol broth	39
Growth on cephalothin	33
Growth on nalidixic agar	35
Growth at 42 and 37°C but not at 25°C	39

TABLE 4. *Agglutination of 21 isolates*

Antisera to:	No. of strains showing agglutination:			
	None	+	++	+++
C. fetus subsp. *fetus*[a]	15	3	2	1[b]
C. fetus subsp. *intestinalis*[c]	14	3	3	1[b]
C. fetus subsp. *jejuni*[d]	6	3	10	2

[a] Serotype A-1, strain 1289.
[b] Isolate K35.
[c] Serotype B, strain 1083.
[d] Serotype C, strain 958.

TABLE 5. *Species identification of 39 isolates by direct fluorescence technique*

Conjugated antisera to:	No. of strains showing fluorescence:		
	None	Weak	Good
C. sputorum subsp. *bubulus* (1 strain)	39		
C. fetus subsp. *fetus* (1 strain)	39		
C. fetus subsp. *intestinalis* (6 strains)	34	4	1
C. fetus subsp. *jejuni* (porcine; AF/58/3)	2[a]	13	24
C. fetus subsp. *jejuni* (human; PB 1/68, PB 1/175)	0	14	25

[a] Isolates V77 and K35.

TABLE 6. *Species identification of 25 isolates by indirect fluorescence*

Antisera to:	No. of strains showing indirect fluorescence:		
	None	Weak	Good
C. fetus subsp. *fetus*[a]	19	5	1
C. fetus subsp. *intestinalis*[b]	20	4	1
C. fetus subsp. *jejuni*[c]	10	10	5

[a] Serotype A-1, strain 1289.
[b] Serotype B, strain 1083.
[c] Serotype C, strain 958.

TABLE 7. *Lytic activity of C. fetus phages on 41 isolates*

Phage[a]	No. of strains showing lysis:			
	None	1-49 plaques	>50 plaques	Complete
I	38	3	0	0
II	37	3	1	0
III	39	1	1	0
IV	39	2	0	0
C	11	17	9	4

[a] Phage types: phages I, II, III, and IV were derived from *C. fetus* subsp. *fetus* or *C. fetus* subsp. *intestinalis* and are active against these subspecies, but do not attack *C. fetus* subsp. *jejuni*. Phage C was derived from *C. fetus* subsp. *jejuni* strain 652/917 and was active against many but not all *C. fetus* subsp. *jejuni* strains from ovine aborted fetuses; the C phage does not attack *C. fetus* subsp. *fetus* or *C. fetus* subsp. *intestinalis*.

Antimicrobial susceptibility. The minimum inhibitory concentrations and measurements of inhibition zones around disks are summarized in Table 8. According to both techniques, the isolates were susceptible to most commonly used antimicrobial agents but resistant to benzylpenicillin, cephalothin, colistin, and trimethoprim. In spite of good overall correlation between the minimum inhibitory concentration and disk diffusion methods, several dis-

TABLE 8. *Antibiotic susceptibility of isolates*

Antibiotic	No. of isolates tested	MICs (μg/ml) for cumulative percentage of susceptible strains			Disk susceptibility test		Interpretation[a]
		95%	50%	10%	Mean zone diameter (mm)	Drug dose per disk (μg)	
Penicillin G	37	8	4	1	8	6	R
Ampicillin	35	4	1	0.25	33	10	S
Cephalothin	37	>128	64	32	6	30	R
Tetracycline	37	0.5	0.25	0.06	48	30	S
Chloramphenicol	38	8	2	1	46	30	S
Erythromycin	35	0.25	0.06	0.03	49	15	S
Streptomycin	38	2	1	0.5	41	10	S
Gentamicin	38	5	2.5	0.125	44	10	S
Clindamycin	38	0.25	0.125	0.06	42	2	S
Colistin	36	16	8	1	9	10	R
Sulfamethoxazole	38	32	8	4	18	50	I
Cotrimoxazole	38	32	8	4	21	TMP, 1.25; SMX, 23.75[b]	I
Trimethoprim	20	>256	>256	>256	6	1.25	R

[a] R, Resistant; S, susceptible; I, intermediate susceptibility.
[b] TMP, Trimethoprim; SMX, sulfamethoxazole.

crepancies were encountered with individual strains which on repetition incriminated the disk diffusion technique as less reliable. This technique is therefore not recommended for routine use.

DISCUSSION

Species identification of *C. fetus*. The individual strains recovered in this investigation share sufficient characteristics with *C. fetus* subsp. *jejuni* to make their identity unequivocal. However, they differ among themselves; therefore, if a single test had been employed for their classification, a number of strains would have been incorrectly identified (Tables 3 to 7). The characteristics that most accurately predicted the subspecies are: production of catalase and H_2S; growth in 1% glycine and at 42°C; and inhibition of growth by 3.5% NaCl and 8% glucose. Tests for inhibition by nalidixic acid or triphenyltetrazolium chloride (9) at the concentrations used were of little help.

Although antigenic analysis was done with antisera against different strains of the subspecies, the direct fluorescence technique gave the most accurate results. However, the tests indicated that several isolates possess antigenic determinants common to all three pathogenic strains of *C. fetus*. Further evidence of the heterogeneity of strains of *C. fetus* subsp. *jejuni* (5) is exemplified by isolate K35, which shares antigenic determinants with *C. fetus* subsp. *fetus* and *C. fetus* subsp. *intestinalis* but is lysed by phage C.

Until now only a single C phage has been available. Phages recovered from isolates with spontaneous plaque formation may enhance our capability to further characterize strains of *C. fetus* subsp. *jejuni*.

Significance of *C. fetus* in human feces. The significantly higher prevalence of *C. fetus* subsp. *jejuni* in 0- to 8-month-old children with diarrhea than in the asymptomatic children strongly suggests that the organism is a causative agent of diarrhea in very young children (Table 2). This contention may well be valid for children throughout the world. Further epidemiological studies with suitably matched controls and evidence of seroconversion are, however, required to firmly establish the role of *C. fetus* subsp. *jejuni* in infantile gastroenteritis.

In asymptomatic children, the prevalence of *C. fetus* subsp. *jejuni* increased dramatically after 9 months of age. With a prevalence of this magnitude it is to be expected that *C. fetus* subsp. *jejuni* will be isolated from a number of patients with diarrhea in whom it is not the causative agent of the illness. Whether the organisms are indicative of a transient infection to which there is no apparent host response or they have colonized the gut on a more permanent basis is unknown.

The high incidence of asymptomatic *C. fetus* subsp. *jejuni* infections observed in this series contrasts strikingly with the lower incidence reported from England (20) and Belgium (7). Whether these differences are real or attributable to differences in methodology is a moot point.

ACKNOWLEDGMENTS

We thank the staff of the Pediatric Clinic, Baragwanath Hospital, and M. Coster and the staff of the Johannesburg

City Health Department for their cooperation; O. Bothma, A. Taubin, and J. Saunders, South African Institute for Medical Research, for bacteriological assistance; R. Robins-Browne for analyzing the results of antibiotic sensitivity testing; and J. W. Foley, National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, for help in serotyping.

This study was supported in part by a grant from the South African Medical Research Council.

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