JOURNAL OF CLINICAL MICROBIOLOGY, June 1986, p. 1039–1043
0095-1137/86/061039-05$02.00/0

Vol. 23, No. 6

Enzyme-Linked Immunosorbent Assays for Virulence Properties of *Campylobacter jejuni* Clinical Isolates

FREDERICK A. KLIPSTEIN,[1,2]* RICHARD F. ENGERT,[1] AND HELEN B. SHORT[2]

Departments of Medicine[1] and Microbiology,[2] University of Rochester Medical Center, Rochester, New York 14642

Received 30 October 1985/Accepted 5 March 1986

To evaluate the capacity of enyzme-linked immunosorbent assays (ELISAs) to identify pathogenic strains among clinical fecal isolates of *Campylobacter jejuni*, 40 consecutively obtained strains from 39 sick patients and 1 asymptomatic person were tested by respective ELISAs for enterotoxin production in culture filtrates and for the invasive virulence antigen of bacterial cells. Of the 40 strains, 14 produced the enterotoxin; 15 strains, two of which were also enterotoxigenic, were invasive; and 11 strains had no detectable virulence property. The presence or absence of these virulence properties was confirmed by the demonstration that viable cells of all 12 randomly selected enterotoxigenic or invasive strains tested, but none of 9 nonpathogenic strains tested, caused fluid secretion in rat ligated ileal loops. All 12 patients examined who were infected with an invasive strain had grossly or microscopically evident blood cells in their stools or both, whereas none of those infected with an enterotoxigenic strain had overtly bloody diarrhea, and only 1 of 8 patients examined had microscopically evident blood cells in the stool. Twelve of the invasive, five of the enterotoxigenic, and three of the nonpathogenic strains also produced small amounts of cytotoxin, but there was no correlation between cytotoxin production and an abnormal response in rat ligated ileal loops. These observations show that enterotoxin production or invasiveness or both can be detected by ELISAs in three-fourths of *C. jejuni* fecal isolates and that there is usually a relationship between the specific pathogenic property of the infecting strain and the clinical mainfestations.

Several different pathogenic properties have been identified among strains of *Campylobacter jejuni*: some strains invade tissue culture cells (22, 23) or the intestinal mucosa (11, 27, 30), some produce an enterotoxin that is functionally and immunologically related to cholera toxin and *Escherichia coli* heat-labile enterotoxin (8, 9, 12, 14–16, 19, 28), some produce a cytotoxin (12, 16, 22, 25, 31; H. Goossens, E. Rummens, S. Cadranel, J.-P. Butzler, and Y. Takeda, Letter, Lancet ii:511, 1985), and some have no detectable pathogenic property whatsoever (16). There is very little information, however, regarding the relationship of the specific virulence property of an infecting strain and the clinical response of the infected host, which may vary from bloody diarrhea, sometimes associated with endoscopic evidence of colitis (2, 17, 18, 20), to watery, secretory-type diarrhea (3, 5, 7, 13) or, in some instances, particularly among children living in developing countries, to no symptomatic response at all (1, 7, 26). The main reason that this relationship has not been evaluated more has been the unavailability until recently of simple in vitro tests capable of identifying the various virulence properties.

Enzyme-linked immunosorbent assays (ELISAs) have now been developed that can identify enterotoxigenic or invasive strains of *C. jejuni* (14, 16, 28). The recent application of these ELISAs to 20 human isolates showed a correlation between the presence or absence of specific virulence properties and (i) the ability of these strains to cause fluid secretion in rat ligated ileal loops and (ii) clinical manifestations of the infected host (16). The number of strains examined, however, was too small to determine whether these ELISAs can consistently detect virulence properties, and the fact that the isolates examined were selected for study because they came from persons with a clearly defined clinical status precluded knowing whether the relationship between virulence properties and clinical response would exist if a large, randomly selected group of cases was studied. To consider these questions, we assayed the pathogenic properties of 40 consecutively cultured *C. jejuni* fecal isolates.

MATERIALS AND METHODS

Bacterial strains. During the 2-year period between 1 September 1983 and 31 August 1985, one blood culture and 50 fecal isolates of *C. jejuni* were obtained in the Clinical Microbiology Laboratory of The University of Rochester Medical Center. Of the fecal isolates, 10 were excluded from this study because 2 were duplicates and no clinical information was available for 8. All 40 fecal isolates studied were positive for hippurate hydrolysis (10).

Bacterial growth. Strains were grown at 42°C in GC medium (Difco Laboratories, Detroit, Mich.) supplemented with 1.0% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) under agitated conditions in the presence of 8% CO_2 (14). The growth of all strains was monitored by determining their optical density at 650 nm at 24 h; values for strains in each of the different pathogenic categories ranged between 0.33 and 0.40. After 24 h of growth, a portion was removed for the processing of viable cells, and 2 mg of polymyxin B per ml was added to the remainder for an additional 10 min of growth. Broth filtrates were obtained by centrifugation at 13,000 × *g* for 10 min followed by passage of the supernatant through a membrane filter (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.); they were stored at

* Corresponding author.

4°C and assayed for enterotoxicity and cytotoxicity within 2 weeks.

ELISA for enterotoxin. Broth filtrates were tested in an ELISA in which 0.1 μg of GM_1 ganglioside (Supelco Inc., Bellafonte, Pa.) was the solid phase and 1 μg of alkaline phosphatase-conjugated affinity-purified goat antiserum to *E. coli* heat-labile enterotoxin was the antibody (16). Optical densities were read with a model EL307 ELISA reader (Bio-Tek Instruments, Burlington, Vt.); a value of ≥0.200 was considered positive.

ELISA for invasiveness. Formalin-killed bacterial cells were used as the solid phase since preliminary studies showed that viable cells react in this assay for only 24 h, whereas Formalin-killed cells react for up to 2 weeks. After separation from the broth supernatant, the cells were resuspended in 1% Formalin in phosphate-buffered saline at room temperature for 30 min; they were then separated by centrifugation and washed once with ELISA coating buffer and, after repeat centrifugation, resuspended at 1/10 of the original culture volume in this buffer for use as the solid phase. The 10-fold concentration of cells was used to assure that there was antigen excess; this was confirmed by the finding that 20-fold concentrations failed to increase the optical density in this assay.

The first antibody was the immunoglobulin fraction of rabbit antiserum to Formalin-killed whole cells of *C. jejuni* invasive strain 79-102 (16). This strain was isolated from a patient with bloody diarrhea; it produces cytotoxin but not enterotoxin, and its viable cells invade enterocytes and cause fluid secretion in rat ligated ileal loops (16). The immunoglobulin fraction was used at a concentration of 3.2 μg/ml (0.63 μg per well), which represented a 1/640 dilution of a stock solution of 2 mg/ml. This was the same dilution as that used in our previous study in which the amount per well was incorrectly reported to be 16.6 μg (16). The second antibody was alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. On the basis of previous experience with this assay (16), optical densities of ≥0.200 were considered positive.

In studies which used absorbed antiserum, the immunoglobulin fraction of rabbit antiserum to whole cells of strain 79-102 was absorbed by exposure to viable cells of either nonpathogenic strain C006 (obtained from an asymptomatic person and negative in rat ligated ileal loops) or invasive strain C018 (obtained from a patient with bloody diarrhea and positive in rat ligated ileal loops) by procedures identical to those described by Pál et al. (24).

Assay for cytotoxin. The HeLa cell assay was performed by the dye-release method described by Gentry and Dalrymple (6). The 50% cell-detachment dilution of broth filtrates was determined as described previously (16).

Rat ligated ileal loops. Broth cultures were centrifuged and reconstituted in growth medium to a 15-fold concentration, and 0.1 ml was instilled for 16 h into each of single 10-cm-long ligated ileal loops of three fasting 175- to 200-g Sprague-Dawley rats as described previously (16). The results reported for each test strain are the mean ± standard error of the mean for the volume/length ratio, expressed in microliters of fluid per centimeter of intestine. The volume/length ratio in 10 rats given either saline or growth medium was consistently <50 μl/cm.

RESULTS

Of the 40 strains tested, 39 came from patients who had gastrointestinal symptoms (Table 1). Thirty-eight patients had no other recognized cause for these symptoms, and one patient (source of isolate C035) was experiencing an exacerbation of colonoscopy-documented Crohn's colitis. One strain (isolate C006) came from a 2-year-old child who had no gastrointestinal symptoms; this strain had no pathogenic properties detected by the ELISAs and did not cause fluid secretion in rat ligated ileal loops (data not included in Table 1).

Of the 39 strains isolated from symptomatic subjects, 14 (36%) produced enterotoxin; none were invasive (Tox^+ Inv^-), but 5 of these strains also produced the cytotoxin. Viable bacteria of all five strains so tested caused fluid secretion in rat ligated ileal loops. None of the patients infected with these strains had grossly bloody stools (information was not available for one), and none of the stools taken from eight patients and examined by microscopy had erythrocytes, although one had leukocytes.

Of the 39 strains isolated from symptomatic patients, 15 (38%) were positive in the ELISA for invasiveness. Six of these strains were randomly selected for testing with absorbed antiserum in this ELISA. The positive response was unchanged when the antiserum was absorbed with bacterial cells of nonpathogenic strain C006, but it was abolished when the antiserum was absorbed with bacterial cells of invasive strain C018 (Table 2). Of the invasive strains, 2 were also enterotoxigenic (Tox^+ Inv^+), and 12 produced the cytotoxin. Viable bacteria of all seven strains tested, including two that did not produce the cytotoxin, caused fluid secretion in rat ligated ileal loops. Of the 15 patients, 7 presented with grossly bloody diarrhea, and examination by microscopy of stools from 11 showed erythrocytes or leukocytes or both present in all cases.

Of the 39 strains isolated from symptomatic patients, 10 (26%), including that from the patient with Crohn's colitis, gave a negative response in both ELISAs (Tox^- Inv^-). Three of these strains produced the cytotoxin. None of the nine strains in this group tested evoked fluid secretion in rat ligated ileal loops. Three of the patients had grossly bloody diarrhea, and all five patients whose stools were examined had erythrocytes or leukocytes or both by microscopy.

DISCUSSION

Of the 39 strains isolated from symptomatic patients, 29 (74%) were identified as either enterotoxigenic or invasive by the ELISAs, and randomly selected strains with either of these properties consistently caused fluid secretion in rat ligated ileal loops. In contrast, none of the 11 strains which had no virulence property detected by the ELISAs caused fluid secretion in rat ligated ileal loops, suggesting that these strains were indeed nonpathogenic. Although polymicrobial infection can occur in persons found to have *C. jejuni* (7, 21, 29), the likelihood that other enteric pathogens were responsible for diarrhea in all 10 of the symptomatic patients who harbored apparently nonpathogenic strains of *C. jejuni* seems remote. A more reasonable explanation is that these strains had lost their virulence property by the time that they were studied. A similar circumstance has been described by Pál et al., who found that some laboratory-derived strains of enteroinvasive *E. coli* lose their ability to produce the virulence antigen responsible for giving a positive response in their respective ELISA for invasiveness and become negative in the Sereny test as well (24; T. Pál, P. Echeverria, D. N. Taylor, O. Sethabutr, and S. Hanchalay, Letter, Lancet **ii**:785, 1985).

The reason for the apparent loss of pathogenic properties of some *C. jejuni* strains isolated from symptomatic persons

TABLE 1. Virulence properties of fecal strains of *C. jejuni* obtained from symptomatic patients

Strain	Patient clinical status						Isolate virulence properties				
					Stool containing[a]:		Cell-free broth filtrate		Bacterial cells		
	Age (yr)	Bloody diarrhea	Fever >101°F (>ca. 38.3°C)	Chills	Erythrocytes	Leukocytes	ELISA enterotoxin[b]	Cytotoxin[c]	ELISA invasiveness[d]	Rat ileal loops (vol/length ratio)[e]	Properties
C003	21	no	no	no	no	no	0.816	0	0.008	267 ± 5	Tox^+ Inv^-
C004	24	no	no	no	NA[f]	NA	1.155	0	0.032	270 ± 10	Tox^+ Inv^-
C008	21	no	yes	yes	NA	no	1.120	1	0.053		Tox^+ Inv^-
C013	31	NA	yes	yes	NA	NA	1.090	0	0.007		Tox^+ Inv^-
C020	24	no	yes	yes	no	NA	0.725	0	0		Tox^+ Inv^-
C024	19	no	no	no	NA	NA	0.709	0	0.032	221 ± 12	Tox^+ Inv^-
C025	53	no	no	no	no	no	1.007	8	0		Tox^+ Inv^-
C029	25	no	no	no	no	no	0.390	0	0.094		Tox^+ Inv^-
C032	8	no	no	no	NA	NA	0.600	3	0.021		Tox^+ Inv^-
C033	6	no	no	no	NA	NA	0.671	0	0		Tox^+ Inv^-
C038	22	no	yes	yes	no	no	0.560	0	0.008	242 ± 18	Tox^+ Inv^-
C043	26	no	no	no	NA	NA	0.595	1	0		Tox^+ Inv^-
C048	48	no	no	no	no	yes	0.415	4	0.053		Tox^+ Inv^-
C050	23	no	no	yes	no	no	0.440	0	0.013	243 ± 7	Tox^+ Inv^-
C002	20	yes	yes	no	NA	NA	0.370	8	0.348		Tox^+ Inv^+
C009	18	yes	yes	no	yes	NA	0	6	0.458	166 ± 13	Tox^- Inv^+
C018	33	yes	yes	no	yes	yes	0	16	0.533	105 ± 5	Tox^- Inv^+
C019	33	yes	yes	yes	yes	NA	0	40	0.277	120 ± 3	Tox^- Inv^+
C022	35	no	yes	no	NA	NA	0.008	4	0.552	173 ± 7	Tox^- Inv^+
C028	3	yes	yes	no	yes	yes	0.008	8	0.521		Tox^- Inv^+
C031	20	no	yes	no	yes	yes	0	5	0.340		Tox^- Inv^+
C034	30	no	yes	yes	yes	yes	0.011	0	0.328	188 ± 17	Tox^- Inv^+
C037	20	yes	no	no	yes	yes	0.587	13	0.330		Tox^+ Inv^+
C039	37	no	yes	no	no	yes	0	6	0.511		Tox^- Inv^+
C040	17	no	yes	no	yes	NA	0.047	4	0.426		Tox^- Inv^+
C044	24	no	no	no	NA	NA	0	3	0.266	114 ± 14	Tox^- Inv^+
C046	2	no	yes	no	NA	NA	0	20	0.370		Tox^- Inv^+
C049	20	no	yes	yes	yes	NA	0	0	0.446	204 ± 13	Tox^- Inv^+
C051	2	yes	yes	no	yes	yes	0.014	0	0.299		Tox^- Inv^+
C001	7	no	no	no	NA	NA	0.012	0	0.002	18 ± 18	Tox^- Inv^-
C005	20	yes	no	no	NA	NA	0.048	0	0.034	23 ± 23	Tox^- Inv^-
C007	29	no	yes	no	NA	NA	0.012	0	0.052	14 ± 14	Tox^- Inv^-
C016	32	no	yes	yes	NA	yes	0	2	0.056	16 ± 12	Tox^- Inv^-
C021	21	no	yes	yes	NA	NA	0.025	0	0.034	10 ± 10	Tox^- Inv^-
C027	22	no	no	yes	no	yes	0.008	3	0.007	28 ± 6	Tox^- Inv^-
C030	38	yes	yes	yes	yes	yes	0.057	0	0	20 ± 10	Tox^- Inv^-
C035	32	yes	no	no	yes	no	0	0	0.006		Tox^- Inv^-
C045	21	no	no	no	NA	NA	0.031	0	0.058	19 ± 19	Tox^- Inv^-
C047	48	no	yes	no	yes	yes	0	1	0.008	28 ± 5	Tox^- Inv^-

[a] Upon examination by microscopy.
[b] Values are the optical density in an ELISA that used GM_1 as the solid phase and affinity-purified goat antiserum to *E. coli* heat-labile enterotoxin as the antibody.
[c] Values are the reciprocals of the maximum dilutions that yielded 50% cell death of HeLa cells.
[d] Values are the optical density in an ELISA that used Formalin-killed whole bacteria as the solid phase and the immunoglobulin fraction of antiserum to Formalin-killed bacteria of invasive strain 79-102 as the antibody.
[e] Fluid secretion expressed as mean ± standard error of the mean of the volume/length ratio in microliters per centimeter after instillation of viable bacteria.
[f] NA, Information not available.

is unknown, and no factor in the processing of the strains could be identified in terms of the time between initial isolation and permanent storage at −60°C (between 3 and 7 days) or the duration of storage (between 1 week and 2 years). One possibility could be plasmid loss, but little is known about plasmid-mediated virulence properties of *C. jejuni* other than preliminary evidence which suggests that its enterotoxin production is plasmid mediated (E. C. Lee, B. A. McCardell, J. M. Madden, and B. T. DeCicco, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, P13, p. 256). The clinical data in the present study suggested that most of the strains found to be nonpathogenic were invasive, and preliminary studies in this laboratory have shown that the invasive virulence antigen detected by the ELISA is located in flagellar preparations (F. A. Klipstein, and R. F. Engert, unpublished data). Another possibility, therefore, could be the loss of flagella, which can occur by phase variation during intestinal carriage (4).

There was a general correlation between virulence properties and clinical manifestations in that all 12 patients examined who harbored an invasive strain had grossly bloody diarrhea or microscopically evident blood cells in their stools or both, whereas none of the 14 patients infected with only an enterotoxigenic strain had grossly bloody

TABLE 2. Effect of absorbing the antiserum used in the ELISA for invasiveness with cells of a nonpathogenic or invasive strain

Strain tested	Optical density in ELISA using antiserum to invasive strain 79-102		
	Unabsorbed	Absorbed with cells of[a]:	
		Strain C006	Strain C018
C009	0.458	0.392	0.092
C022	0.552	0.483	0
C028	0.521	0.421	0
C039	0.511	0.500	0.031
C040	0.426	0.435	0
C049	0.446	0.421	0.016

[a] Strain C006 is nonpathogenic; strain C018 is invasive.

diarrhea and only 1 of the 8 examined had microscopically evident blood cells in the stool. The prevalence of fever was also greater in patients infected with an invasive strain, occurring in 13 of these 15 patients (87%) but in only 4 of the 14 patients (29%) who harbored an enterotoxigenic strain.

As noted previously (16), cytotoxic activity was found to be present principally among the invasive strains in the present study. Although invasive strain 79-102, used to raise antiserum for the invasive ELISA, also produces cytotoxin, it seems most unlikely that antibody to the cytotoxin is involved in the response in this ELISA, since Formalin-killed whole bacterial cells were used both to raise the antibody and as test antigens in the ELISA, whereas the cytotoxin is present principally in broth supernatants. Further, some strains identified by ELISA as being invasive did not produce cytotoxin, whereas others that were enterotoxigenic or nonpathogenic were cytotoxigenic. The role of the cytotoxin in the pathogenesis of the intestinal abnormalities produced by *C. jejuni* infection remains uncertain. Our previous (16) and present results have failed to demonstrate an adverse effect of the cytotoxin in terms of causing fluid secretion in rat ligated ileal loops. Thus, (i) broth supernatants of enterotoxigenic strains cause fluid secretion, but those of invasive or nonpathogenic strains do not, irrespective of whether these strains produce cytotoxin; and (ii) viable bacteria of either enterotoxigenic or invasive strains cause fluid secretion, irrespective of whether they produce cytotoxin, but cytotoxigenic nonpathogenic strains do not.

ACKNOWLEDGMENT

This study was supported by a grant from Johnson and Johnson Biotechnology Center, La Jolla, Calif.

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