BACTERIAL CHARACTERISATION

Cytotoxin detection in *Campylobacter jejuni* strains of human and animal origin with three tissue culture assay systems

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Summary. Cytotoxin (CTX) production in 34 human and 22 animal strains of *Campylobacter jejuni* isolated in Japan and other countries was studied by three assay systems described previously. Furthermore, cholera-like enterotoxin production by these strains was tested by reversed passive latex agglutination (RPLA). CTX titres in the fetal calf serum (FCS) and newborn calf serum (NCS) assays were relatively lower, with a maximum of 4 and 8, respectively, than the maximum of 128 for the serum-free culture (SFC) assay. CTX detection rates were 62, 85 and 100% in human isolates and 64, 77 and 100% in animal isolates for the FCS, NCS and SFC assay systems, respectively. There was no significant difference in the detection rate of CTX between human and animal isolates, or between human isolates from Japan and other countries. With the three assay systems, the strains were divided into four groups from the pattern of CTX detection; 54% of strains gave positive results in all three assay systems, and 9% of them were positive in the SFC assay only. Morphological changes on CHO cells showed distended instead of rounded cells with eight of 21 strains negative in the FCS assay. Cholera-like enterotoxin was not detected in the culture filtrate of any of the strains when tested by RPLA. These results indicate that cytotoxin production by *C. jejuni* is complex as compared with that of other enteric pathogens.

Introduction

Campylobacter jejuni is a common cause of both human and animal diseases.^{1–4} Although the mechanisms of *C. jejuni* pathogenesis have not been fully elucidated, several virulence-related factors such as adherence,^{5–8} invasiveness^{9–11} and production of enterotoxin (ETX)^{12–24} or cytotoxin (CTX)^{25–36} have been associated with the organism. We reported previously the development of three tissue-culture assay systems to detect the CTX produced by *C. jejuni*, and the effect of culture conditions on CTX production was also examined.³⁷ It appeared that the cytotoxicity was dependent on serum added to the tissue culture medium, and that the design of the assay and culture system were important for the detection of cytotoxic activity.

In the present study, the frequency of the production of CTX(s) by *C. jejuni* isolates from human and animal sources in Japan or other countries was determined by these three assay systems.

Materials and methods

Bacterial strains

A total of 34 human and 22 non-human strains of *C. jejuni* was studied for CTX production. All strains of human origin (including one type strain) were isolated from outbreaks of campylobacteriosis in Japan and other countries. Eighteen strains of animal origin (including one type strain) were isolated from faeces of healthy cattle, chickens, a dog and a cat. Four strains were isolated from chicken livers showing necrotic lesions. Strains were suspended in Nutrient Broth No. 2 (Oxoid) containing dimethylsulphoxide (Sigma) 10% and stored at –80°C until used.

Cytotoxin production

Strains were grown on horse blood agar plates for 36 h at 42°C in a gas mixture containing O₂ 5%, H₂ 5%, CO₂ 10% and N₂ 80%. A loopful of bacteria was inoculated into 20 ml of Brucella Broth (BBL Microbiology Systems, Cockeysville, MD, USA) in a flask and the initial optical density at 550 nm (A_{550}) was adjusted to between 0.1 and 0.2. The cultures were incubated at 42°C for 4 days by stationary culture or for 2 days by stirred culture. After incubation, the A_{550} of the whole culture and the number of viable cells were measured and the broth culture was centrifuged at 10000 *g* for 30 min at 4°C. The supernate was filtered through a 0.20- μ m cellulose nitrate membrane (Advantec Toyo Ltd, Tokyo, Japan) and stored at 4°C until tested. The sample was used for toxin detection within 3 days after cultivation.

Assay for cytotoxin

Cytotoxin activity was detected by three tissue-culture assay systems developed previously.³⁷ Briefly, trypsinised Chinese hamster ovary (CHO) cells (Japanese Cancer Resource Bank, Tokyo, Japan) were suspended in Eagle's Minimum Essential Medium (MEM; Nissui Pharmaceutical Co., Tokyo, Japan) containing fetal calf serum (FCS; Flow laboratories Inc., McLean, VA, USA) 20% or newborn calf serum (NCS; Gibco Laboratories, Grand Island, NY, USA) 10%, or ASF 104 Medium (Ajinomoto Co. Ltd, Tokyo, Japan) as a serum free culture (SFC). Volumes of 100 μ l of cell suspensions were placed in a 96-well microtitration plate at 5×10^3 cells/well, and allowed to adhere to the bottom of the wells for 1–3 h. Serial two-fold dilutions of bacteria-free supernate were made in 20 mM phosphate-buffered saline, pH 7.2, and then 100 μ l of the test sample was added to each well, followed by incubation at 37°C for 96 h in air with CO₂ 5%. Wells showing rounding in > 30% of cells were considered positive, and the titre of cytotoxic activity was recorded as the highest dilution of the sample causing morphological changes in the cells. The positive higher titre among the stationary and stirred culture was used as the cytotoxic activity. Brucella broth alone was used as negative control.

Enterotoxin detection

Bacteria-free culture filtrates were assayed for enterotoxin with a *Vibrio cholerae* enterotoxin (CT) and *Escherichia coli* heat-labile enterotoxin (LT) detection kit by reversed passive latex agglutination (RPLA; Denka Seiken Co. Ltd, Tokyo, Japan) as described previously.³⁷ More than 1–2 ng/ml of CT was detectable with this assay kit.

Statistical analysis

Significant differences in the frequency of CTX production among isolates from different sources were analysed by the χ^2 test.

Table I. Differences in CTX production in stationary and stirred culture conditions and different assay systems

CTX production in		Number of strains giving CTX production pattern in (n = 56)		
stationary culture	stirred culture	FCS	NCS	SFC
–	+	9	1	0
+	–	1	20	6
+	+	25	25	50
–	–	21	10	0

Results

Effect of culture conditions

CTX production in stationary and stirred cultures was compared (table I). With the FCS assay system, nine strains gave negative results in the stationary culture but positive results in the stirred culture. Conversely, in the NCS and SFC assay systems, 20 and six strains, respectively, gave negative results in the stirred culture but positive results in the stationary culture.

Frequencies of CTX and CT-like enterotoxin production

Profiles of CTX production in *C. jejuni* isolates from human and animal sources are summarised in table II. The data show only the highest titre of both the stationary and stirred cultures. CTX titres in the FCS and NCS assays were relatively lower with a maximum of 4 and 8, respectively, than the maximum of 128 for the SFC assay. The percentages of cultures producing CTX were 62, 85 and 100% in human isolates and 64, 77 and 100% in animal isolates for the FCS, NCS and SFC assay systems, respectively. Furthermore, the percentage of CTX-positive strains of human origin isolated in Japan were 52, 86 and 100% and from other countries were 77, 85 and 100% for the FCS, NCS and SFC assay systems, respectively. There was no significant difference ($p > 0.05$) in the rate of detection of each CTX assay between human and animal isolates, or between human isolates from Japan or other countries. The frequencies of CTX detection and activity in the three assay systems were higher in isolates from chickens with hepatic lesions than from other animals.

The strains were divided into four groups according to the pattern of CTX detection in the three assay systems (table III). About 50% of human and animal strains exhibited cytotoxic activities in all three assay systems. The strains showing cytotoxicity in both the FCS and SFC but not in the NCS assay, or in the SFC assay only, constituted a minor group.

In this study, the cell rounding morphological appearance in tissue culture was used as the criterion of cytotoxicity. In the FCS assay system, eight of 21 strains judged to be negative produced slight distensions of CHO cells (figure).

Table II. Profiles of cytotoxin production by *C. jejuni* isolates from human and animal sources

Strain no.	Source	A ₅₅₀ *	Viable cells* (log ₁₀ cfu/ml)	CTX titre†			Countries (received from)
				FCS	NCS	SFC	
HP 4941	Human diarrhoea	0.70	7.06	—	2	2	Japan (Dr T. Itoh)
HP 5072		0.52	6.20	—‡	—	4	
HP 5075		0.65	7.00	2	2	8	
HP 5083		1.04	7.77	2	—	8	
HP 5084		0.54	6.57	—‡	2	4	
HP 5085		0.91	6.86	—‡	2	16	
HP 5090		0.65	7.76	2	—	8	
HP 5093		0.73	6.71	—‡	2	8	
HP 5094		0.64	6.66	—	2	4	
HP 5095		0.60	6.41	2	2	8	
HP 5096		0.67	7.01	2	2	8	
HP 5100		1.18	8.48	2	4	128	
HP 5110		0.65	6.69	—	2	8	
HP 5112		0.96	7.33	2	2	8	
HP 5113		0.96	6.07	2	4	32	
HP 5117		0.63	6.25	2	2	8	
HP 5121		0.46	6.20	2	2	8	
HP 5122		0.91	6.88	—	2	16	
HP 5124		0.53	7.91	—	2	8	
CF-85-46		0.90	7.17	2	2	16	
CF-85-48		0.63	6.25	—‡	2	4	
LCDC 4483		0.92	8.48	2	2	8	Canada (Dr H. Lior)
LCDC 3969		0.75	6.81	2	—	4	
LCDC 4182		1.76	8.36	2	4	16	
LCDC 6704		0.72	7.41	2	—	32	
CCUG 7755		0.79	8.21	2	2	8	Sweden (Dr G.-B. Lindblom)
CCUG 6960		0.29	7.12	2	2	4	
CCUG 7800		0.81	7.64	—‡	2	8	South Africa (Dr A. S. Greeff)
ST 23		0.90	7.48	2	4	32	
ST 52		0.70	7.40	—‡	2	16	
ST 116		0.88	7.07	2	4	16	
ST 136SP		0.84	7.57	2	4	16	Mexico (Dr G. M. Ruiz-Palacios)
INN 180		0.65	7.10	—	2	8	
JCM 2013	Type strain (human)	0.40	8.43	2	2	8	
LMG 6444	Type strain (bovine)	0.60	7.44	—	—	16	
C1-16	Bovine faeces	0.67	6.81	—	—	8	Japan Collection of Microorganisms LMG culture collection Japan (this laboratory)
KA-14SK		0.57	7.03	2	2	8	
C6-154		0.70	6.92	2	—	16	
O-46		0.46	6.41	—	—	8	
C1-9S		0.77	7.20	—	2	16	
C5-16		0.67	6.92	—	2	16	
C1-1-2		0.78	7.12	—	—	8	
2639-2		1.19	7.26	2	4	16	
CC-1		0.87	6.97	—	2	8	
B-1		0.84	7.55	2	4	16	
B-3	Chicken faeces	1.23	7.57	2	4	32	
B-4		1.30	7.88	2	4	64	
B-5		1.20	7.14	2	4	16	
B-6		1.34	6.98	2	4	16	
B-10		1.14	7.00	2	4	16	
BL101		1.40	7.87	2	4	128	
BL103		1.35	8.06	4	8	128	
BL107		1.80	8.58	4	8	128	
BL109	Chicken hepatitis	1.43	8.11	4	8	128	
A1-35-a		0.59	6.78	2	2	16	
TZ-24-b	Dog faeces	0.85	6.25	—‡	2	16	
	Cat faeces						

No CT-like enterotoxin in the culture filtrate was detected in all strains tested.

*The value of A₅₅₀ and viable cells in stationary cultures incubated at 42°C for 4 days are indicated.

†The results show only the highest titre of the stationary and stirred cultures.

‡Morphological changes on the CHO cells which showed distending instead of rounding.

CT-like enterotoxin was not detected in the culture filtrate of any of the strains by RPLA.

Discussion

The appropriate three assay systems for the detection of *C. jejuni* cytotoxin with CHO cells were

described previously.³⁷ In this study, the frequencies of CTX production in *C. jejuni* strains isolated from man and animals were examined under optimal conditions.

The production of CTX was greatly influenced by bacterial culture conditions. The rate of CTX detection in stirred culture was higher than that in stationary culture in the FCS assay system. Conversely, station-

Table III. Pattern of CTX production in human and animal isolates in the three assay systems

CTX produced in assay with			Number (%) of strains giving CTX production pattern		
FCS	NCS	SFC	Human isolates (n = 34)	Animal isolates (n = 22)	Total (n = 56)
+	+	+	17 (50.0)	13 (59.1)	30 (53.6)
+	—	+	4 (11.8)	1 (4.5)	5 (8.9)
—	+	+	12 (35.3)	4 (18.2)	16 (28.6)
—	—	+	1 (2.9)	4 (18.2)	5 (8.9)

ary culture was better than stirred culture for the detection of CTX in the NCS and SFC assay systems. These results showed that both the stationary and stirred cultures had to be performed for the detection of CTX in all the three assay systems. However, it was found that bacterial growth did not necessarily correspond to CTX detection since cytotoxic activity was detectable even among the strains giving low growth yields (table II). The CTX titres in the FCS and NCS assays were relatively low, but the titre in the SFC assay was higher with a maximum of 128, and CTX was detected in all strains by the SFC assay. Several researchers^{25, 30, 35} have also demonstrated low CTX titres in human and animal isolates. Guerrant *et al.*³⁰ reported that only a low level of CTX activity was obtained even though highly concentrated cells were lysed by the addition of polymyxin B for detecting CTX. One possible explanation for the low toxicity in the FCS and NCS assays is the presence of some substances in the serum acting as inhibitors, or the toxin may be only very poorly secreted *in vitro*. However, if there is low CTX production *in vivo*, it may be compensated by factors such as adherence or invasiveness.⁵⁻¹¹

Although there was no significant difference in the incidence of CTX production between the strains from human and animal sources or from Japan and other countries in each assay system, chicken isolates,

especially isolates from necrotising hepatitis in broiler chickens, revealed higher activity than other isolates. It was interesting that the strain isolated from the liver lesion produced a high level of CTX. On the other hand, all of the human strains tested in this study were isolated from outbreaks of campylobacteriosis. The relationship between CTX production in human isolates and clinical signs requires further studies.

The strains were divided into four groups according to the pattern of CTX detection in the three assay systems. The possibility of different cytotoxic factors being detected in the three assay systems was demonstrated in a previous study,³⁷ i.e., differences in the sensitivity of the tissue-culture cells or in the cytotoxic activity caused by different bacterial culture conditions in the three assay systems. These findings, together with the present data, suggest that several different cytotoxins are produced by *C. jejuni*.

Distensions of the CHO cells instead of rounding of the cells was observed only in the FCS assay. These morphological changes might have been caused by the cytolethal distending toxin (CLDT).³⁸ However, it is unclear whether they are caused by the same toxin. If the cytotoxic activity is high, the cell might show rounding immediately without distending. Johnson and Lior³⁸ reported that the CHO cell was one of the most sensitive cell lines to CLDT, and the morphological change was observed progressively when assayed for up to 96 h. Although observation was continued for 96 h in the present study, the morphological changes were slight and only detectable in low titre. It was considered that the cell assay systems in this study were not suitable for the detection of the CLDT reported by Johnson and Lior.³⁸

The production by *C. jejuni* of an enterotoxin cross-reacting with CT or LT remains controversial. The results in this study were similar to those reported previously.^{35, 37, 39-42} Although attempts were made to detect CT-like enterotoxin in the culture filtrates prepared by other culture procedures such as biphasic culture,²⁵ amino-acid supplementation¹⁶ or polymyxin B treatment,¹⁶ it was not possible with the RPLA

Figure. CHO cells: **a**, after incubation for 72 h with Brucella broth (control); **b**, after incubation for 72 h with culture filtrate of *C. jejuni* HP5084 previously incubated at 42°C for 4 days without stirring. Bar = 100 μm.

method (unpublished data). Moreover, the supernate reacted with the latex beads coated with normal rabbit IgG as negative control when polymyxin B was added to the cell suspension. We reported³⁷ that our observations of CT-like enterotoxin production by reference strains of *C. jejuni*, including strain LCDC 4182, differed from the original reports.^{25, 26} McFarland and Neill³⁵ also failed to detect cytotoxic activity from strain LCDC 4182, which was reported to produce cytotoxic toxin.²⁵

Expression of the toxic activity of *C. jejuni* is

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