NEW DIAGNOSTIC METHODS

Development of a tissue culture assay system for *Campylobacter jejuni* cytotoxin and the influence of culture conditions on cytotoxin production

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Summary. A tissue culture assay system to detect *Campylobacter jejuni* cytotoxin was developed, and the effect of culture conditions on cytotoxin production was examined. Broth-culture filtrates from clinical isolates were used. The expression of cytotoxicity was dependent on the presence of serum in the culture medium. Although the titre of cytotoxin was low, the greatest cytotoxicity was revealed when newborn calf (NCS), adult bovine or goat serum was added to minimum essential medium. Moderate or weak cytotoxicity was observed when fetal calf (FCS), swine or human serum was added, and no cytotoxicity was seen with the addition of horse, rabbit, cat or chicken serum. Furthermore, the cytotoxic titre in serum-free culture (SFC) for Chinese hamster ovary (CHO) cells was higher than that with serum supplementation. CHO cells showed the highest sensitivity in FCS, NCS and SFC assay systems, whereas HeLa, Vero, HEP-2 and Intestine 407 cells were sensitive only in the NCS assay. Brucella broth was excellent for cytotoxin production, but iron supplementation had no effect. Cytotoxic expression in the three assay systems differed between stationary and stirred cultures. Our results on cytotoxin production by reference strains of *C. jejuni* did not agree with the original reports. These findings suggest that several different cytotoxins may be produced by *C. jejuni*, and that the assay system may be important for the expression of cytotoxic activity.

Introduction

Campylobacter jejuni is recognised as a major causative agent of human diarrhoeal disease^{1–5} and numerous attempts have been made to clarify the mechanism by which *C. jejuni* expresses virulence. It has been shown that potential virulence factors include adherence,^{6–9} invasiveness^{10–11} and production of enterotoxin^{12–24} or cytotoxin,^{25–35} but their contribution to pathogenesis has not been clearly demonstrated.

C. jejuni is known to produce at least three toxins—a heat-labile cytotoxic enterotoxin, a cytotoxin and a cytolethal distending toxin.³⁶ Cytotoxin has been detected in several different strains of *C. jejuni* and has been confirmed to be toxic to Chinese hamster ovary (CHO), Vero, HeLa, HEP-2, Intestine 407 (Int 407) and MRC-5 tissue culture cells. However, the biological, biochemical and genetic characterisation of these holotoxins remains controversial.

Purification and characterisation of this toxin is needed to define its involvement in the pathogenesis of *C. jejuni* infection but little is known about the effect of culture conditions on toxin production and there is not yet an optimal cell assay system for its detection. In the present study, tissue culture cells were used and appropriate culture conditions were developed for the assay of cytotoxin production by *C. jejuni*.

Materials and methods

Bacterial strains

The characteristics of the nine strains of *C. jejuni* used in this study are shown in table I. These strains were isolated from outbreaks of campylobacteriosis. Strains LCDC4483, CCUG7755 and HP5113 were tested in greater detail. The strains were suspended in Nutrient Broth No. 2 (Oxoid) containing dimethylsulphoxide (Sigma) 10% and stored at –80°C before use.

Table 1. Strains of *C. jejuni* studied

Strain no.	Received from	Reference no.
HP 5113	T. Itoh	5
CCUG 7755	G.-B. Lindblom	22
LCDC 4483	H. Lior	25
LCDC 4182		
LCDC 3969		
LCDC 6704		
ST 23	A. S. Greeff	26
ST 116		
ST 136sp		

Cytotoxin production

Each strain was grown on horse blood agar for 48 h at 37°C in an atmosphere of O₂ 5%, H₂ 5%, CO₂ 10% and N₂ 80%. A loopful of bacterial growth was inoculated into 20 ml of Brucella Broth (BB; BBL Microbiology Systems, USA) in a flask, and the initial optical density (A₅₅₀) was adjusted to between 0.1 and 0.2. The cultures were incubated at 37°C or 42°C for 2 or 4 days under stationary or stirred (100 rpm) conditions. After incubation, the number of colony forming units (cfu)/ml and A₅₅₀ were measured. The broth culture was centrifuged at 10000 *g* for 30 min at 4°C and filtered through a 0.20-µm cellulose nitrate membrane (Advantec Toyo Ltd, Tokyo, Japan). The supernate was stored at 4°C before testing.

Cell lines for cytotoxin detection

CHO, HeLa, Vero and C6 cells were obtained from the Japanese Cancer Resources Bank (JCRB, Tokyo). Int 407 and HEp-2 cells (ATCC strains) were purchased from Dainippon Pharmaceutical Co., Ltd, Japan. CHO cells were principally used for cell assays when the conditions for cytotoxin detection were being measured. All cells were maintained in 50-ml culture bottles in Eagle's Minimum Essential Medium (MEM; Nissui Pharmaceutical Co., Ltd, Tokyo) supplemented with 200 mM glutamine, sodium bicarbonate 7.5% and fetal calf serum (FCS; Gibco Laboratories, USA) 10%.

Cytotoxin assay

To examine the effect of serum on cytotoxic activity, FCS, newborn calf serum (NCS; Gibco), adult bovine serum (ABS; Nippon Biotest Laboratories, Tokyo), goat (Cosmo-bio Co., Ltd, Tokyo), horse (Flow Laboratories Inc., USA), swine (Cosmo-bio), rabbit (Cosmo-bio), chicken (Flow Laboratories), human (supplied by our laboratory) or cat (supplied by our laboratory) serum were used for the cytotoxic assay with CHO cells. Trypsinised cells were resuspended in MEM with FCS 10%, and the supernate was aspirated after centrifugation. The cell medium was then replaced with MEM containing 10% of each serum

described above. After washing with the replaced medium, 100 µl of the cell suspension were placed in each well of a 96-well microtitration plate at 5 × 10³ cells/well, and the cells were allowed to adhere to the bottom of the wells for 1–3 h. Then 100 µl of bacteria-free supernate were added to each well, followed by incubation at 37°C for 48 h in air with CO₂ 5%. Cytotoxic effects were assayed at pre-determined times by phase-contrast microscopy, and wells in which > 30% of the cells showed rounding were considered to show a positive result. BB alone was used as a negative control.

To examine the effect of serum concentration, FCS or NCS was added to MEM at concentrations ranging from 1 to 30% for preparation of CHO cell suspensions and then cytotoxic activity was determined.

ASF 104 medium (Ajinomoto Co., Ltd, Tokyo) as a serum free culture (SFC) was also used for cytotoxicity assay. The CHO cell suspension was centrifuged and washed with ASF 104 medium, placed in a 96-well microtitration plate and assayed for cytotoxic activity as described above.

Vero, HeLa, Int 407, HEp-2 and C6 cells were used for comparison of sensitivity to cytotoxin in three different media comprising of MEM supplemented with FCS 20% or NCS 10% or ASF 104.

Detection of enterotoxin

Bacteria-free culture filtrates were also 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). Equal volumes of sample and suspension of latex beads coated with rabbit anti-CT antibody were mixed in a 96-well microtitration plate and kept overnight at room temperature. Latex beads coated with normal rabbit IgG were used as a negative control. More than 1–2 ng/ml of CT was detectable with this assay kit.

Effect of culture conditions on cytotoxin production

Growth media. *C. jejuni* HP5113 was grown at 42°C for 4 days in five different media: BB, Mueller-Hinton Broth (MHB; Difco), casamino acid yeast extract broth (CYE),²⁰ biphasic culture system (BPC)²⁵ and medium 199 tissue culture medium (M199; Nissui). Furthermore, ferric chloride, ferrous sulphate or Iso-Vitale X (BBL) was added to BB as an iron source according to the report of McCardell *et al.*²⁰ Serial two-fold dilutions of the test samples in 20 mM phosphate-buffered saline (PBS), pH 7.2, were added to each well, and the titre of cytotoxic activity was recorded as the highest dilution of the sample causing morphological changes in the cells.

Growth conditions. To determine the optimal culture conditions, *C. jejuni* HP5113 was grown at 37° or 42°C for 6 days with or without stirring in BB. Bacterial growth was monitored by measuring the A₅₅₀ of the

Fig. 1. CHO cells (a) after incubation for 24 h with BB (control) and (b) after incubation for 24 h with culture of *C. jejuni* strain HP 5113. Bar = 100 μ m.

Table II. Effect of serum from different species on cytotoxic activity

Test sample	Cytotoxic activity with indicated serum addition*									
	FCS†	NCS†	ABS†	Goat	Swine	Human	Horse	Rabbit	Cat	Chicken
Culture filtrate of										
LCDC 4483	+‡	+++	+++	+++	—	+	—	—	—	—
CCUG 7755	+	+++	+++	+++	++	+	—	—	—	—
HP 5113	++	+++	+++	+++	+	—	—	—	—	—
Brucella broth alone	—	—	—	—	—	—	—	—	—	—

*See *Materials and methods* for explanation of abbreviations.

†Each serum was added to tissue culture medium at 10%.

‡Percentage of rounded cells is indicated as: —, 0–30%; +, 31–60%; ++, 61–80%; +++, 81–100%.

whole culture and viable cells (cfu/ml), and then the cytotoxic activity was measured by the three toxin assay systems with the CHO cell line.

Results

Effect of cell assay conditions

Serum from different species. The strongest cytotoxic activity was revealed when NCS, ABS or goat serum was added to MEM (fig. 1 and table II). Moderate or weak toxicity was detected when FCS, swine or human serum was added, and no cytotoxic effect was indicated after addition of other sera. The same data were

obtained even if different lots of serum were used for the assay.

Serum concentration. The cytotoxic effect was dependent on serum concentration. Higher cytotoxic activity was detectable when FCS was added to MEM at > 20% and the cytotoxic effect was expressed after incubation for 24 h. In contrast to FCS addition, CHO cell cultures supplemented with NCS > 5% revealed morphological changes within 24 h after treatment with the culture filtrate.

Serum-free medium. The cytotoxin titre in the serum-free assay system was higher than that in the serum-supplemented assay system (table III). Notably, the culture filtrate of HP5113 showed a titre of 32 in SFC.

Sensitivity of different cell lines. Comparative studies of the sensitivity of different cell lines were performed with CHO, Vero, HeLa, HEP-2, Int 407 and C6, and the three assay systems. CHO and C6 were most sensitive cell lines in all the assay systems, whereas Vero, HeLa, HEP-2 and Int 407 cells were sensitive only in the NCS assay system (table IV).

Table III. Comparison of cytotoxin titre in three assay systems

Strain* no.	Titre in indicated assay		
	SFC	FCS	NCS
LCDC 4483	8	2	2
CCUG 7755	8	2	2
HP 5113	32	2	4

*Each strain was cultivated in BB at 42°C for 4 days without stirring.

From these data, the most appropriate conditions for detection of cytotoxin were considered to be achieved with CHO cells as indicator cells and with three assay systems consisting of FCS 20% or NCS 10% added to MEM, and ASF 104 medium as a

Table IV. Comparison of cytotoxic effect against different cell lines

Test sample	Cell assay system	Cytotoxic activity against indicated cells					
		CHO	C6	HeLa	Vero	INT 407	HEp-2
Culture filtrate of HP 5113*	FCS	+++†	+++	—	—	—	—
	NCS	+++	+++	+++	+++	+++	+
	SFC	+++	+++	+	—	—	—
Brucella broth alone	FCS	—	—	—	—	—	—
	NCS	—	—	—	—	—	—
	SFC	—	—	—	—	—	—

*HP 5113 strain was cultivated in BB at 42°C for 4 days without stirring.

†Percentage of rounded cells is indicated as: —, 0–30%; +, 31–60%; ++, 61–80%; +++, 81–100%.

Table V. Cytotoxin production by *C. jejuni* HP 5113* in different growth media

Growth medium	A ₅₅₀ after cultivation	Titre of indicated assay system		
		FCS	NCS	SFC
Brucella broth (BB)	0.96	2	4	32
Casamino yeast extract broth	0.80	2	4	16
Mueller-Hinton broth	0.72	2	2	16
Biphasic medium	0.86	2	2	4
M199	0.40	< 2	< 2	< 2
BB supplemented with				
FeCl ₃ 0.02 µg/ml	0.91	2	4	16
1 µg/ml	0.98	2	4	16
FeSO ₄ 0.1 µg/ml	0.91	2	4	16
1 µg/ml	0.91	2	4	16
IsoVitalex 1%	0.91	< 2	< 2	16

*Strain HP 5113 was cultivated at 42°C for 4 days without stirring.

serum-free medium, as it was doubtful whether the same cytotoxin would be detectable in each assay system.

Influence of culture conditions on cytotoxin production

Effect of growth media. Five media were evaluated for cytotoxin production. The cytotoxin titres of BB and CYE cultures filtrates from the same strain were the same, but the M199 culture filtrate had no cytotoxic effect. MHB and BPC were inferior to BB and CYE for cytotoxin production. Cytotoxic activity was not increased by using BB supplemented with ferric chloride and ferrous sulphate for enterotoxin production as reported by McCardell *et al.*²⁰ In contrast, cytotoxic activity was depressed by addition of IsoVitalex 1% without the SFC assay system (table V).

Culture conditions. To determine the optimal culture

Fig. 2. Time course of bacterial growth (a) and bacterial turbidity (below); ●, stationary; ○, stirred. b, cytotoxic titre in stirred (upper) and stationary culture (below); ■, FCS; ▨, NCS; ■, SFC.

Table VI. Discrepancy in the profile of toxin production between the original reports and the assay systems used in this study

Strain* no.	Profile of toxin production in					
	original reports ^{25, 26}		this experiment			
	Enterotoxin	Cytotoxin	RPLA	FCS	NCS	SFC†
LCDC 4483	+	+	—	2	2	8‡
LCDC 4182	+	—	—	2	4	16
LCDC 3969	—	+	—	2	< 2	4
LCDC 6704	—	—	—	2	< 2	32
ST 23	+	+	—	2	4	32
ST 116	+	—	—	2	4	16
ST 136sp	—	—	—	2	4	16
CCUG 7755	+	Unknown	—	2	2	8
HP 511	Unknown	Unknown	—	2	4	32

*Each strain was cultivated in BB at 42°C for 2 days with stirring or for 4 days without stirring.

†See *Materials and methods* for explanation of abbreviations.

‡Cytotoxin titre.

conditions, comparative studies were performed with or without stirring at 37°C and 42°C. Both bacterial growth and optical density in the stationary culture were greater than in the stirred culture (fig. 2). The combination of cytotoxic expression in the three assay systems differed between stirred and stationary cultures. Cytotoxic activity in the NCS and SFC assay systems was observed in the stationary culture filtrate at an earlier stage of culture. In contrast, the activity in the FCS assay was detected more easily in the stirred culture filtrate after incubation for 24 h. Culture temperature had no obvious effect on toxic activity.

From these data, we considered that the optimal culture conditions for cytotoxin production were achieved with BB as the growth medium and by culturing the bacteria at 42°C for 4 days in a stationary culture.

Cytotoxin and enterotoxin production by Campylobacter isolates

The results of cytotoxin and CT-like or LT-like toxin detection from *C. jejuni* clinical isolates are summarised in table VI. RPLA assay showed that enterotoxin that was cross-reactive with CT or LT was not produced by any strains. Our data on the cytotoxin produced from reference strains did not agree with the original reports.^{25, 26}

Discussion

Cytotoxins have been demonstrated in human and non-human strains of *Campylobacter* and determined to be toxic to HeLa, Vero, HEp-2, Int 407, MRC-5, and CHO cell lines.^{25–35} However, it is difficult to determine the characteristics of the cytotoxins, as the procedures used for preparing samples of cytotoxins

differ among laboratories. This confusion appears to be attributable either to the presence of several different cytotoxins or to the lack of a reliable assay system for studying cytotoxins of *Campylobacter* spp. This study demonstrated that the production and detection of cytotoxin was greatly influenced by the culture conditions and assay system employed. Many researchers have adopted a tissue culture system for detection of cytotoxin, and FCS has usually been used for serum supplementation. However, when we used FCS, we were unable to detect cytotoxic activity in Vero, HeLa, HEp-2 or Int 407 cell cultures. In contrast, cytotoxic activity was observed on all cell lines employed when NCS was added to MEM. Furthermore, a high level of cytotoxic activity on CHO cells was observed with the SFC assay system. These findings suggest that the substance amplifying or inhibiting the cytotoxic activity, or both, may be present in serum. Goossens *et al.*,²⁸ Mahajan *et al.*,³⁴ and Florin *et al.*³⁵ adopted the NCS assay system for cytotoxin detection, but used different media and procedures for cytotoxin production, and it is still not clear whether the same cytotoxin was detected by each researcher. McCardell *et al.*²⁹ reported that heat-stable cytotoxin was detected when the concentration of FCS in the tissue culture medium was reduced to 0.7%, and that the cytotoxicity was inactivated by normal rabbit serum. In our experiments, the cytotoxicity was also inactivated when normal rabbit, horse, cat or chicken serum was added to MEM for CHO cell culture, but we failed to detect any cytotoxicity when FCS 1% was added.

There is a possibility that several different cytotoxins are produced by *C. jejuni*. In the present study, different cytotoxins may have been detected in each of the three assay systems, because it is difficult to consider that these differences in cytotoxic expression are attributable to the sensitivity of the cells to the cytotoxin in each assay system. The combination of cytotoxin expression in the three assay systems varied

according to bacterial culture conditions, i.e., with or without stirring. The characteristics of crude cytotoxin described in previous reports differed with respect to heat stability,^{27, 29} molecular mass^{29, 31, 34} and cytotoxicity against cultured cells.^{25, 27, 28, 35} Cytotoxin production by the reference strains in this study did not correspond to that in the original reports.^{25, 26} It is possible that our assay systems for *C. jejuni* cytotoxin might detect a different type of cytotoxin from the original reports.

The best conditions for cytotoxin production in the NCS and SFC assay systems were growth for 4 days in BB at 37°C or 42°C without stirring. In contrast, stirred cultures were better than the stationary culture for detection in the FCS assay system. Further study is required to clarify whether cytotoxic activity in the three assay systems is an expression of different toxins. The characteristics and the role of these factors in pathogenesis of *C. jejuni* infection remain to be determined.

An enterotoxin produced by *C. jejuni* has been detected by three methods—cytotoxic effect on CHO cells,^{12, 13, 15, 16, 19, 21, 22, 26, 35} the GM₁ ganglioside en-

zyme-linked immunosorbent assay^{14, 16–18, 20, 21, 23, 24} or neutralisation test based upon immunologic cross-reactivity with anti-CT or anti-LT antibody.^{12, 16, 19} In this study, we attempted to detect the enterotoxin by RPLA with anti-CT antibody. However, we were unable to detect CT-like enterotoxin despite the fact that the reference strains had originally been shown to produce the enterotoxin by a CHO assay. One explanation for these discrepant results was that the culture condition examined in this study was not suitable for enterotoxin production. Johnson and Lior reported that the response of CHO cells to a cytolethal distending toxin produced by *Campylobacter* strains was possibly misinterpreted as the heat-labile enterotoxin.³⁶ Further study is required to resolve these discrepancies, as several researchers have been unable to find enterotoxin production by *Campylobacter* strains.^{37–39}

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