Detection of Serum-Dependent Cytotoxic Activity of *Campylobacter jejuni* and its Characteristics

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ABSTRACT. We previously set forth appropriate three assay systems using Chinese hamster ovary cells to detect the *Campylobacter jejuni* cytotoxin. Although we could not reach a conclusion because the cytotoxins shown in this study were not purified, at least three different cytotoxins were detected in these assay systems. The first cytotoxin detected in the presence of fetal calf serum (FCS) was heat-labile and the molecular weight (Mw) was estimated at 50–100 k by ultrafiltration. The second cytotoxin detected in the presence of newborn calf serum (NCS) was heat-stable and Mw was estimated at 0.5–3.0 k. The third cytotoxin detected in serum-free culture (SFC) assay was heat-labile and non dialyzable. However, Mw was not estimated since the low Mw and heat-stable cytotoxin was also detected in this assay. The cytotoxic activity detected in FCS and NCS assays, but not that detected in SFC assay, was completely abolished by treatment with a reducing agent. In contrast, the cytotoxicity detected in both FCS and NCS assays was not inactivated by such an enzyme as trypsin, lipase, neuraminidase, and β -galactosidase. When the filtrate was heated at 100°C to inactivate the heat-labile cytotoxin, the cytotoxic activity was detected in the NCS assay but not in FCS assay. However, when NCS was added to this heated filtrate, the cytotoxicity was restored in FCS assay. Furthermore, when normal rabbit serum (NRS) was added, no cytotoxicity was restored. The cytotoxic activity in SFC assay was completely inactivated with FCS or NRS. These findings suggest that the cytotoxic activity is dependent on serum added to tissue culture medium and that the substance amplifying and/or inhibiting the cytotoxic activity may be present in serum. — **KEY WORDS:** *Campylobacter jejuni*, characteristic, cytotoxin, serum, tissue culture.

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Campylobacter jejuni has been recognized as an important enteric pathogen throughout the world [3, 5, 36] and great efforts have been made by many researchers to clarify the pathogenic mechanism with *in vivo* or *in vitro* models [37]. It is considered that the toxin produced by *C. jejuni* is one of the most important factors causing illness. Although the mechanism by which these toxins cause diarrhea is not clearly understood, *C. jejuni* is known to produce two types of toxins. One is a cytotoxic enterotoxin [2, 6, 7, 10, 16, 20–22, 26, 27, 34, 35] which is immunologically related to both cholera toxin and heat-labile *Escherichia coli* enterotoxin, and the other is a cytotoxin [1, 8, 9, 11, 13, 23, 25, 28, 30, 31, 38]. Although many reports have been published on *C. jejuni* cytotoxin, it is difficult to determine whether the identical toxin was detected, because the procedures for its production and detection have differed among researchers. Furthermore, the cytotoxin was not produced in high concentrations and the characteristics were complicated by loss of the cytotoxic activity during purification by classical techniques [15]. Consequently, the results are conflicting. We previously reported that an appropriate assay system using tissue culture to detect the cytotoxin produced by *C. jejuni* had been developed, and the effect of culture conditions on cytotoxin production was examined [29]. The study demonstrated that the production and detection of cytotoxin was greatly influenced by the culture conditions and assay system employed. We developed three assay systems that use different tissue culture media for Chinese hamster ovary (CHO) cells, i.e., minimum essential medium (MEM) supplemented with 20%

fetal calf serum (FCS) or 10% newborn calf serum (NCS), and serum-free culture (SFC) medium. Furthermore, our results on the cytotoxin produced by the reference strains in the three assay systems did not agree with toxin production in the original reports. In this study, we attempted to characterize the crude cytotoxin in the culture filtrate of *C. jejuni*, and to determine whether the identical cytotoxin was detected in the three assay systems. We determined also the effect of serum on cytotoxic activity in each of the three assay systems.

MATERIALS AND METHODS

Bacterial strains: *C. jejuni* strain BL107 isolated from a necrotic lesion of chicken hepatitis was used in this study since this strain gave the highest titer of all strains tested in our laboratory. The strains were suspended in Nutrient Broth No. 2 (Oxoid Ltd., England) containing 10% dimethylsulfoxide (Sigma Chemical Company, U.S.A.) and stored at -80°C until tested.

Cytotoxin production: *C. jejuni* was grown on horse blood agar plates for 24 hr at 42°C under a gas mixture containing 5% O₂, 5% H₂, 10% CO₂ and 80% N₂. A loopful of bacteria was inoculated into 20 ml of Brucella broth (BBL Microbiology Systems, Cockeysville, MD) in a flask and the initial optical density at 550 nm was adjusted to 0.1 to 0.2. The cultures were incubated at 42°C for 4 days stationarily or for 2 days with stirring (100 rpm). The final optical density after these culture conditions usually ranged from 1.0 to 1.5. The broth culture was centrifuged at 10,000

× g for 30 min at 4°C and filtered through a 0.20-μm cellulose nitrate membrane (Advantec Toyo Ltd., Tokyo). The supernatant was stored at 4°C until testing. The sample was used for characterization within three days after the preparation of the culture filtrate.

Assay for cytotoxin: Cytotoxic activity was detected with three tissue culture assay systems developed previously [29]. In brief, trypsinized CHO cells (supplied by Japanese Cancer Resource Bank, Tokyo) were suspended in Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co., Tokyo) containing 20% fetal calf serum (FCS, Flow Laboratories, Inc., McLean, VA) or 10% newborn calf serum (NCS, Gibco Laboratories, Grand Island, NY), or ASF 104 medium (Ajinomoto Co., Ltd., Tokyo) as a serum-free culture (SFC). A volume of 100 μl containing 5×10^3 cells of cell suspensions was placed in each well of a 96-well microplate, and allowed to adhere to the bottom of the well for 1 to 3 hr. Then 100 μl of bacteria free supernate was added to each well, followed by incubation at 37°C for 72 hr under 5% CO₂ in air. The wells showing rounding by more than 30% of the cells were considered positive. Brucella broth alone was used as a negative control. The cytotoxic titer was indicated as the reciprocal of the highest of twofold dilutions giving positive cytotoxicity.

Characteristics of the cytotoxic factors:

(1) **Heat lability:** Culture filtrates were heated for 30 min at 56, 70, 100 (boiling) or 121°C (heating in an autoclave). After cooling, the cytotoxic effect on CHO cells was determined in the three assay systems.

(2) **pH lability:** Culture filtrate was adjusted by addition of 2N HCl or 2N NaOH to cover the pH range from 1 to 10, and incubated at 37°C for 1 hr. After incubation, the overall pH was then re-adjusted to 7.0 and cytotoxin assay was performed.

(3) **Molecular weight estimation:** Molecular weights (Mw) of cytotoxic factors were estimated by ultrafiltration and dialysis. Ultrafiltration of 20 ml of the filtrate was performed in an Amicon model 8050 (Amicon Inc., U.S.A.) unit fitted with a Diaflo ultrafilter consisting of YC05 (0.5 k cutoff), YM1 (1 k cutoff), YM3 (3 k cutoff), YM10 (10 k cutoff), YM30 (30 k cutoff), XM50 (50 k cutoff), YM100 (100 k cutoff) and XM300 (300 k cutoff). The filtrate through each membrane was assayed for the cytotoxic activity in the three assay systems. Dialysis was also undertaken with a cellulose tube (Spectra/Por® 1; Serva Feinebiochemica GmbH & Co., Germany) with a cutoff of 6 to 8 k. The filtrate was dialyzed for 24 hr at 4°C with two changes of 10 mM phosphate buffered saline (pH 7.2) and determined for the cytotoxic titer.

(4) **Effects of enzymes and reducing agents:** The filtrate was treated with 1 mg/ml of trypsin (1:250, Difco Laboratories, Detroit, MI, U.S.A.), 1 U/ml of lipase (Sigma Chemical Co., St. Louis, MO), neuraminidase (Sigma) or β-galactosidase (Boehringer Mannheim GmbH, Mannheim, Germany). The filtrates were treated with 0.25 mg/ml of dithiothreitol (Nacalai Tesque Co., Tokyo), sodium metabisulfite (Nacalai Tesque), or L-cysteine

monohydrochloride (Wako Pure Chemical Industries, Osaka) as a reducing agent. Each reagent was prepared at 20-fold concentration as the final concentration. The mixture was incubated at 37°C for 1 hr, and the cytotoxic titer was compared with untreated filtrates.

(5) **Effect of serum:** Normal rabbit serum (NRS; Cosmo-bio Co., Ltd., Tokyo), FCS or NCS was added to the filtrate or the SFC assay system at 10%, and the cytotoxicity was determined. The cytotoxic activity in the culture filtrate heated at 100°C for 30 min or dialyzed as described above before serum addition was determined.

(6) **Immunological cross-reactions:** The immunological cross-reactions were determined by reversed passive latex agglutination (RPLA), enzyme immunosorbent assay (ELISA) or the neutralization test. Bacteria-free culture filtrate was assayed for *Vibrio cholerae* toxin (CT)-*Escherichia coli* heat-labile enterotoxin (LT) or *E. coli* Shiga-like toxins (SLT) I and II with RPLA kits (Denka Seiken Co., Ltd., Tokyo). The cross-reaction with *E. coli* heat-stable enterotoxin (ST) was determined with an ELISA assay kit (Denka Seiken). For the neutralization test, rabbit antiserum against *C. jejuni* heat-labile antigen of BL107 strain was prepared according to the method of Lior [24] for serotyping. The filtrate was incubated with antiserum at 1:20 for 1 hr at 37°C before being added to the cells.

RESULTS

Heat lability: The cytotoxicity was completely inactivated in the FCS assay system at higher than 100°C, but it was highly stable in the NCS assay even at 121°C. The cytotoxic activity detected in the SFC assay was mostly heat-labile and partially heat-stable (Table 1).

pH lability: The pH lability of *C. jejuni* cytotoxin is shown in Table 2. Although the cytotoxic activity in the FCS and SFC assay systems were completely lost at pH below 3.0, a low activity was detected in NCS assay.

Estimation of Mw: As shown in Table 3, the Mws of the cytotoxin detected in the three assay systems were different from one to another. The cytotoxin detected in the FCS assay was nondialyzable and estimated at 50 to 100 k. That detected in NCS assay was dialyzable and estimated at 0.5 to 3 k. In contrast, that detected in SFC assay was mostly nondialyzable but Mw was not necessarily uniform in

Table 1. Heat lability of *C. jejuni* cytotoxic effect on CHO cells

Sample treated (°C)	Cytotoxic titer		
	FCS assay	NCS assay	SFC assay
unheated	4	8	128
56	2	8	128
70	2	8	128
100	<2	8	16
121	<2	4	8

ultrafiltration.

Effect of enzyme and reducing agents: The cytotoxic activity was hardly changed upon treatment with any of the enzymes used in this study in the FCS or NCS assay system (Table 4). However, the activity decreased by about 50% when the filtrate was treated with trypsin, but it was not judged as intact negative. The effect of enzyme in SFC assay could not be determined since the cytotoxicity was observed as positive even when an enzyme solution other than β -galactosidase was added to the cells as a negative control. Treatment of the filtrates with a reducing agent at 0.25 mg/ml completely abolished the cytotoxic activity in the FCS and NCS assay systems but not in the SFC assay.

Effect of serum: Addition of FCS, NCS or NRS to the filtrate did not affect the cytotoxic activity when CHO cells had already been cultivated with FCS or NCS. However, when the filtrate was dialyzed to remove low Mw cytotoxin, the cytotoxic activity was recovered with FCS added to the NCS assay system (Table 5). When the filtrate was heated at 100°C to inactivate heat-labile cytotoxin, the cytotoxic activity was restored by addition of NCS to FCS assay. In contrast, when NRS was added, no cytotoxicity was restored. Furthermore, when the filtrate was dialyzed and heated to

exclude both heat-labile and low Mw cytotoxins, the cytotoxic activity was not restored by addition of NCS to FCS assay nor by addition of FCS to NCS assay. The cytotoxic activity in SFC assay was completely depressed with FCS or NRS, and the titer reduced from 1:128 to 1:8 by addition of NCS (Table 6).

Immunological cross-reactions: No immunological cross-reaction was detected between the epitopes of *C. jejuni* cytotoxins and CT, LT, SLT (I and II) or ST with the RPLA or ELISA assay kit. Reference toxins were detectable at 1–2 ng/ml and 10 to 20 ng/ml with the RPLA and ELISA kits, respectively. The cytotoxic activity was not neutralized with the antibody against heat-labile antigen in FCS or NCS assay systems. The neutralization test in SFC assay could not be performed since the antiserum, prepared from the rabbit abolished the cytotoxic activity in the SFC assay system using NRS.

Table 4. Effects of enzymes and reducing agents on the cytotoxic activity

Sample treat with	Cytotoxic titer		
	FCS assay	NCS assay	SFC assay
Trypsin	2	8	ND ^{a)}
Lipase	4	8	ND
Neuraminidase	4	8	ND
β -galactosidase	4	8	128
Dithiothreitol	<2	<2	128
Sodium metabisulfite	<2	<2	128
L-cysteine	<2	<2	128
Untreated	4	8	128

a) Not determined.

Table 5. Effects of heating, dialysis and addition of serum on the cytotoxic activity in the FCS and NCS assay systems

Treatment of culture filtrate		Cytotoxic titer	
		FCS assay	NCS assay
Unheated	Nondialyzed	4	8
	+FCS ^{a)}	4	8
	+NCS	4	8
	+NRS	2	8
	Dialyzed ^{b)}	2	<2
	+FCS	2	2
	+NCS	2	<2
	+NRS	2	<2
Heated ^{c)}	Nondialyzed	<2	8
	+FCS	<2	8
	+NCS	4	8
	+NRS	<2	8
	Dialyzed	<2	<2
	+FCS	<2	<2
	+NCS	<2	<2
	+NRS	<2	<2

a) 10% Serum was added to the culture filtrate.

b) Culture filtrate was dialyzed at 4°C for 24 hr.

c) Culture filtrate was heated at 100°C for 30 min.

Table 2. Effect of pH on cytotoxic activity

pH range	Cytotoxic titer		
	FCS assay	NCS assay	SFC assay
1–2	<2	2	<2
2–3	<2	2	<2
3–4	2	4	32
4–5	4	8	64
5–6	4	8	128
6–7	4	8	128
7–8	4	8	128
8–9	4	8	128
9–10	2	8	64
Untreated	4	8	128

Table 3. Estimation of the molecular weight

Mw cutoff (kDa)	Cytotoxic titer ^{a)}		
	FCS assay	NCS assay	SFC assay
0.5	<2	<2	<2
1	<2	2	16
3	<2	4	8
10	<2	4	8
30	<2	4	8
50	<2	4	32
100	4	8	128
300	4	8	128
Dialysis ^{b)}	2	<2	64
Untreated	4	8	128

a) Cytotoxic titer of the culture filtrate passed through the ultrafiltration membrane is shown.

b) The culture filtrate was dialyzed for 24 hr.

Table 6. Effects of heating, dialysis, and addition of serum on cytotoxic activity in the SFC assay system

Treatment of culture filtrate		Cytotoxic titer after addition of			
		None	FCS ^{a)}	NCS ^{a)}	NRS ^{a)}
Unheated	Nondialyzed	128	<2	8	<2
	Dialyzed ^{b)}	64	<2	<2	<2
Heated ^{c)}	Nondialyzed	16	<2	8	<2
	Dialyzed	4	<2	<2	<2

a) 10% Serum was added to the culture filtrate.

b) Culture filtrate was dialyzed at 4°C for 24 hr.

c) Culture filtrate was heated at 100°C for 30 min.

DISCUSSION

Although we failed to reach a conclusion because the cytotoxins were not purified, some evidence suggested that at least three different types of cytotoxin were detected in the culture filtrate of *C. jejuni* by using three different culture media for CHO cells for detecting the cytotoxic activity. The first cytotoxin detected in the FCS assay system was heat-labile with an Mw estimated at 50–100 k. The second cytotoxin detected in the NCS assay was heat-stable with an estimated Mw of 0.5–3.0 k. The third cytotoxin detected in SFC assay was most heat-labile and non-dialyzable but the Mw range could not be determined by ultrafiltration. Although the pH lability was similar among the assay systems, other properties were very different. The cytotoxic activity in FCS and NCS but not in SFC assay was abolished upon treatment with a reducing agent such as L-cysteine, sodium metabisulfite, and dithiothreitol. MEM and Brucella broth also contained L-cysteine and sodium metabisulfite at a concentration of 0.031 and 0.1 mg/ml, respectively, but the cytotoxic activity was not inhibited at either concentration in these assay systems.

The possibility of different cytotoxic factors detected in the three assay systems was demonstrated by our previous data [29], i.e., the differences in the sensitivity to the tissue culture cells and in the cytotoxic activity with the bacterial culture conditions in the three assay systems. We demonstrated also that the cytotoxic activity was dependent on serum added to the tissue culture medium [29]. Further examinations were designed to determine the effect of serum added to the culture filtrate or SFC assay on the cytotoxic activity. Serum added to the filtrate did not affect the cytotoxic activity when CHO cells had already been cultured in MEM with FCS or NCS, whereas that in the SFC assay was completely depressed by FCS or NRS added. Interestingly, when NCS was added to the heated filtrate in which cytotoxic activity was no longer detected in FCS assay, the cytotoxic activity was restored, but it was abolished after dialysis (Table 5). It was considered that the restoration of the cytotoxicity in FCS assay may have been caused by activation by NCS added.

Furthermore, when the filtrate was dialyzed to remove

the low Mw cytotoxin, the cytotoxic activity was detectable in the FCS assay but not in the NCS assay system. These results also indicate that the cytotoxin detected in FCS or NCS assay may have been activated by the specific serum. However, we failed to determine whether the cytotoxins detected in this study were proteins or some other substance since the cytotoxic activity was not inactivated or neutralized with any enzyme or did not cross-react with antibodies against reference toxins or bacteria-associated antigen. Although data were shown only for the *C. jejuni* strain BL107, all of the characteristics mentioned above were obtained also with culture filtrates of strains HP5113, LCD4483 and CCUG7755 reported previously [29].

A question has been raised whether the cytotoxins detected in this study are identical to those reported by other researchers. It is difficult to compare the characteristics of the cytotoxins since the procedures used for preparing and detecting the cytotoxins differ among laboratories. *C. jejuni* has been reported to produce heat-labile [9, 12, 13, 16, 38] and/or heat-stable cytotoxin [14, 28]. The heat-labile cytotoxin was inactivated with trypsin and Mw was estimated at 30 to 100 k [13, 25, 31]. Although Mw of the heat-labile cytotoxin detected in this study was in the same range, the trypsin sensitivity did not agree with other reports. The cytotoxin detected in FCS assay may have been different from that detected with a bacterial culture medium supplemented with IsoVitale X or sodium metabisulfite [4, 16] because we failed to detect it under the same culture conditions [29].

The cytotoxin detected in NCS assay was heat-stable, had a low Mw and was inactivated with a reducing agent but not with trypsin. A few studies [14, 28] on heat-stable cytotoxins of *C. jejuni* have been performed, but there is no reports on cytotoxin with Mw of 0.5 to 3.0 k. Among the researchers [9, 11, 25] who adopted the NCS assay system for *C. jejuni* cytotoxin detection, only Mahajan and Rodgers [25] reported that Mw was 68 k, but the others did not report the characteristics of the cytotoxins. Lam [23] did not demonstrate such properties as Mw or trypsin sensitivity of the heat-stable cytotoxin, either.

We have not been able to determine whether one or more components were included in the toxin detected in SFC assay since the Mw was not necessarily uniform in ultrafiltration. The cytotoxic activity was mostly heat-labile and completely abolished with FCS or NRS but not with IsoVitale X [29], reducing agents or by dialysis with 6 to 8 k cutoff membrane. These findings support the assumption that the heat-labile cytotoxin detected in the SFC assay is distinct from that detected in the FCS or NCS assay system. Mw of the heat-labile cytotoxin detected in SFC assay might be high. The heat-stable and low Mw cytotoxin detected in the NCS assay might also be detectable in SFC assay because the cytotoxic activity was not completely abolished with NCS. The fact that the cytotoxic activity was not detectable when NCS was added to the culture filtrate after dialysis also supports these findings. McCardell *et al.* [28] reported that heat-stable cytotoxin with Mw 70 k, was

detected when the concentration of FCS in the tissue culture medium was reduced to 0.7%, and that the cytotoxicity was inactivated with NRS. These characteristics resembled those of the cytotoxin detected in the SFC assay, but we failed to detect any cytotoxicity when 1% FCS was added [29].

Other reports have been published concerning cytotoxic or cytolytic activities of *C. jejuni*, i.e., cytolethal distending toxin [17], hepatotoxin [18, 19], and hemolysin [14, 32]. However, we could not compare our data with those of these toxins since their purification and characterization have not been completed. Florin and Antillon [9] also pointed out that it was impossible to deduce from published reports how many cytotoxins had been detected. It is considered that such a confusing status is attributable to the presence of several different toxins, genetic regulation to express the cytotoxicity, or lack of a reliable assay system for studying cytotoxins of *Campylobacter*. It is important for solving the controversy as indicated by Ruiz-Palacios [33] that reference strains producing toxins and details of methods would become easily exchangeable between researchers.

We reported that cytotoxic activity was detectable in NCS assay but not in the FCS assay when Vero cells were used, while it was detectable in both assay systems when CHO cells were used [29]. Such differences in sensitivity between tissue culture cells have also been reported by another researchers [16, 38]. There are two possibilities concerning these differences. First, as mentioned by Florin and Antillon [9], two different cytotoxins, Vero-active and non-Vero-active, are produced. Second, this discrepancy may have been derived from the serum for tissue culture used by laboratories. We usually confirmed that the cytotoxic activity of the heat-inactivated culture filtrate was always negative in the FCS assay using different lots of FCS, because the activity was found to vary among FCS lots (unpublished data). We previously reported that the cytotoxic activity of *C. jejuni* was inactivated by addition of horse serum [29]. Horse serum used for detecting cytotoxin may affect the results of detecting *C. jejuni* toxin because horse serum was usually used for Y-1 cell culture with FCS. It is important that lots of serum used for the cytotoxin detection are checked in future experiments. *C. jejuni* strains which produced both cytotoxin and cytotoxic enterotoxin demonstrated that the culture filtrate was cytotoxic to HeLa and Vero cells, and enterotoxin in the same culture filtrate was detected by morphological changes, such as elongation of CHO cells or rounding of Y-1 cells [4, 16]. However, it was reported that the cytotoxin of *C. jejuni* had cytolethal activity on CHO cells [11–13, 25] and Y-1 cells [1]. These findings, together with our data, suggest that the cytotoxic activity was dependent on serum added to tissue culture medium and that the substance amplifying and/or inhibiting the cytotoxic activity may be present in serum. If the cytotoxins are related to pathogenesis, such factors on the host side as mediators amplifying the toxic activity may be important in causing illness. To clarify the role of these cytotoxins in pathogenesis, purification and further

characterization are currently under way in our laboratories.

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