EXOTOXIN PROFILES OF CAMPYLOBACTERS ISOLATED IN MALAYSIA

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Abstract. Approximately 57% of clinical and 33% of poultry isolates examined produced a cytotoxin. Cytotoxic activity was detected in 25 (50%) isolates of *Campylobacter* of which 12 were isolated from bloody diarrhea and 9 from watery stools. The cytotoxin titers were low, ranging from 2 to 16. The crude filtrates from 50 *Campylobacter* isolates showed no cytotoxic effect in Vero cells, no fluid accumulation in suckling mice and no hemolytic activity.

INTRODUCTION

Campylobacter spp is now recognized as a common cause of human enteritis worldwide, particularly in developing countries (Butzler *et al,* 1992). Symptoms of *Campylobacter* infections are generally those of gastrointestinal disorder, for example, watery diarrhea and a dysentery-like syndrome, but rarely those of extraintestinal infections. The underlying mechanisms of the various symptoms caused by *Campylobacter* have been unclear despite the many studies that have been carried out (Walker *et al,* 1986). One important mechanism by which bacterial enteropathogens induce diarrhea is through the production of potent toxins such as enterotoxin, exotoxin and hemolysin. To understand the pathogenesis of *Campylobacter* gastroenteritis, we attempted to identify various toxin products of *Campylobacter* that might contribute to the symptoms.

MATERIALS AND METHODS

Bacterial isolates

Fifty *Campylobacter* isolates (41 clinical and 9 poultry) were used in this study. Of the clinical strains, 23 and 17 were isolated from watery and bloody stools, respectively, and one isolate was from a patient with fever but without diarrhea. *C. jejuni* were identified by biochemical tests and confirmed using a specific DNA probe (Korolik *et al,* 1988).

Preparations of crude culture filtrates

A large inoculum of *Campylobacter* cells was suspended in 2 ml of trypticase soy broth to a final concentration of 10^{10} organisms/ml and incubated at 42°C for 48 hours with occasional mixing by inversion. The culture was then spun down at 6,000 rpm for 5 minutes and the supernatant was filtered through a 0.22 µm Whatman puradisc membrane. The culture filtrates were assayed for the presence of cytotoxic activity, heat-stable toxins and hemolytic activity.

Cytotoxic activity test

Monolayers of HEP-2 or Vero cells were established in flat-bottomed 96 well tissue culture plates by incubation at 37°C for 18-24 hours. The growth medium was then gently removed from each well and replaced by 50 µl of maintenance medium. This was followed by 50 µl of the culture fitrates which had been warmed to 37°C prior to testing. The plates were incubated at 37°C for 24 hours. After the incubation, detached cells, medium and filtrates were removed by vigorous shaking, and the monolayers washed three times with PBS. The remaining cells were fixed for 10 minutes with 2% formaldehyde in PBS. The fixative was removed and the plates were stained for 20 minutes with 0.13% crystal-violet in 50% ethanol-2% formaldehyde-PBS. After removing excess stain by rinsing the plates five times with sterile distilled water, the plates were air-dried. For quantitation the stain was eluted from each well with 100 µl of 95% ethanol. The amount of crystal-violet eluted was measured by determining the absorbance at two wavelengths (570 nm and 630 nm) with an automated ELISA plate reader (Dynatech, USA). The amount of dye eluted is proportional to the number of epithelial cells that remain attached to the plate and correlates with the number of viable cells (Gentry and Dalrymple, 1980). The toxin dilution which resulted in 50% cell detachment (CD_{50}, *ie.* dye

uptake) was chosen as an appropriate endpoint for the assay.

To determine the cytotoxic titer, equal volumes of the culture filtrate and maintenance medium (containing 1% FBS) were mixed and two-fold serial dilutions prepared. These were then inoculated onto HEp-2 or Vero cells. The titer was defined as the reciprocal of the highest dilution of the culture filtrate at which ≥ 50% cell death was detected by the dye release method.

Cell detachment (%) was calculated as:

$$1 - \frac{\text{mean absorbance of 3 test wells}}{\text{mean absorbance of three control wells}} \times 100\%$$

Heat stable (ST) toxin test

This assay was performed by the suckling mouse assay as described by Dean *et al* (1972).

Hemolytic activity tests

The presence of hemolytic activity in the culture filtrates was determined by an agarose hemolysis and a microplate assay. Wells of 2 mm diameter were punched into blood agarose plates containing 1% agarose and 2% washed erythrocytes. An aliquot of 5 µl of culture filtrates was added to each of the wells and the plates incubated at 42°C for 24 hours. Filtrates that showed zones of hemolysis ≥ 3 mm in diameter were considered positive.

Two-fold dilutions of the filtrates were prepared and added to a microtiter plate with V-bottomed wells containing 50 µl of PBS. To each dilution, 100 µl of a 1% suspension of washed erythrocytes was added and the plates incubated at 42°C for 18-24 hours. The hemolytic titer was expressed as minimal hemolytic units (MHU) which was the reciprocal of the highest dilution at which hemolysis was detected. An MHU value ≥ 2 was considered positive.

RESULTS

Cytopathic effect (CPE) on the HEp-2 cells was observed with the culture supernatant from 25 (50%) *Campylobacter* isolates (22 clinical and 3 poultry). Cell rounding associated with nuclear pyknosis and cell shrinkage were some of the distinctive features of the CPE observed (Fig 1 a,b) which began to ap-pear 24 hours post-incubation and gradually became more extensive at 36-48 hours post-incubation. Twelve of the seventeen isolates from bloody stools produced cytoxin, whereas only 9 (39%) of the 23 isolates from watery stools were cytotoxin positive (Table 1). There was no significant correlation between cytotoxin production and *Campylobacter* biotypes (Table 2).

Fig 1a–Normal confluent HEp-2 cell monolayer after 24 hours incubation with sterile culture broth (crystalviolet stain; × 1000).

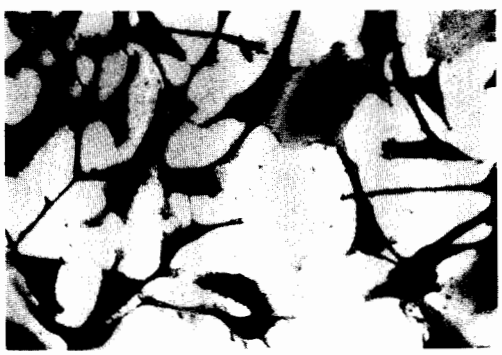

Fig 1b–HEp-2 cell monolayer showing cytopathic effect due to *Campylobacter* cytotoxin. Note the cell rounding and cells that are refractile with several filamentous tendrils extending from them after 24 hours incubation (crystalviolet stain; × 1,000).

Table 1

Detection of cytotoxin production.

Type of stools	CTOX$^+$	CTOX$^-$	Total
Watery stool	9 (39%)	14 (60%)	23
Bloody stool	12 (71%)	5 (29%)	17

CTOX$^+$: cytotoxin detected as determined by ≥ 50% cell destruction by the dye release assay

CTOX$^-$: no cytotoxin detected as determined by < 50% cell destruction by the dye release assay

Note : cytotoxic activity was detected from the crude filtrate of a strain isolated from a patient with fever but no diarrhea

Table 3 shows the biological activity of crude culture filtrates from 25 cytotoxin positive *Campylobacter* isolates. No morphological transformation was observed on Vero cells for all of the 50 culture filtrates, and no diarrhea was observed in suckling mice in the ST-toxin assay. All the cytotoxin positive culture filtrates tested resulted in a ratio of intestinal weight to remaining body weight of less than 0.07 (Table 3). Hemolytic activity was not observed in any of the culture filtrates by agarose hemolysis or microplate assays (Table 3).

DISCUSSION

The method used for the detection of cytotoxic activity in bacterial culture filtrates in this study was simple, fast and allowed a large number of samples to be tested. The effect of the toxin observed in cultured cells was apparently cytotoxic in nature as it became more pronounced with time and affected cells failed to recover. Approximately 57% of the clinical isolates and 33% of the poultry isolates produced cytotoxin . The production of cytotoxin by the poultry isolates implied that they have the potential to be pathogenic and cause gastroenteritis in humans.

Among the clinical isolates, 12 (71%) of 17 obtained from bloody stools, 9 (39%) of 23 isolated from watery stools and a single strain from an individual who had fever but no diarrhea, caused cytopathic effects in HEp-2 cells (Table 1), indicating that the cytotoxin may play a major role in the production of bloody diarrhea. It has been shown that diarrhea induced by cytotoxin-positive isolates was more severe compared to that of cytotoxin-negative isolates (Pang *et al*, 1987).

Table 2

Biotypes of *Campylobacter* and cytotoxin production.

Isolates	CTOX$^+$	CTOX$^-$
Clinical		
C. jejuni	17 (57%)	13 (43%)
biotype I	8 (42%)	11 (58%)
biotype II	9 (82%)	2 (18%)
C. coli	3 (60%)	2 (40%)
untypable isolates	2 (33%)	4 (67%)
Total	22 (54%)	19 (46%)
Poultry		
C. jejuni	3 (33%)	4 (67%)
biotype I	2 (50%)	2 (50%)
biotype II	1 (33%)	2 (67%)
C. coli	0 (0%)	2 (100%)
Total	3 (33%)	6 (67%)

CTOX$^+$: cytotoxin detected as determined by ≥ 50% cell destruction by the dye release assay

CTOX$^-$: no cytotoxin detected as determined by < 50% cell destruction by the dye release assay

Cytotoxin was produced by 57% of the *C. jejuni* and 60% of the *C. coli* examined (Table 2). These figures are low compared to those from Canada where 69% and 78% of *C. jejuni* and *C. coli,* respectively, were found to produce cytotoxin (Johnson and Lior, 1984). However, the percentage of Campylobacters producing cytotoxin varies from country to country

Table 3

Biological activities of *Campylobacter* crude culture filtrates.

	Type of stools	Titer[a] at 37°C	Heat inactivation[b] 56°C	Heat inactivation[b] 100°C	Trypsin[c] sensitivity	Hemolysin[d] production	Vero cell[e] assay	ST[f] assay
C. jejuni biotype I								
24	no diarrhea	2	+	+	–	–	–	0.060
T10	watery	4	+	+	+	–	–	0.055
71	watery	4	+	+	+	–	–	0.063
78	watery	8	+	+	+	–	–	0.049
25	watery	8	+	+	–	–	–	0.061
40	bloody	2	+	+	–	–	–	0.065
29	bloody	16	+	+	–	–	–	0.070
30	bloody	8	+	+	+	–	–	0.058
C. jejuni biotype II								
63	watery	16	+	+	–	–	–	0.065
26	watery	4	+	–	–	–	–	0.059
86	watery	4	+	+	+	–	–	0.060
20	bloody	8	+	+	–	–	–	0.066
18	bloody	8	+	+	+	–	–	0.059
74	bloody	4	+	–	–	–	–	0.062
28	bloody	4	+	–	–	–	–	0.065
44	bloody	4	+	–	–	–	–	0.061
7	bloody	4	+	+	+	–	–	0.057
C. coli								
87	watery	2	+	+	+	–	–	0.062
37	bloody	4	+	+	–	–	–	0.060
66	bloody	8	+	–	–	–	–	0.057
Untypable strains								
72	watery	2	+	–	–	–	–	0.068
36	bloody	4	+	–	–	–	–	0.061
Poultry								
6A		4	+	+	–	–	–	0.057
C13		2	+	–	–	–	–	0.053
11R		4	+	+	–	–	–	0.062

a Twofold serial dilutions of culture filtrate made in maintenance medium were tested for cytotoxic acctivity. The reciprocal of the highest dilution of culture filtrates causing ≥ 50% HEp-2 cell detachment in the dye release assay is expressed as titer.

b Culture filtrates were heated at 56°C or 100°C for 30 minutes prior to testing. Detachment of ≥ 50% of HEp-2 cells considered positive.

c Culture filtrates were incubated with a final concentration of 0.1% (w/v) trypsin for 1 houres at 37°C prior to testing. Detachment of ≥ 50% of HEp-2 cells was considered positive.

d The hemolytic activity of the culture filtrates was determined by the microplate assay. A minimal hemolytic unit (MHU) ≥ 2 was considered as positive.

e The morphological transformation or destruction of Vero cell monolayers were observed. Rounding or detachment of ≥ 50 % of the cells was considered positive.

f An aliquot of 0.1 ml of culture filtrate was inoculated intragastrically into suckling mice that were subsequently left at room temperature for 3 hours. A ratio of intestinal weight to remaining body weight > 0.083 was considered positive.

(Perez-Perez *et al,* 1989; Klipstein *et al,* 1985; Moore *et al,* 1988). This marked variability may be due to different toxigenic strains and because different methods were used in preparing the culture filtrates (McCardell *et al,* 1986).

The cytotoxin titers detected were low, ranging from 2 to 16 (Table 3) which was in agreement with other studies (Pang *et al,* 1987; Moore *et al,* 1988; Perez-Perez *et al,* 1989). This low level of cytotoxic activity observed was reported to be consistent with the lack of high titer cytotoxin in fecal filtrates from patients with *Campylobacter* enteritis (Cover *et al,* 1990).

No cytotoxin was detected in 29% of *Campylobacter* isolated from bloody stools (Table 1) indicating that either the level of cytotoxin produced was too low to be detected *in vitro* or other virulence factors might be involved in causing bloody diarrhea. In addition, *in vitro* growth conditions may not be optimal or even suitable for toxin expression in some isolates. Isolates from symptomatic patients can also lose their pathogenic properties upon storage (Bok *et al,* 1991).

Although watery diarrhea is always associated with the production of enterotoxin and bloody diarrhea with the production of cytotoxin, the detection of cytotoxin from 39% of the isolates causing watery diarrhea may be due to the fact that the strains were isolated at the initial stage of the infections in which the production of cytotoxin was too low to cause bloody diarrhea. At this stage, other factors, such as disruption of the microvilli of the intestinal epithelium, may play a greater role. It is also possible that the cytotoxic activity was not expressed *in vivo.*

Although Vero cells have been described as the most sensitive for the detection of cytotoxin (Johnson and Lior, 1984), the 50 strains tested were found to be negative for this factor (Table 3). This is in agreement with other studies (Wong *et al,* 1983; Wadstrom *et al,* 1983) where cytotoxins were not detected using Vero cells. This negative result may be due to the fact that the cytotoxins were cell specific and the enterotoxin observed previously (RuizPalacios *et al,* 1983; Johnson and Lior, 1984, 1986) was not present in the crude culture filtrates.

The suckling mouse assay for the detection of heat-stable (ST) enterotoxins was negative in previously published studies (Wadstrom *et al,* 1983; Ruiz-Palacios *et al,* 1983; Johnson and Lior, 1984) as well as in this study. The results therefore further support the general consensus on the absence of ST-like enterotoxin production by Campylobacter. However, it is also possible that certain factors essential for enterotoxin production have been overlooked. These include insensitivity of the assay to detect very low levels of ST-like enterotoxin; inadequate conditions (nutrients, temperature, pH, aeration) for enterotoxin production; differences in host specificity in which specific receptors are required for the elucidation of the enterotoxin.

Although no hemolytic activity was detected in the bacterial culture filtrates, another study using live bacterial suspensions of *Campylobacter* isolates detected hemolytic activity (Tay *et al,* manuscript in preparation). The *Campylobacter* hemolysin could be produced in such low amounts as not to be detectable in the supernatants or the intact cell is required for the production of hemolytic activity.

The role of the toxins in the pathogenesis of *Campylobacter* enteritis remains unclear as some of the clinical isolates were negative for both types of toxin. Many strains isolated from stool specimens from patients with enteritis have been reported not to produce either enterotoxin or cytotoxin (Fricker and Park, 1989). However, these strains may produce other toxins, such as cell-associated toxins (Moore *et al,* 1988; Akhtar and Huq, 1989) or a cytotoxin-enterotoxin which has been described for some enteric pathogens (Ketyi *et al,* 1979; Cumberbatch *et al,* 1979) for the induction of watery or bloody diarrhea in *Campylobacter* infections.

ACKNOWLEDGEMENTS

This work forms part of the M Med Sc thesis of the first author and was supported by the University of Malaya and the Ministry of Science, Technology and Environment, Malaysia (R &D) grant # 3/077/01.

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