POTENTIAL PATHOGENiC MECHANISMS OF ANIMAL-PASSED VIRULENCE-ENHANCED STRAiNS OF CAMPYLOBACTER JEJUNI/COLI

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Abstract

In order to study the potential pathogenic mechanisms, live cultures, culture-f ilterates and Polymyxin B released extracts from original and animal-passed strains of C jejunh/coli were compared by a neonatal mouse infection and in-vitro Vero-Cell cytotoxicity. The cell fractions from mouse-passed strains elaborated strong cytotonic and cytotoxic activity causing elongation, granular appearance and lysis of Vero-Cells. Only weak enterotoxic activity Was associated with the original strains. Partial neutralization of the cytotonic activity by mono-clonal anticholera toxin (CT) suggested immunological relationship between the toxins which was also demonstrated by Ouchterlony immunodiffusion using polyclonal anti-CT serum. The in-vivo significance of CT like and other toxic materials produced by Campy/obacter strains during animal-passage remains to be assessed (JPMA 37 : 97, 1987).

INTRODUCTION

Although Campylobacter jejuni is one of the leading causes of acute bacterial diarrhea among persons living in temperate climate as well as travelers[1], very little is known about the pathogenic mechanisms of this bacterium. C. jejuni has been shown to attack the gut epithelial cells, rapidly multiply causing diarrhea and in some cases invade the blood stream[2]. In most of the enteropathogenic bacteria, attachment is due to the presence of fimbriae, invasion is mediated by an unknown property of gram-negative cell envelope and diarrhea is mediated by the production of a specific toxin (s) [3].
C jejuni has been shown to attach to epithelial cell lines, Hela, mt. 407 and brush borders isolated from jejunum of piglets[4,5] however, the presence of fimbriae on the bacterium have not been reported. The role of flagella in attachment and virulence of C. jejuni is yet to be assessed. The organism is clearly invasive as shown by the presence of blood and leukOcytes in the stools of infected persons and experimental animals[1,6] even though invasiveness in the. Sereny test has not been' successful[4].
Although the typical clinical infection in the human does not suggest the production of a classical enterotoxin like that of Vibrio ch~olerae and todate, most of the standard assays for the production of toxins have been negative with C. jejuni, Ruiz Placio, Klipstein et al have recently reported that some strains of C. jejuni produce a toxin immunologically identical to the cholera toxin (C.T) [7,8].
We have recently reported on a consistently reliable mouse model of gastroenteritis resembling the human disease caused by C. jejuni. This model utilised virulence enhancing iron dextran, mucin and animal passage to express virulence of 4 strains of C. jejuni. The unpassed strains of C jejuni produced only mild diarrhea in neonatal mice and no deaths occurred. During the animal passage the LD5 o decreased from $l0^{10}$to 10^5 CFU. All strains became sufficiently virulent to. kill mice after 3 to 4 passages and all strains caused 60% mortality after 3 to 6 passages in weanling BALB/c mice[6].
In order to investigate the potential pathogenic mechanism (s) of C. fejuni strains causing death and

diarrhea in mice, live cultures, culture filtrates, whole cell sonicates and polymyxin B released extracts from the original and animal passed strains of C. jejuni were compared by neonatal mouse assay, and vero cell cytotoxicity for the presence of C.T. like enterotoxin or other factors responsible for the pathophysiology of Campylobacter infections.

MATERIALS AND METHODS

Preliminary Animal Handling

Neonatal (3 to 10 d old) and weanling (3 to 6 d old) BALB/c mice of both sexes were maintained on antibiotic free feed (Charles River Mouse Chow, Agway, Syracause, N.Y.) and tap water. Neonatal mice were separated from their mothers for 3h prior tO the challenge. Before intragastric (IG) or intraparitoneal (IP) inoculation, fecal specimens of representative mice in each group were suspended in sterile distilled water and cultured in Brucella broth (Difco; with 2% agar (Bacto Agar, Difco) and 5% laked horse blood with Blaser's antibiotic supplements (Oxoid, Columbia, MD). Fecal cultures collected and analysed on 2 consecutive days to ensure that mice were not carriers of C. jejuni or related species of Campylobacter.

Bacterial Cultures:

Campylobacter strains and sources are listed in Table 1.

TABLE I
Source and Designation of Strains Used.

Strain	Organism	Source
JCH 667	C. jejuni	Chicken (Dr. N. J. Stern Meat Science Research, USDA).
ATCC 29428	C. jejuni	Clinical
G7	C. jejuni	Clinical (Ms. C. Bachurski, Georgetown Univ. Hospital, Washington, DC)
ATCC 456	C. coli	Pig

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These strains were identified by standard criteria[8] and grown on medium described above for 24h at 37°C or 42°C under a 5% 02, 10% CO_2 and 85% N_2 0environment. All strains had been passed at least twice on the lab media. Cultures were maintained frozen (-70°C) in Brucella broth containing 20% glycerol for use as stocks and subsequent compariSons.

Preparation of the Antiserum:

Weaning (6 to 8 weeks old) BALB/c mice were immunized to raise specific antisera against all four original and animal passed strains of C jejuni by the method of Mageaue and Roberson[9]. Five days after the last injection, mice were bled from the tail. Serum was harvested and stored at -20°C until used. Monoclonal antibody against C. je/uni strain ATCC 29428 and Cholera toxin were kindly provided by Sara Woolery (Meat Science Research Lab, USDA, Beltsville MD) and 'Elaine Rommers (Dept. of Microbiology. Univ. of MD, College Park, MD).

Intraparetoneal pasage:

All four clinical and food isolates of C. jejuni were subjected to serial intraparitoneal (IP) passages in weanling (3 — 6 weeks) according to the method of Kazmi et al[6].

Virulence enhancement testing in Neonatal mice:

Groups of neonatal (3 to 10 d) mice were challenged with original and animal passed virulence enhanced strains of C. Jejuni as described in a previous report[6] (Tabie II). The mice were observed daily for diarrhea and other signs of illness and were weighed daily until they died or diarrhea ceased. Mild diarrhea was exhibited as moist unformed stools. Severe diarrhea was evidenced as watery feces with mucus, anal soiling and fluid accumulation in the GI tract.

Preparation of the Cell free supernatants and concentrates:

Animal passed virulence enhanced strains of C. jejuni causing severe diarrhea in mice and the original unpassed strains were grown in Mueller Hinton broth (Difco) with Blaser's growth supplements in tissue culture flasks incubated for 24h in a shaker bath at 42°C. The cells were removed by centrifugation at 6000xg for 20 minutes at 4°C and the supernatants were sterilized by passage through a 0.22 um pore size membrane (Millipore Corp., Bedford, Mass.). The resulting supernatant was dialyzed against PBS (pH 64) and concentrated 10 fold by perevaporation at 4°C and again filter sterilized. For polymyxin B treated supernatant, polymyxin B sulfate at a conc. of 100 or 1000 lU/ml was added to the whole culture which was then incubated for an additional 15 or 30 minutes at 42°C under agitation. The supernatant was then obtained by centrifugation, sterilized and stored at 4 C till used. Cell free lysate was prepared by sonification of washed cells on ice with a Branson Sonifier (model S-75; Bronson Instruments Co., Danbuiy, Conn) and sterilized by filtration. Cell-free filtrate was also collected from sick animals infected with animal passed and original-strains of C. jejuni.

Toxin Assays:

(i) Animal inoculation: All 3 cell free concentrates from IP infected and IG infected animals, Polymyxin B treated culture, culture filtrate without any treatment cell-free sonicates were introduced intra-gastrically (0.5 ml) into different groups of mice (6 neonatal mice/gp) and the animals were observed for 7 days for signs of diarrhea or disease. One animal from each group was sacrificed every alternate day to see any accumulation of fluid in the intestine or other overt symptoms.

(ii) Tissue Culture Assay: Both heated (at 56 C for 30 mm.) and unheated cell free filtrates described as above were used for assaying cytotoxicity on vero cells. Vero cells were grown on medium 199 (M.A. Bioproducts) containing 10% fetal calf serum, 100 ug penicillin, 100tg streptomycin, and 5% HEPES buffer (25mM) at 37°C in a 5% CO2 incubator 24h. Confluent monolayers in 96 well plates were obtained by dispensing 0.2ml/well of a suspension of 105 cells/mi. Two-fold dilutions of Campy cell-free supernatant, sonicates and supernatant obtained after Polymyxin B treatment of test strains were made with an eight channel pipette using M 199 as diluent. Cholera toxin (Sigma, St. Louis) was used as a positive control and Medium 199 and Mueller Broth were included as negative controls.

To perform the assay, medium from the 96 well plate was aspirated using sterile tips and a multiple channel pipette. With fresh tips 100 ul of each test dilution was delivered to vero cells. Two rows in each 96 well plate were filled with M 199 and one row received two-fold dilutions of cholera toxin and another row had only sterile Mueller Hinton growth medium. Duplicate plates were incubated at 37°C in CO2 incubator and observed after 30 mm and 24h before and after changing the medium. Each well was observed at 40X under an inverted microscope to determine the percentage of cells affected, and at

100X for typical morphological alterations (elongation or lysis) of Vero cells. To determine neutralization of Cytotoxic activity, C. jejuni ceil.free supernatants were preincubated at 37°C for 1 hour with an equal volume of diluted cholera toxin (CT) and Campylobacter monoclonal antisera.

Ouchterlony Double Diffusion Test:

To study the immunological cross-reactivity between the different components ofC. jejuni strains and to establish a relationship between the extracted antigen of C. jejuni and cholera toxin, Ouchterlony double diffusion was employed. Molten 1% Agarose gel (Sea Kem, FMC Corpora. tion, Rockland, ME), in phosphate buffered Saline (PBS), pH 7.2, with 0.02% sodum azide as preservative, was poured into 55 mm plastic petri dishes (Falcon Plastjcs, Cockeysville, MD) and allowed to solidify. A 7-hole template was used to cut wells for applying samples. Equal concentrations of cell-free supernatants, sonicates, outer membrane proteins or cholera toxin were placed in the outside wells, and the central well was fified with specific antiserum. (Anti. CT or Anti. C. jejuni or Anti-Shiga toxin).

Plates were sealed and reagents were allowed to diffuse for 8 days at 25°C in a humidified chamber. The gels were then washed for one day with several changes of 0.85% Saline. The agar was dried to a thin film, proteins were fixed in 5% glacial acetic acid for 5 min and then stained with Coomassie brilliant blue R250 dye and destained using several changes of 40% Methanol and 10% acetic acid.

RESULTS

In-Vivo Virulence Enhancement Testing

IG infected neonatal mice groups which received C. jefuni in iron dextran developed signs of disease usually by day 5, manifesting severe diarrhea with increased mucus discharge and reduced weight gain when compared to controls receiving supplements or Brucella broth. At least one or two animals in each trial had blood in their stools. Six of the 14 infants infected with strain G7 (6th animal passage) had leukocytes and erythrocytes in Giemsa stained stool smears. Of the 58 Iieonatal animals challenged with original strain in iron dextran,36 developed mild diarrhea, exhibited as moist unformed feces and none died. In contrast, of the 57 neonatal mice infected orally with animal-passed strains in iron dextran, 50 developed severe diarrhea defined as watery feces with mucus, anal soiling and fluid accumulation in the intestine and 12 of them died6. The remaining animals recovered by 2 weeks after the onset of diarrhea6.

Toxin Production By C. jejuni isolates:

Intragastric inoculation of culture supernatants of original and animal passed strains of C. fefuni or with the peritoneal washings collected from mice dying of an IP infection with the same strain did not result in death or any signs of disease. Infected animals did not show significant fluid accumulation in the intestine but IN-VITRO tissue culture assay using VERO cells for the detection of toxic factors in untreated, Polymyxin B sulfate treated cell-free supernatant and whole-cell sonicates of strain ATCC 29428 (Table-II)

TABLE – II
DD_{50}a Values in neonatal Mice challenged intragastrically with original and virulence enhance Strains of *C. jejuni*/Coli.

Strain/ passage	Iron dextran	Supplement Mucin	Brucella broth
G7 (0)	1×10^{11} (no diarrhea)[b]	1×10^{11} (ND)[c]	1×10^{11} (no diarrhea)
G7 (6)	3×10^{9} (5 days)	1×10^{9} (4.3 days)	1×10^{10} (6.5 days)
ATCC 29428 (0)	2×10^{11} (ND)	2.1×10^{11} (ND)	3×10^{11} (no diarrhea)
ATCC 29428 (6)	2×10^{11} (5 days)	1×10^{9} (5.5 days)	2×10^{10} (8 days)
JCH 665 (0)	1×10^{11} (ND)	1×10^{11} (ND)	1×10^{11} (no diarrhea)
JCH 665 (3)	3×10^{9} (5 days)	1×10^{10} (5.2 days)	1×10^{10} (7 days)
456 (0)	1×10^{11} (ND)	(ND)	1×10^{10} (no diarrhea)
456 (5)	3×10^{10} (5.5 days)	(ND)	2×10^{11} (6 days)

[a]DD_{50} = Diarrheal dose 50 number of CFU required to produce diarrhea in 50% of mice. Eight mice used for each passage and treatment.

[b]Average number of days for onset of signs.

[c]ND = Not determined.

indicated the presence of cytotonic activity seen as elongation and death of cells inoculated with the untreated fuiterate of C. jejuni but not with the uninoculated control broth; strains jCH 477 (5), G7(7) and 45 6(5) exhibited a cytotoxic activity (Table-III)

TABLE III

Cytotoxic Effects of C. jejuni Extracts on Vero Cells.

Strain (passage)	Elongation[a] culture Super-natants	polymyxin B released factors	Granular[a] Lysis culture Sonicates
JCH (5)	++	+++	ND
ATCC 29428 (5)	++++	+	++
G7 (0)	+	++	++
G7(7)	++++	++++	
456 (5)	+	+++	+
456 (0)	+	+	
Controls			
CT[b] 50 ug/ml	++++	+	ND
Medium 199	—	—	—

[a]Refer to morphological changes of Vero Cells 30 min to 24 h after addition of samples.

[b]Cholera Toxin, Sigma, St. Louis.

++++ = 90% elongated or lysed.

+++ = 75–90% elongated or lysed.

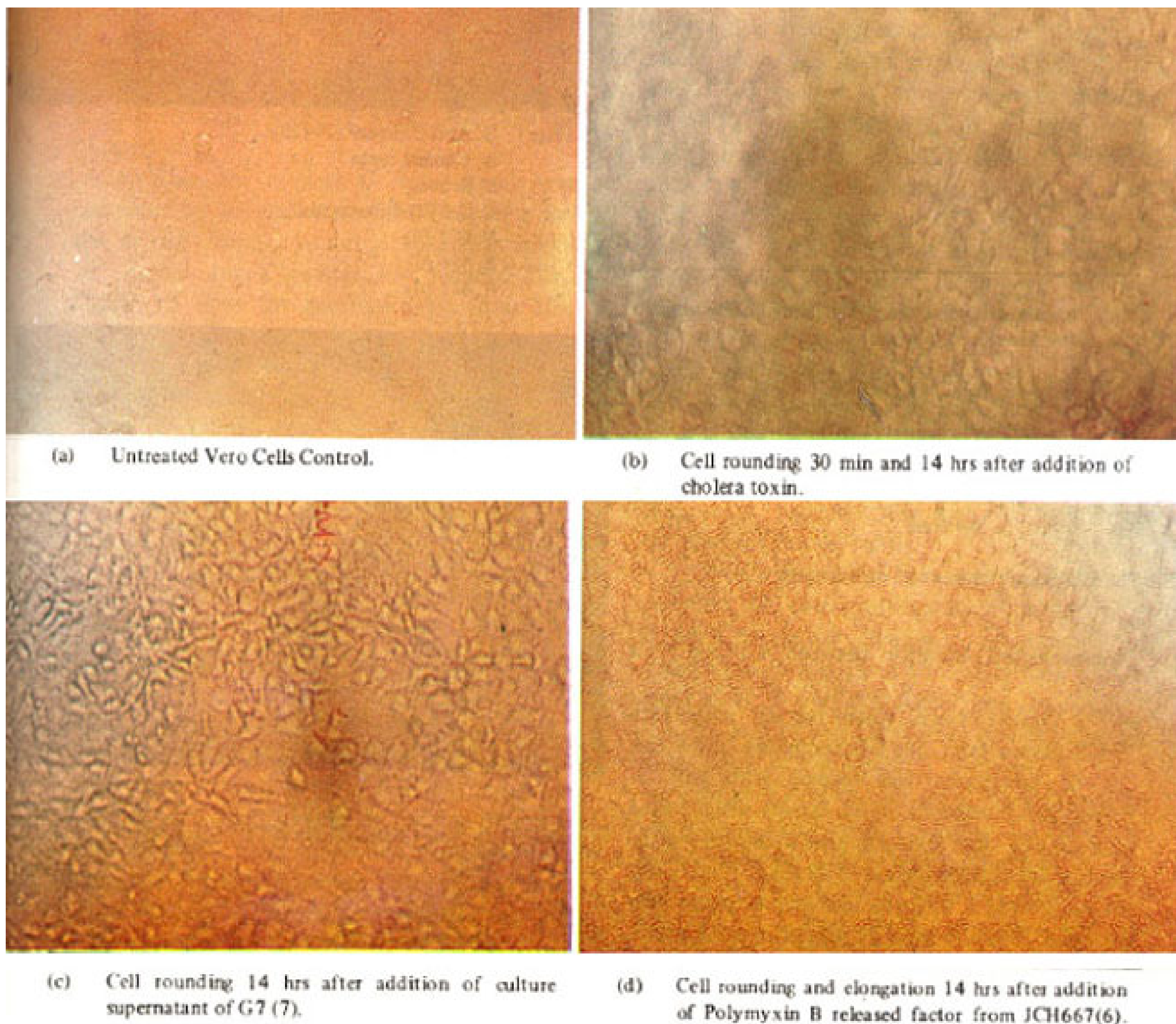
++ = 25–75% elongated or lysed.

\+ = upto 25% elongated or lysed.

ND = Not done

The results are averages of 3 experiments.

defined as cell-rounding, granular appearance of monolayer and cell-lysis (Figure I).



(a) Untreated Vero Cells Control.

(b) Cell rounding 30 min and 14 hrs after addition of cholera toxin.

(c) Cell rounding 14 hrs after addition of culture supernatant of G7 (7).

(d) Cell rounding and elongation 14 hrs after addition of Polymyxin B released factor from JCH667(6).

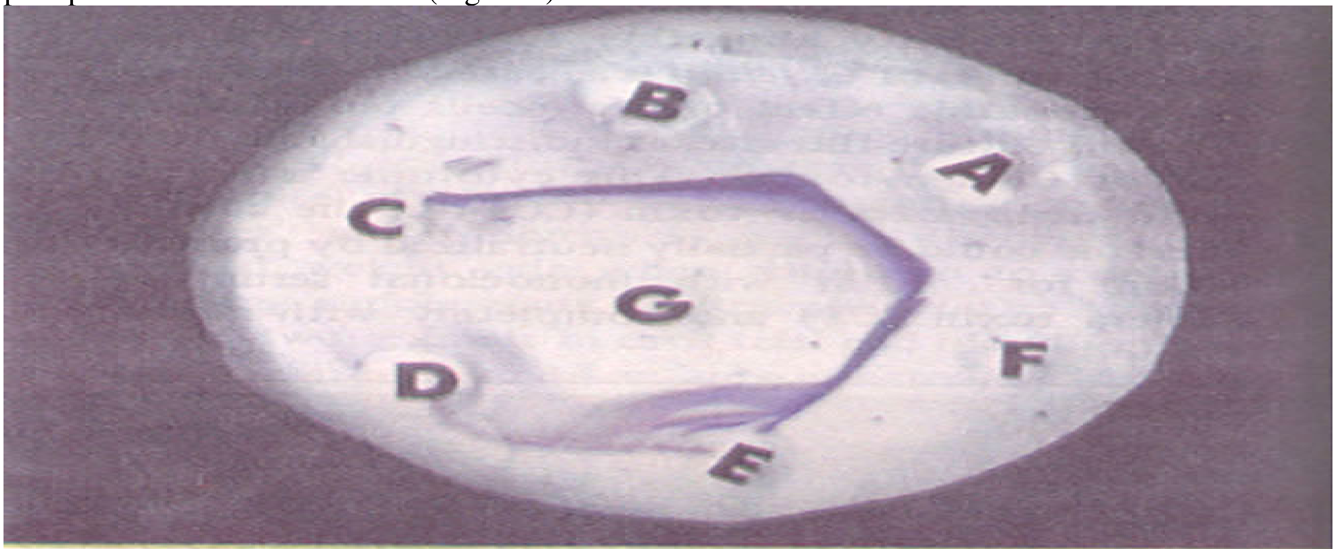
Figure 1 (a, b, c, d). Effect of C. Jejuni toxic factor(s) on tissue culture cells.

By contrast, very weak cyto. toxic and cytotonic activity was noted with undiluted cell-free supernatants and sonicates prepared from unpassed isogenic strains of C.jejuni. When cell-free supernatants of G7(7), jCH(5) and 456(5) strains were, heated at 56°C for 30 min cytopathic effects could still be observed. However, treatment at 100°C for 30 mm abolished the toxic activity altogether suggesting that the toxic factor(s) of C. jejuni isa heatlabile substance. The cytopathic effect on Vero-cells produced by C jefuni culture-filterates is indistinguishable from that of Cholera-toxin. This cytotonic effect of crude campylobacter toxin (CCT) in the culture filterates could be partially neutralized by preincubation for 1 hr with monoclonal serum to Cholera toxin (CT) and completely with monoclonal antibody against ATCC 29428 strain of C fejuni (1:256 Anti CT; 1:512 Anti C. jejuni). Bacterial sonicates exhibited a weak cytopathic effect on VERO-CELLS, suggesting that the toxic factor is most probably an extracellular product (exocytotoxin) rather than an endotoxin. Polymyxin B treated cell-free filterates could not be neutralized by monoclonal Anti-Cholera toxin antibody suggesting that the toxin released by this treatment is not related to CT.

Immunological Identity Between CT and CCI

Cholera toxin when reacted with anti-29428 gave a faint line of precipitate but a fine line of precipitate was formed with anti-CT. Polymyxin B extracts of all strains when tested with anti-CT sera did not

give a line of partial identity but culture filtrate of 29428 (0)and G7(7) strains showed a diffused line of precipitate near the anti-CT well (Figure 2)



Plaţe I G. Anti C. Jejuni (29428).
A. Cholera taxin
B. Saline
C. G 7 (7) Supernatant.
D. G 7 (0) "
E. 456 (0) "
F. 456 (6) "

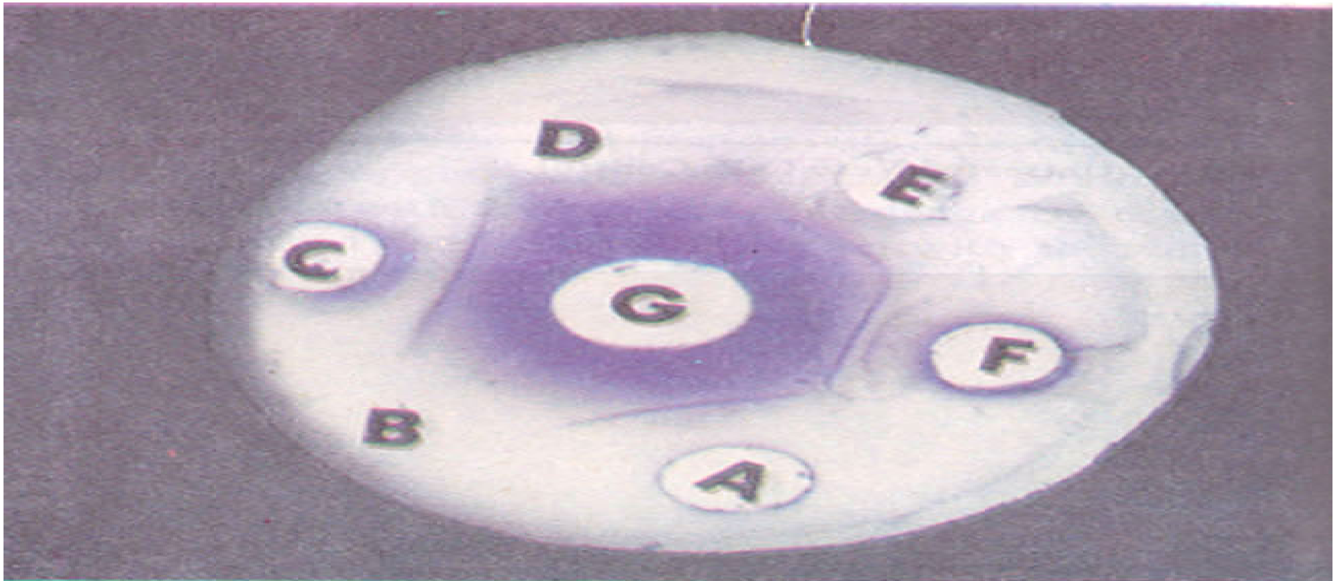


Plate II G. Anti-Cholera Toxin (CT)
A. CT
B. CT
C. Saline
D. G7 (0) Supernatant
E. 456 (0) Sonicate
F. 456 (6) Sonicate

Figure 2. Stained precipitin pattern resulting from two dimensional immunodiffusion in agarose gel.

suggesting cröss-reactivity.

DISCUSSION

The results provided here on 2 clinical and 2 food isolates of C. jejuni indicate that intraparitoneal passage of these strain with virurence enhancing substances can maximize their virulence potential. When immuno competent neonatal BALB/c mice (6 d old) are challenged with these animal passed strains they develop severe diarrhea and other signs similar to that seen in man. This experimental neonatal mice infection is characterized by an incubation period of 1 to 5 days which is similar to human infection. The mortality rate like the human was not extremely high in this neonatal model as only 12 out of 50 developed severe diarrhea and the remaining animals recovered within 2 weeks after the onset of diarrhea.

When the original C. jejuni strains were compared by IG challenge of neonatal mice with the animal passed, virulence enhanced strains, we[6] found animal passage had reduced DD50 gable II) dose for neonatal mice from 10^{11} CPU to 10^{9}.

Among neonatal mice challenged with 10^{11}CFU of the original strain in iron-dextran few of the animals developed a very mild diarrhea with no other signs of ifiness. Two strains used were isolated from diarrhea! stools of patients. Although these strains would be expected to be fully virulent on initial inoculation with supplements, they failed to induce diarrhea in neonatal mice.

Iron has been reported to be a key component in the pathogenesis of C. jejuni[6]. But the molecular mechanisms of iron-transport in C jejuni and how iron affects the expression of virulence of certain strains have yet to be investigated. McCardell et al[10] have recently reported that Campylobacter toxin yield was enhanced under excess iron-conditions. Strains previously negative for toxin production became producers when grown under increased iron-condition. Enhanced expression of virulence by Campylobacter isolates during animal passage with irondextran could be due to the greater toxin producing capacity which may help the bacterium to establish a better infection than the weakly toxigenic unpassed strain of C jejuni. In order to associate virulence of the strains causing, severe diarrhea, fluid loss, intraluminal blood accumulation in neonatal mice with a specific toxic or cellular component (s) in C jejuni strains, various cellular components of both animal passed and virulence enhanced strains were compared using different techniques.

Neither C jejuni culture filtrates nor the filtrates of peritoneal exudate from mice dying of IP infection exhibited toxicity or rapid fluid accumulation in the intestine, when given intragastrically, suggesting an absence or an insufficient amount of a toxic component- to cause any reaction. This observation has also been reported by Yirios et al[11] using BALB/c mice(l8). As confirmation we tested cellfree supernatants, cell sonicates and polymyxin B treated cell free filtrate in a vero cell assay. Culture-filterates of mouse-passed C. jejuni strains ATCC 29428(5) and G7(7) resulted in strong cytotonic influence on VERO CELLS which could be inhibited by preincubation of sterile filterates with monoclonal antisera to Cholera-toxin indicating an immunological relationship between CCT and CT. When grown and tested under the same conditions described above, supernatant of isogenic unpassed strains produced only a weak cytotoxic response on VERO CELLS.

A different toxic factor in the polymyxin B treated cell extracts, particularly of strain 456 (5) and JCH(5) exhibited a cytotoxic activity on VERO CELLS similar to that described by Blankenship et al[12] - This cytopathic effect could not be neutialized by preincubation of treated cell extracts by antiserum to CT, LT (E. Coil) or Shiga-toxin, suggesting this factor to be different than the CT related factor.

Various studies have suggested that the organism is enteropathogenic in man by its invasive properties. Evidence to support this observation comes from clinical studies such as rapid rise of antibody titers post infection[13], the recovery of organisms from patients blood in acute phase of the disease[14] Attempts to detect the production of a heat stable or a heat labile enterotoxin analogous to those produced by Vibrio Choierae and certain strains of E. Coli have not been successful[4]. The typical clinical infection

in humans does not suggest the production of a classical enterotoxin like that of Vibrio Cholerae .. Nonetheless Ruiz-Placios & others[7,8,15] have reported that some strains produce a toxin immunologically identical to cholerae toxin. Supporting this idea we did see partial neutralization of the. cytopathic effects of Ozmpylobacter extracts on vero cells by preincubation with anti-cholera toxin. However, only a faint line of precipate was formed when CT was reacted with Anti C. jejuni serum and otherwise in an Ouchterlony double diffusion (Figure 1 ,2). Similarities in biological activities of cholera toxin and Campylobacter toxin (s) were also demonstrated by morphological changes in the tissue culture assays and by fluid accumulation and diarrhea in neonatal mice infected by viable cells of C. jejuni. The role of C jejuni toxins in the pathophysiology of C. jejuni induced diarrhea has not been established. It seems quite possible that Cam pylobacter has at least two different components to account for the broad spectrum of clinical symptoms and the crude Campylobacter enterotoxin which we detected in culture supernatants may account for the mild watery diarrhea observed in Campylobacter infection, or it is possible that a Campylobacter toxin (s) exists as a complex expressed in the host as a manifestation of differential release. The failure of some laboratories to identify enterotoxin like activity of C jejuni in the past was probably due to their suboptimal culture media, growth conditions, and confining tissue culture assays to sonicated Cell lysate which we found yielded only weak cytopathic effect. However Polymyxin B treated cell extracts produced a greater cytotoxic effect causing granular lysis of vero-cells (Table — III). This is probably due to the release of toxin from periplasm of the cell as suggested by Klipstein et al[16] . The dysentery-like symptoms often observed during infections with Campylobacter spp. may be due to this cytotoxin factor.

The cytotoxin and the enterotoxin are apparently separate moieties as we and others[14,16] have found them to occur separately or together. Klipstein et al[16] were able to neutralize secretory response due to C. jejuni enterotoxin in ligated rat ileal loop passively by anti serum to LT; however this antisera could not neutralize the cytotoxin's action on VERO and Hela cells. Although a number of points need to be clarified concerning physical properties and production of the cytotoxin, the evidence, presented here using limited number of strains have advanced our understanding of potential virulence factor (s) although not establishing its role in an infected animal.

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