Enterotoxigenicity of chicken isolates of *Campylobacter jejuni* in ligated ileal loops of rats

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Summary. Ligated ileal loops in rats of the Charles-Foster strain, weighing 200–250 g and aged 7–8 months, provided a sensitive and reproducible means of testing the enterotoxigenicity of *Campylobacter jejuni*. All of 16 chicken isolates caused fluid accumulation comparable to that produced by toxigenic *Vibrio cholerae* O1, strain 569B. However, 11 of the isolates required one to three consecutive passages through the rat gut before doing so. The amount of fluid produced increased after each passage. Of three culture media tested, brucella broth of pH 6.7 supported the highest degree of enterotoxigenesis. Filtrates of cultures of all 16 chicken isolates in this medium were as effective as viable *C. jejuni* in producing fluid in ileal loops. The enterotoxin was neutralised completely by cholera antitoxin diluted 1 in 160, indicating its close immunobiological relationship to cholera toxin.

Introduction

Campylobacter jejuni is one of the commonest causes of diarrhoea in many countries (Walker *et al.*, 1986) and has also been implicated as a cause of traveller's diarrhoea (Speelman *et al.*, 1983). Some strains from patients with diarrhoea produce heat-labile enterotoxin (LT) (Ruiz-Palacios *et al.*, 1983; Johnson and Lior, 1984; McCardell *et al.*, 1984). Partial antigenic and other similarities between this enterotoxin and the LT of both *Vibrio cholerae* O1 and *Escherichia coli* have also been demonstrated (Ruiz-Palacios *et al.*, 1983; Klipstein and Engert, 1984, 1985; McCardell *et al.*, 1984). Chickens were found to be the commonest reservoir of *C. jejuni* (Harris *et al.*, 1986), but there is no published information on the enterotoxigenicity of chicken isolates. The objectives of the present study were to determine whether (1) chicken isolates cause fluid accumulation in ligated ileal loops of rats, (2) consecutive passages through the rat gut enhance the enterotoxigenicity of non- or weakly toxigenic strains, and (3) the enterotoxin of chicken isolates is neutralised by cholera antitoxin.

Materials and methods

Organisms

Sixteen strains of *C. jejuni* biotype 2 isolated from the intestines of broiler chickens from different sources were

identified by standard tests (Smibert, 1978; Skirrow and Benjamin, 1980) and preserved in a stock medium of nutrient broth containing glycerol 10% and FBP (ferrous sulphate, sodium bisulphite and sodium pyruvate) supplement 0.025% at –90°C (George *et al.*, 1978), each strain being distributed into five tubes. None of the strains had undergone more than two subcultures before being tested for enterotoxin production. *Vibrio cholerae* strain 569B maintained in our laboratory served as a positive control in initial experiments.

Media

Preliminary tests were made with various modifications of Brucella Broth (Gibco) to find the best medium for toxin production. The modifications were (1) the adjustment of pH to different values between 5 and 10, (2) addition of L-asparagine 0.25%, L-serine 0.25% and L-glutamic acid 0.25%, and (3) addition of polymyxin B, 4 IU/ml (Ruiz-Palacios *et al.*, 1983). Brucella broth alone at pH 6.7 supported the maximum degree of toxigenesis and of growth as judged by optical density. No significant increase in the yield of toxin was observed on the addition of polymyxin B or on the readjustment of pH to 6.7 after amino acid supplementation.

Preparation of culture filtrates

Five or six colonies of each *C. jejuni* strain grown for 48 h in a candle jar at 42°C on Campy-BAP (Hi-Media) medium with antibiotic supplement (Blaser *et al.*, 1979) were inoculated into 10 ml of Brucella broth, pH 6.7, contained in 50-ml conical flasks. The cultures were incubated for 72 h in a candle jar without shaking. After

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centrifugation, the culture supernates were filtered through millipore membranes of 0.22- μ m average pore diameter, dispensed in small volumes and stored at 4°C (Ruiz-Palacios *et al.*, 1983). Culture filtrate was prepared only when live cells of a strain caused fluid accumulation in the rat ileal loop either before or after passage through the gut of rats. Culture filtrate of the positive control strain *V. cholerae* 569B was prepared in Richardson's medium by a method described earlier (Sanyal *et al.*, 1984).

Ileal loop tests in adult rabbits

Live cells of the 16 *C. jejuni* isolates were tested in New Zealand albino rabbits by the method of Annapurna and Sanyal (1977). The strains, grown in Brucella broth for 18 h, were injected into rabbit ileal loops in doses of 1.0 ml containing 10^{6-7} cfu. The known toxigenic *V. cholerae* strain 569B grown in Brain Heart Infusion Broth (Difco), and unseeded Brucella broth, served as positive and negative controls respectively. Culture filtrates of all the strains prepared in Brucella broth of pH 6.7 were tested in a similar way in 1-ml doses. Each test was done in triplicate.

Ileal loop tests in rats (Charles-Foster strain)

Albino rats weighing 100–250 g were used to examine the production of enterotoxin by *C. jejuni* strains. Faecal specimens of the rats were cultured for *C. jejuni* for three consecutive days before the tests, and all yielded negative results. The rats were starved for 48 h but given water *ad libitum*. The intestines of each rat were exposed by abdominal incision under light ether anaesthesia and 6–8 loops of 5 cm length at 2 cm intervals were tied, starting from the ileo-caecal junction. Live cells and culture filtrates were injected into these loops as in the rabbit gut loop tests (above), except that the dose was 0.5 ml. *V. cholerae* strain 569B and Brucella broth served as positive and negative control material respectively. The rats were killed after 18 h and the length and fluid content of each loop were measured. Each experiment was done in triplicate.

Consistent and reproducible results with both live cells and culture filtrates were obtained in rats weighing 200–250 g and aged 7–8 months; rats weighing less than 200 g gave inconsistent results. Live cells or culture filtrates of *C. jejuni* strain 9 consistently caused fluid accumulation in the range of 0.5–0.7 ml/cm of gut in 10 initial experiments. In subsequent experiments, therefore, it replaced *V. cholerae* 569B as the positive control strain.

Passage in rat ileal loops

Consecutive passages of the *C. jejuni* strains that caused little or no accumulation of fluid in initial experiments were made in rat gut loops as described earlier (Shankar *et al.*, 1982; Sanyal *et al.*, 1984). In short, such strains

were subcultured from the gut loops on Campy-BAP plates and incubated in a candle jar at 42°C for 48 h. Five to six colonies of each strain were grown in Brucella broth at 42°C for 18 h in a candle jar and 0.5 ml volumes were inoculated again into rat loops. The process was continued until good positive responses were obtained.

Determination of the optimum loop-reacting dose

Cell-free culture filtrates of the *C. jejuni* strains numbered 2, 5, 9, 10 and 13 were injected in volumes ranging between 0.1 and 0.6 ml into rat ileal loops to find the dose causing a maximal reaction (Annapurna and Sanyal, 1977). Filtrates in doses of 0.25 ml caused fluid accumulations of about 0.5 ml/cm of gut. This amount of fluid was no greater when larger volumes of culture filtrate were injected (fig. 1). The 0.25 ml dose of filtrate was used in neutralisation tests with cholera antitoxin in rat loops.

Neutralisation of *C. jejuni* enterotoxin by cholera antitoxin in rat ileal loops

Five strains (2, 5, 9, 10 and 13) were tested in rat loops by a method used earlier in rabbits (Dubey and Sanyal, 1978; Sanyal *et al.*, 1984). Culture filtrate (0.25 ml) was mixed with 0.25 ml of dilutions of anti-CT (obtained from NIH, USA) in PBS of pH 7.2 as described earlier (Dubey and Sanyal, 1978; Lahiri *et al.*, 1982; Sanyal *et al.*, 1984). The mixtures were incubated at 37°C in a water bath for 30 min and then tested in rat ileal loops to find the highest dilution of cholera antitoxin that would completely neutralise enterotoxin activity. Culture filtrate (0.25 ml) mixed with an equal volume of PBS served as a positive control inoculum.

Fig. 1. Determination of optimum loop reacting dose—accumulation of fluid with different doses of culture filtrates. The results are the means ($\pm$ SD) for filtrates of five cultures.

Results

Ileal loop tests

In rabbit gut loops, neither live cells nor culture filtrate of any *C. jejuni* strain caused fluid accumulation, but mild to severe haemorrhage was sometimes observed. *V. cholerae* strain no. 569B and Brucella broth invariably gave positive and negative reactions respectively.

In initial experiments with rat ileal loops, live cells of only five of the 16 *C. jejuni* strains caused fluid accumulation comparable with that of the known toxigenic strain (569B) of *V. cholerae*. Of the 11 strains that gave negative results initially, four became positive after a single passage through rat gut and four and three after two and three passages, respectively. Fluid production was enhanced by each consecutive passage and after the third the volume was ≥ 0.5 ml/cm of gut. Filtrates caused accumulations of fluid in the range from 0.25–0.7 ml (table), i.e., volumes similar to those produced by live cells.

Neutralisation of *C. jejuni* enterotoxin by cholera antitoxin

The highest dilution that completely neutralised the enterotoxic activity of culture filtrate (0.25 ml) of strains 2, 5, 9, 10 and 13 was 1 in 160 (fig. 2). There was proportionately less neutralisation (i.e., more fluid accumulation) with higher serial dilutions of cholera antitoxin (fig. 3).

Table. Enhancement of the fluid-producing effect of *C. jejuni* culture filtrates by passage through rat ileal loops

Strain no.	Fluid produced (ml/cm of gut)	
	before passage	after passage
1	0	0.30–0.50
2	0	0.44–0.55
3	0	0.20–0.30
4	0	0.35–0.45
5	0.25–0.30	0.50–0.70
6	0	0.20–0.40
7	0	0.30–0.45
8	0	0.30–0.50
9	0.20–0.25	0.50–0.70
10	0.25–0.30	0.45–0.55
11	0	0.30–0.35
12	0	0.15–0.35
13	0.25–0.30	0.40–0.45
14	0	0.25–0.40
15	0	0.30–0.35
16	0.18–0.25	0.30–0.40

Fig. 2. Neutralisation of *C. jejuni* enterotoxin by cholera antitoxin. The results are the means for filtrates of five cultures.

Fig. 3. Neutralisation of *C. jejuni* enterotoxin by cholera antitoxin. Loop 1: positive control, 0.25 ml of culture filtrate mixed with 0.25 ml of PBS. Loops 2–7: 0.25 ml of culture filtrate mixed with 0.25 ml of 1 in 220, 1 in 200, 1 in 180, 1 in 160, 1 in 140 and 1 in 120 dilutions of cholera antitoxin respectively. Loop 8: negative control, 0.5 ml of Brucella broth.

Discussion

The observation that New Zealand albino rabbits were not susceptible to either live cells or culture filtrates of the chicken isolates of *C. jejuni* is difficult to explain. Our earlier studies with diarrhoeal isolates of *C. jejuni* from children yielded similar results (unpublished data). The explanation put forward by Ruiz-Palacios *et al.* (1983) that rabbits lack receptors for *C. jejuni* toxin seems untenable in the light of a later experiment by Klipstein and Engert (1985) in which the B sub-unit of *C. jejuni* toxin reacted with GM₁ ganglioside in an enzyme-linked immunosorbent assay. Age, weight or breed of the animal may, however, play an important role.

Ruiz-Palacios *et al.* (1983) first reported that some, but not all human diarrhoeal isolates of *C. jejuni* caused accumulation of fluid (0.28 ± 0.05 ml/cm of gut) in ligated rat ileal loops. However, no details of the weight and age of the rats, the length and number of loops per animal, and the size of the inocula were given. Walker *et al.* (1986) concluded that no satisfactory experimental model was available for *C. jejuni*. The present study showed that in Charles-Foster rats weighing 200–250 g and aged 7–8 months, at least eight gut loops could be made, and that these were consistently susceptible to *C. jejuni* organisms and culture filtrates, both of which stimulated fluid production (0.2–0.7 ml/cm of rat gut). Of several media examined for enterotoxin production, the best was Brucella broth, pH 6.7, without additives.

All of 16 chicken strains of *C. jejuni* were enterotoxigenic, and some, though not all, caused fluid accumulation comparable to that caused by the toxigenic strain 569B of *V. cholerae*. Strain-to-strain and loop-to-loop variation in fluid production can be due to strain variation in the amount of toxin released and to biological variations (Shankar *et al.*, 1982). *C. jejuni* strain 9 was exceptional in

that after passage it invariably caused outpouring of fluid at least as great as that caused by the highly toxigenic strain 569B of *V. cholerae*.

Eleven of 16 strains produced little or no fluid until passaged 1–3 times in the gut of rats. Fluid production increased after each passage. The effects of passage on enterotoxigenicity have been observed with *V. cholerae* non O1 (Singh and Sanyal, 1978; Shankar *et al.*, 1982), *Aeromonas hydrophila* (Annapurna and Sanyal, 1977), *Plesiomonas shigelloides* (Sanyal *et al.*, 1980), *V. fluvialis* (Agarwal and Sanyal, 1981), *V. mimicus* (Sanyal *et al.*, 1983), CT-gene negative *V. cholerae* O1 (Sanyal *et al.*, 1984) and *Klebsiella pneumoniae* (Raychaudhury *et al.*, 1984). Possibly passage assists the expression of a repressed toxin gene (Singh and Sanyal, 1978). Earlier reports dealing with human diarrhoeal isolates of *C. jejuni* indicated that some or all were non-toxigenic (Ruiz-Palacios *et al.*, 1983; Wadstrom *et al.*, 1983). The present study suggests that all *C. jejuni* strains are potentially enterotoxigenic. The importance of domestic pets (Walker *et al.*, 1986) and chickens (Harris *et al.*, 1986) in human infections with *C. jejuni* is recognised. In developing countries close association between man and animals may lead to a cycle of infection that increases toxigenicity. A similar enhancement of other virulence factors of *C. jejuni* has been suggested by Newell *et al.* (1985).

Antisera to cholera toxin or *E. coli* LT inhibit the cytotoxic effects of diarrhoeal isolates of *C. jejuni* (Klipstein and Engert, 1984; McCardell *et al.*, 1984). The present study shows that the enterotoxin produced by chicken isolates can be completely neutralised by cholera antitoxin diluted 1 in 160, indicating the close immunobiological similarity of the two toxins.

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