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The virulence of clinical and environmental isolates of *Campylobacter jejuni*

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SUMMARY

The virulence of *Campylobacter jejuni* and *C. coli* isolated from various water sources was compared with that of clinical strains by *in vitro* assays of adhesion, invasion and cytotoxicity to HeLa cells. Variation in degree of attachment was observed, but this did not appear to be related to strain source, However, water strains were less invasive and less cytotoxic to HeLa cells than clinical strains as shown by immunofluorescence and electron microscopy.

These differences were particularly evident between clinical and water isolates of the same serotype and biotype implicated in an outbreak of campylobacter enteritis in a school. The enhanced virulence of the clinical isolates, possibly induced by passage, was confirmed by colonization tests on infant mice.

INTRODUCTION

Campylobacter jejuni and *C. coli* are the most frequently reported cause of acute bacterial gastroenteritis in the U.K. (Skirrow, 1982). Although the disease is generally self-limiting, the estimated 600,000 cases per annum (Skirrow, 1982) command considerable medical and financial resources. Most cases of campylobacter enteritis are sporadic, but epidemiological studies of outbreaks indicate that milk, water and poultry may be major environmental sources (Blaser, Taylor & Feldman, 1983).

C. jejuni and *C. coli* have been isolated from various water sources (Knill, Suckling & Pearson, 1982) but it is not known whether all such strains are pathogenic. Indeed, it has been suggested that the asymptomatic carriage of *C. jejuni* in humans in developing countries (Rajan & Mathan, 1982) reflects the non-pathogenic nature of some of these environmental strains. In order to test this hypothesis we compared the virulence of several campylobacter strains isolated from river and sea water with that of strains isolated from patients with diarrhoea. Included in the study were environmental and clinical isolates obtained from a previously described outbreak of campylobacter enteritis (Palmer *et al.* 1983).

Table 1. *Source and serotype of bacterial strains*

Strains	Source	Biotype	Penner serotype
	Environmental		
24842*	Storage tank	*C. jejuni* 2	6
24843*	Storage tank	*C. jejuni* 2	6
E2/16/10	River	*C. jejuni* 2	7
E1/16/10	River	*C. jejuni* 2	7
E1/13/11	River	*C. coli*	7
H1/19/9	Sea	*C. jejuni* 2	7
	Clinical		
81116*	Human	*C. jejuni* 2	6
81117*	Human	*C. jejuni* 2	6
03571	Human	*C. coli*	U.T.
68869	Human	*C. jejuni* 1	30
00081	Human	*C. jejuni* 1	N.T.
00167	Human	*C. coli*	N.T.
00241	Human	*C. jejuni* 2	N.T.

* From outbreak of campylobacterosis.
U.T. untypable; N.T., not typed.

MATERIALS AND METHODS

Bacterial strains

The strains used are described in Table 1. Clinical strains were isolated from faeces on antibiotic selective medium (Skirrow, 1977) in microaerobic conditions at 42 °C. Environmental strains were isolated from water, after selective filtration, as previously described (Knill, Suckling & Pearson, 1982).

Strains 24842 and 24843 were isolated from separate samples of drinking water which was the putative source of infection in an outbreak of campylobacter enteritis at a school (Palmer *et al.* 1983). Strains 81116 and 81117 were isolated from two patients with diarrhoea who had drunk this water. All four strains, which could not be distinguished by serotyping or biotyping, were included in this study to assess variations in virulence that might have arisen as a result of human passage.

All strains were subcultured on blood agar (2% agar, 5% defibrinated horse blood) for 24 h at 37 °C. Strains were stored in liquid nitrogen in 1% proteose peptone with 10% glycerol. In most cases they were subcultured only once before use, but strains E2/16/10, E1/16/10, E1/13/11 and H1/19/9 had been subcultured several times before these studies. Strains were biotyped by the method of Skirrow & Benjamin (1980) and serotyped by the method of Penner & Hennessy (1980).

Attachment to and invasion of HeLa cells detected by immunofluorescence

Preparation of antisera. Rabbits were immunized intramuscularly with 0·5 ml of a formalin-treated (0·3% formalin) suspension of *C. jejuni* strain 81116 (optical density of the bacterial suspension at 540 nm = 10·0) in Freund's complete adjuvant. Immunity was boosted at 14-day intervals with subcutaneous injections of the same antigen in Freund's incomplete adjuvant. The resulting antisera cross-reacted with all the strains used in this study.

HeLa cell (Flow Laboratories Ltd) monolayers (2×10^5 cells per well) were cultured in a 24-well tray (L.H. Engineering Ltd, Stoke Poges) on 13 mm glass cover-slips for 18 h at 37 °C in 5 % carbon dioxide in minimum essential medium (MEM) (Gibco-Europe Ltd) containing 10 % foetal calf serum. A suspension of $2{\cdot}8 \times 10^7$ c.f.u. of bacteria was added to each well and centrifuged on to the cells at 1100 g for 20 min. Tests were then incubated for 1 h at 37 °C. The cover-slips were washed twice in phosphate-buffered saline (PBS) and incubated with rabbit anti-campylobacter antiserum (1:100 dilution) with or without prior fixation in acetone (10 min at 4 °C). Acetone fixation made cells permeable to antibody, so that intracellular as well as surface-associated bacteria were detected; unfixed cells allowed only surface-associated bacteria to be detected (Kihlstrom, 1977). The presence of bound rabbit IgG was detected with goat anti-rabbit IgG coupled to FITC (1:25 dilution; Tenovus Labs, Southampton). The number of campylobacters associated with each cell was counted for 500 cells and the proportion of intracellular bacteria was calculated as n fixed $- n$ unfixed/n fixed $\times 100$.

Invasion of HeLa cells detected by electron microscopy

Because the number of intracellular organisms observed by immunofluorescence was small, electron microscopy was used to confirm the results.

HeLa cells were cultured and incubated with campylobacters as described above, except that Thermanox cover-slips (L.I.P. Equipment and Services Ltd, Shipley) were used instead of glass cover-slips. After incubation for periods up to 1 h, the cells were fixed in 2 % glutaraldehyde in 0·1 M Sorenson's phosphate buffer, pH 7·4 for 1 h at 20 °C then washed in the same buffer and postfixed in 1 % osmic acid in Palade's acetate buffer for 30 min at 20 °C and washed. The fixed cells were then stained with 3 % uranyl acetate for 30 min at 20 °C. Internalized organisms were distinguished from surface-associated organisms by labelling the cell surface acidic glycoproteins with electron-dense colloidal thorium (1 % thorium colloid in 3 % acetic acid applied for 18 h at 4 °C). Truly intracellular organisms were surrounded by an unlabelled vacuolar membrane. After washing in 3 % acetic acid, the stained monolayers on the cover-slips were dehydrated through a series of acetone solutions and embedded in Spurr Resin with orientation on a pre-polymerized Spurr resin block. After polymerization the cover-slips were removed by rapid contact with a hot block and 50 nm sections were cut along the monolayer with a diamond knife. These thin sections were stained with lead citrate and viewed on a Philips 201 transmission electron microscope at 60 kV.

Cytotoxicity assay

HeLa cells (1×10^4 cells in 100 μl of MEM) were added to 100 μl MEM per well in a 96-well tray (L.H. Engineering Ltd). The cells were incubated overnight at 37 °C in 5 % carbon dioxide, then the medium was removed and the monolayers washed once with PBS to remove dead cells and cell debris. Doubling dilutions of a campylobacter suspension (optical density at 260 nm was 0·1) in HEPES-buffered MEM with 10 % foetal calf serum were added to the wells (200 μl per well), which were then incubated for 18 h at 37 °C. The medium was removed and 50 μl of 0·034 % neutral red in HEPES-buffered saline (0·9 % sodium chloride with 30 mM HEPES, 1 % foetal calf serum, 0·01 % calcium chloride and 0·01 % magnesium chloride) was added to each well. After a further period of incubation (1·5 h at 37 °C)

Table 2. *Attachment to and penetration of HeLa cells*

Strains	Percentage of cells with one or more associated bacteria (± s.d.)	Average number of bacteria/cell		Intracellular bacteria (%)
		Fixed	Unfixed	
	Environmental			
E2/16/10	32·6±4·8	0·9	0·9	1
E1/16/10	88·8±14·9	—	—	—
24842	84·5±8·6	5·1	3·8	26
24843	43·5±10·1	1·2	0·8	34
	Clinical			
03541	95·7±6·4	6·4	2·9	54
81116	83±16·4	4·1	1·0	76
68869	26·6±2·6	—	—	—
00081	84·5±8·9	—	—	—
00241	70±12·3	—	—	—
00167	87·4±15·6	—	—	—

the neutral red was removed and the monolayer washed three times in HEPES-buffered saline to remove any detached cells. The remaining cells were dissolved in 175 μl of 1% sodium dodecyl sulphate (SDS) in 0·1 M-NaOH and the optical density read on a microELISA reader (Dynatech Laboratories Ltd, Billingshurst) fitted with a 450 nm filter. Each experiment was done in quadruplicate with controls containing medium only. The cells remaining attached to the substratum were stained with the neutral red. The percentage kill was calculated as

$$\frac{\textit{optical density of the treated cells}}{\textit{optical density of the untreated cells}} \times 100.$$

Colonization of infant mice

Five-day-old Balb/c mice were inoculated intragastrically with 1×10^7 c.f.u. of strain 81116 or strain 24842 as previously described (Newell & Pearson, 1984). The infants were separated from the dams for 2h before and 30 min after the inoculation. Segments of the gut were collected and homogenized, and the number of campylobacters present determined by viable counts on antibiotic selective medium (Skirrow, 1977) containing 2% agar. Colonization was followed over a 10-day period. At least three but usually six mice were used for each point.

RESULTS

Attachment to and invasion of HeLa cells

Significant variation in attachment was observed between strains as determined either by the proportion of HeLa cells with associated bacteria or by the average number of bacteria per cell (Table 2). This variation in attachment occurred despite the centrifugation of bacteria on to the cell surface and did not appear to relate to the source of the strain. All strains except E2/16/10 gave higher counts with fixed cells than unfixed cells, which indicated that there had been some invasion. The two clinical strains tested showed this to a greater degree than the three water

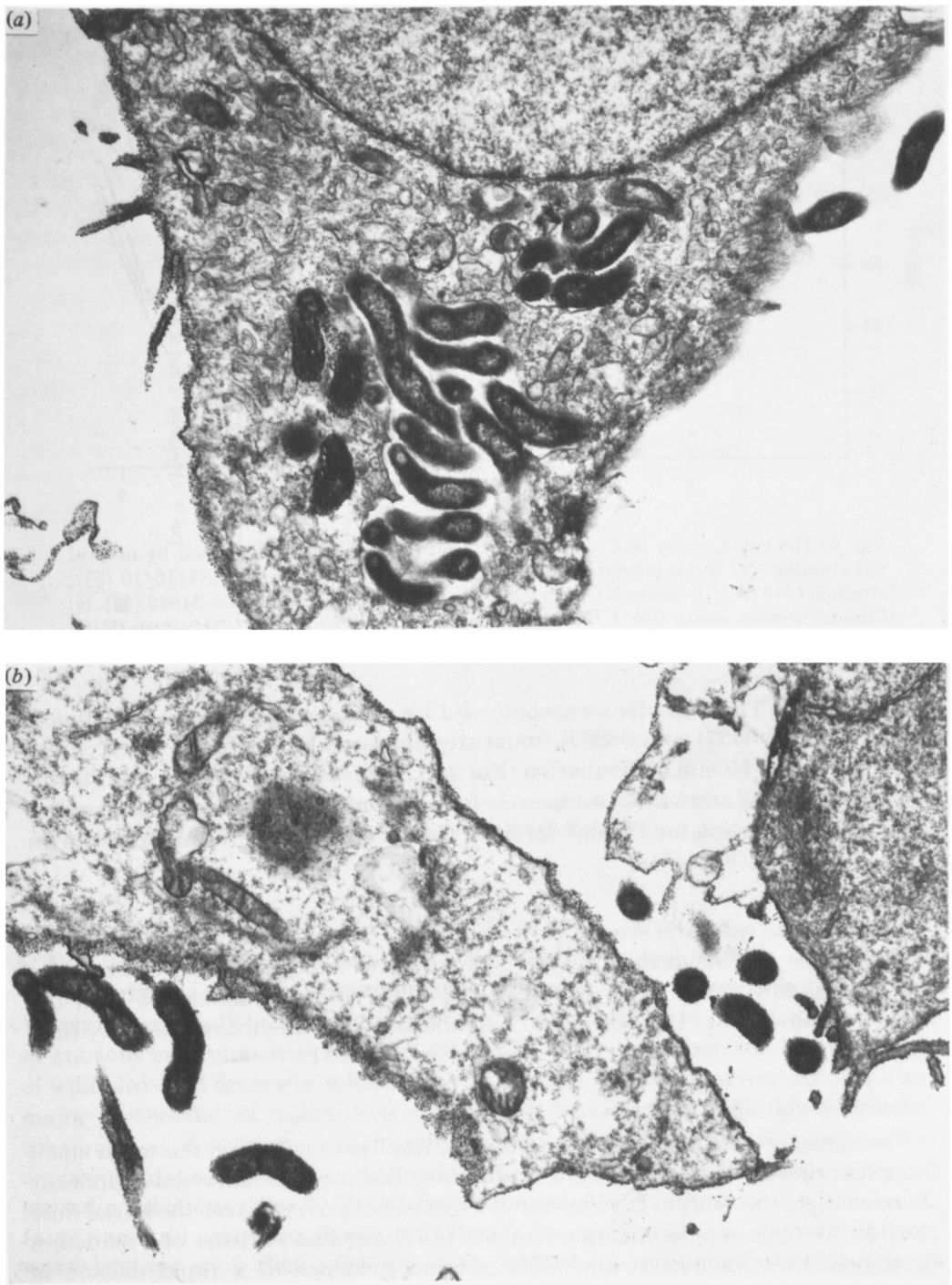

Fig. 1. Attachment and penetration of HeLa cells by *C. jejuni*. (*a*) Strain 81116. Note the presence of organisms in membrane-bound vacuoles unlabelled with thorium indicating penetration. (*b*) Strain E2/16/10. Not the close association of organisms with the thorium-labelled cell surface but the absence of penetration.

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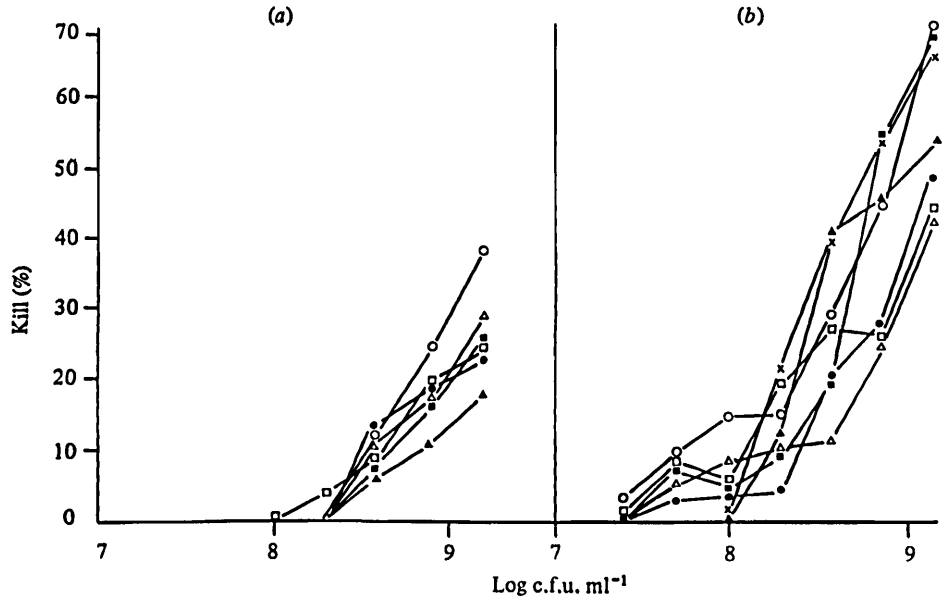

Fig. 2. The cytotoxicity of *C. jejuni and C. coli* to HeLa cells determined by neutral red staining. (*a*) Environmental isolates strain E2/16/10 (●); strain E1/16/10 (□); strain H1/19/9 (△); strain E1/13/11 (○); strain 24843 (▲) and strain 24842 (■). (*b*) Clinical isolates strain 03571 (■); strain 00081 (△); strain 00241 (○); strain 00167 (●); strain 68869 (□); strain 81117 (▲) and strain 81116 (x).

strains tested. These results were confirmed by electron microscopy. The clinical strains (81116, 03571 and 68869) frequently appeared in unlabelled intracellular vacuoles within 10 min of incubation (Fig. 1*a*), whereas the environmental strains (E2/16/10, 24843 and 24842) were rarely found in unlabelled intracellular vacuoles even after incubation for 1 h and despite a close association of the organisms with the cell surfaces (Fig. 1*b*).

Cytotoxicity

The river and sea water strains showed a poorer cytotoxic activity towards HeLa cells than the clinical strains (Fig. 2). Comparison of the cytotoxicity of the water (24842 and 24843) and clinical (81116 and 81117) isolates associated with the outbreak showed that the former had a significantly lower cytotoxic activity than the latter.

Colonization of infant mice

The clinical strain 81116 and the water isolate 24842 colonized the lower small intestine, caecum and colon of 5-day-old mice. Both strains showed a significant increase (up to 50-fold) in the number of organisms recovered over the initial dose (Fig. 3). At 10 days post-infection strain 81116 showed no evidence of a reduction in colonization whereas strain 24842 showed evidence of a rapid decline in colonization. Excretion of strain 81116 continued for at least 30 days after infection, as determined from faecal pellets, whereas excretion of strain 24842 was terminated by the 13th day after infection.

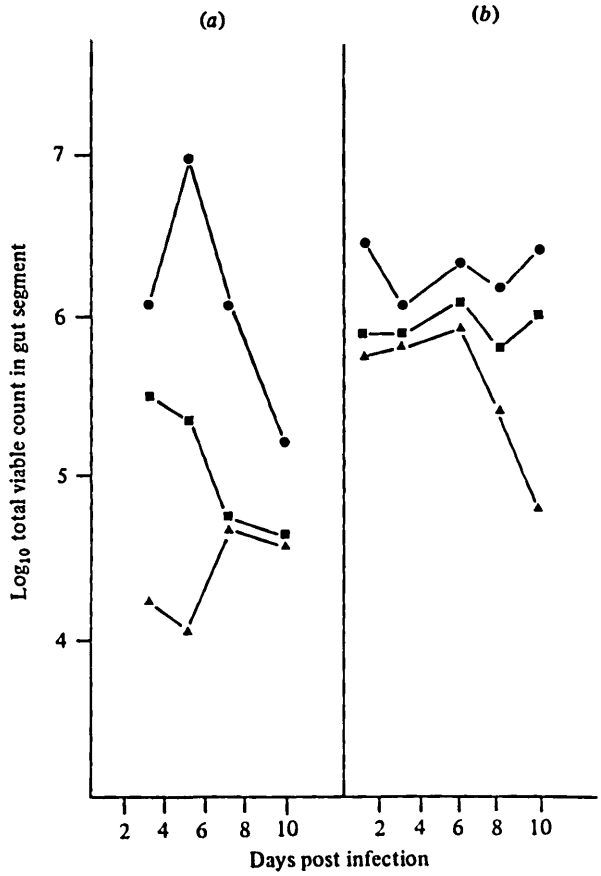

Fig. 3. Colonization of infant mice with (*a*) environmental strain 24842 and (*b*) clinical strain 81116. ●, Colon; ■, caecum; ▲, lower small intestine.

DISCUSSION

Despite the prevalence of campylobacter enteritis in man, little is known of the relative importance of various source(s) of infection or the means of transmission. Water can certainly be a source, and as campylobacters can survive for a long time in aqueous environments (Blaser *et al.* 1980), water contaminated by the faeces of wild birds and domestic animals may be a major natural reservoir. At least two major outbreaks of campylobacter enteritis have been attributed to the consumption of contaminated municipal water supplies, and the consumption of environmental water has been blamed for the occurrence of smaller outbreaks and sporadic infections (Blaser, Taylor & Feldman, 1983). *C. jejuni* has been isolated from river and sea water (Knill, Suckling & Pearson, 1982) and stored drinking water (Palmer *et al.* 1983). Pearson *et al.* (1983) have recognized 21 serotypes of *C. jejuni* and *C. coli* among 110 water isolates. Only 13 of these serotypes had been previously identified with human infections, and their distribution was very different from that of 45 clinical strains. This suggests that environmental campylobacters are non-pathogenic. The presence of non-pathogenic campylo-

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bacters in the environment may account for the high incidence of asymptomatic carriage in domestic and wild animals (Newell, 1984) and in humans in developing countries (Rajan & Mathan, 1982).

Campylobacters isolated from water are biochemically indistinguishable from clinical isolates of *C. jejuni* and *C. coli*. Moreover, they have similar protein profiles as shown by SDS-polyacrylamide gel electrophoresis (unpublished data), in particular a 43K–46K variable major outer membrane protein and a 62K flagella protein which are characteristic of clinical isolates of *C. jejuni* and *C. coli* (Newell, McBride & Pearson, 1984). Yet in our studies the water isolates were not as virulent as the clinical isolates.

Although clinical strains of *C. jejuni* show significant variations in attachment to cells in culture (Manninen, Prescott & Dohoo, 1982), attachment appears to be followed by penetration of the cells and concomitant cell death (Manninen, Prescott & Dohoo, 1982; Newell & Pearson, 1984). In this study the environmental strains did not penetrate HeLa cells as successfully as the clinical strains. Electron microscopy confirmed that the clinical strains rapidly invaded HeLa cells whereas few intracellular organisms were observed with the water strains.

Although Johnson & Lior (1984) reported the production of a heat-labile cytotoxin by most human and non-human isolates of *C. jejuni* and *C. coli*, the cytotoxic activity detected in our studies was thought to reflect invasive activity rather than cytotoxin production (Newell & Pearson, 1984). Cytotoxic activity, as determined by the neutral red viability staining technique, was significantly lower in the water isolates than the clinical isolates. Interestingly this was most pronounced in the otherwise matching water and clinical isolates from the presumptive water-borne outbreak (Palmer *et al.* 1983) and is consistent with the electron microscopic observations. These isolates also differed in their ability to colonize the infant mouse gut.

Colonization of both water and clinical isolates was similar to that previously reported for other clinical isolates (Field *et al.* 1981; Newell & Pearson, 1984) but the water isolate showed a decline in colonization of the gut segments long before the clinical isolate.

Presuming that the water strains (24842 and 24843) were the source of the human outbreak strains (81116 and 81117) these studies suggest that virulence, as measured by colonization *in vivo* and invasion *in vitro*, is enhanced by passage. This is consistent with the recent studies by Kazmi, Roberson & Stern (1983) and Field, Underwood & Berry (1984) on animals.

Although the mechanism(s) by which campylobacters cause enteritis have yet to be established, the clinical features suggest invasion and/or production of a toxin (Newell, 1984). For most enteric pathogens attachment is a prerequisite for invasion and effective delivery of toxins to the intestinal epithelial cells (Giannella, 1981). Thus adhesiveness to cell cultures has been used as a measure of virulence; and since the invasion of tissue culture cells correlates with the capacity of enteropathogenic bacteria to invade intestinal epithelial cells *in vivo* (Giannella *et al.* 1973), it is likely that the activity of these campylobacter strains towards the cell cultures is a reflexion of their pathogenicity.

In vivo models of campylobacteriosis have been difficult to establish (Newell, 1984). Although the colonization of the infant mouse gut by clinical strains of

C. jejuni and *C. coli* may involve invasion of the intestinal mucosa and induce an antibody response (Newell & Pearson, 1984) it is rarely accompanied by overt disease (Field *et al.* 1981). In this model colonization is a reflexion of adhesion to the intestinal mucosa, motility and proliferation of the organism (Newell, McBride & Dolby, 1983).

Using several models of pathogenesis for *C. jejuni* infections we have established that campylobacters isolated from water are less virulent than clinical isolates. Although there is evidence for passage-induced enhancement of virulence we cannot eliminate the possibility that non-pathogenic campylobacters exist in the environment. Further investigations are in progress to determine the mechanisms of pathogenesis of *C. jejuni* and *C. coli* and identify virulence markers for the detection of pathogenic campylobacters in the environment.

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