Comparison of virulence-associated *in vitro* properties of typed strains of *Campylobacter jejuni* from different sources

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Campylobacter jejuni is a major cause of human diarrhoeal disease, but specific virulence mechanisms have not been well defined. This blinded study was undertaken with 40 *C. jejuni* isolates from different sources to determine their haemolytic, cytotoxic and adhesion and invasion activities towards mammalian cells. The results were correlated with source of isolation and genetic makeup by amplified fragment length polymorphism (AFLP) typing. The isolates had variable degrees of haemolytic activity against rabbit erythrocytes and cytotoxicity towards CaCo-2, HeLa and Vero cells. The data indicated that the haemolytic and cytotoxic activities were due to separate factors. A range of cytotoxicity was exhibited, whereby some strains had no activity against the target cells and others had activity against all three cell lines. Certain strains had activity against CaCo-2 cells but little or no activity against the other cells, while others exhibited the opposite phenotype. The data suggested that the cytotoxicity assay with the different cell lines may have detected more than one cytotoxin. A wide variation between isolates was observed for both adherence and invasion with all three cell lines, yet, overall, the strains showed a significantly greater invasion capacity for CaCo-2. There was no clear relationship between source of isolation or disease manifestation and possession of statistically significantly higher levels of particular virulence-associated factors although, in some cases, a correlation between cytotoxicity and cell invasion was evident. Five AFLP clusters, each representing two to eleven isolates with similar profiles, were observed at the 90% similarity level. Some AFLP groups contained isolates with a common serotype, but each group had *C. jejuni* isolates from more than one source with the exception of group IV, which contained only human isolates. Isolates with high cytotoxic activity against CaCo-2 cells were confined to groups I, III and IV and a group of unrelated strains (U). Group II isolates had uniformly low cytotoxicity. Isolates in groups I, V and U were more invasive for CaCo-2 cells than isolates in groups II, III and IV. The strain differences in cytotoxicity or invasion did not correlate with source of isolation.

Received 19 December 2006

Accepted 26 February 2007

INTRODUCTION

Campylobacter jejuni is a major cause of human enteritis and diarrhoeal disease. Virulence mechanisms have been attributed to motility, adherence, cytotoxin and enterotoxin production and invasion of the gut mucosa (Griffiths & Park, 1990; Ketley, 1997; Wassenaar & Blaser, 1999). However, which determinant(s) plays the major role in perturbation of the normal absorptive capacity of the intestinal epithelium that causes the transient watery diarrhoea, and/or blood and leukocytes sometimes found in stools, is still unclear. The disease features could also be attributed to an inflammatory response (Everest *et al.*, 1993; Hickey *et al.*, 1999; Mellits *et al.*, 2002; MacCallum *et al.*, 2005). *Campylobacters* enter the host by the oral route and colonize the distal ileum and colon. Adhesion to the epithelial cell layer (Konkel & Joens, 1989; Konkel *et al.*, 1992) can be followed by invasion of epithelial cells, but this has been reported to be strain-dependent, since some strains adhered to tissue culture cells without invasion, while the capacity of others for invasion was 10^2 – 10^4 times higher (Everest *et al.*, 1992; Oelschlaeger *et al.*, 1993). A significant correlation was reported between invasive ability and the presence of colitis in patients from which isolates were obtained (Everest *et al.*, 1992).

Haemolysin activity has been reported from several laboratories, including our own study (Hossain *et al.*, 1993), but whether this factor is related to other reported cytotoxic activities is not known. Low-level cytotoxic activity of *C. jejuni* culture filtrates or polymyxin B-released cell extracts affecting the integrity of mammalian cells has been assayed by the cytopathic changes in cultured cells, but this has produced only semiquantitative data (Wassenaar, 1997; Pickett, 2000). In a previous report, the cytotoxic activity of cell extracts and culture supernates from four *C. jejuni* strains was assayed against HeLa and Vero cells with a quantitative dye-reduction assay (Coote & Arain, 1996). Here, a blinded study of the haemolytic and cytotoxic activities, adhesion and invasion properties and amplified fragment length polymorphism (AFLP) typing of 40 *C. jejuni* isolates from different sources is reported, in an attempt to correlate laboratory activities towards mammalian cells, which arguably might be involved in pathogenicity, with genetic makeup and source of isolation.

METHODS

Bacterial strains and growth conditions. Thirty coded *C. jejuni* isolates were provided by one of us (R.J.O.) and designated COL 1–30. COL 7 was the type strain NCTC 11168^T. Ten coded *C. jejuni* isolates were provided by another of us (F.J.B.) and designated RPH1–10 (Table 1). These isolates were serotyped (Penner & Hennessy, 1980; Owen *et al.*, 1995) and ribotyped (Fitzgerald *et al.*, 1996). The source of the isolates is included in Table 1. The *C. jejuni* isolates were subcultured on blood agar base (Oxoid) supplemented with 5% (v/v) defibrinated sheep blood (Gibco) and Blaser-Wang selective supplement (Oxoid) and incubated at 42 °C in anaerobic jars under a gas atmosphere of 5% O₂, 15% CO₂ and 80% N₂ (BOC). The isolates were identified by standard tests including

colonial morphology, Gram stain, motility, positive oxidase and catalase tests, lack of growth at 25 °C and positive hippurate test (Ullmann, 1979). After primary isolation, the isolates were subcultured only once to minimize cultural changes, after which samples of each, stored at –70 °C in Brucella broth (Oxoid) and glycerol (60:40 v/v), were used for a limit of five subcultures before a new stored culture was used. For growth in liquid medium, a loopful of the *C. jejuni* growth on agar medium was inoculated into 100 ml Brucella broth and incubated in an anaerobic jar, under the same microaerophilic atmosphere as above, at 42 °C in an orbital shaker at 100 r.p.m.

Haemolysis assay. *C. jejuni* isolates were grown in Brucella broth as described above for 48 h, after which time the cells were centrifuged at 1500 g for 20 min at 4 °C and then resuspended to an OD₆₅₀ of 2.0 in PBS, and 50 µl of doubling dilutions of the cell suspension added to 50 µl PBS was distributed into the wells of 96-well microtitre plates. To each well was added 100 µl freshly collected rabbit red blood cells (RRBC), washed three times in PBS and resuspended to a concentration of 1% (v/v) in PBS. The mixture was incubated at 42 °C for 2 h. The plates were centrifuged at 1500 g for 10 min and 100 µl supernate was removed from each well and the A₅₄₀ was measured in an Anthos 2001 microplate reader. RRBCs lysed with 1% (v/v) Triton X-100 served as a positive control and an RRBC suspension with PBS alone was used as a negative control. Results were expressed as the reciprocal of the highest dilution that produced 50% haemolysis.

Preparation of *C. jejuni* extracts for cytotoxin assays. Broth cultures, prepared as described above, were centrifuged at 1500 g for 20 min at 4 °C. The cell pellets were resuspended to an OD₆₅₀ of 2.0 in PBS containing 0.15% (w/v) polymyxin B (Sigma) to promote the release of cell-associated material. Polymyxin was used previously to demonstrate cytotoxin production by *C. jejuni* (Guerrant *et al.*, 1987). After incubation at 37 °C for 30 min, 1.0 ml of the treated cells was centrifuged at 2500 g for 20 min and the supernates were passed through a 0.45 µm filter (Acrodisc; Gelman Sciences); the filtrates were stored at –20 °C until required. Each isolate was tested in duplicate at least three times on separate occasions in the cytotoxicity assay.

Preparation of mammalian cell suspensions. HeLa (human epithelial cervical carcinoma) and Vero (African green monkey kidney) cells (Flow Laboratories) were propagated as monolayers within plastic tissue-culture flasks in Eagle's minimal essential medium (E-MEM; Flow Laboratories) supplemented with 2 mM L-glutamine and 10% (v/v) fetal calf serum (both from Flow Laboratories). Penicillin and streptomycin (both at 100 IU ml^{–1}; Sigma) were added to prevent bacterial contamination. Cell monolayers were detached by the addition of a trypsinizing solution [0.05% (w/v) trypsin, 0.02% (w/v) EDTA; Flow Laboratories] and gentle tapping of the flask. Cell aggregates in the suspension were dispersed by sterile-pipetting before the cells were washed in fresh medium and suspended to a final viable cell density of 5×10^4 cells ml^{–1}. Human colon adenocarcinoma (CaCo-2) cells were obtained from the European Collection of Animal Cell Cultures (ECACC; CAMR, Porton Down, UK). Cells were propagated in E-MEM containing 10% (v/v) fetal calf serum, 1% (v/v) L-glutamine, 1% insulin-transferrin-selenium, 1% (v/v) penicillin/streptomycin and 1% (v/v) non-essential amino acids (Flow Laboratories). A confluent monolayer of CaCo-2 cells in an 8.0 cm² flask was obtained within 5–7 days, with regular changes of medium. Cells were detached and cell suspensions prepared as described above.

Cytotoxicity assay. An assay based on toxin inhibition of the ability of active mitochondria in cultured mammalian cells to reduce a tetrazolium dye was used (Coote & Arain, 1996). Each well of a flat-bottomed, 24-well microtitre plate (Flow Laboratories) was loaded

Table 1. *Campylobacter jejuni* isolates

Sources of isolation are indicated in the isolate code as follows: B, bovine; C, chicken; H, human; M, milk; O, ovine. Penner heat-stable serotypes (determined according to Owen *et al.*, 1995) are indicated by the prefix 'HS'. 16S rDNA ribotypes (determined according to Fitzgerald *et al.*, 1996) are indicated by the prefix 'H'.

Code	Reference no.	Host and place of isolation*	Serotype	<i>HaeIII</i> 16S rDNA ribotype
COL1 H	C124/93	Human (61 y, m) diarrhoea; UK (Ashford PHL)	HS1	H1
COL2 B	C404/93	Bovine carcass, abattoir; UK (Lancaster Univ.)	HS1	H1
COL3 B	C406/93	Bovine carcass, abattoir; UK (Lancaster Univ.)	HS1	H1
COL4 C	C711/93	Chicken caecum; UK (Exeter PHL)	HS1	H1
COL5 C	C724/93	Chicken carcass, skin; UK (Exeter PHL)	HS1	H1
COL6 H	C6/95	Human diarrhoea; UK (Sunderland Royal Infirmary)	HS1	H1
COL7 H	NCTC 11168 ^T	Human faeces; UK (Worcester Hospital)	HS2	H1
COL8 H	C48/92	Human faeces; UK (Lancaster Hospital)	HS1	H1
COL9 H	A709/92	Human faeces; South Africa (hospital)	HS1	H1
COL10 H	C34/93	Human (f) persistent diarrhoea	HS1	H1
COL11 H	C35/93	Human (79 y, f) severe anaemia; UK (Ashford PHL)	HS1	H1
COL12 C	C85/93	Chicken caecum; UK (Exeter PHL)	HS1	H1
COL13 B	C379/93	Bovine, farm site; UK (Lancaster Univ.)	HS1	H1
COL14 H	C571/93	Human faeces, diarrhoea; UK (Guy's Hospital)	HS1	H1
COL15 H	C547/94	Human bloody diarrhoea; UK (Cardiff PHL)	HS2	H1
COL16 H	C638/94	Human (31 y, f) no details; UK (Cardiff PHL)	HS2	H1
COL17 H	C637/94	Human (4 y, f) no details; UK (Cardiff PHL)	HS4c	H1
COL18 H	C637/94	Human (22 y, m) diarrhoea; UK (Cardiff PHL)	HS2	H1
COL19 H	C649/94	Human diarrhoea; UK (Harrogate NHS)	HS41	No data
COL20 H	C720/94	Human (54 y, m) diarrhoea; UK (Ashford PHL)	HS15	H6
COL21 B	C34/92	Bovine (12 weeks) faeces; UK (Central Veterinary Lab.)	HS1	H1
COL22 H	C662/94	Human (2 y, m) diarrhoea; UK (Cardiff)	NT	H1
COL23 H	C584/94	Human (36 y, f) diarrhoea; UK (Cardiff)	HS53	H4
COL24 H	C614/94	Human (30 y, m) diarrhoea; UK (Cardiff)	HS53	H4
COL25 H	C641/94	Human (18 y, f) diarrhoea and vomiting; UK (Cardiff)	HS11	H15
COL26 H	C656/94	Human (22 y, f) diarrhoea; UK (Cardiff)	HS11	H15
COL27 H	C648/94	Human (60 y, m) diarrhoea; UK (Harrogate)	HS11	No data
COL28 O	C403/93	Ovine; UK (Lancaster)	HS2	H1
COL29 B	C185/93	Bovine faeces; UK (Compton)	HS2	H1
COL30 H	C156/92	Human (2 y) diarrhoea; UK (Lincoln)	HS2	H1
RPH1 C	2529/1991	Poultry meat; UK	HS2	H5
RPH2 C	2523/1991	Poultry meat; UK	HS2	H25
RPH3 C	2608/1991	Poultry meat; UK	HS4c	H5
RPH4 C	306/1990	Poultry meat; UK	HS1	H1
RPH5 H	313/1990	Human enteritis; UK	HS1	H1
RPH6 H	337/1990	Human enteritis; UK	HS1	H1
RPH7 C	2561/1990	Poultry meat; UK	HS2	H1
RPH8 H	1915/1990	Human enteritis; UK	HS2	H1
RPH9 H	3222/1990	Human enteritis; UK	HS4c	H4
RPH10 M	2258/1991	Milk; UK	HS23	H10

NT, Non-typeable.

*f, Female; m, male; y, years of age; PHL, Public Health Laboratory.

with 200 µl mammalian cell suspension and incubated at 37 °C overnight in an atmosphere of 5 % CO₂:95 % air (BOC) to allow the cells to adhere. A portion of the growth medium (100 µl) was discarded and replaced with 100 µl of a twofold dilution of the *C. jejuni* polymyxin B extract in E-MEM. Duplicate wells of each extract were set up: cells exposed to polymyxin B-treated Brucella broth and cells treated with 1 % (v/v) Triton X-100 served as negative (100 % live cells) and positive (0 % live cells) controls, respectively. After 24 h

incubation of the cell monolayers in the presence of bacterial cell extracts, 20 µl of 0.5 % (w/v) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) in PBS was added to each well and incubation was continued for a further 4 h. The tetrazolium ring of MTT is cleaved by dehydrogenase activity to produce a dark-blue formazan product. The overlying medium was removed, the formazan product was solubilized by addition of 100 µl 0.04 M HCl in DMSO and the A₅₄₀ was measured in an Anthos 2001

plate reader. Cytotoxicity (expressed as percentage cell death) was calculated by the formula:

$$100 \times \left[1 - \frac{A_{540}(\text{test}) - A_{540}(\text{positive control})}{A_{540}(\text{negative control}) - A_{540}(\text{positive control})} \right]$$

Adhesion and invasion assay. The *C. jejuni* isolates were grown in Brucella broth, as described above, harvested and washed once with sterile Dulbecco A PBS (10 mM, pH 7.3; Oxoid) and resuspended in E-MEM containing 10% (v/v) fetal calf serum and 2 mM L-glutamine and adjusted to 1×10^9 cells ml^{-1} . Mammalian cell monolayers (CaCo-2, HeLa or Vero) in 24-well tissue-culture plates were seeded at a concentration of 1×10^5 cells per well. After 24 h, or when a monolayer of cells had formed, the growth medium was removed, the cell monolayer was washed twice with sterile warmed (37 °C) E-MEM and 1×10^9 *C. jejuni* cells in 1.0 ml E-MEM were added per well in duplicate tests. Infected cell cultures were incubated at 37 °C for 3 h in an atmosphere of 5% CO_2 /air, after which the cell monolayers were washed five times with E-MEM to remove non-adherent bacteria, before the addition of either fresh E-MEM containing 100 µg gentamicin ml^{-1} (Sigma) to measure invasive organisms alone or E-MEM alone for the estimation of adherent plus invasive organisms. After 2 h further incubation at 37 °C, all monolayers were washed five times with E-MEM and the bacteria were released by lysing the mammalian cells for 5 min with 1.0 ml 1% (v/v) Triton X-100 in PBS per well. Tenfold dilutions of the released bacteria were made in sterile water and viable counts were made on blood agar containing selective growth supplements. The number of c.f.u. of adherent cells alone was determined as c.f.u. of non-gentamicin culture (i.e. adherent + internalized c.f.u.) – c.f.u. of gentamicin-treated culture (i.e. internalized).

AFLP typing. AFLP genotyping was carried out according to Siemer *et al.* (2004, 2005). Briefly, genomic DNA prepared using Easy-DNA kit (Invitrogen) was simultaneously digested with a 'rare'- and a 'frequent'-cutting restriction enzyme and site-specific adapters were ligated onto the ends of the fragments. The fragments were amplified by PCR in which the primer complementary to the 'rare' restriction site/adaptor sequence was fluorochrome-labelled. The PCR products were separated on an ABI 377 automated sequencer (Applied Biosystems) (Kokotovic & On, 1999). Gel images were pre-processed by GENESCAN version 3.1 (Applied Biosystems). The restriction enzymes used were *Bgl*II and *Csp*6I (Siemer *et al.*, 2004) and fragments of 50–400 bases were included in the data analysis. Numerical analysis of the AFLP patterns was achieved with the Dice similarity coefficient and the UPGMA clustering algorithm in Bionumerics version 2.5 (Applied Maths) (On & Harrington, 2000).

Statistical analysis. Statistical analysis of the cytotoxicity, adhesion and invasion and AFLP results of the COL and RPH strains was done with Minitab 12 (Minitab Ltd) using the procedures of Wardlaw (2000). When the isolate codes were unblinded at the end of the laboratory experiments, COL 28, an ovine isolate, and RPH 10, a milk isolate, were excluded from statistical analyses of the results and the AFLP dendrograms as these were single-source isolates. Statistical significance was defined as $P < 0.05$.

RESULTS

Haemolytic activity

None of the cell-free broth-culture supernates had haemolytic activity against washed RRBCs in the microtitre plate assay. However, the *C. jejuni* cells possessed variable

degrees of haemolytic activity against RRBCs (Table 2). The type strain, NCTC 11168^T (=COL 7), possessed little haemolytic activity, but COL 15, 16, 25 and 28 and RPH 5 and 9, all but one being human isolates, showed titres of 16 (Table 2). COL 15 and 16, indicated by AFLP analysis to be a matched pair of isolates from the same source outbreak in Cardiff (see below), had the same level of haemolytic activity and similar phenotypes for the other virulence-associated factors (Table 2). The three non-haemolytic strains from a human source, COL 6, 23 and 27, were isolated from patients with diarrhoea (Table 1).

Cytotoxic activity

None of the cell-free *C. jejuni* culture supernates had cytotoxic activity in the MTT assay. Polymyxin B extracts of all isolates were tested for cytotoxic activity against CaCo-2, Vero and HeLa cells (Table 2). None of the assays caused 100% cytotoxicity of the test cells over the 24 h period, indicating that the toxin activity was weak. The data showed a wide variation in toxic activity among the *C. jejuni* isolates. The cell extracts caused death of 0–77% of CaCo-2 cells and 0–88% of HeLa cells, but were in general less active against Vero cells (0–33% death). When the values for each group were displayed as Minitab point plots with 'jitter' (Wardlaw, 2000), a range of activities from high to low values was seen for CaCo-2 cells, whereas activities against Vero cells and, to a lesser extent, HeLa cells were clustered in the lower range (Fig. 1a). For CaCo-2 cells, there was no significant correlation between the isolate source and the level of cytotoxin production (Fig. 1b). Extracts from isolates COL 4–6, 8, 11–14, 22 and 23 showed good activity against all target cells, but cell-specific cytotoxic activity was apparent with other cell extracts. For example, COL 1 and 30 and RPH 3–9 were essentially inactive against both HeLa and Vero cells but showed high activity against CaCo-2 cells. Conversely, COL 3, 15 and 16 were inactive against CaCo-2 cells, but active against HeLa or Vero cells. COL 7 (=NCTC 11168^T) had only slight activity against CaCo-2 and Vero cells and was inactive against HeLa cells.

Adherence and invasion

Adherence by *C. jejuni* isolates varied between $10^{6.9}$ c.f.u. ml^{-1} (0.8% of input bacteria) for CaCo-2 cells to $10^{2.85}$ c.f.u. ml^{-1} (0.0001% of input bacteria) for Vero cells (Table 2). Variation between strains was most pronounced with Vero and HeLa cells, but strains adhered to CaCo-2 cells at 0.01–0.8% of input bacteria (Table 2). Minitab point plots with 'jitter' showed a trend for the *C. jejuni* isolates to adhere more effectively to CaCo-2 cells while exhibiting a generally lower and similar adherence pattern to HeLa and Vero cells (Fig. 2a). Apart from COL 8, 19 and 20, more adherence was generally obtained with CaCo-2 cells than with HeLa and Vero cells (Table 2). Similarly, the capacity for cell invasion was most pronounced with CaCo-2 cells and significantly greater overall than the invasive capacity

Table 2. Haemolytic titres, cytotoxic activity and adhesion and invasion assays for the *C. jejuni* isolates

Isolate codes are explained in Table 1. In the adhesion (Adh.) assay, results are expressed as $\log_{10}$ c.f.u. ml^{-1} of adhered bacteria. For the invasion (Inv.) assay, the values represent the percentage of adherent organisms that invaded the cell lines. Both sets of data are the means of three duplicate tests. No organisms were detected in 12 controls inoculated with killed bacteria.

<i>C. jejuni</i> isolate	Haemolysis titre	Cytotoxicity (% dead cells)			CaCo-2		HeLa		Vero	
		CaCo-2	HeLa	Vero	Adh.	Inv.	Adh.	Inv.	Adh.	Inv.
COL 1 H	2	77	4	5	5.61	2.3	3.93	3.6	5.69	0.2
COL 2 B	2	23	G*	14	5.59	0.8	3.78	15.5	4.96	0.1
COL 3 B	2	0	27	17	5.77	2.3	4.10	5.2	4.59	4.1
COL 4 C	4	14	11	25	5.95	1.4	5.23	0.5	5.40	0.7
COL 5 C	1	21	45	10	5.59	2.7	3.18	19.3	4.88	3.9
COL 6 H	0	17	36	27	5.00	10.3	3.63	23.0	3.90	75.0
COL 7 H	1	2	G	3	5.04	3.4	4.73	0.7	4.85	10.4
COL 8 H	2	10	29	22	4.93	1.2	5.08	0.3	5.78	0.4
COL 9 H	2	11	0	18	5.79	0.3	4.04	1.9	3.30	1.9
COL 10 H	4	21	0	2	6.90	0.1	3.23	25.3	3.52	0.8
COL 11 H	2	8	15	14	4.76	0.9	3.20	0	4.36	41.3
COL 12 C	4	52	47	7	5.65	0.3	4.00	3.5	4.93	6.2
COL 13 B	2	26	77	15	4.93	2.6	3.80	5.7	4.48	17.6
COL 14 H	4	24	66	21	5.08	46.8	3.54	16.9	4.99	43.8
COL 15 H	16	0	35	18	4.96	3.1	3.97	0.8	4.57	13.5
COL 16 H	16	G	G	23	5.51	0.1	5.58	0.04	5.25	2.4
COL 17 H	4	61	G	33	5.50	0.4	5.18	0.02	4.70	3.6
COL 18 H	8	22	65	0	5.59	2.4	4.49	2.4	3.84	2.9
COL 19 H	1	G	G	G	4.08	78.4	5.60	0.7	4.15	17.9
COL 20 H	2	G	G	16	3.74	39.3	6.04	0.5	4.00	24.0
COL 21 B	1	45	0	14	6.67	6.3	4.15	32.1	5.11	0.7
COL 22 H	1	15	50	11	5.14	44.6	4.83	4.2	4.92	2.0
COL 23 H	0	28	61	32	4.17	40.5	3.85	18.3	3.45	10.8
COL 24 H	1	25	25	6	5.08	96.6	5.18	1.2	4.60	2.0
COL 25 H	16	23	8	5	4.14	12.1	3.53	58.8	3.58	8.4
COL 26 H	1	59	0	15	4.63	32.6	3.51	25.9	3.30	22.5
COL 27 H	0	40	0	8	4.59	17.5	4.04	10.9	3.26	20.0
COL 28 O	16	46	10	26	4.79	12.3	4.15	4.4	3.30	10.0
COL 29 B	1	58	13	3	4.93	7.4	3.86	4.8	3.79	7.8
COL 30 H	1	62	G	5	4.30	16.0	3.45	64.3	2.85	35.5
RPH 1 C	1	38	19	4	4.77	73.9	3.30	24.0	4.88	2.3
RPH 2 C	4	30	13	G	5.93	4.1	3.26	12.8	5.04	5.9
RPH 3 C	2	47	G	G	4.64	24.8	3.30	26.7	4.65	1.9
RPH 4 C	1	50	G	G	5.75	8.4	3.96	1.3	5.78	0.13
RPH 5 H	16	47	7	G	5.97	5.0	4.74	1.4	4.65	1.5
RPH 6 H	4	28	G	G	6.38	7.5	4.86	0.5	4.38	9.6
RPH 7 C	0	32	G	G	6.46	0.2	4.78	0.5	5.43	0.8
RPH 8 H	2	30	G	6	6.87	6.8	4.97	1.0	4.62	3.2
RPH 9 H	16	52	G	G	5.69	32.7	3.49	14.8	5.30	1.1
RPH 10M	0	20	14	G	5.20	0.9	4.08	1.6	5.78	1.1

*G, An increase in growth of cells was noted.

exhibited towards HeLa and Vero cells ($P < 0.0001$) (Fig. 2a). Nevertheless, although, for example, isolates COL 19 and 24 and RPH 1 had a considerably greater capacity to invade CaCo-2 cells compared with both HeLa and Vero cells, conversely, isolates COL 6, 25 and 30 had a greater capacity to invade either HeLa or Vero cells compared with

CaCo-2 cells (Table 2). There was no significant correlation between source of an isolate and the level of adhesion or invasion in CaCo-2 cells (Fig. 2b). Tukey's pairwise comparisons indicated no significant differences between human, bovine or chicken isolates for adherence or invasion of any of the cell lines.

Fig. 1. Cytotoxic activity of *C. jejuni* isolates. (a) Values obtained from polymyxin extracts of individual isolates (Table 2) against CaCo-2, HeLa and Vero cells. (b) Values obtained against CaCo-2 cells for isolates from different sources. Point plots of individual values were separated by 'jitter' of 0.1.

AFLP fingerprinting

The *C. jejuni* isolates were all typeable by the AFLP protocol. The repeatability among independent duplicate profiles was tested ($n=34$) and the mean similarity (S) was 99.3 % (SD 1.2 % S). A dendrogram based on profiles is shown in Fig. 3. Five clusters (I–V, comprising 31 isolates) each representing two to eleven isolates with similar profiles were observed at the 90 % S level. The remaining seven isolates displayed clearly distinct profiles. Within the five clusters, eight groups of two to four isolates had identical profiles, as indicated by the vertical terminal lines in the dendrogram. COL 28, the ovine isolate, was in cluster III and RPH10, the milk isolate, had a distinct profile (data not shown).

A conservative evaluation of the dendrogram results showed that six pairs of isolates (COL 2 and 3; COL 4 and 5; COL 10 and 11; COL 12 and 14; COL 15 and 16; and COL 13 and RPH 6) were identical. Profile similarity between isolates suggested that the following five groups of isolates may be clonally related: (i) COL 2, 3, 13 and 22 and RPH 6; (ii) COL 7 and 29; (iii) COL 10–12 and 14 and RPH 4 and 5; (iv) COL 15, 16 and 30; and (v) COL 25–27.

Group IV contained only strains isolated from a human source and groups I and V contained isolates from only

Fig. 2. Adhesion (Adh.) and invasion (Inv.) of the *C. jejuni* isolates. (a) Values obtained for individual isolates (Table 2) are shown for CaCo-2, HeLa and Vero cells. (b) Values obtained for adhesion (left panel) and invasion (right panel) with CaCO-2 cells for isolates from different sources. Point plots of individual values were separated by 'jitter' of 0.025.

two sources, human and chicken (Fig. 4). Group II contained isolates from only human and bovine sources, whereas group III, which contained the reference strain NCTC 11168^T (=COL 7), contained isolates from all three sources, although they were mainly human isolates (Fig. 4). Although there was some clustering according to source, the numbers of isolates in some groups were small. Some isolates for which the typing had indicated identity were from the same source, COL 2 and 3 (bovine), COL 4 and 5 (chicken), COL 10 and 11 (human), COL 15 and 16 (human) and COL 25 and 26 (human), but two sets which had identical AFLP patterns (100 % similarity) were from different sources: COL 12 (chicken) and COL 14 (human) and COL 13 (bovine) and RPH 6 (human) (Table 1 and Fig. 3), implicating chicken and cattle as potential sources of sporadic human infection.

AFLP and concordance with other typing information

Penner serotype and 16S ribotype information were available for almost all the isolates (Table 1). The serotype and ribotype were the same for all pairs of identical isolates characterized by the AFLP protocol. Furthermore, ribotype

Fig. 3. AFLP groupings of the *C. jejuni* isolates (Siemer *et al.*, 2004). Clusters (indicated as e.g. 1#) were defined at 90% similarity. Asterisks indicate strains that were not grouped into clusters.

Fig. 4. *C. jejuni* isolates classified by source of isolation and AFLP groups. U, Unrelated. Point plots of individual values were separated by 'jitter' of 0.025 on both axes.

H1 was largely equivalent to clusters I, II and III, which comprised all serotype 1 and all but two of the serotype 2 strains which constituted cluster V (Fig. 3). The serotype and ribotype information also lends support to the suggested clonal relationships between isolates.

Virulence-associated properties within AFLP clusters

There was no significant correlation between possession of high haemolytic, cytotoxic or invasive activities of the isolates with particular genotypic clusters. A comparison of Minitab point plots with 'jitter' for the cytotoxicity and invasive properties of isolates showed that each AFLP group contained strains with a range of cytotoxic and invasive phenotypes against CaCo-2 cells (Fig. 5a, b). However, although only five isolates were grouped in AFLP group II, all exhibited low toxicity towards CaCo-2 cells (Fig. 5a), and four of these, COL 2, 3 and 13 and RPH 6, had both low cytotoxic and invasive capacities against CaCo-2 cells (Table 2).

DISCUSSION

In agreement with other reports (Pickett *et al.*, 1992; Hossain *et al.*, 1993; Misawa *et al.*, 1995a; Tay *et al.*, 1995), whole cells of several of the isolates examined here were weakly haemolytic. Parkhill *et al.* (2000) reported that the genome of NCTC 11168^T contained coding regions for putative contact-dependent haemolysins. The haemolysis assay used here was not designed specifically to detect contact haemolysis, and NCTC 11168^T (=COL 7) possessed little haemolytic activity under our assay conditions. For those strains exhibiting the highest haemolytic titres, COL 15, 16, 25 and 28 and RPH 5 and 9, it was not possible to attribute this activity clearly to cytotoxicity measured against CaCo-2, Vero or HeLa cells (Table 2). Thus, although four of the six isolates (COL 25

Fig. 5. Cytotoxic (a) and invasive (b) activities against CaCo-2 cells of *C. jejuni* isolates within AFLP groups. U, Unrelated. Point plots of individual values were separated by 'jitter' of 0.05.

and 28 and RPH 5 and 9) had quite marked cytotoxic activity against CaCo-2 cells, COL 15 and 16 did not and were only marginally more active against HeLa or Vero cells. Conversely, some isolates that possessed high cytotoxic activity against CaCo-2 cells, such as COL 1, 17, 29 and 30, had very low haemolytic titres. These data would thus suggest that the haemolytic and cytotoxic activities detected were due to separate factors.

A range of cytotoxic activities was exhibited by the strains, with some having no activity against any of the target cells. Of particular note was the very low cytotoxicity exhibited by the type strain NCTC 11168^T. This is at variance with a previous study (Coote & Arain, 1996), which demonstrated cytotoxicity of NCTC 11168^T against HeLa and Vero cells. The reason for this discrepancy is unclear, but the strain NCTC 11168^T was obtained from different sources in the two studies and the assays, although similar, differed in procedure. In this study, some strains showed activity against all three target cells, whereas there was obvious isolate variation with others in the ability to produce cytotoxic activity against the three target cell lines when they were grown and assayed under identical conditions. Thus, certain strains showed good activity against CaCo-2 cells and little or no activity against the other cell lines, while other isolates exhibited the opposite phenotype

(Table 2). These data suggest that the assays using the different cell lines may have detected more than one cytotoxin, as noted previously (Wassenaar, 1997; Pickett, 2000). Overall, the results corroborate previous reports where low-level cytotoxicity was reported against CHO (Chinese hamster ovary), HeLa, Vero, J774 (mouse macrophage-like) and Int 407 (human intestinal epithelial) cells, but variation in cytotoxic activity between *C. jejuni* strains was noted (Guerrant *et al.*, 1987; Pang *et al.*, 1987; Moore *et al.*, 1988; Misawa *et al.*, 1995b; Lee *et al.*, 2000; Nadeau *et al.*, 2003). This report extends these observations to include CaCo-2 cells, proposed as a more suitable target cell for *C. jejuni* (Everest *et al.*, 1992), and provides a more quantitative assay than those used in previous work, which, with the exception of Nadeau *et al.* (2003), who also used the dye-reduction assay described here, relied on cytopathic changes to cultured mammalian cells that required prolonged incubation periods and provided only semi-quantitative data. Statistical analysis showed that, as a group of 38 isolates, the source showed no statistical significance with regard to cytotoxic capacity. For example, within the human isolates, cytotoxic activities varied between 0 and 77 % for CaCo-2, 0 and 33 % for Vero and 0 and 66 % for HeLa cells. A similar range of activities was found with bovine and chicken isolates. Other workers noted no significant difference in the extent of cytotoxin activity between human and animal isolates (Misawa *et al.*, 1995b; Nadeau *et al.*, 2003), although Lee *et al.* (2000), in a study of ten strains from human or chicken sources, found cytotoxin producers to be mainly human isolates.

It is unlikely that the cytotoxic activity described here can be attributed to cytolethal distending toxin (CDT). This toxin has been shown to interfere with cell-cycle progression in certain mammalian cells, including HeLa, CHO and CaCo-2 cells, by promoting DNA damage, but cell death occurs after more than 48 h exposure to the toxin (Pickett, 2000; Lara-Tejero & Galan, 2002). A CDT-deficient mutant of *C. jejuni* 81-176 still elicited cytopathic effects on HeLa, Vero and CaCo-2 cells (Purdy *et al.*, 2000; A. Mohammadzadeh, R. Parton and J. G. Coote, unpublished observations). Purdy *et al.* (2000) also reported that a CDT-deficient mutant of *C. jejuni* NCTC 11168^T did not have any residual cytotoxic activity against HeLa or Int 407 cells, which is in agreement with results reported here, where the type strain NCTC 11168^T (=COL 7) exhibited little cytotoxic activity against HeLa and CaCo-2 cells. In addition, the low activity shown by strain NCTC 11168^T (=COL 7) against Vero cells (Table 2) was not reduced further by mutants deficient in the putative haemolysin *tlyA* (Cj05888) or phospholipase *pldA* (Cj0183) (A. Mohammadzadeh, R. Parton and J. G. Coote, unpublished observations; mutants kindly supplied by P. Everest, Veterinary College, Glasgow University).

As with cytotoxic activity, a wide variation between individual isolates was obtained for both adherence and invasion with all cell lines (Table 2). Overall, the strains showed greater adherence and significantly greater invasion

capacities with human colonic CaCo-2 cells than with human epithelial HeLa or monkey kidney Vero cells. Thus, although the relevance of *in vitro* studies to the behaviour of bacteria *in vivo* is open to question, this demonstrates the importance of an *in vitro* cell culture model for epithelial cell adhesion and invasion that resembles more closely the cell type that campylobacters interact with *in vivo*. Previous work using CaCo-2 cells indicated that more invasive strains may correspond to those that cause colitis and more severe disease symptoms (Everest *et al.*, 1992). Studies with infant rhesus monkeys showed that the primary mechanism of colitis by strain 81-176 was associated with invasion of the bacteria into the absorptive epithelial cells in the colon (Russell *et al.*, 1993).

Cytotoxicity, adherence and invasion may be responsible, individually or in combination, for the manifestations of *C. jejuni*-induced enteritis, including diarrhoea and inflammation. In some cases, a correlation between cytotoxic activity and the capacity to invade cells was evident. Thus, COL 14, 23, 26, 27 and 30 and RPH 1, 3 and 9 showed high cytotoxic and invasive activities against CaCo-2 cells (Table 2). All these COL isolates and RPH 9 were from human cases of diarrhoea or enteritis, but RPH 1 and 3 were poultry isolates. With other strains, for example COL 1, 12, 13, 17, 18, 21 and 29 and RPH 2 and 4–8, high cytotoxic activity was accompanied by a low invasive capacity for CaCo-2 cells. These strains were from human, chicken and bovine sources. For COL 19 and 20 (from human cases of diarrhoea), the converse was true, these strains exhibiting high invasive capacity and no detectable cytotoxicity for CaCo-2 cells (Table 2). For our isolates as a whole, there was no clear relationship between source of isolation or disease manifestation and possession of statistically significantly higher levels of particular virulence-associated factors assayed in these studies (Table 2, Figs 1b and 2b).

Whole-genome AFLP detection is an effective tool for identifying genetic relatedness of *C. jejuni* isolates. Profiles clustering at similarity values >90 % have been shown to identify groups of strains with epidemiological or clonal significance (On & Harrington, 2000; Siemer *et al.*, 2004). Five groups of strains were identified that clustered together at or just below this similarity value (Fig. 3). When the source of the isolates was compared with the AFLP clusters, it was clear that each group had *C. jejuni* isolates from more than one source, with the exception of group IV, which contained only human isolates (Fig. 4). The placement of strains in the AFLP groups may provide an indication of shared characteristics. Other studies have shown genetic relatedness between individual isolates from a single source, either human strains or strains from non-human reservoirs (On *et al.*, 1998; Duim *et al.*, 1999; Fitzgerald *et al.*, 2001; Dingle *et al.*, 2002; Schouls *et al.*, 2003; Siemer *et al.*, 2004). Our study also showed that four of the five groups contained strains of the same serotype (groups I and II, serotype 1; group IV, serotype 11; and group V, serotype 2). Group III contained isolates of serotypes 1 and 2. This is similar to previous reports, where

several AFLP clusters contained strains assigned to a single serotype (Desai *et al.*, 2001; Siemer *et al.*, 2004). The data confirm genetic heterogeneity of *C. jejuni* and support the existence of distinct groups or clusters, some of which contain isolates with a common serotype.

The relationship of the AFLP groups to the expression of virulence-associated factors can also be assessed. It was reported previously that highly CHO cell-cytotoxic isolates were distributed in two clusters identified by PFGE (Nadeau *et al.*, 2003), but our data supported this to only a limited extent, as isolates having high cytotoxic activity against CaCo-2 cells were present in groups I, III, IV and a group of unrelated strains (U) identified by AFLP analysis (Fig. 5a). Group II isolates had uniformly low cytotoxicity. Similarly, in a recent report (On *et al.*, 2006), CDT and haemolysin activities were not significantly different between two clusters of strains differentiated on the basis of whole-genome microarray analysis of interstrain gene polymorphism. However, the authors did detect a significant difference between the clusters with regard to survival under aerobic conditions, a phenotype which was not tested here. Assessment of the capacity of groups to invade CaCo-2 cells indicated that isolates in groups I, V and U had a noticeably greater capacity overall to invade the cells than isolates in groups II, III and IV (Fig. 5b). These differences in cytotoxic activity or invasive capacity did not, however, relate in any obvious way to source of isolation except that group IV, which contained isolates with higher cytotoxic and invasive capacities, contained isolates from human sources only. To some extent, these data support other reported work, where the potential for invasion was significantly higher for human than for poultry isolates (Nadeau *et al.*, 2003). The phenotypic data, together with the genotypic AFLP analysis, emphasize the heterogeneity of *C. jejuni* isolates, and our data overall do not support the idea that clonally related isolates have common *in vitro* virulence characteristics. This is in keeping with other work that has shown genetic plasticity by whole-genome DNA comparisons (Wassenaar *et al.*, 1998; Dorrell *et al.*, 2001; Suerbaum *et al.*, 2001; de Boer *et al.*, 2002; Leonard *et al.*, 2003; On *et al.*, 2006) and single-nucleotide polymorphisms that arose between clonal variants of the type strain NCTC 11168^T that resulted in differences in gene expression and the capacity to colonize chicks (Gaynor *et al.*, 2004). There is a requirement to examine strains by *in vivo* methods to obtain a clearer picture of *C. jejuni* virulence.

ACKNOWLEDGEMENTS

D. E. S. S.-T. and J. G. C. wish to thank the UK Department of Health for a contract grant to support part of this work.

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