Virulence spectra of typed strains of *Campylobacter jejuni* from different sources: a blinded *in vivo* study

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Campylobacter jejuni is a major cause of human diarrhoeal disease, but specific virulence mechanisms have not been well defined. The aims of the present blinded study were to measure and compare the *in vivo* properties of 40 serotyped, biotyped and genotyped *C. jejuni* isolates from different sources and genetic makeup. An 11-day-old chick embryo lethality assay, which measured embryo deaths and total viable bacteria over 72 h following inoculation of bacteria into the chorioallantoic membrane, revealed a spectrum of activity within the *C. jejuni* strains. Human and chicken isolates showed similar high virulence values for embryo deaths while the virulence of the bovine isolates was less pronounced. A one-way ANOVA comparison between the capacity of the strains to kill the chick embryos after 24 h with cytotoxicity towards cultured CaCo-2 cells was significant ($P=0.025$). After inoculation with a *Campylobacter* strain, mouse ligated ileal loops were examined histologically and revealed degrees of villous atrophy, abnormal mucosa, dilation of the lumen, congestion and blood in lumen, depending on the isolate examined. A 'total pathology score', derived for each *C. jejuni* strain after grading the pathology features for degree of severity, showed no apparent relationship with the source of isolation. Some relationship was found between amplified fragment length polymorphism groups and total ileal loop pathology scores, and a one-way ANOVA comparison of the mouse pathology scores against total chick embryo deaths after 72 h was significant ($P=0.049$).

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INTRODUCTION

Campylobacter jejuni is a major cause of human gastrointestinal illness. The mechanisms by which *C. jejuni* causes disease are not fully understood, but host inflammatory responses would seem to be important. Intestinal cells, in response to the presence of *C. jejuni*, release pro-inflammatory cytokines which recruit neutrophils and macrophages to the site of infection (Hickey *et al.*, 1999, 2000; Bakhiet *et al.*, 2004; Hu & Hickey, 2005; MacCallum *et al.*, 2006). Disruption of electrolyte and fluid absorption functions (MacCallum *et al.*, 2005) is likely to contribute to inflammation and diarrhoea and allow influx of neutrophils. Inflammatory responses with infiltration of neutrophils together, in some instances, with the presence of

intracellular organisms within the intestinal mucosa after invasion of colonic epithelium were features of microscopic histology of *C. jejuni* enterocolitis in humans (Butzler & Skirrow, 1979; van Spreeuwel *et al.*, 1985; Black *et al.*, 1992) and in animal models (Newell, 2001). The severity of these symptoms was reported to be strain-related (Black *et al.*, 1992; Walker *et al.*, 1992; Everest *et al.*, 1993).

Laboratory *in vivo* challenge models for *C. jejuni* have been proposed for many animal species, as reviewed by Newell (1984, 2001), but there is no satisfactory model that mimics human disease (Newell, 2001). An adequate mouse model has yet to be established as *C. jejuni* colonizes the mouse gastrointestinal tract after oral administration but does not cause subsequent gastroenteritis (Blaser *et al.*, 1983; Newell, 2001). Everest *et al.* (1993) used the ileal loop assay in rabbits and recorded acute inflammatory responses after 18 h exposure to *C. jejuni*, but the rabbit test would be expensive to adopt as a routine test for pathogenicity. The chick embryo assay was adopted by Field *et al.* (1986) for

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Abbreviation: AFLP, amplified fragment length polymorphism.

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use with *C. jejuni*. The organisms were inoculated onto the chorioallantoic membrane of 11-day-old chick embryos and it was reported that strains varied in their ability to kill the embryos. Previously, Coote *et al.* (2007), in a blinded study, compared *C. jejuni* strains from human, bovine and chicken sources with a range of *in vitro* assays. The aim of the present blinded study was to compare the *in vivo* virulence-associated properties of the same group of *C. jejuni* strains.

METHODS

Bacterial strains and growth conditions. Thirty coded *C. jejuni* isolates were provided by Dr R. Owen, Health Protection Agency, Colindale, London, UK, and designated COL 1–30. COL 7 was later identified as strain NCTC 11168. Ten coded *C. jejuni* isolates were received from Dr F. Bolton, Public Health Laboratory Service, Royal Preston Hospital, UK, and designated RPH 1–10. The serotype, ribotype, amplified fragment length polymorphism (AFLP) group and source of isolation of the strains were reported previously (Coote *et al.*, 2007) (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). For growth in liquid medium, *C. jejuni* growth on agar medium was inoculated into 100 ml Brucella broth and incubated in an anaerobic jar, under the same microaerophilic atmosphere, at 42 °C in an orbital shaker at 100 r.p.m.

Eleven-day-old chick embryo lethality assay. This was based on the procedure of Field *et al.* (1986). Hens' eggs containing 11-day-old embryos (kindly provided by Marshalls Food Products, Edinburgh, UK) were incubated in a rotary incubator at 37 °C in a humid atmosphere for 24 h to allow the eggs to adapt before inoculation. The *C. jejuni* strains, grown in Brucella broth with supplements, were harvested by centrifugation at 5800 g for 10 min, washed and resuspended in sterile PBS to an OD₆₀₀ of 0.2, a cell density of $\sim 1 \times 10^9$ ml⁻¹. Each of five eggs was inoculated with 100 µl bacterial suspension containing a high dose (1×10^6 – 1×10^7 c.f.u.) or low dose (1×10^3 – 1×10^4 c.f.u.) of *C. jejuni*, or 100 µl PBS alone. A small hole at the air sac and another above the chorioallantoic membrane were formed with a fine dental drill. Air was released from the air sac with a suction bulb to drop the chorioallantoic membrane so that the inoculum could be injected through the second drill hole. Both holes were sealed with sterile adhesive tape and the eggs were candled twice a day. Each strain was graded on a scale of 1–5 dependent on the number of embryo deaths caused by the high (H) and low (L) doses of bacterial inoculum over a 72 h period. The number of deaths caused by each strain in the 10 inoculated eggs was graded from 5.0 for 5H deaths and 5L deaths to the lower grade of 0.5 for 1H death and 1 or 0L deaths. Dead embryos were removed at 24, 48 and 72 h into 9.0 ml sterile water and completely emulsified in a sterile Silverson mixer. Embryos that survived for 3 days were killed at 4 °C for 2 h and emulsified. Viable counts from the embryos were determined by plating dilutions of the homogenized embryo onto blood agar followed by incubation at 42 °C under microaerophilic conditions, as described above. Each lethality assay was done twice.

Ligated ileal loop assays in mice. Adult mice were anaesthetized with halothane M & B (2-bromo-2-chloro-1,1,1-trifluoroethane; RMB Animal Health). A laparotomy was carried out and three intestinal loops were prepared with small interloops, to prevent contamination from one loop to another, by the procedure described

by Stewart-Tull *et al.* (2004). Each *Campylobacter* strain was inoculated in 0.1 ml PBS containing 1×10^7 – 1×10^8 c.f.u. of an overnight culture into each of two mice, into two ligated loops proximal in one mouse and distal in the other to the ileo-caecal junction; the third distal loop was inoculated with 0.1 ml sterile PBS. Each animal was injected with 0.05 ml 0.05% (w/v) Valium (diazepam injection BP 20 mg per 4.0 ml ampoule; Roche) after the operation. After 18 h, the mice were euthanased with halothane and gross changes in the loops, such as fluid accumulation, were recorded before the infected loops were placed in 10% (v/v) formalin for histological examination. After fixation and embedding, 10 µm thickness cross-sections of the loops were cut and stained with haematoxylin and eosin. Three transverse sections were examined from each loop and histological changes were recorded.

Statistical analyses. Statistical analyses were done with Minitab 12 according to the procedures of Wardlaw (2000). Statistical significance was defined as $P < 0.05$.

RESULTS AND DISCUSSION

The 40 *C. jejuni* strains had previously been used in a blinded study to characterize their properties in a series of *in vitro* assays (Coote *et al.*, 2007). Their serotype, ribotype and AFLP group, haemolytic activity, cytotoxicity and adhesion and invasion activity against CaCo-2, HeLa and Vero cells were established. Whole-genome AFLP detection is an effective tool for identifying genetic relatedness of *C. jejuni* isolates. Profiles clustering at similarity values >90% identified groups of strains with epidemiological or clonal significance (On & Harrington, 2000; Siemer *et al.*, 2004). Five AFLP clusters, each representing 2–11 isolates with similar profiles, were observed at the 90% similarity level (Coote *et al.*, 2007). The results of the *in vitro* study emphasized the heterogeneity of the isolates. There was no clear relationship between source of isolation or disease manifestation and possession of statistically significant higher levels of particular virulence-associated factors, although in some cases, a correlation between cytotoxicity and cell invasion was evident. Isolates with high cytotoxic activity against CaCo-2 cells were confined to AFLP groups I, III, 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 present blinded study was undertaken to compare the behaviour of the strains in different *in vivo* models of infection and to determine whether a similar spectrum of virulence-associated properties was apparent. In the absence of a suitable animal model which mimics human disease, a mouse toxicity test, a chick embryo lethality assay and an ileal loop assay in mice were studied for comparison.

In a preliminary study, a mouse toxicity test was undertaken whereby 7-day-old CD-1 mice were inoculated intraperitoneally with $\sim 1 \times 10^8$ c.f.u. ml⁻¹ of an individual *Campylobacter* strain and weight loss was checked three times daily. As the majority of mice failed to respond to the *C. jejuni* challenge, the model was not considered suitable for discriminating between the *C. jejuni* strains, and

Table 1. Features of *C. jejuni* isolates and data from the chick embryo lethality assay

For the chick embryo assay, low-dose ($1 \times 10^3 - 1 \times 10^4$ c.f.u.) or high-dose ($1 \times 10^6 - 1 \times 10^7$ c.f.u.) inocula of *C. jejuni* strains were used. 'Total dead embryos' refers to the number of dead embryos from five eggs during the 72 h incubation period. Viable counts recovered from each set of five embryos are expressed as mean $\log_{10}$ c.f.u. 'Grade' refers to an arbitrary scale indicating the degree of severity of infection derived from the number of embryo deaths recorded.

Strain code no.	Place of isolation	Host	HS type*	HaeIII 16S rRNA ribotype†	AFLP group‡	Chick embryo assay				
						Total dead embryos		Mean log ₁₀ c.f.u.		Grade
						Low dose	High dose	Low dose	High dose	
COL 1	UK, Ashford PHL	Human (61 years, male) diarrhoea	1	H1	I	5	5	9.42	7.69	5.0
COL 2	UK, Lancaster Univ.	Bovine carcass, abattoir	1	H1	II	0	5	5.92	8.96	4.5
COL 3	UK, Lancaster Univ.	Bovine carcass, abattoir	1	H1	II	2	5	7.34	7.89	4.5
COL 4	UK, Exeter PHL	Chicken caecum	1	H1	I	4	3	6.44	9.20	4.0
COL 5	UK, Exeter PHL	Chicken carcass, skin	1	H1	I	2	5	4.06	7.47	4.5
COL 6	UK, Sunderland R.I.	Human diarrhoea	1	H1	III	3	3	6.59	5.94	3.0
COL 7	UK, Worcester Hosp.	Human faeces (?)	1	H1	III	5	5	7.20	5.79	5.0
COL 8	UK, Lancaster Hosp.	Human faeces	1	H1	I	2	2	4.01	5.76	1.5
COL 9	S. Africa Hosp.	Human faeces	1	H1	I	3	3	5.15	5.32	3.0
COL 10	UK, Ashford PHL	Human (female) persistent diarrhoea	1	H1	I	4	4	6.79	6.86	4.0
COL 11	UK, Ashford PHL	Human (79 years, female) severe anaemia	1	H1	I	2	5	6.30	6.22	4.5
COL 12	UK, Exeter PHL	Chicken caecum	1	H1	I	4	4	7.42	8.92	4.0
COL 13	UK, Lancaster Univ.	Bovine, farm site	1	H1	II	0	3	5.98	9.42	2.5
COL 14	UK, Guys Hospital	Human faeces, diarrhoea	1	H1	I	3	5	6.08	4.75	5.0
COL 15	UK, Cardiff PHL	Human bloody diarrhoea	2	H1	III	5	4	7.12	6.07	4.0
COL 16	UK, Cardiff PHL	Human (31 years, female), no details	2	H1	III	2	4	6.57	7.93	3.5
COL 17	UK, Cardiff PHL	Human (4 years, female), no details	4	H1	U	5	5	6.86	6.95	5.0
COL 18	UK, Cardiff PHL	Human (22 years, male) diarrhoea	2	H1	III	5	3	5.60	4.87	5.0
COL 19	UK, Harrogate NHS	Human diarrhoea	41	No data	U	5	5	5.28	7.84	5.0
COL 20	UK, Ashford PHL	Human (54 years, male) diarrhoea	15	H6	U	5	5	7.00	7.50	5.0
COL 21	UK, C Vet Lab.	Bovine (12 weeks) faeces	1	H1	III	2	3	4.22	4.94	2.5
COL 22	UK, Cardiff	Human (2 years, male) diarrhoea	NT	H1	II	3	1	5.06	6.81	1.0
COL 23	UK, Cardiff	Human (36 years, female) diarrhoea	53	H4	U	3	4	8.54	6.77	4.0
COL 24	UK, Cardiff	Human (30 years, male) diarrhoea	53	H4	U	1	1	6.45	5.38	0.5
COL 25	UK, Cardiff	Human (18 years, female) D & V	11	H15	IV	3	3	5.38	5.74	3.0
COL 26	UK, Cardiff	Human (22 years, female) diarrhoea	11	H15	IV	1	3	5.80	6.59	2.5
COL 27	UK, Harrogate	Human (60 years, male) diarrhoea	11	No data	IV	1	3	5.92	5.75	2.5
COL 28	Lancaster	Ovine	2	H1	–	3	5	6.85	6.22	5.0
COL 29	UK, Compton	Bovine faeces	2	H1	III	2	5	5.84	7.38	4.5
COL 30	UK, Lincoln	Human (2 years) diarrhoea	2	H1	III	1	5	5.98	6.85	4.5
RPH 1	UK	Poultry meat	2	H5	V	2	4	5.32	7.56	3.5
RPH 2	UK	Poultry meat	2	H25	V	4	3	9.35	7.84	3.0
RPH 3	UK	Poultry meat	4	H5	U	3	4	4.98	7.53	4.0

Table 1. cont.

Strain code no.	Place of isolation	Host	HS type*	HaeIII 16S rRNA ribotype†	AFLP group‡	Chick embryo assay			
						Total dead embryos		Mean log ₁₀ c.f.u.	
						Low dose	High dose	Low dose	High dose
RPH 4	UK	Poultry meat	1	H1	I	5	2	5.36	6.34
RPH 5	UK	Human enteritis	1	H1	I	4	2	4.29	8.24
RPH 6	UK	Human enteritis	1	H1	II	2	4	6.93	6.36
RPH 7	UK	Poultry meat	2	H1	III	3	3	5.94	6.58
RPH 8	UK	Human enteritis	2	H1	III	1	5	3.79	4.12
RPH 9	UK	Human enteritis	4	H4	U	1	4	3.24	7.01
RPH 10	UK	Milk	23	H10	–	4	5	4.40	5.47

*HS, Penner heat-stable serotypes (determined according to Owen *et al.*, 1995). NT, Not typed.

†16S rRNA ribotypes (determined according to Fitzgerald *et al.*, 1996) are indicated by the prefix 'H'.

‡AFLP group refers to AFLP fingerprint profiles with similarity values >90% (Coote *et al.*, 2007).

consequently, after a small trial, the test was removed from the study.

Chick embryo lethality assay

The number of dead embryos was recorded daily within the 3-day test after inoculation of eggs with the high-dose and low-dose inocula. The *C. jejuni* strains exhibited a range of lethality grades from 0.5 to 5.0 (Table 1). None of the embryos died in the control eggs inoculated with PBS alone. A comparison of the grades for the chick embryo deaths with the source of isolation of the *C. jejuni* strains indicated that human and chicken isolates displayed essentially similar levels of virulence while bovine isolates appeared to be less virulent. When total dead embryos were compared with the source of isolation of the *C. jejuni* strains, little discrimination was apparent with the high-dose inocula, but the low-dose inocula again indicated similar high virulence values for the human and chicken isolates while those for the bovine isolates were less pronounced (see Supplementary Fig. S1 in JMM Online). However, only five bovine isolates were represented and more strains would need to be examined to confirm this relationship. A comparison of grades of chick embryo deaths with AFLP groups showed that each AFLP group contained strains with a range of grades, but, although AFLP group IV had a majority of strains with lower grades compared to the other groups, this was not statistically significant. This group contained three clonally related human isolates that possessed moderate cytotoxic and invasive properties (Coote *et al.*, 2007).

The mean viable counts at 72 h obtained from the five embryos from the low and high inocula were also recorded (Table 1). The 10 egg embryo observations, taken together for each *C. jejuni* strain, indicated that there was a significant difference among all of the strains, $P < 0.001$, when analysed by one-way analysis of variance test (ANOVA). Significant differences were found between the recovery of *C. jejuni* organisms from dead embryos given the low and high inocula, ANOVA $P < 0.0001$. However, the one-way ANOVA was not significant for c.f.u. recovered from dead chick embryos compared to the source of each strain or its AFLP group. A comparison of embryo deaths to the final c.f.u. recovered from harvested embryos showed that the c.f.u. values were significantly affected by the 'death day' ($P < 0.0001$), i.e. death at 24, 48 or 72 h, as there was a steady increase in c.f.u. as the days of the test advanced. It was clear that with the low-dose inoculum, *in vivo* growth of the strain was necessary to cause death of embryos before 72 h. Eggs inoculated with the high dose of virulent strains caused embryo deaths at 24 and 48 h with the remainder dead by 72 h. Where the embryos died within 24 h, there was generally little increase in the viable count per embryo compared to that of the original inoculum and there was a significant correlation ($P = 0.025$; Supplementary Fig. S2) between the capacity of strains to cause embryo death at 24 h and the cytotoxicity

Fig. 1. Histological appearance of representative cross-sections of ligated ileal loops from mice. (A, B) Control intestine injected with PBS (magnification $\times 4$ for A and $\times 20$ for B). (C–H) Test loops injected with *C. jejuni* strains as follows: C, COL 25 $\times 4$, dilation of lumen and atrophy of villi grade 0.5; D, RPH 1 $\times 20$, ibid. grade 3.0; E, COL 11 $\times 20$, villous atrophy grade 3.0 and proteinaceous exudates in lumen; F, COL 7 $\times 4$, villous atrophy grade 3.0 and blood in lumen; G, COL 12 $\times 20$, polymorphs and necrosis of villous tip; H, RPH 3 $\times 4$, necrosis of the mucosa with blood in the lumen. Mouse sections: (a) lumen; (b) mucosa; (c) muscularis; (d) villus; (e) crypt; (f) villous atrophy; (g) proteinaceous exudates in lumen; (h) blood in lumen; (i) polymorphs; (j) necrosis of villous tip.

Table 2. Histological pathologies of transverse sections of mouse ileal loops inoculated with the *C. jejuni* strains

Each effect was graded for severity from 1.0 to 3.0 and the sum of the grades created a 'total pathology score' for each strain.

Strain*	Source†	AFLP group	Haemorrhage in the loop	Dilated lumen	Villous stunting or total atrophy	Submucosal oedema	Oedema of lamina propria	Congestion of mucosa	Polymorphs in lumen and mucosal epithelium	Blood in lumen	Necrosis of villi	Necrosis of mucosa and haemorrhage	Total Path Score
COL 1	H	I	1	2	2	2	1	3	—	—	—	—	11.0
COL 2	B	II	—	2	1.5	2	1	—	—	—	—	—	6.5
COL 3	B	II	2	2	2	—	—	3	3	—	—	—	12.0
COL 4	C	I	—	2	2	—	—	—	—	—	—	—	4.0
COL 5	C	I	—	1	1	—	—	—	3	—	—	—	5.0
COL 6	H	III	—	2	2	—	—	—	—	—	—	—	4.0
COL 7	H	III	—	2	3	2	2	—	—	3	—	—	12.0
COL 8	H	I	1	2	1	—	—	3	3	3	—	—	13.0
COL 9	H	I	1	3	2	—	—	3	3	—	3	—	15.0
COL 10	H	I	1	2	2	2	2	3	—	3	—	—	15.0
COL 11	H	I	0.5	2	3	—	—	—	—	—	—	—	5.5
COL 12	H	I	2	1	1	—	—	—	3	3	3	—	13.0
COL 13	C	II	—	2	2	2	2	—	3	3	—	3	17.0
COL 14	B	I	1	2	2	—	2	3	3	3	—	3	19.0
COL 15	H	III	1.5	2	2	—	—	—	—	—	—	3	8.5
COL 16	H	III	—	2	2	—	—	—	—	3	3	—	10.0
COL 17	H	U	—	1.5	2	—	1	3	—	—	—	3	10.5
COL 18	H	III	1	1.5	2	—	1	—	—	3	—	3	11.5
COL 19	H	U	1	2	1	1	1	—	3	—	—	—	9.0
COL 20	H	U	1	2	1	—	1	—	—	3	—	—	8.0
COL 21	B	III	—	1	1	—	—	—	—	—	—	—	2.0
COL 22	H	II	1	2.5	2.5	—	—	—	—	—	—	—	6.0
COL 23	H	U	—	—	—	—	—	—	—	—	—	—	0
COL 24	H	U	—	—	—	—	—	—	—	—	—	—	0
COL 25	H	IV	0.5	0.5	0.5	—	—	—	—	3	—	—	4.5
COL 26	H	IV	—	2	2	—	2	3	—	3	3	3	18.0
COL 27	H	IV	1	2	2	—	—	—	3	—	3	—	11.0
COL 28	O	III	1	—	—	—	—	—	—	—	—	—	1.0
COL 29	B	III	1	1	1	—	—	—	—	—	—	—	3.0
COL 30	H	III	0.5	2	1	—	—	—	—	—	—	—	3.5
RPH 1	C	V	3	3	3	—	—	—	3	—	3	—	15.0
RPH 2	C	V	1	2	1	—	2	—	3	—	3	—	12.0
RPH 3	C	U	2	2	2	—	0.5	—	—	3	—	—	9.5
RPH 4	C	I	—	2	1	0.5	0.5	—	3	—	—	—	7.0
RPH 5	H	I	3	3	3	—	—	—	—	—	—	—	9.0
RPH 6	H	II	2	1	1	—	—	3	—	—	—	3	10.0
RPH 7	C	III	—	—	—	—	—	—	—	—	—	—	0
RPH 8	H	III	—	1	—	—	—	—	—	—	—	3	4.0
RPH 9	H	U	1	—	—	—	—	—	—	—	3	—	4.0
RPH 10	M	U	2	—	—	—	—	—	—	—	—	—	2.0
Controls			1/25	3/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	0/25	

*COL, Colindale; RPH, Royal Preston Hospital.

†H, Human; B, bovine; C, chicken; M, milk.

of strains against CaCo-2 cells cultured *in vitro* (Coote *et al.*, 2007).

Histological examination of mouse ileal loop tissues after exposure to *C. jejuni* strains

The quality of the pathology in the sectioned loops was reproducible and the histological examination of the control loops inoculated with PBS revealed a normal mucosa (Fig. 1A, B). Villi were tall and slender, and approximately twice the width of the crypts, and the simple columnar epithelium with goblet cells and Paneth cells was easily defined. There were a few mononuclear cells scattered throughout the lamina propria, but in some sections clusters of polymorphs were scattered on the serosal surface. The latter finding was not unexpected in view of the recent surgical intervention. The histological findings were consistent in all three sites examined in the control loops.

Histology revealed a spectrum of activity within the *C. jejuni* strains. Two basic lesions were observed in the majority of loops inoculated with a *Campylobacter* strain, namely, dilation of the lumen and atrophy of the villi. The affected dilated loops became thin-walled due to excess fluid accumulation that caused the mucosa to become stretched. The extent of this effect was graded from 1.0 to 3.0 for severity (Table 2). Examples of the grades are illustrated in Fig. 1(C, D). The fluid pressure affected the villi, which were shortened in relation to the crypts, graded 1.0–2.0, or, where there was total villous atrophy, graded 3.0 (Table 2 and Fig. 1E). Nine of the loops were congested and in 12 there was free blood in the lumen. Each of these conditions was given a pathology grade of 3.0 and for some strains both changes were recorded in the histological sections (Table 2). In general, these observations were associated with the concomitant dilation of the lumen or villous atrophy (Fig. 1E, F). There was no evidence of a vasculitis or even congestion in the lamina propria. Polymorphonuclear leukocytes were free in the lumen of 12 cases, and in five of these there was necrosis of the tips of villi (Table 2) (Fig. 1G). This latter change was also recorded in a further three cases in which numbers of polymorphs were not found in the lumen. Oedema of the mucosa or the lamina of the villi was recorded in 14 loops. In other ileal loop sections, there was total collapse and necrosis of the mucosa with blood in the lumen (Fig. 1F, H). Lymphocytolysis was noted in Peyer's patches in some inoculated loops, although the lymphoid area was not consistently found in all sections. No evidence of intracellular bacteria was seen, but bacteria were noted in the centre of the loops in many cases, including controls. Further tests would be required to confirm that these were *Campylobacter* organisms. The pathology findings in the mouse ileal loop sections were graded from 1 to 3 and addition of these grades created a total pathology score for each of the *C. jejuni* strains (Table 2).

A one-way ANOVA analysis of mouse total pathology scores against source of the strains or against previously reported CaCo-2 invasion and cytotoxicity data (Coote *et al.*, 2007) yielded *P*-values of 0.094 and 0.075, respectively. A comparison of the pathology scores with AFLP groups (Coote *et al.*, 2007) recorded high pathology scores for AFLP groups I, II IV and V and low scores for groups III and U, the untypable group (Supplementary Fig. S3). In our previous study (Coote *et al.*, 2007), isolates from groups I, III, IV and U were generally more cytotoxic against CaCo-2 cells, but isolates in groups I, V and U were more invasive for these cells. In addition, although AFLP group IV isolates induced lower chick embryo deaths than other groups, this was not reflected by the mouse pathology scores. Significantly, a comparison of total mouse pathology scores with total chick embryo deaths gave an ANOVA value of *P*=0.049. The single strain, RPH 10, isolated from milk and the single ovine strain, COL 28, had high grades in the embryo egg assay (Table 1) but low pathology scores (Table 2). Further investigation of strains from these sources and from other species, e.g. cats and dogs, is warranted.

Conclusions

Our data showed that the *in vivo* chick embryo assay and mouse ileal loop assay pathology scores discriminated between the various *C. jejuni* strains. Although there was no clear relationship between the ileal loop pathology scores and the source of the *C. jejuni* strains or the *in vitro* cytotoxicity and invasion data reported previously (Coote *et al.*, 2007), a relationship between AFLP groups and pathology scores was apparent, where members of group III and a group of unrelated strains (U) exhibited lower scores than members of groups I, II, IV and V. Of note, however, was a significant correlation (ANOVA value *P*=0.025) between the capacity of strains to cause chick embryo death at 24 h and *in vitro* cytotoxicity effects on CaCo-2 cells, and a significant relationship (ANOVA value *P*=0.049) between total chick embryo deaths and total pathology scores. This might suggest that the chick embryo assay could be used to advantage as a first-line *in vivo* experimental approach to assess the virulence capacity of *C. jejuni* strains. As such, it would offer an alternative to the ethical and technical difficulties involved in the application of whole animal or ileal loop assays. Fertile eggs are relatively cheap, easy to handle and can be used in statistically significant numbers. It could be a useful adjunct to other *in vivo* procedures that are more technically difficult, especially for high throughput screening of multiple isolates.

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