Galleria mellonella as an infection model for *Campylobacter jejuni* virulence

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Larvae of *Galleria mellonella* (Greater Wax Moth) have been shown to be susceptible to *Campylobacter jejuni* infection and our study characterizes this infection model. Following infection with *C. jejuni* human isolates, bacteria were visible in the haemocoel and gut of challenged larvae, and there was extensive damage to the gut. Bacteria were found in the extracellular and cell-associated fraction in the haemocoel, and it was shown that *C. jejuni* can survive in insect cells. Finally, we have used the model to screen a further 67 *C. jejuni* isolates belonging to different MLST types. Isolates belonging to ST257 were the most virulent in the *Galleria* model, whereas those belonging to ST21 were the least virulent.

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INTRODUCTION

Campylobacter jejuni is recognized as the leading cause of bacterial gastroenteritis across the developed world; the World Health Organization estimates that 1% of the population of Western Europe is infected with campylobacters each year. It is thought that for each reported case, a further nine go unreported (Humphrey *et al.*, 2007), thus, based on the reported figures for 2009 from the Health Protection Agency, this would mean that there were in excess of 500 000 cases in England and Wales alone. Furthermore, beyond the initial diarrhoeal disease, *C. jejuni* may also cause post-infection complications including irritable bowel syndrome, meningitis and Guillain–Barré syndrome plus its variant Miller Fisher syndrome (Janssen *et al.*, 2008; van Doorn *et al.*, 2008).

Despite having been first identified as a causative agent of diarrhoea in 1977 (Skirrow, 1977), *C. jejuni* pathobiology remains poorly understood, with its core virulence determinants remaining elusive. A major contributing factor in the lack of determination of these factors has been the absence of a suitable infection model for *C. jejuni*. Previous models have included a ferret diarrhoeal model (Fox *et al.*, 1987), a chick colonization model (Wassenaar *et al.*, 1993) and a colostrum-deprived piglet model (Babakhani *et al.*, 1993). However, in common with other mammalian and

avian models, their widespread use has been limited by factors such as cost, ease of use, reproducibility and ethics (Newell, 2001).

We recently reported that larvae of the lepidopteran insect *Galleria mellonella* (Greater Wax Moth) are susceptible to infection by *C. jejuni* and can be used to screen for virulence genes (Champion *et al.*, 2010). In this study, we aimed to characterize *G. mellonella* as a *C. jejuni* infection model and to screen a panel of multilocus sequence typed (MLST) *C. jejuni* field isolates for virulence in *G. mellonella*. Different MLST groups were chosen to cover types detected in the main food-producing animals, the environment and clinical disease.

METHODS

Strains and cultures. All bacterial strains and mutants used in this study are shown in Table 1. *C. jejuni* strain 11168-H is a hypermotile variant of the sequenced strain NCTC 11168 that readily colonizes chickens (Jones *et al.*, 2004; Karlyshev *et al.*, 2002). *C. jejuni* strains were cultured on either blood agar Skirrows actidione (BASA) plates or Columbia agar plates (CBA) supplemented with 5% (v/v) horse blood in anaerobic jars in an atmosphere of 6% O₂/10% CO₂ (CampyPak; Oxoid) for 48 h at 37 °C.

For infections, bacteria were subcultured into 6 ml Mueller–Hinton (MH) broth (Oxoid) and grown under microaerobic conditions for 24–48 h at 37 °C, 150 r.p.m. The bacteria were then adjusted to OD₅₉₀ 1.0 in PBS (0.1 M, pH 7.2) for infections, equivalent to 1 × 10⁸ c.f.u. ml⁻¹. Infections at lower doses were adjusted accordingly.

Abbreviations: GFP, green fluorescent protein; H&E, haematoxylin and eosin; MLST, multilocus sequence typed.

Table 1. Bacterial strains used in this study

Strain	Origin	MLST group
11168-H	Human	ST43/CC21
11168-O	Human	ST43/CC21
pREM5 11168H	Human	ST43/CC21
81116	Human	ST267/CC283
81176	Human	ST913/CC42
01/51	Human	ST626/CC21
01/43	Human	ST257/CC257
93/372	Pet	ST21/CC21
94/229	Poultry	ST45/CC45
99/97	Human	ST45/CC45
99/118	Cow	ST21/CC21
99/188	Human	ST21/CC21
99/189	Human	ST45/CC45
99/194	Cow	ST45/CC45
99/197	Human	ST45/CC45
99/201	Cow	ST48/CC48
99/202	Cow	ST45/CC45
99/212	Human	ST45/CC45
99/216	Human	ST45/CC45
99/242	Poultry	ST45/CC45
A1/CF/12	Poultry	ST257/CC257
A6/T2/15	Poultry	ST257/CC257
A8/35/15A	Poultry	ST257/CC257
C1/C/2	Poultry	ST257/CC257
C120/2	Poultry	ST257/CC257
C132/1	Poultry	ST19/CC21
C3/T/25	Poultry	ST257/CC257
C5/T2/8	Poultry	ST257/CC257
C85-4-99-5	Cow	ST262/CC21
C500-1-99-2	Cow	ST48/CC48
C559-3-99-2	Cow	ST262/CC21
D2/27/3	Poultry	ST48/CC48
D2/T/8	Poultry	ST48/CC48
D2/T/95	Poultry	ST48/CC48
D5-20-9A	Poultry	ST262/CC21
EX1182	Environmental	ST262/CC21
EX1286	Poultry	ST262/CC21
MB1	Poultry	ST48/CC48
MB2	Poultry	ST48/CC48
MB3	Poultry	ST48/CC48
MB4	Poultry	ST19/CC21
MB5	Poultry	ST19/CC21
MB6	Poultry	ST19/CC21
MB7	Poultry	ST262/CC21
MB8	Poultry	ST48/CC48
MB9	Poultry	ST257/CC257
MB10	Poultry	ST19/CC21
MB12	Poultry	ST21/CC21
MB13	Poultry	ST21/CC21
MB14	Poultry	ST21/CC21
MB15	Poultry	ST45/CC45
MB16	Poultry	ST48/CC48
MB17	Poultry	ST262/CC21
MB18	Poultry	ST21/CC21
Ps308	Pig	ST51/CC403
Ps549.1	Pig	ST403/CC403

Table 1. cont.

Strain	Origin	MLST group
Ps623	Pig	ST552/CC403
Ps762	Pig	ST270/CC403
Ps830	Pig	ST403/CC403
Ps838	Pig	ST403/CC403
Ps843	Pig	ST403/CC403
Ps849	Pig	ST403/CC403
Ps852	Pig	ST270/CC403
Ps857	Pig	ST270/CC403
S39-2-99-3	Sheep	ST21/CC21
S87-4-99-3	Sheep	ST262/CC21
S120-4-99-4	Sheep	ST45/CC45
S216-5-99-1	Sheep	ST257/CC257
S372-5-99-4	Sheep	ST21/CC21
S379-8-99-1	Sheep	ST262/CC21
S435-3-99	Sheep	ST262/CC21
S499-1-99-5	Sheep	ST19/CC21
S585-3-99	Sheep	ST19/CC21

G. mellonella virulence assays. *G. mellonella* larvae were purchased from Live Foods UK and maintained on wood chips at 15 °C. The infection of larvae was carried out as previously described (Champion *et al.*, 2010) using a micro-injection technique whereby 10 µl *C. jejuni* was injected into the haemocoel via the right foreleg, using a Hamilton syringe. Larvae were then incubated at 37 °C and survival and macroscopic appearance were recorded at 24 h post-infection. PBS injected and uninfected controls were used. For each, experimental groups of ten *G. mellonella* larvae were infected.

Association of *C. jejuni* with haemocytes. A group of three *G. mellonella* larvae was infected as above with 10⁶ c.f.u. of *C. jejuni* 11168-H and incubated at 37 °C for 24 h. The larvae were chilled on ice for 20 min before aseptic removal of the bottom 2 mm of the body. The haemocoel was drained from each larva into sterile microcentrifuge tubes and centrifuged at 200 g for 5 min. The supernatant, which was the haemolymph, was transferred to a separate sterile microcentrifuge tube. The pelleted haemocytes were resuspended in 100 µl sterile distilled water, and pipetted up and down ten times to lyse the cells. Serial dilutions of both haemolymph and haemocytes were plated out on CBA to enumerate bacteria.

Histopathology. *C. jejuni*-infected and uninfected larvae (five per group) were fixed by immersion in 10% (v/v) neutral buffered formalin for 3–7 days. For light microscopy, larvae were blocked by a longitudinal section dividing the animal into two pieces and smears were air-dried and stained with Gram-Twort. The larvae (20%) were blocked into 11 transversal sections serially from the cranial to the caudal extremities of the larvae. Sections were embedded in paraffin wax and routinely stained with haematoxylin and eosin (H&E) for microscopic examination.

Investigation of *C. jejuni* morphology following infection of *G. mellonella*. A green fluorescent protein (GFP)-tagged *C. jejuni* strain, pREM5 11168H GFP (donated by Andrey Karlyshev, Kingston University, Surrey, UK), was cultured under microaerobic conditions on MH agar. It was then subcultured into MH broth as before and incubated for 24 h at 37 °C under microaerobic conditions. An inoculum was prepared at OD₅₉₀ as previously described above. Five *G. mellonella* larvae were infected with 10 µl of the prepared inoculum, and a further five were inoculated with 10 µl PBS. The larvae were incubated at 37 °C for 3 h before being chilled on ice for

5 min. They were then swabbed with 70 % ethanol prior to the aseptic removal of the bottom 2 mm of the body as previously described. One of each larval set was drained separately; the other four of each set had their haemocoel combined. This combined haemocoel was centrifuged at 500 g for 5 min to pellet the haemocytes, and 10 µl of the supernatant (haemolymph) was dropped onto a slide. A further 10 µl from the non-centrifuged haemocoel and 10 µl from the overnight *C. jejuni* culture were also dropped onto separate slides. Slides were examined using a Zeiss LSM 510 META confocal microscope.

Cell culture. J774A.1, a murine monocyte macrophage-like cell line, was obtained from the American Type Culture Collection (reference TIB-67), and cultured in Dulbecco's Modified Eagle Medium (from Fisher Scientific) supplemented with 10 % fetal bovine serum at 37 °C. SF9, a lepidopteran cell line, was donated by Richard ffrench-Constant (University of Exeter, UK), and cultured in Grace's Insect Medium (from Fisher Scientific) supplemented with 10 % fetal bovine serum at 27 °C. The cells were seeded at 2×10^5 cells in 6-well tissue culture plates and then incubated at the appropriate temperature for 24 h under 5 % CO₂ prior to infection with *C. jejuni*.

Bacterial infection of cultured cells. *C. jejuni* 11168-H was cultured on a CBA plate and harvested from an overnight culture by rolling a moistened swab over the plate; cells were resuspended in PBS. The OD₅₉₀ was measured and the inoculum was prepared at a multiplicity of infection (m.o.i.) of 10 in L-15 medium (from Fisher

Scientific) before being added to both macrophages and insect cells (three replicates). The macrophages were incubated at 37 °C for 1 h; the insect cells were incubated at 27 °C for 1 h. Following incubation, the inoculum in each well was replaced with L-15 medium containing 50 µg gentamicin ml⁻¹, and the plates were then incubated at the appropriate temperature for a further 1 h. The medium was removed and the cells were incubated in L-15 containing 10 µg gentamicin ml⁻¹ for approximately 16 h.

The cells were then washed three times with PBS, and 1 ml cold sterile water was added to each well. The cells were mechanically lysed to release intracellular bacteria, and c.f.u. were determined after plating out serial dilutions on CBA plates and incubating microaerobically at 37 °C.

RESULTS

C. jejuni induces histopathological changes in *G. mellonella*

To better understand the fate of *C. jejuni* inoculated into *G. mellonella*, larvae challenged with 10⁶ c.f.u. of some well-characterized human isolates were fixed in neutral buffered formalin at 24 h post-infection and sectioned for histopathology. Fig. 1 shows H&E-stained sections of uninfected

Fig. 1. H&E-stained sections of *G. mellonella*. (a) Non-infected larva (bar, 100 µm); (b) control (0.1 M PBS inoculation) larva (bar, 100 µm); (c) larva infected with *C. jejuni* 81116 and incubated at 37 °C (bar, 250 µm); (d) larva infected with *C. jejuni* 81116 and incubated at 42 °C (bar, 100 µm). Structures are annotated as follows: a, adipose bodies; b, bacterial colonies; c, cuticle; h, haemolymph; p, pigmented structures.

and infected larvae. Bacteria were observed in the haemocoel and sections of gut from infected larvae, but were absent in sections from uninfected controls. There was evidence of damage to the midgut, with apoptotic cells and loss of integrity to the gut wall in the infected larvae. This damage was not visible in the control sections. Other tissues (fat body, muscle, nervous tissue) appeared undamaged in infected larvae and uninfected controls. Pigmented nodules were also present in infected larvae, and bacteria were associated with these nodules. These nodules were not visible in the uninfected control larvae.

The observed bacteria were coccoid rather than having the characteristic spiral form associated with *C. jejuni*. To investigate whether these coccoid bacteria were actually *C. jejuni*, *G. mellonella* larvae were infected with GFP-tagged *C. jejuni*; the haemocoel was collected and centrifuged at low speed to sediment haemocytes, which are often autofluorescent. Comparisons with *C. jejuni* from an overnight culture showed that these bacteria had the expected morphology, but bacteria in haemolymph were of a coccoid nature (Fig. 2). Similar observations of haemolymph from control larvae inoculated with PBS showed no fluorescence at all.

To investigate the site of replication, haemocoel was collected and centrifuged at low speed to sediment haemocytes. The number of bacteria found in the resuspended cell pellet (4.1×10^6 c.f.u.; SEM 2.98×10^6) was broadly similar to the number found in the haemolymph (7.7×10^6 c.f.u.; SEM 2.3×10^6).

A macro scoring system was used to examine whether there was a correlation between the colour of the larvae and the presence of bacteria in the gut or body cavity (Table 2; Fig. 3). There was a significant association between macro colour and the presence of bacteria in the body cavity ($P < 0.001$, Kruskal–Wallis non-parametric test). No associations were made between the location of the bacteria and temperature at which they had been grown, or location and the strain of *C. jejuni* used to inoculate the larvae.

An insect cell line and mammalian macrophages are comparable in their response to challenge with *C. jejuni*

Insect (SF9) and mammalian (J774A.1) cells were infected at an m.o.i. of 10 with *C. jejuni* 11168-H, and monitored at 4 h and 24 h post-infection. In J774A.1 macrophages, bacterial numbers declined 100-fold by 4 h post-infection (Fig. 4). However, the bacterial numbers then remained approximately constant in the macrophages at 24 h post-infection. There was a broadly similar pattern of survival in the SF9 cell line. Bacterial numbers decreased 1000-fold during the first 4 h of the infection, but there was an approximate 10-fold increase in bacterial numbers between 4 h and 24 h (Fig. 4). This increase was statistically analysed using a Student's *t*-test and found to be significant ($P < 0.05$).

Fig. 2. Confocal microscope images of GFP-tagged *C. jejuni* pREM5 11168H. (a) *C. jejuni* from an overnight broth culture; (b) *C. jejuni* in the haemolymph of infected *G. mellonella* after a 3 h incubation.

Differences in virulence were observed in *G. mellonella* between *C. jejuni* MLST complexes

To investigate whether there was an association between MLST type and virulence, larval survival was recorded following challenge with 67 *C. jejuni* strains belonging to different MLST types (Fig. 5). It was observed that there was variation within MLST groups as well as between them. There was a significant difference ($P = 0.0002$) between the ability of ST21 and ST257 strains to cause disease. Overall, strains belonging to ST21 showed the least virulence in the model, whilst strains belonging to ST257 were the most virulent.

DISCUSSION

We have previously demonstrated that *G. mellonella* larvae can be used to screen for virulence of *Campylobacter* genes (Champion *et al.*, 2010). In this study, we have characterized the *G. mellonella* model and demonstrated that it can

Table 2. Macro scores for different *C. jejuni* strains in terms of colour, presence of bacteria in the larval gut and presence of bacteria in the larval body cavity

G. mellonella was incubated at different temperatures. A score of 3 for macro colour refers to fatality. ND, No data.

<i>C. jejuni</i> strain	Incubation temperature (°C)	Animal no.	Macro colour	Bacteria in gut	Bacteria in cavity	Mean <i>Galleria</i> survival (%)
01/51	37	1	0	3	0	80
		2	2	3	3	
		3	3	3	3	
		4	3	3	3	
		5	3	2	2	
	42	1	2	3	3	82
		2	2	3	2	
		3	2	2	1	
		4	2	3	3	
		5	2	3	3	
11168-O	37	1	1	2	3	27
		2	1	3	3	
		3	2	3	3	
		4	2	3	3	
		5	3	3	3	
	42	1	1	3	2	80
		2	2	2	2	
		3	2	1	2	
		4	2	3	3	
		5	2	3	3	
11168-H	37	1	1	2	3	0
		2	1	2	1	
		3	2	3	3	
		4	2	1	0	
		5	3	3	2	
	42	1	0	2	0	ND
		2	1	3	1	
		3	1	2	1	
		4	2	3	1	
		5	3	3	3	
81116	37	1	1	2	0	90
		2	1	3	0	
		3	3	3	3	
		4	3	3	3	
		5	3	3	3	
	42	1	1	2	2	65
		2	1	2	0	
		3	2	3	2	
		4	2	2	0	
		5	3	1	3	
81176	37	1	1	2	0	90
		2	1	2	0	
		3	2	2	0	
		4	2	2	1	
		5	3	2	3	
	42	1	2	2	2	75
		2	2	3	3	
		3	2	3	3	
		4	3	2	3	
		5	3	3	3	
PBS		1	0	1	0	100
		2	0	2	0	

Table 2. cont.

<i>C. jejuni</i> strain	Incubation temperature (°C)	Animal no.	Macro colour	Bacteria in gut	Bacteria in cavity	Mean <i>Galleria</i> survival (%)
Uninfected		3	0	2	0	100
		4	0	2	0	
		5	1	1	0	
		1	0	1	0	
		2	0	1	0	
		3	0	1	0	
		4	0	2	0	
		5*	1	3	3	

*Autolysis.

be used as an infection model to provide data about pathology and intracellular survival.

Similarities between invertebrate and mammalian humoral and cellular innate immune responses are exploited when using insects as virulence models. For example, *G. mellonella* possesses a cuticle that acts in the same physical barrier capacity as mammalian skin (Kemp & Massey, 2007). Once the cuticle has been breached, *G. mellonella* induces a humoral response, producing soluble factors such as antimicrobial peptides (Mullett *et al.*, 1993). In parallel with a humoral response, *G. mellonella* induces a cellular response to invading micro-organisms. Insect haemocytes phagocytose bacteria in a manner similar to that of mammalian neutrophils and produce a respiratory burst (Bergin *et al.*, 2005). Thus, the response of *G. mellonella* to infection with *C. jejuni* is likely to have similarities to the response of humans.

In the initial experiment, larvae were infected with human *C. jejuni* isolates 11168-H, 11168-O, 81116, 81176 and 01/51. These were selected as they are well characterized in a

number of other animal models; invasion and toxin data are also available for them. It would not have been possible to fix *G. mellonella* for all the strains used later on, as this would have been time-consuming and expensive with no guarantee of any further data.

Histopathology of infected larvae demonstrated that bacteria are found in the haemocoel and in the gut and that extensive tissue damage occurs in the latter. This pathology may be caused by haemocytes in the gut tissue, which have ingested bacteria circulating in the haemocoel and then produced responses such as the release of free radicals and peroxide, causing the visible tissue damage. The presence of pigmented nodules, which are aggregations of haemocytes around foreign bodies, indicates a vigorous immune response to infection (Lackie, 1980). The observed colour change in infected larvae correlating with the presence of bacteria in the body cavity is a product of melanogenesis; this process is thought to protect

Fig. 3. Macroscopic evaluation of Wax Moth larvae following infection with *C. jejuni*: white, score 0; orange, score 1; black, score 3.

Fig. 4. Chart showing the mean numbers of *C. jejuni* 11168-H recovered from J774.1A murine macrophages and SF9 insect cell line at 4 h (shaded bars) and 24 h (white bars) post-infection ($n=3$). 0 h (black bars) represents the initial inoculum.

endogenous tissues within the cavity from systemic damage resulting from pathogen killing (Nappi & Christensen, 2005).

Within the haemocoel, cell-associated and free bacteria were found. *C. jejuni* is primarily an extracellular pathogen; however, intracellular survival has been hypothesized to play an important role in its pathogenesis (Kiehlbauch *et al.*, 1985; Hickey *et al.*, 2005; Young *et al.*, 2007). Reproducible *in vitro* infection models that mimic pathogenesis

The bacterial numbers recovered from the macrophages remained consistent between 4 h and 24 h; there was survival within the macrophages. This is consistent with previous studies (Kiehlbauch *et al.*, 1985; Hickey *et al.*, 2005). However, there was a significant increase in *C. jejuni* recovered from the insect cell line at 24 h compared to 4 h. Thus, it is possible that the bacteria not only survived within the cells, but also replicated.

C. jejuni strains can be classified by MLST complexes. A number of studies have sought to establish whether there is a link between MLST type and the development of post-infectious complications (Dingle *et al.*, 2001; Nielsen *et al.*, 2010; Islam *et al.*, 2009). It was noted that the ST22 complex is over-represented in isolates from patients who have contracted Guillain–Barré syndrome; no Guillain–Barré-related isolates have been shown to carry ST45, despite it being a common sequence type (Dingle *et al.*, 2001; Nielsen *et al.*, 2010). No sequence types have been found to be exclusive for clinical outcomes (Islam *et al.*, 2009). This supports the findings of Manning *et al.* (2003), who studied a large number of *C. jejuni* isolates and found that in terms of MLST types, the populations of veterinary and human isolates overlapped; it was suggested that most veterinary sources should be considered reservoirs of pathogenic campylobacters. However, these studies did not assess whether bacteria from different MLST types exhibited different levels of virulence. Recent studies have suggested that there may be associations between *C. jejuni* MLST type and virulence factors (Habib *et al.*, 2009; de

Haan *et al.*, 2010). We observed that when bacteria selected as representatives of major MLST groups were put through the *G. mellonella* model, MLST type 257 strains were significantly more virulent than the MLST type 21 set. MLST type 257 is mainly associated with poultry and clinical isolates. MLST type 21 is common in all food-producing animals; the strains used here are also all of clonal complex (CC) 21, which is one of the four most common CCs in human disease. The reduced virulence of these isolates in the model may thus appear anomalous, but Habib *et al.* (2010) suggest that the abundant prevalence of *C. jejuni* of CC21 may be a result of its increased tolerance of stresses encountered during the human food chain. A less virulent but more stress-tolerant strain would thus be encountered more frequently than a more virulent strain that did not tolerate such stresses to the same extent. The convenience of the *G. mellonella* model allows for high throughput screening to assay for the differences in virulence. Such a model could provide preliminary data when considering food security issues.

This study has sought to further characterize *G. mellonella* as a model for *C. jejuni* infection, and suggests that, since the bacteria convert to a coccoid form once within the insect, it may be used to provide opportunities for further study of this morphological change. The model may also prove useful in investigating the *in vivo* intracellular survival of *C. jejuni* within macrophages, an area of some dispute. In particular, the model allows screening for natural variations in the virulence of *C. jejuni* field isolates, which would prove invaluable for tracking particularly virulent strains in the food chain.

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