**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–Barre syndrome plus its variant Miller Fisher syndrome (Janssen et al., 2008; van Doorn 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.

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 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.

**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.

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

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4Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK

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.
Table 1. Bacterial strains used in this study

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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 10^6 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 microscopic examination.
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 2×10⁵ 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
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, Kruskall–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).

**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.

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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

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Table 2. cont.

<table>
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<tr>
<th><em>C. jejuni</em> strain</th>
<th>Incubation temperature (°C)</th>
<th>Animal no.</th>
<th>Macro colour</th>
<th>Bacteria in gut</th>
<th>Bacteria in cavity</th>
<th>Mean <em>Galleria</em> survival (%)</th>
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*Autolysis.

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**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).

The bacteria observed in the larval sections were not identified, but were only present in *Campylobacter* infected larvae. The bacterial cells were coccoid rather than spiral; however, *C. jejuni* that has become intracellular converts rapidly from the spiral form to the coccoid form (Kiehlbauch et al., 1985). There is some debate about how this change affects the bacteria (Moore, 2001). Adaptation to the coccoid form is generally seen as a response to stress, such as starvation or oxidative stress (Harvey & Leach, 1998). Some studies, such as that of Moran & Upton (1986), have reported that the coccoid form is thus degenerative. However, it has also been reported that coccoid *C. jejuni* becomes viable but non-culturable, with the potential to still act as an infectious agent (He & Chen, 2005). Reproducible *in vitro* infection models that mimic pathogenesis in vivo have been used to study *C. jejuni* intracellular survival in epithelial cells (De Melo et al., 1989; Watson & Galán, 2008). However, reports of *C. jejuni* intramacrophage survival *in vitro* are conflicting. Some groups indicate that *C. jejuni* is killed by macrophages (Watson & Galán, 2008); others suggest that the bacteria survive within the macrophage (Day et al., 2000; Hickey et al., 2005). In this study, bacterial infection of different cell lines was undertaken to establish whether there was a difference in response between mammalian macrophages and an insect cell line. It was uncertain as to whether *C. jejuni* would survive intracellularly in the SF9 insect cell line under tissue culture conditions. However, although the levels of *C. jejuni* recovered from the insect cell line were approximately ten times lower than those recovered from murine macrophages at 4 h post-infection, it is clear from the data presented here that the bacteria did invade the cells and survive within them.

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.

These observations are consistent with the hypothesis that *C. jejuni* enters insect haemocytes during *in vivo* model infections of *G. mellonella*. This intracellular persistence may allow the bacteria to avoid, or at least reduce the impact of, host antimicrobial defences. Nevertheless, the fact that at least some bacteria provoked the formation of melanized nodules is not surprising as it has previously been shown that nodule formation is associated with phagocytosis (Dean et al., 2004).

*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

![Graph of G. mellonella survival following challenge with C. jejuni of different MLST types.](http://jmm.sgmjournals.org/667)

**Fig. 5.** Graph of *G. mellonella* survival following challenge with *C. jejuni* of different MLST types. Each data point represents the mean percentage larval survival (n=10). Horizontal lines represent the mean for each MLST type.
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.

ACKNOWLEDGEMENTS

We thank Peter Splatt (University of Exeter) for his technical assistance with confocal microscopy.

REFERENCES


Kiehlbauch, J. A., Albach, R. A., Baum, L. L. & Chang, K.-P. (1985). Hyperphagocytic haemocytes: a representative 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.

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