Histopathological and ultrastructural studies of a mouse lung model of Campylobacter jejuni infection

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Campylobacter jejuni is a major cause of diarrhoea in humans. However, the pathogenesis of C. jejuni diarrhoea is poorly understood due to the lack of a good animal model of infection. Many animals have been tried with limited success, but a mouse lung model of infection has been found to be satisfactory previously; however, the lung pathology of this model has not been studied. For the purpose of characterizing the histopathological and ultrastructural lesions in the lung of the mouse pulmonary model of C. jejuni infection, C. jejuni strain 81-176 or sterile PBS was intranasally inoculated into BALB/c mice. The infection resulted in a mild illness only, and in an initial predominance of polymorphonuclear cells, followed by the accumulation of macrophages and later the prominence of epithelioid cells. Focal peribronchial pneumonia appeared on day 3, granuloma-like reaction on day 4 and bronchopneumonia on day 5 post-infection. These features developed until day 5 post-infection, but were less consistent afterwards when histopathology was monitored up to 9 days post-infection. Intracellular structures resembling bacteria were observed on days 3 and 5 post-infection, but not on day 7 post-infection. On days 3 and 5 post-infection, degenerative changes were also observed by transmission electron microscopy. The histological changes were not associated with acid-fast bacteria or any fungal elements. The infection was systemic as C. jejuni was isolated from blood and all organ homogenates (lung, spleen, liver, and small and large intestines) at 24 h post-infection. Thereafter, the organism was recovered from the intestine only, thus indicating its predilection for this location. This characterization of pathology should contribute to a better understanding of the animal model and pathogenesis of C. jejuni infection.

INTRODUCTION

Campylobacter jejuni is a major cause of diarrhoea in humans (Blaser, 1997; Tauxe, 1992; Taylor, 1992). The infection can manifest as acute watery diarrhoea or severe inflammatory diarrhoea (Blaser, 1997; Maki et al., 1979; van Vliet & Ketley, 2001), but asymptomatic infections occur in children in developing countries (Taylor, 1992). Some patients develop extraintestinal manifestations (Skirrow & Blaser, 2000). Late-onset complications following Campylobacter infection include reactive arthritis (Hannu et al., 2004) and Guillain–Barré syndrome (Nachamkin et al., 2000; Tsang, 2002). C. jejuni has been shown to cause tissue inflammation in human studies (Black et al., 1988; Maki et al., 1979) and in animal models of infection (Hodgson et al., 1998; Sestak et al., 2003).

Due to the lack of a suitable animal model, the pathogenesis of C. jejuni infection is poorly understood. As there are many inherent histological and immunological similarities between the lung and the intestine, a mouse lung model for studying many aspects of intestinal pathogens, such as Vibrio cholerae, Shigella flexneri and C. jejuni (Baqar et al., 1996; Fullner et al., 2002; Van De Verg et al., 1995), has been established. However, the pathology of lung lesions caused by C. jejuni has not been characterized for this model (Baqar et al., 1996). The objectives of this study were to evaluate the pathological lesions of the mouse pulmonary model of C. jejuni infection by histology and electron microscopy of the lung, and to study distribution of the organism in various organs. We also hypothesized that this model would reflect...
the inflammatory and disseminating nature of the infection in humans (Blaser, 1997).

METHODS

Mice. Outbred BALB/c mice from Animal Resource Centre, Faculty of Medicine, Kuwait University, were used. The mice were housed in cages with sterile bedding of wood shavings (Special Diet Services), and were given a standard pelleted diet (Special Diet Services) and filtered tap water. The mice were free of Campylobacter and parasitic infections as evidenced by screening of three consecutive stool filtrates (Oxoid) and BHI broth supplemented with 1 % yeast extract in a gas pack (Oxoid) in a jar with activated palladium catalyst at 42 °C. The organism was stored in Brucella broth (Becton Dickinson) and BHI broth supplemented with 1 % yeast extract (Becton Dickinson). The plates were incubated under microaerophilic conditions generated with a Campy gas pack (Oxoid) in a jar with activated palladium catalyst at 42 °C for 48 h. The organism was stored in Brucella broth (Becton Dickinson) containing 15 % glycerol (Sigma–Aldrich) at −70 °C.

In vivo passages of C. jejuni 81-176 in mice. This C. jejuni 81-176 strain does not cause a natural infection among laboratory mice, and therefore needs to be adapted first to establish a consistent infection in mice (Baqar et al., 1996). The C. jejuni strain was cultured on BA (Oxoid), suspended in PBS, pH 7.2, to ~10^8 c.f.u. ml^-1 and injected into the ileal loops of fasted adult BALB/c mice. One day later, the material that accumulated within the loops was injected into other mice, and this was repeated until the strain underwent four passages (Chattopadhyay et al., 1991). Then, C. jejuni was recovered on Campy blood agar plates (Oxoid) and subcultured on BA (Oxoid). This organism was then passed through the intranasal route (Baqar et al., 1996). The lungs of 6–8-week-old BALB/c mice were collected 24 h post-infection, homogenized in PBS and cultured on BA. The resulting growth was then used to inoculate other mice and this was repeated until the strain underwent three passages. The lung homogenates of the last set of mice were plated on BA (Oxoid) and charcoal agar (Oxoid) to detect the presence of C. jejuni. Colonies identified as C. jejuni were stocked in Brucella broth with 15 % glycerol (Sigma–Aldrich) at −70 °C. Our previous attempts to passage C. jejuni by the intravenous route (using spleen homogenate) (Baqar et al., 1996) and the direct intranasal route (using lung homogenate) have failed.

Intranasal inoculation. The mouse ileum- and lung-adapted C. jejuni 81-176 strain was cultured on BA (Oxoid) for 24 h. The growth was suspended in 1 ml brain heart infusion (BHI) broth (Oxoid) supplemented with 1 % yeast extract (Becton Dickinson). The suspension was grown on a biphasic medium with BHI agar slant (Oxoid) and BHI broth supplemented with 1 % yeast extract in a microaerophilic atmosphere at 42 °C for 24 h. The bacteria in the broth were pelleted by centrifugation at 2147 g at 4 °C for 30 min in a benchtop centrifuge with a 1.19 rotor (Beckman GS-6R). The pellet was suspended in 1 ml PBS and recentrifuged under the same conditions in a centrifuge (Ependorff). The resulting pellet was suspended in 300–400 µl PBS (Baqar et al., 1996). Then, 6–8-week-old BALB/c mice were anaesthetized by injecting xylazine and ketamine intraperitoneally as described by Fullner et al. (2002). The test mice were intranasally inoculated with 30 µl culture (~4 × 10^7 c.f.u.), and the control mice were inoculated with 30 µl sterile PBS (Baqar et al., 1996; Fullner et al., 2002).

Illness monitoring. On a daily basis for up to 5 days, 20 mice (2 test mice and 2 control mice per day) were monitored to determine signs of illness and assess the degree of mortality using 5 criteria that were scored on a scale of 1 to 5 (worst to best) (Fullner et al., 2002). Total health rate was then calculated as the mean scores of these five criteria (Fullner et al., 2002).

Histopathological studies. In a group of 36 mice, histopathological studies were carried out on a daily basis for up to 9 days, 2 mice were sacrificed from each group by neck dislocation. Both lungs were removed under aseptic conditions and kept in 10 % buffered formalin for 24 h. The lung tissue was dehydrated, kept in xylene and processed in paraffin. Sections were cut at 5–10 µm thickness, and stained with haematoxylin and eosin. In addition, sections were processed and stained with Ziehl–Neelsen (ZN) stain, periodic acid–schiff with diastase digestion (PAS/D) stain, and Brown and Hopps (BH) (modified tissue Gram) stain (Bancroft et al., 1994). Histological evaluation was done by a pathologist in a blinded fashion.

Transmission electron microscopy (TEM) studies. On days 1, 3 and 5 post-infection, two mice were sacrificed from each group. Pieces of lung tissue were removed from each animal and kept in 3 % glutaraldehyde for 3 h. The tissues were then washed in Millonig’s phosphate buffer and post-fixed in 1 % osmium tetroxide for 3 h. After fixation, the tissues were dehydrated in graded ethanol, treated with propylene oxide for 30 min, embedded in graded araldite mixture and blocks were prepared. For histological and topographical identification, 1 µm semi-thin sections were cut by glass knife and stained with toluidine blue/borax solution. For ultrastructural analysis, serial ultrathin sections (100 nm) cut by DuPont diamond knife (DuPont) were used. The sections were collected onto copper grids and stained with uranyl acetate followed by lead citrate. The ultrastructure of the stained sections was examined by 1200 EX II (JEOL) transmission electron microscope (Bozzola & Russel, 1999; Robinson et al., 1987).

Bacteriological studies. On days 1, 3 and 5 post-infection, mice (four test mice and four control mice) were anaesthetized. Then, blood (approx. 1 ml) was collected from the heart using a 27G needle syringe into sterile Eppendorf tubes. The mouse was sacrificed by neck dislocation and the abdomen was opened under aseptic conditions in a laminar flow hood. Organs — lung, spleen, liver, and small intestine and large intestine — were collected into pre-weighed sterile Eppendorf and test tubes, and weights were determined on an electronic balance. Then, all organs were cut into pieces, and subjected to manual homogenization using a pestle and mortar. All organ homogenates were suspended in PBS (1 ml for spleen, liver and lung, and 2 ml for small and large intestines). Serial dilutions of each organ homogenate (tenfold for small and large intestine, and twofold for lung, liver and spleen) were made in sterile PBS. Also, twofold serial dilutions were made for blood. A total of 50 µl of these dilutions from the organ homogenates and blood were plated on Charcoal agar (Oxoid). The plates were incubated under microaerophilic conditions at 42 °C for 48 h, and the number of colonies were counted and recorded. C. jejuni was identified by Gram stain, motility and oxidase test (Nachamkin, 2003). Colony counts were expressed as c.f.u. (100 mg tissue)^−1 and c.f.u. (ml blood)^−1. The health monitoring, and studies of pathology and bacteriology, were done twice using a total of 112 mice. The animal experiments were carried out as per institutional guidelines.

RESULTS AND DISCUSSION

Illness monitoring. C. jejuni 81-176 did not cause severe systemic illness, including death, in mice. The total health scores (4.4–4.2)
did not deteriorate throughout the 5 days of infection, though a breakdown of the illness scores illustrated worsening of lung appearance and improvement of other scores, such as ocular character and mobility, as shown in Table 1.

When the mouse pulmonary model was established for *C. jejuni* infection, prior *in vivo* passage through the intravenous route was performed and the recovered organisms from the spleen were used for the pulmonary infection (Baqar *et al.*, 1996). Our attempts to passage the strain six times through the intravenous route failed. One possible explanation for this is the quick clearance of bacteria from the bloodstream. We do not know the history of *in vitro* passage of *C. jejuni* 81-176. If the strain was subjected to multiple *in vitro* passages, it would have a reduced its colonizing ability (Field *et al.*, 1991). The relatively lower virulence of this strain in our hands, as determined by its inability to cause more serious disease and death, as reported by Baqar *et al.* (1996), might be related to its reduced colonizing ability. We have successfully passed *C. jejuni* 81-176 via mouse ileal loops and then indirectly through the intranasal route. This is the first time that this has been performed for *C. jejuni* 81-176 in mice. Other researchers have used the ileal loop passage with rats (Chattopadhyay *et al.*, 1991) and the intranasal passage for other pathogens (Essig *et al.*, 2000; Islam *et al.*, 1994).

It has been reported that *C. jejuni* results in severe systemic illness with significant mortality rate within 6 days post-infection (Baqar *et al.*, 1996). This was not observed in our study, although the infection was established and disseminated. As already demonstrated, bacterial dissemination into the spleen and liver might occur without the appearance of clinical signs of severe illness (Vučković *et al.*, 1998). Differences in the passage history of the strain [ileal loop followed by intranasal passage in our study versus intravenous route by Baqar *et al.* (1996)] might possibly have influenced the lethality of the resultant infection. Campylobacteriosis in humans is not a lethal infection per se (Smith & Blaser, 1985), and a significant proportion of human campylobacteriosis results in mild manifestations or remains asymptomatic (Skirrow & Blaser, 2000). Thus, severe illness scores are not a pre-requisite for the model of *C. jejuni* infection.

### Histopathological studies

**Haematoxylin and eosin staining.** The lungs of the control mice showed focal non-expansion that persisted for up to 9 days (Fig. 1a). However, the lungs of mice inoculated with *C. jejuni* resulted in patchy interstitial inflammation with oedema and neutrophil infiltration into the alveolar spaces on day 1 (Fig. 1b) and 2 post-infection. This was followed by patchy peribronchial consolidation from day 3 to day 5, where the initial predominance of polymorphonuclear cells was followed by the accumulation of macrophages (Fig. 1c). *C. jejuni* infection induced bronchopneumonia on day 5 where macrophages were found in the bronchioles and alveolar spaces, and lymphocytes were observed (Fig. 1d, e). In addition, a granuloma-like reaction developed and epithelioid cells were most prominent on day 4 (Fig. 1f).

Thus, the maximal pathology seemed to have occurred on days 3–5 post-infection.

On day 6, there was no difference between the test and the control mice. Beyond day 6, the histological changes seemed to be less extensive, but bronchopneumonia was still observed on day 7 (Fig. 1g). Furthermore, haemorrhage and oedema were observed. This was extensive and consistent from day 1 to day 9 in test mice. In control mice, these features were similar to or less extensive than in test mice. Thus, these changes were regarded as non-specific and unrelated to *C. jejuni* infection because of the following reasons: (i) other investigators have also noted minor histopathological changes in the lungs of sham-inoculated control mice, even while using specific pathogen-free mice, probably related to the aspiration of PBS under anaesthesia (Fullner *et al.*, 2002); (ii) we discounted the possibility of mycobacterial and fungal infection of the mice by ZN staining and PAS/D staining, respectively (see below); (iii) bacterial structures were seen by BH staining in test mice only (see below); (iv) histology of the lungs of three randomly selected mice, from the colony, whose lungs were not inoculated with any material, appeared normal without any evidence of inflammation (data not shown). These rule out infection of mice used in our studies with extraneous contaminants.

**ZN and PAS/D staining.** ZN and PAS/D staining were performed to exclude the involvement of other microorganisms, such as acid-fast mycobacteria and fungi. Histopathological examination of lung tissue stained with ZN and PAS/D indicated the absence of acid-fast bacteria and fungi, thus excluding their association with the pathological lesions.

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<th>Table 1. Illness scores of mice monitored for 5 days post-inoculation</th>
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<td><strong>Criteria</strong></td>
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<td>Scruffiness</td>
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<td>Mobility</td>
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<td>Ocular character</td>
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<td>Breath rate</td>
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<td>C</td>
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<td>Lung appearance</td>
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<td>C</td>
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<tr>
<td>Total health score*</td>
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T, Test mice; C, control mice.

*Mean of score of the five criteria.
BH staining. Lung tissues from test and control mice sacrificed on days 3, 5 and 7 were also stained with BH stain. Histopathological examination of such stained tissues of mice sacrificed on day 3 and day 5 post-infection revealed the presence of bacteria-like structures within cells that were focally distributed, whereas the lungs from the control mice did not show such structures either on day 3 or on day 5 (Fig. 2a, b, c). In the lungs of test mice, the macrophages had clear foamy cytoplasm on day 7 (Fig. 2d).

The exact cause of the focal non-expansion observed in the lungs of control mice is not clear. It is likely that the mice had aspirated the normal flora of the respiratory tract along with PBS, and thus developed a mild reaction to these organisms, similar to the findings in other studies (Fullner et al. 2002).

We observed a predominance of polymorphonuclear cells and macrophages in the lung tissues of test animals similar to the findings of other studies where *C. jejuni* stimulated macrophage and neutrophil responses in the intestinal lavage fluid upon intraperitoneal infection (Mixter et al., 2003). Also, the intranasal inoculation of *C. jejuni* induced features of innate immunity as early as day 1 (the earliest time point tested) in concordance with the findings for intranasal inoculation with *V. cholerae* (Fullner et al., 2002; Haines et al., 2005) and *Shigella* (Van De Verg et al., 1995). The test mice in our study developed peribronchial pneumonia and bronchopneumonia similar to the results of studies of the intranasal model of *V. cholerae* (Fullner et al., 2002), *S. flexneri* (Phalipon et al., 1995) and *Shigella sonnei* (Mallett et al., 1995). However, these features (e.g. bronchopneumonia) were observed at earlier time points.
elsewhere (at time point \( \leq 24 \) h post-infection (Fullner et al., 2002; Mallett et al., 1995; Phalipon et al., 1995) compared to our study (5 days post-infection). Similarly, (activated) macrophages have been observed at time points as early as 24 h (Fullner et al., 2002) or 48 h (Van De Verg et al., 1995), but were observed on day 3 onwards in our study.

One interesting observation was the development of a granuloma-like reaction, which was similar to the early stages of the granulomatous reaction reported in the liver and spleen after the intravenous inoculation of *Salmonella typhimurium* in mice (Mastroeni et al., 1995). The cause of the continued activation of macrophages and the development of epithelioid cells is not known. The inducer of macrophage activation might possibly be a virulence factor, such as LPS, that might be associated with dead bacteria as in shigellosis (Phalipon et al., 1995), or the superoxide stress defence of *C. jejuni*, which plays a role in its intracellular survival (Hickey et al., 2005; van Vliet & Ketley, 2001). Moreover, Mastroeni et al. (1995) demonstrated that the induction of cytokines, such as tumour necrosis factor alpha, might possibly contribute to the formation of granuloma-like lesions in salmonellosis, and this might provide an alternative explanation for our findings.

**TEM**

For further determination of lung pathology, lung tissues were processed for TEM. No significant changes were observed in comparison to the control on day 1 post-infection. However, significant degenerative changes were observed after 3 and 5 days in the lung tissue of test mice as compared to that of the control mice. In the lung tissue of test mice, the cytoplasm appeared granular, whereas the cells of the control lung tissue had normal structures (Fig. 3). The significant degenerative changes observed

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**Fig. 2.** (a) The lung was devoid of infiltrates in control mice on day 3. (b, c) Macrophages with intracytoplasmic bodies were observed in test mice on days 3 and 5, respectively, indicated by arrows. (d) Foamy intra-alveolar macrophages without intracytoplasmic bodies were observed in test mice on day 7, indicated by an arrow. All cells are BH stained. Magnification \( \times 100 \).

**Fig. 3.** TEM showed that there were significant degenerative changes in the lung tissue of test mice (b), where the cells had a granular cytoplasm, indicated by an arrow (magnification \( \times 4000 \), whereas the lung tissue from control mice sampled at the same time point (a) showed organized organelle structures in the cells, indicated by an arrow (magnification \( \times 3000 \)).
under TEM might reflect the ability of C. jejuni to destroy infected cells, as C. jejuni has been shown to induce apoptosis (Zhu et al., 1999), as well as the necrosis of tissues (Butler et al., 1987).

**Bacteriological studies**

C. jejuni was not detected in the organs of PBS-inoculated controls. Bacterial counts were performed on days 1, 3 and 5 post-infection. After intranasal inoculation of C. jejuni, two patterns of infection were seen (Table 2). The first one was in the lungs, blood, liver and spleen, where C. jejuni was isolated after 1 day, but was not detected on day 3 and 5. The second pattern of infection occurred in the small and large intestines where C. jejuni was isolated on days 1, 3 and 5 post-infection.

The intranasal inoculation resulted in systemic dissemination into different organs (lungs, liver and spleen) most probably via the bloodstream. The rapid clearance that was observed by day 3 has also been demonstrated when C. jejuni was inoculated orally (Blaser et al., 1983), intraperitoneally (Pancorbo et al., 1994) and intranasally (Van De Verg et al., 1995).

Our results demonstrated that C. jejuni colonized the intestine for an extended period (up to 5 days) compared to other organs. Intestinal colonization was also reported in mice when other routes of inoculation were used (Hodgson et al., 1998; Baqar et al., 1996; Blaser et al., 1983) and was similarly observed in humans, where C. jejuni colonizes the large intestine and the terminal part of the small intestine for periods of up to 16 days (van Vliet & Ketley, 2001).

In our study, intra-cytoplasmic structures resembling bacteria were found in the lung tissues of test animals. Their focal distribution, their presence in locations of maximal histological changes and their absence in control lung tissues sampled at the same time points would exclude the possibility of these structures being non-specific. Immunohistochemistry might further clarify whether those structures reflect newly phagocytosed particles undergoing destruction by the macrophages (Wassenaar et al., 1997) or prolonged intracellular survival of C. jejuni (Hickey et al., 2005). When these structures were observed in the intranasal model of Shigella infection, they were thought to be LPS associated with dead bacteria present (Phalipon et al., 1995). Therefore, the presence of these structures in low numbers might be caused by intense inflammatory reaction, and might suggest the dual role of inflammation as a defence strategy and a mechanism that promotes invasion of C. jejuni, similar to what has been suggested in shigellosis (Phalipon et al., 1995). By day 7, the bacteria-like structures disappeared, the macrophages had cleared cytoplasm and at the same time the areas of inflammation tended to diminish, indicating the clearance of infection, the resolution of the inflammation and hence the importance of macrophages in the elimination of C. jejuni (Wassenaar et al., 1997; Pancorbo et al., 1999). Longer monitoring would indicate the time point when the lung returns to normal.

It has been observed that chicken-passaged C. jejuni strains colonized the birds at lower inocula than non-passaged strains (Cawthraw et al., 1996; Ringoir & Korolik, 2003). Moreover, passaged strains have slower clearance in animals than non-passaged strains (Field et al., 1991; Ringoir & Korolik, 2003). Likewise, to establish infection in our study, the challenge strain had to be adapted previously by passage through the intestine and lung. This would have introduced changes in the organism that are difficult to determine. However, it has been shown that DNA profiles and reactions to heat-stable antigens do not change following in vivo passage (Nielsen et al., 2001). Moreover, lung is more aerated than intestine. Since C. jejuni is a microaerophilic organism, lung could be less favourable than intestine to establish infection. Nevertheless, this is a cheaper and widely available model than some recently described cytokine knockout mouse intestinal models, which are expensive, need special care and are not widely available (Mansfield et al., 2007; Watson et al., 2007).

The mouse lung infection model has demonstrated the inflammatory and systemic nature of C. jejuni infection in accordance with our hypothesis. The organism also showed a predilection for intestinal colonization. This study has characterized the intranasal model of infection with regard to other organs. Intestinal colonization was also reported in mice when other routes of inoculation were used (Hodgson et al., 1998; Baqar et al., 1996; Blaser et al., 1983) and was similarly observed in humans, where C. jejuni colonizes the large intestine and the terminal part of the small intestine for periods of up to 16 days (van Vliet & Ketley, 2001).

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**Table 2.** Distribution of C. jejuni in organs of infected mice (T) compared to control mice (C)

Mean colony counts are presented in the form of c.f.u. (100 mg tissue)\(^{-1}\) or c.f.u. (ml blood)\(^{-1}\) for four test mice and four control mice for days 1, 3 and 5 post-infection. SD is shown in parentheses.

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<th>Day</th>
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<th>Colony count in the:</th>
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<td>Lung</td>
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<tr>
<td>1</td>
<td>T</td>
<td>244.8 (190.1)</td>
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to pathology in the lungs. This additional information will be valuable for studying the virulence, pathogenesis and immunity of *C. jejuni* infection in this model.

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