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Experimental *Legionella longbeachae* infection in intratracheally inoculated mice

Ivana Gobin,[1] Milorad Susa,[1] Gabrijela Begic,[1] Elizabeth L. Hartland[2] and Miljenko Doric[1]

Correspondence
Ivana Gobin
ivanagobin@yahoo.co.uk

[1]Department of Microbiology and Parasitology, Medical Faculty, University of Rijeka, B. Branchetta 20, 51000 Rijeka, Croatia

[2]Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3010, Australia

This study established an experimental model of replicative *Legionella longbeachae* infection in A/J mice. The animals were infected by intratracheal inoculation of 10^3–10^9 c.f.u. *L. longbeachae* serogroup 1 (USA clinical isolates D4968, D4969 and D4973). The inocula of 10^9, 10^8, 10^7 and 10^6 c.f.u. of all tested *L. longbeachae* serogroup 1 isolates were lethal for A/J mice. Inoculation of 10^5 c.f.u. *L. longbeachae* caused death in 90 % of the animals within 5 days, whilst inoculation of 10^4 c.f.u. caused sporadic death of mice. All animals that received 10^3 c.f.u. bacteria developed acute lower respiratory disease, but were able to clear *Legionella* from the lungs within 3 weeks. The kinetics of bacterial growth in the lungs was independent of inoculum size and reached a growth peak about 3 logarithms above the initial inoculum at 72 h after inoculation. The most prominent histological changes in the lungs were observed at 48–72 h after inoculation in the form of a focal, neutrophil-dominant, peribronchiolar infiltration. The inflammatory process did not progress towards the interstitial or alveolar spaces. Immunohistological analyses revealed *L. longbeachae* serogroup 1 during the early phase of infection near the bronchiolar epithelia and later co-localized with inflammatory cells. BALB/c and C57BL/6 mice strains were also susceptible to infection with all *L. longbeachae* serogroup 1 strains tested and very similar changes were observed in the lungs of infected animals. These results underline the infection potential of *L. longbeachae* serogroup 1, which is associated with high morbidity and lethality in mice.

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INTRODUCTION

Legionella longbeachae was classified as a member of the family *Legionellaceae* in 1981 (McKinney *et al.*, 1981). There are two serogroups of *L. longbeachae* and serogroup 1 is most commonly associated with disease in humans (Bibb *et al.*, 1981; Cameron *et al.*, 1991). The pathogen has been identified as a cause of lower respiratory infection in both normal and immunocompromised individuals (O'Connor *et al.*, 2007; Phares *et al.*, 2007). In Europe and the USA, *L. longbeachae* is rarely implicated in human disease, but the organism appears to be responsible for up to half of the cases of legionellosis in Australia (Lanser *et al.*, 1990; Lim *et al.*, 1989; Steele *et al.*, 1990) and Thailand (Phares *et al.*, 2007). Epidemiological exploration of the source of *L. longbeachae* serogroup 1 has indicated potting soil as the major reservoir of infection (Cameron *et al.*, 1991; Steele *et al.*, 1990). *L. longbeachae* serogroup 1 was isolated from commercial potting mixes and the soil of potted plants in Australia, the USA and Japan (CDC, 2000; Hughes & Steele, 1994; Koide *et al.*, 1999). Therefore, it is believed that transmission of *L. longbeachae* serogroup 1 from the environment to humans may occur via inhalation of aerosols from potting soils containing the organism. To investigate the virulence of different *Legionella* species, several *in vitro* and some *in vivo* experimental models have been developed. *L. longbeachae* serogroup 2 has been shown to replicate in guinea pig macrophages and A/J, C57BL/6 and DBA/2 mouse peritoneal macrophages (Izu *et al.*, 1999). *L. longbeachae* serogroup 1 replicates in U937 cells (Doyle & Heuzenroeder, 2002) but is unable to replicate in Mono Mac 6 cells, in *Acanthamoeba castellanii* (Neumeister *et al.*, 1997) or in *Hartmannella vermiformis* (Wadowsky *et al.*, 1991). Recently, it was shown that *L. longbeachae* serogroup 1 has a different intracellular life cycle to *Legionella pneumophila*, where, unlike *L. pneumophila*, the *L. longbeachae*-containing vacuole possesses markers of both early and late endosomes (Asare & Abu Kwaik, 2007). It has also been shown that a *L. longbeachae* serogroup 1 clinical isolate was capable of causing death and disease in guinea pigs exposed to an aerosol dose of the organism, similar to the observation with *L. pneumophila* (Cianciotto *et al.*, 1990). Furthermore, Doyle *et al.* (2001)

showed that there are distinct virulence groupings of *L. longbeachae* serogroup 1 strains based on the severity of disease produced in a guinea pig model. Previous studies have shown that the inbred mouse strains C57BL/6, BALB/c, C3H/HeN and DBA/2 are resistant to *L. pneumophila* infection, and that only A/J mice are susceptible (Brieland *et al.*, 1994; Izu *et al.*, 1999). In inbred mouse strains, permissiveness to intracellular replication of *L. pneumophila* is associated with polymorphisms in the *Naip5* allele and flagellin-mediated early activation of caspase 1 in mouse macrophages (Molofsky *et al.*, 2006; Wright *et al.*, 2003). In contrast to *L. pneumophila*, different mouse strains, including A/J, BALB/c and C57Bl/6, are susceptible to *L. longbeachae* infection, and therefore the genetic susceptibility of mice to infection by *L. longbeachae* is apparently independent of the allelic polymorphism of *Naip5* (Asare *et al.*, 2007). In this study, we examined pathological changes in the murine lung caused by experimental *L. longbeachae* infection to improve our knowledge of the pathological mechanisms implicated in infection. Through this work, we have established a reliable murine model of legionellosis caused by *L. longbeachae* serogroup 1 to aid further investigations into the pathogenesis of *L. longbeachae* infections.

METHODS

Animal care. Female and male specific-pathogen-free, 6–10-week-old A/J, BALB/c and C57BL/6 mice were used in the experiments. The animals were obtained from the Central Animal Facility of the Medical Faculty (University of Rijeka, Croatia), kept in plastic cages and given standard laboratory rodent food and water *ad libitum*. The experiments were conducted in accordance with the guidelines found in the International Guiding Principles for Biomedical Research Involving Animals. The Ethical Committee at the University of Rijeka approved all of the animal experiments described here.

Bacterial inoculum. The three strains of *L. longbeachae* serogroup 1 (D4968, D4969 and D4973) were clinical isolates from California, Oregon and Washington State, USA, and were kindly provided by Dr Yousef Abu Kwaik (Department of Microbiology and Immunology, University of Louisville, USA; CDC, 2000). Bacteria were stored at −80 °C in 10 % glycerol broth. *Legionella* cells were used directly from buffered charcoal yeast extract agar plates (BCYE-α; Merck). A stock solution containing approximately 2×10^{10} c.f.u. *Legionella* ml^{-1} was used for survival experiments and 10^8 c.f.u. ml^{-1} for other experiments, and was used immediately. The number of viable bacteria in the inoculum was confirmed by enumeration of the bacteria on BCYE-α agar.

Animal inoculation. Mice were inoculated intratracheally according to a previously described protocol (Brieland *et al.*, 1994; Susa *et al.*, 1998). Briefly, the mice were anaesthetized by intraperitoneal injection of ketamine (2.5 mg per mouse) and the trachea was isolated. A 50 µl water suspension containing a total of 10^3–10^9 c.f.u. was inoculated directly into the trachea. Control animals were mock-infected with heat-inactivated bacteria or inoculated with sterile water.

Assessment of virulence and quantification of *L. longbeachae* in mice. For enumeration of bacterial load at the indicated time points following inoculation, the mice (three animals per group) were euthanized by cervical dislocation. Independent experiments were performed at least three times. To enumerate viable bacteria in the lungs of infected mice, the organs were removed aseptically, minced and homogenized with 5 ml sterile water, centrifuged and resuspended in 1 ml sterile water. Determination of c.f.u. in the different tissues was carried out by using a serial dilution method. Results were expressed as the number of c.f.u. per lung homogenate.

Histopathological analysis and immunostaining. Following euthanasia, the lungs were instilled with buffered 4 % paraformaldehyde for 10 min and placed into 4 % paraformaldehyde for fixation. Fixed lungs were mounted in paraffin, and sections were cut and prepared for staining. Sections from the lungs were stained with haematoxylin and eosin. Alternatively, the hydrated lung sections were treated with 3 % hydrogen peroxide in 70 % methanol to block endogenous peroxidase. Slides were then incubated with an appropriate dilution of primary antibody (rabbit anti-*L. longbeachae* polyclonal serum, kindly provided by Dr Yousef Abu Kwaik). Controls were performed in which non-specific rabbit serum was substituted for the primary antibody to ensure the specificity of the staining. The slides were incubated with a biotin-labelled secondary anti-rabbit antibody (Vectastain Elite ABC kit; Vector Laboratories) for an additional 60 min, washed and incubated with streptavidin–peroxidase for 20 min. Visualization was achieved by incubation with 0.05 % 3.3′-diaminobenzidine tetrahydrochloride. The slides were counterstained with Mayer's haematoxylin.

Evaluation of histology and immunohistochemistry. The sections were analysed using an Olympus BX51 microscope. Histology and immunohistology were analysed at magnifications of ×100, ×400 and ×1000. The figures show representative examples from five to seven experiments using three mice per group.

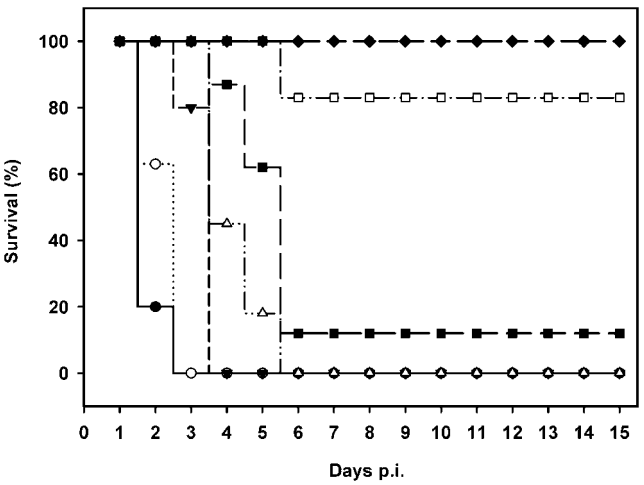

Fig. 1. Survival of A/J mice inoculated intratracheally with 10^3 (◆), 10^4 (□), 10^5 (■), 10^6 (△), 10^7 (▼), 10^8 (○) or 10^9 (●) c.f.u. of *L. longbeachae* (isolate D4968). The death of animals is plotted as percentage survival rate against the number of days after exposure to bacteria. The graph represents a typical result obtained from five to ten animals per group. The experiments were repeated at least three times for each isolate of *L. longbeachae* (D4968, D4969 and D4973). Statistical groupings were assigned based on time to death.

Statistical analysis of data. The number of bacteria in each experiment was estimated by dividing the sum of bacterial counts for different dilutions by the sum of the dilution factors. Datasets were analysed using the Kruskal–Wallis comparison test followed by Student's *t*-test. Significance was assigned where $P < 0.05$.

RESULTS AND DISCUSSION

Assessment of virulence of *L. longbeachae* serogroup 1 isolates

Experimental animals (five to ten animals per group) were challenged with 10^3–10^9 c.f.u. *L. longbeachae* (Fig. 1). Irrespective of the *L. longbeachae* isolate (D4968, D4969 or D4973) used for inoculation, the inocula of 10^9, 10^8, 10^7 and 10^6 c.f.u. *Legionella* were lethal for all tested mice. In groups of mice challenged with 10^5 c.f.u. of each *L. longbeachae* strain, approximately 10 % of the animals eventually cleared the infection and survived. An inoculum of 10^5 c.f.u. *L. longbeachae* therefore appeared to be at the limit of control by the host immune system. An inoculum of 10^4 c.f.u. had a moderate effect, although sporadic death of mice was still observed in each test group. Mice infected with 10^3 c.f.u. of each *L. longbeachae* strain developed a respiratory infection but all survived. In contrast to the results of Doyle *et al.* (2001), we did not observe differences in virulence between the tested strains. However, *in vivo*, *L. longbeachae* showed at least a 100-fold higher infectivity potential than *L. pneumophila* for A/J mice (Susa *et al.*, 1998). Such a low inoculum leading to a lethal infection is, to our knowledge, unusual for *Legionella* species. Interestingly, whilst most other *Legionella* infections are associated with a contaminated water source, *L. longbeachae* resides in soil, and our data suggest that the aspiration of even very low doses of *L. longbeachae* from soil may lead to infection in humans.

Replication of *L. longbeachae* serogroup 1 in the lungs of mice

In order to determine the *in vivo* growth kinetics of different *L. longbeachae* serogroup 1 isolates in the lungs of experimental mice (A/J, BALB/c and C57BL/6), the animals were inoculated with 10^3 or 10^5 c.f.u. bacteria and the

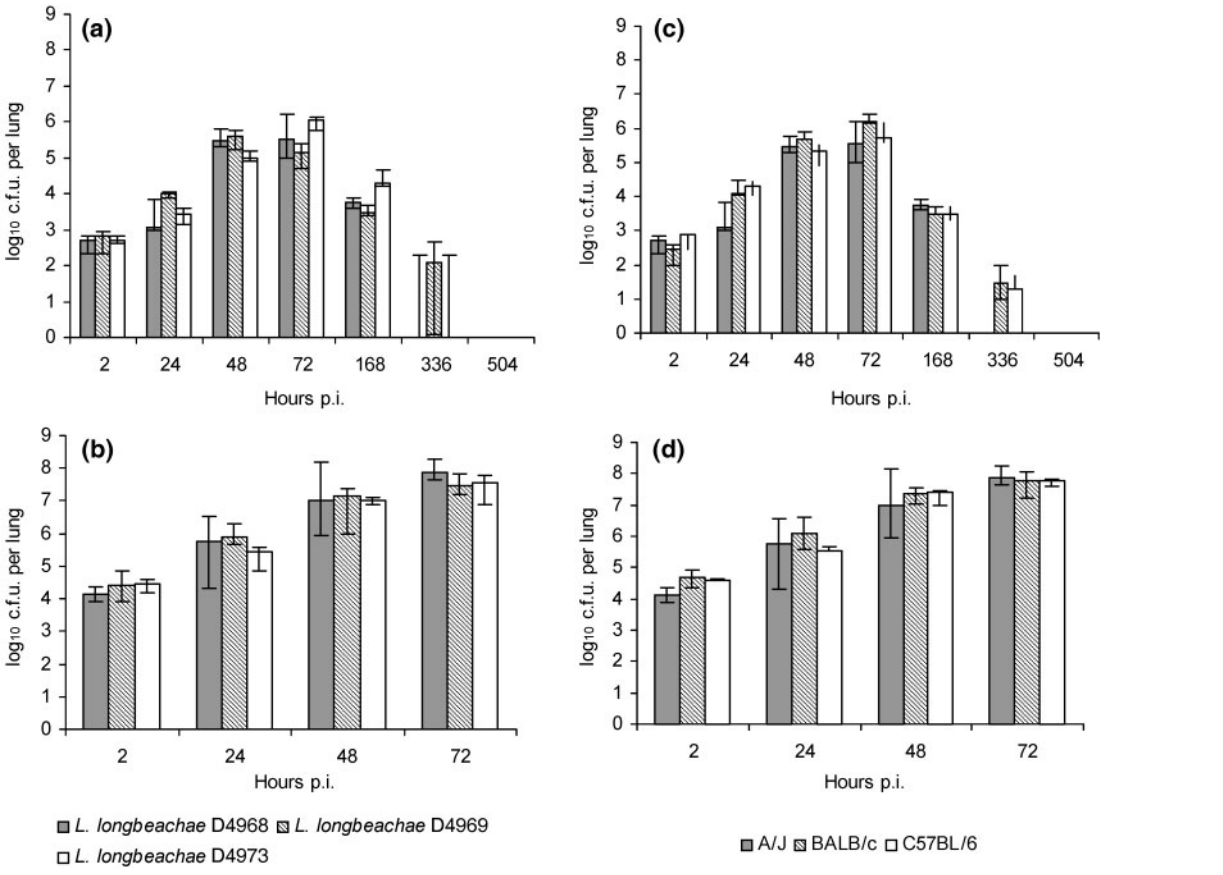

Fig. 2. (a, b) *In vivo* growth of different *L. longbeachae* isolates (D4968, D4969 and D4973) in the lungs of A/J mice inoculated with 10^3 c.f.u. (a) and 10^5 c.f.u. (b). (c, d) *In vivo* growth of *L. longbeachae* D4968 in the lungs of A/J, BALB/c and C57BL/6 mice inoculated with 10^3 c.f.u. (c) and 10^5 c.f.u. (d). The graphs show representative c.f.u. data obtained from three lungs of three to five experiments and are presented as the median value and the range of maximal and minimal values.

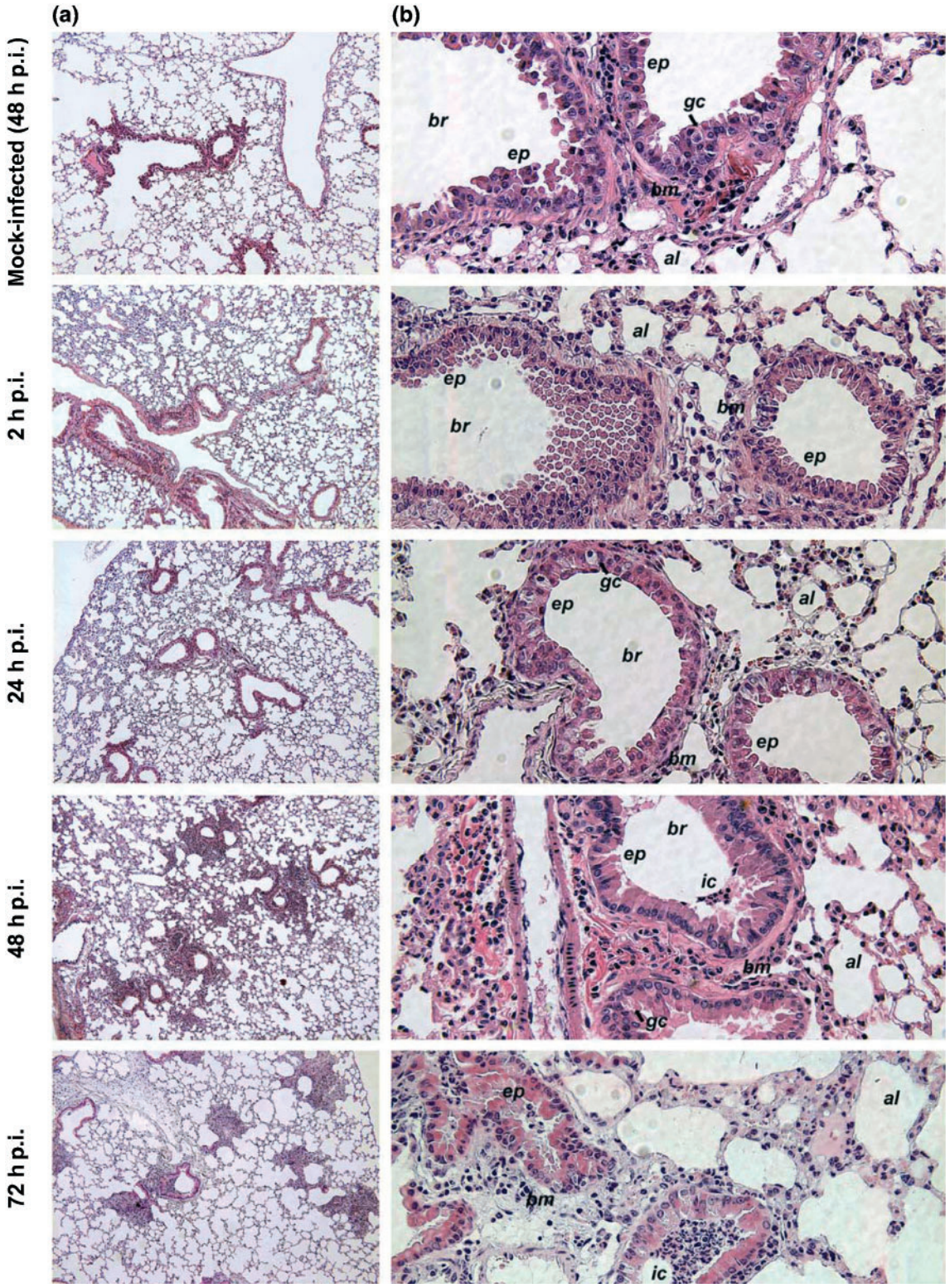

Fig. 3. Representative histopathology of the lungs during infection of A/J mice with 10^5 c.f.u. viable or heat-inactivated (mock infection) *L. longbeachae* serogroup 1 (strain D4968). Experiments were repeated three times. The sections were analysed under ×100 (a) or ×400 (b) magnification. The lungs of mock-inoculated mice did not show any histopathological changes at the analysed time points. al, alveolar spaces; bm, basement membrane; br, bronchioli; ep, bronchiolar columnar epithelium; gc, goblet cell; ic, inflammatory cells.

number of c.f.u. in the lungs was determined at different time points (three mice per group) (Fig. 2). Within the lungs, all tested strains of *L. longbeachae* showed exponential growth during the first 3 days post-infection (p.i.) (Fig. 2a, b). Mice infected with 10^3 c.f.u. were able to reduce the bacterial burden in the lungs below the detection level of the assay (Fig. 2a, c). However, in some animals infected with 10^3 c.f.u., we were able to detect *L. longbeachae* in the lungs for up to 21 days after intratracheal inoculation (data not shown). Again, there were no significant differences between the tested strains of *L. longbeachae* serogroup 1 (Fig. 2c, d). In addition, irrespective of the initial inoculum size or genetic background of the mouse strain, the *in vivo* growth index of *L. longbeachae* was approximately 1000, which was measured by comparison of bacterial yield in the lung 72 and 2 h after inoculation. As already demonstrated, *L. longbeachae* serogroup 1 replicates equally well in the lungs of A/J, BALB/c and C57BL/6 strains of mice (Asare *et al.*, 2007). In summary, we demonstrated that all of the tested *L. longbeachae* strains caused a productive pulmonary infection after inoculation of only 10^3 c.f.u.

Histopathological changes in the lungs during infection with *L. longbeachae* serogroup 1

In this study, we characterized lung lesions caused by *L. longbeachae* serogroup 1. All strains tested caused very similar changes in the lungs of A/J, BALB/c and C57BL/6 mice (data not shown). Fig. 3 shows the lung histology of A/J mice after intratracheal inoculation with 10^5 c.f.u. viable or heat-inactivated *L. longbeachae* serogroup 1 D4968. Two hours after intratracheal inoculation, the first changes were seen in the columnar epithelium of the bronchioles. The epithelial cells were rounded on the apical side, reduced in size and had partially lost their pseudostratified morphology. The muscularis mucosa of the basement membranes of the bronchioles was rarefied and enlarged. No infiltration was seen in the muscularis mucosa or in the peribronchiolar regions or luminal spaces of the bronchioles. At 72 h after intratracheal inoculation of *L. longbeachae* serogroup 1, the columnar epithelium was destratified and reduced to columnar basal epithelial cells. The smooth muscle layers of the muscularis mucosa were thickened, infiltrated with polymorphonuclear and

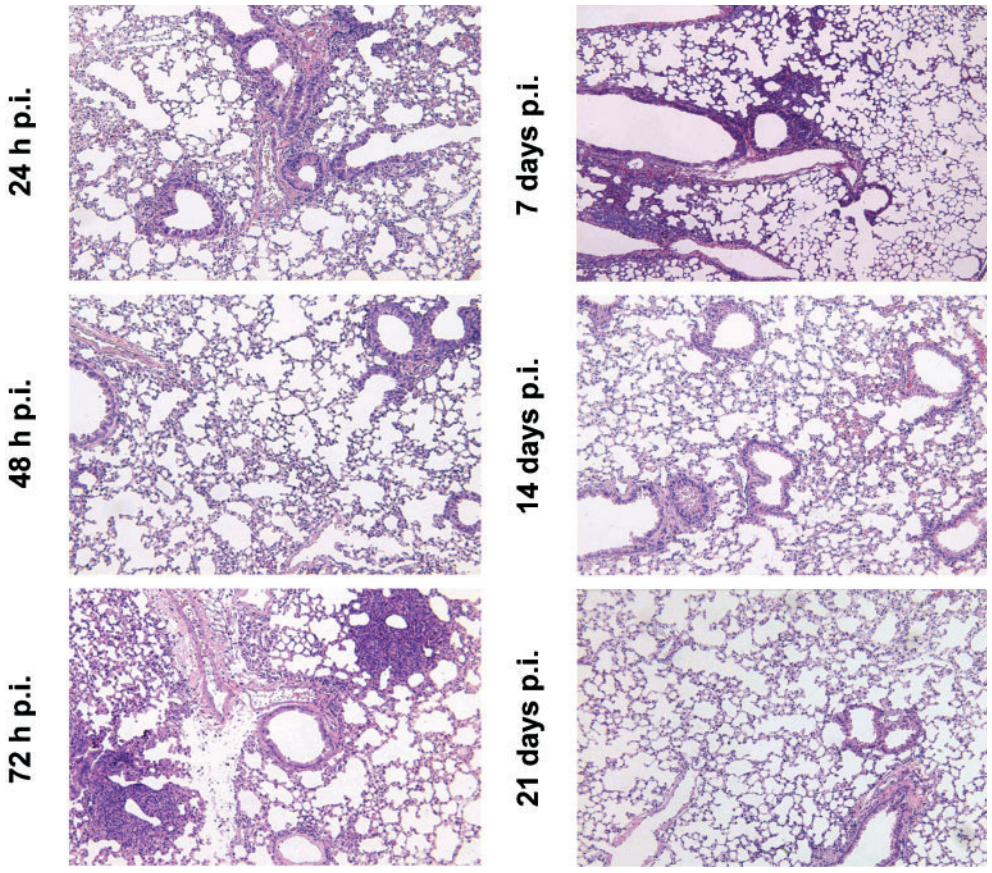

Fig. 4. Representative analysis of lung histopathology during an infection of A/J mice inoculated with 10^3 c.f.u. viable *L. longbeachae*. The sections were stained with haematoxylin and eosin. At the indicated time points, the histology was analysed under ×100 magnification.

mononuclear cells, and showed necrotic degeneration. The peribronchiolar and interstitial spaces of the alveoli close to the bronchioles were predominantly infiltrated with neutrophils, but there was no progression of infiltration towards the alveolar sacs or to the centrifugal interstitial spaces. The inflammatory cells were recruited only around the bronchioles, giving an impression of focal infiltration. The luminal sites of the bronchioles were also infiltrated with polymorphonuclear leukocytes. Blood vessel walls and intimas were thickened and infiltrated with inflammatory cells. As the lethality of mice receiving 10^5 c.f.u. *L. longbeachae* serogroup 1 (strain D4968) was very high, it was not possible to observe the late pathological changes in their lungs. Therefore, we also followed the histopathological changes in the lungs of mice inoculated with 10^3 c.f.u. *L. longbeachae* serogroup 1 (strain D4968) (Fig. 4). Although the changes were delayed, they were very similar to those of animals receiving an inoculum of 10^5 c.f.u., i.e. initial morphological changes in the epithelial cells between 24 and 48 h p.i., and focal peribronchiolar inflammatory cell infiltration with basement membrane injury 3–7 days p.i. (Fig. 4). Restoration of lung parenchyma was observed not earlier than 14–21 days after infection and correlated with the ability of mice to control the *in vivo* growth of *L. longbeachae* serogroup 1 in the lungs. To our knowledge, this is the first report that *L. longbeachae* serogroup 1 causes bronchopneumonia in different mouse strains. Recently, histopathological examination of the lungs of patients who had died of *L. longbeachae* infection indicated a purulent bronchopneumonia (Kümpers *et al.*, 2008). This is in contrast to *L. pneumophila* pneumonia in A/J mice. Brieland *et al.* (1994) showed that *L. pneumophila* caused changes mainly in the alveoli, and that the bronchi and bronchioles were essentially normal.

Immunohistochemistry for detection of *L. longbeachae* serogroup 1 (strain D4968) in lung parenchyma

The pathological changes in lung parenchyma after intratracheal inoculation of *L. longbeachae* serogroup 1 suggested that the changes correlated with the localization of bacteria. Therefore, we performed immunohistochemistry of the lungs to determine the exact localization of *L. longbeachae*. Two hours after intratracheal inoculation of 10^5 c.f.u. *L. longbeachae* (strain D4968), single stained cells, most probably macrophages engulfing the bacteria, were detected in close proximity to the respiratory epithelium. With progression of infection, the positively stained cells in the lung parenchyma increased in number. Unfortunately, we were not able to observe whether *L. longbeachae* serogroup 1 also resided within alveolar epithelial cells. In the late stages of infection, *L. longbeachae* was detected within the focal infiltrates of the bronchioli or inside their lumina (Fig. 5). However, we were not able to detect any infiltrating cells with *L. longbeachae* either within the basement membranes of the bronchioles or outside the focal peribronchiolar infiltrates. The acute injury of lower respiratory epithelium 2 h after inoculation of bacteria and early co-localization of *L. longbeachae* with the respiratory epithelium surface structures may indicate a host–pathogen interaction responsible for the morphological rearrangement.

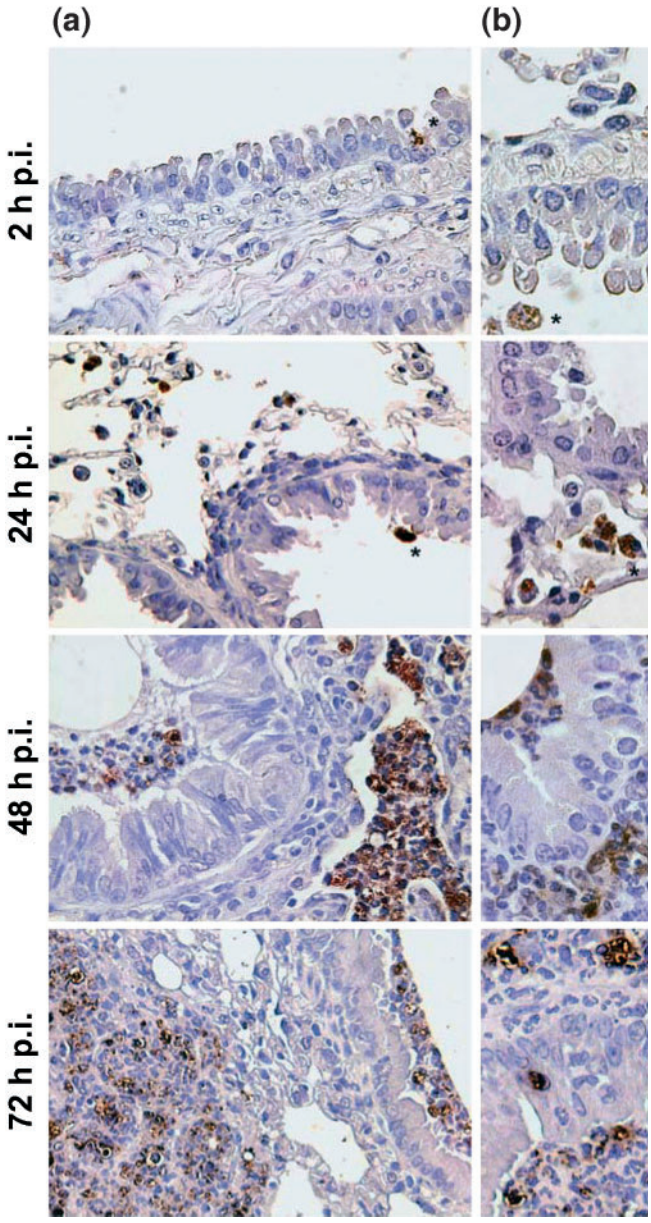

Fig. 5. Immunohistology of the lungs from A/J mice 2–72 h after intratracheal inoculation of 10^5 c.f.u. *L. longbeachae*. Representative lung slices were stained with rabbit polyclonal anti-*L. longbeachae* serum, and *L. longbeachae* was visualized using diaminobenzidine (brown) and counterstained with Mayer's haematoxylin. Magnification: (a) ×400; (b) ×1000. At the higher magnification, intracellular stained bacteria can be visualized. Positively stained cells are indicated by an asterisk. The lungs of mock-inoculated mice did not react with serum at any time point analysed (not shown).

In conclusion, *L. longbeachae* serogroup 1 is a highly virulent pathogen in mice. The bronchopneumonia caused by *L. longbeachae* serogroup 1 was not restricted to a particular genetic background of the host mice. Within 3 days, *L. longbeachae* serogroup 1 can cause a severe bronchopneumonia characterized by a focal, predominantly neutrophil infiltration of peribronchiolar spaces and bronchiolar lumina, with degeneration of bronchiolar epithelia and basement membranes. Although mice are not natural hosts of *L. longbeachae*, our data showed that mice are a useful surrogate model for human infection. A drawback of studying host responses to *L. pneumophila* infection has been the restriction of this species to the A/J mouse background. In contrast, it will be possible to study the pathogenesis of disease caused by *L. longbeachae* serogroup 1 strains and the importance of particular immune responses to *L. longbeachae* infection in several mouse backgrounds, in particular C57BL/6 mice, for which there are many knockout and transgenic lines available. Although the ability of *L. longbeachae* serogroup 1 to infect a range of mouse strains may reflect additional or different virulence factors to *L. pneumophila*, the fact that serogroup 1 strains of *L. longbeachae* are largely non-motile may also contribute to the increased virulence of this species over *L. pneumophila*. It is well documented that mutants of *L. pneumophila* that lack flagellin have increased virulence in A/J mice and can also infect C57BL/6 mice, similar to *L. longbeachae* serogroup 1 (Molofsky *et al.*, 2006). In summary, our study characterized an intratracheal mouse infection model for *L. longbeachae*. This information will be of great value for studying the virulence and pathogenesis of *L. longbeachae* infections in comparison with *L. pneumophila*.

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