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Source / Izvornik: **Sanitarno inženirstvo, 2008, 2, 4 - 9**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:184:407557

Original scientific paper

Comparison of Virulence of *L. longbeachae* to other *Legionella* Strains in an Animal Model

Received: 12. 2. 2008.
Accepted: 6. 4. 2008.

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ABSTRACT:
Legionella species are one of the causing agents of bacterial pneumonia. *Legionella pneumophila* accounts for the vast majority of cases in most of the world, with *L. micdadei* ranking distantly second. Most of the studies has been focused on the understanding the pathogenesis of pneumonia caused by *L. pneumophila*. Little is knows about the virulence of *L. longebeachae* although it's leading cause of legionellosis in Australia. The aim of study was to determine the virulence of *L. longbeachae* in comparison to different *Legionella* strains including *L. pneumophila*, *L. micdadei* and *L. steigerwaltii.* An animal model of intratracheal infection was established on A/J mice. Our results showed that all the mice that received the dose of 10^5 CFU of *L. pneumophila, L. micdadei, L. longbeachae* serogroup 2 or *L. steigerwaltii* survived and cleared the infection while the mice infected with the same dose of *L. longbeachae* serogroup 1 developed severe bronchopneumonia and died within five days. Taken together, among all tested strains the most virulent one for mice was *L. longbeachae* serogroup 1.

KEY WORDS:
Pneumonia, *Legionella* spp, Mice

INTRODUCTION

Legionella species are one of the most common ethiological agents of bacterial pneumonia. There are 48 species and more than 70 distinct serogroups in the genus *Legionella*. About 20 *Legionella* species have been associated with human disease. *L. pneumophila* is the first isolated species, and the leading cause of legionellosis in Europe and The United States. *L. micdadei* is the second most commonly isolated member of *Legionella*, which infects immunocompromised hosts primarly [1,2]. *L. longbeachae* serogroup 1 is the most frequent causative agent of bacterial pneumonia in Australia, which contrasts with its low incidence in the rest of the world [3,4]. *L. longbeachae* serogroup 2 rarely cause disease at humans [5]. *L. steigrwaltii* hasn't been associated with human disease [6].

Most of *Legionella* species inhabits aquatic environments in amoeba as their primarily host [7,8]. Thus, whereas *L. pneumophila* and *L. micdadei* infection occurs through the inhalation of contaminated aerosols, *L. longbeachae* occurs through contaminated potting soil, which is their natural environment [9,10]. After the infection legionella enters and multiplies within the alveolar macrophages. The intracellular infection of mammalian and protozoan cells with different *Legionella* strains have been addressed before [6,11,12]. Our previous studies showed a high infectivity potential of *L. longbeachae* serogrupe 1 in a mice model [13]. There are just a few papers that described the virulence of different *Legionella* strains in an animal model of infections [13-15]. However, these studies have examined only limited number of strains. In this study, we compared virulence of *L. longbeachae* serogroup 1 with the virulence of *L. pneumophila*, *L. micdadei*, *L. longbeachae* serogroup 2 or *L. steigerwaltii*.

L. pneumophila and *L. micdadei* infection occurs through the inhalation of contaminated aerosols, *L. longbeachae* occurs through contaminated potting soil, which is their natural environment.

METHODS

Bacterial strains

L. longbeachae serogroup 1 (clinical isolate), *L. longbeachae* serogroup 2 (clinical isolate), *L. pneumophila* (AA100), *L. micdadei* (clinical isolate), *L. steigerwaltii* (environmental isolate) have been used in all of the experiments. All strains were kept frozen at -80°C in sterile tap water containing glycerol 10% v/v.

Infection of mice and quantitation of bacteria in the lung tissue

Male and female pathogen free A/J mice 6 – 8 weeks old were used in whole experiment. All mice were housed in the animal facility at University of Rijeka. The experimental protocol was approved by the institutional animal care and use committee of the Ministry of Science, Education and Sports of Republic of Croatia.

For the preparation of the intratracheal inoculation, *Legionella* strains were grown on BCYE agar plates for 3-5 days. The mice were inoculated intratracheally with 50 μl of bacterial suspension (10^5 CFU/mouse) or sterile tap water (control) as has been described previously [15]. At

specific time points (2, 24, 48, and 72 hours) after intratracheal inoculation of *Legionella*, mice were humanely euthanized. The lungs were removed, and the bacteria were cultured on BCYE agar for 3-5 days. The number of CFU in the lungs was determined by plate dilution method using BCYE agar. For survival assay, the mice were observed for 14 days after infection.

Histopathological analysis

The mice were humanely sacrificed 72 hours after inoculation. Before lung removal, the pulmonary vasculature was perfused with 10 ml of saline containing 5 mM EDTA, via the right ventricle. The excised lungs were inflated and fixed in 10% neutral formalin for 24 h, dehydrated, and embedded in parafin. Sections (5 μm) were cut and stained with hematoxylin-eosin and examined for pathologic changes by light microscopy.

RESULTS AND DISCUSSION

Previous studies have shown that among inbred mice strains, A/J mice is the only inbred mice strain susceptible to *L. pneumophila* infection, while all the other strains are resistant [16]. In this report, we have addressed great differences in mortality rate of A/J mice inoculated with different legionella. As shown previously in our recent paper, as low as 10^5 CFU of *L. longbeachae* were lethal to mice [13]. In this study we compared effect of other *Legionella* strains implicated in the dose of 10^5 CFU/mouse (Fig. 1). Our results showed that all the mice infected with *L. pneumophila*, *L. longbeachae* serogroup 2, *L. micdadei* and *L. steigerwaltii* survived the infection, while 90% of the animals infected with *L. longbeachae* serogroup 1 died within 7 days after infection (Fig. 1). In comparable studies the LD_{50} and LD_{90} for A/J mice inoculated with *L. pneumophila* and *L. micdadei* were 10^7 and 10^9 CFU respectively (data not shown). We concluded that, *L. longbeachae* serogroup 1 caused a more severe infection in A/J mice compared to other *Legionella* strains

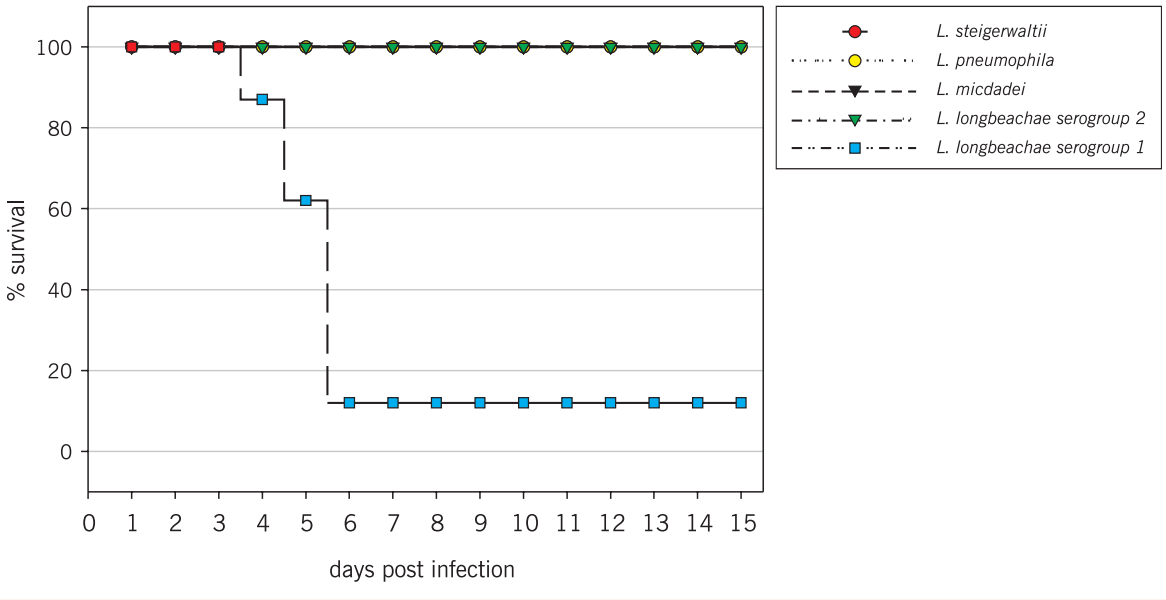


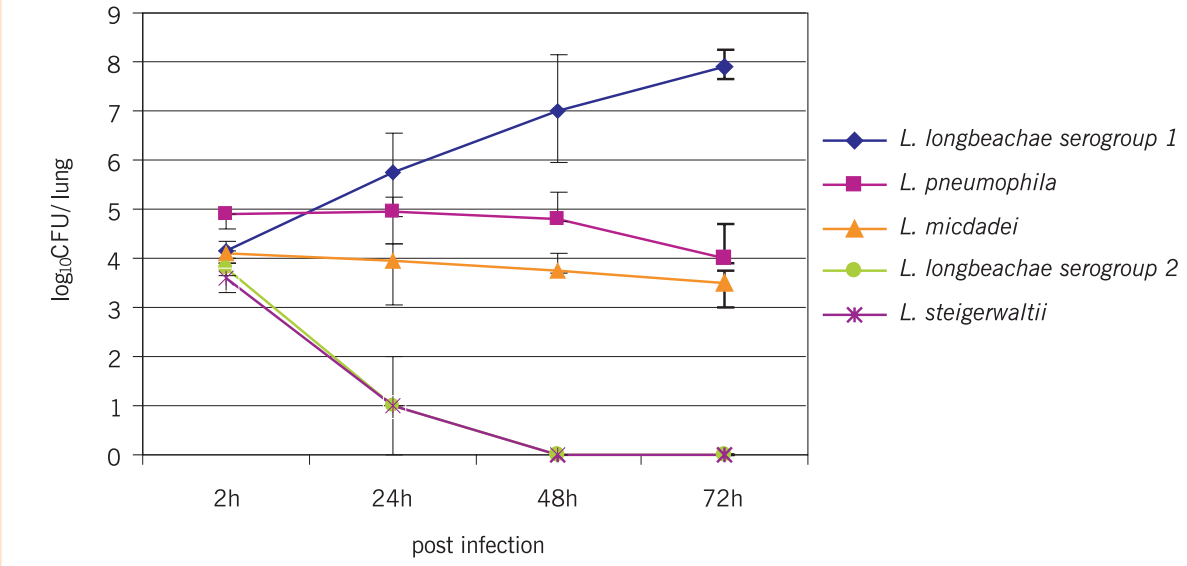
Figure 1. Mortality assay.
The A/J mice were infected with 1 x 10^5 CFU of *L. longbeachae* serogroup 1, *L. pneumophila, L. longbeachae* serogroup 2, *L. micdadei* or *L. steigerwaltii*. Lethality was monitored over 14 days. The experiment was done in triplicate with 10 mice per group.

(*L. pneumophila, L. longbeachae* serogroup 2, *L. micdadei* and *L. steigerwaltii*).

The genetic susceptibility has been attributed to a polymorphism in the neuronal apoptosis inhibitory (naip5) allele of chromosome 13 [17]. *L. longbeachae* serogroup 1 has been shown to cause disease and death in guinea pigs exposed to an aerosol infection [18]. Previous studies have shown that in contrast to *L. pneumophila, L. micdadei* fails to replicate intrapulmonarly in A/J mice [14]. Our recent results showed that susceptibility of mice to infection by *L. longbeachae* is independent of polymorphism in the naip5 allele [13]. To determine that different lethality of mice was due to different replication of bacteria in the lungs, A/J mice were infected with the dose of 10^5 CFU of *L. longbeachae* serogroup 1, *L. longbeachae* serogroup 2, *L. pneumophila, L. micdadei* or *L. steigerwaltii* and the number of CFU was determined over a 72 h period. As shown in Fig. 2 we detected rapid replication of *L. longbeachae* serogroup 1 in the lungs of A/J mice. At 72 hours after infection of the A/J mice with *L. longbeachae* serogroup 1 the number of bacteria in the lungs reached 10^7 – 10^8 CFU per mice (Fig. 2). In contrast, *L. pneumophila* and *L. micdadei* did not replicate in the lungs of A/J mice and the numbers of bacteria were constant during 72 hours (10^4 – 10^5 CFU/lung) (Fig. 2). In addition, A/J mice cleared infection with *L. longbeachae* serogroup 2 and *L. steigerwaltii* already 24 hours after inoculation (Fig. 2). The results of this study showed that the clearance of *L. pneumophila* infection was dose dependent which was not the case with the infection caused by *L. micdadei*. Our previous results showed that infection of A/J mice with the dose of 10^6 *L. pneumophila* resulted in an increase in the number of bacteria in the lung by 100 fold within 2-3 days of infection [13]. In contrast to *L. pneumophila, L. micdadei* failed to replicate intrapulmonary in A/J mice with that dose infection as well [14]. In summary, our results showed that in contrast to *L. longbeachae* serogroup 1, all tested strains could not efficiently multiply in the lungs of A/J mice after intratracheal inoculation of 10^5 bacteria/mouse.

Figure 2. Growth kinetics.

The A/J mice were intratracheally inoculated with 10^5 CFU of *L. longbeachae* serogroup 1, *L. pneumophila*, *L. longbeachae* serogroup 2, *L. micdadei* or *L. steigerwaltii*. Lungs were harvested, and the number of CFU in the lungs was determined at the indicated time points. The results are representative of three independent experiments and error bars represent the range from minimal to maximal values.



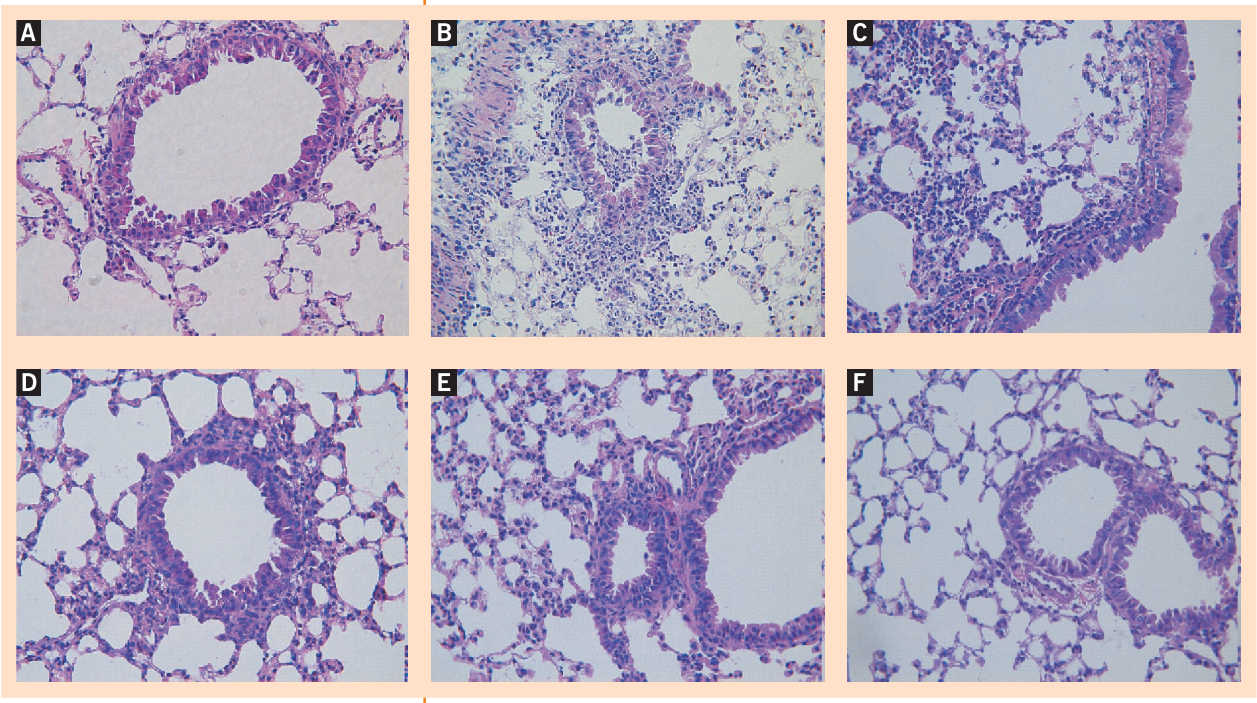


Figure 3. Pulmonary histopathology.

Control A/J mice were intratracheally inoculated with sterile water (A) while other A/J mice were inoculated with 10^5 CFU/mouse of *L. longbeachae* serogroup 1 (B), *L. pneumophila* (C), *L. micdadei* (D), *L. longbeachae* serogroup 2 (E) or *L. steigerwaltii* (F). At 72 hours p.i. the lungs were harvested, fixed in formalin, dehydrated, and embedded in parafin. Sections were cut and were stained with hematoxylin-eosin and examined for pathologic changes by light microscopy. We used magnification of 400x. The results are representative of three independent experiments.

The A/J mice have been used by many investigators to study the pathogenesis of legionelosis. Most of the studies undertaken to understand pathogenesis of *Legionella* have focused on *L. pneumophila*. We and others have shown that *L. pneumophila* caused alveolar pneumonia in the A/J mice with the dose of 10^6 CFU/ mouse [15,19-21]. Some other studies showed that *L. longbeachae* serogroup 1 is capable of causing severe acute pneumonia in guinea pigs [18.]. In this study we examined the histopathological changes in the lungs of A/J mice after intratracheal inoculation of 10^5 of different legionella/mouse at 72 h after infection (Fig. 3). Our results showed that *L. longbeachae* serogroup 1 caused a severe bronchopneumonia characterized with a focal, predominant-neutrophil infiltration of peribronchiolar spaces and bronchiolar lumina as well as degeneration of bronchiolar epithelia (Fig. 3). In contrast, *L. pneumophila* caused inflammation of the cells in the interstitial spaces (Fig. 3). *L. micdadei* caused mild infiltration of the cells in the interstitium while *L. longbeachae* serogroup 2 and *L. steigerwaltii* caused minimal pathohistological changes in the lungs of infected animals (Fig. 3). In conclusion, the infection dose of 10^5 bacteria/mouse caused histopathological changes in the lungs of mice infected only with *L. longbeachae* while in all other tested strains only mild to minimal pathological changes were observed.

In summary, we have shown that lethality, intrapulmonary replication, pulmonary histopathology by *L. longbeachae* serogroup *1, L. longbeachae* serogroup 2*, L. pneumophila, L. micdadei* and *L. steirgewaltii* are different in an A/J model of infection. In this model *L. longbeachae* serogroup 1 seems to be the most virulent *Legionella* species.

Acknowledgment

This study was financially supported by the Ministry of Science, Education and Sport of Croatia (Project 062-0621273-1275).

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