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Systemic Character of Legionnaires' Disease – A Murine Model*

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Summary

In contrast to lung infection caused by *Legionella pneumophila*, little is known about the pathogenesis of this disease in other organs. In this study, we analyzed the number of colony forming units (CFU) of legionellae not only in lungs but also in EDTA plasma, liver, spleen and kidneys. The number of CFU was determined 2, 24, 48, 72 and 168 h after intratracheal inoculation. Results showed that the inflammatory response was mostly pronounced in lungs. Legionellae, however, were also found in EDTA plasma and all the other investigated organs. The duration of infection was most protracted in lungs, with persistence for at least 168 h. In the remaining organs, legionellae were found for a maximum of 72 h after inoculation. Besides the culture methods used for detection of CFU we also used LightCycler (LC) PCR to confirm the presence of bacteria in the blood of intratracheally infected mice. By this method the bacterial DNA could be detected during the first two days of post infection.

Key words: Legionnaires' disease, systemic disease, animal model, real-time polymerase chain reaction

Introduction

Legionnaires' disease was first recognized during an outbreak of pneumonia involving delegates to the 1976 American Legion convention at a Philadelphia hotel. In January 1977, a Gram-negative bacterium was isolated and designated *Legionella pneumophila* (*1*). *L. pneumophila* is a facultative intracellular bacteria naturally found in water as a parasite within protozoa. Pathogens that are able to survive in the environment for extended periods tend to be relatively virulent. The virulence of legionellae may be increased by replication in amoeba (*2*,*3*). Transmission to humans occurs by inhalation of contaminated aerosols generated by numerous man-made devices, such as showerheads, air-conditioners, mist machines in grocery, and medical and dental equipment (*4*,*5*).

As diagnostic methods have improved and epidemiology of the reservoir has been investigated, legionellae have been found to be a common cause of community-acquired and nosocomial pneumonia (*6*). Although the lungs are the most common sites of infection with

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legionellae, an important subset of patients have infection at extrapulmonary sites. Dissemination of *L. pneumophila* is incompletely understood due to the lack of an efficient animal model for studying the systemic character of the disesase.

The aim of this study was to investigate the systemic character of *L. pneumophila* infection in A/J mice, induced by intratracheal inoculation of virulent bacteria. The CFU in the organs was determined. We compared the culture to a molecular assay for qualitative detection of *L. pneumophila* in EDTA plasma of A/J mice.

Materials and Methods

Animals

Female pathogen-free A/J mice, 8 to 9 weeks of age, were used for all experiments. They were housed in our animal facility according to standard guidelines.

Bacteria

The *L. pneumophila* Philadelphia strain 1, serogroup 1, (35133; American Type Culture Collection, Rockville, MD) was cultured on BCYE plates (Merck, Darmstadt, Germany) for 24 to 36 h. Bacteria were washed by centrifugation in sterile saline at 4 °C and resuspended for the appropriate concentration ($1.0 \cdot 10^7$ CFU).

Inoculation of animals

Intratracheal inoculation of mice was done according to the previously described protocol (*7*,*8*). Briefly, the mice were narcotized by intraperitoneal injection of ketamine (2.5 mg/mouse). A total of 50 μL of the bacterial suspension in sterile water ($1.0 \cdot 10^7$ CFU) was inoculated directly into the trachea using a 26-gauge needle followed by 10 to 20 μL of air. The skin incision was surgically closed. Control animals were inoculated with saline only.

Quantitation of L. pneumophila in mice

Mice were humanely sacrificed 2, 24, 48, 72 or 168 h after the intratracheal inoculation. Subsequently, lungs, spleen, liver, and kidneys were aseptically excised, minced, and homogenized in a tissue homogenizer with 5 mL of sterile distilled water. Blood was taken aseptically from the heart. All the blood was collected in EDTA 3.0 mL tubes. Within 2 h of drawing the blood, tubes were centrifuged at 1500 g for 20 min at room temperature. After centrifugation, plasma aliquots were prepared, and were immediately frozen at –70 °C until testing or whole blood collection tubes were frozen at –70 °C without prior preparation. The number of CFU in the organs was determined by the plate dilution method using BCYE agar. After 5 days of incubation at 35 °C and CO_2 5 %, the colonies were counted and the results were expressed as the number of CFU per organ homogenate and EDTA plasma, respectively. Contamination of homogenates was excluded by culturing an aliquot of organ homogenate on Mueller-Hinton blood agar for 3 days. All experiments were repeated at least 3 times using 3 mice.

DNA extraction

A fully automated DNA extraction protocol, the MagNA Pure LC DNA Isolation Kit III for Bacteria and Fungi (Roche Applied Systems, Mannheim, Germany) was employed. Prior to the start of the automated extraction, bacteria lysis buffer 130 μL, internal control (IC) 2.5 μL (50 copies) and proteinase K 20 μL were added to 100 μL of sample material, followed by a 10-min incubation at 65 °C. After this, the DNA extraction protocol was done according to the manufacturer's instructions. The elution volume was set to be 100 μL.

Primers and probes

Oligonucleotides deduced from the published sequence of the 16S rRNA gene of the *Legionella* genome were used (*9*,*10*). For detection of the target sequence, hybridization probes (TIB MOL-BIOL, Berlin, Germany) were labeled with LC Red 640 at the 5' end and with fluorescein at the 3' end (Table 1). For the detection of the IC, hybridization probes (TIB MOLBIOL) were labeled with LC Red 705 at the 5' end and with fluorescein at the 3' end.

Real-time PCR on the LightCycler instrument

The real-time PCR was performed on the LightCycler instrument (Roche Applied Systems). The PCR master mix contained Fast Start Master DNA Hybridization Probes reaction mix 2 μL, $MgCl_2$ 2.4 μL (final concentration c=4 mM), forward and reverse primers, 0.2 μL each (final concentration c=0.5 μM), Leg FL and Leg LC hybridization probes, 0.2 μL each (final concentration c=0.2 μM), Neo-LC Red 705 and Neo FL of neo-hybridization probes, 0.2 μL each (final concentration c=0.2 μM) and PCR-grade sterile water to a final volume of 15 μL. A 5 μL aliquot of extracted sample was added to 15 μL of PCR master mix in each LC glass capillary. After this, LC capillaries were sealed, inserted into the specially designed LC Carousel (Roche Applied Systems), and centrifuged with 3000 rpm for 15 s. Finally, the LC Carousel was placed into the LC instrument.

The cycling protocol was run as follows: one cycle at 95 °C for 7 min followed by 55 cycles consisting of denaturation at 95 °C for 3 s, annealing at 62 °C for 10 s, and elongation at 72 °C for 20 s. The capillaries were then cooled at 40 °C for 2 s. After the final cycle, the melting curve was started at 50 °C and the thermal chamber temperature was slowly raised to 82 °C. Fluoroscence curves were analyzed with the LC software (Ver3.5.3). Channel F2 was selected for calculation of crossing points of the target sequence, done by the automated second derivative maximum method. Channel F3 was selected for calculation of crossing points of the internal control (IC), done by the automated second derivative maximum method.

Results and Discussion

In this study, a replicative *L. pneumophila* lung infection was assessed *in vivo*, using a murine model of Legionnaires' disease in A/J mice inoculated with virulent bacteria. The rate of clearance of *L. pneumophila* from the lungs was determined by the culture of tissue homoge-

Table 1. Oligonucleotides used for real-time PCR assay

Primers and probes (sequences)	GeneBank accession no. (positions amplified)	Length (nucleotides)	G+C w/%	Melting t/°C
Primers				
Leg For (5´-AGGGTTGATAGGTTAAGAGC)	M159157 (451-470)	20	45	49.8
Leg Rev (5´-CCAACAGCTAGTTGACATCG)	M159157 (818-837)	20	50	53.4
Legionella and neo-specific hybridization probes				
Leg FL (5´-GTGGCGAAGGCGGCTACCT-FL)	M159157 (722-740)	19	68.4	64.4
Leg LC (5´-LC Red640-TACTGACACTGAGGCACGAAAGCGT)	M159157 (748-772)	25	52	65.3
Neo FL (5´- GCTGCATACGCTTGATCCGGCT-FL)	V00618 (516-537)	22	59.1	66.0
Neo LC (5´-LC Red705-CCTGCCCATTCGACCACCAAGC)	V00618 (539-560)	22	63.6	68.6

nates (Fig. 1). A mean of $3.4 \cdot 10^6$ CFU was recovered from the lungs of mice 2 h after the intratracheal inoculation of *L. pneumophila*. The maximum number of CFU was reached after 48 h. From this timepoint onwards, the number of bacteria decreased continuously. At 168 h after inoculation, bacteria (mean, $2.5 \cdot 10^3$ CFU) were still detectable in the lungs.

To determine whether these mice developed a disseminated *L. pneumophila* infection additionally, spleen, liver and kidneys of the infected mice were taken for analysis. At 2, 24 and 48 h after the intratracheal inoculation, *L. pneumophila* was recovered from all of these organs (Fig. 1). The maximum number of CFU was reached after 24 h. At 48 h after the inoculation, the number of bacteria decreased in all the investigated organs. At 72 h after the inoculation, bacteria could not be found in spleen nor in kidneys. In contrast, legionellae were always found in liver at that timepoint. This finding may correspond to the clinical presentation of Legionnaries' disease with a slight elevation of bilirubin and transaminases. Kupffer cells might serve as a suitable reservoir for legionellae. At 168 h after intratracheal inoculation, bacteria could not be detected in any of the investigated organs except for the lungs.

Extrapulmonary legionellosis is a rare event, but the clinical manifestations are often dramatic (*11*). Legionellae have been found in cases of pancreatitis, peritonitis, pyelonephritis and endocarditis (*12,13*). Dissemination apparently occurs through bacteremia. To analyze this possibility, we tested the blood of infected animals on legionellae using two different methods: culture and real-time PCR. *Legionella pneumophila* was found in the blood of A/J mice at 2, 24 and 48 h after the infection by real-time PCR (Fig. 2). Culture method gave comparable results.

Culture has been considered as the gold standard for diagnosis of *Legionella* infections. A drawback of the culture is that the results are not available for several days. In this study, a real-time LC PCR assay for qualitative detection of *Legionella* DNA in EDTA plasma was evaluated. For this assay, oligonucleotides derived from the 16S rRNA gene were employed. Specificity of 16S rRNA gene primers for the genus *Legionella* used in the

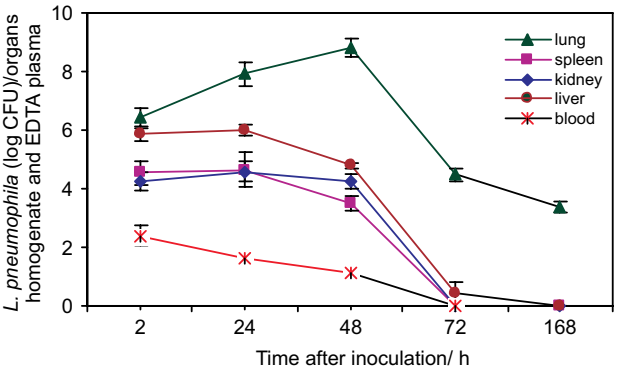

Fig. 1. Kinetics of colony counts in lung, liver, spleen, kidney and blood in A/J mice inoculated intratracheally with $1.0 \cdot 10^7$ CFU of *L. pneumophila*. Error bars represent standard deviation of the mean

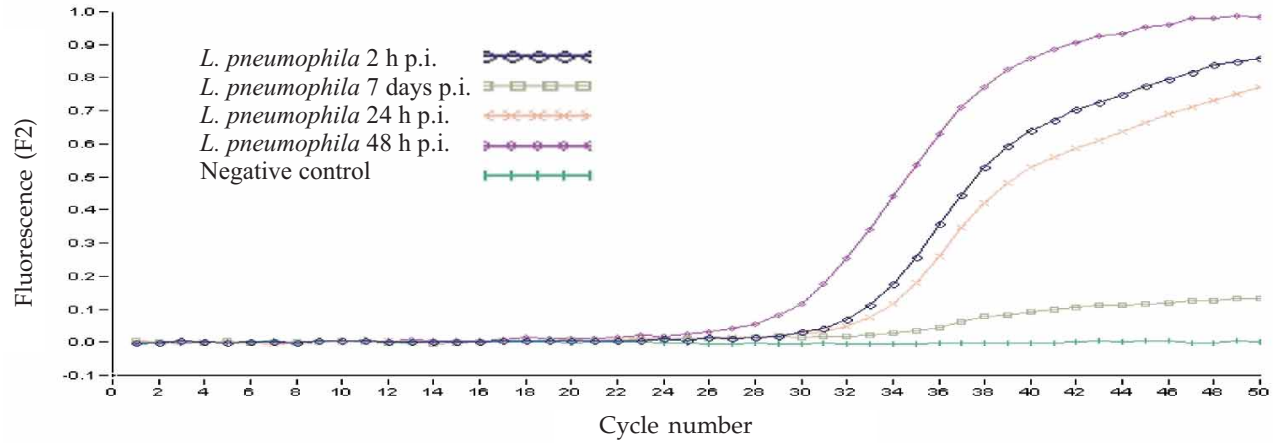

Fig. 2. Fluorescence versus cycle number plots of EDTA plasma samples containing *L. pneumophila* serogroup 1. DNA was detected on fluorescence channel F2 and the corresponding internal control-specific amplification products in channel F3 (not shown)

present study has recently been described (*14–16*). The 16S rRNA gene is more suitable as target gene than 5S rRNA because it exists in multiple copies per genome and thus improves the sensitivity and specificity of the PCR assay (*17*). Amplification may fail because of the interference with PCR inhibitors. It is, therefore, useful to incorporate an IC in every molecular assay (*18*). The IC used in this study was co-extracted with samples and co-amplified with the same primers used for the target DNA.

In conclusion, Legionnaires' disease does not only include lung infection, but also dissemination of legionellae throughout the body. Infection of liver, spleen and kidneys was detected after intratracheal inoculation of *L. pneumophila*. When inoculating $1.0 \cdot 10^7$ CFU, the infection can still be seen in the lungs 168 h later, while the duration of the infection seems to be shorter in other organs. Legionellae can also be detected in EDTA plasma by both, culture and molecular techniques. The employed real-time PCR assay offers advantages including easy and quick performance and reduction of the risk of cross-contamination.

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Sustavna obilježja legionarske bolesti – na modelu miša

Sažetak

Za razliku od plućnih infekcija koje uzrokuje *Legionella pneumophila*, malo je poznato o patogenezi ove bolesti u drugim organima. U radu je proučavan broj bakterija *Legionella pneumophila* (CFU), ne samo u plućima nego i u EDTA-plazmi, jetri, slezeni i bubrezima. CFU je određen 2., 24., 48., 72. i 168. sata nakon intratrahealne inokulacije. Dobiveni rezultati pokazuju da je upalni proces bio najizraženiji u plućima. Međutim, Legionellae su pronađene i u EDTA-plazmi i u ostalim ispitanim organima. Infekcija je najdulje trajala u plućima, i to najmanje 168 sati. U ostalim organima, Legionellae su pronađene najdulje 72 sata nakon inokulacije. Da bi se dokazala prisutnost bakterija u krvi intratrahealno inficiranih miševa, osim metode uzgoja, primijenjena je i metoda »LightCycler PCR«. Ovom metodom bakterijska DNA može se dokazati u prva dva dana nakon infekcije.