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Microbes and Infection 17 (2015) 755–760

www.elsevier.com/locate/micinf

Severe pneumonia due to *Parachlamydia acanthamoebae* following intranasal inoculation: a mice model

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Received 17 June 2015; accepted 24 August 2015
Available online 1 September 2015

Abstract

Parachlamydia acanthamoebae is an obligate intracellular bacterium naturally infecting free-living amoebae. The role of this bacterium as an agent of pneumonia is suggested by sero-epidemiological studies and molecular surveys. Furthermore, *P. acanthamoebae* may escape macrophages microbicidal effectors. Recently, we demonstrated that intratracheal inoculation of *P. acanthamoebae* induced pneumonia in 100% of infected mice. However, the intratracheal route of infection is not the natural way of infection and we therefore developed an intranasal murine model.

Mice inoculated with *P. acanthamoebae* by intranasal inoculation lost 18% of their weight up to 8 days post-inoculation. All mice presented histological signs of pneumonia at day 2, 4, 7, and 10 post-inoculation, whereas no control mice harboured signs of pneumonia. A 5-fold increase in bacterial load was observed from day 0 to day 4 post-inoculation. Lungs of inoculated mice were positive by *Parachlamydia*-specific immunohistochemistry 4 days post-inoculation, and *P. acanthamoebae* were localized within macrophages.

Thus, we demonstrated that *P. acanthamoebae* induce a severe pneumonia in mice. This animal model (i) further supports the role of *P. acanthamoebae* as an agent of pneumonia, confirming the third Koch postulate, and (ii) identified alveolar macrophages as one of the initial cells where *P. acanthamoebae* is localized following infection.

Keywords: Intracellular bacteria; *Parachlamydiaceae*; Pneumonia; Animal model

1. Introduction

Parachlamydia acanthamoebae is an obligate intracellular *Chlamydia*-related bacteria belonging to the *Chlamydiales* order. This bacteria was first isolated within amoebae during a humidifier related fever outbreak in USA [1]. The researchers investigating this outbreak also identified an association between the presence of anti-*Parachlamydia* antibodies and pneumonia. This was the first hint in favour of a potential pathogenic role of *P. acanthamoebae* in humans [1]. Then, sero-epidemiological studies and molecular surveys provided additional evidences of the role of *P. acanthamoebae* as an agent of pneumonia (reviewed in Refs. [2,3]). For example, presence of anti-*Parachlamydia* antibodies was associated with aspiration pneumonia [4], whereas parachlamydial DNA was amplified by PCR from patients with bronchiolitis or community-acquired [5–8].

Noteworthy, *P. acanthamoebae* is able to (i) circumvent the microbicidal effectors of the human macrophages, (ii) to

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http://dx.doi.org/10.1016/j.micinf.2015.08.007

replicate within these immune cells, and (iii) to induce their apoptosis [9,10]. In addition, *P. acanthamoebae* is able to persists within pneumocytes and fibroblasts [11], making the lung one likely target organ for infection following exposure to *P. acanthamoebae*. Thus, *P. acanthamoebae* represents an emerging agent of pneumonia, and an animal model is warranted to confirm the 3rd and the 4th Koch postulates.

It was already demonstrated that intratracheal inoculation of *P. acanthamoebae* induced a severe pneumonia in 100% of infected mice [12]. However, the intra-tracheal route is not a natural route of infection. Consequently, we developed a new murine model of respiratory tract infection based on intranasal inoculation.

2. Material and methods

2.1. Bacterial strain and preparation of the inocula

P. acanthamoebae strain Hall's coccus was grown in amoebae and purified as described [11]. Such amoebal co-culture is not permissive to *Mycoplasma* [13], excluding then any risk of *Mycoplasma* contamination. Concentration of living *P. acanthamoebae* in PBS was determined as described [12]. Bacteria used as a negative control were heat-inactivated during one hour at 90 °C. Mock control was prepared by the same purification process except that the amoebae were not infected with *P. acanthamoebae*.

2.2. Experimental model of infection

Fourteen mice were inoculated intranasally as previously described [14], with 2.5×10^8 living and heat-inactivated *Parachlamydia*, respectively. Groups of 2 mice were sacrificed at days 0, 2, 4, 7, 10, 14, and 21 days post-inoculation (n = 14 for each group). The right and left lung, as well as the spleen, were aseptically removed and divided in five equal parts for further investigations (see below). A blood sample for serology was obtained at the time of the sacrifice. Mice inoculated with PBS (n = 4) were used as negative controls and sacrificed 7 and 21 days post-inoculation. An additional negative control was performed using a mock preparation and mice (n = 2) were sacrificed 7 days post-inoculation. All animal experiments were approved by the Local District Government and carried out in adherence to German regulations for protection of animal life (permit: 33.42502-05/940).

2.3. Histopathology

Lungs were fixed in 4% formalin buffer, paraffin embedded, and stained with haematoxylin-eosin (H&E). Stained sections were assessed for the type and degree of pneumonia. Pneumonia was considered as interstitial when an exudate composed of inflammatory cells was seen in the alveolar interstitium, as alveolar or peribronchoalveolar when the exudate was seen within the alveolar or peribronchoalveolar space, and finally the pneumonia was considered as confluent when the exudate was seen within both interstitial and alveolar spaces.

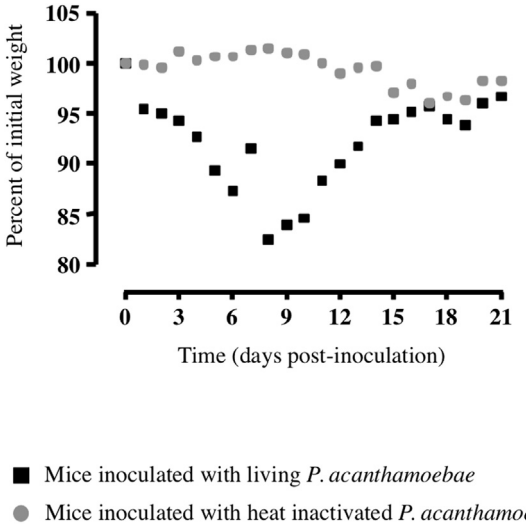

Fig. 1. Intranasal inoculation of *Parachlamydia acanthamoebae* leads to mice body weight loss. Body weight variation curves of C57BL/6 mice inoculated with $2.5.10^8$ living *Parachlamydia acanthamoebae* (triangle) or with the same load of heat-inactivated bacteria (square).

2.4. Immunohistochemistry

Paraffin sections were investigated as already described previously [15] for the presence of parachlamydial antigens by using a specific polyclonal rabbit anti-*Parachlamydia* antibody raised against purified elementary bodies, and for the presence of macrophages using a monoclonal rat anti mouse F4/80 antibody (Catlag Medsystems, Buckingham, UK), according to manufacturer's instructions.

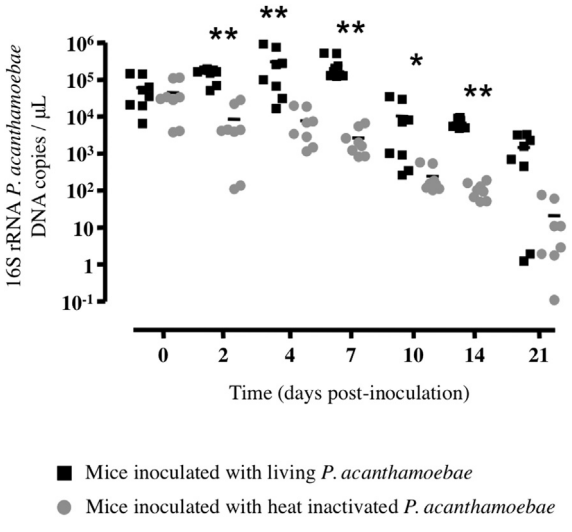

Fig. 2. Bacterial load in lungs of mice infected with *Parachlamydia acanthamoebae*. Quantification of the bacterial load in the lungs of mice inoculated with living *P. acanthamoebae* (black square) compared to mice inoculated with heat-inactivated bacteria (grey circle). P-value <0.01 and P-value<0.05 are depicted as ** and *, respectively.

2.5. Real-time PCR assay

Parachlamydial DNA was extracted from the lungs using the AquaPure Genomic DNA extraction Kit (BioRad Laboratories, Rheinach, Switzerland) according to the manufacturer's instructions. Real-time PCR was performed as described previously [16], to quantify the number of parachlamydial 16S rRNA gene copies.

2.6. Amoebal co-culture

Lungs were homogenized in a mortar and suspended in PBS. Tissue suspension was processed as described previously for amoebal co-culture [13]. Briefly, samples were inoculated on *Acanthamoeba castellanii* seeded in 24-well microplates (Corning, NY, USA), centrifuged at 1790 × g, and incubated at 32 °C. Each well was screened daily for amoebal lysis and after 6 days of subculture, the presence of *P. acanthamoebae* in all amoebal co-cultures was tested using a specific real-time quantitative PCR as previously described [16].

2.7. Serology

Two-fold diluted mice sera were tested by immunofluorescence for antibody reactivity against purified *P. acanthamoebae* as previously described [17]. Fluorescein isothiocyanate-coupled (FITC) anti-mouse immunoglobulin antibody (BioRad) was used as a secondary antibody.

2.8. Statistical analysis

The t-test was used to compare the genomic copies/5 µl between mice infected with living *P. acanthamoebae* and mice inoculated with heat-inactivated bacteria. Statistical analyses were performed with GraphPad (GraphPad Software, San Diego, CA, USA).

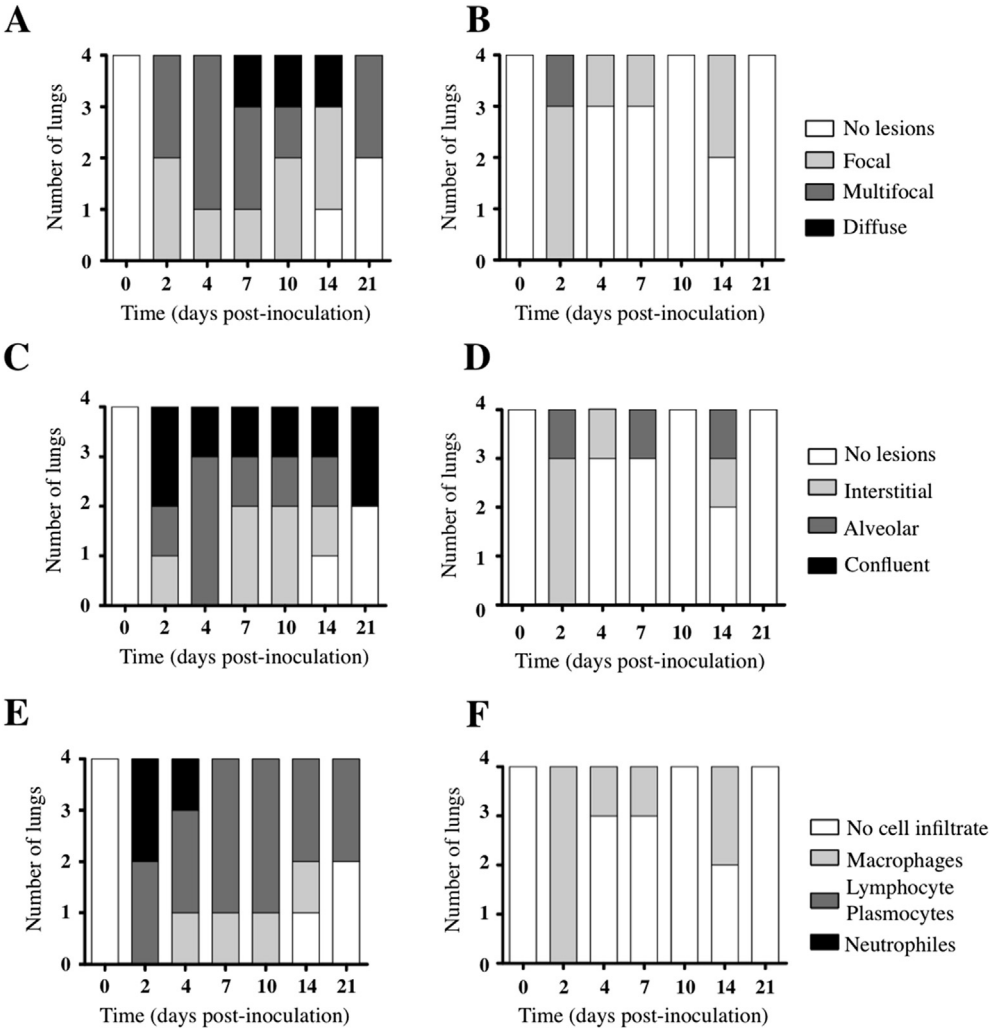

Fig. 3. Lung lesions and inflammation induced by intranasal inoculation of *Parachlamydia acanthamoebae*. A) Degree of extension of the lesions in mice lungs at different time post inoculation of *Parachlamydia acanthamoebae.* C) Inflammation degrees in lungs of mice at different time post inoculation of *Parachlamydia acanthamoebae*. E) Type of cells infiltrated in lungs of mice at different time post inoculation of *Parachlamydia acanthamoebae*. B), D), and F) are the respective mock controls.

3. Results

3.1. Clinical impact of mice infection

Following intranasal inoculation of 2.5×10^8 living *P. acanthamoebae*, mice lost 18% of their weight up to 9 days post-inoculation. Then mice started to recover weight and survived up to 21 days (Fig. 1). In contrast, mice inoculated intranasally with heat-inactivated bacteria showed no weight loss up to 21 days (Fig. 1).

3.2. Bacterial load and bacterial viability

In both groups, no death was recorded. Then, we assessed the bacterial load in the lungs by using a specific quantitative real-time PCR, revealing 100% positive up to 21 days post-inoculation in infected mice. At day 0, this bacterial load was of 10^5 bacteria/5 µl of DNA, and it increased about 5 times up to 4 days post-inoculation. We also observed a low bacterial load of about 10^2 bacteria/5 µl at 2 and 4 days post-inoculation, within spleens of infected mice. Conversely, bacterial load (determined as copies of genomic DNA) in lungs of mice inoculated with heat-inactivated bacteria decreased continually up to 21 days post-inoculation (Fig. 2). To know if bacteria recovered from lungs were viable, we inoculated amoebal cells using infected lung lysates, and we were able to isolate living *P. acanthamoebae* from lungs of infected mice up to 14 days post-inoculation. Conversely, no *P. acanthamoebae* were recovered from any lung taken from the control mice (Data not shown).

3.3. Histopathological lesions

In all mice infected with viable *P. acanthamoebae*, lung histopathology was characterized by focal to multifocal lesions at day 2 and day 4 post-inoculation. These lesions were characterized as interstitial to confluent pneumonia, with purulent exudates composed of neutrophils. At that time, mice inoculated with heat-inactivated bacteria presented few focal lesions of interstitial pneumonia, with few macrophages. From day 7 to day 21, lung histopathology was characterized by focal to diffuse lesions of interstitial to confluent pneumonia, with lymphocytes/plasmocytes exudates. At that time, interstitial and alveolar pneumonia was observed in 3 of 16 lungs of mice inoculated with heat-inactivated bacteria. Mock control mice did not exhibit any signs of pneumonia (Figs. 3 and 4).

3.4. Presence of P. acanthamoebae within the lesions

Lungs of mice inoculated with living *P. acanthamoebae* were positive for *Parachlamydia*-specific immunohistochemistry from day 0 to day 14 post-inoculation (Fig. 5A) with a

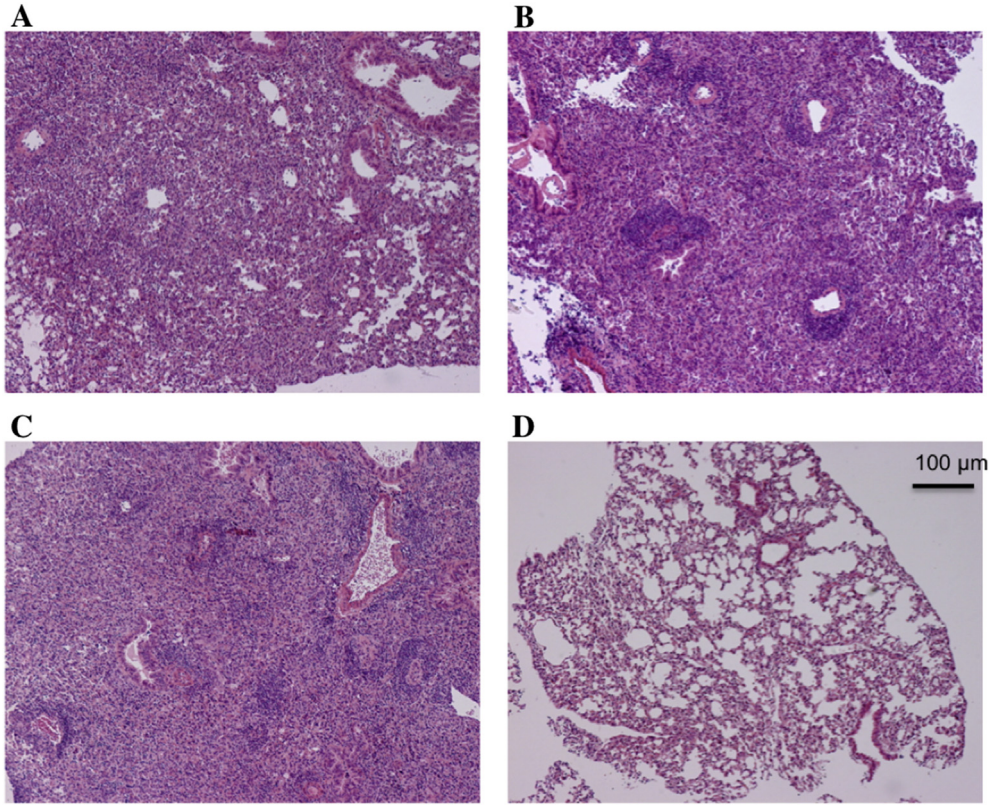

Fig. 4. Lung histopathological damages induced by intranasal inoculation of *Parachlamydia acanthamoebae*. A) Lung histopathology of a mouse infected with living *P. acanthamoebae* 10 days post-inoculation (magnification 100×). Mouse presenting an interstitial pneumonia with macrophages. B) Lung histopathology of a mouse infected with living *P. acanthamoebae* 10 days post-inoculation (magnification 100×). Mouse presenting a confluent pneumonia with monocytes and macrophages. C) Lung histopathology of a mouse infected with living *P. acanthamoebae* 2 days post-inoculation (magnification 100×). Mouse presenting a confluent pneumonia with monocytes, macrophages and neutrophils. D) Lung histopathology of a mock control mouse (magnification 100×).

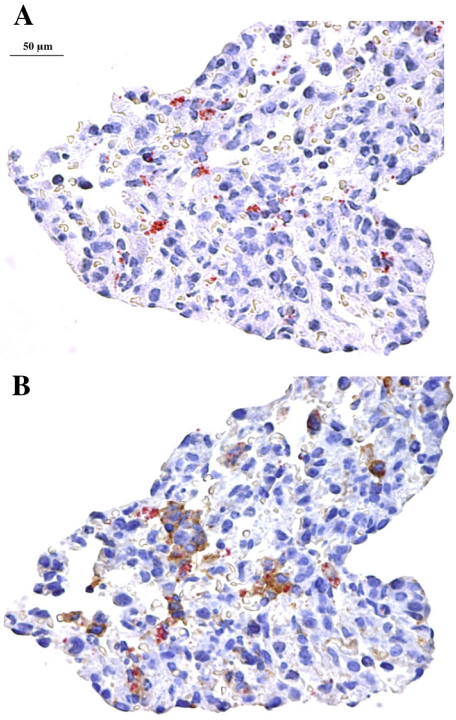

Fig. 5. *Parachlamydia acanthamoebae* are associated with immune cells within infected lungs. A) Positive immunohistochemical staining against *Parachlamydia acanthamoebae* in the lung of a mouse infected with living *P. acanthamoebae*, magnification 400×. B) Positive immunohistochemical staining against monocytes/macrophages in the lung of a mouse infected with living *P. acanthamoebae*, magnification 400×.

staining intensity that was correlated with qPCR results (i.e. larger amount at day 4). Interestingly, we could observe, on adjacent paraffin sections, a colocation between *P. acanthamoebae* and macrophages at day 7 post-inoculation (Fig. 5A and B).

3.5. Serological response

Mice sera were tested by immunofluorescence for anti-*Parachlamydia* antibody reactivity. Serology was negative (antibody titer < 1/32) from day 0 to day 7 in mice inoculated with living *P. acanthamoebae*. On day 10 and 14, positive serology was observed in mice inoculated with living bacteria (Fig. 6). Conversely, serology was negative from day 0 to day 14 in all mice inoculated with heat-inactivated bacteria. This was somehow expected since a similar result was already observed after intratracheal inoculation of heat-inactivated *P. acanthamoebae* [12]. Moreover, no anti-*Parachlamydia* antibody was detected in PBS- or mock-inoculated controls.

4. Discussion

In the present work, we demonstrated that following intranasal inoculation, *P. acanthamoebae* induce a severe pneumonia in mice. This is in line with a previous murine model showing that *P. acanthamoebae* may cause pneumonia in 100% of mice after intratracheal inoculation [12]. Intranasal

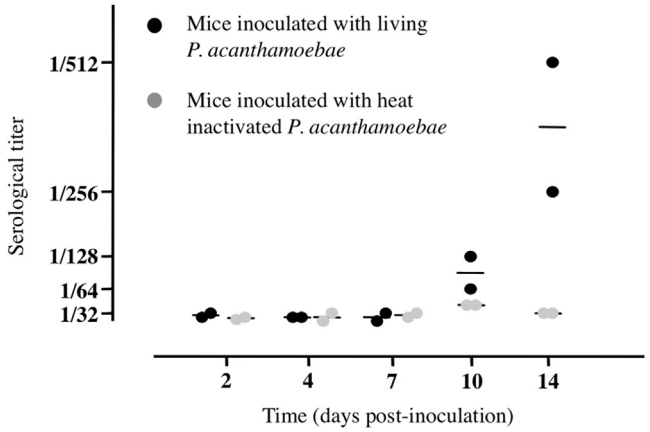

Fig. 6. Antibody response after intranasal inoculation of *Parachlamydia acanthamoebae*. Serological titer of specific anti-*Parachlamydia* antibodies in mice inoculated with *Parachlamydia acanthamoebae* (black dots), and mice inoculated with heat inactivated bacteria (grey dots).

inoculation is less invasive and more relevant to natural respiratory tract infection. After inoculation of 2.5×10^8 bacteria, we could recover about 10^5 bacteria in the lungs at early time post infection, which actually should be considered as the real infecting dose. Signs of lethargy and ruffled fur were associated with a body weight loss of 18% of initial mice's weight. This is similar to what has been described in murine models of *Chlamydia pneumoniae* respiratory tract infections [18,19].

Noteworthy, we observed an increase in the bacterial load up to 7 days post-inoculation. Then, bacteria were progressively cleared. Two time periods were associated with the recruitment of different immune cells at the infection sites. Thus, up to day 7, pneumonia was severe and infected lungs were full of neutrophils. Then, from day 7 to day 21 post-infection, there were many mononuclear infiltrates. This suggests that *P. acanthamoebae* might stimulate migration of neutrophils and monocytes, like *Chlamydia pneumoniae* [20]. Clearance of *P. acanthamoebae* was very slow as observed for *C. pneumoniae* [19], or *Chlamydia psittaci* [21], in similar mice models, and we observed a systemic dissemination since bacterial DNA was detected in the spleen of infected mice up to 7 days post-inoculation. Interestingly, between 14 and 21 days post-inoculation, we observed a persistence of parachlamydial DNA with 10^3 DNA copy number/5 µl in the lungs. This DNA could correspond to residual DNA of dead bacteria, or DNA from alive and persistent *P. acanthamoebae*. The latter hypothesis is in accordance with the described, *in vitro* and *in vivo*, persistence of other members of the *Chlamydiales* order [22–24]. Moreover, the systemic dissemination of *P. acanthamoebae* observed here, and the documented role of *P. acanthamoebae* in bovine abortion [15,17,25], suggest that the respiratory tract might be a primary route of infection for subsequent *P. acanthamoebae* placenta infections.

Since *P. acanthamoebae* is emerging as a new pathogen of medical and veterinary importance, this new murine model might be very useful to characterize the pathogenesis of

infections due to *P. acanthamoebae*, and to assess the impact of specific genetic background by using specific knockout mice [26]. Finally, given the growing body of evidence supporting the pathogenic role of *P. acanthamoebae* as an agent of lung infections [27], the murine model we developed may be useful to test *in vivo* the susceptibility of *P. acanthamoebae* to antibiotics such as doxycycline and macrolides, which were already identified to be effective *in vitro* [28].

Conflict of interest

The authors declare to have no conflict of interest.

Acknowledgments

We thank Sebastien Aeby for technical support. Research of G. Greub's group is supported by the SNSF grant n°141050, that covers the salary of L. Pilloux.

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