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Deposition pattern of aerosolized *Legionella* using an *ex vivo* human-porcine respiratory model

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ABSTRACT

Legionella are bacteria responsible for severe lung pathologies. However how they enter and are deposited within the respiratory tract remains poorly documented. Data using animal testing led to the establishment of mathematical models allowing the estimation of aerosol dispersion risks. But direct extrapolation to humans is questionable and experimental models more physiologically representative of the inhalation route are welcome. The aim of this study was to develop a model as close as possible to the human anatomy and physiology allowing determining the deposition pattern of aerosolized *Legionella* while limiting *in vivo* experiments. To that purpose, we adapted the chimeric respiratory tract model we previously developed. This original model consisted of a replica of the human upper respiratory airways made by additive manufacturing connected to *ex vivo* porcine lungs ventilated by passive expansion, as for humans in physiological conditions. These experiments didn't imply specific animal sacrifices as pigs were bred for human consumption and lungs were considered as wastes by the slaughterhouse. Fluorescent *Legionella* were aerosolized and visualized using Cellvizio® Lab (probe-based confocal fluorescence microscope). *Legionella* were found in the whole respiratory tract. Broncho-alveolar lavages were also performed and the amount of *Legionella* reaching the thoracic region was quantified by culture and qPCR. *Legionella* were found preferentially in the left upper lobe compared to the right lower lobe. To our knowledge, it is the first time that experiments mimicking so closely human exposure by inhalation are performed while limiting animal experiments and providing a model for further *Legionella* infectious risk assessment.

1. Introduction

Legionella are Gram-negative bacteria found in various aquatic environments, especially hot water sources including domestic water systems and showers, cooling towers, decorative fountains, hot tubs, etc. (Anses, 2014; Burillo et al., 2017; Khodr et al., 2016; Phin et al., 2014). *Legionella* are usually spread through water droplets in the air. After inhalation by humans, bacteria are conveyed through the tracheobronchial tree deep into the alveoli where they infect macrophages and lung epithelial cells, potentially causing pneumonia called legionellosis including Legionnaires' disease and Pontiac fever (Burillo et al., 2017; Khodr et al., 2016; Phin et al., 2014). *Legionella pneumophila,* especially from the serogroup 1 (*Lp1*), is the main species responsible for pathologies (about 90% of the cases) (Burillo et al., 2017; Khodr et al., 2016; Phin et al., 2014). The incidence of legionellosis is estimated between 8000 and 18000 new cases each year in the United States and between 1200 and 1500 in France (Campèse et al., 2015; WHO, 2016).

The monitoring of *Legionella* consists of their detection in water samples by culture on selective glycine, vancomycine and polymyxine B charcoal yeast extract (GVPC) agar (quantification as colonies forming units (CFU) per liter, as defined by the AFNOR NF T90-431 or ISO 11731-2 standards) or by quantitative PCR (qPCR, as number of genome units (GU) per liter, as defined by the AFNOR NF T90-471 or ISO/TS 12869 standards) (Burillo et al., 2017; Légifrance, 2010). However, it is difficult to correlate the number of detected *Legionella* to an infectious risk probably due to the presence in the water samples of viable but non-culturable (VBNC) *Legionella* and to a lesser extent because of PCR and culture inhibitors (Allegra et al., 2008; Allegra et al., 2011a,b). Regulatory preventive thresholds were established empirically (O'Brien and Bhopal, 1993) based on the observation of legionellosis epidemics when a correlation between patient and environment was possible (less than 16% of the cases) or based on *in vivo* studies. The

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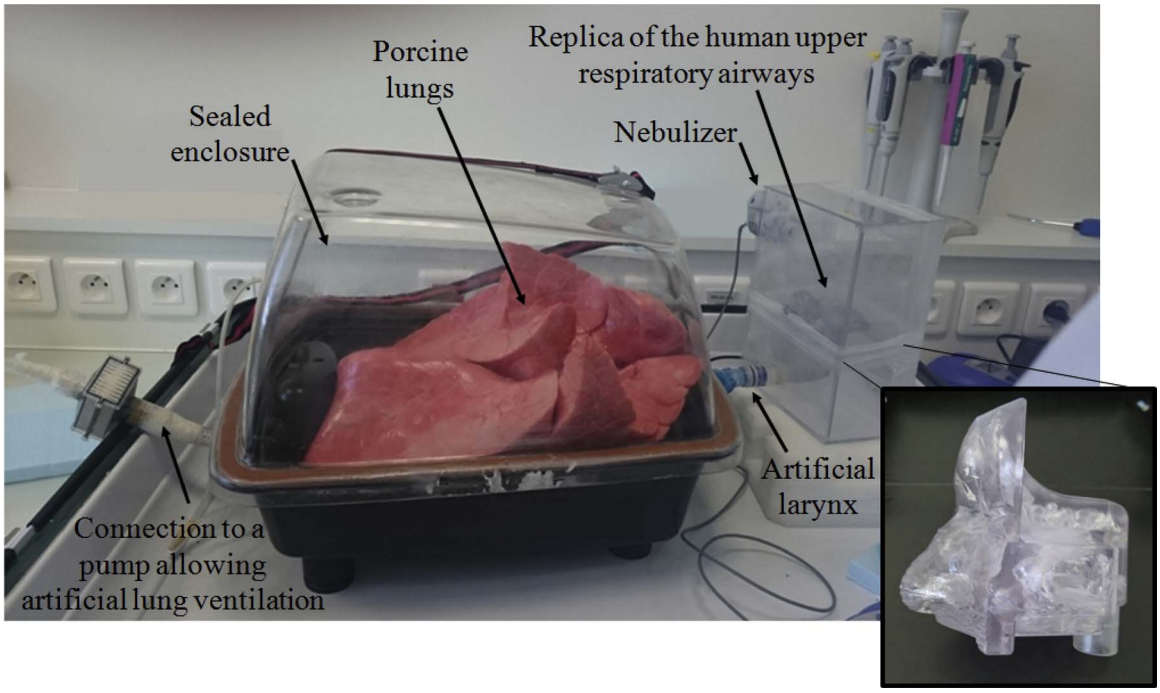

Fig. 1. Illustration of the experimental setting. In the inset, enlargement of the upper respiratory airways replica.

way infectious doses are determined depends on the reservoir studied (water or aerosols) and/or on the models used (such as animals). Mathematical models allowing the estimation of aerosol dispersion risks have been described but it is not possible to directly extrapolate their results to humans (Armstrong and Haas, 2008; Armstrong and Haas, 2007a,b). Therefore, adapted models and methodologies are required to improve *Legionella* detection and quantification to revisit or refine the infectious doses obtained from animal models. Moreover, inhalation of aerosols should be used, more representative of a physiological route than intratracheal or intraperitoneal injections or instillations. As a matter of fact, to date, how *Legionella* may enter and be deposited in the respiratory tract upon aerosol generation remains poorly documented (Górny et al., 1999; Thomas et al., 2008).

In this context, the aim of the present study was to propose an experimental model, as close as possible to the human anatomy and physiology, allowing determining the deposition pattern of aerosolized *Legionella* within the respiratory tract. These data are important to determine a real risk of inhalation exposure by the assessment of the infectious dose reaching the respiratory barrier. One of our main concerns to reach this objective was to limit animal experiments in accordance with the 3R concept introduced by Russell and Burch (Russell and Burch, 1959). Indeed, we paid attention to replace, reduce and refine our research approach by developing an alternative and very innovative experimental model. To that purpose, we adapted and optimized a chimeric respiratory model we previously developed and validated for other researches in the aerosol therapy field (Perinel et al., 2017). It consisted of a complete respiratory tract including a 3D replica of the human upper respiratory airways (nasal cavity, pharynx, sinuses) made by additive manufacturing allowing oro-nasal breathing. This human replica was connected to *ex vivo* porcine lungs through a home-made artificial larynx. It is important to mention that lungs did not come from animals bred for scientific research but from animals bred for human consumption. They were provided by a slaughterhouse as in France lungs are not fit for human consumption and are consequently considered as organic wastes. It means that no animals were sacrificed specifically for this study since we used, for scientific research, organic wastes that are currently discarded by slaughterhouse. Lungs were ventilated by passive expansion as in human physiological ventilation allowing to reproduce inhalation exposure to aerosols (El Merhie et al., 2016; Le Guellec et al., 2014; Leclerc et al., 2014a; Perinel et al., 2016, 2017). Previously, we characterized *Legionella* aerosols generated upon nebulization using a fluorescent *Legionella* strain (Allegra et al., 2016). This strain was aerosolized in the chimeric model as ventilation was ongoing and then visualized using the Cellvizio® Lab technology (probe-based confocal fluorescence microscope). Broncho-alveolar lavages (BAL) were also performed to quantify the amount of *Legionella* reaching the thoracic region. To our knowledge, it is the first time that experiments mimicking so closely a real human physiological exposure were performed while limiting animal experiments.

2. Materials and methods

2.1. Legionella airborne suspensions

A *Legionella pneumophila* serogroup 1 (*Lp1*) species (responsible for LD) was used in this study. Bacteria are fluorescent as they were transfected with a GFP-containing plasmid and thus express the green fluorescent protein (GFP) (Cormack et al., 1996; Köhler et al., 2000). It was provided by Centre National de Référence des Légionelles (CNRL-Lyon) as their 008 strain. In this study, the strain is identified as *Lp1-008*. Thanks to the GFP, *Legionella* deposition was followed (Köhler et al., 2000; Unal and Steinert, 2006). *Legionella* were plated on BCYE agar (Buffered Charcoal Yeast Extract, SR0110C, Oxoid, France) supplemented with 8 mg/mL chloramphenicol (Sigma Aldrich, France) (BCYE + Chl) to select the bacteria bearing the GFP-containing plasmid.

For the nebulization process, suspensions were prepared from *Legionella* cultivated in BCYE + Chl for 72 h at 37 °C under a 5% CO_2 atmosphere to get bacteria with similar morphology to pathogenic *Legionella* present in aquatic reservoirs (Robertson et al., 2014). 8 mL of calibrated *Legionella* suspension were prepared in sterile normal saline (0.9% NaCl) water at a final concentration of 2×10^7 CFU/mL. An optical density of 0.2 at 600 nm (Biomate TM3; Avantec, Illkirch, France) was taken as reference for a bacteria suspension containing 10^8 CFU/mL. 5 mL of this suspension were placed in the nebulizer (see the aerosol generation section below) and aerosolized during lung