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Effect of *Parachlamydia acanthamoebae* on pulmonary function parameters in a bovine respiratory model

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ABSTRACT

The aim of this study was to evaluate pulmonary dysfunction induced by experimental infection with *Parachlamydia acanthamoebae* in calves. Intrabronchial inoculation with *P. acanthamoebae* was performed in 31 calves aged 2–3 months old at two different challenge doses of 10^8 and 10^{10} inclusion-forming units (IFU) per animal. Control animals received heat inactivated bacteria. The effects on pulmonary gas exchange were determined by arterial blood gas analysis and haemoximetry during the 7 days post inoculation (DPI). For pulmonary function testing (PFT), impulse oscillometry, capnography, and measurement of O_2 uptake were undertaken in spontaneously breathing animals 7 and 3 days before inoculation and were repeated until 10 DPI.

In the early phase after challenge (1–3 DPI), mild hypoxaemia occurred, which was accompanied by a significant reduction in both tidal and alveolar volumes (each related to bodyweight, BW). In parallel, expiratory flow rate and specific ventilation (i.e. minute ventilation related to O_2 uptake) were significantly increased. Minute and alveolar ventilations (each related to metabolic BW) increased significantly due to higher respiratory rates, lasting until 4 and 5 DPI, respectively. Oxygen uptake was slightly reduced during the first 2 days after challenge, but increased significantly during the recovery phase, from 4 to 8 DPI. No deterioration in respiratory mechanics or acid–base balance was observed. Respiratory infection with 10^{10} IFU *P. acanthamoebae* per calf induced mild respiratory dysfunction, mainly characterised by hypoxaemia. The study's findings do not indicate severe pathophysiological consequences of *P. acanthamoebae* infection on pulmonary function in the bovine host.

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Introduction

Chlamydiae are recognised as pathogens that cause several diseases of clinical significance. In addition to the *Chlamydiaceae*, there are eight families representing the '*Chlamydia*-like' organisms, forming the order *Chlamydiales*. During the past two decades, more emphasis has been placed on investigating the pathogenic potential of recently recovered species (Corsaro and Greub, 2006; Taylor-Brown et al., 2015). Increasing attention has focussed on *Parachlamydia acanthamoebae*, in terms of its pathogenicity and zoonotic potential (Borel et al., 2010), since there is evidence that this particular organism might represent an emerging pathogen of clinical significance (Greub and Raoult, 2002). *P. acanthamoebae* has been implicated in causing abortion in cattle (Borel et al., 2007; Ruhl et al., 2009; Blumer et al., 2011; Wheelhouse et al., 2012) and has also been linked to miscarriage in humans (Baud et al., 2009).

P. acanthamoebae is an obligate intracellular bacterium, which is potentially pneumopathogenic and is suspected to be involved in lower respiratory tract infections in humans (Greub, 2009; Lamoth and Greub, 2010; Lamoth et al., 2011) as well as in cattle (Wheelhouse et al., 2013). This organism was found to invade pneumocytes and macrophages in vitro (Greub et al., 2003; Casson et al., 2006) and its modulation of intracellular trafficking enables *P. acanthamoebae* to escape the microbicidal response induced by activated macrophages (Greub et al., 2005). In mice, intratracheal inoculation with *Parachlamydia* resulted in a 50% pneumonia-associated mortality after 5 days (Casson et al., 2008a).

A preliminary investigation into the pathogenesis of *P. acanthamoebae* in calves, based on intrabronchial inoculation, revealed dose-dependent effects on the respiratory tract, accompanied by an increased acute phase response and pathological changes in pulmonary tissues (Lohr et al., 2015). While the previous study focussed on clinical signs, inflammatory mediators and pathological changes, information on the effects of a *P. acanthamoebae* infection on respiratory function remained to be elucidated. The present study was undertaken in parallel to the one reported by Lohr et al. (2015), with the specific aim of investigating pulmonary function following

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experimental infection with *P. acanthamoebae* (see Appendix: Supplementary Fig. S1). It was hypothesised that intrabronchial challenge with *P. acanthamoebae* would affect pulmonary gas exchange in calves in a dose-dependent manner (Study 1). In a subsequent trial, focussing on the challenge dose/s that induced significant gas exchange restrictions, the underlying pulmonary dysfunction was explored, with respect to ventilation, respiratory mechanics and changes in the acid–base equilibrium (Study 2).

Materials and methods

Legislation and ethical approval

This study was carried out in accordance with the guidelines set out by the European Union for the protection of animals used for scientific purposes (Directive 2010/63/EU). The protocol was authorised by the Committee on the Ethics of Animal Experiments and the Protection of Animals of the State of Thuringia, Germany (approval number: 04-001/12; date of approval: 13 July 2012, extended 14 December 2012).

Animals

In this prospective study, 31 conventionally raised, Holstein-Friesian male calves were enrolled. The animals originated from one local farm. The herd of origin was assessed for the absence of chlamydial antigen and serum antibodies, employing *Chlamydiaceae*-specific PCR (Pantchev et al., 2010) and indirect ELISA (IDEXX Laboratories), respectively. Following routine entry screening (Reinhold et al., 2012), prophylactic treatment and quarantine, calves were used for the experiments. Throughout the study period, calves were reared under standardised conditions. All calves were necropsied either during ($n = 8$) or at the end of the study period (see Appendix: Supplementary Fig. S2). The protocol used for necropsy examination has been published previously (Lohr et al., 2015).

Preparation of bacteria used for inoculation and intrabronchial administration

Culture and purification of *P. acanthamoebae* strain Hall's coccus was performed as previously described (Casson et al., 2006). The inoculation procedure was performed by bronchoscopy under general anaesthesia, as described previously (Prohl et al., 2014; Lohr et al., 2015).

Study 1 design: Pulmonary gas exchange based on arterial blood gas analysis and haemoximetry

Calves ($n = 22$) were separated into three groups according to the parachlamydial challenge dose administered (see Appendix: Supplementary Fig. S2). In Group 1 ($n = 8$), animals were 42 ± 2 days old, with a bodyweight (BW) of 67.5 ± 4.8 kg. They were inoculated with 10^8 inclusion-forming units (IFU) of heat inactivated *P. acanthamoebae* (controls). Group 2 ($n = 8$; 41 ± 6 days old, BW 67.9 ± 7.9 kg) was challenged with 10^8 IFU per calf of viable *P. acanthamoebae*. Group 3 ($n = 6$; 44 ± 5 days old, BW 71.5 ± 5.4 kg) were inoculated with 10^{10} IFU per calf of viable *P. acanthamoebae*.

Starting 5 days before challenge, the respiratory rate (RR) of each animal was assessed during daily physical examination. The study groups were investigated for challenge-dose-related differences in pulmonary gas exchange by evaluating inter- and intraindividual changes in parameters of arterial blood gas analysis (BGA) and haemoximetry. To enable daily collection of arterial blood samples until 7 DPI, an indwelling catheter was implanted into the abdominal aorta of the paralumbar region of each calf 1 day before challenge as previously described (Ostermann et al., 2013). Complementary samples of peripheral venous blood were drawn twice before and daily after inoculation until 7 DPI. Throughout the study, up to three animals per group were subjected to necropsy at predetermined time points, thus continuously reducing the number of calves enrolled in testing (see Appendix: Supplementary Fig. S2).

Study 2 design: Non-invasive pulmonary function testing

This part of the study included nine calves (50 ± 4 days old; BW 80.1 ± 4.1 kg) and was designed as a longitudinal study. Each calf served as its own control, i.e. baseline data before challenge were compared to the data assessed after infection. The challenge dose was 10^{10} IFU of viable *P. acanthamoebae* per calf. Before exposure, calves were adapted to both the equipment and the procedure of pulmonary function testing (PFT). Individual baseline values of PFT parameters were obtained twice prior to inoculation. After challenge, PFT was repeated at 2, 3, 4, 7, 8, and 10 DPI (see Appendix: Supplementary Fig. S2). Individual BWs were measured prior to each PFT session. The RR was counted manually during daily physical examination starting 7 days prior to inoculation and lasting throughout the study. Additionally, samples of peripheral venous blood were collected daily starting 7 days prior to inoculation through 10 DPI before morning feeding to evaluate possible changes in acid–base balance.

Blood gas analysis and haemoximetry

Analyses of arterial and venous blood samples were performed using a blood gas analyser (ABL 725, Radiometer) and a haemoximeter (OSM 3, Radiometer) as described previously (Ostermann et al., 2013). Body temperature of each calf was assessed daily, immediately before blood collection. Partial pressures of O_2 and CO_2 as well as pH (all analysed at 37 °C) were automatically corrected for individual body temperature by the software implemented in the blood gas analyser. The BGA data were classified with reference to the previously published values of healthy calves, comparable in age and constitution (Reinhold and Födisch, 1993; Uhlig and Gorzny, 1993; Ostermann et al., 2013).

Pulmonary function testing

The main principles of PFT, the appropriate devices and test protocols were adopted from previous work (Ostermann et al., 2014). Briefly, after allowing an adaption to the devices and surroundings, three non-invasive PFT techniques (Jaeger-CareFusion) were applied consecutively at each time point to conscious calves breathing spontaneously: (1) impulse oscillometry (IOS) assessing respiratory mechanics; (2) volumetric capnography measuring the CO_2 concentration against exhaled volume; and (3) rebreathing system assessing O_2 uptake. All PFT parameters were calculated using the software included in the three systems. When applicable, PFT parameters were corrected either by BW or metabolic BW ($MBW = BW^{0.75}$ [$kg^{0.75}$]) in order to capture changes due to parachlamydial infection only and to ignore the influence of somatic growth throughout the period of investigation (see Appendix: Supplementary Table S1).

Exclusion of parachlamydial colonisation and coinfection

Potential respiratory coinfections with mycoplasma (ELISA Kit for *Mycoplasma bovis*, Bio-X-Diagnostics), *Pasteurella* spp. or *Mannheimia* spp. (bacteriological culture) were excluded by testing nasal swabs obtained during entry (21 days before inoculation) and at the day of necropsy. Additionally, the presence of chlamydial antigen was excluded by *Chlamydiaceae*-specific quantitative real-time PCR (Pantchev et al., 2010) in nasal, ocular and faecal swabs, taken at the same time points including 0 DPI. Simultaneously, *Parachlamydia*-specific real-time PCR (Casson et al., 2008b) was utilised confirming the absence of *Parachlamydia* prior to challenge (0 DPI) in all groups, and during the study (at 2, 4, 7 DPI, respectively) in the control group (Lohr et al., 2015).

Positive results were sporadically detected in nasal swabs, specifically *Mannheimia haemolytica* in 3/31 (10%) calves 3 weeks before challenge and in 4/31 (13%) at the day of necropsy. At this time point one calf also tested positive for *Mycoplasma bovirhinis*. However, bacteriological culture of lung tissue samples obtained at necropsy at the end of the two studies did not reveal any colonisation of the lower respiratory tract with *Mycoplasma*, *Pasteurella* or *Mannheimia* spp.

Statistical analysis

Data analysis was performed using Statgraphics Centurion XVI (version 16.1.18, Statpoint Technologies) and MedCalc (version 13.1.0, MedCalc Software). Data that were normally distributed are presented as mean ± standard deviation (SD); non-parametric data are presented as median (range), where range is the difference between maximum and minimum. Non-parametric tests were applied for intergroup comparison in Study 1, i.e. the Mann–Whitney–Wilcoxon U test was used for two independent samples and the Kruskal–Wallis (K–W) test for multiple independent samples. Wilcoxon signed-rank (W) test was utilised for intragroup comparison. In Study 2, data originating from the same calves and collected over multiple time points were analysed using repeated measures ANOVA. P values < 0.05 were considered significant. Post hoc testing was conducted by Bonferroni's multiple comparison procedure with a level of significance set at $P < 0.10$.

Results

Study 1: Arterial blood gas analysis

Prior to challenge, the baseline values of arterial partial pressures of O_2 and CO_2 (PaO_2, 12.16 ± 0.90 kPa and $PaCO_2$, 6.29 ± 0.41 kPa) did not differ significantly between groups ($P > 0.05$). PaO_2 did not change significantly over time in control calves or those challenged with a dose of 10^8 IFU per calf ($P > 0.05$). Median PaO_2 decreased by 6.41% (0.85 kPa) at 1 DPI compared to baseline in animals exposed to 10^{10} IFU of *P. acanthamoebae* per calf (Fig. 1A; W test; $P = 0.04$). Compared with the controls and low-dose-challenged group, the median PaO_2 remained significantly different at 2 DPI in the high-dose-challenged group ($P = 0.03$). The fraction of oxyhaemoglobin (O_2Hb) and oxygen saturation (sO_2) was lower

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