RESEARCH ARTICLE

A bovine model of a respiratory *Parachlamydia acanthamoebae* infection

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Chlamydia-like organisms such as *Parachlamydia*, have been identified in connection with disease in a range of hosts, although proving causation is difficult. This study demonstrated that a suitable concentration of *Parachlamydia acanthomoebae*, administered directly to the lungs of healthy calves resulted in a mild respiratory illness. This advances the understanding of the potential pathogenic role of this organism in animal hosts.

ABSTRACT

The aim of this study was to evaluate the pathogenicity of *Parachlamydia (P.) acanthamoebae* as a potential agent of lower respiratory tract disease in a bovine model of induced lung infection. Intrabronchial inoculation with *P. acanthamoebae* was performed in healthy calves aged 2–3 months using two challenge doses: 10^8 and 10^{10} bacteria per animal. Controls received 10^8 heat-inactivated bacteria. Challenge with 10^8 viable *Parachlamydia* resulted in a mild degree of general indisposition, whereas 10^{10} bacteria induced a more severe respiratory illness becoming apparent 1–2 days post inoculation (dpi), affecting 9/9 (100%) animals and lasting for 6 days. The extent of macroscopic pulmonary lesions was as high as 6.6 (6.0)% [median (range)] of lung tissue at 2–4 dpi and correlated with parachlamydial genomic copy numbers detected by PCR, and with bacterial load estimated by immunohistochemistry in lung tissue. Clinical outcome, acute phase reactants, pathological findings and bacterial load exhibited an initial dose-dependent effect on severity. Animals fully recovered from clinical signs of respiratory disease within 5 days. The bovine lung was shown to be moderately susceptible to *P. acanthamoebae*, exhibiting a transient pneumonic inflammation after intrabronchial challenge. Further studies are warranted to determine the precise pathophysiologic pathways of host-pathogen interaction.

Key words: *Chlamydia*-like organism; large animal model; *Parachlamydia*; pneumonia

INTRODUCTION

As the majority of community-acquired pneumonia in humans fails to be diagnosed aetiologically (Marrie *et al.*, 1996; Cilloniz *et al.*, 2012), the quest for potential causative agents continues more than ever. Besides *Chlamydia* spp., *Chlamydia*-like organisms are thought to be concurrently causative in the incidence of community-acquired pneumonia in humans (Greub and Raoult, 2002; Corsaro and Greub, 2006;

Haider *et al.*, 2008). Current literature indicates that *Parachlamydia acanthamoebae*, a well-studied agent among the represented species of *Chlamydia*-like organisms, has a pneumopathogenic potential (Marrie *et al.*, 2001; Greub, 2009). The incidence of *P. acanthamoebae*-associated pneumonia is further supported by epidemiological, serological and molecular studies (Lamoth and Greub, 2010; Bousbia *et al.*, 2013). Employing free-living amoebae as natural host and vector, *P. acanthamoebae* potentially co-occurs with *Acanthamoeba* spp., benefitting from their ubiquitous dissemination (Horn, 2008). Additionally, the natural exposure of humans to this obligate intracellular bacterium was shown by isolating parachlamydial-infected *Acanthamoeba* spp. from nasal mucosa of healthy volunteers (Amann *et al.*, 1997).

Furthermore, resistance against human macrophages was indicated when *in vitro* studies revealed that *Parachlamydia* enters and replicates within macrophages, inducing their death by apoptosis (Greub *et al.*, 2003). In these cells, intracellular digestion is disabled by preventing the acquisition of lysosomal hydrolases (such as cathepsin D) to the *Parachlamydia*-containing vacuole (Greub *et al.*, 2005b). Pneumocytes and lung fibroblasts have also been shown to be susceptible to *Parachlamydia*, allowing prolonged survival of the bacteria after internalization (Casson *et al.*, 2006). Moreover, intratracheal inoculation of BALB/c mice resulted in a fulminant bronchopneumonia and led to a pneumonia-associated mortality of 50% within 5 days (Casson *et al.*, 2008b). All these studies suggested the respiratory system as one target organ. Although *Parachlamydia* has not yet been definitely identified as a naturally occurring pneumopathogen in cattle, susceptibility became evident when parachlamydial DNA was detected in diagnostic lung samples originating from cases of cattle pneumonia (Wheelhouse *et al.*, 2013). Additionally, the abortigenic role of *P. acanthamoebae* has been recently identified in cattle after demonstrating the presence of the pathogen in placental samples by quantitative real-time PCR (qPCR), immunohistochemistry (IHC) and electron microscopy (Borel *et al.*, 2007; Blumer *et al.*, 2011; Wheelhouse *et al.*, 2012), supporting the susceptibility of cattle to *P. acanthamoebae*. Wild ruminants have also been identified to host *Parachlamydia* spp. in different internal organs and shedding them with faeces and ocular discharge (Regenscheit *et al.*, 2012).

Closely related to *Parachlamydiaceae*, different species of *Chlamydiaceae* are established naturally occurring pathogens in cattle (Reinhold *et al.*, 2011) with their zoonotic potential currently being the subject of investigation (Wheelhouse and Longbottom, 2012). A bovine model of experimentally induced lung infection was recently introduced and validated to assess the pathogenesis of *Chlamydia psittaci* (Reinhold *et al.*, 2012). This sophisticated large animal model was found suitable for studying the pathogen-host interactions *in vivo* and under biologically relevant conditions, complementing the numerous murine models in terms of respiratory pathophysiology.

As several lines of evidence support the role of *P. acanthamoebae* as a potential agent of lower respiratory tract infection, the present study was aimed to assess the pathogenic potential of *P. acanthamoebae* to the bovine lung in order to extrapolate a predictable severity of illness. To specify the character of an acute parachlamydial lower respiratory tract infection on a dose–response relationship, clinical outcomes and both systemic and local markers of inflammation were studied within the period of acute onset up to 7 days post inoculation (dpi). To verify the fulfillment of Koch's postulates, emphasis was placed on the detection, quantification and recovery of the pathogen from infected individuals as well as the assessment of dissemination and possible shedding of the pathogen.

MATERIALS AND METHODS

Legislation and ethical approval

This study was carried out in strict accordance with European and National Law for the Care and Use of Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments and the Protection of Animals of the State of Thuringia, Germany (Permit Number: 04-001/12). All experiments were conducted in a containment of biosafety level 2 under supervision of the authorized institutional Agent for Animal Protection. Bronchoscopy for pathogen inoculation was performed under general anaesthesia. Throughout the study, every effort was made to minimize animal suffering.

Animals

In this prospective, single-blinded and double-controlled study, 39 conventionally raised male calves (Holstein–Friesian) were enrolled. Animals originated from one local farm without any history of *Chlamydia*-associated health problems. In advance, the herd of origin was randomly checked for chlamydial antigen and antibodies by *Chlamydiaceae*-specific PCR and indirect ELISA, respectively. After purchasing the calves at an age of 21 ± 5 days (mean $\pm$ SD) and a body weight of 54.8 ± 6.1 kg, they were kept in quarantine for 22 ± 1 days. Following entry screening, animals were treated with iron(III)-hydroxide-dextran-complex solution (10 mg Fe^{3+} kg^{-1} body weight, subcutaneously, belfer®; belapharm GmbH & Co. KG, Vechta, Germany) and marbofloxacin (10 mg kg^{-1} body weight, intramuscularly, Forcyl®; Vétoquinol SA, Cedex, France). Both substances were administered prophylactically on a single shot basis 22 ± 1 or 20 ± 1 days prior to inoculation, respectively. After confirming a clinically healthy status, calves were challenged. Throughout the entire study, animals were reared under standardized conditions (temperature: 18–20°C, relative humidity: 60–65%) and in accordance with international guidelines for animal welfare. Nutrition included commercial milk replacers and coarse meal. Water and hay were supplied *ad libitum*. None of the given feed contained antibiotics.
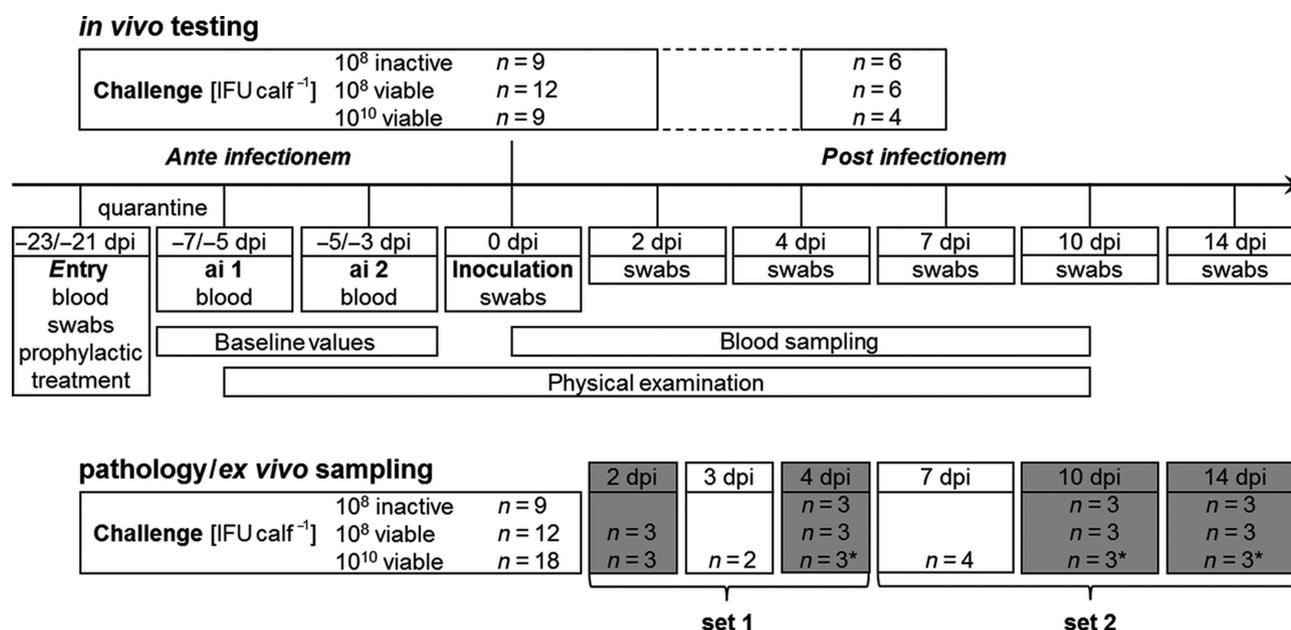
Study design

For *in vivo* testing, 30 calves were randomly allocated to three study groups according to the challenge dose.

Group 1 ($n = 9$, age 39–46 days, 67.0 ± 4.7 kg body weight): 10^8 inclusion-forming units (IFU) per calf of heat-inactivated *P. acanthamoebae* (controls).
Group 2 ($n = 12$, age 33–52 days, 67.6 ± 7.6 kg body weight): 10^8 IFU per calf of viable *P. acantamoebae* (low dose).
Group 3 ($n = 9$, age 38–51 days, 70.5 ± 6.3 kg body weight): 10^{10} IFU per calf of viable *P. acanthamoebae* (high dose).

Starting 5 days before challenge, a physical examination of each animal was performed daily throughout the study. Venous blood samples (Primavette S®; KABE Labortechnik GmbH, Nuembrecht-Elsenroth, Germany) were drawn at two individual time points before inoculation (baseline values) and once a day thereafter. To assess the presence and shedding of *Parachlamydia*, ocular, nasal and rectal swabs (eSwab™; COPAN Italia S.p.A., Brescia, Italy) were also taken twice before challenge as well as 2, 4, 7, 10 and 14 dpi (Fig. 1). Three animals of each group inoculated with viable *Parachlamydia* underwent necropsy at 2, 4, 10 and 14 dpi for extensive pathological, histological and immunohistochemical evaluation. Additional *ex vivo* samples of the 10^{10} IFU-challenged group were taken at accessory time points, that

in vivo testing

Figure 1. Study design. Flow chart indicates procedures and sampling over time. In total, 39 individuals were enrolled; 30 were included in *in vivo* testing during the corresponding time span. All animals were subjected to gross pathology (extent of altered lung tissue) as well as to *ex vivo* sampling (BALF, tissue for PCR and co-culture). The additional individuals (*$n = 9$) received the same challenge under equal conditions but were exclusively subjected to pathological evaluation and *ex vivo* sampling. Grey labelled fields indicate time points and number of animals for detailed pathomorphologic examination (histopathology and immunohistochemistry). ai, *ante inoculationem*; dpi, days *post inoculationem*.

is 3 dpi ($n = 2$) and 7 dpi ($n = 4$), in order to assess *ex vivo* findings in shorter subsequent time periods. Therefore additional calves ($n = 9$) comparable in age and body weight were challenged with 10^{10} IFU of *P. acanthamoebae* to replace the calves already sacrificed at the odd time points (3 and 7 dpi). Challenge was performed using the same protocol, shifted at a different time point. These animals were necropsied at 4, 10 and 14 dpi (Fig. 1) but were not included in *in vivo* testing. Analysis of *ex vivo* samples was performed within two sets of time points for each group (*set 1*: 2–4 dpi and *set 2*: 7–14 dpi). Due to a limited number of animals, time points were grouped to perform statistical analysis (inter-individual comparison). This grouping only applied to data collected during post mortem evaluation. Individuals of each group were allocated randomly to post mortem assessment.

Only the veterinarian who performed the challenges and took care of the animals was aware of the challenge type. As parameters assessed *in vivo* (e.g. body temperature, respiratory rate, heart rate) were measured numerically, the response to challenge can therefore be regarded as 'objective'. The assessors were blinded to all specimens collected *in vivo* or *ex vivo*.

Preparation of the parachlamydial inoculum and intrabronchial administration

Parachlamydia acanthamoebae Hall's coccus strain (Birtles *et al.*, 1997) was used for inoculation. Culture and purification of *P. acanthamoebae* was performed as previously described (Casson *et al.*, 2006). Briefly, bacteria were grown within *Acanthamoeba castellanii* (ATCC® 30010™) in peptone-yeast extract-glucose broth at a temperature of 32°C. Cultures were harvested by centrifugation and suspended in Page's amoeba saline after 6 days of incubation. Inoculation doses, that is IFU, were titrated using a lysis test (Greub *et al.*, 2003). Defined doses of 10^8 and 10^{10} IFU of *Parachlamydiae* were finally suspended in

8 mL of SPG medium [sucrose (glucose): 75 g, phosphate: 87 mL Na_2HPO_4 0.2 M, 13 mL NaH_2PO_4 0.2 M, L-glutamic acid: 0.72 g to a total volume of 100 mL] and stored at $-80°C$. Bacteria used as a negative control were inactivated for 1 h at 90°C.

For inoculation, non-fed calves were anaesthetized by injecting xylazine (0.2 mg kg^{-1} body weight, Rompun® 2%; Bayer Vital GmbH, Leverkusen, Germany) followed by ketamine (1.3 mg kg^{-1} body weight, ketamine 10%; bela-pharm GmbH & Co. KG) intravenously and were placed in right lateral recumbency. A flexible video endoscope (Veterinary Video Endoscope PV-SG 22-140; KARL STORZ GmbH & Co.KG, Tuttlingen, Germany) and a Teflon® tube catheter (2 mm diameter, 175 cm length; KARL STORZ GmbH & Co. KG) were used for intrabronchial application. Inoculation doses of either viable 10^8 or 10^{10} IFU per calf, or heat-inactivated 10^8 IFU per calf of *P. acanthamoebae* were administered into eight defined bronchi of the lung as described previously (Reinhold *et al.*, 2012; Prohl *et al.*, 2014). Each calf was inoculated intrabronchially with 6 mL of a total of 8 mL inoculum containing either viable or heat-inactivated *Parachlamydia* at the given quantity. The remaining 2 mL were aerosolized intranasally with a custom-build actuator (3-mL syringe attached to a pharyngeal spray applicator).

Clinical scoring

The state of health was assessed daily by physical examination capturing clinical parameters of each calf according to a semi-quantified scoring system. Basically, general condition, the respiratory system, the cardiovascular system, and other relevant organ systems, for example the gastrointestinal tract, were subjected to close investigation starting 6 ± 1 days before inoculation through to 10 dpi. As the main target of observation, for the respiratory score breathing frequency, occurrence of dyspnoea, ocular and nasal discharge, and the presence of cough were taken into account. Deflections were assigned score points with

Table 1. Tissues sampled during necropsy according to type of preservation and further examination.

Tissue/Organ	Histology	Immunohisto-chemistry	Quantitative real-time PCR	Amoebal co-culture	Differentiating microbiology
Type of fixation and preservation	Formalin/Paraffin	Formalin/Paraffin	Native/−20°C	Native/−80°C	Native/4°C
Lung lesions left	X	X	XXX	X	
Lung lesions right	X	X	XXX	X	XX
Lung intact	X	X	X		
Ln. mediastinalis	X	X	X		
Ln. tracheobronchialis	X	X	X		
Ln. retropharyngealis medialis	X	X	X		
Conjunctiva	X		X		
Tonsilla pharyngealis	X		X		
Nasal mucosa	X		X		
Trachea	X				
Spleen	X		X		
Liver	X		X		
Kidney	X		X		
Heart muscle	X		X		
Aorta	X		X		
Valva artrioventricularis sinistra	X		X		
Ln. poplitealis	X				
Synovialis	X		X		
Jejunal Peyer's patches	X				
Ileal Peyer's patches	X				

Samples were obtained from each calf, respectively. Count of crosses (×) indicate number of individual samples. *Ln., Lymphonodus.*

respect to severity which summed up to a total clinical score reflecting the overall health status of the animal. Criteria for evaluation of clinical signs and the corresponding scoring system have been described in detail elsewhere (Reinhold *et al.*, 2012).

Analysis of acute-phase reactants

Serum was harvested from venous blood samples by centrifugation (3120 *g*; 15 min, 15°C), and was stored at −80°C until analysis. Quantitative analysis of lipopolysaccharide binding protein (LBP) was performed using a bovine-specific enzyme-linked immunosorbent assay (ELISA; Ostermann *et al.*, 2013a). The ELISA protocol used to determine lactoferrin serum concentrations has been described previously (Schroedl *et al.*, 2003). For comparison, baseline values were obtained from all animals before inoculation.

White blood cell count and differentiation

Whole blood samples were collected in potassium–EDTA Primavette S® (KABE Labortechnik GmbH) and were processed, counted and differentiated as previously described (Ostermann *et al.*, 2013b).

Necropsy and tissue samples

Animals were euthanized according to the time point of the set they were enrolled in (Fig. 1). Under conditions of deep anaesthesia (pentobarbital-sodium, 83.7 ± 8.0 mg kg^{-1} body weight, intravenously; Release®; WdT eG, Garbsen, Germany), the trachea was exposed and large clamps were placed distal to the larynx to prevent contamination of the airways by blood or gastric contents. Subsequently, the animals were sacrificed by exsanguination. The thoracic cavity was opened, the lung removed and pulmonary lesions were recorded. After collection of bronchoalveolar lavage fluid (BALF, described below), tissue samples (2–3 mm^3 in size) were collected from representative sites of each lung lobe for detection of *Parachlamydia* by PCR and parachlamydial recovery by amoebal co-culture. Preferentially, locations with macroscopic lesions were sampled and for intra-individual control, intact lung tissue was utilized. The same locations were sampled on a larger scale for histology and IHC. A complete necropsy was then performed and further samples of additional tissue were collected for molecular detection of *Parachlamydia*. Table 1 provides a complete overview of the specimens obtained during necropsy.

Collection of BALF *ex vivo* and BALF analyses

Immediately after exsanguination, BALF was obtained from freshly exenterated lungs. At two different locations (*Lobus caudalis sinister, Lobus medius*) three subsequent washes using 20 mL of 37°C warm sterile saline solution (0.9 % NaCl) for each instillation (in total 120 mL; 60 mL per lung lobe) were applied using glass syringes and a custom-made catheter inserted through the trachea. BALF recovered by aspiration was collected in siliconized glass bottles (SL-2 Sigmacote®, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and immediately placed on ice. The recovery rate of BALF was 66.7 ± 6.5% (mean ± SD) and did not differ significantly between groups (*P* = 0.27). Cellular and liquid proportions were separated by centrifugation (300 *g*; 20 min) resulting in BALF supernatant and a cell pellet.

BALF cytology
Total cell count of native BALF and differentiation of leucocyte populations was performed according to an earlier description (Reinhold *et al.*, 2012). Sediment preparations of 200 leucocytes were classified using oil immersion and a × 100 magnification.

Total protein
Concentrations of total protein were measured in BALF supernatant using commercially available modified Lowry Protein

Assay Kit (Pierce, Rockford, IL). Each sample was analyzed in duplicate.

Eicosanoids

Liquid chromatography–tandem mass spectrometry (LC-MS-MS) was used to analyze concentrations of 6–keto–prostaglandin $F_{1\alpha}$, thromboxane B_2, PGE_2, leucotriene B_4, 5–hydroxyeicosatetraenoic acid (HETE), 12–HETE and 15–HETE, 12,13–dihydroxyoctadecanoic acid (12,13–DiHOME) and 13–dihydroxyoctadecanoic acid (13–HODE) in BALF supernatant. The detailed procedure is outlined in Reinhold et al. (2012).

Gross pathology, histopathology and immunohistochemistry

Distribution, extent and quality of pulmonary lesions were estimated and recorded. Tissue samples for histology and IHC were immersed in 3.5% neutral buffered formalin and stored at 4°C until being embedded in paraffin blocks. Lesions were evaluated in haematoxylin and eosin-stained sections. To perform IHC staining, Parachlamydia were labelled using an indirect immunoperoxidase method. A specific polyclonal rabbit anti-Parachlamydia antibody (SZ3210) raised against purified elementary bodies (Casson et al., 2008b) was applied as a primary antibody to investigate paraffin sections for parachlamydial antigen. Sections were deparaffinated in xylene and rehydrated through graded ethanol to water. Endogenous peroxidase activity was blocked by immersion with a methanol hydrogen peroxide solution for 25 min. For antigen retrieval, sections were pre-digested with 0.05% proteinase K (Merck KGaA, Darmstadt, Germany) for 5 min at 37°C. Following incubation with normal goat serum diluted 1 : 10 in a 12.5% bovine serum albumin in Dulbecco's phosphate-buffered saline solution (BSA/DPBS) for 20 min, slides were incubated with the primary antibody diluted 1 : 500 in BSA/DPBS for 60 min. A horseradish peroxidase-conjugated goat anti-rabbit IgG (AffiniPure; Jackson ImmunoResearch Europe Ltd, Cambridgeshire, UK) was utilized as a secondary antibody, the sections of which were incubated at a 1 : 50 dilution in BSA/DPBS for 60 min. Finally, slides were developed in diaminobenzidine for 10 min and counterstained with haematoxylin. A sample that tested strongly positive for Parachlamydia by qPCR was used as a positive control. Tissue sections of animals inoculated with heat-inactivated Parachlamydia served as negative controls.

Processing of swabs, BALF cells and tissue: pathogen detection and quantification by real-time PCR

Swabs were sampled at locations and time points mentioned above, and were stored in Amies (1967) medium at 4°C until being processed. For preparation of DNA extraction, 200 μL medium of each sample were centrifuged at 20 000 g for 5 min, creating a cell pellet. Tissue specimens designated for molecular antigen detection (Table 1) were kept native at −20°C, and separated BALF cells at −80°C. Parachlamydial DNA was extracted from tissues, BALF cells and recovered pellets using the Wizard® SV Genomic Purification System (Promega Corporation, Madison, WI) according to the manufacturer's instructions. Following the extraction procedures, the presence of P. acanthamoebae was detected by qPCR targeting a 103–bp part of the 16S rRNA-encoding gene as described earlier (Casson et al., 2008a). Alternatively, a StepOne Plus(R)™ qPCR System (Applied Biosystems, Rotkreuz, Switzerland) was used for genomic amplification and detection of the products. Each sample was amplified

in duplicates. Inhibition controls, negative PCR mixture controls, and extraction controls were tested systematically. To allow quantification, a plasmid containing the target gene was used (Casson et al., 2008). Samples were considered to be positive that had a cycle threshold (C_t) value of < 35.0 and as being questionable positive when straddling thresholds between 35.0 and 45.0. If no amplification occurred, samples were considered to be negative, and duplicates creating only one C_t value were retested. Genomic copy numbers resulting from left and right lung lesions of each individual were averaged for further calculation.

Re-isolation by amoebal co-culture

Specimens of altered lung tissue or, if unavailable, from predefined sites were sampled during necropsy and stored immediately at −80°C until being processed. Samples were prepared by adding 1 mL of sterile PBS and homogenized in gentleMACS™ C Tubes using a corresponding Dissociator (4000 r.p.m. for 15 s; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Details of the following procedure of amoebal co-culture have been reported elsewhere (Jacquier et al., 2013). To prove that the amoebal lysis that occurred was due to parachlamydial growth, 50-μL samples of the homogenates as well as of the first passage were tested by Parachlamydia-specific qPCR (both DNA extraction and qPCR were conducted using the same protocol as for tissue samples and swabs).

Exclusion of co-infections

The herd of origin was known to be unaffected by bovine herpes virus 1 (BHV-1) and bovine virus diarrhoea/mucosal disease virus (BVDV). Additionally, after entry into the study, immunohistological examination of ear biopsies using the monoclonal antibody BVD/CD16 against the nonstructural antigen proteins confirmed the absence of BVDV (Cay et al., 1989), indicating that none of the calves enrolled in the study was immunocompromised by persistent BVDV infection. Absence of Chlamydia spp. (ELISA test; IDEXX GmbH, Ludwigsburg, Germany) and Mycoplasma bovis (ELISA Kit for M. bovis; Bio-X-Diagnostics, Jemelle, Belgium) was confirmed by serology at the time of entry. Additionally, absence of chlamydial antigen was confirmed by Chlamydiaceae-specific qPCR in nasal, ocular and faecal swabs taken before inoculation and at necropsy. Nasal swabs taken at the same time points were tested for relevant respiratory co-pathogens (Mycoplasma, Pasteurella and Mannheimia spp.). Positive results were sporadically seen for Mycoplasma haemolytica in 3/30 of cases (10%) before challenge and in 1/30 of cases (3%) at necropsy. In addition, one individual was positive for Mycoplasma bovirhinis. However, bacteriological examination of lung tissue samples obtained during necropsy did not reveal any colonization of relevant pneumopathogens. Furthermore, viral respiratory co-pathogens, that is bovine respiratory syncytial virus (BRSV), parainfluenza 3 virus (PI–3), adenovirus type 3, BHV–1 and BVDV (Bio-X respiratory penta ELISA Kit; Bio-X-Diagnostics) were also excluded by serological screening.

Before prophylactic antimicrobial treatment, in faecal samples of 3/10 animals (10%), non-specified Eschericha coli were detected. Oocytes of Cryptosporidium spp. were detected sporadically in 5/30 (17%) and numerously in 2/30 (7%) calves. Other relevant enteric parasites were absent in faecal smearings. Additional routine microbiological screening revealed that all animals were negative for Salmonella infections (faecal swabs).

Statistical analysis

Data analysis was performed using PASW STATISTICS 17.0 (IBM) and STATGRAPHICS CENTURION XVI (Version 16.1.18; Statistical Graphics Corporation). Data featuring normal distribution are presented as mean ± standard deviation, and data with non-normal or unknown distribution are given as median (range). Normal distribution was determined by utilizing the Shapiro-Wilk test, visual inspection of histograms, normal Q–Q plots and boxplots as well as considering skewness and kurtosis. For comparison of normally distributed data, parametric tests were applied to compare means, that is the *t*-test for two independent samples or ANOVA, and the multiple range test for multiple independent samples (inter-group comparison). Non-parametric methods were used for inter-group comparison of medians of data showing non-normal distribution, that is Mann–Whitney–Wilcoxon (*U*) test for two independent samples and Kruskal–Wallis test for multiple independent samples. For dependent sample (intra-group) comparison, a paired *t*-test was used for normally distributed data and a Wilcoxon signed-rank (*W*) test was performed for data showing non-normal distribution. The lowest achievable probability level was 95% and *P*-values ≤ 0.05 were considered statistically significant.

RESULTS

Clinical signs and corresponding scoring

As shown in Fig. 2, inter-group comparison revealed that animals exposed to 10^{10} IFU of *P. acanthamoebae* presented a total clinical score significantly higher compared with animals exposed to 10^8 IFU as well as the control group over a period up to at least 4 dpi. The total clinical score of calves receiving a challenge dose of 10^{10} IFU increased significantly after inoculation compared with the baseline score obtained before challenge, with a peak value of 3.0 (7.0) score points [median (range)] at 1 dpi. Total score values remained significantly high in this group up to 7 dpi, with a slight decrease towards 5 dpi (Fig. 2). Controls and animals challenged with 10^8 IFU showed a slight elevation of the total clinical score after inoculation as well. However, deviations were not significantly different to each other over time. Besides respiratory score and general condition, the cardiovascular system contributed proportionally the major part to the sum of the total clinical score (Supporting Information, Table S1).

10^8 IFU inactive

Apart from non-specific ocular and nasal discharge, no clinical signs of respiratory disease became apparent. Very mild signs of systemic inflammation, such as elevated heart frequency and mucosal hyperaemia, were shown upon inoculation. The general condition of all animals remained mainly undisturbed throughout the study.

10^8 IFU live

Animals presented clinical signs of an impaired state of health to a minor degree throughout observation, characterized by mild signs of systemic inflammation. Notably, a slight elevation in median rectal temperature up to 38.8 (1.45)°C, hyperaemic conjunctiva and oral mucosa, enlarged submandibular lymph nodes as well as non-specific diarrhoea contributed to the clinical outcome.

10^{10} IFU live

In response to the challenge, calves exhibited respiratory illness showing a significantly elevated breathing frequency [+24.4 (62.7)%] culminating at 2 dpi and spontaneous coughing. The median rectal temperature rose instantly after inoculation up to 39.5 (2.05)°C and was significantly elevated compared with both other groups at 1 dpi (*P* < 0.01). Furthermore, there was moderate impairment of health throughout the study, characterized by cardiovascular signs of systemic inflammation such as hyperaemic conjunctiva and oral mucosa. Submandibular lymph nodes were enlarged and tended to be painful upon palpation. From 2 to 7 dpi, all calves (9/9) presented signs of an altered state of health. Signs of respiratory disease were absent from 6 dpi (Table S1).

Markers of inflammation tested in blood

White blood cell count

Total leucocytes in peripheral venous blood did not differ significantly either before or after inoculation between all three groups (data not shown). However, the proportion of band neutrophils increased continuously in both groups inoculated with viable *Parachlamydia* after exposition. The increase was faster and more distinct in calves challenged with 10^{10} IFU. A significant difference was evident at 1 dpi by inter-group comparison
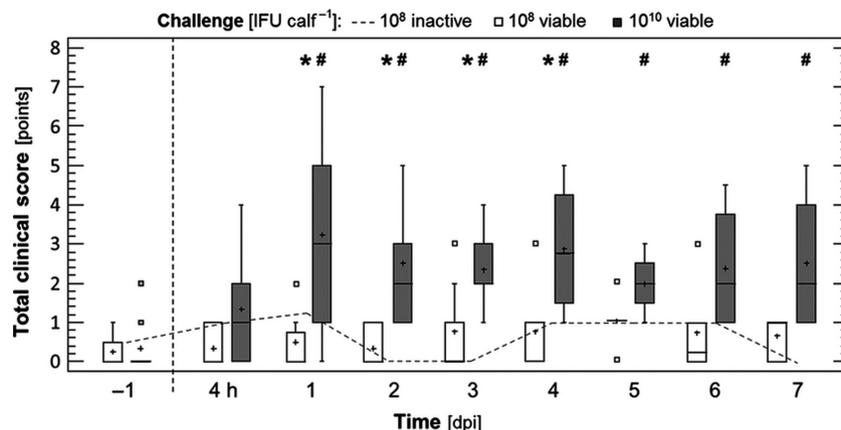

Figure 2. Development of total clinical score over time. Data are given as boxplots for groups inoculated with viable *Parachlamydia* and as time sequence plot (dashed line) representing medians of controls, respectively. Vertical hatched line indicates time point of inoculation at 0 dpi. The first dataset after pathogen application was captured 4 h *post inoculationem*. The Kruskal–Wallis test revealed significant differences (*) between groups. Score points of animals challenged with 10^{10} IFU were significantly higher at given time points (#) compared with baseline values (*W* test). *P* ≤ 0.05; dpi, days *post inoculationem*.

Table 2. Cytological differentiation and quantities of cell populations present in BALF at 2–4 dpi.

BALF cells	Unit	Control	Challenge group		Kruskal–Wallis test (P)
		10^8 IFU inactive ($n = 3$) Median (range)	10^8 IFU viable ($n = 6$) Median (range)	10^{10} IFU viable ($n = 8$) Median (range)	
Total cell count	10^8 L^{-1}	4.00 (1.30)	3.80 (6.40)	4.45 (7.60)	0.90
Cell differentiation absolute					
Alveolar macrophages	10^8 L^{-1}	3.80 (1.40)	3.55 (6.20)	3.95 (4.30)	0.94
Neutrophil granulocytes	10^8 L^{-1}	0.06 (0.10)	0.21 (0.33)	**0.41**↑ (3.51)	0.02
Unsegmented	10^8 L^{-1}	0.00 (0.02)	**0.05**↑ (0.25)	**0.22**↑ (0.86)	0.02
Polymorph nuclear	10^8 L^{-1}	0.04 (0.10)	0.10 (0.21)	0.24 (2.76)	0.04
Lymphocytes	10^8 L^{-1}	0.00 (0.02)	0.03 (0.09)	0.05 (0.17)	0.30
Cell differentiation relative					
Alveolar macrophages	%	96.0 (3.0)	94.3 (7.0)	**85.8**↓ (30.0)	0.02
Neutrophil granulocytes	%	1.5 (2.5)	4.0 (6.0)	**11.3**↑ (31.0)	0.03
Unsegmented	%	0.0 (0.5)	**1.5**↑ (2.5)	**6.0**↑ (7.5)	0.02
Polymorphonuclear	%	1.0 (2.5)	2.0 (4.0)	7.4 (24.0)	0.04
Lymphocytes	%	0 (0.5)	0.5 (2.0)	1.0 (2.5)	0.36

Data is given as median (range). *Kruskal–Wallis* test: $P \leq 0.05$ indicates significant differences between medians of all groups. Bold values indicate a significant increase (↑) or decrease (↓) compared with controls (*U*-test: $P \leq 0.05$).

[1.00 (6.00) vs. 0.00 (3.00)%; $P = 0.03$] and over time, reaching a maximum of 2.50 (2.00)% at 4 dpi, when compared with corresponding baseline values (0.67 (1.67)%; $P = 0.03$). Additionally, the proportions of neutrophilic granulocytes [44.00 (46.00)%] and segmented neutrophils [43.00 (40.00)%] were also significantly high in the high dose group compared with the group receiving 10^8 IFU [31.50 (22.00) and 31.50 (21.00)%, respectively] as well as with controls [31.00 (28.00) and 30.50 (27.00)%, respectively] at 1 dpi ($P < 0.02$). The proportion of lymphocytes decreased with increasing parachlamydial challenge dose at the same time (data not shown).

Lipopolysaccharide binding protein
Only individuals inoculated with viable *Parachlamydia* developed a positive deviation of serum LBP concentration; controls did not exhibit any significant increase over time. In animals exposed to 10^{10} IFU, serum LBP reached its peak concentration of 15.7 (69.5) μg mL^{-1} [median (range)] at 1 dpi. In comparison with baseline data [5.5 (34.6) μg mL^{-1}], a significant increase was evident at 3 dpi ($P \leq 0.05$). Compared with this relative elevation of + 247.48 (1116.28)% in response to the highest challenge dose, serum LBP concentrations in calves challenged with a pathogen load of 10^8 IFU were lower, but were still significantly elevated until 4 dpi (intra-group comparison to baseline data; $P < 0.03$; data not shown).

Lactoferrin
In animals receiving viable *Parachlamydia*, comparable baseline concentrations of lactoferrin were present in blood before challenge [145.51 (221.00) or 150.87 (169.33) ng mL^{-1} in calves exposed to 10^8 or 10^{10} IFU, respectively]. Intra-individual comparison revealed a significant increase of lactoferrin concentration in both the 2- to 3-dpi groups, but without statistically relevant differences between the two groups. Calves challenged with 10^{10} IFU exhibited their highest lactoferrin concentration at 2 dpi [241.66 (140.18) ng mL^{-1}; $P = 0.01$], whereas calves exposed to 10^8 IFU showed a significant increase at 3 dpi [212.29 (244.96) ng mL^{-1}; $P = 0.04$]. After this, lactoferrin concentrations declined, reaching baseline data at 7 dpi. Interestingly, even controls showed a similar time trend and significant increases of lactoferrin at 2 and 3 dpi.

Local biomarkers of pulmonary inflammation in BALF

Total cell count
In the first set of necropsies (2–4 dpi), the proportion of neutrophils increased with increasing challenge dose, that is the percentage of neutrophils in BALF was significantly higher in animals challenged with 10^{10} IFU than in controls or animals challenged with 10^8 IFU (inter-group comparison; Table 2). Accordingly, the proportion of alveolar macrophages decreased in favour of the increasing number of neutrophils. At the second set of necropsies (7–14 dpi) the high dose-inoculated group showed significantly more band neutrophils per mL BALF than the low dose group ($P = 0.04$; data not shown). Total cell count did not differ markedly between any of the three groups at either sampling time.

Total protein
In BALF supernatant, medians of protein concentrations ranged between 199.3–232.9 (2–4 dpi) and 197.3–261.9 μg mL^{-1} (7–14 dpi), and were not significantly different between groups or time points.

Eicosanoids
15–HETE concentrations increased with increasing parachlamydial challenge and were comparable within each group at different time points (Fig. 3). Data obtained at 7–14 dpi still revealed a significantly higher 15-HETE concentration in BALF supernatant of calves exposed to 10^{10} IFU than in controls.

Other eicosanoids assessed in BALF supernatant, that is LTB$_4$, PGE$_2$, 6–keto PGF$_1\alpha$, LXA$_4$, 5–HETE, 12–HETE, 9,10–DiHOME, 12,13–DiHOME and 13–HODE, did not show any statistically relevant difference related to group or time point (data not shown).

Pulmonary lesions and histopathology

From a macroscopic perspective, foci of pneumonic inflammation were visible in both study groups challenged with viable *Parachlamydia*. The distribution of foci corresponded predominantly with the sites of inoculum application (Fig. 4a). This means that mainly the caudal parts of the left and right cranial lobe (Fig. 4b), the right middle and accessory lobe as well as both basal lobes were affected (Fig. 4c). Some controls (3/9)
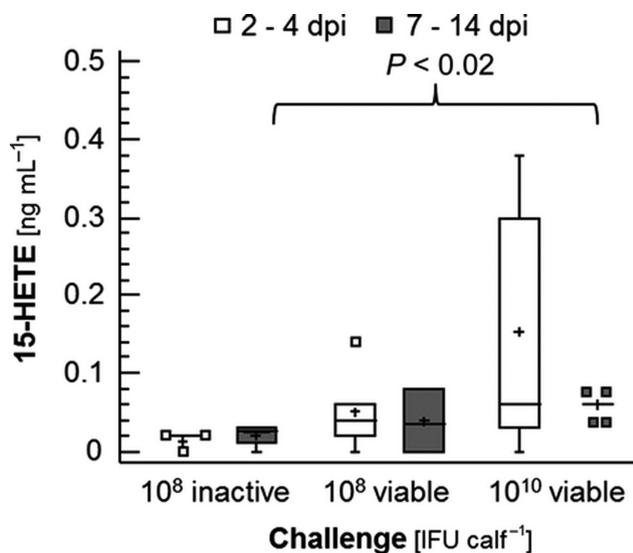
Figure 3. 15-HETE concentration assessed in BALF supernatant. A *U*-test revealed significant inter-group differences between calves challenged with 10^{10} IFU and controls at 7–14 dpi at the given *P*-level.

Table 3. Extent of lung lesions according to challenge dose and time point.

Challenge	Lung lesion (%)					
(IFU per calf)	2–4 dpi		7-14 dpi			U-test (P)
10^8 inactive	0.0 (0.7)	(n = 3)	0.0 (0.0)	(n = 6)		0.24
10^8 viable	1.1 (2.7)	(n = 6)	0.6 (1.8)	(n = 6)		0.17
10^{10} viable	6.6 (6.0)	(n = 8)	1.3 (3.0)	(n = 10)		<0.001
Kruskal–Wallis test	P < 0.002		P < 0.007			

Data is given as median (range). Inter-group comparison was conducted by *Kruskal–Wallis* test and intra-group comparison by *U-tes*

challenged calves decreased in extent significantly compared with the lesions found in the low dose-challenge group and controls. Calves necropsied at the later time points (set 2) showed a significantly greater inter-group discrepancy in affected lung tissue compared with the early time points (set 1). An overview of estimated degrees of altered lung tissue according to challenge and time point is given in Table 3.

The following histological findings refer to calves exposed to 10^{10} IFU of viable *P. acanthamoebae*. In general, the histological character of lesions varied between foci inter- as well as intra-individually. A severe, circumscribed purulent or fibrinous bronchopneumonia, adjacently coexisting at the same time or showing transition, was confirmed at 2 dpi. Alveoli were extensively filled with fibrin and/or neutrophils (Fig. 5b). Besides bronchi-associated lymph nodes, *Lymphonodus (Ln.) bronchotrachealis* in particular, showed a moderate purulent lymphadenitis. Animals necropsied at 4 dpi presented a profound subacute bronchopneumonia in an early stage of resolving. An increased number of macrophages were present in the alveolar exudate and a considerable lymphocytic infiltration occurred in perivascular spaces, interlobular septa and in the area surrounding the
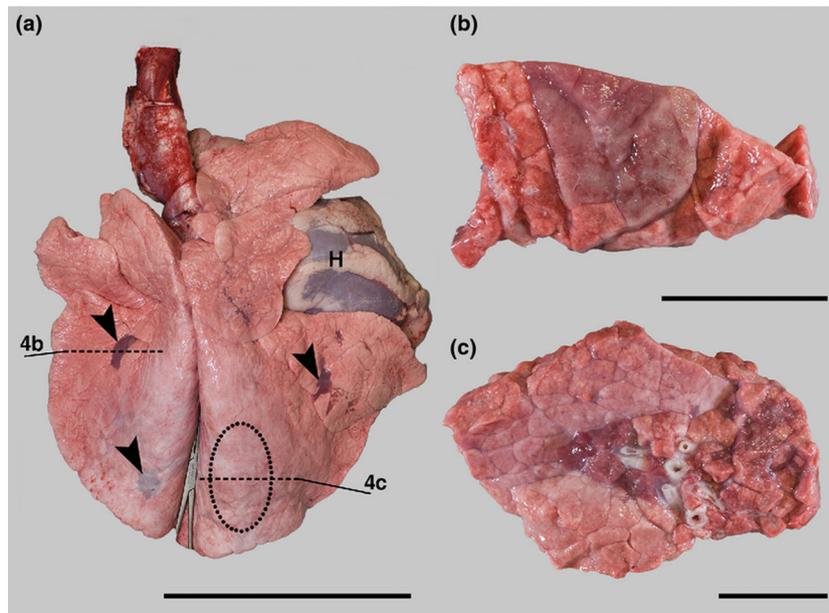
developed a few small foci of soft dark-red pulmonary tissue in both apical and basal lobes of <1%, referring to tissue volume of the entire lung. Low dose-challenged individuals showed altered tissue in a ratio of 1.1 (2.7)% at 2–4 dpi (set 1) and 0.6 (1.8)% at 7-14 dpi (set 2). The greatest degree of macroscopic lung lesions were exhibited by animals challenged with 10^{10} IFU within set 1, which added up to 6.6 (6.0)%, and animals within set 2, amounting to 1.3 (3.0)%. In a dose-dependent manner, the extent of lung lesions was significantly higher in calves receiving 10^{10} IFU than calves inoculated with 10^8 IFU of *Parachlamydia* or controls at all time points observed. Over time, lesions of high dose-

Figure 4. Distribution and extent of macroscopic pulmonary lesions 2 dpi in a calf inoculated with 10^{10} IFU of viable *Parachlamydia acanthamoebae*. (a) Dorsal view of the lung and heart (H). Superficially distinguishable pneumonic lesions are present as red discoloration in the medial lobe and the cranial and caudal aspect of the left caudal lobe (arrowheads). In addition, lesions are present deep in the pulmonary tissue under the area outlined by the dotted circular line. The position of the transversal sections shown in (b) and (c) are indicated by hatched lines. (b) Section through lesion in the cranial part of the left caudal lobe: several lobules are affected by fibropurulent bronchopneumonia. Scale bar: 2 cm. (c) Section through lesion deep in the right caudal lobe: fibrinopurulent lesions are distributed around larger bronchi. They do not extend to the surface of the lung. Scale bar: 2 cm.
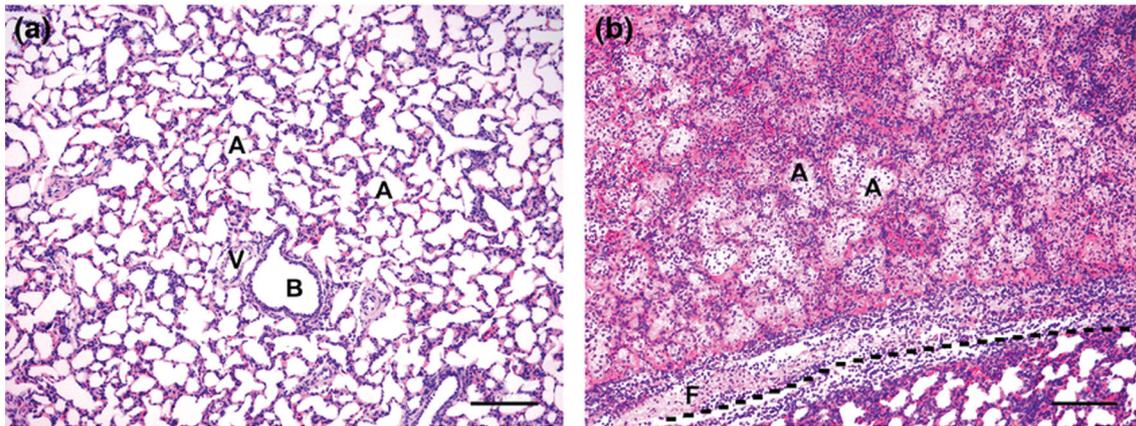
Figure 5. Histology of pulmonary tissue in a calf at 2 dpi. (a) Control: Morphologically normal pulmonary tissue with alveoli (A) separated by thin interalveolar septa, bronchioli (B) and blood vessels (V). H&E stain. (b) 10^{10} IFU of viable *Parachlamydia acanthamoebae*: Pulmonary lobule with severe acute fibrinopurulent bronchopneumonia (above hatched line) is separated by an interlobular septum (hatched line) from adjacent intact pulmonary tissue (below hatched line). Alveoli (A) are filled with fibrin, neutrophils and few macrophages. Lymphatics in interlobular septa are dilated by fibrin thrombi (F). H&E stain. Scale bars: 200 μm.

lower airways. At this time point, a mild purulent lymphadenitis was still present. At 10 and 14 dpi, a stage of progressive pulmonary regeneration was observed, indicated by persistent, circumscribed *bronchiolitis obliterans* and concomitant multifocal enlargement of interalveolar septa by infiltrating histiocytes and lymphocytes. Perivascular and peribronchial lymphocytic infiltration were of varying degree. In calves receiving viable 10^8 IFU of *Parachlamydia,* lesions were assessed as mild acute focal catarrhalic to purulent bronchopneumonia at 2 dpi, followed by a mild subacute multifocal purulent to histiocytic bronchopneumonia and pulmonary abscesses at 4 and 10 dpi. In control animals, intact lung tissue predominated (Fig. 5a). One individual showed a minimal lesion distinguished as a mild multifocal subacute catarrhal-purulent bronchopneumonia affecting the cranial parts of the cranial lung lobes only. All other lesions seen in controls were confirmed as superficial atelectasis.

Immunohistochemistry

In general, considerable labelling was found in both groups of animals challenged with viable *Parachlamydia* and was predominantly associated with histological lesions of lung tissue. Low numbers of *Parachlamydia*, detected as small granular structures, were present in two of three (67%) individuals of the low dose-challenge group at 2 dpi, whereas all three (100%) individuals tested positive at 4 dpi. At both time points, specific labelling was associated with macrophages and neutrophils, and at 2 dpi with alveolar epithelial cells, additionally. There was no specific labelling detected at 10 dpi for this group, even in the pulmonary abscesses.

In calves inoculated with 10^{10} IFU, mostly fibrinous foci tested positive for parachlamydial antigen compared with sites of purulent inflammation. Intact tissue did not exhibit specific labelling. Massive granular, parachlamydial labelling was present in altered lung tissue of all (100%) high dose-challenged individuals at 2 dpi, and were thus considered strongly positive. Granules were different in extent and distribution and were seen especially in association with neutrophil granulocytes, alveolar macrophages and alveolar epithelial cells (Fig. 6b). At 4 dpi, all (100%) samples tested positive with moderate multifocal labelling and were also detected in altered lung tissue. Granules were located either extracellularly or intracellularly within alveolar macrophages. One of three (33%) animals tested positive at

10 dpi, showing pathogen structures in a sporadic manner only. Controls did not show *Parachlamydia*-specific labelling (6a). Tissue of respiratory tract-associated lymph nodes was negative in all groups (data not shown).

Real-time PCR

Swabs
All swabs examined for the presence of parachlamydial antigen tested negative. Throughout the observation period, it was not possible to detect any genomic amplification of parachlamydial DNA, regardless of the origin of the sample or the challenge group tested (not shown).

Tissue and BALF cells
There was molecular evidence of the genomic presence of *P. acanthamoebae* in samples of lung tissue, BALF cells and respiratory tract-associated lymph nodes obtained from animals of both groups inoculated with viable *Parachlamydia* at both investigated time points, that is set 1 and set 2 (Fig. 7). C_t values and corresponding genomic copy numbers are shown in Table 4. In controls, there was no *Parachlamydia*-specific genomic copy detectable at any time (not shown). As Fig. 7a indicates, inter-group comparison revealed parachlamydial genomic copy numbers in altered lung tissue that were significantly higher, as shown by the increasing challenge dose at the given time points. Intra-group comparison indicated a significant decrease of genomic copy numbers over time in both study groups. Significantly more genomic copies were also detected in samples originating from lung lesions compared with samples recovered from intact lung tissue of the same individual (not shown). In set 2, BALF cells deriving from the high dose-challenge group contained significantly more genomic copies of *P. acanthamoebae* compared with the low dose group. A significant decrease of parachlamydial DNA over time was shown by intra-group comparison for isolated BALF cells originating from low dose-inoculated calves (Fig. 7b). Parachlamydial DNA load in respiratory tract-associated lymph nodes, that is *Lnn. tracheobronchiales*, *Lnn. mediastinales* and *Lnn. retropharyngeales* was always higher in 10^{10} IFU challenged individuals than in the ones exposed to 10^8 IFU (Fig. 7c). Significance was evident within set 1. In all other tissues sampled there was no relevant parachlamydial DNA detectable by qPCR (Table 1).
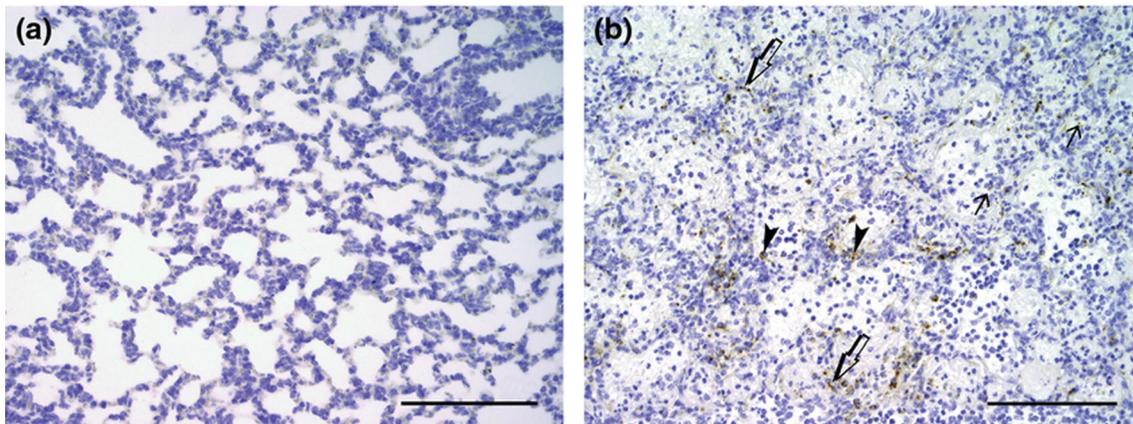
Figure 6. Immunohistochemistry of pulmonary tissue in a calf at 2 dpi. (a) Control: There is no *Parachlamydia*-specific staining by the indirect immunoperoxidase method. (b) 10^{10} IFU of viable *Parachlamydia acanthamoebae*: Parachlamydial inclusions are unevenly distributed within lesions. They are present in alveolar epithelial cells (open arrows), macrophages (arrowheads) and neutrophils (arrows). Indirect immunoperoxidase method. Scale bars: 200 μm.

Amoebal co-culture

Re-isolation of *Parachlamydia* was possible up to 7 dpi (set 2) from lung tissue samples originating from individuals inoculated with viable bacteria. Positive results for co-culture were seen in 7/9 (78%) animals challenged with a dose of 10^{10} IFU. Parachlamydial replication was evident in 4/9 (45%) cases, with all positives sampled at set 1. Furthermore, *Parachlamydia* were recovered by culture from 10/12 (83%) calves exposed to 10^8 IFU; replication could be shown for 3/12 (25%) individuals only. Controls were all negative for parachlamydial recovery and replication in amoebal co-culture (data not shown).

DISCUSSION

To our knowledge this study was the first to investigate experimentally the pathogenicity of *P. acanthamoebae* as an agent of lower respiratory tract infection in an animal model other than the mouse. Concerning the challenge doses of *P. acanthamoebae*, the applied quantities (10^8 IFU and 10^{10} IFU per calf, respectively) were based on observations made in previous studies. As the anticipated individual suffering was extrapolated from the dose–effect relation observed in the mouse model, a minimal number of animals were enrolled in the study due to ethical criteria. In mice, a mortality of 50% was observed at 5 dpi when challenged with 2.5×10^8 IFU per mouse intratracheally (Casson *et al.*, 2008b). Additionally, a dose-dependent severity of illness was observed in a methodologically comparable bovine model of *C. psittaci* infection. Here, animals exhibited respiratory syndromes accompanied by a very severe systemic indisposition when challenged with a dose of 10^9 IFU per calf, whereas the medium alone did not affect the animal's respiratory health (Reinhold *et al.*, 2012).

In the present study, dose-dependent effects were observed with most investigated parameters. The total clinical score of calves exposed to viable *P. acanthamoebae* increased in direct relation to increases in the challenge dose. As shown by the moderately impaired state of health after inoculation, both groups receiving viable *Parachlamydia* developed a mild respiratory course of disease, the extent of which depended on the administered pathogen load. From 1 to 4 dpi, animals exposed to a challenge dose of 10^{10} IFU showed signs of an initiating, apparent respiratory illness that were less severe and even not apparent in calves challenged with 10^8 IFU and in

controls, respectively. Over time, that is beginning from 4 dpi, high dose-inoculated calves recovered from signs of respiratory indisposition and cardiovascular signs (e.g. hyperaemic conjuctiva and oral mucosa), whereas other organ systems (e.g. enlarged mandibular lymph nodes) contributed to the major proportion of the total clinical score. This observation accounts for a shift from a non-specific acute response of the respiratory system directed against the instilled viable *Parachlamydia* towards an attenuated systemic immune response against the replicative pathogen. With respect to pathogenicity, calves exposed to a dose of 10^{10} IFU exhibited a clinical outcome much less severe than in calves challenged with 10^8 IFU of *C. psittaci* (Reinhold *et al.*, 2012). *Parachlamydiaceae* lack most of the chlamydial major outer membrane proteins (Horn *et al.*, 2004), possibly explaining its relative lower virulence. However, the type 3 secretion system of *Parachlamydia* appeared necessary to infect its natural amoebal host (Greub *et al.*, 2009; Croxatto *et al.*, 2013), which may be one reason for a stronger host reaction shown in mice challenged with 2.5×10^8 IFU of *Parachlamydia* (Casson *et al.*, 2008b).

In fact, white blood cell count of infected calves did not show a considerable deviation in total leucocytes, indicating a low activity of the innate immune response against the applied bacteria. However, proportions of the differentiating leucocyte populations were shifting slightly, driven mainly by an increase in neutrophil granulocytes. An initiating regenerative left shift was induced by increasing proportions of band neutrophils in calves infected with 10^{10} IFU.

Controls challenged with inactivated bacteria did not exhibit any deterioration of clinical health or inflammatory markers, which excludes non-specific effects of disengaged LPS originating from destroyed parachlamydial cell walls. Increased serum LBP in groups challenged with viable bacteria indicated the involvement of LPS in the host reaction against live *Parachlamydia*. In contrast, *in vitro* experiments have shown that viable *P. acanthamoebae* neither induce secretion of proinflammatory cytokines, such as interleukin (IL)–1α, IL–6 and tumour necrosis factor (TNF)α, or trigger the oxidative burst which allows *Parachlamydia* to escape the microbicidal response of human macrophages (Greub *et al.*, 2005a). In this study, LBP expression was significantly higher after parachlamydial challenge. However, the increase in LBP levels turned out to be much lower compared with fulminant *C. psittaci* pneumonia (Ostermann *et al.*, 2013a) and experimental *M. haemolytica*
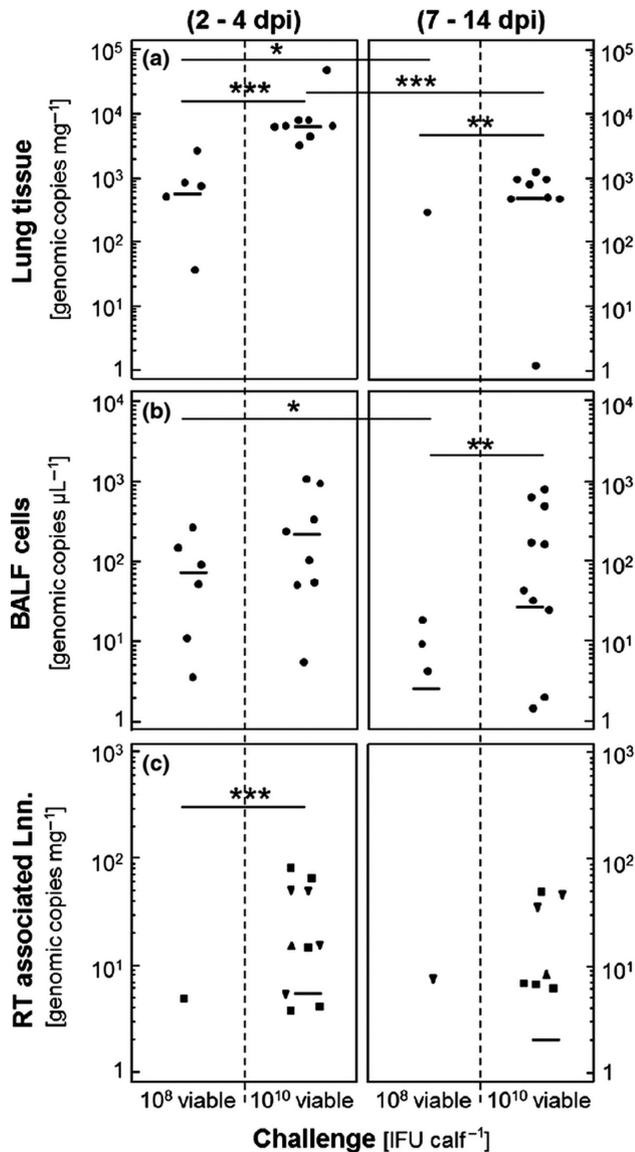
Figure 7. Genomic copy numbers of *Parachlamydia acanthamoebae* detected in tissue according to challenge and time point of sampling. Pathogen load in altered lung tissue (a), isolated BALF cells (b) and respiratory tract (RT)-associated lymph nodes (c) was maximal in calves inoculated with 10^{10} IFU of *P. acanthamoebae*. Data are expressed as individual animals. Corresponding medians are indicated as solid bars. Controls were all negative and are not shown. A *U*-test revealed significant differences between groups at $^*P < 0.03$, $^{**}P < 0.02$ and $^{***}P < 0.01$. *Lnn., lymphonodi.* (c) ■ *Lnn. mediastinales* ▼ *Lnn. tracheobronchiales* ▲ *Lnn. retropharyngeales*.

infection (Schroedl *et al.*, 2001). In the latter case, LBP was proved to be a sensitive and rapid marker of Gram-negative respiratory tract infection in cattle. Lactoferrin concentration, which is in general drastically increased during infection, did not show markedly deviations in inter-group comparisons. It is mainly dependent on neutrophil infiltration and its subsequent release due to LPS detection lowers the iron concentration locally or binds LPS directly (Vogel, 2012). However, a rising proportion of neutrophils in BALF according to the increasing parachlamydial challenge dose but without showing a significant increase in lactoferrin response supports the assumption that the presence and LPS-neutralizing activity of lactoferrin depends on the

presence and concentration of other LPS-capturing proteins such as LBP (Elass-Rochard *et al.*, 1998).

Parameters characterizing local inflammation (BALF cytology and eicosanoids in BALF supernatant) showed contradicting dose-dependent responses. On the one hand, influx of neutrophils due to the parachlamydial challenge caused a shift in the proportion in the composition of the BALF cell population, in line with findings seen in other animal models of respiratory parachlamydial or chlamydial infection (Del Rio *et al.*, 2000; Casson *et al.*, 2008b; Reinhold *et al.*, 2008, 2012). These findings were correlated to the proportional increase of band neutrophils in peripheral venous blood due to the migration of neutrophil granulocytes to the site of infection and the continuous supply through granulopoiesis. Surprisingly, total protein levels in BALF supernatant did not show a pronounced dose-dependent increase, as seen in experimental *C. psittaci* infection as well as in naturally acquired chlamydial infections in calves (Jaeger *et al.*, 2007).

A pathogenic link between chlamydial infections and activation of the cyclooxygenase-mediated arachidonic acid cascade has been shown *in vitro* for multiple cell types (Krausse-Opatz *et al.*, 2004; Rupp *et al.*, 2004; Fukuda *et al.*, 2005) as well as *in vivo* for experimental *C. psittaci* infection (Reinhold *et al.*, 2012). Besides 15–HETE, eicosanoids measured in BALF supernatant did not exhibit significant dose-dependency. Together with the absence of a response of BALF total protein concentration, this supports the idea of a low inflammatory response against viable *Parachlamydia*.

Differences in the incidence and extent of lung lesions elicited by induced respiratory infection correlated well with the severity of clinical outcomes. Intranasally aerosolized *Parachlamydia* did not affect the outcome of the histological, IHC and molecular investigations for neither the nasal mucosa nor the *tonsillae pharyngeales*, suggesting that these are not the primary sites of pathogen entrance. IHC revealed the presence of a large amount of *Parachlamydia* located intracellularly. The absence of cross-reactivity of the rabbit anti-*Parachlamydia* antibody against other *Chlamydia*-like organisms has been tested and proved in previous work (Casson *et al.*, 2007; Borel *et al.*, 2009). The predominant occurrence of *Parachlamydia*-specific granules within neutrophils and alveolar macrophages contributes to the conclusion that these cells represent the first line of the innate immune response and are important target cells of parachlamydial infection. Epithelial cells in general have been described as being the first and major targets of chlamydial infection (Rasmussen *et al.*, 1997). This was rarely the case for *Parachlamydia*, although type 2 pneumocytes and lung fibroblasts have been proposed to be permissive to *P. acanthamoebae*, representing a niche for sustained survival (Casson *et al.*, 2006). Indeed, the alveolar epithelium of viably challenged individuals did label specifically in IHC at 2 dpi only, suggesting that epithelial cells were targeted primarily by *P. acanthamoebae* but did not serve as a location of persistent infection.

Results from qPCR, testing samples of lung lesions and intact tissue, supported the IHC findings. Being the more sensitive method, the molecular presence of *Parachlamydia*-specific DNA in both groups of inoculated animals was evident throughout the study. Additionally, *Parachlamydia* was recovered from altered lung tissue of infected animals for a prolonged period using amoebal co-culture. The decreasing load of parachlamydial DNA in all specimens tested indicates a subsequent pathogen clearance over time. However, molecular detection of specific DNA as well as cultural re-isolation of *P. acanthamoebae* even up to 9 dpi suggests a replicative activity or at least a

Table 4. Genomic copy numbers and corresponding C_t values according to challenge dose and time point.

	Challenge group				
	10^8 IFU per calf viable		10^{10} IFU per calf viable		
	Set 1	Set 2	Set 1	Set 2	*Kruskal–Wallis test (P)*
Lung tissue					
GC (mg^{-1})	620 (0; 2709)	0 (0; 296)	6623 (3197; 37985)	490 (0;1118)	< 0.003
C_t	32.9 (30.1; 35.4)	32.0	28.8 (25.5; 30.1)	31.8 (31.7; 34.9)	
RT-associated lymph nodes					
GC (mg^{-1})	0 (0; 2)	0 (0; 3)	5 (2; 45)	2 (0; 17)	< 0.04
C_t	39.6	39.1	38.2 (36.2; 39.7)	38.6 (35.9; 40.8)	
BALF cells					
GC (μL^{-1})	72 (4; 270)	2 (0; 18)	121 (56; 1078)	103 (1; 741)	< 0.02
C_t	31.6 (29.8; 37.2)	35.6 (34.4; 36.8)	31.2 (27.4; 36.8)	31.5 (28.3; 38.7)	

Values of genomic copy numbers (GC) and cycle threshold (C_t) values are given as medians (minimum; maximum). As for C_t values, only data of samples generating detectable results were included. Data representing respiratory tract (RT)-associated lymph nodes were averaged from all appendant lymph nodes. Set 1 refers to time period 2–4 dpi and set 2 to 7-14 dpi. P-values represent the highest confidence interval in multi-sample comparison with all three challenge groups at both time periods, respectively (*Kruskal–Wallis* test). Controls were all negative and are not shown.

prolonged presence of the pathogen after recovery from apparent clinical signs of indisposition.

As calves were raised conventionally, all results are related to immunocompetent individuals. For this reason it is not surprising that differentiating bacteriology of nasal swabs revealed the sporadic presence of potential respiratory co-pathogens. In earlier serological studies targeting parachlamydial antibodies, the association with immunocompromised hosts also hints at a rather opportunistic character of this amoebal endosymbiont (Corsaro and Greub, 2006). The impact of immunosuppression will need to be addressed in further studies.

The fact that there is no molecular evidence of parachlamydial dissemination after inoculation in organs other than altered lung tissue or associated lymph nodes supports the findings of absent nasal, ocular or faecal shedding. Due to rapid clearance by the nasal mucosa there was no molecular detection of *Parachlamydia* possible even after local application to the nasal cavity. Inter-individual transmission of *P. acanthamoebae* also could not be demonstrated by co-housing infected and naive mice. Interestingly, in infected mice, dissemination was shown by detecting and re-isolating *Parachlamydia* from the spleen (Casson *et al.*, 2008b). Up to date it has not been possible to recover *Parachlamydia* by co-culture from any respiratory sample originating either from a human or animal field infection. Furthermore it is not known to what extent cattle are exposed to *P. acanthamoebae* in their natural surroundings. From this perspective, study of seroprevalence is important to establish a basis for further research.

CONCLUSION

In summary, the calf was found to be a suitable mammalian host to evaluate pathogenicity of *P. acanthamoebae* for the bovine lung *in vivo*. Intra-bronchial challenges of 10^8 and 10^{10} IFU per calf resulted in a mild respiratory illness with a dose-dependent degree of severity. All infected animals which were not subjected to earlier necropsy were fully recovered by the end of the study at 14 dpi. Signs of respiratory illness were already absent from 7 dpi. In comparison with another study of induced respiratory infection with *C. psittaci* using the same bovine model under similar conditions, the clinical outcome, parameters of systemic and local inflammation as well as pathological findings all demonstrated that *P. acanthamoebae* is less pathogenic towards the respiratory system of the bovine host.

STATEMENT

Part of the data was presented at the 54th Conference of the German Society for Pneumology (DGP) Hannover, Germany, 2–5 March 2013, the 2nd European Meeting on Animal Chlamydiosis and Zoonotic Implications (EMAC–2) Jena, Germany, 13–14 June 2013 and the annual meeting of the Veterinary Comparative Respiratory Society (VCRS) in association with the 5th World Equine Airway Symposium (WEAS) Calgary, Canada, 15–17 July 2013.

AUTHORS' CONTRIBUTION

G.G. and P.R. contributed equally as senior authors.

Conflict of interest statement. None declared.

SUPPLEMENTARY DATA

Supplementary material is available at Pathogens and Disease online.

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