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Evaluation and pathophysiological characterisation of a bovine model of respiratory
***Chlamydia psittaci* infection**

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„Die Welt ist groß und Erkenntnis lauert überall."

G. Brem*

Meinen Eltern und Stephan

in Liebe und Dankbarkeit gewidmet

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1 Introduction

1.1 General background

Zoonoses, regardless whether emerging or classical, are not only a challenge for human public health, but also cause significant economic losses in animal husbandry (Christou 2011). Thus, interdisciplinary research networks dealing with zoonotic infectious pathogens were established by the German Federal Ministry of Education and Research (BMBF). *Chlamydiales*, an order of gram-negative, obligate intracellular bacteria, include several species associated with well-documented and suspected zoonotic risks (Wheelhouse and Longbottom 2012). Until recently, research was hampered by the lack of genetic systems and cell-free culturing applicable to chlamydiae (Wang et al. 2011; Omsland et al. 2012). So the overall aims of the network "Zoonotic *Chlamydiae*" included (i) the elucidation of molecular pathogenesis, (ii) the improvement of diagnostic tools, and (iii) the evaluation of medical treatment for human chlamydial infections. An animal model mimicking closely the relevant characteristics of human disease is still a key element for understanding the underlying mechanisms of disease pathogenesis. Moreover, a valid animal model is a prerequisite for development and efficacy testing of therapeutics and vaccines (Kahn et al. 2009).
The present work is an integral part of the above mentioned zoonotic network ('Subproject Two') and focused on the introduction and characterisation of a biologically relevant animal model. The main objective was the profound examination and evaluation of both pathogenetic and epidemiological aspects of infection with *Chlamydia* (*C.*) *psittaci*. This chlamydial species is known to be capable of inducing airborne respiratory disease in animals and humans (Rohde et al. 2010). Given the zoonotic potential of *C. psittaci*, an integrated ONE HEALTH approach (Kahn et al, 2012), beneficial to human and veterinary medicine, as well as epidemiological research, appeared straightforward.

The following paragraphs will familiarise the reader with the key knowledge necessary to understand the peculiarities of chlamydiae and respiratory *C. psittaci* infection. General pulmonary functions and peculiarities of the bovine species and their respiratory tract are described, as they are essential for understanding the expected benefits and potential applications of the developed animal model. After defining the tasks of this thesis, the overall study design is outlined to explain the concept underlying the four experimental studies included in this thesis.

1.2   Chlamydiae

In the further text spelling of taxonomy-related terms is based on current recommendations differentiating strictly between terms related to the taxonomic units (always capitalised C, italics) and latinised common descriptions (no italics, lower case, capitalised C only at the beginning of a sentence, Horn 2011; Kuo et al. 2011; Bavoil et al. 2013).

1.2.1   Taxonomic classification

Chlamydiae are non-motile obligate intracellular bacteria with only distant relationships to other bacterial divisions (Gupta 2001; Stephens 2003). Their cell wall contains lipopolysaccharide (LPS) with a family specific epitope, and an additionally branched Kdo-oligosaccharide is *C. psittaci*-specific (Kdo is 3-deoxy-alpha-d-manno-oct-2-ulosonic acid, Gerstenbruch et al. 2010). However, the absence of peptidoglycan in chlamydial cell walls is a distinctive feature to typical gram-negative bacteria (Horn 2011). Further peculiarities include a small genome (1 – 2.4 Megabases) which most probably resulted from reductive co-evolution within their eukaryotic host cells (Tan and Bavoil 2012). As the most characteristic feature, they share a unique biphasic developmental cycle (Abdelrahman and Belland 2005; Horn 2011), which takes, dependent on the chlamydial species, 40 – 80 hours *in vitro* (Schoborg 2011).

The Phylum *Chlamydiae* contains only one class, *Chlamydiia,* and one order, the *Chlamydiales,* which is currently comprised of eight families. The family *Chlamydiaceae* currently includes only the accepted genus *Chlamydia* (Kuo et al. 2011) and, thus, all classical known chlamydial pathogens which are summarised in Table I-1. The former genus name *Chlamydophila* is no longer in use (Tan and Bavoil 2012), but the fundamental subdivision into nine species (Tab. I-1) suggested by Everett et al. in 1999 is still a valid reference. Literature published prior to 1999 should be regarded critically with respect to the identity of chlamydial species. The addition of two new species, resulting from the isolation of non-classified strains in bird samples during the last decade, is currently under consideration (Gaede et al. 2008; Laroucau et al. 2009; Gasparini et al. 2011; Sachse et al. 2012; Sachse et al. in press).

Chlamydia spp. are closely related pathogens sharing a core set of 668 conserved proteins, which amounts to two-thirds of the genome. New evidence suggests that human chlamydial isolates have been acquired in our evolutionary past from animal hosts (Tan and Bavoil 2012). Currently, relatively little is known regarding the chlamydial factors involved in virulence, host interaction or host specificity (Voigt et al. 2012), mainly due to the fact that applicable genetic systems and cell-free culturing have been developed only recently (Wang et al. 2011; Omsland et al. 2012).

Table I-1: Overview about *Chlamydia* (*C.*) *species* and their pathogenetic relevance.

Species	Common hosts	Pathogenetic significance
C. abortus	small ruminants (sheep, goat)	• abortions ('ovine enzootic abortion', 'enzootic abortion of ewes') • asymptomatic infections are frequent • **zoonotic potential!**
C. caviae	guinea pig	• conjunctivitis uro-genital infections
C. felis	cat	• pneumonia, rhinitis, conjunctivitis • asymptomatic infections are frequent • **zoonotic potential!**
C. muridarum	(laboratory) mouse	• pneumonia
C. pecorum	cattle, small ruminants (sheep, goat)	• enteritis, encephalitis, pneumonia, polyarthritis, uro-genital infections, mastitis, abortions and fertility disorders • asymptomatic infections are frequent
C. pneumoniae	human	• respiratory disease
C. psittaci	all avian species	• respiratory disease ('psittacosis', 'ornithosis', 'parrot disease') • asymptomatic infections are frequent • **zoonotic potential!**
C. suis	pig	• enteritis, conjunctivitis, mastitis, pericarditis, pneumonia, polyserositis, uro-genital infections • asymptomatic infections are frequent
C. trachomatis	human	• trachoma (eye infection in tropical countries) • sexually transmitted uro-genital infections
Proposed species (Sachse et al. in press)		
C. avium sp. nov.	psittacine birds, pigeons	• probably facultative pathogen • zoonotic potential unclear
C. gallinacea sp. nov.	chicken, turkey	• asymptomatic infections are frequent • zoonotic potential unclear

1.2.2   Chlamydial developmental cycle and persistence

The infectious form of chlamydiae is represented by small, rigid-walled, environmentally-resistant elementary bodies (EBs; Abdelrahman and Belland 2005; Kuo et al. 2011). The doctrine of EBs being metabolically dormant has to be reconsidered as recent expression analysis of messenger ribonucleic acid (mRNA) has revealed high levels of metabolic and biosynthetic activity in EBs (Omsland et al. 2012). *In vitro*, chlamydiae are able to invade a variety of cell types. *In vivo*, the infection of epithelial cells is a perquisite for inducing infection, disease and persistence in the host species (Rasmussen et al. 1997; Stephens 2003). The adherence of EBs to susceptible host cell surfaces is mediated by a variety of different adhesion-receptor combinations (reviewed by Hackstadt 1999; Dautry-Varsat et al. 2005; Campbell and Kuo 2006). By means of a type III secretion system, *Chlamydia* injects effector proteins into the epithelial cells (Beeckman et al. 2008) and initiates extensive remodelling of the actin cytoskeleton at the bacterial attachment site, resulting in the engulfment of EBs by plasma membrane extensions (Dunn and Valdivia 2010). The involvement of clathrin in subsequent endocytosis was reported to be dependent on the chlamydial species (Korhonen et al. 2012). Intracellular survival is enabled by inhibition of the phagolysosome fusion and establishment of a membrane-bound vacuole, termed inclusion (Eissenberg and Wyrick 1981; Eissenberg et al. 1983). Within this inclusion, EBs differentiate via intermediate bodies into metabolically active but non-infectious reticular bodies (RBs), which then multiply several times in succession by binary fission (Hackstadt et al. 1997; Abdelrahman and Belland 2005; Schoborg 2011). Due to dependency on host-derived molecules and a regular exchange of molecules across the inclusion membrane, chlamydiae are often referred to as parasites (Entrican et al. 2004). After reorganisation of RBs into intermediate bodies (IBs) and finally to EBs the latter will be released by either host cell lysis or extrusion of the inclusion enabling a new infectious cycle (Hybiske and Stephens 2007). A third morphologically and metabolically distinct form, so-called aberrant bodies (ABs), may evolve as a phenotype of RBs due to disruption of the cycle under stressful growth conditions (e.g. nutrient deprivation, immunological responses, antibiotics, oxidative stress or viral co-infections; Goellner et al. 2006; Wyrick 2010; Prusty et al. 2012). So far ABs have been generally accepted as viable but non-infectious among chlamydiologists (Schoborg 2011). *In vitro* ABs are not cultivable but infection is revivable when living conditions improve (Hogan et al. 2004; Goellner et al. 2006). Despite strong evidence of persistence *in vitro* (reviewed by Hogan et al. 2004; Wyrick 2010), electron-microscopic identification of ABs in vital mammalian model organisms is rare (Pospischil et al. 2009; Phillips Campbell et al. 2012). It has not yet been determined whether or not chlamydiae enter this developmental state in order to establish chronic host infections (Schoborg 2011).

The term 'persistence', in literature ambiguously used for both the *in vitro* and *in vivo* phenomena, does not necessarily connote the aberrant RB phenotype *in vivo* (Wyrick 2010). However, latency or persistence is a key concept in chlamydial pathogenesis (Stephens 2003; Longbottom et al. 2013). In this thesis it describes a state of infection during which the host immune response does not eliminate the pathogen, resulting in continuing presence and probable damage to the host (Casadevall and Pirofski 2000; Stephens 2003).

1.2.3 *Chlamydia psittaci*

A zoonotic *C. psittaci* outbreak was first described in 1879 by Jakob Ritter and in the early 19th century, outbreaks were regularly associated to the import of exotic pet birds (Pospischil 2009). In this pre-antibiotic era, human psittacosis was fatal in 15 % to 20 % of cases (Rohde et al. 2010). Today, *C. psittaci* infections occur worldwide with incidences and distribution varying largely with the present bird species and sero- or genotype of the pathogen, respectively (Andersen et al. 1997; Longbottom and Coulter 2003). *C. psittaci* infections are notifiable in many countries, but the number of reported human cases is thought to be underestimated, due to inadequate epidemiological coverage and insufficient diagnostic testing (Harkinezhad et al. 2009b; Rohde et al. 2010). Infections with this pathogen were referred to as psittacosis, parrot fever or ornithosis, as an attempt to include the involved bird species into the disease description. Avian chlamydiosis was proposed as a unifying term (Andersen et al. 1997). In the near future this term might also become too unspecific, because it would include new, recently suggested chlamydial species which are also hosted by birds (Sachse et al. in press). Moreover, the host range of *C. psittaci* has been expanded to wild and domestic non-human mammals with the improvement of polymerase chain reaction (PCR)-based diagnostic tools.

1.2.3.1 Serovars, genotypes and further genome analysis

The subdivision of *C. psittaci* into eight serovars was based on the use of monoclonal antibodies against the major outer membrane protein (MOMP; Andersen et al. 1997). Today, the analysis of the MOMP encoding *ompA* gene provides a faster and more sensitive method for species subdivision. Analysis of the naturally occurring *ompA* gene sequences by either sequencing (Everett et al. 1999), genotype-specific real-time PCR (Geens et al. 2005) or microarray led to the recognition of at least 15 *ompA* genotypes (Sachse et al. 2008).
All serovars and genotypes should be considered to be transmissible to humans (Geens et al. 2005; Beeckman and Vanrompay 2009; Harkinezhad et al. 2009a). Six classically known genotypes (A-F) were reported to be mainly associated with certain types of birds, whereas another two genotypes were isolated from cattle or muskrats. An overview about serovars, genotypes, associations with the predominant host species and the documentation of zoonotic transmission is given in Table I-2. Genotype A, however, was also shown to occur in cattle, sheep, goats, horses and pigs (Pantchev et al. 2010). The genetic diversity does not seem to be

fully assessed by these 15 genotypes, thus further subdivision patterns were proposed (Laroucau et al. 2008; Sachse et al. 2008). Recently, multi-locus sequence typing has generally confirmed the association between genotype and bird host species, but also revealed an expanded genome diversity and host range (Pannekoek 2012). Seven bovine *C. psittaci* isolates clustered together with those of rabbits, rats and psittacine birds, suggesting host species jumps (Pannekoek 2012). These findings have been supported by comparative whole genome phylogenetic analysis, revealing frequently switching hosts and genome-wide recombination (Read et al. 2013).

Table I-2: Serotypes and genotypes of *C. psittaci*
(modified according to Stephens 2003; Sachse et al. 2008; Stewardson and Grayson 2010).

Serovar	Genotype	Predominant host order: Examples	Human infection documented
A	A	Psittaciformes: budgerigars, cockatiels, parakeets	Yes
B	B	Columbiformes: pigeons, doves	Yes
C	C	Anseriformes: ducks, geese, swans	Yes
D	D	Galliformes: turkeys, pheasants, chickens	Yes
E	E	Struthioniformes: ostriches, rheas. Also in pigeons and ducks, low virulence disease in turkeys	Yes
F	F	Isolated from a single parakeet and turkey only	Yes
WC	G	Cattle	No
M56	H	Rodents: muskrat; snowshoe hare	No
n.d.	E/B	Ducks	Yes
n.d.	1V	*n.d.	No
n.d.	6N	*n.d.	No
n.d.	Mat116	*n.d.	No
n.d.	R54	*n.d.	No
n.d.	YP84	*n.d.	No
n.d.	CPX0308	*n.d.	No

Abbreviations: n.d. not defined, *n.d. avian origin, but host range is not defined.

1.2.3.2 Importance of *C. psittaci* infections

C. psittaci is listed as a class B bioterrorism agent in Germany and other countries (Klee et al. 2003; Maidhof 2007; Centers for Disease Control and Prevention 2013) which are by definition, moderately easy to disseminate, result in low mortality rates, and require considerable demands on the public health services. Once distributed, organisms can remain viable in organic material (e.g. feather dust) for over a month (Smith et al. 2010).

C. psittaci infections in birds

C. psittaci was detected in at least 360 wild and domestic bird species, so that generally all avian species are considered to be susceptible (Kaleta and Taday 2003; Pospischil et al. 2010). *C. psittaci* infection may induce acute, subacute, chronic or subclinical (inapparent) infections with respiratory, gastrointestinal and systemic clinical signs (reviewed by Kaleta and Taday 2003; Longbottom and Coulter 2003). Incubation period (5 days to 10 weeks), disease severity, morbidity (5 – 80 %), and mortality rates (1 – 30 %) depend on dose, virulence of the infecting strain, host species and its immune competence (e.g. determined by maturity or concurrent illnesses; Andersen et al. 1997; Pospischil et al. 2010). Infection and disease with *C. psittaci* cause economic losses to the poultry industry due to increased mortality, reduced performance and the usual necessity of antmicrobial treatment.

C. psittaci infections in humans

Human *C. psittaci* infections are frequently associated with leisure or occupational exposure to birds (Stewardson and Grayson 2010), but even brief or unexpected exposure to contaminated bird excretions may suffice to transmit the pathogen, e.g. by lawn-mowing in gardens polluted by bird droppings (Williams et al. 1998; Telfer et al. 2005; Dickx et al. 2010; Smith et al. 2010). The majority of human infections are mild or subclinical (Moroney et al. 1998), wherefore reported cases are thought to be underestimated (Harkinezhad et al. 2009a; Rohde et al. 2010). Clinical signs are usually reminiscent of influenza, with headache, myalgia, fever, and mild cough, but can also cover a broad range of life-threatening complications including endocarditis, myocarditis, glomerulonephritis, hepatitis, pancreatitis, or meningoencephalitis. According to literature, cases leading to multiorgan failure and death are relatively rare today (reviewed by Beeckman and Vanrompay 2009; Rodolakis and Mohamad 2010; Rohde et al. 2010; Stewardson and Grayson 2010). However, *C. psittaci* is a well-known cause of 'atypical' community-acquired pneumonia (CAP). Pneumonia is referred to as 'atypical' when it is accompanied by additional systemic reaction. *C. psittaci* causes only about 1 % of atypical pneumonia cases (Gacouin et al. 2012) with specific local epidemiological factors influencing the incidences (Bartlett et al. 1998). Factors associated with disease severity in humans are rather suggested than investigated (Crosse 1990; Moroney et al. 1998) because it is difficult to distinguish severe psittacosis from other severe forms of

pneumonia (e.g. Legionaire's disease) when diagnosis is solely based on clinical, radiological and biological presentation (Gacouin et al. 2012).

C. psittaci in non-human mammals

The role of non-avian *C. psittaci* strains has yet not been evaluated with respect to their zoonotic potential (Reinhold et al. 2011), which is seemingly rare from these sources (Stewardson and Grayson 2010). Likewise, the pathogenetic role is not readily clear. *C. psittaci* infections have been associated with recurrent airway obstruction or chronic obstructive pulmonary disease in horses (Theegarten et al. 2008) and with reproductive disorders in pigs (Kauffold et al. 2006). *C. psittaci* is regularly detected in cattle and sheep (Borel et al. 2006; Twomey et al. 2006; Kauffold et al. 2007; Kemmerling et al. 2009; Pantchev et al. 2010; Lenzko et al. 2011), and experimental challenge of calves was shown to induce respiratory disease (Bednarek and Niemczuk 2005). Additionally the pathogen was found in companion, laboratory and wild animals, i.e. in dogs (Sako et al. 2002; Sprague et al. 2009), ferrets (Read et al. 2013), rabbits and rats (Pannekoek 2012), wild boar (Hotzel et al. 2004), red deer (Di Francesco et al. 2012), muskrats & snow hare (Longbottom and Coulter 2003).

1.2.3.3   Transmission within and between species

Shedding via faeces, ocular or nasal secretions can occur intermittently in birds with or without signs of clinical disease. It can be activated by stress factors, such as transportation, rehousing, overcrowding, chilling, breeding, poor nutrition or concurrent diseases (Longbottom and Coulter 2003; Harkinezhad et al. 2009a).

The primary route of infection for birds and humans is inhaling aerosolised bird excreta , but direct contact, ingestion and blood-sucking ectoparasites have also to be taken into account (Longbottom and Coulter 2003; Harkinezhad et al. 2009a; Pospischil et al. 2010).

There is evidence for the possibility of human-to-human transmission (Ito et al. 2002; McGuigan et al. 2012) but it is thought to be a rare event (Longbottom and Coulter 2003).

The organism is environmentally labile but can remain infectious for over a month if protected by organic debris (e.g., litter and faeces, Smith et al. 2010).

With respect to the zoonotic potential all known serovars or genotypes should be considered transmissible to humans (Beeckman and Vanrompay 2009; Harkinezhad et al. 2009a). The zoonotic potential of non-avian *C. psittaci* strains is thought to be minimal, but, to date, reliable data about the excretion and transmission of *C. psittaci* in mammalian hosts are lacking.

1.2.3.4   Diagnostic investigation

Culturing of *Chlamydiae* is relatively difficult, with a sensitivity of at best 60 % to 80 % (Ward 2002). In case of *C. psittaci*, targeted culturing is restricted to biosafety level 3 laboratories. Dead chlamydiae, e.g. after inappropriate sampling, transport or storage, as well as the aberrant RB phenotype are not cultivable at all. In contrast, PCR-based detection methods are fast, highly sensitive and specific, but do not allow any conclusion about viability. Serologic discrimination between infections with *C. psittaci* and other chlamydial species is currently not possible with commercially available enzyme-linked immunosorbent assays (ELISA). However, there are attempts to increase specificity and reduce cross-reactions by the use of synthetic proteins.

1.3   Bovines as a suitable model species

Interpreting the results of animal models, one must be aware of the major structural and functional differences between the model and the target species (Pabst 2008). These differences may not only cause limitations of the transferability of results to other species, but might also mirror pathophysiological mechanisms more clearly due to an increased level of sensitivity.

The reasons for choosing calves as a suitable host to model respiratory *C. psittaci* infections in the mammalian lung are introduced in STUDY 1 with regard to susceptibility, genetics, immunobiology and some aspects of the respiratory physiology. The evaluation of the present animal model rests largely on different aspects of the lung function. Therefore, the following paragraphs provide more detailed information about peculiarities in structure and function of the bovine lung.

1.3.1   Structural and functional peculiarities of the bovine respiratory tract

Interspecies comparison of the sub-gross lung anatomy resulted in the classification of the bovine lung as a so-called 'Type I lung' which are characterised by extremely well-developed lobulation, a thick pleura, the presence of terminal bronchioles, infrequently observed respiratory bronchioles and a lack of collateral airways (McLaughlin et al. 1961; Kirschvink and Reinhold 2008).

1.3.1.1   Segmental anatomy

Lungs of cattle show well-developed lobation (schematically depicted in Supplement Figure S2 of STUDY 1) and lobulation with secondary lobules distinctly separated from each other by complete fascial sheaths and covered by a thick pleura (Robinson 1982). Each segment and subsegment represents a closed functional unit, consisting of one supplying bronchus which is accompanied in parallel by its functional blood vessels (McLaughlin et al. 1961). The high degree of compartmentalisation results in a large amount of interstitial tissue (McLaughlin et

al. 1961; Robinson 1982), which is partly responsible for a low specific lung compliance and high lung tissue resistance to airflow compared to other mammals (Lekeux et al. 1984a). The required breathing activity in cattle is, therefore, rather high compared to other species (Veit and Farrell 1978; Gallivan et al. 1989a). The pronounced segmentation enables sharp demarcation of inflammatory processes, but is − in combination with a lack of collateral airways − extremely disadvantageous under conditions of obstructive disorders (Reinhold 1997).

1.3.1.2   Collateral ventilation

The phenomenon of collateral ventilation is defined as ventilation via passages or channels that bypass the standard airway branching pattern (Mitzner 1991; Voshaar 2008). Bypassing structures considered for the human lung include alveolar pores ("pores of Kohn", Kohn 1893), bronchiole-alveolar communications ("channels of Lambert", Lambert 1955), and interbronchial connections ("channels of Martin", Martin 1966). Collateral ventilation has been shown to prevent regional alveolar hypoxia and thus help to maintain the ventilation-perfusion balance (Kuriyama et al. 1984). The bovine lung, however, lacks structures enabling collateral ventilation (McLaughlin et al. 1961). Thus obstructions occurring in bovine respiratory diseases frequently result in the development of atelectases (Caswett and Williams 2007). Under conditions of partial obstruction, "air trapping" might develop due to incomplete deflation prior a new inflation. This condition can deteriorate further when pressure of active expiration overcomes the intraluminal pressure so that small airways temporarily collapse. Thus not only ventilation itself is impaired, but overdistension of alveoli may also result in obstructive emphysema (Reinhold 1997).

1.3.1.3   Lung size and gas exchange capacity

In relation to body weight, the bovine species has only a small lung volume and a small alveolar surface area, thus the percentage of ventilated lung tissue per breath (29.0 %) is increased compared to other species (e.g. cat 7.5 %, man 11.0 %, horse 14.3 %, Veit and Farrell 1978). Also in relation to body weight, bovines have, at least compared to horses, an increased basal oxygen consumption (Gallivan et al. 1989b).
The exchange of oxygen and carbon dioxide across the alveolo-capillary membrane is not only dependent on the ventilation and alveolar surface area, but also on the number of pulmonary capillaries and diffusion per alveolar section. It has been shown that the number of capillaries per alveolar section is low in the bovine lung (Berg 1982), which may act as a limiting factor of the gaseous exchange in case of pulmonary disease.

1.3.1.4   Alveolar ventilation and dead space volumes

Only a part of the tidal volume (Vt) reaches peripheral pulmonary tissues, where the gas exchange takes place: this is the alveolar ventilation. The pulmonary gaseous volume not

involved in gas exchange is called dead space volume (Vd) and has to be subdivided in two portions: (i) anatomical dead space volume (Vd_{anat}) and (ii) alveolar dead space (Vd_{alv}; Lekeux and Art 1993). Vd_{anat} is determined by the size of the conducting airways, i.e. nose, mouth, pharynx, larynx, trachea, bronchi and non-respiratory bronchioles, where inspired air is humidified and warmed up (Comroe 1965). The absolute volume of Vd_{anat} is invariable except for a growth related increase. In contrast, Vd_{alv} represents a variable volume present in alveoli which are not involved in gas exchange (Lekeux and Art 1993) due to inhibited perfusion of diffusion.

In cattle, the ratio between dead space volume and tidal volume (Vd/Vt) increases with age from 40 % – 55 % (calf) to 75 % (adult cattle) due to anatomy and length of the tracheobronchial tree. Compared to other species, this ratio is comparable to horses (49 – 75 %), but higher than in dogs (33 %) or pigs (53 – 59 %) for example (Gallivan et al. 1989b; Kirschvink 2008).

1.3.1.5 Reactivity of pulmonary vessels

Alveolar hypoxia is known to elicit a fast but reversible vasoconstriction of small pulmonary vessels, especially small precapillary arteries in the mammalian lung (described at first by Euler and Liljestrand 1946; Reinhold and Höchel 2005). Hypoxic pulmonary vasoconstriction (HPV) is an autonomous physiological phenomenon in healthy lungs and a regional adjusting mechanism in alveolar hypoventilation (Reinhold and Höchel 2005; Robinson 2007b). Increased vascular resistance resulting in diversion of blood from hypo-ventilated towards better ventilated regions of the lung is the only mechanism to maintain ventilation-perfusion balance in species lacking collateral ventilation (Kuriyama et al. 1984). Compared to other domestic animals, bovines have an increased amount of smooth muscles in the media of small pulmonary arteries, enabling a vigorous vasoconstriction (Robinson 2007b). Many vasoactive substances (e.g. histamine, angiotensin, catecholamines, arachidonic acid metabolites) are probably involved into the modulation of hypoxic vasoconstriction (Robinson 2007b). Due to this peculiarity in the pulmonary vasculature, bovines are highly susceptible to develop pulmonary hypertension in conditions of either hypoventilation or inflammation.

1.3.1.6 Mucosal-associated lymphoid tissue of the bovine respiratory tract

In contrast to murines, bovines and humans do not have mucosal-associated lymphoid tissue (MALT) at the bottom of the nasal cavity. Instead, lymphatic tissue of Waldeyer ring is constitutively organised as tonsils (Liebler-Tenorio 2007). Within the lung, bronchus-associated lymphoid tissue (BALT) is absent in neonatal calves and increases progressively with age until it declines in aged adult cattle (Anderson et al. 1986).

1.3.2   Age dependent peculiarities

Lung morphology in mature new-born calves is structurally comparable to adult bovines, because − in contrast to rats and mice − prenatal lung development already includes the development of alveoli (de Zabala and Weinman 1984; Reinhold et al. 2007a; Tschanz 2007). With increasing postnatal age, ongoing alveolarisation, as well as growth in the number and surface of bronchiolar diameters (Castleman and Lay 1990), continues up to a body weight of about 300 kg. During this period, lung function parameters generally correlate more closely with body weight than with age (Lekeux et al. 1984a; Gustin et al. 1988a). From a translational point of view, lung volumes, airflows and respiratory mechanics are comparable between adult humans and the lung of calves weighting about 50 – 100 kg (Kirschvink and Reinhold 2008).

1.3.3   Assessment of pulmonary functions in calves

The main function of the lung is gas exchange, i.e. the delivery of oxygen (O_2) to and the removal of carbon dioxide (CO_2) from the organism. Pulmonary ventilation, perfusion, diffusion, and distribution (i.e. the ratio between ventilation and perfusion) are components of this process. The delivery of oxygen-rich air is determined by the effort exerted by the respirartory muscles, the elasticity of the lung and chest wall, and the flow resistance of the airways (Robinson 1982). Deflation is usually facilitated by elastic restoring forces of lung tissue. In addition chest wall and expiratory muscles do assist, mainly under conditions of forced expiration.

The application of pulmonary function tests (PFTs) in veterinary science is restricted by the collaboration of the animals, especially when they are meant to be conscious and to breathe spontaneously. Nevertheless a wide range of PFTs has been successfully adapted to calves (summarised in Tab. I-3).

In the present study, lung functions were evaluated by (i) assessing the pulmonary gas exchange function (using arterial blood gas analysis and haemoxymetry) and by (ii) non-invasive PFTs, i.e. impulse oscillometry, volumetric capnography and Helium (He) rebreathing for measurement of the functional residual capacity (FRC). All methods were adapted from human medicine and had been previously evaluated for their use in conscious calves (Tab. I-3). Due to their non-invasiveness, repeated measurements and an intra-individual study approach were possible under spontaneous breathing conditions without causing harm to the calves.

Table 3: Methods to assess pulmonary functions in unsedated calves (modified according to Lekeux et al. 1993).

PFT-method	Assessed parameters (examples)	Assessed pulmonary function	References
Blood gas analysis	$p(a)O_2$, $p(a)CO_2$	gas exchange	(Donawick and Baue 1968; Gustin et al. 1988c; Nagy et al. 2002)
Spirometry	RR, Vt, Vmin	ventilatory pattern	(Spörri and Zerobin 1964; Bureau et al. 2001)
CO breathing (multiple breath)	CO transfer factor	gas exchange	(Kneucker 2008)
He dilution (wash in, multiple breath)	FRC	ventilation	(Kneucker 2008; Kneucker et al. 2008)
N_2 wash out (multiple breath)	FRC pulmonary N_2 clearance delay	ventilation distribution	(Gallivan and Mcdonell 1989)
Volumetric capnography	end-tidal cCO_2, dead space volumina, curve shape	ventilation distribution	(Reinhold et al. 2007b; Reißig 2007)
Lung scintigraphy	inhalation-to-perfusion ratio	distribution	(Gustin et al. 1988b; Verhoeff et al. 1988)
Tidal breathing flow-volume loops	loop shape indices	resp. mechanics	(Lekeux et al. 1988)
Oesophageal pressure measurement and **Pneumotachography**	Total pulmonary resistance, and dynamic lung compliance, RR, Vt, Vmin	resp. mechanics ventilatory pattern	(Lekeux et al. 1984a; Lekeux et al. 1984b; Lekeux et al. 1984c)
Monofrequent oscillation technique	oscillatory resistance	resp. mechanics	(Reinhold et al. 1992; Reinhold et al. 1996)
Forced oscillation technique	Rrs, Xrs	resp. mechanics	(Gustin et al. 1988d)
Impulse oscillometry	Rrs, Xrs	resp. mechanics	(Reinhold et al. 1996; Reinhold et al. 1998a; Reinhold et al. 1998b; Smith et al. 2005)

Methods used in the present study are highlighted in boldface.

Abbreviations: c concentration, CO carbon monoxide, CO_2 carbon dioxide, FRC functional residual capacity , He Helium, N_2 nitrogen, $p(a)O_2$ partial pressure of oxygen in arterial blood, $p(a)CO_2$ partial pressure of carbon dioxide in arterial blood, PFT pulmonary function test, resp. respiratory, RR respiratory rate, Rrs respiratory resistance, Vmin volume of minute ventilation, Vt tidal volume, Xrs respiratory reactance.

1.4 Aims of the project

In accordance with the ONE HEALTH concept (Kahn et al. 2012), the overall aim of this project was to introduce and to evaluate an integrated respiratory *C. psittaci* animal model, advantageous for both human and veterinary medicine. The working hypothesis was that calves, representing natural hosts for *C. psittaci*, are well suited to model respiratory chlamydial infections of the mammalian lung. Secondly, respiratory large animal models may offer a great clinical translational potential (Ballard-Croft et al. 2012).

During the introduction of a respiratory *C. psittaci* infection model in calves, the objectives were

 I. Development of a highly reproducible endoscopic application pattern and a challenge dose titration to evaluate doses of *C. psittaci* inducing mild, moderate and severe acute respiratory disease (STUDY 1)

 II. *In vivo* verification of the dose dependency with respect to consequences for the pulmonary gas exchange, innate immunity and acute-phase reaction (STUDY 2)

 III. Clinical and immunological characterisation of the long-term course of an acute experimental versus naturally acquired *C. psittaci* infection and evaluation of possible transmission routes (STUDY 3)

 IV. Pathophysiological characterisation of pulmonary dysfunctions and acid-base imbalances induced by an acute respiratory *C. psittaci* infection (STUDY 4)

1.5 Design of the project

In order to clarify the aforementioned issues, a total of 69 calves (aged $42 - 64$ days) were included in the four studies. Aims, materials and methods, groups and durations of the studies are summarised in Table I-4. Generally the project was subdivided into two major sections. (i) In order to evaluate dose response relationship (STUDIES 1 and 2), 24 calves were allocated to six different groups (Tab. I-4): Four groups were challenged with different doses of viable *C. psittaci* ($10^6 - 10^9$ inclusion-forming units (ifu)/calf, n = 14) and two groups served as controls to exclude non-specific influences of medium and cell or pathogen components (n = 10). (ii) Course and pathophysiological consequences of an acute respiratory *C. psittaci* infection were assessed in a long-term follow-up study (STUDIES 3 and 4). Clinical signs, immune response and pathogen excretion were assessed prior to and after experimental challenge with 10^8 ifu/calf (n = 21) and were compared to a group of naïve calves (n = 3), which acquired the infection naturally after socialisation with the infected animals (STUDY 3). In addition, pulmonary (dys)function and acid-base imbalances induced by *C. psittaci* were assessed in comparison to a control group challenged with uninfected cell culture (n = 21). Further details of animals, groups, sampling and necropsy time points are given in the corresponding studies.

Table 4: Aims, structures, materials and methods of STUDIES 1 to 4.

Study	Aim	Ending	Doses		n	Materials and Methods

STUDY 1:
 	
A bovine model of respiratory *Chlamydia psittaci* infection: Challenge dose titration
	Establishing and evaluating the application of different *C. psittaci* doses and control media with respect to clinical and pathological signs as well as immunological host response	3 dpi
		
		(individual animals up to 14 dpi)
			viable *C. psittaci*	10^6 ifu/calf	4	*in vivo*:
						• clinical examination (scoring)
						• immunoblotting of ssAB from blood sera
						ex vivo:
						• BALF analysis
						  - cytology
						  - markers of inflammation
						  - PCR detection and quantification of *Chlamydiae*
						  - immunoblotting of ssAB
						• gross pathology
						• histopathology
						• immunohistochemistry
						• PCR-based quantification of chlamydial antigen in the lung

				10^7 ifu/calf	4	
				10^8 ifu/calf	4	
				10^9 ifu/calf	2	
			controls	ink-spiked cell culture medium	4	
				UV-inactivated 10^8 ifu/calf	6	

STUDY 2:
 	
Dose-dependent effects of *Chlamydia psittaci* infection on pulmonary gas exchange, innate immunity and acute-phase reaction in a bovine respiratory model
	Evaluation of pathophysiological consequences (gas exchange, acid-base equilibrium) and immunological reactions with respect to different doses of *C. psittaci* and to control media	3 dpi	viable *C. psittaci*	10^6 ifu/calf	4	*in vivo*:
						• examination of the respiratory system (scoring)
						• blood analysis
						  - arterial blood gases
						  - electrolytes
						  - metabolites
						  - haemoxymetry
						  - WBC and differentiation
						  - biomarkers of acute-phase reaction

				10^7 ifu/calf	4	
				10^8 ifu/calf	4	
				10^9 ifu/calf	2	
			controls	ink-spiked cell culture medium	4	
				UV-inactivated 10^8 ifu/calf	6	

Table I-4: continued

Study	Aim	Ending	Doses		n	Materials and Methods

STUDY 3:
 	
Infection, disease, and transmission dynamics in calves after experimental and natural challenge with a bovine *Chlamydia psittaci* isolate
	Evaluation of routes and the possibility of transmission of *C. psittaci* from experimentally challenged calves to naïve calves. Assessment of the differences after experimental and natural exposure with respect to the presence of the pathogen in blood and excretions, clinical signs, acute phase and immune response on a long-term basis	35 dpi	viable *C. psittaci*	10^8 ifu/calf	21	*in vivo*:
						• clinical examination (scoring)
						• blood analysis
						  - quantitation of *Chlamydiaceae* by rt-PCR and species detection
						  - WBC and differentiation
						  - biomarker of acute-phase reaction (LBP)
						• rt-PCR based detection of *C. psittaci* in excretions
						  - ocular, nasal and rectal swabs
						  - exhaled breath
						  - room air
						ex vivo:
						• recultivation of the pathogen from lung tissue
						• strain identification

			naïve sentinel calves	exposure to experimentally challenged calves	3	

STUDY 4:
 	
Evaluation of pulmonary dys-functions and acid-base imbalances induced by *Chlamydia psittaci* in a bovine model of respiratory infection
	Quantification and differentiation of pulmonary dysfunctions and acid-base imbalances induced by acute *C. psittaci* infection	14 dpi	viable *C. psittaci*	10^8 ifu/calf	21	*in vivo*:
						• non-invasive pulmonary function tests
						  - impulse oscillometry
						  - volumetric capnography
						  - He dilution (FRC measurement)
						• assessment of acid-base variables from venous blood and calculation of Stewart variables (strong ion approach)

			controls	uninfected cell culture	21	

Abbreviations: BALF broncho-alveolar lavage fluid, dpi days post inoculation, FRC functional residual capacity, He Helium, ifu inclusion forming units, LBP lipopolysaccharide-binding protein, n number of calves, PCR polymerase chain reaction, rt-PCR real-time polymerase chain reaction, ssAB strain-specific antibodies, WBC white blood cell count

2   Publications and disclosure of personal contributions

STUDY 1

Reinhold, P., **Ostermann, C.**, Liebler-Tenorio, E., Berndt, A., Vogel, A., Lambertz, J., Rothe, M., Rüttger, A., Schubert, E., Sachse, K. (2012):

A Bovine Model of Respiratory *Chlamydia psittaci* Infection: Challenge Dose Titration.

PLoS One 7 (1): e30125.

DOI: 10.1371/journal.pone.0030125

STUDY 2

Ostermann, C., Schroedl, W., Schubert, E., Sachse, K., Reinhold. P. (2013):

Dose-dependent effects of *Chlamydia psittaci* infection on pulmonary gas exchange, innate immunity and acute-phase reaction in a bovine respiratory model.

The Veterinary Journal 196 (3): 351-359.

DOI: 10.1016/j.tvjl.2012.10.035

STUDY 3

Ostermann, C., Rüttger, A., Schubert, E., Schroedl, W., Sachse, K., Reinhold, P. (2013):

Infection, Disease, and Transmission Dynamics in Calves after Experimental and Natural Challenge with a Bovine *Chlamydia psittaci* Isolate.

PLoS One 8(5): e64066.

DOI: 10.1371/journal.pone.0064066

STUDY 4

Ostermann, C., Linde, S., Siegling-Vlitakis, C., Reinhold, P.:

Evaluation of pulmonary dysfunctions and acid-base imbalances induced by *Chlamydia psittaci* in a bovine model of respiratory infection.

Multidisciplinary Respiratory Medicine 9 (1): 10.

DOI: 10.1186/2049-6958-9-10

Personal contributions are listed on the front page of every study.

2.1   STUDY 1:
A Bovine Model of Respiratory *Chlamydia psittaci* Infection:
Challenge Dose Titration

Personal contributions:

- Development of the inoculation pattern and clinical scoring system
- Animal experiments:
    - bronchoscopy and inoculation of the challenge strain or control medium
    - clinical examination of the calves
    - participation in the collection of blood and swab samples, preparing and counting blood cells
    - performing blood gas analysis and haemoxymetry
    - anaesthesia prior necropsies, assistance during necropsies and preparation of broncho-alveolar lavage fluid *ex vivo*
- Analyses of clinical data
- Preparation of parts of the manuscript:
    - clinical scoring (materials and methods, results)
    - intrabronchial inoculation (materials and methods)
    - description of the study design
- Preparation of the following tables and figures:
    - Figure 1. Development of the total clinical score over time
    - Supplement Figure 2. Scheme of intra-bronchial inoculation
    - Supplement Table 2. Clinical Scoring

PLoS ONE

A Bovine Model of Respiratory *Chlamydia psittaci* Infection: Challenge Dose Titration

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Abstract

This study aimed to establish and evaluate a bovine respiratory model of experimentally induced acute *C. psittaci* infection. Calves are natural hosts and pathogenesis may resemble the situation in humans. Intrabronchial inoculation of *C. psittaci* strain DC15 was performed in calves aged 2–3 months *via* bronchoscope at four different challenge doses from 10^6 to 10^9 inclusion-forming units (ifu) per animal. Control groups received either UV-inactivated *C. psittaci* or cell culture medium. While 10^6 ifu/calf resulted in a mild respiratory infection only, the doses of 10^7 and 10^8 induced fever, tachypnea, dry cough, and tachycardia that became apparent 2–3 days post inoculation (dpi) and lasted for about one week. In calves exposed to 10^9 ifu *C. psittaci*, the respiratory disease was accompanied by severe systemic illness (apathy, tremor, markedly reduced appetite). At the time point of most pronounced clinical signs (3 dpi) the extent of lung lesions was below 10% of pulmonary tissue in calves inoculated with 10^6 and 10^7 ifu, about 15% in calves inoculated with 10^8 and more than 30% in calves inoculated with 10^9 ifu *C. psittaci*. Beside clinical signs and pathologic lesions, the bacterial load of lung tissue and markers of pulmonary inflammation (i.e., cell counts, concentration of proteins and eicosanoids in broncho-alveolar lavage fluid) were positively associated with ifu of viable *C. psittaci*. While any effect of endotoxin has been ruled out, all effects could be attributed to infection by the replicating bacteria. In conclusion, the calf represents a suitable model of respiratory chlamydial infection. Dose titration revealed that both clinically latent and clinically manifest infection can be reproduced experimentally by either 10^6 or 10^8 ifu/calf of *C. psittaci* DC15 while doses above 10^8 ifu *C. psittaci* cannot be recommended for further studies for ethical reasons. This defined model of different clinical expressions of chlamydial infection allows studying host-pathogen interactions.

Introduction

The obligate intracellular bacterium *Chlamydia* (*C.*) *psittaci* is the causative agent of psittacosis, a widespread infection in psittacine birds and domestic poultry [1–3]. Transmission of *C. psittaci* to humans and the zoonotic potential of this infection were first documented in the 19th century. Outbreaks of human *C. psittaci* infections still occur [4,5], but the number of reported cases today is thought to be underestimated due to inadequate epidemiological coverage and insufficient diagnostic testing [6,7]. During the last decade, *C. psittaci* has also been regularly detected in non-avian domestic animals, i.e. swine, horses, dogs, cattle, and sheep [8–13]. Although serological data from the 1990s already indicated chlamydioses in domestic animals as a relevant source of infection for humans [14], *C. psittaci* strains of non-avian origin have not been in the focus of extensive research. Both their pathogenic role in large animals and their zoonotic potential to humans have remained elusive to date.

In natural hosts, clinical outcomes of *C. psittaci* infection range from clinical silence to severe or even life-threatening illness, suggesting that host-pathogen interactions are important to the pathogenesis. Psittacosis in birds is known as a systemic disease of acute, protracted, chronic or subclinical course. Psittacosis in humans is recognized mainly as a respiratory infection initially reminiscent of an influenza-like illness and atypical pneumonia, but may also manifest as a fulminant course including myocarditis, hepatitis, and encephalitis [15–17]. Diversity of chlamydial infection expression in calves ranges from acute respiratory illness, keratoconjunctivitis or polyarthritis [18–20] to clinically inapparent infections in the majority of herds [13]. Despite clinical silence, chlamydial infections in young cattle were found to be associated with long-lasting respiratory dysfunctions [21] indicating pathogenetic involvement of the respiratory system in ''asymptomatic'' bovine chlamydiosis.

Relevant animal models of chlamydial infections are needed to answer open questions about (i) the pathogenetic role of non-avian

C. psittaci in the mammalian lung with respect to different clinical outcomes, and (ii) transmission routes of this potentially zoonotic agent between different hosts. As calves represent natural hosts for chlamydiae [20,22,23] they offer the possibility to analyze host-pathogen interactions under natural conditions. In contrast, artificial murine models imperfectly recapitulate many aspects of infectious diseases due to host restriction in non-typical hosts [24]. Furthermore, the following peculiarities in genetics, immunobiology and respiratory physiology reveal species-specific aspects that suggest large-animal models becoming an obligatory complement to widely used murine models.

Genetics

The bovine genome, fully sequenced in 2009, more closely resembles the human genome than that of mice and rats [25]. Comparative analyses further revealed that sequences of bovine proteins are generally more similar to human orthologs than are rodent orthologs [26]. In general, recent data about genome diversity confirmed that the mouse genome is much more rearranged than that of most other taxa [27].

Immunobiology

With respect to the genetically determined regulation of defense mechanisms, significant differences exist between species (reviewed by [28]). For example, interleukin-8 (IL-8) plays a significant role in human inflammatory processes. In the mouse genome, the *il-8* gene is missing; but it does exist in the genome of dogs, pigs, sheep, and cattle. The protein encoded in cattle even exhibits a high cross-species activity with human IL-8 [29,30]. Further significant differences between murine and human innate and adaptive immune response are related to such important aspects as the Toll receptors, inducible NO synthase, Fc-Receptors, immune globulin subsets or immune mediators (summarized by [31,32]). Particularly for chlamydial infections, marked host-adapted differences in the IFN-gamma response have been recently discovered comparing mice and humans [24].

Respiratory Physiology

Considering the murine lung as a model for human respiratory diseases, one has to be aware of numerous structural and functional peculiarities (summarized by [33,34]). The most important differences include the branching pattern of the bronchi (monopodial pattern in mice *versus* dichotomous pattern in larger mammalian lungs) and the lack of bronchial vessels in mice. Due to the latter, several steps of leukocyte infiltration in the bronchial wall will be completely different compared to larger mammalian lungs. Furthermore, Clara cells are present in about 50% of airways in mice but are rare in conducting airways of humans and other larger species where goblet (mucus) cells dominate. This difference significantly influences production of mucus and consequently the function of mucociliary clearance as an important defense mechanism to eliminate inhaled particulate antigens.

That mice do not faithfully reproduce pathophysiological aspects of human pulmonary disease (due to many significant differences in lung anatomy, respiratory physiology, and pulmonary immunology) has been shown for airway epithelium repair and regeneration, asthma, cystic fibrosis, various cancers, and various pulmonary infections - for example tuberculosis or MRSA [35–43]. In contrast, lung volumes, airflows and respiratory mechanics are comparable between adult humans and calves due to comparable body weights (50–100 kg), and the bovine lung is particularly suited to mirror pulmonary dsyfunctions [39].

The current study was undertaken to establish and evaluate a bovine respiratory model of experimentally induced *C. psittaci*

infection because calves are likely to resemble more closely than mice the situation in humans and also because chlamydial infections play an important role in cattle. As data on dose-response-relationships of chlamydial infections in the bovine respiratory system were absent, dose titration of the inoculum was the main goal of this study. Clinical outcomes, markers of pulmonary inflammation, lung pathology, recovery of chlamydiae and humoral response were assessed after intrabronchial challenge of doses between 10^6–10^9 inclusion forming units (ifu) per animal. Results of this study reveal that both clinically latent and clinically manifest *C. psittaci* infection can be reproduced experimentally. This defined model of a predictable severity of illness is essential for further research to understand the underlying pathogenetic mechanisms of different clinical phenotypes of chlamydial infection, and to clarify details about dissemination, shedding and transmission of *C. psittaci* as it relates to the clinical picture.

Results

1. Clinical signs

Control calves challenged with either cell culture medium (n = 4) or the inactivated *C. psittaci* strain (n = 6) did not exhibit any clinical sign of respiratory illness (Fig. 1A). In calves exposed to viable *C. psittaci*, the total clinical score increased with increasing doses of inoculum (Fig. 1B). Clinical illness manifested as respiratory signs and was confirmed by the respiratory score that contributed to about 50% to the general clinical score (data not shown). Clinical illness was most evident 2–3 days post inoculation (dpi). As examples, body temperatures and respiratory rates measured at the peak of clinical signs (i.e. 48–72 hours pi) are shown in Figure 2. Beside fever and respiratory illness, the following dose-dependent clinical signs were evident:

- In calves challenged with **10^6** ifu (n = 4), mild diarrhea and spontaneous cough occurred without apparent affect on appetite, feed intake or general behavior. Nasal or ocular discharge was not observed.

- Calves exposed to **10^7** ifu (n = 4) or **10^8** ifu (n = 4) developed clinical illness of similar severity characterized by fever (Fig. 2A), tachypnea (Fig. 2B) and mild tachycardia (medians [ranges] of heart rates: 90 [68–120] beats min^{-1} for 10^7 ifu/ calf; 82 [72–108] beats min^{-1} for 10^8 ifu/calf). In most of these calves, appetite and milk intake was reduced at 2–3 dpi, and diarrhea was seen in a few animals. In all calves, dry cough occurred while nasal and ocular discharges were rarely seen. In general, the period of 2–3 dpi was accompanied by reduced general activity (dullness).

- The most severe clinical picture was present in two calves challenged with **10^9** ifu. Within the period 2–3 dpi, general behavior was mostly depressed and accompanied for 6–12 hours by apathy, inability to stand up, tremor, markedly reduced appetite or complete feed rejection with or without diarrhea. Heart rate increased to approximately 160% compared to baseline data (median [range]: 106 [88–124] beats min^{-1}). Dry cough was present while nasal and ocular discharges were rarely seen. Due to severity of clinical illness, the two calves were euthanized 3 dpi and no further calves were exposed to 10^9 ifu.

In the three groups challenged with 10^6, 10^7, or 10^8 ifu of *C. psittaci*, clinical signs returned to baseline within one week after challenge (data not shown).
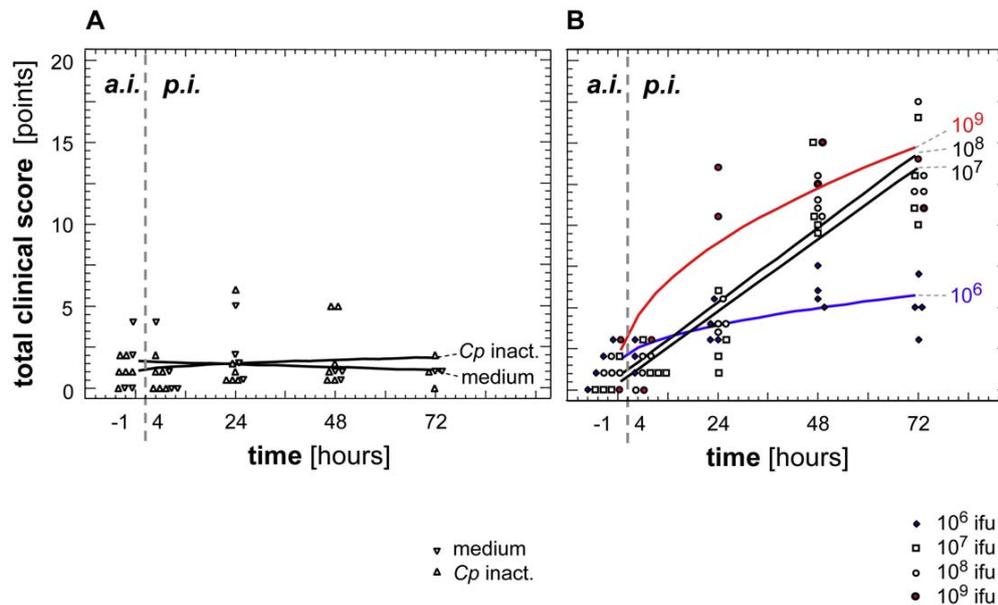
Figure 1. Development of the total clinical score over time. Data are given as regression lines and individual data according to the best fitting regression model per group. In control calves, no significant changes of total clinical score occurred after inoculation of medium or inactivated chlamydiae (panel A). In calves experimentally inoculated with different doses of viable *C. psittaci*, scores of clinical illness increased with challenge doses (panel B). Equations of regression, coefficients of correlation, R-squared values, and probability levels are given in Table 1.
doi:10.1371/journal.pone.0030125.g001
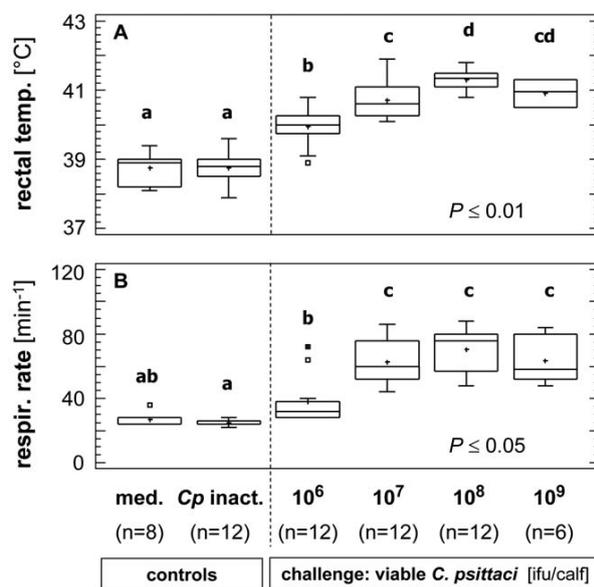
Figure 2. Rectal temperature and respiratory rate measured 48–72 hours post inoculation (i.e. peak of clinical signs). In calves experimentally inoculated with different doses of viable *C. psittaci*, both rectal temperature (panel A) and respiratory rate (panel B) were significantly increased while no significant changes were seen in control calves. Data are given as Box-and Whisker Plots based on 2 or 3 measurements per calf in controls or infected animals, respectively. Different letters indicate significant differences between groups at given *P*-level (multiple range test).
doi:10.1371/journal.pone.0030125.g002

2. Biomarkers of pulmonary inflammation in BALF

Results of BALF cytology obtained during the acute phase, i.e. 2–3 dpi, are summarized in Table 2. Total cell count in BALF was higher in calves challenged with viable chlamydiae compared to controls, and counts increased with challenge dose. Although absolute numbers of all cell types (i.e. alveolar macrophages, granulocytes, and lymphocytes) contributed to elevated total cell counts in calves challenged with viable chlamydiae, the most significant increase was attributed to neutrophil granulocytes, particularly unsegmented ones. Percentages of the three cell types revealed that the relative amount of alveolar macrophages decreased significantly in a dose-dependent manner because the percentage of mainly unsegmented neutrophil granulocytes increased.

BALF cytology of the calves that had been exposed to viable *C. psittaci* and necropsied at 7 dpi still showed dose-dependent effects. For example, total cell counts 7 days after exposure to 10^6, 10^7, and 10^8 ifu were still 4.8, 7.0, and 8.2×10^8/L, respectively. In calves sacrificed at 14 dpi, BALF cytology did not differ from those of control calves (data not shown).

The concentration of total protein in BALF supernatant was <300 µg/mL in controls as well as in groups challenged with 10^6 or 10^7 ifu. Protein concentration in BALF increased in the group challenged with 10^8 ifu, and was dramatically elevated after inoculation of 10^9 ifu of *C. psittaci* (Fig. 3A).

As shown in Figure 3B as a typical example, eicosanoids, i.e. thromboxan B2 (TXB2), prostaglandin E2 (PGE2), and hydro-xyeicosatetraenoic acids (15-HETE, 12-HETE), were almost undetectable in BALF supernatants of the control animals but became measurable in calves challenged with doses above 10^6 ifu and attained highest concentrations in BALF samples of calves exposed to 10^9 ifu.

Table 1. Assessment of regression lines of the clinical scores given in Figure 1.

Challenge	Best fitting regression model	Coefficient of correlation	R-squared	
medium	linear [Y = a+b*X]	−0.13	1.57%	P>0.10
Cp inactivated	square root-X model [Y = a+b*sqrt(X)]	0.20	4.08%	P>0.10
10^6 ifu/calf	square root-X model [Y = a+b*sqrt(X)]	0.78	61.62%	**P<0.001**
10^7 ifu/calf	Linear model [Y = a+b*X]	0.91	83.36%	**P<0.001**
10^8 ifu/calf	Linear model [Y = a+b*X]	0.96	91.40%	**P<0.001**
10^9 ifu/calf	square root-X model [Y = a+b*sqrt(X)]	0.88	77.54%	**P<0.001**

doi:10.1371/journal.pone.0030125.t001

3. Pulmonary lesions and detection of chlamydiae

3.1. Gross lesions. Bronchopneumonia was seen in all calves exposed to viable *C. psittaci*, but in none of the calves inoculated with cell culture medium or inactivated *C. psittaci*. Distribution of lesions was consistent with the sites where inoculum had been applied. Thus, the most extensive involvement was seen in the middle lobe and in the left and right basal lobes (Fig. 4). Especially in the basal lobes, lesions were often not readily visible at the surface, but located deep within the tissue (Fig. 4). At the time point of most pronounced clinical signs, i.e. 3 dpi, the extent of lesions was below 10% of pulmonary tissue in calves inoculated with 10^6 and 10^7 ifu *C. psittaci*. The proportion increased to about 15% in calves inoculated with 10^8 and to more than 30% in calves inoculated with 10^9 ifu *C. psittaci*.

In calves inoculated with 10^6, 10^7 and 10^8 ifu *C. psittaci* necropsied at 7 dpi, lesions were still present in the basal lobes, but at 14 dpi lesions had almost resolved.

3.2. Histological lesions and detection of chlamydial inclusions by immunohistochemistry. Neither histological lesions nor chlamydial inclusions were seen in the calves inoculated with medium or with inactivated chlamydiae. In the calves challenged with viable chlamydiae, the presence of *C. psittaci* inclusions was mainly restricted to altered pulmonary tissue, and macroscopic lesions were confirmed by histology as follows:

At 3 dpi, purulent bronchopneumonia was seen predominantly in calves inoculated with 10^6 and 10^7 ifu *C. psittaci*. Small foci with fibrinous exsudate and necrosis were seen only in severely affected lobes. The number of chlamydial inclusions was low, and had a multifocal distribution. Chlamydial inclusions were seen in alveolar epithelial cells. After inoculation of 10^8 ifu *C. psittaci*, fibrinopurulent bronchopneumonia with multifocal areas of necrosis and pleuritis was frequently observed. The number of chlamydial inclusions was further elevated and the inclusions were often associated with neutrophils and macrophages. In calves inoculated with 10^9 ifu *C. psittaci*, areas of necrosis were more extensive and numerous chlamydial inclusions were found throughout the altered tissues.

At 7 dpi, an increased number of alveolar macrophages and mild lymphohistiocytic infiltrates occurred, indicating organization of pneumonic lung tissues. Extensive areas of necrosis were seen in the calf inoculated with 10^8 ifu *C. psittaci*, multifocal areas in the calf inoculated with 10^7 ifu *C. psittaci* and none in the calf inoculated with 10^6 ifu *C. psittaci*. Chlamydial inclusions were numerous in areas of necrosis, but infrequent in those of organization.

At 14 dpi, lesions had resolved in the calf inoculated with 10^6 ifu *C. psittaci*. The lung of the calf that had received 10^7 ifu *C. psittaci* had multiple areas with thickened interalveolar septae, alveolar epithelial cell type II hyperplasia and lymphocytic

infiltrates. Few chlamydial inclusions were found overall, but there were a few foci with groups of macrophages containing chlamydial inclusions.

4. Quantification of chlamydial antigen in the lung

Examination of lung tissue by real-time PCR revealed that genome copy numbers of *C. psittaci* in lung tissue at 3 dpi increased with the challenge dose (Figure 5). Seven days after challenge, copy numbers of the pathogen were still dose-dependent but already significantly reduced compared to 3 dpi. Fourteen days post inoculation, less than 30 copies/mg were detectable in lung tissues of calves challenged with viable chlamydiae.

In lung lymph nodes, similar dose-dependent effects were seen at 3 dpi, but absolute copy numbers per mg of lymph node tissue were much lower compared to those found in lung tissue (data not shown). In cell pellets of BALF, highest copy numbers were seen at 3 dpi in the two calves challenged with the highest dose of 10^9 ifu/calf (621 and 1611 copies per 10^4 BALF-cells), while hardly any *C. psittaci* were found in BALF cells of the other groups at any time point.

5. Humoral response

Specific antibodies against the challenge pathogen were detected mainly in the group of animals exposed to 10^8 ifu *C. psittaci*, where reactive bands were detected in serum on day 7 after inoculation (Fig. S1). Sera from the groups infected with 10^7 and 10^6 ifu failed to show a specific immune reaction in the first 11 days after challenge. Immunoblot analysis of BALF supernatants showed only a weak reaction for the group exposed to 10^8 ifu on 7 dpi (Fig. S1). Since the two calves challenged with 10^9 ifu had been sacrificed already at 3 dpi, no data of their specific humoral response is available.

Discussion

1. Model validity

To the best of our knowledge, this is the first study assessing dose response effects to the pathogen *C. psittaci* in a domestic animal model of respiratory infection. Despite the known disadvantages in terms of cost, time consumption and limited availability of immunological and molecular tools compared to widely used murine models, calves were selected because (1) bovine chlamydiosis closely resembles the situation in a natural host [21,23], (2) the *C. psittaci* isolate (strain DC 15) used to establish the model originated from a calf, and (3) the bovine lung is more relevant than the mouse to model human functional consequences of ventilatory disorders due to its segmental anatomy and the lack of collateral airways [39]. Furthermore, domestic animal models are especially advantageous because they can be

Respiratory *C. psittaci* Infection Model

Table 2. Quantity of cells present in broncho-alveolar lavage fluid (BALF) 2–3 days post inoculation in calves experimentally inoculated with different doses of viable *C. psittaci* (*Cp*) and in control calves.

		Control Groups		Challenge Groups				Kruskal-Wallis test
		Medium	*Cp* inactivated	*Cp* 10⁶ ifu	*Cp* 10⁷ ifu	*Cp* 10⁸ ifu	*Cp* 10⁹ ifu	
		(n = 4)	(n = 6)	(n = 2)	(n = 2)	(n = 2)	(n = 2)	
BALF cells	Unit	Med [min;max]	Med [min;max]	Med [min;max]	Med [min;max]	Med [min;max]	Med [min;max]	
Total Cell Count	10^8/L	4.23 [1.08; 4.73]	3.38 [2.73; 4.43]	**4.83** ↑ [4.58; 5.08]	**6.28** ↑ [5.25; 7.30]	**8.72** ↑ [7.68; 9.75]	**11.69** ↑ [9.08; 14.30]	*P* = **0.02**
Cell differentiation absolute								
alveolar macrophages	10^8/L	2.94 [0.96; 4.44]	2.33 [2.10; 4.20]	3.45 [3.10; 3.80]	3.84 [3.31; 4.48]	4.51 [3.76; 5.27]	**5.00** ↑ [4.72; 5.29]	*P* = 0.19
neutrophil granulocytes	10^8/L	0.27 [0.03; 1.81]	0.49 [0.04; 0.95]	1.16 [0.55; 1.78]	**2.15** ↑ [1.68; 2.63]	**3.89** ↑ [3.30; 4.49]	**5.43** ↑ [2.00; 8.87]	*P* = **0.04**
unsegemented	10^8/L	0.07 [0.00; 0.13]	0.17 [0.04; 0.25]	**0.71** ↑ [0.50; 0.91]	**1.01** ↑ [0.63; 1.39]	**2.96** ↑ [2.30; 3.61]	**4.63** ↑ [1.82; 7.44]	*P* = **0.01**
polymorph nuclear	10^8/L	0.20 [0.01; 1.69]	0.31 [0.00; 0.89]	0.45 [0.05; 0.86]	**1.15** ↑ [1.05; 1.24]	0.94 [0.88; 1.00]	0.81 [0.18; 1.43]	*P* = 0.42
lymphocytes	10^8/L	0.14 [0.00; 0.32]	0.13 [0.08; 0.21]	0.22 [0.20; 0.23]	**0.28** ↑ [0.26; 0.29]	0.31 [0.00; 0.61]	1.25 [0.14; 2.36]	*P* = 0.45
Cell differentiation relative								
alveolar macrophages	%	89.5 [47; 94]	79.5 [66; 95]	72.0 [61; 83]	**61.5** ↓ [60;63]	**51.5** ↓ [49;54]	**44.5** ↓ [37;52]	*P* = 0.11
neutrophil granulocytes	%	6.5 [2;45]	16.5 [1;30]	23.5 [12;35]	**34.0** ↑ [32;36]	**44.5** ↑ [43;46]	42.0 [22;62]	*P* = 0.17
unsegemented	%	2.5 [0; 3]	5.0 [1;7]	**14.5** ↑ [11;18]	**15.5** ↑ [12;19]	**33.5** ↑ [30;37]	**36.0** ↑ [20;52]	*P* = **0.01**
polymorph nuclear	%	4.5 [1;42]	10.0 [0; 28]	9.0 [1;17]	18.5 [17;20]	11.0 [9;13]	6.0 [2;10]	*P* = 0.71
lymphocytes	%	6.0 [0; 8]	4.0 [3;5]	4.5 [4;5]	4.5 [4;5]	4.0 [0; 8]	13.5 [1;26]	*P* = 0.99

Med = median. Kruskal-Wallis test: *P* ≤ 0.05 indicates significant differences between medians of all groups. *W* test: *P* ≤ 0.067 indicates significant differences between two groups. Medians highlighted in bold increased (↑) or decreased (↓) significantly in comparison to controls '*Cp* inactivated'. Data of the two control groups did not differ significantly.
doi:10.1371/journal.pone.0030125.t002
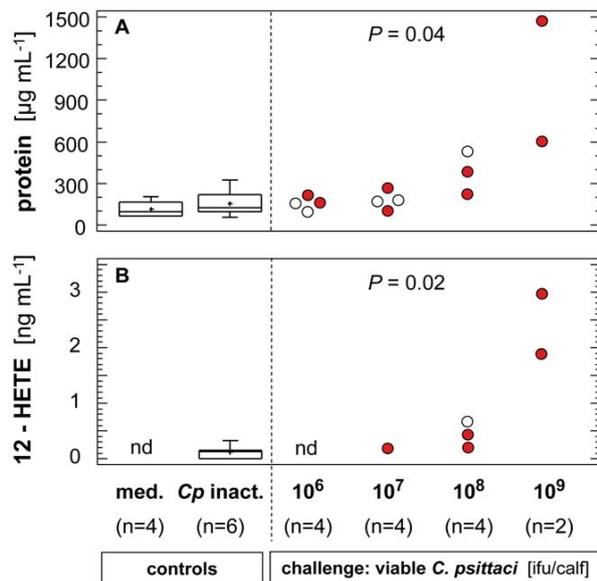
Figure 3. Markers of pulmonary inflammation assessed in broncho-alveolar lavage fluid (BALF). Both concentration of total protein (A) and 12-HETE (B) were maximal in calves inoculated with 10^9 ifu of *C. psittaci*. Data for control groups are from 2 and 3 dpi combined (Box and Whisker Plots). Data for calves challenged with different doses of viable *C. psittaci* are given on an individual basis for time points when calves were sacrificed (filled circles: 3 dpi; open circles: 7 dpi and 14 dpi). Kruskal-Wallis test revealed significant differences between groups at given *P*-level.
doi:10.1371/journal.pone.0030125.g003

Figure 4. Distribution and extent of pulmonary lesions at day 3 pi in a calf inoculated with 10^9 ifu of *C. psittaci*. Dorsal view of the lung and heart (H). Pneumonic lesions present as dark red discolorations (>) in the apical lobes, middle lobe and basal lobes. Note distension of the basal lobes due to severe bronchopneumonia in the inferior segments (hatched lines). Bar = 5 cm.
doi:10.1371/journal.pone.0030125.g004

used as dual-purpose models that benefit both agricultural and biomedical research [44,45]. From an epidemiological point of view, infectious diseases in farm animals are useful biological models to provide empirical data that aids infectious disease modeling and to advance our understanding of infectious disease dynamics and control for human populations [46].

Due to ethical criteria of animal protection, calves included were limited to the lowest number essential to document inherent differences in host responses (4 calves per challenge dose 10^6–10^8 ifu/calf; 2 calves per challenge dose 10^9 ifu/calf). This small animal number was sufficient because large animals offer the great advantage of enabling the characterization of functional, inflammatory and morphological changes in a multi-factorial within-subject approach.

With respect to the pathogen, previous models of respiratory '*C. psittaci* infection' in domestic animals published more than 20 years ago have to be critically scrutinized on the basis of current taxonomy. For instance, isolates of *C. psittaci* from ovine pneumonia were inoculated either endobronchially in red deer [47] or intratracheally in pigs [48] to produce pneumonia. Experimentally induced pneumonia by intratracheal inoculation of different strains of the old *Chlamydia psittaci sensu lato* (now comprising the species of *C. abortus*, *C. felis*, *C. caviae* and *C. psittaci*) was also reported for pigs and calves [49–51]. From today's perspective, in the light of two recent revisions of the taxonomy of *Chlamydiales* [52,53], it is doubtful that those models actually used the species currently defined as *C. psittaci*.

2. Dose-dependent effects of *C. psittaci* in the host

Clinical signs of illness increased with challenge doses in all calves exposed to viable *C. psittaci*. While 10^6 ifu/calf resulted in

mild clinical signs only, the doses of 10^7 and 10^8 induced clinically apparent illness that became visible 2–3 dpi. Comparing the latter two doses, the clinical picture induced by 10^8 ifu was more reproducible. Doses above 10^8 ifu *C. psittaci* cannot be recommended for further studies for ethical reasons.

According to BALF cytology, increasing numbers of cell types capable of phagocytosis and antigen presentation were recruited with increasing challenge doses of *C. psittaci*. The early recruitment of neutrophils is in line with results obtained after aerogeneous *C. suis* infection in pigs [54] and in a murine model of *Chlamydia* infection [55]. In our model, predominantly juvenile unsegmented neutrophils were recruited as the first line of defense in a dose-dependent manner. To a smaller extent, alveolar macrophages and lymphocytes also contributed to the increase of cells in broncho-alveolar compartments.

Concentrations of total protein in BALF indicated severity of inflammation and increased permeability of pulmonary vessels for challenge doses above 10^7 ifu/calf. Increases in protein concentration >600 µg/mL BALF as seen after inoculation of 10^8 ifu of *C. psittaci* are in accordance with data published for calves with naturally acquired chlamydial infections [21]. Dramatically elevated protein concentrations in BALF samples of the two calves exposed to 10^9 ifu of *C. psittaci* demonstrated dramatic loss of integrity of the alveolo-capillary barrier in the lung, which is line with the particularly severe clinical outcome.

The pathogenetic link between chlamydial infection and activation of the arachidonic acid (AA) cascade *via* the cyclooxygenase (COX)-mediated pathway has been shown *in vitro* for multiple cell types such as epithelial cells, peripheral blood mononuclear cells, human monocytes, and antigen-presenting
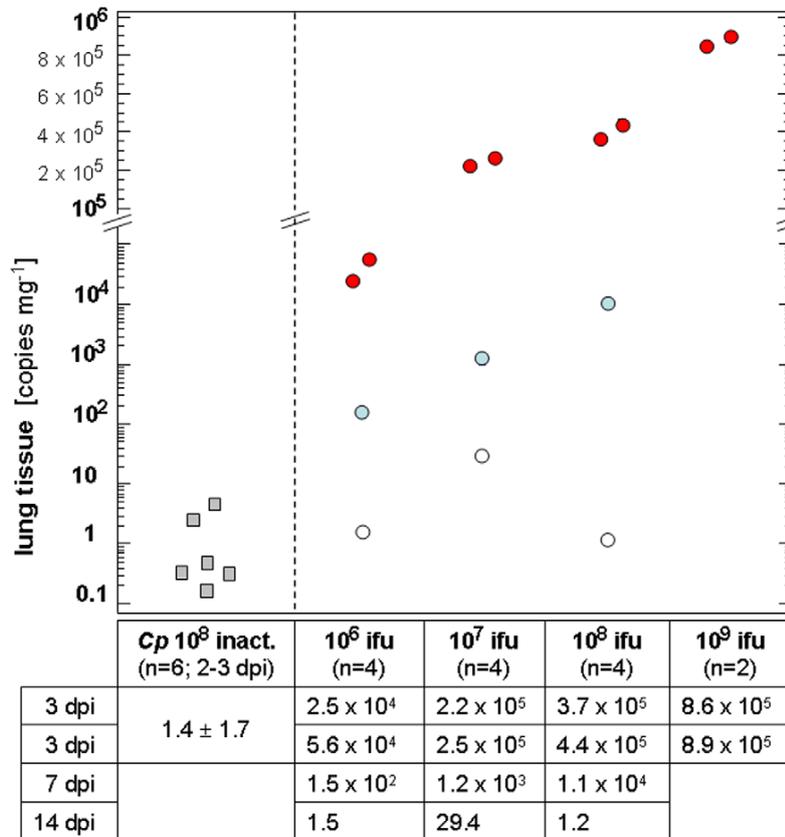
Figure 5. Numbers of inclusion-forming units (ifu) of *C. psittaci* in lung tissues. Data are expressed as individual animals. Boxes indicate control calves euthanized 2–3 dpi after inoculation of UV-inactivated 10^8 ifu. Circles represent calves challenged with viable bacteria of different doses. Infected calves were sacrificed 3 dpi (red circles), 7 dpi (blue circles) and 14 dpi (open circles). Copy numbers in lung tissue represent the mean of 4 samples analyzed per lung (two of left caudal lobe and two of right caudal lobe).
doi:10.1371/journal.pone.0030125.g005

dendritic cells [56–59]. In our model, eicosanoids produced by this pathway were increasingly obvious with increasing challenge doses indicating that the AA cascade became more intensively involved in pulmonary host response as the chlamydial inoculum increased. This finding is in good agreement with *in vitro* data obtained in human monocytes showing that the amount of synthesized eicosanoids was dependent on the chlamydial multiplicity of infection [60]. While *in vitro* studies focused mainly on PGE2, *in vivo* data of our study revealed that concentrations of at least four eicosanoids (TXB2, 15-HETE, 12-HETE, and PGE2) increased with chlamydial load in lung tissue.

Differences in the quantity of pulmonary tissue affected by pneumonia were well correlated with the severity of clinical signs. A dose-dependent increase in the number of pulmonary lesions and in the type of lesions was observed. There was a continuous change from purulent to fibrino-exsudative lesions and in the extent of necrosis. Similar changes may be seen after deposition of foreign material in the lung causing aspiration pneumonia. However, since lesions occurred after inoculation with viable chlamydiae only, they are most likely a consequence of the replicating bacteria. Early organization of pulmonary lesions was seen at 7 dpi. Chlamydiae were still present in areas of inflammation, but had been cleared from areas of organization. At 14 dpi, reconstitution was complete in calves that had received 10^6 ifu of *C. psittaci*. In calves that had received the higher doses,

areas of necrosis had not yet been completely organized and chlamydiae could still be found in these areas.

Specific antibodies against *C. psittaci* occurred in both blood and BALF about 7 dpi, but only in calves exposed to the challenge dose of 10^8 ifu. Lower challenge doses did not induce a measurable specific humoral response within two weeks after inoculation. Whether humoral response to lower challenge doses requires a longer time or whether challenge doses below 10^8 ifu are not sufficient to induce humoral response has yet to be elucidated.

3. Detection of the pathogen: Localization and time-dependence

Within 14 days after intrabronchial challenge with viable *C. psittaci*, different kinds of swabs (nasal, ocular, rectal) collected on a daily basis were unsuitable to detect the challenge strain by PCR (data not shown). In lung tissue, however, *C. psittaci* was detected by real-time PCR, and increasing copy numbers of the challenge strain were found in correspondence to increasing challenge doses until 7 dpi. Furthermore, we were able to recover the challenge strain in cell culture from lung tissues obtained at necropsy (data not shown).

The presence of chlamydial inclusions assessed by immuno-histochemistry was restricted to altered pulmonary tissue while

alterations mainly surrounded the eight locations of inoculation. Distribution of any infection or inflammation in the bovine lung is spatially hampered due to a very high degree of lobulation and segmentation of pulmonary tissue and the lack of collateral airways [39,61,62]. Thus, dissemination of infection was impossible *via* connective tissue septa between pulmonary segments and could only happen if infectious particles or droplets would be transported to other segments by airflow. This however, is less likely for obligate intracellular pathogens such as chlamydiae. Consequently, consolidated lobules could be found adjacent to healthy lobules within the same lung lobe.

4. Exclusion of effects mediated by lipopolysaccharide or liquid instillation

Inoculation of cell culture medium did not result in any clinical sign or pulmonary lesion, excluding significant host response to instillation of 6 ml liquid per lung.

To address the question to what extent chlamydial lipopolysaccharide (LPS) induced either local effects in the lung or general clinical signs, we included a second control group that was inoculated with UV-inactivated *C. psittaci* at the highest acceptable dose (10^8 ifu). Compared to controls exposed to cell culture medium only, calves exposed to inactivated chlamydiae did not express any significant difference in any parameter assessed in this study. Consequently, clinical signs of respiratory disease and local effects of inflammation induced in the lung required viability of the pathogen. The time course of alterations in calves challenged with doses 10^6 to 10^8 ifu is consistent with the duration of at least one chlamydial developmental cycle in the host cells (initial clinical signs occurred about 48 h after challenge). In calves exposed to 10^9 ifu of *C. psittaci*, signs of general clinical illness occurred earlier (less than 24 h after challenge) which might indicate involvement of toxic products from the pathogen or stronger release of inflammatory mediators by the host. For challenge doses 10^6 to 10^8 ifu per calf, however, involvement of LPS effects in the pathogenesis could be excluded.

5. Conclusions

The calf was found to be a suitable mammalian host to establish and evaluate an *in vivo* model of experimental respiratory infection by *C. psittaci*. Intra-bronchial challenges between 10^6 to 10^9 ifu/calf resulted in dose-dependent pulmonary and systemic host reactions ranging clinically from mild to severe. For further studies, only doses between 10^6 and 10^8 ifu per animal are recommended, depending on the clinical outcome to be achieved. While 10^6 ifu of strain DC 15 per animal will lead to a mild or even subclinical infection, 10^8 ifu per animal causes reproducible clinically manifest disease and predictable humoral response.

This domestic animal model will add valuable information to the current knowledge about chlamydial infections obtained from other studies (laboratory animal or cell culture models). It may be used to address the following questions with relevance for both human and veterinary medicine:

1. To study pathogenetic details of *C. psittaci* infection at the tissue level, i.e. the interplay between intracellular chlamydial infection and host cell responses.
2. To verify consequences of *C. psittaci* infection at the organ level, i.e. pulmonary dysfunctions in the host.
3. To characterize long-term host-pathogen interactions *in vivo*.
4. To assess the spread and shedding of the organism in order to understand the dissemination of the pathogen within the host

and transmission routes between animals, as well as from animals to humans.
5. To evaluate the usefulness and efficacy of prophylactic and therapeutic options in order to control chlamydioses in livestock and, perhaps, eliminate chlamydial infections in human patients.

Materials and Methods

1. 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: 04-002/07). All experiments were done in a containment of biosafety level 2 under supervision of the authorized institutional Agent for Animal Protection. Bronchoscopy to inoculate the pathogen was strictly performed under general anesthesia. During the entire study, every effort was made to minimize suffering.

2. Animals

In this prospective and double-controlled study, 24 conventionally raised calves (Holstein-Friesian, male) were included. Animals originated from one farm without any history of *Chlamydia*-associated health problems. Before the study, the herd of origin was regularly checked for the presence of chlamydiae by the National Reference Laboratory for Psittacosis. Calves were purchased at the age of 16 to 26 days weighing between 48 and 76 kg (57 ± 6; mean $\pm$ SD). After a quarantine period of at least 20 days and confirmation of a clinically healthy status, animals were included in the study.

Throughout the entire study, animals were reared under standardized conditions (room climate: 18 to 20°C) 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.

3. Study design

At the age of 45–54 days, 14 calves weighing 70.8 ± 4.3 kg were inoculated with *C. psittaci* whereas another 10 calves (body weight: 71.6 ± 7.2 kg) served as controls. By bronchoscope, four challenge groups received four different infection doses of live *C. psittaci* containing the following amounts of inclusion-forming units (ifu) in 6 mL stabilizing medium SPGA (containing **s**accharose, **p**hosphatile substances, **g**lucose and bovine **a**lbumin; [63]: 10^6 (n = 4), 10^7 (n = 4), 10^8 (n = 4), and 10^9 (n = 2) ifu per animal, respectively. Controls received either 6 mL containing 10^8 ifu of inactivated strain DC 15 (n = 6) or cell culture medium colored by ink solution (5 mL per animal; dilution: 1:5).

Animals exposed to 10^6–10^8 ifu of were euthanized and necropsied 3, 7 or 14 days post inoculation (dpi), while the two calves exposed to 10^9 ifu were sacrificed 3 dpi. Controls were euthanized 2 and 3 dpi. Broncho-alveolar lavage was performed, and lungs were examined and sampled to assess lesions and presence of *C. psittaci*.

Before inoculation until necropsy, each calf underwent daily clinical examination. In addition, blood samples were collected daily to monitor humoral immune response. Thus, venous blood was collected from the jugular vein before morning feeding using 9.0 mL plastic syringes (S-Monovette, Sarstedt AG & CoKG,

Nuembrecht, Germany). Serum was harvested by centrifugation and stored at −20°C until analyzed.

4. Preparation of bacteria used for inoculation

4.1. Live chlamydiae. Strain DC 15 was isolated at FRIEDRICH-LOEFFLER-INSTITUT (Jena, Germany) from an aborted calf fetus in 2002. The isolate was classified as *C. psittaci* genotype A-VS1 by DNA microarray testing and *omp*A gene sequencing [64]. Chlamydiae were propagated in buffalo green monkey kidney (BGM) cell culture using standard procedures [65]. Frozen stocks of strain DC15 were diluted to the required titer in stabilizing SPGA medium and used as antigen in the present trial.

4.2. Inactivated chlamydiae. Six-well cell culture plates were filled with 7-mL portions of stabilizing medium containing 10^8 ifu of *C. psittaci* DC15. Inactivation was achieved by 4.5-h exposure on a UV Transilluminator plate (UVP Inc. CA, Upland, CA) and simultaneous irradiation from a UV lamp installed above the vessel. While 6 mL were preserved as a single-animal dose to be inoculated, the remains of about 1 mL were left for subsequent examination of viability of these preparations. Cell culture passages using immunofluorescence confirmed the inability of treated chlamydial bodies to re-enter a developmental cycle.

5. Intrabronchial administration

For intrabronchial challenge, the non-fed calf was anesthetized with xylazin (0.2 mg/kg bodyweight, Rompun 2%, Bayer Vital GmbH, Leverkusen, Germany) and ketamine (1.7 ± 0.3 mg/kg bodyweight, Ursotamin, Serumwerk Bernburg AG, Bernburg, Germany); both injected intravenously at time intervals of approximately 3 min.

For inoculation, a flexible video endoscope of 140 cm working length and outer diameter of 9 mm was used (Veterinary Video Endoscope PV-SG 22–140, KARL STORZ GmbH & Co.KG, Tuttlingen, Germany). The endoscope was inserted through a metal tubular speculum (diameter: 3.5 cm, length: 35 cm) placed into the calf's mouth. Defined doses of the freshly prepared *C. psittaci*-suspension or cell culture medium, respectively, were administered at eight defined locations in the lung (Fig. S2) using a Teflon tube (inner diameter 1 mm, outer diameter 2 mm, 175 cm length, dead space: 1.4 mL) that was inserted through the working channel (diameter 2.2 mm) of the endoscope.

6. Clinical Scoring

Clinical observations were recorded twice daily and included feed intake, rectal temperature, respiratory rate, and the presence or absence of clinical signs of diarrhea or respiratory disease, such as cough or nasal discharge. In addition, the appearance of oral mucosa, conjunctivae, skin, hair and dyspnea were assessed daily, and the heart rate was counted. Extremities, umbilicus and *Lnn. mandibulares* were palpated and inducement of cough was tested (by a short compression of the larynx). Results were summarized using a 49-point clinical score (Table S1) consisting of sub-scores for general condition (max. 8 points), respiratory system (max. 17 points), cardiovascular system (max. 13 points) and other organ systems (max. 11 points).

7. Necropsy and tissue samples

At the end of the study, all animals were euthanized. Under conditions of deep anesthesia (pentobarbital-sodium, 770 ± 123 mg/10 kg bodyweight, 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 lung was removed, macroscopic lesions recorded and samples collected from each lung lobe. Sites with macroscopic lesions were preferentially sampled. Aliquots of each sample were used for histological and immunohistological examination and detection of *C. psittaci* by PCR. Then a complete necropsy was performed.

8. Collection of broncho-alveolar lavage fluid and BALF analyses

Broncho-alveolar lavage fluid (BALF) was obtained from freshly exenterated lungs immediately after exsanguination. At three different locations (*Lobus caudalis dexter, Lobus medius, Lobus caudalis sinister*) three subsequent washes using 20 mL of ice-cold cell buffer (140 mM NaCl; 2.8 mM KCL; 10 mM $Na_2HPO_4 \times 12H_2O$) for each instillation (in total 180 mL; 60 mL per lung lobe) were installed using glass syringes and a catheter inserted through the trachea. BALF obtained by aspiration was immediately placed on ice. The BALF recovery was $55 \pm 6\%$ (mean $\pm$ SD) and did not differ between groups. Cells and supernatant of BALF were separated by centrifugation ($300 \times g$; 20 min).

BALF cytology — Absolute number of leukocytes in BALF was determined by cell counting using traditional 'NEUBAUER chambers'. To quantify leukocyte populations, 400 µL of native BALF were placed on glass slides. The cellular sediments were fixed with 100% methanol for 10 min and subsequently stored at −20°C. For microscopic examination, the cell sediments were stained according to PAPPENHEIM (HemaDiff, bioanalytic GmbH, Umkirch/Freiburg, Germany), and the percentages of leukocyte populations (lymphocytes, macrophages, unsegmented and polymorphonuclear neutrophil granulocytes) were determined by counting a total of 100 cells. The absolute cell numbers of the leukocyte subsets in BALF were calculated based on the absolute number of leukocytes and the percentages of leukocyte populations.

Total protein — Concentrations of total protein were measured in BALF supernatant using commercially available modified Lowry Protein Assay Kit (Pierce, Rockford IL, USA). Each sample was analyzed in duplicate.

Eicosanoids — Liquid Chromathography – Tandem Mass Spectrometry (LC-MS-MS) was used to analyze concentrations of TXB2, PGE2, 15-HETE, and 12-HETE in BALF supernatant. Lipid mediators and the deuterated standards were purchased from Cayman Chemical (Ann Arbor, USA). Solvents and reagents (water, methanol, acetonitrile, formic acid and ammonium acetate) were LC-MS grade from Fisher Scientific (Loughborough, United Kingdom). After adding internal standards, the samples were filtrated and directly analyzed using an Agilent 1200 HPLC system coupled with an Agilent 6460 Triplequad mass spectrometer with electrospray ionisation. HPLC conditions were as follows: Zorbax Stable Bond 3.5 µm, 2.1×150 mm column, injection volume 20 µL, flow rate 0.4 mL/min, elution gradient from 10% (v/v) acetonitrile to 90% in 10 min, held for another 10 min. Analysis of lipid mediators was performed with Multiple Reaction Monitoring in negative mode. Results were calculated using the Agilent Mass Hunter Software.

9. Gross pathology, histopathology, immunohistochemistry

Distribution, extent and quality of macroscopic pulmonary lesions were recorded. Tissues collected at necropsy were fixed in 3.5% neutral buffered formalin for 24 h and embedded in paraffin. Lesions were evaluated in hematoxylin- and eosin-stained paraffin sections. Chlamydiae were labeled in paraffin sections by

indirect immunoperoxidase method using the anti chlamydial-LPS antibody ACI-P500 (Progen, Heidelberg, Germany) as primary antibody and peroxidase-labeled sheep anti-mouse IgG (NA 931, GE Healthcare Europe GmbH, Freiburg, Germany) as secondary antibody. Sections were pre-digested with 0.05% proteinase K (Merck, Darmstadt, Germany) for antigen retrieval.

10. Detection and quantification of chlamydiae using real-time PCR

Samples of lung tissue (2 of left caudal lobe, 2 of right caudal lobe), lung lymph nodes, and BALF-cells were subjected to DNA extraction using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) following the instructions of the manufacturer. One µl of the final eluate was used as template in real-time PCR testing for the family *Chlamydiaceae* [66] and the species of *C. psittaci* [67].

11. Immunoblotting

Immunoblotting was applied to both sera and BALF to detect specific antibodies. Lysates of partially purified elementary bodies of *C. psittaci* strain DC15 were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using a standard protocol. BGM cell lysates were included as controls. Prior to electrophoresis, the protein content had been determined using the Bradford reagent (Sigma, Hamburg, Germany), so that equal amounts of protein, i.e. 5 µg per lane, could be run from each sample. Semi-dry electroblotting was used to transfer the separated bands onto polyvinylidene difluoride membranes (PVDF, Amersham Biosciences, NJ, USA). Subsequently, membranes were blocked with 5% (weight/volume percent; w/v) skimmed dried milk (Roth, Karlsruhe, Germany) in TBS-T (10 mM Tris-HCl, 0.15 M NaCl, 0.1% Tween-20, pH 7.4) for 1 h and probed overnight with serum or BALF supernatant (both 1:50 dilution in TBS-T). Thereafter, incubation of Protein G conjugated to horseradish peroxidase (HRP Calbiochem, Nottingham, UK) was generally performed in 1% (w/v) bovine serum albumine (Serva, Heidelberg, Germany) in TBS-T. The blots were stained by adding the HRP substrate chloronaphthol (Sigma, Hamburg, Germany) and photographed using a G:Box imager and GeneSnap software (Syngene, Cambridge, UK).

12. Exclusion of co-infections

The herd of origin was known to be free of bovine herpes virus 1 (BHV-1) and bovine virus diarrhoea/mucosal disease virus (BVDV). Routine microbiological screening revealed that all animals were negative for Salmonella infections (fecal swabs) and relevant enteric parasites (fecal smearing). To verify relevant respiratory co-pathogens, the presence of *Mycoplasma*, *Pasteurella* or *Mannheimia* spp. was evaluated in nasal swabs taken immediately before challenge and before necropsy as well as in lung tissue samples obtained during necropsy. Neither *Mannheimia haemolytica* nor *Mycoplasma bovis* was detected in any sample. *Pasteurella multocida* and *Mycoplasma bovirhinis* was detected at least once in a nasal swab from 4 of 24 calves (17%) or 8 of 24 calves (33%), respectively, but never in any lung tissue sample. By serology, systemic infection with *Mycoplasma bovis* could be excluded (ELISA Kit for *Mycoplasma bovis*, Bio-X-Diagnostics, Jemelle, Belgium). Serology was also used to check for viral co-pathogens i.e. 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,). Only maternal antibodies (with titers decreasing in the course of the study) were seen against

BRSV (24/24), PI-3 (24/24), and adenovirus type 3 (23/24). In addition to serology, ear biopsies were examined for the presence of BVDV by immunohistochemistry [68]. All biopsies were negative for BVDV antigen indicating that none of the calves was immunocompromised by persistent BVDV infection.

During the quarantine period, all 24 calves included were checked serologically for antibodies against chlamydiae (ELISA test; IDEXX GmbH, Ludwigsburg, Germany). While 23/24 were serologically negative prior to inoculation, one calf (later challenged with inactivated chlamydiae) revealed an unexpected positive test result.

13. Statistical analysis

Data with normal distribution are presented as mean and standard deviation (SD) while data with non-normal or unknown distribution are given as median and range (minimum-maximum). Box and Whisker Plots represent lower and upper quartile values (box) with median and mean (+). Whiskers extend from each end of the box to the most extreme values within 1.5 interquartile ranges. Outliers are data beyond the ends of the whiskers. Regression analyses according to the best fitting model were performed to calculate regression lines for the development of total clinical scores over time per group.

For multiple sample comparison of normally distributed data, multiple range test (parametric test) was used to compare means. Kruskal-Wallis test (non-parametric test) was applied to multiple samples with non-normal distribution to compare medians. To compare the medians of two groups, Mann-Whitney-Wilcoxon W test was used. In the latter, the lowest achievable probability level was 93% due to small sample sizes (n) between $n = 2$ and $n = 6$ per group. Thus, *P*-values below $P \leq 0.07$ were accepted as statistically significant. For all tests, *P*-levels are given with the results.

Supporting Information

Figure S1 Dose titration and time course of the humoral immune response to *C. psittaci* infection in calves. Whole-cell proteins of *C. psittaci* DC15 were separated by SDS-PAGE. Development of the specific antibody response at three different infectious doses in serum (A) and BALF supernatants (B) were analyzed by immunoblotting (no BALF samples from 14 dpi available). Molecular mass markers (kD) are indicated on the right. (TIF)

Figure S2 Scheme of intra-bronchial inoculation. (TIF)

Table S1 Clinical Scoring. (DOC)

Author Contributions

Conceived and designed the experiments: PR ELT AB KS. Performed the experiments: PR CO ELT AB AV JL MR AR ES KS. Analyzed the data: PR CO ELT AB AV JL AR KS. Contributed reagents/materials/analysis tools: PR CO ELT AB AV JL MR AR ES KS. Wrote the paper: PR CO ELT AB AR ES KS.

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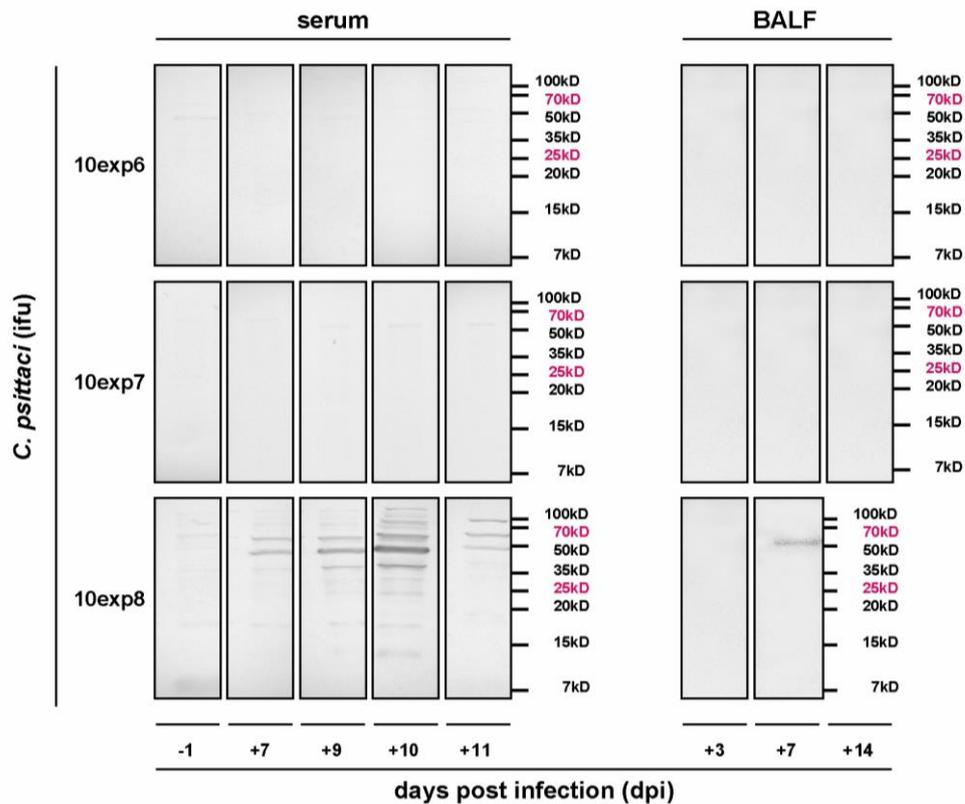
Supplement Figure 1. Dose titration and time course of humoral immune response to *C. psittaci* infection in calves. Whole-cell proteins of *C. psittaci* DC15 were separated by SDS-PAGE. Development of the specific antibody response at three different infectious doses in serum (A) and BALF supernatants (B) were analyzed by immunoblotting (no BALF samples from 14 dpi available). Molecular mass markers (kD) are indicated on the right.
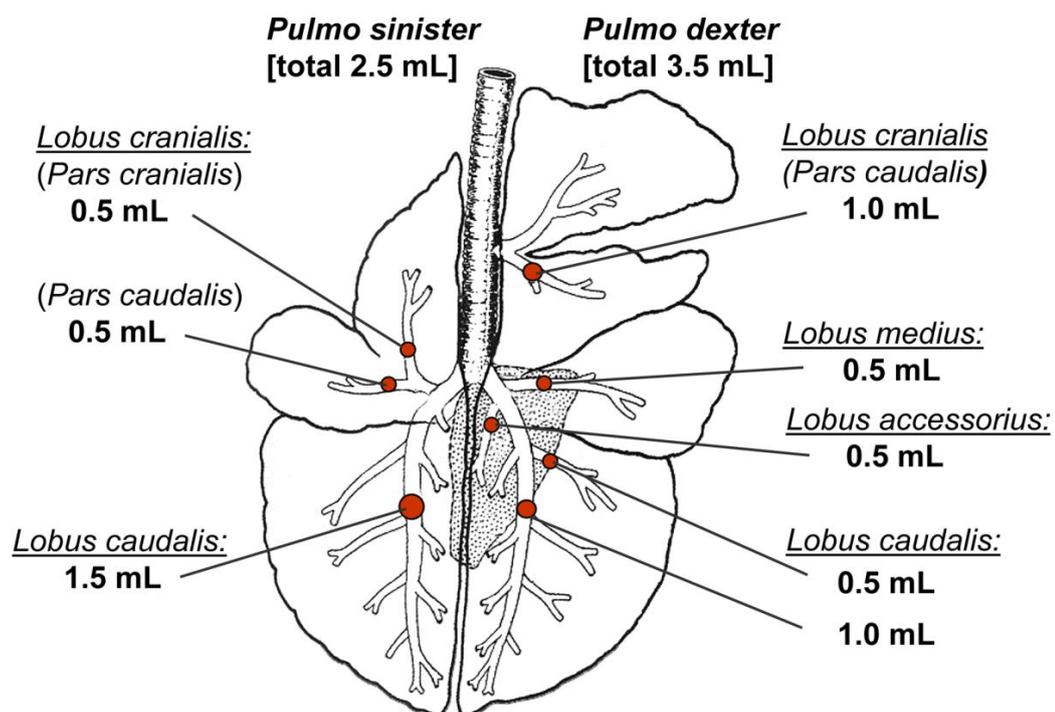
Supplement Figure 2. Scheme of intra-bronchial inoculation.

Supplement Table 1: Clinical Scoring

		Score [points]				
		0	**1**	**2**	**3**	**max.**
General Condition	appetite (feed intake)	normal	reduced	no intake	-	**2**
(max. 8 points)	conduct/behaviour	lively and vigilant	dull: rests a lot	very dull: prefers to lie	apathetic/moribund	**3**
	rectal temperature	≤ 39.5 °C	39.6 – 40.0 °C	40.1 – 40.5 °C	≥ 40,6 °C	**3**
Respiratory System	respiratory rate	≤ 30 per min	31-40 per min	41-50 per min	≥ 50 per min	**3**
(max. 17 points)	nasal discharge	none	mild/watery	moderate/mucous	severe/purulent	**3**
	ocular discharge	none	mild/watery	moderate/mucous	severe/purulent	**3**
	spontaneous cough	none	rarely	occasionally	frequently	**3**
	induced cough	none	single cough	bout of coughing	-	**2**
	dyspnoea	none	slight effort	moderate effort	severe effort	**3**
Cardiovascular System	heart rate	≤ 100 beats/min	101-120 beats/min	121-140 beats/min	≥ 141 beats/min	**3**
(max. 13 points)	conjunctivae	pink, wet, smooth, glossy	mild hyperaemia or anaemia	hyperaemia or anaemia	plus 1 point for each yellowish, altered, surface dry	**5**
	oral mucosa	pink, wet, smooth, glossy	mild hyperaemia or anaemia	hyperaemia or anaemia	plus 1 point for each yellowish, altered, surface dry	**5**
Other Organs	faeces	pasty	semi solid	liquid	watery	**3**
(max. 11 points)	skin and hair	skin and hair intact	hairless areas	decubitus	-	**2**
	umbilicus	not thickened or painful	omphalitis	-	-	**1**
	extremities (articulations)	normal	mono- or oligoarthitis	polyarthritis	-	**2**
	Lnn. mandibulares (size)	not enlarged	low-grade enlarged	high-grade enlarged	-	**2**
	Lnn. mandibulares (algesia)	not painful	painful	-	-	**1**

2.2   STUDY 2:

Dose-dependent effects of *Chlamydia psittaci* infection on pulmonary gas exchange, innate immunity and acute-phase reaction in a bovine respiratory model

Personal contributions:

- Animal experiments:
    - assistance during arterial catheterisation and catheter care
    - bronchoscopy and inoculation of the challenge strain or control medium
    - clinical examination of the calves
    - participation in the collection of blood and swab samples, preparation and counting blood cells
    - performing blood gas analysis and haemoxymetry
    - anaesthesia prior necropsies and assistance during necropsies
- Data analyses:
    - clinical data (score values)
    - blood gas analysis
    - haemoxymetry
    - electrolytes
    - metabolites
    - white blood cells
- Drafting of the manuscript
- Preparation of the following figures and tables:
    - Figure 1. Development of the respiratory score in control calves (a) and in calves experimentally inoculated with different doses of viable *C. psittaci* (b) over time.
    - Figure 2. Percentages of different types of white blood cells
    - Table 1. Analysis of blood samples: parameter, methods and sampling times
    - Table 2. Results of arterial blood gas analysis and haemoxymetry
    - Table 3. Concentrations of electrolytes and metabolites in venous blood
    - Table 4. Absolute cell count of venous blood smears
    - Supplement Table 1. Assessment of the respiratory score

2.3 STUDY 3:

Infection, Disease, and Transmission Dynamics in Calves after Experimental and Natural Challenge with a Bovine *Chlamydia psittaci* Isolate

Personal contributions:

- Animal experiments:
    - bronchoscopy and inoculation of the challenge strain
    - clinical examination of the calves
    - participation in the collection of blood and swab samples, preparation and counting blood cells
    - anaesthesia prior necropsies and assistance during necropsies
- Statistical analyses of raw data provided by co-authors:
    - PCR analyses and sequencing: K. Sachse
    - immunoblotting: A. Rüttger
    - recultivation and differential diagnostic tests: E. Schubert
    - concentrations of acute-phase proteins: W. Schrödl
- Drafting of the manuscript
- Preparation of the following figures and tables:
    - Figure 2. Score of clinical and respiratory health before and after *C. psittaci* challenge
    - Figure 3. Concentration of lipopolysaccharide binding protein (LBP) in peripheral blood before and after *C. psittaci* challenge
    - Figure 4. Relative amounts of white blood cells before and after *C. psittaci* challenge
    - Figure 5. Study design
    - Supplement Table 1. Absolute cell counts of white blood cells

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Infection, Disease, and Transmission Dynamics in Calves after Experimental and Natural Challenge with a Bovine *Chlamydia psittaci* Isolate

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Abstract

Chlamydia (*C.*) *psittaci* is the causative agent of psittacosis, a zoonotic disease in birds and man. In addition, *C. psittaci* has been repeatedly found in domestic animals and is, at least in calves, also able to induce respiratory disease. Knowledge about transmission routes in cattle herds is still deficient, and nothing is known about differences in host response after either experimental or natural exposure to *C. psittaci*. Therefore, our recently developed respiratory infection model was exploited to evaluate (i) the presence of the pathogen in blood, excretions and air, (ii) the possibility of transmission and (iii) clinical symptoms, acute phase and immune response until 5 weeks after exposure. In this prospective study, intrabronchial inoculation of 10^8 inclusion-forming units of *C. psittaci* (n = 21 calves) led to reproducible acute respiratory illness (of approximately one week), accompanied by a systemic inflammatory reaction with an innate immune response dominated by neutrophils. Excretion and/or exhalation of the pathogen was sufficient to transmit the infection to naïve sentinel calves (n = 3) co-housed with the infected animals. Sentinel calves developed mild to subclinical infections only. Notably, excretion of the pathogen, predominantly via feces, occurred more frequently in animals naturally exposed to *C. psittaci* (i.e. sentinels) as compared to experimentally-inoculated calves. The humoral immune response was generally weak, and did not emerge regularly following experimental infection; however, it was largely absent after naturally acquired infection.

Introduction

Psittacosis is a zoonotic disease caused by infection with the obligate intracellular bacterium, *Chlamydia* (*C.*) *psittaci*. The most important route of infection for humans and birds is inhalation of desiccated and aerosolized excreta (urine, feces, ocular, nasal, and respiratory tract secretions) of *C. psittaci*-shedding birds. Symptoms of both human and avian psittacosis encompass the whole range from clinically silent or mild flu-like symptoms to fatal disease [1–6]. Clinically inconspicuous cases of chronic infection in animals, where intermittent shedding of the pathogen continues, can easily be overlooked. Likewise, the frequency of reported human cases is thought to be underestimated [7,8].

With the improvement of diagnostic tools during the last decade, *C. psittaci* was additionally found in various non-human mammals, such as sheep and pigs [9,10], wild boar [11], horses [12,13], dogs [14,15] as well as in cattle [16–20]. Although chlamydial infections in cattle are mostly subclinical, they are very likely associated with reduced performance (e.g. retarded growth of calves, reduced fertility, and decreased milk yield [21,22]). It was shown recently that experimental infection with a bovine *C.*

psittaci strain was capable of inducing acute broncho-pneumonia in calves [23].

Chlamydiae are present in many bovine herds, but reports of cases involving farmers suffering from Chlamydia-caused respiratory disorders or asthma-like symptoms are only anecdotal and suggest the zoonotic potential being minimal compared to avian strains. While more definitive data on the identity and role of molecular virulence factors have been reported recently [24,25], markers for the zoonotic potential of avian and non-avian strains of *C. psittaci* are still unknown. Analysis of complete genome sequences of mammalian *C. psittaci* isolates failed to identify a link between *C. psittaci* genotypes and host species [26]. It has been emphasized that all genotypes and serovars of *C. psittaci* should be considered potentially transmissible to humans [7,27,28].

Until now, routes of infection, shedding and transmission of the pathogen, as well as the long-term cause-effect relationships in bovine *C. psittaci* infection, have not been evaluated under standardized conditions, wherefore we exploited our recently introduced respiratory *C. psittaci* infection model in calves [23]. The aim of the present study was to obtain experimental evidence on routes and mechanisms of shedding, as well as transmission of

the pathogen by socializing naïve sentinel animals with *C. psittaci*-challenged calves. Herein, the course of experimentally induced and naturally acquired infection will be evaluated with respect to clinical outcome, acute-phase reactions, and cellular vs. humoral immune response. Results of the present study support the validity of our hypotheses that (i) the pathogen is transmissible within bovine herds, (ii) *C. psittaci* is able to chronically impair health, and (iii) naturally acquired infections result in a milder course of disease compared to experimentally induced infection.

Results

Quantification of *C. psittaci* in blood and recovery from lung tissue

Examination of venous blood samples after challenge using real-time (rt)-PCR revealed that genomic DNA of *Chlamydiaceae* was found in the peripheral blood of 14 of 21 (67%) experimentally inoculated calves, and in two of the three sentinels. As depicted in Fig. 1, copy numbers per mL blood peaked within the first week after contact to the pathogen with variable maximal copy numbers. Statistical analysis confirmed that *C. psittaci* DNA contents were significantly higher in experimentally challenged calves compared to sentinels (Mann-Whitney rank sum test, $P = 0.03$). Detection of chlamydiae in blood was possible until the end of the study, i.e. 35 days post inoculation (dpi) in inoculated or 31 days post contact (dpc) in sentinels, respectively. Detailed information about significant, time-dependent differences in detected DNA amounts within the inoculated group is given in Table 1. DNA microarray examination of selected samples confirmed that indeed *C. psittaci* was found.

Recovery by cell culture of *C. psittaci* from lung tissue was successful in all experimentally challenged calves euthanized up to day 14. The identity of re-isolates to the challenge strain was exemplarily shown in four instances by sequencing of the ompA locus (data not shown). However, recovery from animals euthanized at day 35 was not successful, neither could the infectious agent be cultured from sentinels (necropsied >30 dpc).

Detection of chlamydial DNA in excretions, exhaled breath and room air

None of the ocular, nasal or rectal swabs taken before inoculation or socialization were PCR positive for *C. psittaci*. Fecal shedding occurred in 5 of 21 inoculated calves (from 3 to 22 dpi) and in all sentinel calves (n = 3, from 5 to 21 dpc). On a cumulative basis, the proportion of positive rectal swabs in sentinels (13/60; i.e. 21.7%) exceeded the detection rate in experimentally challenged calves (positive rectal swabs: 8/152; i.e. 5.3%). However, comparison of the cumulated data is only legitimate for animals surviving up to the end of the study, i.e. 3 inoculated animals versus 3 sentinels. At the end of the study, the probability level of a higher fecal excretion in sentinels was 13,2% (Fisher's exact test). Comparing the groups at given time points confirmed a significantly higher detection rate in sentinels, compared to inoculated calves at 10 dpi (P≤0.05, Fisher's exact test). Later on, the group size of n = 3 was too small to obtain meaningful results. There was only one positive nasal swab at 6 dpi (1/153, i.e. 0.7%) in the inoculated group while all nasal swabs from sentinel calves were PCR negative. None of the ocular swabs in any group was positive.

All room air samples collected before challenge in either the animal rooms (n = 7) or the bronchoscopy room (n = 1) were negative for *C. psittaci*. From a total of 10 room air samples collected from the animal rooms (where the calves were housed after inoculation of *C. psittaci*), the two samples obtained at 1 and 2 dpi, respectively, were still negative, while 4 of 5 samples collected between 3 and 7 dpi were PCR positive. *C. psittaci* could not be detected anymore in the three room air samples collected 28, 30, and 35 dpi from the room where three remaining infected calves and three sentinels were co-housed.

In the three infected calves euthanized at 35 dpi, exhaled breath was screened for the presence of *C. psittaci* consecutively between 14 and 31 dpi. Only 1 of the 3 exhaled breath samples collected at 14 dpi was positive for the pathogen while *C. psittaci* could not be found in any of the samples collected at a later time point. Exhaled breath samples collected from sentinels between 22 and 29 dpc were also negative for *C. psittaci*.
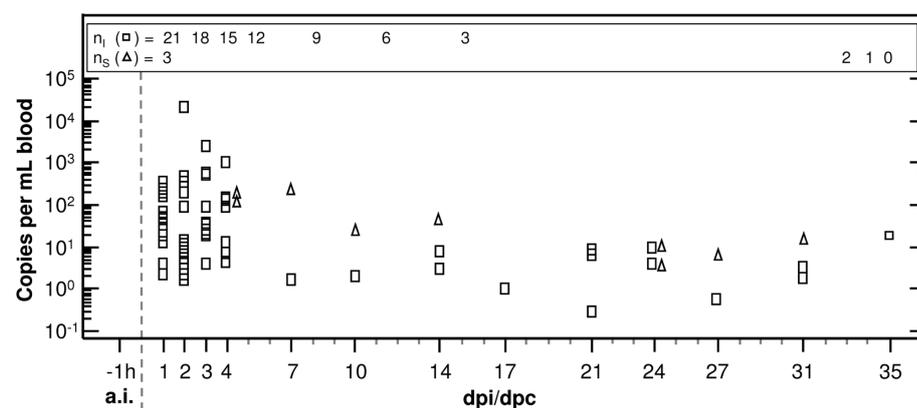
Figure 1. Detection of *C. psittaci* DNA in blood samples. The number of genomic equivalents to inclusion-forming units as determined by quantitative rtPCR was maximal within one week after both experimental challenge (squares) and natural acquisition (triangles). In experimentally challenged calves the maximal number of copies per mL blood varied between different animals to a large extent. Medians of three extractions, each run in duplicate were calculated for each blood sample. Mann-Whitney rank sum test was conducted to compare the amount of chlamydial DNA at different time points within the group of experimentally inoculated calves. Significant *P*-values are given in Table 1. a.i. *ante infectionem*. dpi days post inoculation. dpc days post contact to inoculated calves. h hour. n_I number of inoculated calves. n_S number of sentinels.
doi:10.1371/journal.pone.0064066.g001

Table 1. Significant differences in the amount of chlamydial DNA in blood of calves challenged with 10^8 ifu of *C. psittaci*.

Timepoint	1 dpi	2 dpi	3 dpi	4 dpi	7 dpi	10 dpi	14 dpi	17 dpi	21 dpi	24 dpi	27 dpi	31 dpi	35 dpi
−1h a.i.	***	***	***	***	-	-	*	-	***	***	-	*	-
7 dpi	**	**	**	**	-	-	-	-	**	*	-	*	-
10 dpi	*	*	**	**	-	-	-	-	**	*	-	-	-

Data are given in Figure 1.
Mann-Whitney rank sum test was used to compare the amount of copy numbers per mL blood at different time points. Significant differences are indicated by * $P{\leq}0.05$,
** $P{\leq}0.01$, *** $P{\leq}0.001$. a.i. *ante infectionem*. dpi days post inoculation. h hour.
doi:10.1371/journal.pone.0064066.t001

Clinical outcome

Inoculation of 10^8 ifu of *C. psittaci* per calf resulted in acute respiratory illness, the signs of which were maximal at 2–3 dpi (Fig. 2A). From 4–8 dpi, health improved continuously, although none of the calves recovered completely by the end of this study. Both respiratory and total health scores at days 1 through 8 and day 10 after inoculation differed significantly compared to the mean values before inoculation (Wilcoxon signed-rank test, Fig. 2A, B). Acute clinical signs included elevated heart rates (max. 132 at 2 dpi), respiratory rates (max. 120 at 2 dpi), rectal temperatures (max. 41.6°C at 2 dpi), dry and scattered cough, reduced feed intake and hyperemic conjunctivae. Throughout the course of the study elevated respiratory rates, cough, hyperemic conjunctivae and enlarged mandible lymph nodes continued to occur.

Sentinels remained largely asymptomatic in terms of their general condition (e.g., appetite, conduct, and rectal temperature). The total and respiratory score did not exceed the sum of 6 out of 49 (Fig. 2A) or 4 out of 17 points (Fig. 2B), respectively. However, cough occurred between 7 to 11 dpc and continued intermittently up to the end of the study. Additionally, ocular or nasal discharge occurred, conjunctivae were often hyperemic, and mandibular lymph nodes were repeatedly enlarged between 15 and 29 dpc.

Acute-phase response

The concentration of lipopolysaccharide (LPS)-binding protein (LBP) in experimentally challenged calves rose from a stable baseline level before inoculation (median: 11.7 µg/mL, range: 37.0 µg/mL) to statistically significant elevated values between 1–10 dpi, with a maximum at 2 dpi (median: 121.3 µg/mL, range: 148.8 µg/mL). Towards the end of the study, individual LBP concentrations remained elevated compared to median baseline values before inoculation (Fig. 3).

In sentinels, the concentration of LBP increased between 10–28 dpc, but this increase was transient and mild compared to inoculated calves (Fig. 3).

Systemic cellular response

White blood cells in inoculated calves. The total number of peripheral blood leukocytes (Table S1) increased within the first two days after inoculation and was maximal and significantly elevated at 2 dpi. After dropping below baseline level at 3 and 4 dpi, the number of leukocytes measured between one and two weeks after challenge revealed an ascending tendency, which was significant at 14 dpi compared to baseline data. Individual leukocyte numbers between 17–35 dpi also tended to exceed the pre-inoculation median in the majority of calves.

The early increase of leukocytes in inoculated calves was mainly driven by an increase of both mature and band forms of neutrophils, which where maximal at 2 dpi on a percentage basis and based on absolute numbers (Fig. 4 A, B, Table S1). The absolute number of polymorphonuclear neutrophils as well as their relative amount dropped sharply at 3 dpi and median values were below the initial medians up to 7 or 10 dpi, respectively. From 7 dpi towards the end of the study, the relative number of polymorphonuclear neutrophils tended to rise again and individual values exceeded mostly the initial level at the last days of the study (Fig. 4 B). In contrast, banded neutrophils exceeded the baseline value significantly up to 10 dpi (except at 4 dpi) and returned back to initial levels towards the end of the study (Fig. 4A, Table S1).

The absolute amount of lymphocytes was slightly but significantly reduced between 1–4 dpi and subsequently returned to the initial level (Table S1). On a percentage basis, the clear reduction of lymphocytes at 1 and 2 dpi was followed by an upward trend and a statistically significant maximum at 4 dpi (Fig. 4C). At the final stage, individual values of lymphocytes were generally lower compared to initial values on a percentage basis.

The proportion of monocytes was significantly elevated on 3, 4, 7, and 14 dpi (Fig. 4D). Absolute and relative amounts of monocytes measured hereafter tended to be elevated compared to initial values (Fig. 4D and Table S1).

WBCs in sentinel calves. In sentinels, the total number of blood leukocytes increased constantly up to 10 dpc (individual maximum of 19.25 G/L). Towards the end of the study, they slowly approached the initial level (Table S1). The absolute number of polymorphonuclear neutrophils was clearly elevated between 10–21 dpc and did not reach the initial level up to the end of the study. On a percentage basis, polymorphonuclear neutrophils were elevated in the final period with individual maxima on 7, 24 and 28 dpc (44, 58 and 46%, Fig. 4B). Banded neutrophils peaked between 10–17 dpc in both absolute and relative numbers and returned to initial levels afterwards (Table S1, Fig. 4A). Absolute numbers of lymphocytes and monocytes did not show clear trends up to the end of the study.

Humoral immune response

Western immunoblotting revealed the formation of specific antibodies against the *C. psittaci* challenge strain DC15 in four of the six inoculated calves that were euthanized at 14 and 35 dpi. The onset of the specific humoral immune reaction has been detected between 10 and 14 dpi. In one calf, the antibody level was maintained until the end of the trial whereas in two other calves, antibodies decreased towards the end of the study (data not shown). In sera of sentinel calves, no chlamydia-specific antibodies could be detected at any time point.
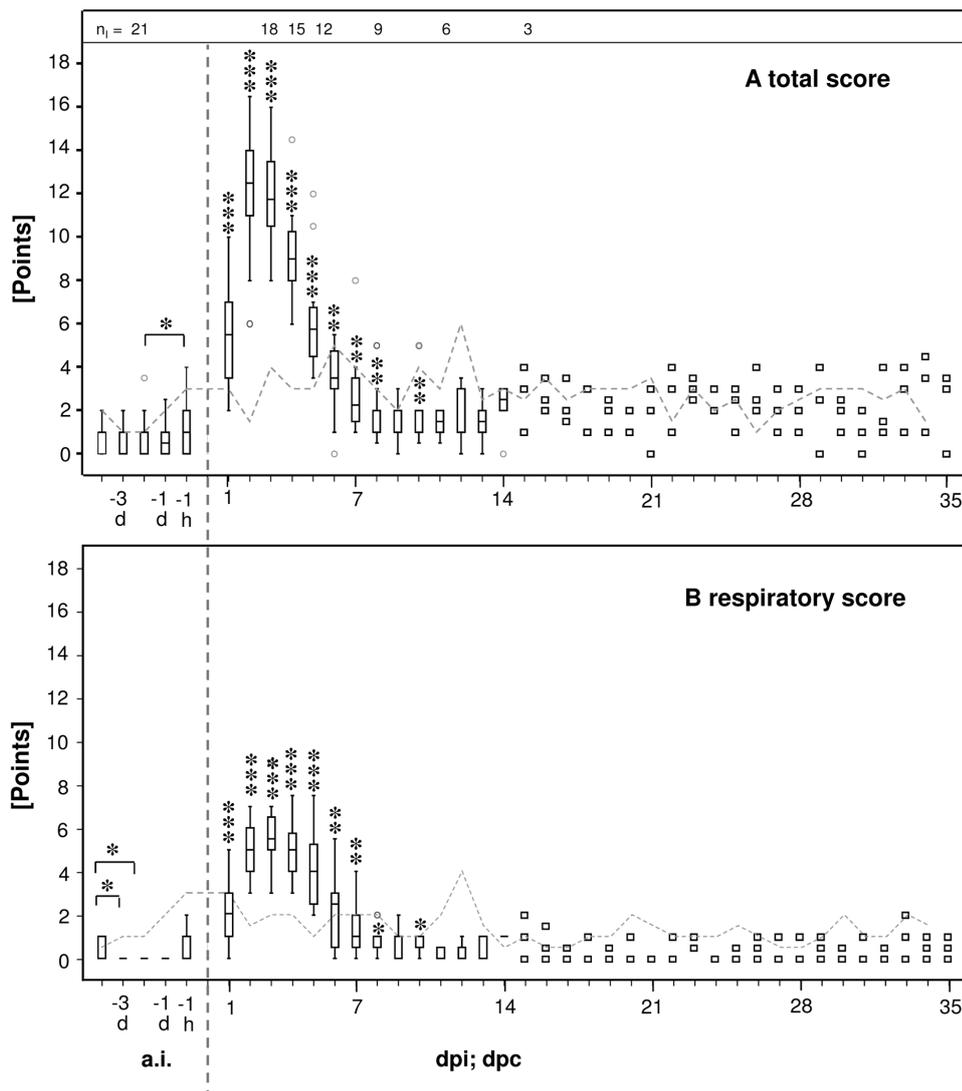
Figure 2. Score of clinical and respiratory health before and after *C. psittaci* challenge. General clinical score (panel A) and respiratory signs (panel B) peaked in the inoculated group 2–3 dpi. Symptoms resolved incompletely. The respiratory score contributed maximally 50% to the general clinical picture presented by the total score. In contrast, natural acquisition of *C. psittaci* infection lead only to mild symptoms in sentinel calves. Based on the group size of calves inoculated with 10^8 ifu/animal (n_I given above panel A) data are given as Box and Whisker plots or as individual data (squares) if n≤3, respectively. The dashed line indicates the maximum of score points achieved in sentinels (n = 3). For statistical analysis, baseline data (averaged per animal) were compared to data obtained at different time points after challenge (Wilcoxon signed-rank test). Values of * $P≤0.05$, ** $P≤0.01$, and *** $P≤0.001$ were considered significant. Outlier values are indicated by small grey circles. a.i *ante infectionem*. d day. dpi days post inoculation. dpc days post contact to inoculated calves. h hour.
doi:10.1371/journal.pone.0064066.g002

Discussion

Horizontal transmission of *C. psittaci*

In the present experimental setting of intrabronchial inoculation of 10^8 ifu *C. psittaci* per calf, clinical signs were induced, bacteremia occurred, pathogens were recovered from lung tissue, and humoral immune response revealed successful infection. The excretions of inoculated calves were sufficient to transmit infection to naïve sentinels as indicated by the presence of the pathogen in their peripheral blood. To the best of our knowledge, this is the first study emulating natural animal-to-animal transmission of *C. psittaci* in bovine herds. More importantly, these findings substan-

tiate the hypothesis that, since *C. psittaci* infection was easily transmissible from calf to calf, humans in close contact with infected animals could similarly be at risk of exposure.

Interestingly, less than one fourth of the calves excreted DNA of the pathogen after experimental infection, but all of naturally contracted sentinel calves did. In both groups, fecal excretion was predominant compared to nasal and/or ocular secretion or exhalation. There are two hypotheses to explain the presence of *C. psittaci* DNA in feces. (i) Mucociliary clearance transported the pathogen to the pharynx where it was subsequently swallowed. As such, *C. psittaci* might pass into the gastrointestinal tract without further interaction or might instead infect the mucosal cells. (ii)
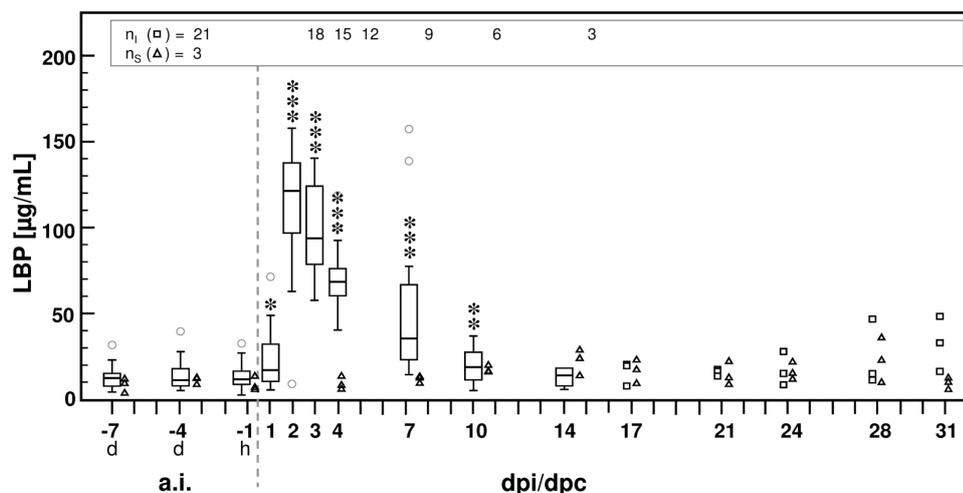
Figure 3. Concentration of lipopolysachharide binding protein (LBP) in peripheral blood before and after *C. psittaci* challenge. After inoculation of *C. psittaci*, LBP concentration was significantly increased until 10 dpi. Based on the group size of calves inoculated with 10^8 ifu/animal (n_I) data are given as Box and Whisker plots or as individual data (squares, if n≤3), respectively. The LBP concentrations obtained in sentinels (n_S, triangles) increased slightly after 7 days post contact (dpc) but did not exceed 35.1 µg/mL (i.e. maximal LBP concentration: at 28 dpc). Due to small sample size no statistical hypothesis was applied to this group. For statistical analysis of experimentally challenged calves, baseline data (averaged per animal) were compared to data obtained at different time points after challenge (Wilcoxon signed-rank test). Values of * P≤0.05, ** P≤0.01, and *** P≤0.001 were considered significant. Outlier values are indicated by small grey circles. a.i *ante infectionem*. d day. dpi days post inoculation. dpc days post contact to inoculated calves. h hour.
doi:10.1371/journal.pone.0064066.g003

The pathogen was spread by bacteremia to the intestinal mucosa, where it infected epithelial cells that were released during the epithelial turn-over into the gastrointestinal tract. Findings of our study provide strong evidence for the first hypothesis because *C. psittaci* DNA was detected in tracheal mucus, but not in epithelial cells of upper airways neither in the intestinal mucosa [29].

Detection rates in rectal swabs of sentinels were four-fold higher compared to inoculated calves indicating that the route of infection might influence the shedding. Aerosolization of dried feces and contaminated dust is considered to be the most common transmission route of psittacosis originating from birds [8]. In the present study, despite complete daily cleaning of the environment, *C. psittaci* was routinely detected in room air during the acute phase of infection (3–7 dpi). In addition, the pathogen was sporadically detectable in exhaled breath even 14 dpi, i.e. at a time point when acute clinical signs had largely dissipated. Although inhalation of bio-aerosols is a likely route of air-borne transmission, alternative possibilities should be taken into consideration. Routes of zoonotic transmission from birds to man include ingestion of contaminated feces, mouth-to-beak contact, bites, open wounds, and blood-sucking ectoparasites [30]. In the present study, transmission via vectors (e.g., insects, parasites) can be ruled out, but group housing with direct contact certainly opened ways of direct (e.g. fecal-oral) transmission through mutual licking. It was reported that the risk of infection with *C. pecorum* and *C. abortus* in calves increased in a quadratic regression with group size, even though calves had physical contact only with their direct neighbours [31].

Chlamydemia

Hematogenic spread of the pathogen has been shown by quantitative rt-PCR after both experimentally induced and naturally acquired infection. In inoculated calves euthanized at 35 dpi, low levels of chlamydial DNA were detectable in peripheral blood up to the end of the study, which suggests an ongoing systemic presence of the pathogen. Although rt-PCR neither allows identification of cell types carrying chlamydiae nor assessment of the viability of the pathogen, we assume that *C. psittaci* behaved similarly as described for *C. pneumoniae* in murine macrophages in vivo [32], where the agent was capable of infecting, surviving and multiplying. While macrophages enabled the dissemination of viable chlamydiae to distant tissue sites, DNA of initially inactivated chlamydiae in macrophages was rapidly degraded. Furthermore, *in vitro* studies revealed an improved long-term survival in macrophages after presence in apoptotic neutrophils [33]. Last but not least, cell-independent transport of elementary bodies (EBs) in blood cannot be excluded, as direct binding of *C. trachomatis* and *C. pneumoniae* EBs to apolipoprotein B containing fractions of plasma lipoproteins (i.e. low and very low density lipoproteins) was shown *in vitro* [34].

Pathogenesis

Clinical outcome. Both character and intensity of clinical signs observed in the 21 calves inoculated with 10^8 ifu of *C. psittaci* were consistent. Thus, the results provide statistically meaningful evidence confirming the general reproducibility of this particular dose in an animal model introduced recently by our group using different doses (10^6–10^9 ifu) [23].

The acute clinical outcome in inoculated calves was maximal between 2 and 3 dpi, and lasted for about one week. During acute illness, the total health score comprised up to 50% signs related to the respiratory score, which indicates strong involvement of the respiratory system. Thus, clinical findings in experimentally infected calves are in accordance with the few cases of acute respiratory disease due to *C. psittaci* infections reported in calves [17,35].

Sentinels acquired the infection, but remained almost clinically inconspicuous. Although obvious signs of illness were lacking in these animals, the few mild clinical signs observed with a certain time delay were comparable to the low clinical score values seen in inoculated calves in the period from 14 dpi onwards. These
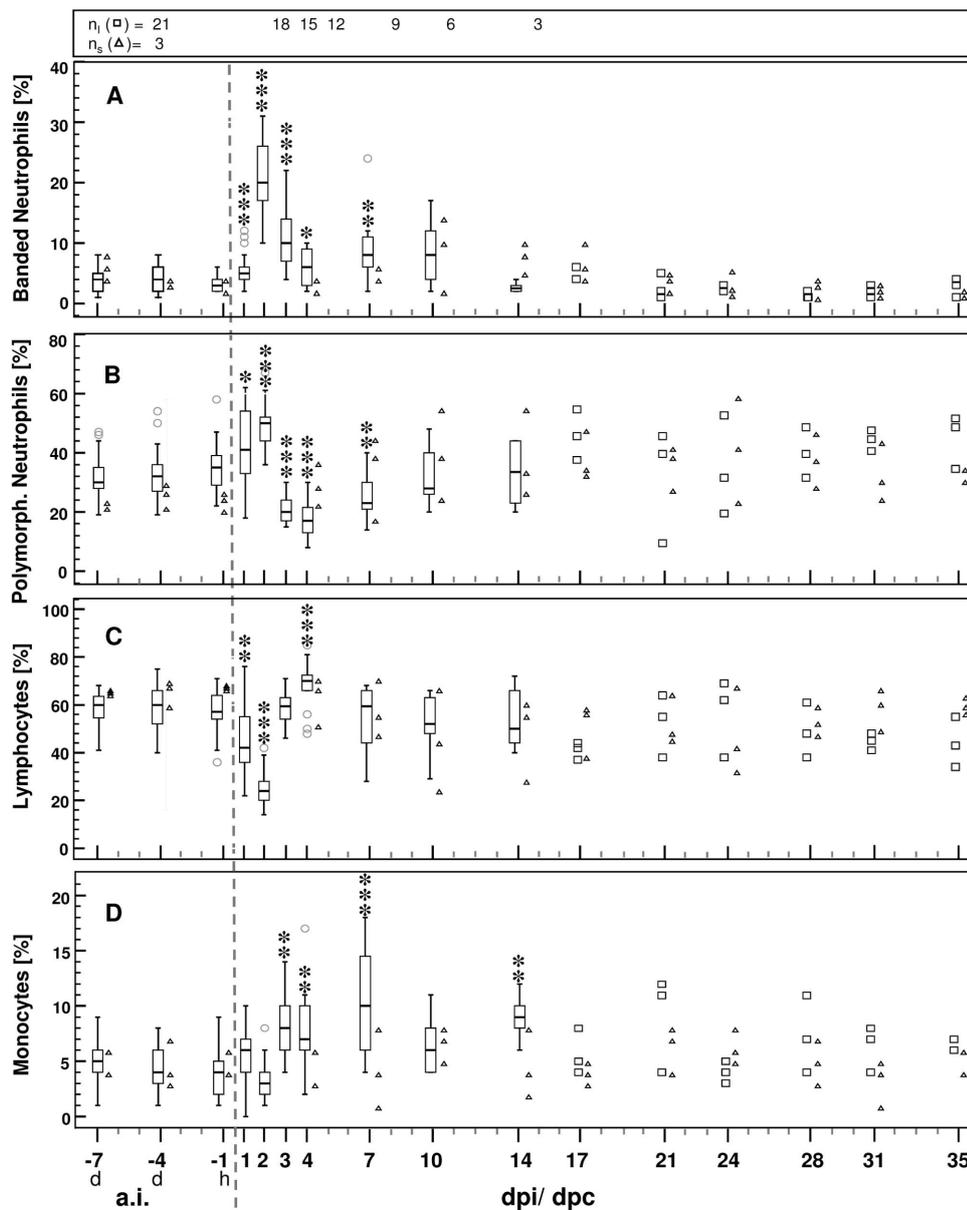
Figure 4. Relative amounts of white blood cells before and after *C. psittaci* challenge. Compared to baseline data, both banded and polymorphnuclear neutrophils increased during the acute phase of infection whilst lymphocytes decreased transiently. Based on the group size of calves inoculated with 10^8 ifu/animal (n_I) data are given as Box and Whisker plots or as individual data (squares, if $n \leq 3$), respectively. For statistical analysis, baseline data (averaged per animal) were compared to data obtained at different time points after challenge (Wilcoxon signed-rank test). Values of * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ were considered significant. Outlier values are indicated by small grey circles. Due to small sample size of sentinel calves (n_S, triangles) no statistical hypothesis was applied to this group. a.i *ante infectionem*. d day. dpi days post inoculation. dpc days post contact to inoculated calves. h hour.
doi:10.1371/journal.pone.0064066.g004

observations support previous findings revealing the negative impact of naturally acquired subclinical *Chlamydia* spp. infections on calf health [36]. Furthermore, observations in the field indicated that the vast majority of widely spread chlamydial infections in cattle remain asymptomatic, but show a clear association to reduced growth rates in calves [22], and performance losses in dairy cows [20,21].

Acute phase and innate immune response. Even the clinically inconspicuous acquisition of the pathogen in sentinel calves resulted in a slight increase of LBP concentration in blood. In calves experimentally challenged with *C. psittaci*, LBP concentrations in peripheral blood were markedly elevated during the acute phase which is in good agreement with LBP concentrations measured previously in blood of calves challenged with an identical dose of either live or inactivated *C. psittaci* [23]. Taking these findings together, LBP can be regarded as a reproducible and dose-dependent LPS-related biomarker in bovine chlamydiosis. Although the functional consequence of an LBP increase

remains to be elucidated, our findings extend the relevance of LBP which was previously described as a fast-reacting sensitive marker of bovine respiratory disease [37,38,39].

In experimentally challenged calves, the leukocytic response of the innate immune system was prompt. Leukocytosis (i.e. >12.0 G/L; [40]) at 2 dpi was mainly driven by the increase of both polymorphonuclear and banded neutrophils, i.e. regenerative left shift in the leukocyte curve. This rapid, distinct increase of neutrophils was followed by a subsequent drop that can be interpreted as recruitment to the site of infection. This interpretation is in accordance with our previous findings concerning fibrinopurulent bronchopneumonia and influx of neutrophils into the lung at 3 dpi [23], as well as with several studies reporting the rapid recruitment of neutrophils in Chlamydia-induced pneumonia [41,42,43,44].

Although acute symptoms subsided, the demand for leukocytes in blood remained elevated towards the end of the study, thus indicating again the shift from the acute phase into a subclinical persisting course. During this subclinical phase, neutrophils and monocytes tended to be elevated on a percentage basis, while relative amounts of blood lymphocytes were lowered, which corresponds in general to the composition of blood cells in naturally chlamydia-infected young cattle [36].

Even though we used Western immunoblotting to test the calf sera, which is more sensitive than currently available enzyme linked immunosorbent assays (ELISA), only two thirds of experimentally challenged calves and none of the sentinels developed chlamydia-specific antibodies. This indicates that the presence of *C. psittaci* DNA in blood was not associated to humoral immune response induction. This observation is not surprising as previous studies demonstrated that only about 60% of naturally *C. abortus* and/or *C. pecorum* infected calves were sero-positive for *Chlamydia* spp. [36] and that seropositivity and chlamydial shedding do not necessarily correlate [18,45]. Both human and veterinary medicine are faced with the problem that chlamydial infections often do not elicit sufficiently high antibody responses, which complicates both development of specific serological tests and development of safe and efficacious vaccines [46].

Materials and Methods

Ethics statement

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: 04-002/07). All experiments were done in a containment of biosafety level 2 under supervision of the authorized institutional Agent for Animal Protection. Bronchoscopy to inoculate the pathogen was strictly performed under general anesthesia. During the entire study, every effort was made to minimize discomfort or suffering.

Animals

Calves originated from a conventional farm without any history of *Chlamydia*-associated health problems. All animals (n = 24) were male and except for one, they all belonged to the Holstein Frisian breed. They were purchased at the age of 14 to 25 days (19.0±3.2, mean±SD) weighing between 46.2 and 71.2 kg (57.1±5.5, mean±SD). After arrival they had a quarantine period of at least four weeks (31±3.2 days, mean±SD).

The study was carried out in the Federal Research Institute for Animal Health (Friedrich-Loeffler-Institut, Jena, Germany) where animals were reared under standardized conditions (completely

daily cleaning, room temperature: 18–20°C, relative humidity: about 60%) and in accordance with international guidelines for animal welfare. Feeding included hay and water ad libitum and individual supplementation with commercial milk replacer and coarse meal. All food products were free from antibiotics.

Exclusion of co-infections. The herd of origin was known to be free from bovine herpes virus 1 (BHV-1) and bovine virus diarrhoea/mucosal disease virus (BVDV). The latter status was confirmed, using BVDV-specific immunohistochemistry of ear biopsies [47]. Serological findings (Bio-X respiratory penta ELISA Kit, Bio-X-Diagnostics) confirmed that animals did not acquire infections with respiratory viruses (i.e. bovine respiratory syncytial virus (BRSV), parainfluenza 3 virus (PI-3) or adenovirus type 3) during the study. To verify the absence of bacterial co-infections (e.g., *Mycoplasma* (*M.*), *Pasteurella* (*P.*), *Mannheimia spp.*), nasal swabs were taken (i) before challenge and (ii) before necropsy. In addition, lung tissue was obtained during necropsy, but neither *P. multocida* nor *M. bovis* were detected in any sample. Colonisation of *Mannheimia haemolytica* was found in two calves euthanized at 2 and 4 dpi (once in lung tissue and nasal swab and once only in a nasal swab) without pathological consequences. In addition, nasal swabs were sporadically positive for *P. multocida* and *M. bovirhinis* (i.e. 4.3% and 14.9% respectively), both are known commensals of the nasal mucosa of healthy calves [48,49]. In two instances, further non-pathogenic agents (*Acholeplasma laidlawii* and *M. canadense*) were detected from nasal swabs. Calves were also checked and found to be negative for the presence of *Salmonella* spp. (via rectal swabs).

Study design

After the quarantine period and confirmation of physical health, all 24 calves aged 42 to 58 days (50.1±3.7 days, mean±SD) and weighting 59.2 to 93.0 kg (74.4±7.2 kg, mean±SD) were included. The entire study lasted from one week before challenge until 5 weeks after exposure.

Twenty-one calves were challenged intrabronchially with 10^8 ifu of a bovine *C. psittaci* strain (DC15) as described elsewhere [23], and were housed in three communicating rooms (n = 7 per room). Three calves served as naïve sentinels, co-housed with the experimentally infected ones by replacing those animals euthanized at 2 dpi. Further necropsies of three calves per time point were performed at 3, 4, 7, 10, and 14 dpi (Fig. 5). After 14 days, the remaining 3 experimentally inoculated calves and the 3 sentinels were housed in one room until the end of the study. While the 3 inoculated calves were euthanized 35 dpi, the sentinels were euthanized at 32, 33, and 34 dpc. All necropsies were performed as described previously [23].

At time points depicted in Figure 5, in vivo sampling included (i) ocular, nasal and rectal swabs for detection of the pathogen and (ii) collection of peripheral blood from the jugular vein in order to asses acute phase response, immune response, and bacteremia. Swabs and blood were generally collected before morning feeding.

Quantitation of *Chlamydiaceae* from blood samples using rt-PCR and species detection

Blood of all calves was tested before and after challenge according to time points given in Fig. 5. Therefore, aliquots of 300 µl of each blood sample were DNA extracted using the Qiagen Stool Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Detection of *Chlamydiaceae* spp. DNA was done on a Mx3000P (Agilent, Waldbronn, Germany) using a TaqMan rt-PCR protocol targeting the 23S rRNA gene with an internal amplification control as described previously [50,51]. Amplification reactions contained 5 µl of DNA extract and were conducted on 96-well plates. A decimal dilution

Inoculation
- 10^8 ifu/calf
- intrabronchial
- n = 21

Co-housing
- healthy sentinel calves
- n = 3

Legend:
a.i. = ante inoculation
x = sampling

10^8 ifu/calf	days post inoculation (dpi)	a.i.	1			7		14		21		28		35
	clinical examination	x x x	x x x x x x x x	x x x x x x x	x x x x x x x	x x x x x x x	x x x x x x x	x x x x x x x						
	swabs	x x x	x x x x x x x x	x x x x x x x	x x x x	x	x	x	x					
	blood	x x x	x x x x x	x	x	x	x	x	x	x	x	x		
	exhaled breath		x	x		x	x	x x	x					
	animals (n)	21	21 18 15 12	9 6	3	3	3							
	necropsy (n)		3 3 3 3	3 3				3						

sentinels	days post contact (dpc)	a.i.	1		7		14		21		28	32
	clinical examination	x x x	x x x x x	x x x x x x x	x x x x x x x	x x x x x x x	x x x x x x x					
	swabs		x x x x x	x x x x x x	x x x x x x	x x x x	x	x	x	x		
	blood	x x x	x	x	x	x	x	x	x	x	x	
	exhaled breath					x	x x	x x x				
	animals (n)	3	3	3	3	3	3	2 1				
	necropsy (n)							1 1 1				

room air	a.i.: n = 7	x x x x x x x x	x				x	x	x

Figure 5. Study design. During this prospective study, three samples were collected before experimental or natural challenge with *C. psittaci* and hereafter on time points given below the timeline for both study groups.
doi:10.1371/journal.pone.0064066.g005

series of defined contents of inclusion-forming units (10^4–10^{-1} ifu of *C. psittaci* strain DC15) was included in each run, and the resulting calibration curve served as the basis for copy calculation by the software MxPro 4.1 (Agilent, Waldbronn, Germany). Three extractions of each sample were tested in duplicate. For further statistical analyses the median of all six runs was used per calf and time point. For identification of the chlamydial species, selected *Chlamydiaceae* positive samples were examined by use of DNA microarray test [52] and a rt-PCR assay for *C. psittaci* [51].

Recultivation of the pathogen from lung tissue and strain identification

For recovery of *C. psittaci*, lung tissue with inflammatory alterations (if present) obtained during necropsy was immediately placed in sterile transport medium for *Chlamydia* spp. (SPGA [53]), and further processed using standard procedures. Briefly macerated and homogenized tissue was used to inoculate BGM cell layers, and chlamydial inclusions were detected by immunofluorescence. For strain identification a 1088-bp segment of the ompA gene comprising all variable domains was sequenced as described [54].

Confirmation of *C. psittaci* in swabs using rt-PCR

Ocular, nasal and rectal swabs were DNA extracted as described previously [55]. All swab samples were run in duplicate using the same rt-PCR methodology [50]. To confirm the presence of *C. psittaci*, *Chlamydiaceae*-positive samples were further examined using species-specific rt-PCR [51]. Selected *C. psittaci* positive samples where confirmed using DNA microarray tube assay [52].

Sampling and analysis of room air and exhaled breath

Stable air was collected as recommended in literature [56] using an AirCheck XR 5000 instrument with standard IOM personal inhalable dust sampler (both SKC Inc., Eighty Four, PA, USA),

which was equipped with gelatine filters of 3 mm pore size (SKC Inc., Eighty Four, PA, USA). The instrument was placed out of reach for the calves and pumped a total of 480 L through the filter at a flow rate of 2 L/min.

To analyze exhaled breath, the gelatine filter assembled IOM sampler was wired to the expiratory side of a Y-shaped inspiratory–expiratory valve, which was adapted to a tightly fitting face mask for calves (Kruuse, Langeskov, Denmark) as shown in Fig. S1. For each exhaled breath collection, the calf in- and expired through the mask for one hour.

Samples of both room air and exhaled breath were collected at time points given in Figure 5. For further PCR analyses, gelatine filters were processed and DNA extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer. To confirm the presence of *C. psittaci*, *Chlamydiaceae*-positive samples were further examined using species-specific rt-PCR [51].

Clinical examination and scoring

Starting 4 days before challenge, clinical examination of each animal was performed daily until the end of the study. Results were summarized using a score system. Criteria for evaluation of clinical signs and the corresponding scoring system have been described elsewhere [23]. Shortly, the total health score consisted of sub-scores reflecting different organ systems. For the respiratory score, breathing frequency, occurrence of dyspnoea, ocular and nasal discharge, and the presence and inducement of cough were taken into account.

Analysis of lipopolysaccharid-binding protein (LBP)

Venous blood was sampled into 9.0 mL plastic syringes (S-Monovettes, Sarstedt AG & CoKG, Nuembrecht, Germany) and serum was harvested by centrifugation (3120 g; 15 minutes, 15°C). Sera were stored at −80°C until analyzed. Concentrations of LBP were measured using an ELISA as described previously [57].

White blood cell analysis

For white blood cell count (WBC), 20 µl of the collected potassium EDTA blood were prepared using Leuko–tic kit (bioanalytic GmbH, Umkirch/Freiburg, Germany). A Neu-bauer-improved hemoxymeter was filled, and WBSs were counted under 100-fold magnification of a bright light microscope. For cell differentiation, air dried blood smears were stained with HemaDiff - Quick Staining Set (bioanalytic GmbH, Umkirch/Freiburg, Germany). One hundred WBCs were differentiated under 400 fold magnification and served as basis to calculate absolute numbers of different cell types.

Antigen preparation, SDS-PAGE and immunoblot

Immunoblotting was performed as described previously [23] for all animals which were included in the study for at least 14 days, i.e. six inoculated animals and all sentinels. Time points of blood sampling are given in Figure 5. Sera were harvested by centrifugation as described above, and stored at −20°C until analyzed.

Statistical methods

SPSS (Version 19.0, IBM Corporation, NY, USA) and Statgratgraphics Centurion XVI (StatPoint Technologies, Inc., VA, USA) were used for statistical evaluation of the data. Normal distribution was tested using Kolmogorov-Smirnov Goodness-of-Fit Test. As the majority of data was not normally distributed, Wilcoxon signed-rank test was carried out to compare baseline data to daily post-inoculation values for the group of inoculated calves. In 'Box and Whisker plots', outlier values (circles) are 1.5–3 times of the length of a box away from the median.

Due to the small number of sentinels (n = 3) a within group approach was not possible. Descriptive data are given as minimum and maximum. Comparison of sentinels to the inoculated group, (per time point or compared to the three inoculated calves surviving to the end of the study) were performed by means of Mann-Whitney rank sum test or by Fisher's Exact test for categorical data (i.e. qualitative PCR analysis of fecal swabs). In case of quantitative rt-PCR of blood, medians of three extractions, each run in duplicate were compared at different time points using Mann-Whitney rank sum test. Generally values of * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ were considered significant.

Supporting Information

Figure S1 Sampling of Exhaled Air. For sampling of exhaled air, calves wore a tightly fitting face mask which was adapted to a Y-shaped inspiratory–expiratory valve. Each animal inspired for one hour through the inspiratory valve (IN) and expired through the expiratory valve (EX) towards the IOM-Sampler. The IOM sampler was assembled with a gelatine filter, which was subsequently DNA extracted and PCR analyzed.
(TIF)

Table S1 Absolute cell counts of white blood cells. The amount of white blood cells is given as median [minimum; maximum] for the inoculated group and as [minimum; maximum] for sentinels (n = 3). Baseline values (mean of data before inoculation) were tested against post inoculation values by means of Wilcoxon signed-rank test. Values of [A] $P \leq 0.05$, [B] $P \leq 0.01$, and [C] $P \leq 0.001$ were considered significant. Arrows indicate significant increase (↑) or decrease (↓) compared to baseline data. dpi days post inoculation. dpc days post contact to inoculated calves. No statistical hypothesis was applied to the sentinel group.
(XLS)

Author Contributions

Conceived and designed the experiments: PR KS. Performed the experiments: CO AR WS ES KS PR. Analyzed the data: CO AR WS ES KS PR. Contributed reagents/materials/analysis tools: CO AR WS KS PR. Wrote the paper: CO AR ES KS PR.

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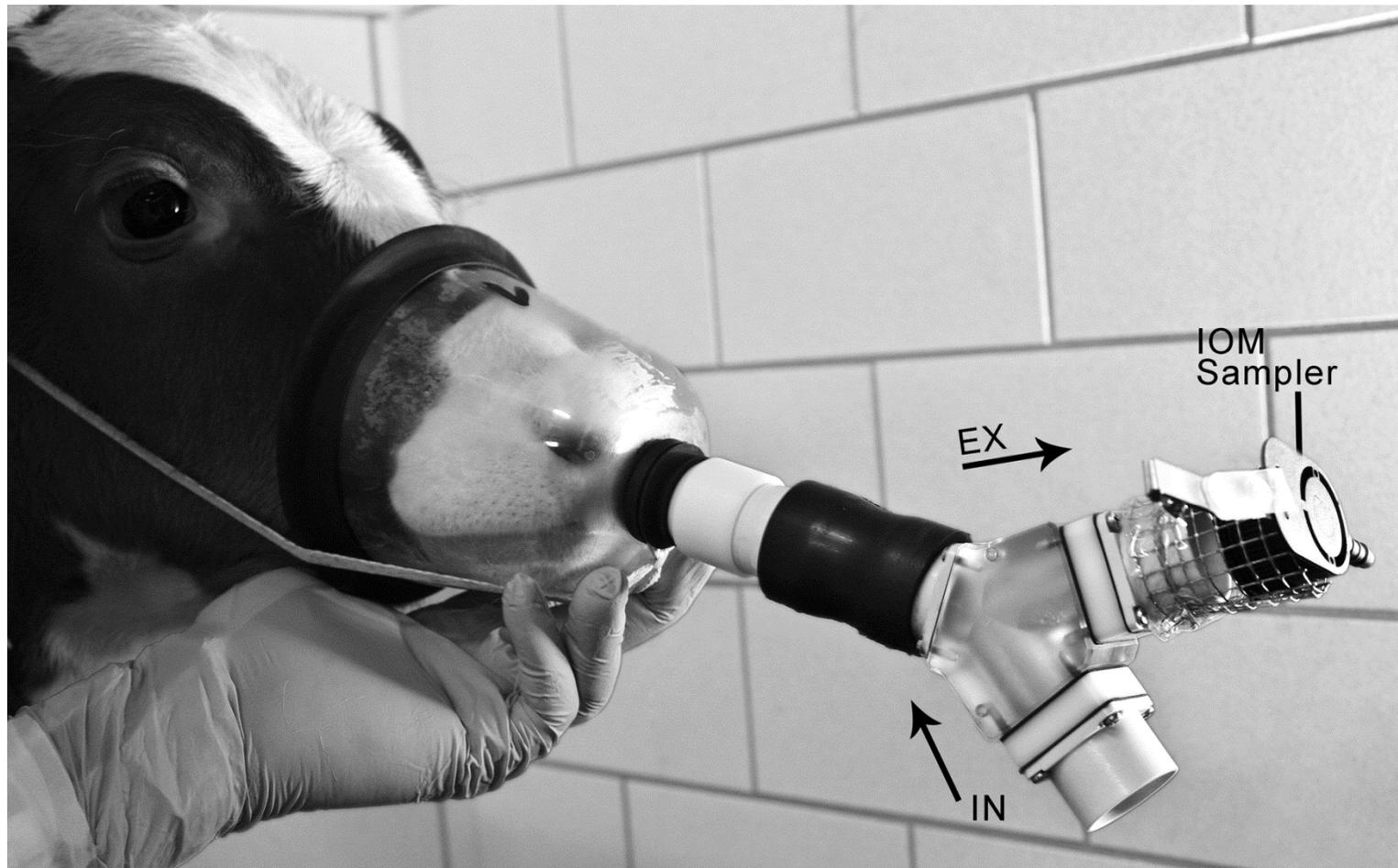
Supplement Figure 1. Sampling of Exhaled Air. For sampling of exhaled air, calves wore a tightly fitting face mask which was adapted to a Y-shaped inspiratory–expiratory valve. Each animal inspired for one hour through the inspiratory valve (IN) and expired through the expiratory valve (EX) towards the IOM-Sampler. The IOM sampler was assembled with a gelatine filter, which was subsequently DNA extracted and PCR analyzed.

Supplement Table 1. Absolute cell counts of white blood cells.

time	Leukocytes [G/L] 10^8 ifu/calf	Sentinels	Banded neutrophils [G/L] 10^8 ifu/calf	Sentinels	Polymorphonuclear neutrophils [G/L] 10^8 ifu/calf	Sentinels	Lymphocytes [G/L] 10^8 ifu/calf	Sentinels	Monocytes [G/L] 10^8 ifu/calf	Sentinels
-7 day	8.39 [6.11; 14.00]	[7.05; 10.85]	0.31 [0.08; 0.84]	[0.40; 0.87]	3.01 [1.64; 4.81]	[1.62; 2.50]	5.17 [2.97; 8.12]	[4.51; 7.05]	0.44 [0.06; 1.07]	[0.42; 0.60]
-4 day	9.3 [7.05; 15.25]	[8.85; 10.35]	0.35 [0.07; 0.92]	[0.27; 0.41]	2.94 [1.49; 6.05]	[1.86; 3.00]	5.81[3.45; 8.61]	[5.96; 6.11]	0.37 [0.09; 1.08]	[0.27; 0.72]
-1 hour	9.8 [6.95; 15.50]	[8.75; 10.05]	0.31 [0.16; 0.56]	[0.18; 0.36]	3.31 [1.56; 5.51]	[1.81; 2.61]	5.52 [3.29; 9.15]	[5.78; 6.83]	0.39 [0.07; 1.17]	[0.30; 0.54]
1 dpi/dpc	12.00 [4.35; 21.10]		**0.57** ↑^{C} [0.10; 1.71]		**4.81** ↑^{A}[0.86; 13.08]		**4.62** ↓^{A} [1.83; 7.56]		**0.70** ↑^{A} [0.00; 1.27]	
2 dpi/dpc	**15.50** ↑^{C} [7.80; 28.25]		**3.54** ↑^{C} [1.09;6.88]		**8.06** ↑^{C} [3.35; 15.26]		**3.70** ↓^{C} [1.79; 7.25]		0.59 [0.11; 1.85]	
3 dpi/dpc	**8.25** ↓^{C} [4.35; 13.30]		**0.69** ↑^{C} [0.29; 2.06]		**1.56** ↓^{C} [0.83; 3.46]		**4.74** ↓^{C} [2.70; 8.13]		0.58 [0.26; 1.33]	
4 dpi/dpc	**7.70** ↓^{C} [4.45; 11.20]	[8.80; 10.65]	0.35 [0.13;0.83]	[0.18; 0.43]	**1.06** ↓^{C} [0.65; 2.69]	[1.94; 3.83]	**5.02** ↓^{B} [3.00; 7.65]	[5.43; 6.16]	0.46 [0.13; 1.90]	[0.35; 0.85]
7 dpi/dpc	8.25 [6.60; 20.50]	[7.95; 11.80]	**0.68** ↑^{B} [0.14;4.92]	[0.32; 0.71]	1.82 [1.19; 7.38]	[1.35; 4.48]	4.85 [3.04; 7.70]	[4.30; 6.49]	0.96* [0.26; 3.10]	[0.12; 0.64]
10 dpi/dpc	10.45 [7.65; 20.25]	[10.80; 19.25]	**0.96** ↑^{A} [0.20; 3.44]	[0.22; 2.70]	3.01 [1.76; 9.72]	[2.59; 10.40]	5.46 [3.67; 8.28]	[4.62; 7.13]	0.61 [0.35; 1.22]	[0.81; 0.96]
14 dpi/dpc	**10.62** ↑^{A} [8.75; 14.40]	[10.95; 18.35]	0.26 [0.18; 0.58]	[0.63; 1.47]	3.61 [2.01; 6.34]	[2.85; 9.91]	5.71 [4.70; 7.60]	[5.14; 6.90]	**1.03** ↑^{A} [0.63; 1.30]	[0.22; 1.47]
17 dpi/dpc	13.65 [12.60; 13.80]	[10.05; 16.20]	0.55 [0.50; 0.82]	[0.46; 1.62]	6.35 [5.19; 6.93]	[3.22; 7.61]	5.73 [4.66; 6.07]	[5.83; 6.50]	0.69 [0.50; 1.09]	[0.30; 0.81]
21 dpi/dpc	9.85 [9.55; 13.15]	[8.95; 13.85]	0.20 [0.13; 0.48]	[0.24; 0.69]	4.53 [0.96; 5.26]	[3.27; 5.68]	6.11 [3.74; 7.23]	[4.30; 7.74]	1.08 [0.53; 1.15]	[0.48; 0.97]
24 dpi/dpc	12.70 [11.05; 13.35]	[9.80; 14.30]	0.25 [0.22; 0.40]	[0.10; 0.61]	4.06 [2.21; 7.08]	[2.25; 5.19]	7.62 [5.07; 7.87]	[2.86; 6.57]	0.51 [0.33; 0.67]	[0.49; 0.98]
28 dpi/dpc	12.50 [11.45; 15.65]	[11.30; 14.10]	0.16 [0.11; 0.25]	[0.00; 0.35]	6.13 [3.66; 6.26]	[4.00; 5.29]	6.98 [4.75; 7.51]	[5.41; 8.44]	1.10 [0.46; 1.38]	[0.35; 1.00]
31 dpi/dpc	13.20 [9.80; 15.20]	[8.50; 13.65]	0.26 [0.15; 0.29]	[0.09; 0.42]	5.94 [4.02; 7.30]	[2.04; 6.06]	5.94 [4.70; 6.23]	[5.61; 8.19]	0.69 [0.53; 1.22]	[0.14; 0.55]
35 dpi/dpc	11.35 [10.15; 17.65]		0.34 [0.10;0.71]		4.97 [3.97; 9.18]		6.00 [4.36; 6.24]		0.68 [0.61; 1.24]	

The amount of white blood cells is given as median [minimum; maximum] for the inoculated group and as [minimum; maximum] for sentinels (n = 3). Baseline values (mean of data before inoculation) were tested against post inoculation values by means of Wilcoxon signed-rank test. Values of ^{A} P≤0.05, ^{B} P≤0.01, and ^{C} P≤0.001 were considered significant. Arrows indicate significant increase (↑) or decrease (↓) compared to baseline data. dpi days post inoculation. dpc days post contact to inoculated calves. No statistical hypothesis was applied to the sentinel group.

2.4   STUDY 4:

Evaluation of pulmonary dysfunctions and acid-base imbalances induced by *Chlamydia psittaci* in a bovine model of respiratory infection

Personal contributions:

- Animal experiments:
    - bronchoscopy and inoculation of the challenge strain or control medium
    - clinical examination of the calves
    - performing lung function measurements
    - participation in the collection of blood, preparation and counting blood cells
    - performing blood gas analysis and haemoxymetry
- Drafting of the manuscript in all parts related to lung function testing
- Preparation of the following figures:
    - Figure 1. Evaluation of dead space volume (Vd) and end-tidal CO_2 concentration using volumetric capnography
    - Figure 2. Respiratory impedance during expiration.
    - Figure 3. Respiratory reactance assessed at 3 Hz during expiration, proximal and distal airway resistance.
    - Figure 4. Variables of the spontaneous breathing pattern.
    - Figure 5. Tidal volume per kg body weight and mean inspiratory airflow.
    - Figure 6. Dead space volume in relation to tidal volume and endtidal concentration of CO_2

Multidisciplinary Respiratory Medicine

ORIGINAL RESEARCH ARTICLE   **Open Access**

Evaluation of pulmonary dysfunctions and acid–base imbalances induced by *Chlamydia psittaci* in a bovine model of respiratory infection

Carola Ostermann[1†], Susanna Linde[1†], Christiane Siegling-Vlitakis[2] and Petra Reinhold[1*]

Abstract

Background: *Chlamydia psittaci* (*Cp*) is a respiratory pathogen capable of inducing acute pulmonary zoonotic disease (psittacosis) or persistent infection. To elucidate the pathogenesis of this infection, a translational large animal model was recently introduced by our group. This study aims at quantifying and differentiating pulmonary dysfunction and acid–base imbalances induced by *Cp*.

Methods: Forty-two calves were grouped in (i) animals inoculated with *Cp* (n = 21) and (ii) controls sham-inoculated with uninfected cell culture (n = 21). For pulmonary function testing, impulse oscillometry, capnography, and FRC (functional residual capacity) measurement were applied to spontaneously breathing animals. Variables of acid–base status were assessed in venous blood using both (i) traditional Henderson-Hasselbalch and (ii) strong ion approach.

Results: Both obstructive and restrictive pulmonary disorders were induced in calves experimentally inoculated with *Cp*. Although disorders in respiratory mechanics lasted for 8–11 days, the pattern of spontaneous breathing was mainly altered in the period of acute illness (until 4 days post inoculation, dpi). Expiration was more impaired than inspiration, resulting in elevated FRC. Ventilation was characterised by a reduction in tidal volume (−25%) combined with an increased percentage of dead space volume and a significant reduction of alveolar volume by 10%. Minute ventilation increased significantly (+50%) due to a compensatory doubling of respiratory rate. Hyperventilatory hypocapnia at 2–3 dpi resulted in slightly increased blood pH at 2 dpi. However, the acid–base equilibrium was additionally influenced by metabolic components, i.e. the systemic inflammatory response, all of which were detected with help of the strong ion theory. Decreased concentrations of albumin (2–10 dpi), a negative acute-phase marker, resulted in a decrease in the sum of non-volatile weak acids (A_{tot}), revealing an alkalotic effect. This was counterbalanced by acidic effects of decreased strong ion difference (SID), mediated by the interplay between hypochloraemia (alkalotic effect) and hyponatraemia (acidic effect).

Conclusions: This bovine model was found to be suitable for studying pathophysiology of respiratory *Cp* infection and may help elucidating functional host-pathogen interactions in the mammalian lung.

Keywords: Acid–base status, Animal model, *Chlamydia psittaci*, Pulmonary function

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Background

Chlamydiae include important respiratory pathogens. In humans, infections with *Chlamydia* (*C.*) *psittaci* are typical examples of pulmonary zoonotic diseases, historically known as psittacosis (parrot fever) or ornithosis (transmitted from poultry). Taking into consideration that chlamydial infections are frequently present in cattle herds [1], the bovine species was also proven to be a natural host for *C. psittaci* [2-6]. Although a bovine model of experimentally induced *C. psittaci* infection was recently introduced by our group [7] we are still far from fully understanding the pathogenesis and consequences of *C. psittaci* infections.

However, addressing open questions by using a large animal model can offer greater clinical translational potential [8] and benefits both, human and veterinary medicine [9]. In this particular model, the respiratory tract as the target organ was chosen because there is still a lack of knowledge regarding the pathophysiology of pulmonary disorders induced by *C. psittaci*. With respect to the clinical outcome, respiratory chlamydial infections are known to be highly variable. Human and avian *C. psittaci* infections may range from clinically silence to acute respiratory and systemic illness. In human medicine, acute 'atypical pneumonia' is a well-known phenomenon in patients that acquired psittacosis due to zoonotic transmission [10-12]. Persistent infection with *C. psittaci*, however, was identified in humans with pulmonary emphysema and/or chronic obstructive pulmonary disease (COPD) as well as in horses with chronic recurrent airway obstruction [13,14] suggesting a pathogenetic link between chronic pulmonary inflammation and persistent infection with chlamydiae. Similar observations were reported for the bovine lung. While an acute outbreak of upper respiratory tract disease in calves was attributed to *Chlamydia* [3], chronic recurrent chlamydial infections in calves remained clinically inconspicuous but were associated with persistent peripheral airway obstruction and chronic pulmonary inflammation [15].

Our defined respiratory model of *C. psittaci* infection in calves offers the possibility to study cause-effect relationships under biologically relevant conditions, i.e. between a pathogen with a clear affinity to the respiratory system and a natural host. This particular study was allocated (i) to identify and to quantify acute respiratory dysfunction induced by *C. psittaci* in a mammalian lung comparable to the human lung in terms of volumes and airflows. To assess lung function parameters typically measured in human medicine, effort-independent and non-invasive pulmonary function techniques common in human pulmonology were applied to conscious and spontaneously breathing calves. (ii) In order to evaluate systemic consequences of pulmonary dysfunctions, acid–base imbalances were quantified and differentiated by assessing metabolites and electrolytes taking both the traditional Henderson-Hasselbalch approach and the new strong ion models [16,17] into account.

The results of this study provide new information regarding the pathophysiology of acute respiratory infection caused by *C. psittaci* with relevance for both veterinary and human medicine taking the ONE HEALTH concept into account.

Methods

Legal conformity and ethics statement

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: 04-002/07). All experiments were done in a containment of biosafety level 2 under supervision of the authorised institutional Agent for Animal Protection. Bronchoscopy to inoculate the pathogen was strictly performed under general anaesthesia. During the entire study, every effort was made to minimise suffering.

Animals

In this prospective and controlled study, 42 conventionally raised calves (Holstein-Friesian breed, male) were included. Animals originated from one farm where each individual calf was fed with maternal colostrum for at least three consecutive meals after birth. In the subsequent period, calves received mixed colostrum until they were purchased at the age of 14 to 28 days weighing between 42.2 and 71.2 kg (56.3 ± 6.8 kg; mean ± SD). The herd of origin was without any history of *Chlamydia*-associated health problems (regularly checked by the National Reference Laboratory for Psittacosis). In the institute calves were reared under standardised conditions (room climate: 18–20°C, rel. humidity: 60–65%) and in accordance with international guidelines for animal welfare. Throughout the entire study, nutrition included commercial whey-based milk replacers and coarse meal. Water and hay were supplied *ad libitum*. None of the given feed contained antibiotics.

Study design

At the age of 42–64 days, 21 calves weighing 73.9 ± 7.4 kg were inoculated with 10^8 inclusion forming units (ifu) of a bovine *C. psittaci* strain (DC 15) per calf, whereas another 21 calves (body weight: 69.3 ± 8.3 kg; mean ± SD) served as controls. Preparation of the challenge strain, procedure of intrabronchial inoculation using a flexible video-bronchoscope and scheme of inoculation at 8 defined localisations in the lung have been described elsewhere [7]. Controls were inoculated with uninfected

Buffalo Green Monkey Kidney cell culture suspended in 6 mL stabilising medium SPGA (containing saccharose, phosphatile substances, glucose and bovine albumin; [18]) using the same methodology.

As illustrated in Table 1, pulmonary function tests (PFT) were performed in 18 *C. psittaci*-infected and in 18 sham-inoculated calves from 7 days *ante inoculation* (a.i.) up to 14 days *post inoculation* (dpi). Body weight (b.w.) was measured individually prior to each lung function test. Prior morning feeding blood samples were collected from the jugular vein starting 1 hour a.i. up to 14 dpi (Table 1). After blood sampling and PFT per day, three calves per group were sacrificed 2, 4, 7, 10, and 14 dpi. Consequently, the number of calves per group decreased continuously from $n = 21$ at the beginnig of the study to $n = 6$ at the end of the study (14 dpi).

Protocol of pulmonary function testing

All PFT measurements were performed in conscious calves breathing spontaneously through a tightly fitting facemask (dead space of facemask: < 100 mL), and in a room with controlled ambient conditions (18–20°C, rel. humidity: 60–65%). After an adaptation period of approximately 5 min, three non-invasive lung function techniques (all JAEGER, CareFusion) were applied consecutively to each animal per time point: (1) impulse oscillometry system to assess respiratory mechanics, (2) volumetric capnography to measure the concentration of exhaled CO_2 against exhaled volume, and (3) re-breathing system to assess FRC (functional residual capacity). All systems were originally produced for human medicine and have been successfully applied to calves previously [19-22]. In each system, a Lilly-type pneumotachograph (mesh resistance: 36 Pa/(L/s)) was used for continuous measurement of airflow (V').

Impulse oscillometry

Complex respiratory impedance, consisting of both respiratory resistance (Rrs) and respiratory reactance (Xrs), was analysed in the frequency range 3 Hz - 15 Hz as described elsewhere [15,22,23]. In addition, proximal airway resistance (Rprox) and distal airway resistance

(Rdist) were calculated [15,21]. Three impulse oscillometry measurements were performed per calf and time point as described by Jaeger et al. (2007) [15]. Duration of one measurement was 60 seconds with 3 test impulses per second (sec), and 32 sampling points after each impulse with a period between two sampling points of 5 ms. Results of three measurements per animal and time point were averaged and these average values were used for further statistical analysis.

Volumetric capnography

Volumetric capnography is the projection of expired CO_2 *versus* expired volume. In a breath-by-breath analysis, 10 exhaled CO_2 curves were registered per calf and time point in triplicate. Dead space volume and end-tidal CO_2 were calculated for each breath as shown in Figure 1. Results of all 30 exhaled CO_2 concentration curves per individual measurement were averaged for further statistical analyses.

Re-breathing method

FRC of the lung was measured by the multiple breath Helium-dilution technique (wash-in), using Helium (He) as test component of the inspired gaseous mixture (inspiratory concentrations: 7–10% He, 35% oxygen, rest nitrogen). The volume of the reservoir (re-breathing) bag was filled with 9 L of the test gas, expired CO_2 was absorbed and O_2 was added when the bag volume decreased. Re-breathing time to perform the test was 2 minutes ± 38 sec (mean ± SD).

All PFT parameters were calculated automatically using the software included in the three systems. For further analysis, the following variables of pulmonary function were taken into account: - complex respiratory impedance in the frequency range 3 Hz – 15 Hz; expressed as respiratory resistance (Rrs) and respiratory reactance (Xrs); each separated for inspiration and expiration, - proximal and distal airway resistance (Rprox, Rdist), - respiratory rate (RR), - time of inspiration and time of expiration (Tin, Tex), - tidal volume (Vt), - volume of minute ventilation (Vmin = Vt∗ RR), - tidal volume in relation to body weight (Vt/kg), - airflow (V') during in- and expiration (V'in, V'ex), - ratio between dead space volume and tidal volume (Vd/Vt), - functional residual capacity of the lung (FRC).

Table 1 Study design

	Animals	−7	−4	−1	+1	+2	+3	+4	+7	+8	+10	+11	+14
		d a.i.	d a.i.	h a.i.	dpi	dpi	dpi	dpi	dpi	dpi	dpi	dpi	dpi
PFT	*C. psittaci*	n = 18	n = 18				n = 18	n = 15	n = 12	n = 9	n = 9	n = 6	n = 6
	Controls	n = 18	n = 18				n = 18	n = 15	n = 12	n = 9	n = 9	n = 6	n = 6
Blood	*C. psittaci*			n = 21	n = 21	n = 21	n = 18	n = 15	n = 12		n = 9		n = 6
	Controls			n = 21	n = 21	n = 21	n = 18	n = 15	n = 12		n = 9		n = 6

d a.i./h a.i., day/hour ante inoculation (baseline); dpi, day post inoculation; PFT, pulmonary function testing.

Figure 1 Evaluation of dead space volume (Vd) and end-tidal CO_2 concentration using volumetric capnography. The rectangle **ABCD** represents the same area as the CO_2-volume-area under the capnographic curve (X) while **BC** represents a constant CO_2 concentration that equals to end-tidal CO_2. In this case, areas p and q are of the same size, and Vd according to Bohr represents the volume without CO_2.

Protocol of acid–base assessment

Jugular venous blood was collected (i) anaerobically in 2 mL polypropylene syringes with lyophilised electrolyte-balanced heparin (PICO 50, Radiometer Copenhagen) for immediate analysis and (ii) in 9.0 mL syringes (S-Monovette, Sarstedt AG & Co) for serum production.

Analysis of jugular venous blood

Heparinised blood samples were transported to the laboratory at room temperature and analysed within 10 min of collection using a combined blood-gas- and electrolyte-analyser (ABL 725, Radiometer), working with manufacturer's standard electrodes. The following factors were measured in the venous (v) blood: pH(v), partial pressure of CO_2 (pCO$_2$(v)), and the plasma concentrations of sodium (cNa^+), potassium (cK^+), calcium (cCa^{2+}) and chloride (cCl^-) by ion-selective potentiometry. Plasma concentrations of glucose (cGlucose) and L-lactate (cL-lactate) were measured in the same equipment using enzymatic electrodes.

Serum biochemical analysis

Serum was harvested by centrifugation (3120 g for 15 min at 15°C) and stored at –20°C until analysed. Serum concentrations of total protein (biuret method) and inorganic phosphate (ammonium-molybdate) were measured spectrophotometrically (Cobas 6000, Roche/Hitachi). Capillary electrophoresis was performed to determine concentrations of albumin and globulin, as well as the globulin spectra ('Capillarys2', Sebia).

Calculated acid–base variables

The following variables were calculated using proprietary equations included in the software of the blood-gas- and electrolyte-analyser: blood pH and pCO$_2$ (v), each corrected for the actual body temperature (BT) of the animal as measured rectally via digital thermometer

before each blood collection (pH $_{BT}$, pCO$_2$(v) $_{BT}$) and traditional variables of acid–base balance, i.e. bicarbonate ($cHCO_3^-$), standard bicarbonate ($cHCO_3^-$(st)), actual base excess (cBase), and standard base excess (cBase (Ecf)). The Henderson-Hasselbalch approach quantifies the unmeasured anion concentration by calculating the anion gap (AG) as follows [24-26]:

$$AG = (cNa^+ + cK^+) - (cCl^- + cHCO_3^-) \quad (1)$$

The strong ion model [16] simplified by Constable [17] provides a novel insight into the pathophysiology of mixed acid–base disorders. This approach is based on the assumption that plasma pH is a dependent variable and as such its value is determined by three independent factors: pCO$_2$, the strong ion difference (SID), and the sum of non-volatile weak acids (A_{tot}) [27]. SID is the difference between the total sum of all strong cation concentrations and the sum of all strong anion concentrations. Strong ions are those that dissociate completely at physiologic pH, existing either as strong cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) or strong anions (principally Cl^- and L-lactate). The most important measured strong ions are Na^+, K^+ and Cl^-, calculated in SIDm$_3$ [28,29].

$$SIDm_3 [mmol/L] = cNa^+ + cK^+ - cCl^{-a} \quad (2)$$

All other electrolytes (measurable or unmeasurable) are involved in the strong ion gap (SIG), the difference between remaining unmeasured cations ($cUC = Ca^{2+}$, Mg^{2+}, and in very low amounts - and therefore negligible - micronutrients e.g. Cu $^{2+}$, Fe^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}, Mn^{2+}) and unmeasured anions ($cUA = SO_4^{2-}$, lactate$^-$, beta-hydroxybutyric acid$^-$, acetoacetic acid$^-$) [30].

$$SIG = cUC - cUA \quad (3)$$

Re-arranging gives:

$$SID = SIDm_3 + SIG \; or \quad (4)$$

$$SID = (cNa^+ + cK^+ + cUC) - (cCl^- + cUA) \quad (5)$$

The SID represents the net charge which must be balanced by charges on the weak acids in the solution for electrical neutrality to be maintained [17]. Acid total (A_{tot}) represents the total amount of non-volatile weak acid present in the system. The law of conservation of mass means that the total amount of A_{tot} in the system must be constant [31]. In plasma, the major non-volatile weak acids present are plasma proteins and phosphates [32,33]. In calves, however, the albumin concentration (cAlbumin) is most important and can be used alone as an estimate of A_{tot} in plasma, the results of which were used to calculate and prepare a gamblegram [34]. A_{tot} and SIG were calculated from both total protein concentration (cProtein total) and cAlbumin and temperature

corrected blood pH using the following equations and pK_a data for calves [25,26,28]:

$$A_{tot\ (Alb)}[mmol/L] = 0.622 \times cAlbumin\ [g/L] \qquad (6)$$

$$A_{tot\ (Prt)}[mmol/L] = 0.343 \times cProtein\ total\ [g/L] \qquad (7)$$

$$SIG = A_{tot}/\left(1 + 10^{(pKa-pH)}\right) - AG \qquad (8)$$

with $pK_a = 7.08$ [$K_a = (0.84 \pm 0.41) \times 10^{-7}$].

$$SIG_{(Alb)}[mmol/L] = cAlbumin\ [g/L]$$
$$\times \left(0,622/\left(1 + 10^{(7,08-pH)}\right)\right) - AG \qquad (9)$$

$$SIG_{(Prt)}[mmol/L] = cProtein\ total\ [g/L]$$
$$\times \left(0,343/\left(1 + 10^{(7,08-pH)}\right)\right) - AG \qquad (10)$$

Statistical methods

Normally distributed data are presented as mean and standard deviation (SD) while data with unknown or non-normal distribution are given as median and range. The analysis of lung function data was performed using PASW (Predictive Analyse Software) Statistics 17.0 (IBM Corporation) and StatgraphicsPlus 4.0 (StatPoint Technologies, Inc.). To compare multiple data with normal distribution, multifactorial analysis of variance (ANOVA) was used with Bonferroni's multiple comparison procedure as *post hoc* test. To compare two unpaired samples, i.e. differences between two groups at one time point, the unpaired *t*-test was used for normally distributed data (comparison of means) while the Mann-Whitney-Wilcoxon *W* test was used for data with unknown or non-normal distribution (comparison of medians).

For analysis of acid–base variables, Matlab (Matlab R2007a, Version 7.4.0.287; The MathWorks, Inc.) was used. Significant changes within each group compared to baseline data were assessed by Wilcoxon signed rank test, while Mann-Whitney-Wilcoxon *W* test was used to identify significant differences between groups at a given time point [35,36]. Since the given p are equal or less than 0.05, there is a statistically significant difference at the 95.0% confidence level. All confidence levels (p) are given with the results.

Results

Respiratory mechanics

Before challenge, complex respiratory impedance assessed by impulse oscillometry was comparable between groups and reproducible within each group (Figure 2; baseline data). After inoculation of *C. psittaci*, Xrs - representing the elastic properties of the lung - decreased significantly at all frequencies (3–15 Hz) compared to control calves. Coevally, respiratory resistance at low frequencies (Rrs ≤ 5 Hz) increased significantly. This effect was stronger during expiration (Figure 2) than inspiration (data not shown). Figure 2 illustrates frequency-dependent courses of respiratory impedance assessed during expiration at selected time points indicating that significant differences in Xrs between groups lasted for at least 11 dpi. Numeric data (given in Table 2 for selected time points corresponding to Figure 2) and within-group analysis over time revealed a continuous growth-related increase in Xrs within the observation period of 21 days in controls that was clearly absent in calves exposed to *C. psittaci*. Instead, Xrs decreased significantly 3 dpi compared to intra-group baseline data before inoculation of *C. psittaci* (Table 2).

Proximal and distal airway resistances (Rprox, Rdist) are given in Figure 3A-B. Calves exposed to *C. psittaci* showed significantly elevated Rprox and Rdist data compared to controls as well as compared to intra-individual baseline data. Interestingly, the increase in Rdist was larger (146%; mean of intra-subject difference between baseline a.i. and 3 dpi) compared to the increase in Rprox (116%), but duration of significantly-elevated airway resistance lasted longer in proximal airways (until 8 dpi) compared to distal airways (4 dpi).

Respiratory pattern

The pattern of spontaneous breathing was characterised by the variables given in Figure 4. In controls, tidal volume (Vt) increased continuously due to growth over time. Averaged respiratory rate (RR) was 28 breathing cycles per minute and did not change during the study. Thus, minute ventilation (Vmin) increased slightly (but not significantly) from 16.5 L (mean –7 days) to 19.4 L (mean 14 dpi). In calves exposed to *C. psittaci*, significant alterations in the respiratory pattern were seen 3–4 dpi compared to baseline data, characterised by a reduction of Vt by 25% and a doubling of RR. Consequently, Vmin increased to about 150% of baseline. These changes were reversed by 10 dpi and, compared to intra-group data before challenge, Vt was significantly increased while RR was significantly decreased.

To eliminate any influence of growth from volumes of respiration, Vt was additionally calculated per kg b.w. (Figure 5A). In calves challenged with chlamydiae, minima of 6.9 and 6.7 mL/kg (means) were measured at 3 and 4 dpi, while averaged Vt per kg b.w. ranged between 8.7 mL/kg and 10.1 mL/kg in controls and in challenged calves at other time points.

With doubling of RR in *C. psittaci*-exposed calves, time of inspiration (Tin) and time of expiration (Tex) were significantly shorter compared to baseline data before challenge (Tin: 0.44 sec 3 dpi compared to 1.0 sec at baseline; Tex: 0.56 sec 3 dpi compared to 1.2 sec at baseline; means).

Figure 2 Respiratory impedance during expiration. Complex respiratory impedance during expiration is expressed as resistance (R) and reactance (X). d a.i., days after inoculation; dpi, days post inoculation; Rrs ex, respiratory resistance during expiration; Xrs ex, respiratory reactance during expiration. Open symbols: control calves. Filled symbols: calves inoculated with *C. psittaci*. Data are expressed as means per group. Significant differences between groups are indicated by *($p < 0.05$), **($p < 0.01$), or ***($p < 0.001$) [unpaired *t*-test]. For reasons of visual clarity, data are expressed as means per group only. Numeric data presenting standard deviation and results of within-group comparison in addition are given in Table 2.

The mean ratio Tex:Tin, however, ranged within each group between 1.2 and 1.3 without changing significantly in any group at any time point (data not shown in detail).

Airflows assessed during inspiration and expiration (V'in, V'ex) revealed that mean inspiratory flow was physiologically higher (0.8 L/s) compared to expiratory flow (0.6 L/s) at baseline in both groups. Due to inoculation of chlamydiae, increases in airflows at time points 3 dpi and 4 dpi were comparable during in- and expiration (about 170% at 3 dpi and 140% at 4 dpi compared to baseline data). Data are only depicted for V'in (Figure 5B).

Alveolar ventilation
The mean ratio between dead space volume and tidal volume per breath (Vd:Vt, Figure 6A) was about 0.54 in controls as well as in calves before challenge with chlamydiae. Three and 4 dpi of *C. psittaci*, the percentage of Vd per breath increased to 64% (Vd:Vt = 0.64) in average indicating that alveolar volume per breath was reduced by about 10%. FRC (i.e. the volume present in the lung at end of spontaneous expiration) was significantly increased by about 500 mL in the *C. psittaci* group. While FRC was 3.0 L (40.5 mL/kg b.w.) at baseline, it was elevated to 3.5 L (45.7 mL/kg b.w.) 3 dpi after inoculation of chlamydiae (ANOVA, LSD, $p < 0.01$). In control animals neither a significant increase of FRC over time was seen nor a decrease of end-tidal CO_2. End-tidal (i.e. alveolar) concentration of CO_2 in exhaled breath decreased significantly from about 5 Vol% (baseline data in both groups) to 4.2 or 4.4 Vol%,

Table 2 Respiratory impedance assessed during expiration in the frequency range 3–15 Hz in calves either challenged with 10^8 inclusion forming units of *C. psittaci* or sham-inoculated controls

		−7 d a.i.	−4 d a.i.	3 dpi	8 dpi	11 dpi	14 dpi
		n = 18 per group	n = 18 per group	n = 18 per group	n = 9 per group	n = 6 per group	n = 6 per group
Xrs ex 3 Hz	*C. psittaci*	−0.018	−0.021	**−0.038**	**−0.040**	**−0.018**	−0.010
		[0.015]	[0.012]	[0.031]	[0.026]	[0.019]	[0.017]
	Controls	−0.026	−0.028	−0.014	- 0.009	0.006	0.004
		[0.026]	[0.028]	[0.017]	[0.019]	[0.015]	[0.011]
Xrs ex 5 Hz	*C. psittaci*	−0.003	−0.009	**−0.045 ↓**	**−0.034**	0.000	0.007
		[0.018]	[0.015]	[0.041]	[0.032]	[0.023]	[0.022]
	Controls	−0.015	−0.015	0.001	−0.008	0.025	0.026
		[0.035]	[0.032]	[0.021]	[0.025]	[0.022]	[0.020]
Xrs ex 10 Hz	*C. psittaci*	0.041	0.027	**−0.031 ↓**	**0.005**	0.045	0.047
		[0.024]	[0.025]	[0.050]	[0.032]	[0.023]	[0.041]
	Controls	0.023	0.032	0.046	0.051	0.077	0.075
		[0.045]	[0.038]	[0.031]	[0.033]	[0.033]	[0.032]
Xrs ex 15 Hz	*C. psittaci*	0.058	0.049	**0.002 ↓**	**0.037**	**0.064**	0.066
		[0.028]	[0.026]	[0.046]	[0.023]	[0.018]	[0.035]
	Controls	0.047	0.050	0.068	0.067	0.089	0.093
		[0.034]	[0.036]	[0.032]	[0.030]	[0.017]	[0.021]
Rrs ex 3 Hz	*C. psittaci*	0.333	0.334	**0.387**	0.355	0.321	0.329
		[0.051]	[0.038]	[0.103]	[0.059]	[0.052]	[0.074]
	Controls	0.335	0.339	0.314	0.303	0.284	0.279
		[0.068]	[0.060]	[0.044]	[0.059]	[0.038]	[0.031]
Rrs ex 5 Hz	*C. psittaci*	0.318	0.319	**0.366**	0.329	0.306	0.319
		[0.047]	[0.036]	[0.096]	[0.046]	[0.048]	[0.071]
	Controls	0.337	0.318	0.300	0.291	0.276	0.272
		[0.060]	[0.050]	[0.040]	[0.056]	[0.031]	[0.033]
Rrs ex 10 Hz	*C. psittaci*	0.319	0.314	0.326	0.306	0.310	0.327
		[0.042]	[0.031]	[0.079]	[0.038]	[0.035]	[0.061]
	Controls	0.328	0.314	0.302	0.302	0.299	0.296
		[0.044]	[0.038]	[0.034]	[0.047]	[0.014]	[0.038]
Rrs ex 15 Hz	*C. psittaci*	0.336	0.321	0.325	0.319	0.330	0.343
		[0.041]	[0.027]	[0.074]	[0.043]	[0.040]	[0.055]
	Controls	0.344	0.339	0.327	0.324	0.335	0.324
		[0.041]	[0.040]	[0.032]	[0.035]	[0.020]	[0.035]

d a.i., days *ante inoculation*; dpi, days post inoculation; Rrs ex, respiratory resistance during expiration; Xrs ex, respiratory reactance during expiration. Data are given as mean [standard deviation]. Significant difference between groups at the given time point are highlighted in bold (unpaired *t*-test, p ≤ 0.05). ↓ indicates a significant decrease compared to baseline data within one group (ANOVA, *post hoc* test: Bonferroni's multiple comparison procedure, p ≤ 0.01).

respectively, at 3–4 dpi in calves exposed to *C. psittaci* (Figure 6B).

Blood gases and acid–base variables
During the acute phase of infection and the development of respiratory illness, pCO_2 in venous blood (pCO_2 (v) $_{BT}$) of calves inoculated with *C. psittaci* was significantly reduced 2–3 dpi while blood pH was significantly increased 2 dpi compared to baseline values within the challenge group and compared to non-infected control calves at the same time points (Additional file 1). In the same time period, both $cHCO_3^-$ (st) and cBase (Ecf)) were significantly elevated 2 dpi while all bicarbonate and base excess data ($cHCO_3^-$, $cHCO_3^-$(st), cBase, cBase (Ecf)) were significantly reduced one day later (3 dpi).

After a significant rise of $SIDm_3$ at 1 dpi, all strong ion differences ($SIDm_3$, $SIDm_4$, $SIDm_5$) also dropped significantly down at 3 dpi compared to baseline data as well

Figure 3 Proximal and distal airway resistance. Calves were either challenged with 10^8 inclusion forming units of *C. psittaci* or sham inoculated (controls). Rdistal, distal airway resistance; Rproximal, proximal airway resistance. Data are expressed as Box-and-Whisker Plots representing lower and upper quartile values (box) with median and mean (+). Whiskers extend from each end of the box to the most extreme values within 1.5 interquartile ranges. Outliers are data beyond the ends of the whiskers. In calves inoculated with *C. psittaci*, # indicates a significant difference at the given time point compared to controls (Mann-Whitney-Wilcoxon *W* test) at a probability level of $p \leq 0.05$. In controls, arrows indicate significant decreases ($\downarrow$) at the given time point compared to data assessed within the group -7 days *ante inoculationem* (a.i.) at a probability level of $p \leq 0.05$ (ANOVA, *post hoc* test: Bonferroni's multiple comparison procedure).

as compared to $SIDm_3$, $SIDm_4$, $SIDm_5$ data assessed in controls (Additional file 2). Calculated anion gap (AG) was significantly elevated within the first 3 days after *C. psittaci* challenge while strong ion gap (SIG) was significantly reduced between 1–4 dpi (Additional file 1). The latter effect was more prominent in SIG $_{(Alb)}$ compared to SIG $_{(Prt)}$ due to significantly diminished A_{tot} $_{(Alb)}$ values between 2–10 dpi compared to baseline data before challenge and compared to non-infected controls (Additional file 3). During the resolution period of clinical signs (about one week after inoculation), $pCO_2(v)$ $_{BT}$ was significantly increased in *C. psittaci* infected calves compared to baseline data before challenge (7 dpi, 10 dpi) and compared to data obtained from non-infected calves (7 dpi) without any marked changes in blood pH. Within the period 7–10 dpi, $cHCO_3^-$, $cHCO_3^-$ (st), cBase, and cBase (Ecf) were significantly increased compared to baseline data and compared to data obtained from control calves (Additional file 1). $SIDm_3$, $SIDm_4$ and $SIDm_5$ started to increase significantly within the infected group at 7 dpi, and were even higher 10–14 dpi (significant in comparison to both baseline values and control calves) (Additional file 2). While the significant reduction in A_{tot} $_{(Alb)}$ lasted until 10 dpi, A_{tot} $_{(Prt)}$ was slightly increased 10–14 dpi in calves exposed to the

pathogen (Additional file 3). Thus, in comparison to non-infected calves, SIG $_{(Prt)}$ of calves exposed to *C. psittaci* was higher at 7 dpi while SIG $_{(Alb)}$ was lower at 10 dpi (Additional file 1).

Serum biochemical analysis and protein electrophoresis
Blood concentration of inorganic phosphate of calves infected with *C. psittaci* decreased significantly between 1–10 dpi (compared to baseline data) with minima between 2–7 dpi that were also significantly lower than in controls (Additional file 3).

Serum albumin concentration was significantly reduced from 2 dpi till 10 dpi in infected calves (compared to baseline values and compared to control calves) with a maximal reduction observed at 4–7 dpi. In contrast, concentration of serum globulins increased significantly over time after experimentally induced infection (different time courses of single globulin fractions as given in Additional file 3). Consequently, the concentration of serum proteins measured in calves infected with *C. psittaci* was, compared to baseline data, significantly decreased at 2–3 dpi and significantly increased at 10–14 dpi. The ratio between albumin and globulin in the infected group, however, was significantly decreasing in the course of the study (Additional file 3).

Figure 4 Variables of the spontaneous breathing pattern. Calves were either challenged with 10^8 inclusion forming units of *C. psittaci* or sham inoculated (controls). RR, respiratory rate; Vmin, volume of minute ventilation; Vt, tidal volume. Data are expressed as Box-and-Whisker Plots representing lower and upper quartile values (box) with median and mean (+). Whiskers extend from each end of the box to the most extreme values within 1.5 interquartile ranges. Outliers are data beyond the ends of the whiskers. In calves inoculated with *C. psittaci*, # indicates a significant difference at the given time point compared to controls (Mann-Whitney-Wilcoxon *W* test) at a probability level of p ≤ 0.01. Arrows (↑ or ↓) indicate significant increases or decreases, respectively, at the given time point compared to data *ante inoculationem* (a.i.) within the group (ANOVA, *post hoc* test: Bonferroni's multiple comparison procedure) at a probability level of p ≤ 0.05 (grey) or p ≤ 0.01 (black).

### cGlucose, cL-lactate and electrolytes

Concentration of blood glucose was significantly reduced during the first 7 days after *C. psittaci* challenge with a lowest group median seen at 3 dpi. In contrast, cL-lactate in venous blood was significantly increased within the period 1–4 dpi with a highest group median seen at 2 dpi. Changes of cGlucose and cL-lactate in *C. psittaci*-inoculated calves were significant in comparison to both baseline data and control calves (Additional file 2). With respect to electrolytes in the peripheral blood, the concentrations of calcium, sodium, and chloride were slightly, but significantly, reduced in inoculated calves after challenge (cCa^{2+}: 2–4 dpi, cNa$^+$: 2–7 dpi, cCl$^-$: 2–14 dpi). Potassium concentration (cK$^+$) was, compared to baseline values and compared to control calves, significantly increased at 1 dpi and significantly decreased at 4 dpi after inoculation of *C. psittaci* (Additional file 2).

Strong ion approach of acid–base disorders

Figure 7 provides an example of the interplay of the aforementioned components during the acute phase (3 dpi) of the disease course. Decreases of cNa$^+$ and cCl$^-$ influenced cHCO$_3^-$ in an opposite manner. Similarly, the decrease of SID (acidotic effect) counterbalanced the decrease of A$_{tot}$, (alkalotic effect), thus effects of these parameters on blood pH (Additional file 1) appeared minimal.

Discussion

This prospective controlled study was undertaken to evaluate pulmonary dysfunctions induced by *C. psittaci* in the mammalian lung along with systemic acid–base alterations and imbalances in electrolytes and metabolites. A bovine model was exploited for the following reasons. (i) The lungs of species lacking collateral airways (predominantly cattle and pigs) are extremely sensitive to functional consequences of any changes in respiratory mechanics [37].

Figure 5 Tidal volume per kg body weight and mean inspiratory airflow. Calves were either challenged with 10^8 inclusion forming units of *C. psittaci* or sham inoculated (controls). b.w, body weight; Vt, tidal volume. Data are expressed as Box-and-Whisker Plots representing lower and upper quartile values (box) with median and mean (+). Whiskers extend from each end of the box to the most extreme values within 1.5 interquartile ranges. Outliers are data beyond the ends of the whiskers. In calves inoculated with *C. psittaci*, # indicates a significant difference at the given time point compared to controls (Mann-Whitney-Wilcoxon *W* test) at a probability level of $p \leq 0.01$. Arrows (↑ or ↓) indicate significant increases or decreases, respectively, at the given time point compared to data *ante inoculationem* (a.i.) within the group (ANOVA, *post hoc* test: Bonferroni's multiple comparison procedure) at a probability level of $p \leq 0.01$.

Thus, the bovine lung does present an advantageous model to assess pathophysiological consequences of both airway obstructions and pulmonary restrictions. (ii) This large animal model offers the great potential to perform non-invasively and almost painless long-term studies allowing a simultaneous within-subject approach of functional changes of both the organ and the systemic level.

In this particular model signs of acute respiratory illness were maximal 2–4 dpi and did not last longer than one week after challenge [38]. Although the severity of illness was mainly driven by respiratory signs, additional systemic reaction, similar to atypical pneumonias in human medicine [39] were observed. The most striking symptoms included dry cough, tachypnea, fever, reduced appetite, and tachycardia [38].

It was also shown earlier that in this model respiratory insufficiency during the acute phase (2–3 dpi) is characterised by hypoxaemia, linked to reduced haemoglobin oxygen saturation, increased alveolar-arterial oxygen partial pressure difference and pulmonary shunt [40]. Due to long time course of the present study the risk of secondary infection after catheterising a representative arterial vessel was not taken, as anticipated advantages with respect to the evaluation of acid–base disorders and PFTs were thought to be minimal.

Pulmonary dysfunctions

Pulmonary function techniques from human medicine were applied to spontaneously breathing animals with body weights comparable to adult humans. Thus basic parameters of pulmonary functions (for example airflows and lung volumes) were more comparable and transferable to human patients compared to results obtained from murine models.

Furthermore, a functional differentiation between airway resistance and tissue mechanics of the lung was possible in the present study, while assessment of compliant properties of the lung in mice would require medical or surgical treatment [41,42]. During the acute period of illness (3–4 dpi) due to *C. psittaci* infection, the pattern of breathing was characterised by a significant decrease in tidal volume and a significant increase in both respiratory rate and airflows (clinically seen as short and rapid breathing cycles, i.e. dyspnoea). Per breath, dead space volume was significantly elevated while alveolar volume was reduced by about 10% indicating alveolar hypoventilation that was confirmed by a decreasing end-tidal concentration of CO_2. However, global hypoventilation was not confirmed. In contrast, partial pressure of CO_2 in peripheral blood decreased, too, indicating hypocapnia due to global hyperventilation. The latter was caused by an increase in

Figure 6 Dead space volume in relation to tidal volume and end tidal concentration of CO_2. Calves were either challenged with 10^8 inclusion forming units of *C. psittaci* or sham inoculated (controls). Vd, dead space volume; Vt, tidal volume. Data are expressed as Box-and-Whisker Plots representing lower and upper quartile values (box) with median and mean (+). Whiskers extend from each end of the box to the most extreme values within 1.5 interquartile ranges. Outliers are data beyond the ends of the whiskers. In calves inoculated with *C. psittaci*, # indicates a significant difference at the given time point compared to controls (Mann-Whitney-Wilcoxon *W* test) at a probability level of p < 0.01. Arrows (↑ or ↓) indicate significant increases or decreases, respectively, at the given time point compared to data *ante inoculationem* (a.i.) within the group (ANOVA, *post hoc* test: Bonferroni's multiple comparison procedure) at a probability level of p ≤ 0.05 (grey) or p ≤ 0.01 (black).

Figure 7 Gamblegram at 3 dpi. Calves were either challenged with 10^8 inclusion forming units of *C. psittaci* or sham inoculated (controls). The thickness of the arrow indicates the strengths of the influence. Na^+ decreased remarkably (8 mmol/L) 3 dpi in *C. psittaci* infected calves, therefore HCO_3^- decreased; but Cl^- decreased clearly (6 mmol/L) too, therefore HCO_3^- should increase. Both influences together Na^+ and Cl^- lead to a minimal decrease of HCO_3^-. Similar reaction were seen in SID and A_{tot}, both decreased (SID = acidotic, A_{tot} = alkalotic), that is why no change occurred in venous pH.

minute ventilation by 50% due to the strong increase in respiratory rate. The elevated minute ventilation was most likely the attempt to compensate for hypoxaemia induced by *C. psittaci* infection as shown previously [40].

Alterations in respiratory mechanics after inoculation of *C. psittaci* included both obstructive and restrictive components and lasted longer than the clinically visible changes in the pattern of respiration. Restriction was assessed by decreasing respiratory reactance (Xrs) which indicates limitations in elasticity or compliance of the lung-thorax system [23]. This loss in elasticity was predominantly a result of inflammatory reactions, such as cell infiltration, accumulations of fibrin and protein-rich fluid or signs of regeneration described for this model in detail elsewhere [7,43]. In the present study, the statistically significant decrease of Xrs at all frequencies (3–15 Hz) in *C. psittaci* challenged calves continued until 11 dpi. Thus, the duration of reduced compliant properties of lung tissue exceeded the presence of acute clinical signs [38] by about one week.

In the acute phase of respiratory illness, the loss of pulmonary compliance was most likely accompanied by stiffness of the peripheral respiratory system due to small airways narrowing or constriction [44]. Indeed, peripheral airflow was limited in calves inoculated with *C. psittaci* compared to control calves during the acute phase of disease which was indicated by an increase of Rrs ≤ 5 Hz and Rdist (significant at 3 dpi and at 3–4 dpi, respectively). The negative frequency dependence of Rrs, i.e. an increase only at low frequencies (Rrs < 5 Hz), is a valid diagnostic tool to identify peripheral airways obstruction in both humans [45] and calves [21]. In addition to obstruction in distal airways, calves experimentally challenged with *C. psittaci* also suffered from obstruction in central or upper airways as indicated by an increase Rprox. These finding are in good agreement with reports in literature associating chlamydial infections in calves with both upper respiratory tract disease [3] and obstruction of peripheral airways [15]. Moreover, it was also shown in experimentally *C. suis* challenged pigs that peripheral airways obstruction during the acute phase (3 dpi) were followed by upper airways obstruction (at 7 dpi). To our knowledge lung function data of humans suffering from acute chlamydial pneumonia are not available, but taking these findings together obstruction of the upper and lower respiratory tract might probably also be involved in pathogenesis of acute chlamydial pneumonia in humans.

In parallel to the presence of airways obstruction, FRC increased significantly at 3–4 dpi. Baseline data of about 40 mL/kg b.w. measured in this study in calves are in good agreement with data reported for the healthy bovine lung in adult cows (38.6 ± 3.1 mL/kg; [46]). After *C. psittaci*-infection, FRC increased significantly by 17%

to 45.7 mL/kg b.w. (3 dpi) which is moderate compared to FRC data reported in cows with severe bronchiolitis and an expanded lung field (56.5 ± 7.7 mL/kg; [47]). In calves, due to the lack of collateral airways, the presence of fibrin, inflammatory cells, detritus and protein rich fluid in the airways and/or alveoli during the acute phase of this model [43] resulted in narrowed peripheral airways which can easily result in the development of trapped air. In the present model the increase in FRC was transient, thus hyperinflation or over-distension of alveoli is indicated rather than the presence of emphysema [48]. As over-distension might reduce the recoil of elastic fibers it is likely that hyperinflation also contributed to reduced lung compliance described above. An increase of FRC was also reported for *C. suis* infected swine [49]. Radiographically-impressive distension of the lung with air is found in cases of *C. trachomatis* pneumonia in children, which despite the mild respiratory symptoms in infancy is associated with obstructive limitations up to 7–8 years after hospitalisation (i.e. increased FRC, forced and peak expiratory flow rates) [50,51]. A long-term impairment of lung function and structure after chlamydial infection was also shown for naturally *Chlamydia*-infected calves [15] and experimentally challenged mice [52]. In human medicine, asthma is a common chronic inflammatory disease of the airways, and the involvement of *C. pneumoniae* in asthma pathogenesis is still largely discussed [53,54].

None of the lung functions assessed in control animals was significantly influenced by intrabronchial inoculation of BGM cell suspension. Pulmonary function data in control calves revealed physiological changes over time due to lung growth and development (gain in body weight during the study was 0.6 kg per day in average). In control calves Xrs increased significantly over time, displaying increasing compliant properties of lung and thorax. These findings are in line with fundamental understanding from the very beginning of veterinary pulmonology showing that lungs are easier to stretch with enhanced body or lung size [55,56]. It has been shown for growing calves that Xrs increased with increasing body weight [57]. During the period of pulmonary maturation (until a body weight of about 300 kg [58,59]) bronchiolar diameters were also shown to increase [60], resulting in decreased airway resistance.

Acid–base imbalances

Compared to other studies [28,61-63] the control values of pH, pCO_2, HCO_3^-, base excess and $A_{tot\ (Alb)}$ or $A_{tot\ (Prt)}$ are in the ranges reported whereas AG and $SIDm_{3,4}$ were lower and $SIG_{(Alb)}$ or $SIG_{(Prt)}$ were higher in absolute values than those described in literature. In calves experimentally infected with *C. psittaci*, most of the effects assessed in venous blood were slight or moderate in

amplitude and were mostly related to either the acute phase (2–4 dpi) or the resolution phase (7–10 dpi) after inoculation of the pathogen. Nevertheless the investigated parameters accurately assessed the influence of *C. psittaci* on the acid–base balance of the host organism.

Partial pressure of CO_2 provides information regarding ventilation or respiratory component of acid–base balance in the Henderson-Hasselbalch equation as well as in the strong ion approach. Despite no access to arterial blood in this study, venous blood was informative enough identifying venous hypocapnia ($pCO_2(v)\downarrow$) 2–3 dpi as a result of hyperventilation (the latter was proved by pulmonary function testing). In general, hyperventilation can be caused primary by stimulation of pulmonary nociceptive receptors related to pulmonary disease and impairment of gas exchange (hypercapnia, hypoxaemia) or secondary for recovery from metabolic acidosis [64]. Natural compensatory mechanisms probably never overcompensate, and as a general rule, the pH will vary in a direction similar to the primary component disorder [65]. Therefore, it is more plausible that hyperventilation occurred to compensate for hypoxaemia, a known consequence of experimentally induced pulmonary disease in this model as reported previously by our group [40]. As a result, blood pH increased slightly 2 dpi. Decreases in both $cHCO_3^-$ and $cHCO_3^-$(st) at 3 dpi, together with decreased cBase and cBase (Ecf), can traditionally be interpreted as compensatory mechanisms to return to normal pH. In the period 7–10 dpi, $cHCO_3^-$, cBase and cBase (Ecf) increased but pH was not influenced. In conformity with the more modern approach, $cHCO_3^-$, cBase and cBase (Ecf) are described as dependent (strong ion) variables that cannot be regulated independently of pCO_2, while SID and A_{tot} are independent variables [66]. Only the independent variables influence the system and they are not influenced by the system. A_{tot} and SID reflect the metabolic system. $A_{tot\ (Alb)}$ decreased 2–10 dpi and produced an alkalotic effect caused by hypo-albuminaemia. Albumin is a negative acute-phase protein, i.e. a marker of inflammation. This finding supplements our previously reported results identifying LBP (lipopolysaccharide binding protein) as a suitable marker of the acute phase in bovines [38,40]. In addition, albumin is the most important buffer in plasma [29]. $A_{tot\ (Prt)}$ was less affected because of hyper(gamma)globulinaemia, a spontaneous immune response. Our findings are in good agreement with data reported recently by Poudel et al. (2012) demonstrating that both the lowered plasma albumin and the increased globulin concentrations were associated with the intensity of *C. pecorum* infection in calves, and were attributed to ongoing systemic inflammation and its detrimental effects on liver function caused by chlamydiae [67].

$SIDm_3$, $SIDm_4$ and $SIDm_5$ decreased 3 dpi (acidotic effect) caused by hyponatraemia (without change in haematocrit; data not shown) which dominated the concurrent hypochloraemia (alkalotic effect). $SIDm_3$, $SIDm_4$ and $SIDm_5$ increased slightly 10–14 dpi due to continuing hypochloraemia and normalised sodium concentrations which led to a mild alkalotic effect as seen at the same time in a slightly increased pH. Effects of increased potassium and lactate were compensated and were not seen in $SIDm_3$. The adaptive retention of acid during sustained hypocapnia is normally accompanied by a loss of sodium into the urine [68]. To maintain the electroneutrality in blood in the presence of hyponatraemia, the $HCO3^-$ concentration must decrease concurrently. The measured values of cNa^+ and base excess agree with studies by Funk (2007) [34], which showed that a decrease of sodium by 10 mmol/L explains a decrease of Base excess by –3 mmol/L.

This experimental study demonstrated again that often multiple acid–base disturbances exist concurrently and that mixed acid–base disturbances traditionally cannot be detected when the blood pH is unchanged. Strong ion theory provides evidence about the presence of acid–base imbalances, but only the selective view on the single parameters, which are required to calculate the strong ion variables, help to understand the complex response of the host organism and interactions between numerous variables.

Conclusions

The present study improved the current understanding of the pathophysiology of respiratory *C. psittaci* infections. Pulmonary dysfunctions and acid–base imbalances assessed by sensitive methods lasted clearly longer than clinically obvious signs and may thus also help elucidating functional host-pathogen interactions in the mammalian lung.

Endnotes

[a]Further analysis are based on calculations on $SIDm_3$. However, to enable a better comparability to other studies $SIDm_4$ and $SIDm_5$ were additionally calculated:

$$SIDm_4[mmol/L] = (cNa^+ + cK^+)-(cCl^- + cL-lactate);$$

$$SIDm_5[mmol/L] = (cNa^+ + cK^+ + cCa^{2+})-(cCl^- + cL-lactate).$$

Results are given in Additional file 2.

Additional files

Additional file 1: Results of blood-gas analysis, bicarbonate concentrations, base excess, anion gap, and strong ion gap. http://respiratory-research.com/imedia/1164744271062177/supp1.xlsx.

Additional file 2: Concentrations of plasma glucose, L-lactate, sodium, potassium, chloride, and calculated strong ion differences (SID). http://respiratory-research.com/imedia/1452059447106217/supp2.xlsx.

Additional file 3: Concentrations of inorganic phosphate and total protein, results of electrophoresis, and calculated values for A(tot). http://respiratory-research.com/imedia/5257028221062177/supp3.xlsx.

Abbreviations

Alb: Albumin; A(tot): Acid total; AG: Anion gap; BT: Body temperature; b.w.: Body weight; Ca^{2+}: Calcium; c: Concentration; cBase (Ecf): Standard base excess; cBase: Actual base excess; Cl$^-$: Chloride; *Cp: Chlamydia psittaci*; dpi: Days post inoculation; FRC: Functional residual capacity; He: Helium; HCO$_3^-$: Bicarbonate; HCO$_3^-$(st): Standard bicarbonate; ifu: Inclusion forming units; K$^+$: Potassium; K$_a$: Acid dissociation constant; m: Number of strong ions measured in plasma; p: Partial pressure; PFT: Pulmonary function tests; pK$_a$: − log $_{10}$K$_a$; Prt: Protein total; Rdist: Distal airway resistance; Rprox: Proximal airway resistance; RR: Respiratory rate; Rrs: Respiratory resistance; SD: Standard deviation; SID: Strong ion difference; SIG: Strong ion gap; Tex: Time of expiration; Tin: Time of inspiration; UA: Unmeasured anions; UC: Unmeasured cations; V: Venous; V'ex: Airflow (V') during expiration; V'in: Airflow (V') during inspiration; Vmin: Volume of minute ventilation; Vt: Tidal volume; Xrs: Respiratory reactance.

Competing interests

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Authors' contributions

CO carried out pulmonary function tests, drafted parts of the manuscript. SL analysed variables of acid–base status, performed statistical analysis of acid–base data, drafted parts of the manuscript. CSV participated in analysis of acid–base variables and drafted parts of the manuscript. PR conceived the study, and participated in its design and coordination, supported statistical analysis of lung function data, and revised the manuscript critically.

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     responds to disruption of acid–base balance by the lung.** *Nephron Physiol*
     2003, **93**:61–66.

Additional file 1. Results of blood-gas analysis, bicarbonate concentrations, base excess, anion gap, and strong ion gap.

		Baseline	1 dpi	2 dpi	3 dpi	4 dpi	7 dpi	10 dpi	14 dpi
		n = 21/group	n = 21/group	n = 21/group	n = 18/group	n = 15/group	n = 12/group	n = 9/group	n = 6/group
pH(v) $_{BT}$	*C. psittaci*	7.38 [7.36-7.41]	7.37 [7.35-7.40]	**7.40 ↑#** [7.36-7.44]	7.37 [7.35-7.41]	7.38 [7.31-7.42]	7.38 [7.36-7.40]	7.39 [7.37-7.40]	7.39 [7.37-7.42]
	Controls	7.38 [7.34-7.40]	7.38 [7.34-7.40]	7.38 [7.33-7.41]	7.37 [7.33-7.40]	7.38 [7.32-7.39]	7.37 [7.35-7.40]	7.38 [7.35-7.39]	7.37 [7.37-7.39]
pCO$_2$(v) $_{BT}$ (kPa)	*C. psittaci*	7.22 [6.59-7.92]	7.22 [6.30-7.80]	**6.88 ↓#** [6.40-7.96]	**6.84 ↓#** [5.89-7.48]	6.97 # [6.37-8.52]	**7.60 ↑#** [7.19-8.12]	**7.47 ↑** [7.15-7.89]	7.43 [7.06-7.64]
	Controls	7.27 [6.74-8.26]	7.22 [6.66-7.64]	7.22 [6.43-7.74]	7.27 [6.68-8.72]	7.35 [6.90-7.89]	7.07 [6.78-8.44]	7.13 [6.80-8.09]	7.22 [6.49-7.85]
cHCO$_3^-$ (mmol/L)	*C. psittaci*	30.30 [27.30-33.60]	29.85 ↓ [26.65-31.65]	30.1 [28.60-33.10]	**27.93 ↓#** [24.15-30.05]	29.15 [27.50-33.50]	**32.23 ↑#** [31.10-34.20]	**32.20 ↑#** [31.00-34.20]	32.35 [31.45-33.20]
	Controls	30.60 [27.45-35.35]	30.10 ↓ [26.90-31.70]	30.50 ↓ [26.05-32.50]	29.93 ↓ [27.40-33.00]	30.45 ↓ [27.00-32.20]	30.05 ↓ [27.30-34.05]	30.00 [28.50-32.85]	30.63 [28.35-32.50]
cHCO$_3^-$ (st) (mmol/L)	*C. psittaci*	28.28 [26.15-30.80]	28.05 [25.65-30.35]	**29.45 ↑#** [27.90-31.85]	**26.90 ↓#** [24.25-29.10]	27.45 [26.45-31.10]	**30.30 ↑#** [29.00-31.90]	**30.00 ↑#** [29.15-32.20]	30.30 [29.10-31.75]
	Controls	28.68 [25.73-31.93]	28.40 [24.90-29.45]	28.75 [24.90-29.85]	27.63 ↓ [25.45-30.25]	28.25 [24.50-29.70]	28.03 ↓ [25.90-30.40]	28.30 [26.70-29.80]	28.78 [26.95-29.80]
cBase (mmol/L)	*C. psittaci*	5.10 [2.50-7.68]	4.85 [2.05-7.20]	**6.15 ↑#** [4.65-8.65]	**3.60 #** [0.65-6.05]	4.25 [3.05-8.25]	**7.20 ↑#** [5.65-8.70]	**6.95 ↑#** [5.90-9.00]	7.18 [6.05-8.25]
	Controls	5.55 [2.33-9.30]	5.25 [1.80-6.65]	5.45 [1.20-7.15]	4.63 ↓ [2.25-7.50]	5.20 [1.35-6.80]	4.95 [2.35-7.95]	5.20 [3.55-7.05]	5.55 [3.65-6.85]
cBase (Ecf) (mmol/L)	*C. psittaci*	5.60 [2.70-8.85]	5.15 [2.25-7.50]	6.25 [4.70-8.90]	**3.73 ↓#** [0.45-6.15]	4.95 [3.20-8.70]	**7.50 ↑#** [6.15-9.25]	**7.50 ↑#** [6.35-9.40]	7.68 [6.55-8.75]
	Controls	6.05 [2.70-10.45]	5.60 ↓ [2.20-7.25]	5.85 ↓ [1.45-7.75]	5.08 ↓ [2.60-8.20]	5.80 ↓ [1.85-7.45]	5.43 ↓ [2.65-8.95]	5.5 [3.95-7.85]	6.05 [4.05-7.60]

Additional file 1: continued

		Baseline	1 dpi	2 dpi	3 dpi	4 dpi	7 dpi	10 dpi	14 dpi
		n = 21/group	n = 21/group	n = 21/group	n = 18/group	n = 15/group	n = 12/group	n = 9/group	n = 6/group
AG (mEq/L)	*C. psittaci*	7.90 [3.95-11.85]	**10.25 ↑#** [5.95-11.30]	9.30 ↑ [5.60-10.95]	10.00 ↑ [5.75-11.10]	9.75 [4.55-12.00]	7.45 # [4.05-9.15]	9.00 ↑ [7.75-10.15]	9.70 [7.75-10.40]
	Controls	7.55 [7.05-10.75]	8.35 [7.05-9.80]	8.25 ↑ [6.05-10.75]	8.43 ↑ [7.35-10.20]	8.35 ↑ [6.85-9.00]	8.35 ↑ [7.40-9.15]	8.60 ↑ [7.35-9.00]	8.28 ↑ [7.30-9.75]
SIG (Prt) (mEq/L)	*C. psittaci*	4.34 [-1.23-9.83]	2.68 ↓ [0.58-7.28]	2.94 ↓ [0.95-6.75]	1.76 ↓ [0.06-7.69]	2.05 ↓ [0.42-7.64]	5.16 # [2.57-7.77]	4.07 [2.31-4.93]	3.11 [2.43-6.15]
	Controls	3.89 [-1.08-7.95]	3.88 [1.85-5.93]	2.75 ↓ [0.73-7.01]	2.98 ↓ [0.73-5.34]	3.85 ↓ [1.31-6.23]	3.61 ↓ [1.70-6.11]	3.45 ↓ [2.44-4.54]	3.72 [2.32-6.00]
SIG (Alb) (mEq/L)	*C. psittaci*	3.24 [-2.22-7.84]	**1.57 ↓#** [-0.12-6.19]	1.64 ↓ [-0.25-5.83]	**0.44 ↓#** [-1.20-5.63]	0.75 ↓ [-1.24-5.74]	3.15 [0.14-5.98]	**1.84 ↓#** [-0.02-2.89]	1.42 [-0.07-4.98]
	Controls	3.78 [-0.40-7.01]	3.39 [2.13-5.93]	2.88 ↓ [0.35-6.75]	2.70 ↓ [1.12-4.97]	3.66 ↓ [1.42-5.17]	3.41 [1.28-5.72]	3.27 ↓ [2.24-5.02]	3.35 [2.14-5.84]

Values were obtained from jugular venous blood of calves experimentally exposed to *C. psittaci* (n = 21) or sham-inoculated controls (n = 21). Data are given as median and [range]. **#** indicates a significant difference between groups at the given time point (Matlab, Mann-Whitney-Wilcoxon *W* test, *P* ≤ 0.05). ↓ ↑ indicate significant decrease or increase, respectively compared to baseline values within each group (Matlab, Wilcoxon signed rank test, *P* ≤ 0.05).

Additional file 3: Concentrations of plasma glucose, L‑lactate, sodium, potassium, chloride, and calculated strong ion difference (SID)

		Baseline	1 dpi	2 dpi	3 dpi	4 dpi	7 dpi	10 dpi	14 dpi
		n = 21/group	n = 21/group	n = 21/group	n = 18/group	n = 15/group	n = 12/group	n = 9/group	n = 6/group
cGlucose (mmol/L)	*C. psittaci*	4.15 [3.40-5.05]	**4.05** ↓# [3.05-4.55]	3.75 ↓# [3.15-4.45]	**3.53** #↓ [3.00-4.05]	3.70 ↓# [3.15-4.50]	3.63 ↓# [2.15-4.80]	3.95 [3.50-4.30]	3.93 [3.20-4.50]
	Controls	4.20 [3.65-4.90]	4.20 [3.50-4.85]	4.45 ↑ [3.75-4.70]	4.33 [3.85-4.85]	4.40 [3.80-4.95]	4.20 [3.70-4.80]	4.15 [3.90-4.65]	3.75 [3.00-4.35]
cL⁻lactate (mmol/L)	*C. psittaci*	0.40 [0.30-0.60]	**0.50** ↑# [0.35-0.75]	**0.80** ↑# [0.50-1.20]	**0.60** ↑# [0.40-0.90]	0.50 ↑# [0.30-2.05]	0.35 [0.30-0.55]	0.40 [0.30-0.50]	0.40 [0.30-0.40]
	Controls	0.40 [0.30-0.45]	0.40 [0.30-0.70]	0.40 [0.30-0.50]	0.40 [0.25-1.50]	0.40 [0.30-0.50]	0.40 [0.25-1.15]	0.40 [0.20-0.45]	0.40 [0.35-0.45]
cNa⁺ (mmol/L)	*C. psittaci*	133.00 [131.50-134.50]	134.00 [129.50-135.00]	**131.00** ↓# [126.00-135.00]	**128.00** ↓# [125.00-129.50]	**128.00** ↓# [126.00-131.00]	130.00 ↓# [117.00-134.00]	133.00 # [129.50-136.00]	133.00 [130.00-136.00]
	Controls	132.75 [130.50-135.25]	133.00 [130.00-135.00]	134.00 ↑ [131.00-137.50]	136.00 ↑ [131.50-139.00]	136.00 ↑ [131.00-139.50]	136.00 ↑ [131.00-138.00]	135.50 ↑ [134.00-136.00]	134.25 [133.00-137.00]
cK⁺ (mmol/L)	*C. psittaci*	4.30 [3.80-5.40]	**4.50** ↑# [4.00-5.75]	4.30 [2.90-4.65]	4.28 [4.10-4.40]	**4.05** ↓# [3.75-4.50]	4.10 # [3.85-4.60]	4.10 [3.80-4.80]	4.15 [3.95-4.30]
	Controls	4.25 [3.90-4.80]	4.20 [3.90-5.30]	4.25 [3.80-5.05]	4.35 [4.00-5.10]	4.30 [3.75-4.85]	4.50 ↑ [3.90-6.30]	4.30 [4.10-5.85]	4.18 [3.90-4.55]
cCa²⁺ (mmol/L)	*C. psittaci*	1.33 [1.28-1.42]	1.33 [1.26-1.38]	**1.28** ↓# [1.22-1.33]	1.33 # [1.25-1.40]	**1.29** ↓# [1.24-1.37]	1.30 # [1.21-1.38]	1.32 [1.28-1.39]	1.32 [1.29-1.39]
	Controls	1.34 [1.27-1.37]	1.35 [1.26-1.40]	1.34 [1.26-1.40]	1.37 ↑ [1.26-1.41]	1.37 ↑ [1.30-1.42]	1.36 ↑ [1.30-1.40]	1.34 [1.30-1.36]	1.34 [1.26-1.36]
cCl⁻ (mmol/L)	*C. psittaci*	99.00 [96.00-103.00]	98.50 [95.50-103.00]	**96.00** ↓# [88.00-101.50]	**95.00** ↓# [93.00-98.50]	**93.50** ↓# [92.00-96.00]	**93.50** ↓# [82.50-100.00]	**95.00** ↓# [92.00-100.00]	**95.50** ↓# [92.00-98.00]
	Controls	98.00 [94.00-103.00]	98.00 ↑ [95.00-105.00]	100.00 ↑ [95.00-107.00]	101.00 ↑ [96.50-106.00]	101.00 ↑ [96.00-106.50]	101.50 ↑ [96.00-106.50]	101.00 ↑ [99.00-104.00]	99.50 [98.00-104.50]

Additional file 3: continued

		Baseline	1 dpi	2 dpi	3 dpi	4 dpi	7 dpi	10 dpi	14 dpi
		n = 21/group	n = 21/group	n = 21/group	n = 18/group	n = 15/group	n = 12/group	n = 9/group	n = 6/group
$SIDm_3$ (mEq/L)	*C. psittaci*	38.30 [34.10-41.30]	**39.55 ↑#** [34.70-41.10]	39.25 [34.85-42.40]	**36.95 ↓#** [34.10-39.85]	38.10 [35.10-42.00]	40.38 ↑ [35.95-42.10]	**41.10 ↑#** [39.00-43.20]	**41.83 ↑#** [40.95-42.20]
	Controls	38.85 [34.85-43.05]	38.15 [35.30-39.60]	38.90 [34.10-41.30]	38.58 [35.85-43.10]	38.60 [35.35-40.25]	38.50 [34.85-42.45]	38.65 [37.25-40.70]	39.75 [36.05-40.40]
$SIDm_4$ (mEq/L)	*C. psittaci*	37.95 [33.80-40.80]	39.05 [34.30-40.50]	38.15 [34.25-41.60]	**36.13 ↓#** [33.60-39.15]	37.55 [34.50-41.55]	39.98 ↑ [35.65-41.80]	**40.70 ↑#** [38.50-42.90]	**41.43 ↑#** [40.60-41.90]
	Controls	38.40 [34.55-42.60]	37.80 [34.60-39.20]	38.50 [33.70-41.00]	38.15 [35.35-41.60]	38.20 [34.95-39.95]	38.08 [34.55-41.30]	38.40 [36.85-40.25]	39.38 [35.65-40.00]
$SIDm_5$ (mEq/L)	*C. psittaci*	39.30 [35.08-42.15]	40.38 [35.56-41.81]	39.42 [35.58-42.84]	**37.47 ↓#** [34.87-40.51]	38.82 [35.75-42.86]	41.27 ↑ [36.95-43.17]	**42.02 ↑#** [39.83-44.21]	**42.73 ↑#** [41.99-43.21]
	Controls	39.73 [35.89-43.95]	39.14 [35.96-40.51]	39.81 [35.04-42.26]	39.55 [36.74-42.98]	39.55 [36.35-41.35]	39.46 [35.89-42.67]	39.70 [38.18-41.59]	40.67 [37.00-41.34]

Values were obtained from jugular venous blood drawn from calves experimentally exposed to *C. psittaci* (n = 21) or sham inoculated controls (n = 21). Data are given as median and [range]. **#** indicates a significant difference between groups at the given time point (Matlab, Mann‑Whitney‑Wilcoxon W test, $P \leq 0.05$). ↓ ↑ indicate significant decrease or increase, respectively compared to baseline values within each group (Matlab, Wilcoxon signed rank test, $P \leq 0.05$).

Additional file 2: Concentrations of inorganic phosphate and total protein, results of electrophoresis, and calculated values for (A_{tot})

		Baseline	1 dpi	2 dpi	3 dpi	4 dpi	7 dpi	10 dpi	14 dpi
		n = 21/group	n = 21/group	n = 21/group	n = 18/group	n = 15/group	n = 12/group	n = 9/group	n = 6/group
cPhosphate inorg. (mmol/L)	*C. psittaci*	2.48 [1.77-3.04]	2.35 ↓ [1.71-2.63]	**1.76 ↓#** [1.50-2.31]	**1.84 ↓#** [1.39-2.13]	**1.87 ↓#** [1.53-2.29]	**2.07 ↓#** [1.84-2.40]	2.22 ↓ [2.10-2.46]	2.42 [1.94-2.62]
	Controls	2.49 [1.76-2.84]	2.34 [1.89-2.69]	2.34 [2.01-2.79]	2.31 [1.92-2.73]	2.30 ↓ [1.94-2.58]	2.42 [2.19-2.75]	2.41 [2.08-2.65]	2.52 [2.08-2.73]
cProtein total (g/L)	*C. psittaci*	54.90 [47.30-62.60]	54.80 [49.70-63.00]	52.50 ↓ [46.20-58.20]	52.05 ↓ [46.20-58.60]	53.20 ↓ [48.60-56.60]	53.80 [42.60-60.00]	**55.10 ↑#** [53.50-59.00]	**56.50 ↑** [53.90-61.90]
	Controls	52.60 [44.90-63.70]	53.60 [43.70-60.80]	52.80 [40.30-67.20]	53.00 [44.70-61.10]	53.10 [44.40-58.60]	52.95 [46.50-59.40]	52.20 [47.70-58.10]	54.30 [46.30-62.00]
cAlbumin (g/L)	*C. psittaci*	27.83 [23.65-30.03]	27.50 [24.90-32.04]	**26.01 ↓#** [22.73-28.46]	**25.56 ↓#** [22.84-28.25]	**25.02 ↓#** [23.22-28.18]	**25.13 ↓#** [18.91-26.76]	**26.20 ↓#** [23.41-27.39]	26.34 [23.34-29.80]
	Controls	28.00 [24.80-34.80]	27.90 [24.30-33.20]	27.40 [22.90-36.40]	27.70 [25.00-32.80]	28.90 [24.50-30.10]	28.55 [24.60-33.40]	27.80 [25.80-33.20]	28.90 [25.10-33.80]
ratio albumin: globulin	*C. psittaci*	1.03 # [0.88-1.19]	1.00 # [0.86-1.24]	**0.97 ↓#** [0.85-1.25]	**0.95 ↓#** [0.85-1.11]	**0.92 ↓#** [0.87-1.08]	**0.83 ↓#** [0.77-0.95]	**0.87 ↓#** [0.76-0.99]	**0.84 ↓#** [0.75-1.15]
	Controls	1.19 [0.89-1.35]	1.15 [0.90-1.35]	1.18 [0.95-1.43]	1.16 [0.99-1.39]	1.17 [1.03-1.42]	1.18 [1.01-1.39]	1.19 [1.04-1.33]	1.19 [1.06-1.22]
cAlpha1-globulin (g/L)	*C. psittaci*	0.95 # [0.82-1.30]	0.97 [0.87-1.23]#	0.90 # [0.81-1.20]	0.96 # [0.81-1.20]	**1.05 ↑#** [0.92-1.18]	**1.12 ↑#** [0.95-1.21]	**1.14 ↑#** [1.07-1.20]	**1.16 ↑#** [1.05-1.31]
	Controls	0.89 [0.79-0.98]	0.86 [0.75-1.01]	0.88 [0.70-1.04]	0.87 [0.76-1.01]	0.85 [0.72-1.03]	0.86 [0.79-0.96]	0.85 [0.80-0.96]	0.90 [0.80-0.94]
cAlpha2-globulin (g/L)	*C. psittaci*	0.24 # [0.17-0.32]	0.25 # [0.19-0.29]	**0.27 ↑#** [0.19-0.32]	**0.29 ↑#** [0.22-0.35]	**0.28 ↑#** [0.26-0.33]	**0.31 ↑#** [0.28-0.43]	**0.27 ↑#** [0.25-0.33]	**0.29 ↑#** [0.25-0.40]
	Controls	0.20 [0.14-0.27]	0.20 [0.12-0.26]	0.20 [0.10-0.25]	0.21 [0.14-0.24]	0.18 [0.12-0.27]	0.20 [0.14-0.27]	0.21 [0.17-0.24]	0.24 [0.15-0.26]

Additional file 2: continued

		Baseline n = 21/group	1 dpi n = 21/group	2 dpi n = 21/group	3 dpi n = 18/group	4 dpi n = 15/group	7 dpi n = 12/group	10 dpi n = 9/group	14 dpi n = 6/group
cBeta-globulin (g/L)	*C. psittaci*	0.51 [0.42-0.64]	**0.54** ↑# [0.47-0.64]	0.53 # [0.46-0.63]	0.50 [0.48-0.61]	0.51 # [0.47-0.57]	0.55 # [0.41-0.58]	**0.57** ↑# [0.54-0.61]	**0.56** ↑# [0.55-0.66]
	Controls	0.48 [0.39-0.65]	0.50 [0.39-0.67]	0.46 [0.38-0.63]	0.47 [0.36-0.65]	0.47 [0.36-0.56]	0.48 [0.39-0.58]	0.46 [0.42-0.51]	0.50 [0.43-0.60]
cGamma-globulin (g/L)	*C. psittaci*	0.92 [0.60-1.38]	0.92 [0.66-1.31]	0.86 ↓ [0.57-1.18]	0.91 ↓ [0.54-1.18]	0.81 ↓ [0.60-1.15]	0.84 [0.62-1.37]	**0.98** ↑# [0.86-1.26]	1.05 [0.71-1.16]
	Controls	0.89 [0.56-1.28]	0.87 [0.51-1.27]	0.89 [0.47-1.24]	0.86 [0.53-1.14]	0.83 [0.55-1.14]	0.88 [0.58-1.09]	0.84 [0.71-1.02]	0.91 [0.66-1.07]
$A_{tot\,(Alb)}$ (mmol/L)	*C. psittaci*	17.31 [14.71-18.68]	17.10 [15.49-19.93]	**16.18** ↓# [14.14-17.70]	**15.90** ↓# [14.21-17.57]	**15.57** ↓# [14.44-17.53]	**15.63** ↓# [11.76-16.64]	**16.29** ↓# [14.56-17.04]	16.38 [14.52-18.53]
	Controls	17.42 [15.43-21.65]	17.35 [15.11-20.65]	17.04 [14.24-22.64]	17.23 [15.55-20.40]	17.98 [15.24-18.72]	17.76 [15.30-20.77]	17.29 [16.05-20.65]	17.98 [15.61-21.02]
$A_{tot\,(Prt)}$ (mmol/L)	*C. psittaci*	18.83 [16.22-21.47]	18.80 [17.05-21.61]	18.01 ↓ [15.85-19.96]	17.85 ↓ [15.85-20.10]	18.25 ↓ [16.67-19.41]	18.45 [14.61-20.58]	**18.90** ↑# [18.35-20.24]	19.38 ↑ [18.49-21.23]
	Controls	18.04 [15.40-21.85]	18.38 [14.99-20.85]	18.11 [13.82-23.05]	18.18 [15.33-20.96]	18.21 [15.23-20.10]	18.16 [15.95-20.37]	17.90 [16.36-19.93]	18.62 [15.88-21.27]

Values were obtained from venous blood sera of calves either experimentally exposed to C. psittaci (n = 21) or sham inoculated controls (n = 21). Data are given as median and [range]. **#** indicates a significant difference between groups at the given time point (Matlab, Mann‑Whitney‑Wilcoxon *W* test, $P \le 0.05$). ↓↑ indicate significant decrease or increase, respectively compared to baseline values within each group (Matlab, Wilcoxon signed rank test, $P \le 0.05$). $A_{(tot)}$ = total plasma non-volatile buffer concentration

Multidisciplinary Respiratory Medicine

CORRECTION   **Open Access**

Correction: Evaluation of pulmonary dysfunctions and acid–base imbalances induced by *Chlamydia psittaci* in a bovine model of respiratory infection

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Correction

Following publication of our article [1], we noticed the following mistakes:

Firstly, the numbers in the headings of the additional files have been reversed. In Additional file 2, cell A1 should read "*Additional file 2*: Concentrations of plasma glucose, L-lactate, sodium, potassium, chloride, and calculated strong ion difference (SID)" rather than "*Additional file 3*: Concentrations of plasma glucose, L-lactate, sodium, potassium, chloride, and calculated strong ion difference (SID)". In Additional File 3, cell A1 should read "*Additional file 3*: Concentrations of inorganic phosphate and total protein, results of electrophoresis, and calculated values for (Atot)" rather than "*Additional file 2*: Concentrations of inorganic phosphate and total protein, results of electrophoresis, and calculated values for (Atot)".

Secondly, in the legend of Figure 2 the term "d a.i." is not an abbreviation of "days after inoculation" and should instead read "days *ante inoculationem*".

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3 Discussion

The project presented in this thesis aimed to establish and evaluate a bovine respiratory model of experimentally induced *C. psittaci* infection. Bovines were chosen as the host because (i) bovine *C. psittaci* infection closely reflects the situation in a natural host, and (ii) the bovine lung is relevant to model functional consequences of ventilatory disorders due to its segmental anatomy and the lack of collateral airways. The overall objective of this thesis was the pathophysiological characterisation of this model. It also evaluated the pathogenetic potential of *C. psittaci* for bovines and potential transmission routes by assessing parameters of health and lung functions, as well as the shedding of this potentially zoonotic pathogen.

A total of 69 conventionally raised Holstein-Friesian calves, grouped according to Table I-4, were included in the four presented studies. Several read out parameters were assessed under differing study set-ups, thus Table D-5 might facilitate a fast, comparative and integrated overview about results obtained from inoculated calves, sentinels and control calves.

3.1 Factors determining, influencing and characterising the model

3.1.1 Methodological aspects

3.1.1.1 Choice of animal species

Reasons for the choice of calves instead of rodents were explained in detail in STUDY 1, and, with respect to lung function testing, in STUDY 4. In brief, calves were chosen because they are natural hosts for *C. psittaci*. Lung size allows direct comparison to humans in terms of airflows and volumes. In contrast to mice models, precise techniques for assessment of airway resistance and tissue mechanics are applicable to calves in a non-invasive manner. Thus repeated pulmonary function testing and an intra-individual long-term approach were successfully conducted (STUDY 4). Peculiarities of the bovine lung structure offer an advantageous sensitivity for the detection of pulmonary disorders.

However, the pronounced segmentation of the bovine lung tissue and the artificial inoculation method limit the significance of data about pathogen spread and propagation within the lung tissue. Another limitation of working with calves is that specifically pathogen-free or genetically defined animals are not available. Thus, relevant co-pathogens were ruled out by monitoring the herd of origin and by testing the purchased calves (STUDIES 1 and 3).

3.1.1.2 Choice of challenge strain and doses

A bovine *C. psittaci* strain (DC15, described in detail in STUDY 1) was chosen in order to obtain results displaying conditions of natural host-pathogen interactions. The choice of a non-avian strain additionally enabled the work to be conducted under conditions of biosafety level 2. Doses of viable *C. psittaci* inoculated per calf ranged between 10^6 to 10^9 ifu, which is higher compared to described mouse models, usually working in a scale about 10^4 to 2×10^6 ifu/mouse (Tang et al. 2010; Bode et al. 2012; Fiegl et al. 2013). However, taking the much larger body size of calves compared to mice into account, this elevation might be considered as moderate. In line with this approach, intranasal challenge models of *C. pneumoniae* and *C. abortus* in rabbits and sheep, respectively, also used higher doses up to 5×10^7 ifu/animal (Fong et al. 1997; Longbottom et al. 2013).

With respect to the results of STUDY 1, chosen doses proved to be suitable to model the mild, moderate and severe acute respiratory disease by 10^6, $10^7 - 10^8$, and 10^9 ifu of *C. psittaci* per calf, respectively.

3.1.1.3 Choice of administration mode

C. psittaci is usually transmitted by the airborne route, thus a direct challenge of the respiratory tract was considered to be the most valuable route of infection. Available methods are aerosol challenge (inhalation) or deposition of a fluid bolus into the respiratory tract. A bolus might be administered intranasally, intratracheally or intrabronchially. For the present model it was decided to apportion 6 mL of the inoculum endoscopically between eight defined bronchi of each calf according to the administration pattern depicted in STUDY 1.

The addition of ink into medium of one control group traced the dissemination of fluid after inoculation (medium control group, STUDY 1). Ink is known to be inert in the lung (Heilmann and Müller 1987) which is in line with our result, that this group developed neither clinical nor pathological signs of pneumonia (STUDY 1). Two to three days post inoculation (dpi) the colour of the inoculum was distributed deeply within the lung, surrounding the peribronchiolar tissue near the sites of inoculation (unpublished). Likewise, the distribution of lung lesions after inoculation of vital *C. psittaci* reflected more the sites of inoculation (STUDY 1), than the picture of naturally occurring bronchopneumonia in bovines which generally affects more cranio-ventral regions of the lung (Caswett and Williams 2007; Panciera and Confer 2010). However, every challenge with viable *C. psittaci* successfully induced bronchopneumonia (STUDY 1, Lambertz 2011) and clinical signs of respiratory disease. The severity increased with rising doses, but within each group the clinical signs were uniform.

In the present model inoculation deeply into the lung was chosen not only for occupational safety (unknown zoonotic potential) but for the benefit of standardisation.

Pathogen inhalation via aerosol might mimic the most lifelike challenge method and although it proved effective in inducing chlamydial pneumonia in pigs (Sachse et al. 2004), a major

disadvantage is that neither the actually deposited dose nor the localisation of infection can be defined. Similarly, the distribution after intranasal or intratracheal inoculation of a fluid bolus is dependent on the breathing pattern and body position during inoculation. Bolus applications into the upper respiratory tract are widely used in rodent models, where − in contrast to large animal models − usually the whole lung is used for further investigations (e.g. Fong et al. 1997; Bode et al. 2012).

In large animal models, however, intrabronchial inoculation is advantageous for two reasons. (i) Areas of pneumotic altered tissue corresponded well with the area used for inoculation (Gogolewski et al. 1987; Narita et al. 2002) and may thus enable an intra-individual comparison (inoculated *versus* non-inoculated lobes) and targeted *in vivo* and *ex vivo* sampling. (ii) Since intrabronchial inoculation bypasses the mucociliary escalator of the trachea and larger bronchi, the potential pathogenicity of pulmonary pathogens can be assessed more clearly and clinical outcome was reported to be less variable compared with aerosol challenge (Potgieter et al. 1984). In other calf models the inoculation was usually restricted to a main bronchus of a caudal lung lobe. To the best of our knowledge, STUDY 1 provides the first description of a detailed allocation of defined volumes to defined bronchi. The use of this defined administration pattern resulted in highly reproducible clinical, pathological and histopathological outcomes. Thus, our results are in line with the described advantages of intrabronchial challenge. In addition, the distribution of the pathogen within the lung was improved compared to classically used one-lobe administration.

3.1.1.4 Study design and implementation

The overall aim of this thesis was the pathophysiological characterisation of a bovine model of respiratory *C. psittaci* infection. Being only one part of a project network, this work contributed to the development of an integrated, physiologically relevant animal model of *C. psittaci* infection. The choice of a large animal model allowed repeated sampling and thus enabled an intra-individual data comparison and the reduction of group sizes. The project was subdivided into two major sections, (i) the evaluation of a dose response relationship (STUDIES 1 and 2) and (ii) a follow-up study investigating the course and pathophysiological consequences of an acute respiratory *C. psittaci* infection (STUDIES 3 and 4). The use of two control groups during the first part of the project enabled exclusion and differentiation of effects related to medium or inactivated chlamydial cell components. The evaluation of challenge doses between 10^6 to 10^9 ifu/calf provided a model covering the range from mild to severe disease. A long-term observation of acute respiratory *C. psittaci* infection during the second part of the project enabled an evaluation of the course and of epidemiological aspects, i.e. excretion and transmission of the pathogen. Investigations on naïve sentinel calves socialised with the infected group revealed the real possibility of transmission as well as the clinically inconspicuous course of natural *C. psittaci* infection.

During both parts of the project a broad range of clinical, immunological, biochemical and pathological read-out parameters were analysed, enabling an integrated evaluation of closely connected pathogenetic processes. To our knowledge the present model is the first construction of such an integrated animal model of respiratory *C. psittaci* infection.

Generally animal models of *C. psittaci* infection are rare for several reasons. (i) Targeted work with this pathogen usually requires biosafety level 3 facilities (except for non-avian strains). (ii) Detection, differentiation and investigation techniques are methodically demanding and usually restricted to specialised laboratories. (iii) *C. psittaci* infections are not considered to pose an acute major threat to human health. Since the refinement of taxonomy in 1999 single aspects of *C. psittaci* infection have mainly been investigated using molecular methods and cell culture models (e.g. Goellner et al. 2006). Only a few studies investigated host-pathogen interactions and immunological aspects using embryonated chicken eggs and mouse models (Bode et al. 2012; Braukmann et al. 2012; Fiegl et al. 2013). By providing relevant tissue samples to a number of network partners the present model served as the basis for an integrated approach elucidating various pathogenetic aspects of *C. psittaci* infection. Obtaining these samples, e.g. for pathologic and immunologic questions (Lambertz 2011; Möhle 2011) required the sacrifice of calves in the course of the study. This reduction of calves resulted in statistical restrictions for *in vivo* investigations later than 14 dpi (n = 3). The number of sentinels was, due to the animal facility size, also restricted to n = 3. Thus data reflecting clinically inconspicuous *C. psittaci* infection − either after natural infection or in the long-term course of disease − are descriptive cues rather than approved evidence.

Due to occupational safety and the necessity of wearing full body protection suits the access to the animal facilities was restricted to a low number of authorised staff. Thus blinding of the *in vivo* parts of the study was not possible. In contrast *ex vivo* analyses of samples were blinded for the investigators. Despite these limitations the study design was adequate for the objectives of the present thesis.

3.1.1.5   Causality assessment and chlamydial detection

Causality between pathogen and disease was traditionally assessed by means of Koch's postulates which can be summarised as follows:

1. The microorganism must be present in diseased organisms but should not be found in healthy organisms.
2. The bacteria must be isolated from the host with the disease and grown in pure culture.
3. The specific disease must be reproduced when the cultured microorganism is inoculated into a healthy susceptible host.
4. The microorganism must be reisolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

Compliance with postulates 1 and 2 is hampered by the nature of intracellular 'stealth pathogens', capable of inducing clinically silent and persistent infections (Breitschwerdt et al. 2013). The more modern 'molecular variant' of these postulates proposed by Falkow (1988) is not applicable to chlamydiae due to the lack of genetic tools. With the recognition of complexity of microbial pathogenesis a further set of postulates has been proposed, but finally it was concluded, that proof of disease causation rests on the concordance of scientific evidence, with Koch's postulates *sensu lato* serving as guidelines for collecting this evidence (Fredericks and Relman 1996).

In the present model, the dependence of various parameters, including clinical and pathological signs, on dose and viability of the pathogen, as well as the high reproducibility, provided evidence that *C. psittaci* infection was the initiating cause. However, methods for chlamydial detection including PCR based methods, recovery by cell culture and sequencing of the *ompA* gene from re-isolates were used to substantiate causality. Whilst pathogen excretion was assessed using a *C. psittaci* specific rt-PCR, targeting the *omp*A gene (Pantchev et al. 2009), a more sensitive *Chlamydiaceae* specific rt-PCR targeting the 23S-rRNA gene (Ehricht et al. 2006) was used to obtain quantitative evidence of the pathogen in regularly sampled blood and lung tissue. Detection of the pathogen in blood was positive for two thirds of calves (10^8 ifu/calf and sentinels, STUDY 3) and the method succeeded in providing a dose-related DNA amount in lung tissue up to 7 dpi. At 7 dpi the DNA content detected in lungs of animals challenged with viable *C. psittaci* was still higher compared with DNA amounts 2 – 3 days after challenge with an inactivated dose, indicative of propagation. However, as specifically pathogen-free calves were not available, the identity of *C. psittaci* was confirmed by analysing selected *Chlamydiacea* positive samples with species-specific methods, i.e. *C. psittaci* rt-PCR or microarray tube (Sachse et al. 2005; Pantchev et al. 2009). Throughout the trials, animals were kept in a self-contained bio-level 2 facility and staff had to wear disposable overalls, so the introduction of another *C. psittaci* strain is highly improbable. Nevertheless, the identity of pulmonary re-isolates to the challenge strain was approved in four instances by *omp*A sequence analysis (STUDY 3). Neither PCRs nor sequencing can provide information about the viability of the pathogen. Recovery from lung tissue samples by cell culture was successful up to 14 dpi but, on the other hand, failed cultivation attempts hereafter do not exclude the presence of *C. psittaci* in form of aberrant bodies. Findings suggestive for persistent infection include ongoing alterations of white blood cell composition and detection of small amounts of chlamydial DNA up to the end of the study in experimentally and naturally challenged calves. It was proposed that secondary infection via apoptotic neutrophils, and propagation of chlamydiae in macrophages, might maintain infection. In contrast it was shown that DNA of defunct chlamydiae is rapidly degraded (Moazed et al. 1998; Rupp et al. 2009).

With respect to the evaluation of transmission routes the assessment of the viability in swabs and air samples would have provided valuable additional information, but recovery from bovine faeces, seemingly the most important source of ambient contamination, is a very difficult task (Sachse[1] 2013, personal communication). Hence, it is still a matter of speculation which source and route enabled transmission of the infection to the naïve sentinel calves. An ultimate proof of infection of sentinels would have included the re-isolation of the challenge strain from blood, organs or excretions.

In conclusion the accordance to Koch's postulates can be summarised as follows:

1. Contrary to postulate number one − but in line with literature − *C. psittaci* was also found in clinically inconspicuous animals, i.e. in sentinels and in calves after overcoming induced acute disease. It has to be emphasised that *C. psittaci* was not found in the control groups (Lambertz 2011).

2. The chlamydial strain used was originally isolated from a bovine host but growth and propagation in pure (i.e. cell-free) culture was not possible due to the obligate intracellular lifestyle of this pathogen. In a narrow sense postulate number two was not met.

3. Every inoculation of *C. psittaci* lead to pathologically proven pneumonia (STUDY 1, Lambertz 2011) and signs of respiratory disease (STUDIES 1 and 3), the severity of which increased with rising challenge dose. In contrast clinical signs were comparable among calves that received the same dose. Thus, postulate number three was completely met.

4. *C. psittaci* was reisolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent. Postulate number four was also completely met.

3.1.2   Biological factors

3.1.2.1   Impact of the challenge dose on severity and course of disease

Disease severity was dependent on the dose during the acute phase of disease

The acute phase (2 − 3 dpi) of respiratory *C. psittaci* infection was clearly dose-related in numerous findings, assessed in STUDIES 1 and 2, including the respiratory and clinical score (Tab. D-5, IA), the extent and quality of lung lesions (Tab. D-5, IIIC), total cell counts in broncho-alveolar lavage fluid (BALF, Tab. D-5, IIIA1), and several variables evaluating the pulmonary gas exchange function (partial pressure of oxygen in arterial blood ($p(a)O_2$), percentage of oxy-haemoglobin in total haemoglobin ($O_2Hb(a)$), percentage of haemoglobin saturated with O_2 in arterial blood ($O_2(a)$), percentage of deoxy-haemoglobin in total haemoglobin ($HHb(a)$) and pulmonary shunt; Tab. D-5, IIIB1).
Clinical signs obtained in the present model covered the range from almost asymptomatic to

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severe disease. These findings are in good agreement with reports in literature associating chlamydial infections in calves with both obvious upper respiratory tract disease (Twomey et al. 2006) and clinically silent infections (Jaeger et al. 2007; Poudel et al. 2012). Moreover, they are in line with the wide severity range of *C. psittaci* infection reported for birds and humans. For human *C. psittaci* infections it was specifically suggested that the degree of exposure might be a factor determining severity. This conclusion was drawn from the recognition that most cases were mild to subclinical, but that life-threatening diseases developed after very close contact, i.e. by administering 'mouth-to-beak' resuscitation to a parrot suffering from *C. psittaci* infection (Crosse 1990; Moroney et al. 1998).

In the present study, striking symptoms of acute respiratory *C. psittaci* infection after a challenge dose of 10^8 ifu/calf resolved within 7 – 10 days, which is quite fast compared with respiratory diseases due to major respiratory pathogens in cattle (Caswett and Williams 2007). However, it is generally in line with the course of disease, reported from a *C. psittaci* mouse model, where disease severity (assessed by weight loss) peaked at 4 dpi and animals recovered within 14 dpi (Fiegl et al. 2013).

The long-term course of infection did not resemble the initial severity of disease

In contrast to the dose-dependent acute phase, a protracted clinically silent course indicated by intermittent mild symptoms, faecal pathogen excretion, and chlamydaemia, followed not only on the heels of acute respiratory disease, but even on natural, initially mild infection (STUDY 3; Tab. D-5, IA, IVA and B). This phenomenon is in line with one of the major problems in chlamydial research; the subclinical nature of infection which has, nevertheless, a long-term negative impact on health in many hosts. For calves, it was reported that asymptomatic chlamydial infections can chronically impair lung function, blood cell counts and composition, gain in body weight and general performance (Jaeger et al. 2007; Reinhold et al. 2008a; Poudel et al. 2012). In human medicine, it was suggested that *C. psittaci* infections might be associated with psoriasis, a chronic skin disease, and several kinds of lymphomas (Stinco et al. 2012). For turkeys, it was also shown that *C. psittaci* infections, whether apparent or not, interact and trigger disease outcome after super-infection with *E. coli* (Van Loock et al. 2006), which is relevant for almost every species. With respect to the present study, respiratory co-pathogens were ruled out, so that the observed acute and long-term effects can be considered to result only from *C. psittaci* infection.

As an overall conclusion, two facets of *C. psittaci* infection in bovines can be distinguished by the presented model; (i) acute clinical disease and (ii) clinically inconspicuous persistent infection.

Table D-5: Overview about results assessed in STUDIES 1 to 4.

Feature	Groups inoculated with viable *C. psittaci* (STUDIES 1-4)	Sentinels (STUDY 3)	Control groups (STUDIES 1, 2 and 4)
I Clinical signs			
A) Clinical and respiratory score (STUDIES 1, 2 and 3)	• positive correlation between challenge dose and clinical manifestation • clinical signs peaked 2 – 3 dpi and subsided mainly within 7 – 10 dpi • incomplete recovery up to 5 weeks after acute respiratory *C. psittaci* infection (10^8 ifu) • respiratory signs correlated positively with the challenge dose and contributed up to 50% to the clinical score • impairment of the general condition and dyspnoea after doses above 10^6 ifu	• unaffected general condition • mild respiratory signs started 7 – 11 dpc and lasted up to the end of the study (31 dpc)	• neither the inoculation of cell culture medium (medium) nor of 10^8 ifu of UV-inactivated C. psittaci (in*Cp*) resulted in respiratory illness or other clinical signs
II Blood analyses: Systemic and humoral host response			

A) Acute phase response c.m. of APPs (STUDIES 2, 3 and 4)	• LBP:
	  - dose related increase 2 – 3dpi
	  - acute *C. psittaci* infection (10^8 ifu) resulted in statistically elevated cLBP compared to baseline data between 1 – 10 dpi with a maximum at 2 dpi
	  - individual cLBP remained elevated up to the end of the study (35 dpi)
	• Hp increased in groups challenged with 10^6 and 10^7 ifu
	• CRP increased in all groups in a comparable manner
	• albumin: decreased significantly between 2 – 10 dpi after acute *C. psittaci* infection (10^8 ifu)
		• LBP increased slightly but clear between 10-28 dpc.
		• Hp and CRP: n.d.
		• albumin: n.d.
			• LBP remained unchanged after medium challenge but increased 2 – 3 dpi of in*Cp* in an amount comparable to the same dose of vital *C. psittaci*
			• Hp and CRP: increased slightly in both control groups (STUDY 2).
			• albumin remained unchanged after the inoculation of medium

Table D-5: continued

Feature	Groups inoculated with viable *C. psittaci* (STUDIES 1-4)	Sentinels (STUDY 3)	Control groups (STUDIES 1, 2 and 4)
B) Serum bio-chemical analysis and protein electrophoresis c.m. in serum (STUDY 4)	10^8 ifu: • inorganic phosphate and albumin were significantly reduced up to 10 dpi • globulins increased significantly over time • protein decreased significantly at 2 – 3 dpi and increased significantly 10 – 14 dpi	n.d.	• no significant alterations in the medium group

C) Acid-base status, metabolites and electrolytes
 	
c.m.
 	
(STUDIES 2 and 4)
	• pH(a) decreased 2 – 3 dpi of 10^9 ifu
	• pH(v) increased 2 dpi of 10^8 ifu
	• glucose decreased maximally 2 – 3 dpi after 10^8 and 10^9 ifu, and remained diminished up to 7 dpi (10^8 ifu)
	• L-lactate increased after doses above 10^6 ifu and remained elevated up to 4 dpi (10^8 ifu)
	• electrolytes:
	  - Na+ and Cl- decreased with rising dose (above 10^6 ifu) during the acute phase (2 – 3 dpi)
	  - After 10^8 ifu cCa2+, Na^+ and Cl^- were slightly but significantly reduced (cCa^{2+}: 2 – 4 dpi, cNa^+: 2 – 7 dpi, cCl^-: 2 – 14 dpi) and K^+ in- and decreased significantly at 1 and 4 dpi, respectively
		n.d.	• pH(a): no changes due to the inoculation of medium or in*Cp*
			• cK^+: slight variations in after inoculation of medium or in*Cp* compared to baseline data

Table D-5: continued

Feature	Groups inoculated with viable *C. psittaci* (STUDIES 1-4)	Sentinels (STUDY 3)	Control groups (STUDIES 1, 2 and 4)
D) Systemic cellular response WBC and differentiation (STUDIES 2 and 3)	• doses above 10^6 ifu: leukocytosis at 2 dpi, mainly driven by an increase of neutrophil granulocytes • at 2 dpi percentage of unsegmented neutrophils increased with rising dose • after a subsequent fall leukocyte and neutrophil counts increased and remained elevated up to the end of the study (35 dpi) • the absolute amount of lymphocytes was slightly but significantly reduced between $1 - 4$ dpi • monocytes tended to be elevated after challenge (significantly at 1, 7 and 14 dpi)	• total number of leukocytes increased constantly up to 10 dpc, and then approached slowly to the initial level • absolute numbers of banded neutrophils were clearly elevated $10 - 21$ dpc and did not reach the initial level up to the end of the study (31 dpc) • no clear trends in the number of lymphocytes and monocytes	• in*Cp*: leukocytes, neutrophils and lymphocytes increased slightly at 2 dpi • medium: unsegmented granulocytes and monocytes increased slightly (1 and 3 dpi; 1 and 2 dpi, respectively)
E) Humoral immune response Immunoblotting (STUDIES 1 and 3)	• 10^6 and 10^7 ifu: no antibody detection (until 14 dpi) • 10^8 ifu: onset 7-14 dpi; about two third of calves (STUDY 3) developed antibodies with titers maintaining or decreasing towards the study end (35 dpi) • 10^9 ifu/calf: n.d.	• no specific antibodies at any time point	n.d.

Table D-5: continued

Feature	Groups inoculated with viable *C. psittaci* (STUDIES 1-4)	Sentinels (STUDY 3)	Control groups (STUDIES 1, 2 and 4)
III Investigations of the lung as a target organ			
A) Pulmonary inflammation 1. *BALF-cytology* (STUDY 1)	• total cell counts increased with rising dose (2 – 7dpi) but were again comparable to control groups at 14 dpi • unsegmented neutrophils contributed predominantly to this increase but also alvolar macrophages, granulocytes and lymphocytes increased	n.d.	• during the acute phase cell counts and composition were comparable between control groups (medium, in*Cp*)
2. *Biomarkers in BALF* c.m. (STUDY 1)	• <u>cPrt</u> in BALF supernatant was comparable to control groups after inoculation of 10^6 or 10^7 ifu, but clearly to dramatically increased after challenge doses of 10^8 or 10^9 ifu, respectively • <u>cEicosanoids</u> (TXB2, PGE2, 12- & 15-HETE) were detectable after doses above 10^6 ifu in amounts increasing with rising challenge dose	n.d. n.d.	• below 300 µg/mL (2 – 3dpi) • <u>cEicosanoids</u> near the detection limit(2 – 3dpi)
B) Pulmonary functions 1. *Efficacy of gas exchange* BGA and haemoxymetry (STUDIES 2 and 4)	<u>Acute phase (2 – 3dpi):</u> • dose-dependent reduction of p(a)O2 (hypoxaemia), $O_2Hb(a)$ and $sO_2(a)$ • dose-dependent increase of HHb(a) and pulmonary shunt • $p(a)CO_2$: slight increase after a low challenge dose (10^6 ifu) and decrease after 10^8 and 10^9 ifu • $pCO_2(v)_{BT}$ reduced after 10^8 ifu • marked increase of A-aO$_2$ after 10^9 ifu • p50 decreased in all groups <u>Resolution period</u> (7 – 10 dpi): increase of $pCO_2(v)_{BT}$	n.d.	• $p(a)O_2$ decreased by less than 0.5 kPa • slightly increased pulmonary shunt and p50

Table D-5: continued

Feature	Groups inoculated with viable *C. psittaci* (STUDIES 1-4)	Sentinels (STUDY 3)	Control groups (STUDIES 1, 2 and 4)

2. *Respiratory mechanics*
 	
 impulse oscillometry
 	
 (STUDY 4)
	10^8 ifu (compared to control calves):
	 • pulmonary restrictions until 11 dpi
	   (significant decrease of Xrs (3 – 15 Hz))
	 • airway obstructions:
	   a) peripheral obstructions 3 – 4 dpi (significant increase
	      of Rrs ≤ 5 Hz at 3dpi and of Rdist (until 4 dpi))
	   b) proximal obstructions until 8 dpi (increase of Rprox)
		n.d.	• continuous growth-related increase of Xrs

3. *Respiratory pattern*
 	
 VC
 	
 (STUDY 4)
	10^8 ifu (compared to control calves):
	 • significant alterations 3 – 4 dpi:
	   reduction of Vt, doubling of RR, increased Vmin,
	   increased inspiratory and expiratory airflows
		n.d.	• continuous growth-related increase of Vt over time and therefore also a slight increase of Vmin

4. *Alveolar ventilation*
 	
 VC and He dilution
 	
 (STUDY 4)
	10^8 ifu:
	 • 3 – 4 dpi: reduced alveolar volume per breath
	   (i.e. increased Vd:Vt ratio)
	   and reduced end-tidal cCO_2
	 • 3 dpi: elevated FRC
		n.d.	• no significant alterations after inoculation of medium

C) Pulmonary lesions
 	
 1. *Gross pathology*
 	
 (STUDY 1)
	Acute phase (3 dpi):
	 • bronchopneumonia at sites consistent with the
	   inoculation pattern
	 • extent of lesions increased with rising challenge doses
	   (10^6 and 10^7 ifu: 10%, 10^8 ifu: 15%, 10^9 ifu: 30%)
	 Successive recovery of lesions was almost completed at 14 dpi (doses between 10^6-10^8 ifu)
		n.d.	• no lesions due to the inoculation of medium or in*Cp*

Table D-5: continued

Feature	Groups inoculated with viable *C. psittaci* (STUDIES 1-4)	Sentinels (STUDY 3)	Control groups (STUDIES 1, 2 and 4)
C) Pulmonary lesions 2. *Histopathology* (STUDY 1)	• <u>Acute phase (3 dpi):</u> extent and quality of lesions increased with rising challenge dose from purulent bronchopneumonia with small foci of fibrinous exsudate and necrosis (10^6 and 10^7 ifu) to fibrinopurulent bronchopneumonia with frequent multifocal areas of necrosis and pleuritis (10^8 ifu and even more severe after 10^9 ifu) • <u>subsequent organisation</u> of pneumotic lung tissue indicated by increased numbers of macrophages and lympho(histio)cytic infiltrates	n.d.	• no lesions due to the inoculation of medium or in*Cp*
IV Pathogen detection			
A) Peripheral blood rt-PCR (STUDY 3)	• positive for two thirds of calves (10^8 ifu) • copy numbers peaked within the first week • low amounts detectable up to the study end (35 dpi)	• positive for two thirds of calves • low amounts detectable up to the study end (31 dpc)	n.d.
B) Excretions and ambient contamination *C. psittaci* PCR (STUDY 3)	• faecal swabs: Positive in 5 of 21 calves (3 – 22 dpi) • nasal swabs: only one positive sample (0.7%) at 6 dpi • ocular swabs: no positive samples • exhaled breath: one positive sample at 14 dpi • room air: 4 of 5 samples collected between 3 – 7 dpi were positive	• faecal shedding in 3 of 3 calves, from 5 to 21 dpc • exhaled breath, ocular and nasal swabs: all samples were negative	n.d.

Table D-5: continued

Feature	Groups inoculated with viable *C. psittaci* (STUDIES 1-4)	Sentinels (STUDY 3)	Control groups (STUDIES 1, 2 and 4)
C) Lung tissue histopathology (STUDY 1)	• chlamydial inclusions in epithelial cells, mainly restricted to altered pulmonary tissue • amount of inclusions increased with the challenge dose and was higher in necrotic tissue compared to areas of organisation • foci with groups of macrophages containing chlamydial inclusions were found during the organisation of pneumotic lung tissue.	n.d.	• no chlamydial inclusions
quantitative rt-PCR (STUDY 1)	• genome copy numbers increased with the challenge dose (3 and 7 dpi) but decreased from 3 to 7 to 14 dpi	n.d.	n.d.
D) Recovery from lung tissue and strain identification cell culture and sequencing (STUDY 3)	• successful in all calves challenged with 10^8 ifu and sacrificed up to 14 dpi • identity of re-isolates to the challenge strain was exemplarily shown in four instances by sequencing of the *omp*A locus	• not successful	n.d.

Abbreviations: (a) in arterial blood, APPs acute phase proteins, BGA blood gas analysis, $_{BT}$ corrected for body temperature, *C. Chlamydia*, c concentration, Ca^{2+} calcium, c.m. concentration measurement, CO_2 carbon dioxide, dpc days after contact (to the group inoculated with 10^8 ifu/calf), dpi days post inoculation, HETE hydroxyeicosatetraenoic acid, ifu inclusion-forming units of *C. psittaci* administered per calf, in*Cp* 10^8 ifu of UV-inactivated *C. psittaci*/calf, K^+ potassium, medium cell culture medium, n.d. not determined (if data are available they are not included in STUDIES 1 – 4), O_2 oxygen, p partial pressue PGE2 prostaglandin E2, Prt total protein, (v) in venous blood, TXB2 thromboxan B2, VC volumetric capnography, WBC white blood cell count.

3.1.2.2 Impact of chlamydial viability and dose on initiation of disease

Inflammation and host response were dependent on the viability of the pathogen rather than on LPS-effects

Generally, LPS of gram-negative bacteria is known to be a potential activator of the immune system. In order to rule out effects mediated by such cell wall components, an UV-inactivated dose of 10^8 ifu/calf was administered to a control group of six calves (STUDIES 1 and 2). According to the prevailing pathophysiological concept, chlamydia's first and major target cells on mucosal surfaces are epithelial cells which initiate and sustain the immunological host response by secretion of proinflammatory chemokines (Rasmussen et al. 1997; Stephens 2003). It was shown *in vitro* that chlamydial LPS failed to induce an increased epithelial cytokine secretion (Rasmussen et al. 1997). Although cytokine production was not measured in the presented studies, the absence of pulmonary inflammation and clinical signs after challenge with an inactivated dose of *C. psittaci* is in line with this concept.

It was also reported, that proinflammatory chlamydial compounds, like heat shock protein 60 (HSP60) and MOMP, are only expressed after entering and propagating in host cells, indicating a selective pathway regulation associated with invasion and multiplication (Rupp et al. 2004). Although neither gene expression nor chlamydial components were determined, this information may provide an explanation why, in the present model, inflammation and host response were dependent on the viability and the dose of the pathogen.

3.1.2.3 Involvement of MALT into pathogenesis

It has only recently been reported how host-pathogen interactions in the upper respiratory tract might be involved in chlamydial pathogenesis. It has been hypothesised that MALT in the ovine nasopharyngeal tract is likely to play a role in the development of latency. Latency is a key element in the pathogenesis of ovine enzootic abortion (OEA) which is caused by *C. abortus* (Longbottom et al. 2013). As described for *C. psittaci,* transmission routes of *C. abortus* include ingestion and inhalation (Pospischil et al. 2010). While oral administration of 5 x 10^9 ifu of *C. abortus* per ewe induced latency and placental infection (Gutierrez et al. 2011) an even lower dose (5 x 10^7 ifu) stimulated protective immunity after intranasal administration (Longbottom et al. 2013). Although the structure of nasal-associated lymphoid tissue (NALT) in bovines is not as yet particularly defined (Liebler-Tenorio and Pabst 2006), there is the suggestion that it plays a role in bovine infectious diseases (Zhang et al. 2006). Unfortunately, as this potential effect was unknown at the time the studies were conducted, the inoculation scheme was developed without taking it into account.

3.2 Complex pathogenetic and pathophysiological implications and their consequences for the bovine host

The project led to a fundamental understanding of pathogenetic stages and pathophysiologic mechanisms of *C. psittaci* infection in a natural mammalian host. With respect to the complexity of pulmonary dysfunctions induced by *C. psittaci* infection on respiratory mechanics, this model may also serve as a basis for human medicine. In severely diseased patients, blood analysis is the only way to assess disease severity because pulmonary function tests cannot be performed. The following discussion will, therefore, focus on the host response accessible by means of blood analysis and on the complexity of pathogenesis and pathophysiology in the mammalian lung.

3.2.1 Innate and adaptive immune response

3.2.1.1 Acute phase reaction

Acute phase proteins (APPs) are produced in hepatocytes in response to proinflammatory cytokines resulting from tissue injury or inflammation. They function as a part of the early nonspecific immune defence, participating in a variety of functions to aid tissue repair (Jones and Allison 2007).
C-reactive protein (CRP), haptoglobin (Hp) and lipopolysaccharide-binding protein (LBP) are positive APPs, i.e. proteins that increase in plasma after tissue damage or inflammation. In contrast albumin belongs to the negative APPs characterised by decreasing concentrations. The decrease of the albumin concentration, detected 2 – 10 dpi, influenced the acid-base equilibrium and will therefore be discussed in section 3.2.2.3.

In the present model, concentrations of CRP increased slightly in control groups, and in a comparable, dose independent manner in all groups challenged with different doses of viable *C. psittaci* (Study 2, Tab. D-5, IIA). In line with these findings, it was reported that determining CRP concentrations in cattle is of limited value (Murata et al. 2004; Petersen et al. 2004). Although CRP is frequently tested and often increased in human respiratory disease, its diagnostic value is also controversial (van der Meer et al. 2005). In severe cases of human *C. psittaci* infections, however, the concentration of CRP has been reported to be clearly increased (Gacouin et al. 2012) and to be a useful to follow-up parameter during the most critical phase of disease (Wichert et al. 2000). These findings are in contrast to results obtained from the present model, but might be explained with major species differences in the acute-phase response (Petersen et al. 2004; Eckersall and Bell 2010).

The function of Hp is capturing free haemoglobin to prevent both iron loss and kidney damage during haemolysis (Abdullah et al. 2012). In the present model, a clear increase in the Hp concentration was only observed after administering lower doses of viable *C. psittaci* (i.e. 10^6 and 10^7 ifu/calf). Therefore, Hp offered no potential to predict the severity of clinical disease or to the extent of hypoxaemia or pulmonary lesions (STUDIES 1 and 2, Tab. D-5, I, IIA,

IIIC). It is known from literature that Hp expression is stronger in cattle than it is in man (Petersen et al. 2004), but the diagnostic value in bovine respiratory disease is controversial (Gray et al. 1996; Wittum et al. 1996; Young et al. 1996; Heegaard et al. 2000; Angen et al. 2009; Tothova et al. 2010). To date there are no reports suggesting a predictive power of Hp concentration in cases of human chlamydial pneumonias, which might support the conclusion of not being an useful marker of respiratory *C. psittaci* infection.

It was shown that LBP plays an essential role in the pulmonary immune response to gram-negative bacterial infection by using LBP knockout mice (Hemmila et al. 2006). Its function is to help in presenting LPS to CD14 receptors and toll-like receptor 4 (TLR4), which is used for signalling.

Seemingly in line with this essential role, it was shown in the present model that the LBP concentration increased with rising challenge dose (STUDY 2, Tab. D-5, IIA).

In addition, LBP concentrations were comparable between groups challenged with the same dose of either life or inactivated *C. psittaci*. It was therefore concluded, that the LBP concentrations reflected the LPS load, but were clearly not related to the clinical outcome.

The minimal effect on clinical outcome and blood cell composition might be explained as follows: Like other LPS, chlamydial LPS (cLPS) was shown to use TLR4 for signalling, but unlike other LPS, activation was strictly dependent on CD14 positive cells, i.e. on monocytes and macrophages (Heine et al. 2003). Macrophages within the lung tissue provide the cellular 'first–line defence', as they degrade pathogen-containing cells and detritus. Their cytokine production in response to cLPS was shown to be reduced by a factor about 10, compared to other typical endotoxins (Heine et al. 2003). This weak biological activity of cLPS was supposed to result from low binding affinities for LPS-recognition molecules, such as CD14 and LBP (Tsutsumi-Ishii et al. 2008). Moreover, the cytokine pattern secreted by macrophages was shown to differ in response to challenge with either dead or viable chlamydiae (Entrican et al. 1999).

In conclusion, the minimal effects of inactivated *C. psittaci* in the present model are likely to be attributed to the failure of epithelial cells – the primary target cells of chlamydial infection − to respond to cLPS (due to the absence of CD14 surface expression) and to the weak biological activity of cLPS. Although the pathophysiological consequences of an LBP increase remain to be elucidated, the results of the present model are in line with the literature, accepting LBP as a useful marker of lower respiratory tract diseases − associated to gram-negative bacteria − in both bovines (Schroedl et al. 2001; Nikunen et al. 2007) and humans (Hopstaken et al. 2009; Zobel et al. 2012).

3.2.1.2   Cellular immune response

Haematology indicated a prompt, but transient, increase of leukocytes, seen as mainly neutrophil-driven leucocytosis at 2 dpi in groups challenged with 10^7 or 10^8 ifu/calf. At 2 dpi, the percentage of unsegmented neutrophils in blood increased with rising dose, indicating an increased demand and a regenerative left shift. Neutrophils, monocytes and lymphocytes were recruited into the lung as indicated by their increased numbers in broncho-alveolar lavage fluid (BALF). Cell counts in BALF again correlated positively with the challenge dose up to 7 dpi. Young and mature neutrophils provided a first-line of defence. While banded neutrophils decreased back to their initial levels, the amount of polymorphonuclear neutrophils and monocytes remained elevated until 5 weeks after acute *C. psittaci* infection, i.e. the end of the long-term study (STUDY 3, Tab. D-5, IID).

Our findings are in line with others, reporting a fast and distinct recruitment of phagocytic cells to the site of chlamydial infection followed by 'cleaning up' of macrophages (e.g. Gieffers et al. 2004; Bai et al. 2005; Reinhold et al. 2008b; Rupp et al. 2009).

Generally, a proinflammatory response at the attacked tissue site aims at the resolution of infection. However, in case of chlamydial infection, it was shown that the chemokine production by infected epithelial cells continues (Rasmussen et al. 1997). It was therefore suggested that the advantage of ongoing inflammation is likely to facilitate intra-host spread (Stephens 1999). It was shown that *C. pneumoniae* can, in the course of pneumonia, infect and survive within granulocytes (Gieffers et al. 2004), which are subsequently engulfed by macrophages acting as a vector able to enter the whole body via the bloodstream (Moazed et al. 1998; Rupp et al. 2009). An increased ongoing demand of neutrophils and monocytes during the subclinical phase of disease (STUDY 3, Tab. D-5, IID) corresponds very well with these observations. Moreover, it is also in line with the blood cell composition of naturally *Chlamydia*-infected calves (Reinhold et al. 2008a). Ultrastructural observations of another work group in the present model can additionally sustain the theory of the 'monocyte vector shuttle': 2 – 4 dpi chlamydial inclusions filled with RBs, IBs and EBs were found in alveolar epithelial cells type 1, while IBs and EBs were seen free in the cytoplasm or in phagolysosomes of neutrophils and alveolar macrophages in calves inoculated with 10^8 ifu/calf (Möhle 2011). Finally, chlamydial DNA was also detected in blood up to the end of the long-term study at 35 dpi (STUDY 3, Tab. D-5, IVA). This finding again sustains the proposed maintenance of infection on a long-term basis, because DNA of defunct *Chlamydiae* was shown to be rapidly degraded (Moazed et al. 1998; Rupp et al. 2009).

3.2.1.3   Humoral immune response

In the present model strain-specific antibodies were detected by immunoblotting, which is more sensitive and specific than commercially available ELISAs. Specific antibodies, indicating successful infection, occurred in blood of two thirds of calves challenged with 10^8 ifu. The onset of humoral response was about 7 – 14 dpi (STUDIES 1 and 3 Tab. D-5, IIE). During the challenge

dose titration, individual animals challenged with doses below 10^8 ifu/calf were kept beyond the acute phase, but did not develop a measurable humoral response. Hence, it is still unclear whether lower challenge doses would induce the development of specific antibodies or if the onset of humoral response requires longer than two weeks after challenge (STUDY 1, Tab. D-5, IIE). Interestingly, in blood of naturally contracted sentinels no antibodies were detected up to 5 weeks after contact to acutely *C. psittaci* diseased calves. In calves experimentally challenged with 10^8 ifu, the detected humoral immune response was generally weak, did only emerge in two thirds of animals, and was sometimes already decreasing towards the study end at 35 dpi. These results are in line with the literature reporting that antibodies against *Chlamydia spp.* were detected in only about 60 % of naturally *C. abortus* and/or *C. pecorum* infected calves (Reinhold et al. 2008a). It is similarly in line with literature that seropositivity was not correlated to chlamydial shedding, which occurred in only 24 % of calves inoculated in STUDY 3 (Tab. D-5, IVB; Wehrend et al. 2005; Kauffold et al. 2007). Both human and veterinary medicine are faced with the problem that chlamydial infections often do not elicit sufficiently high antibody responses, which complicates both development of specific serological tests and development of safe and efficacious vaccines (Igietseme et al. 2011).

3.2.1.4 How immunological interactions might mediate sequelae of *C. psittaci* infections

In the present model, chlamydial DNA and increased levels of monocytes and LBP were shown in blood of calves up to the end of the study at 32 - 35 days after challenge with a dose of 10^8 ifu/calf or an undetermined dose acquired by natural infection. These findings indicate ongoing host-pathogen interactions. They are in line with literature suggesting that asymptomatic chlamydial infections in bovines are largely mediated by metabolic stress on the liver by circulating inflammatory mediators (Ahluwalia et al. 2010; Kaltenboeck 2013). Consequences of macrophage activation by low-level, but persistent, endotoxaemia (i.e. cLPS and LBP) together with oxidised low-density lipoproteins are suggested to play a role in the pathogenesis of atherosclerosis (Wiesner et al. 2010), for which an involvement of chlamydial infections is still largely disputed (reviewed by Joshi et al. 2013). Results of those studies are mostly based on seroepidemiological investigations, not differentiating between *C. psittaci* and *C. pneumoniae* antibodies. Thus the particular role of *C. psittaci* was largely left out of consideration. In birds and dogs however *C. psittaci* was detected in atherosclerotic altered vessels (Sako et al. 2002; Schenker and Hoop 2007; Pilny et al. 2012) and in our model, aorta samples were positive for *Chlamydiaceae* in 5 of 21 calves experimentally inoculated with *C. psittaci* (Lambertz 2011). It was confirmed that indeed *C. psittaci* was found by means of DNA microarray (Lambertz 2011). In conclusion results of this bovine model are in good agreement with the suggested concept of *C. psittaci* as a 'stealth pathogen'.

3.2.2   Pathogenetic interactions in the bovine lung

Pulmonary dysfunctions − with respect to their time course and in comparison to knowledge from literature – have been discussed in detail in STUDIES 2 and 4, so the following text will rather focus on the mechanisms and interactions leading to functional consequences within the respiratory system. Figure D-1 summarises these proposed interactions, the complexity of the pathogenetic network and pathophysiological consequences. It will be explained in more detail in the following sections (3.2.2.1. – 3.2.2.3.)
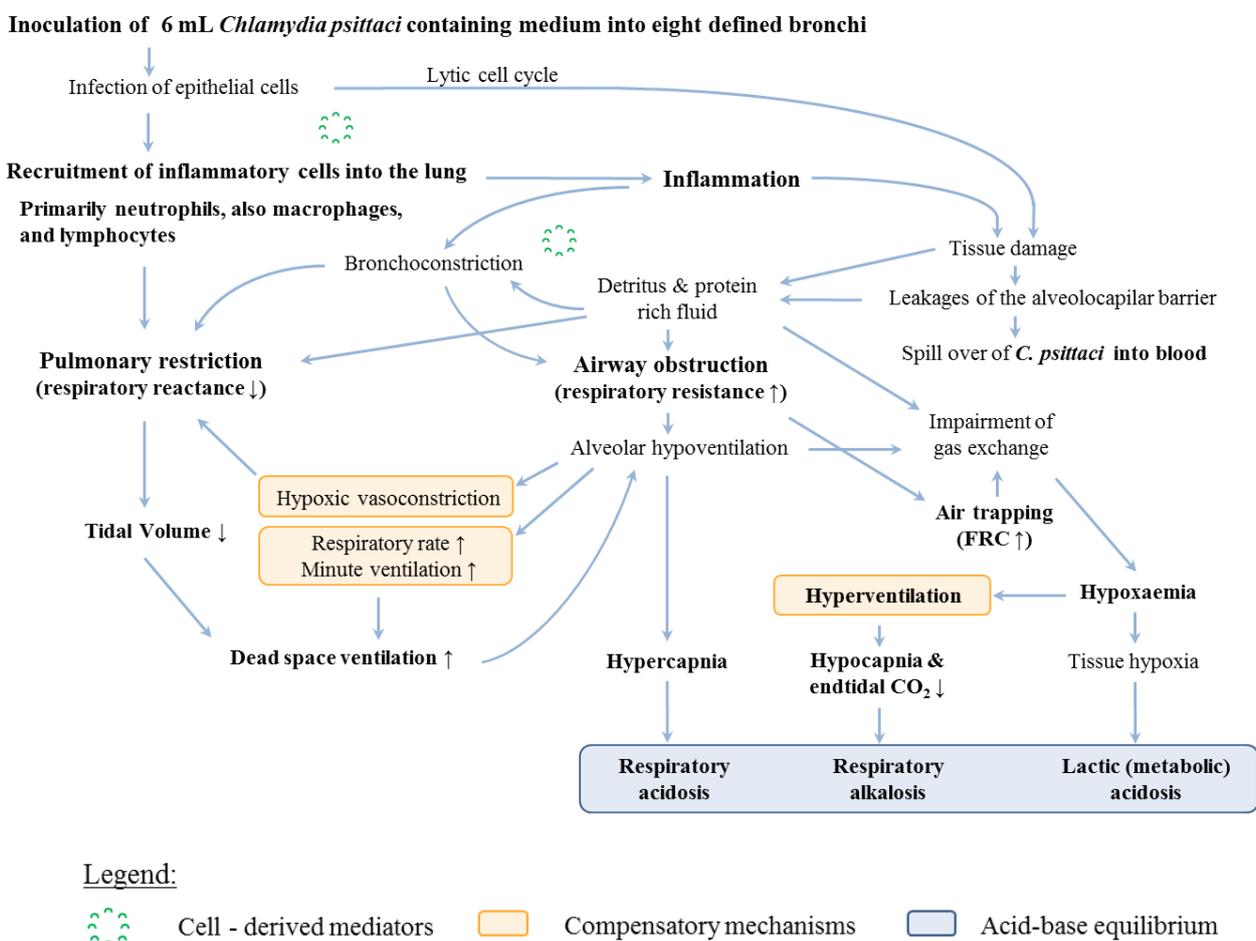
Figure D-1: Pathogenesis and pathophysiology of *Chlamydia psittaci* infection in the bovine lung. Key factors, mechanisms and interactions determining the pathogenesis and pathophysiology of *Chlamydia psittaci* infection in the bovine lung are summarised on the basis of results assessed in STUDIES 1 − 4 (bold face) and information from literature. Abbreviations: *C. Chlamydia*; FRC functional residual capacity.

3.2.2.1   Local pulmonary infection and inflammation

The inoculation of 6 mL viable *C. psittaci*-containing medium into eight defined locations of the lung resulted in infection and replication of the pathogen in pulmonary epithelial type I cells (Möhle 2011). This finding is in line with the prevailing pathophysiological concept wherein the acute host response to *Chlamydia* at mucosal surfaces is primarily initiated and sustained by epithelial cells, the first and major targets of chlamydial infection (Rasmussen et al. 1997). After infection with viable chlamydiae, epithelial cells secrete a range of proinflammatory chemokines (Stephens 2003) in order to activate the inflammation cascade. Macrophages within the respiratory system are important in metabolising arachidonic acid to its inflammatory metabolites via lipoxygenase and cyclooxygenase (COX) pathways resulting in the production of prostaglandins (PG), thromboxanes (TX), and leukotrienes (Bertram et al. 1989) of which PGE2, TXB2, 15- hydroxyeicosatetraenoic acid (HETE) and 12-HETE were shown to be increased in a dose dependent manner after challenge with viable *C. psittaci* (STUDY 1, Tab. D-5, IIIA2). Arachidonic acids and prostaglandins play also a role in lung injuries induced by pathogen-associated molecules including LPS (Soethout et al. 2002; Alba-Loureiro et al. 2004). The role of LPS has been discussed in detail in section 3.1.2.2., but in summary chlamydial LPS (cLPS) is known to be much less potent compared to other typical endotoxins (Heine et al. 2003; Tsutsumi-Ishii et al. 2008). Other proinflammatory compounds like heat shock protein 60 (HSP60) and MOMP are only expressed after entering and propagating in host cells (Rupp et al. 2004). This explains why, in the present model, inflammation and host response were largely dependent on the viability of the pathogen. Proinflammatory mediators initiate vasodilation, reduced blood flow and increased capillary permeability in order to enable inflammatory cells and fluid to pass into the attacked tissue (Thacker 2006). The severity of inflammation and increased permeability of pulmonary vessels were also indicated by an increase of total protein concentration in BALF after challenge doses above 10^7 ifu/calf (STUDY 1, Tab. D-5, IIIA and C). The presence of protein rich fluids was additionally shown by histology (Lambertz 2011). Recruitment and accumulation of phagocytic and reactive immune cells from the blood into the lung has been shown in STUDIES 1 and 2 (white blood cell count, BALF and histopathology, Tab. D-5, IID, IIIA and C) and for the long-term experiments in other PhD theses (Lambertz 2011; Möhle 2011).

Phagocytes − in the present study first and foremost neutrophils (shown in BALF and by histopathology, Tab. D-5, IIIA and C) − take up bacteria and debris but also release enzymes that further damage the tissues, chemokines that attract other immune cells (here macrophages and lymphocytes), and cytokines which further increase the inflammation (Thacker 2006). Additionally, in case of chlamydial infection, tissue damage and further proinflammatory activation is likely to be caused by lysis of infected cells during the release of chlamydial EBs (Rasmussen et al. 1997). Tissue damage and leakages of the alveolo-capillary membrane (Möhle 2011) might have enabled a spillover of the pathogen into the bloodstream, because chlamydial

DNA could be detected in blood (STUDY 3, Tab. D-5, IVA). Another mechanism enabling pathogen spread within the host is associated with macrophages serving as vector, as discussed in detail in the section 3.2.1.2.

3.2.2.2   Impact of infection and inflammation on the respiratory system and on pulmonary functions

Regardless of the mechanism, the accumulation of fluid, cells and debris within the alveoli and the lung tissue causes several interacting factors diminishing lung function in various ways. Although the oxygen transfer across the alveolar-capillary membrane was not quantified in the present project, it is likely that the presence of these materials in the alveolar lumen directly reduced the gas exchange. According to Fick's law of diffusion, complete airway obstruction and atelectases reduce the surface area capable of exchanging gases, and fluid accumulation in the alveolar spaces elongates the diffusion barrier.

The main consequence of an impaired gas exchange, the reduction of the oxygen partial pressure in arterial blood ($p(a)O_2$), was shown to be dependent on the dose of viable *C. psittaci*. Nevertheless, hypoxaemia, i.e. $p(a)O_2 < 11$ kPa (Reinhold and Höchel 2005) occurred due to all doses used for challenge with viable *C. psittaci* (STUDY 2, Tab. D-5, IIIB1). The extent of functional impairment was also clearly indicated by the dose-dependent decrease of arterial oxygen saturation ($sO_2(a)$) and the percentage of oxyhaemoglobin in total haemoglobin ($O_2Hb(a)$). Additionally the pulmonary shunt (i.e. the percentage of non-arterialised blood after passage through the lung) increased in line with increasing challenge dose. These dose-dependent indicators of disease severity were linked to the extent and quality of lesions assessed in STUDY 1 (Tab. D-5, IIIC).

In line with the accumulation of fluid, cells and debris, the origin of the observed oxygen deficiency was an impaired diffusion and/or distribution rather than reduced ventilation alone, because the alveolar arterial oxygen partial pressure difference (A-aO_2) was increased and hyperventilatory hypocapnia was observed after higher *C. psittaci* doses (i.e. 10^8 and 10^9 ifu/calf, STUDY 2, Tab. D-5, IIIB1 and C).

However, in line with the maximal extent of pulmonary inflammation at 3 – 4 dpi (Lambertz, 2011) the analysis of respiratory mechanics and breathing pattern revealed peripheral airway obstruction and reduced alveolar ventilation during the acute phase of disease (STUDY 4, Tab. D-5, IIIB2 and 3). Reduced alveolar ventilation worsens the already reduced oxygen delivery and may thus also contribute to additional death of alveolar epithelial type 1 cells, which are known to be intolerant to reduced oxygen supply (Jain and Sznajder 2005). In species lacking collateral alveolar ventilation, the transit of debris from the alveoli through the narrow lumen of the bronchiole exacerbates inflammatory obstruction (Caswett and Williams 2007). Expiration, representing a mainly passive retraction, was more impaired than inspiration, thus complete deflation was hindered and air was trapped. This 'trapped air' led to an overdistension

of alveoli, concomitantly measurable as elevated FRC at $3 - 4$ dpi (STUDY 4, Tab. D-5, IIIB4). Cough, representing a forced exhalation, occurred frequently during acute respiratory *C. psittaci* infection and is likely to have supported obstructions and FRC increase due to increased intrapleural pressure and resulting dynamic small airway collapse (Robinson 2007a). From a functional perspective, the resulting overdistension of alveoli caused an additional elongation of the space where gases moved only slowly by diffusion and thus complicated the gas exchange. The involvement of ventilatory disorders in the pathogenesis of respiratory *C. psittaci* infection was also indicated by the slight increase of $p(a)CO_2$ after low challenge doses (10^6 ifu/calf and even more slight after 10^7 ifu/calf, STUDY 2, Tab. D-5, IIIB1). In contrast to O_2, which can be reduced as a consequence of impaired diffusion, ventilation, perfusion or distribution, the gas exchange of CO_2 is, due to a 20-fold higher solubility coefficient, only impaired under conditions of reduced ventilation.

A further important consequence of inflammation is the development of pulmonary restriction. Restriction was assessed by decreasing respiratory reactance (Xrs), which is indicative of limitations in elasticity or compliance of the lung-thorax system. (Smith et al. 2005). In the present model, the respiratory reactance was statistically significantly reduced up to 11 dpi in calves suffering from experimentally induced acute *C. psittaci* pneumonia (STUDY 4, Tab. D-5, IIIB2). Lung compliance, usually mainly determined by the retractive forces of elastic fibres and the surface tension of surfactant within the alveoli, was most probably reduced due to multi-causal events. At first the accumulation of cells within the tissue itself reduced the elasticity. Secondly, bronchoconstriction mediated by stimulation of tracheobronchial irritant receptors (parasympathetic reflex-bronchoconstriction) or in response to inflammatory mediators, stiffened and obstructed trachea, bronchi and bronchioles (Robinson 2007a). It is very likely that irritant receptors were stimulated because accumulated mucus was found in the tracheal lumen of calves suffering from acute respiratory *C. psittaci* infection in amounts decreasing in the course of disease (Lambertz 2011). This result is again in line with the observation that proximal resistance (Rprox) was significantly increased up to 8 dpi, indicating proximal airway obstruction (STUDY 4, Tab. D-5, IIIB2). Obstructions of peripheral airways were, however, only detected during the acute phase of disease (STUDY 4, Tab. D-5, IIIB2). The consequences of these obstructions are alveolar hypoventilation and concomitant pulmonary vasoconstriction (HPV) in hypoventilated alveolar areas. Increased vascular resistance deteriorated the loss of compliant pulmonary properties. This ultimately decreased the tidal volume (Vt) during the acute phase of respiratory disease (STUDY 4, Tab. D-5, III2 and 3). Clinically reduced Vt and compensatory increase of the respiratory rate (RR) and volume of minute ventilation (Vmin) were seen as short, rapid breathing cycles (i.e. dyspnoea), which were characterised by an increased percentage of dead space volume (Vd) per breath.
In conclusion, the pathways of induced pulmonary dysfunctions assessed in the present model interacted in a complex network.

3.2.2.3   Impact of tissue damage and pulmonary dysfunctions on acid-base equilibrium

Pulmonary functions do interact with acid-base equilibrium in two different ways. On the one hand, pulmonary dysfunctions associated with changes in $p(a)CO_2$ will change blood pH. On the other hand, the lung may provide compensatory mechanisms to stabilize blood pH if changes occur from the metabolic site. In our model the most important consequence of impaired lung function and reduced gas exchange was hypoxaemia, which occurred in all groups challenged with different doses of viable *C. psittaci* (STUDY 2, Tab. D-5, IIIB1). *In vivo*, reduced $p(a)O_2$ is recognised by peripheral chemoreceptors (*glomerae carotica et aortica*) which drive respiration via the *Nervus* (*N.*) *vagus* and *N. glossopharyngeus* (parasympathetic nervous system). Intensified respiration may lead to an increased CO_2 exhalation, thus pCO_2 decreased in alveoli (seen as reduced endtidal CO_2) as well as in arterial blood (hypocapnia; STUDIES 2 and 4, Tab. D-5, IIIB1 and 4). The ‚loss‘ of CO_2 led to a shift of the buffer equilibrium, which is associated with a consumption of protons and an increase in blood pH when this effect is strong. Due to the respiratory origin of this alkalotic effect, it is also called 'respiratory alkalosis'. Although hypocapnia was observed $2 - 3$ days after inoculation of higher *C. psittaci* doses (10^8 and 10^9 ifu/calf, Tab. D-5, IIIB1), it was not accompanied by an elevated blood pH (Tab. D-5, IIC), indicating the involvement of counterbalancing influences on the acid-base equilibrium.

While blood pH was not altered after 10^8 ifu/calf, a higher challenge dose resulted in a decrease of blood pH. From a classical perspective on the acid-base equilibrium there are two directions of causality. (i) Reduced blood pH might result from alveolar hypoventilation and consecutive hypercapnia (i.e. respiratory acidosis), which is in contrast to the observed hypocapnia. (ii) The inability to cover the oxygen demand of peripheral tissues may result in the necessity of anaerobe metabolism, indicated by an increased production of L-lactate and protons (i.e. a reduced pH, 'metabolic acidosis', STUDY 2, Tab. D-5, IIC).

This traditional approach of the acid-base equilibrium according to Henderson-Hasselbalch is largely based on alterations in the proton concentrations, indicated by pH. New strong ion models, taking the influence of electrolytes and non-volatile weak acids additionally into account, are a useful tool to assess mixed acid-base disorders (Stewart 1983; Constable 1997). In the present model, it was shown that chloride and sodium concentrations decreased in a dose-dependent manner during the acute phase of disease ($2 - 3$ dpi, STUDY 2, Tab. D-5, IIC). These electrolytes are attributed to the strong ions, evaluated by the strong ion difference (SID). They are characterised as independent variables which are not influenced secondary to changes in pCO_2 (Kellum 2000). In line with this suggestion, the most likely reason for the dose-dependent decrease of chloride and sodium concentrations in blood was lung tissue damage. It includes the destruction of endothelial cells expressing angiotensin-converting enzyme (ACE), and consequently a lack of angiotensin II which usually stimulates the proximal tubular resorption of sodium and chloride (Stockham and Scott 2008). The interplay of

hypochloraemia (alkalotic effect) and hyponatriaemia (acidic effect) resulted in a reduction of SID (acidic effect) during the acute phase of disease (1 – 3 dpi). This acidic effect was not reflected by a decreased pH because it was counterbalanced by the alkalotic effect of a reduced albumin concentration and the concomitant decrease of the sum of non-volatile weak acids (A_{tot}). Albumin, being the most important buffer in plasma (Constable and Stämpfli 2005) was reduced up to 10 dpi (STUDY 4, Tab. D-5,IIB), and the observed reduction is in line with its description of being a negative acute phase marker in cattle (Cray et al. 2009).

In conclusion, the consideration of SID and A_{tot} realised, for the first time, the evaluation of metabolic components within mixed acid-base disorders due to acute respiratory chlamydial infection.

3.3 Excretion and transmission of *C. psittaci* in bovines

Following experimental challenge, *C. psittaci* was predominantly excreted via faeces (5 of 21 calves challenged with 10^8 ifu/calf) but was also sporadically detectable in room-air, exhaled breath and nasal secretions. The extent of ambient contamination due to *C. psittaci* shedding sufficed for successful transmission to sentinel calves. The latter was indicated by the presence of *Chlamydiaceae* in blood (2 of 3 sentinels) and by repeated faecal shedding (3 of 3 sentinels). The observation that faecal excretion was even higher after natural *C. psittaci* infection might imply an influence of the acquisition mode on pathogen excretion, similarly as on the pathogenesis discussed in section 3.1.2.3. However, with only three sentinels in the study, this hypothesis remains to be statistically proven. The use of PCR-based methods did not allow evaluation of viability and infectivity, and recovery of chlamydial species from bovine faeces, seemingly the most important source of ambient contamination, is a very difficult task (Sachse 2013[2], personal communication). Hence it still unclear which source contained infectious EBs capable of transmitting the infection to naïve sentinel calves.

Nevertheless, frequent and intermittent shedding by clinically inconspicuous hosts (STUDY 3) is a well-known phenomenon in birds and is accepted as a hazard for human health (Harkinezhad et al. 2009a). For clinicians, contact with birds is one of the most important diagnostic cues, but 20 – 25 % of patients suffering from psittacosis do not mention contact with birds during anamnesis (Gacouin et al. 2012). With respect to uncommon sources of human psittacosis, one should also be aware of the evidence for human to human transmission (Ito et al. 2002; McGuigan et al. 2012). Moreover, host species jumps of *C. psittaci* are suggested by the phylogenetic proximity of bovine, rabbit and rat strains with psittacine strains (Pannekoek 2012). With the present model, the zoonotic potential of non-avian *C. psittaci* strains was not assessed, but as animal-to-animal transmission was possible, zoonotic transmission cannot be ruled out.

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3.4   Conclusions and directions for further research

Our studies provided new insights into the pathophysiological complexity of respiratory
C. psittaci infections in the mammalian lung. Different doses, natural infection and long-term
assessment after acute disease reveal the two facets of *C. psittaci* infection: clinically overt acute
disease *versus* almost asymptomatic persistent infection.

Whilst conducting the studies, multiple defined samples were obtained in addition for either
project partners and for asservation. These samples may serve as a basis for further in-depth
investigations addressing open questions on the pathogenesis and host-pathogen interactions.
The presented model focused on the pathophysiology within the bovine host, but it would also be
worth to specify the development of the pathogen within the host, e.g. by analysis of gene
expression patterns in lung tissue, obtained at different time points. Furthermore analysis of
cytokines involved in bovine pulmonary inflammation after *C. psittaci* infection will add
valuable information to complete the picture of pathogenesis (currently in work by collaborating
partners).

Obtained samples might also support the development and evaluation of new diagnostic tools.
Especially the development of sensitive, species-specific serologic tests, requiring defined sera
for evaluation, would improve both epidemiological risk assessment and individual diagnosis in
man and animals.

Generally this biologically relevant model is suitable for further studies focusing on vaccine
development and treatment evaluation. Under conditions of antimicrobial treatment the
occurrence of ABs would particularly be worth to be investigated. For future studies, the
developed intrabronchial administration pattern enables targeted endoscopic *in vivo* sampling of
infected areas, e.g. cytobrush, biopsy or broncho-alveolar lavage. The safety of obtaining those
samples was evaluated in human medicine before (Elston et al. 2004). Due to the potential
involvement of MALT (particularly NALT) in chlamydial pathogenesis, in further studies spray
application of a small volume of the inoculum into each nostril should be conducted in addition
to the current administration pattern.

With respect to the pathogenetic role of *C. psittaci* it would be interesting to evaluate effects of
co-infections with other relevant respiratory pathogens. Cell culture co-infection models of
Human Herpes Simplex Virus and *C. trachomatis* demonstrated already interactions, e.g. the
induction of chlamydial ABs (Deka et al. 2007; Vanover et al. 2010) and raise the question of
veterinary relevance of chlamydia-herpes virus co-infections in animals (e.g. galline Herpes
Virus 2 + *C. psittaci* or bovine Herpes Virus 1 + *C. pecorum* or *C. psittaci*).

With respect to the zoonotic potential of non-avian *C. psittaci* strains, the present model was able to elucidate that transmission within the bovine species is possible, implicating that also zoonotic transmission might be possible. A useful ONE HEALTH approach would be the investigation of the prevalence in cattle herds and relation to the prevalence and lung health in humans in regular contact with these herds.

4   Summary

Chlamydia (*C*.) *psittaci* is a gram-negative, obligate intracellular bacterium, capable of inducing respiratory disease and persistent infection. The host range of this zoonotic pathogen includes not only birds and man, but also various wild and domestic mammals. Knowledge about pathogenesis and functional consequences of *C. psittaci* infection in the mammalian lung has remained elusive to date.

The present project aimed to develop, to evaluate and to characterise a bovine model of experimentally induced respiratory *C. psittaci* infection which might be beneficial for both human and veterinary medicine. Bovines were chosen as the host because (i) bovine *C. psittaci* infection closely reflects the situation in a natural host, and (ii) the bovine lung is relevant to model functional consequences of ventilatory disorders due to its segmental anatomy and the lack of collateral airways. Moreover, the pathogenetic potential of *C. psittaci* for bovines and potential transmission routes were evaluated by assessing clinical and immunological variables of health and lung function as well as the shedding of this potentially zoonotic pathogen.

A total of 69 Holstein-Friesian calves aged 6 – 9 weeks were included in four separated studies (STUDY 1 – 4) and were challenged as follows:

- Intra-bronchial application of viable *C. psittaci*, strain DC 15 (n = 35)
- Intra-bronchial application of UV-inactivated *C. psittaci*, strain DC 15 (n = 6)
- Intra-bronchial application of cell culture medium (n = 25)
- Naïve calves (i.e. sentinels) were socialised with acutely diseased animals due to experimentally induced *C. psittaci* infection (n =3).

Each intra-bronchial application was performed according to a previously developed protocol. In brief, a total volume of 6 mL inoculum/calf was endoscopically administered and distributed in a standardised way into 8 defined bronchi of each animal.

In STUDIES 1 & 2, dose response relationships were evaluated up to 3 days post inoculation (dpi). Thus, 14 calves received different doses of viable *C. psittaci* (10^6 – 10^9 inclusion forming units (ifu)/calf). The use of two control groups enabled the separated evaluation of effects mediated by cell culture medium (n = 4) and chlamydial cell components (10^8 UV-inactivated ifu/calf, n = 6).

In STUDIES 3 & 4, time courses and pathophysiological consequences of an acute respiratory *C. psittaci* infection induced by 10^8 ifu/calf (n = 21) were assessed in a follow-up study ending at 35 dpi. In addition to the assessment of intra-individual time courses within each calf, a control group was challenged by cell culture medium only (n = 21) for inter-subject comparison. Furthermore, a group of naïve calves (sentinels, n = 3) was socialised with the infected animals, and aimed at evaluating risks and routes of transmission as well as the course of natural infection.

In the different studies, systemic host response was assessed by clinical signs, variables of innate immune response, acute phase proteins, and acid-base parameters including electrolytes and metabolites in the peripheral blood. To evaluate pulmonary inflammation, the concentrations of eicosanoids and total protein were measured in broncho-alveolar lavage fluid (BALF) in addition to BALF cytology and pathological as well as histological characterisation of lung lesions. Pulmonary function tests included arterial blood gas analysis, haemoxymetry as well as the assessment of respiratory mechanics, alveolar ventilation and the pattern of breathing. For the latter, non-invasive pulmonary function methods (originally adapted from human medicine and previously evaluated for calves) were used, i.e. impulse oscillometry, volumetric capnography, and helium dilution re-breathing test. Excretion and distribution of the pathogen was assessed by specific nucleic acid-based detection assays (real-time PCR, microarray).

The results obtained in the 4 STUDIES can be summarised as follows. Administration of the viable *C. psittaci* strain succeeded in inducing reproducible infections. Disease outcome was largely dose-dependent. While 10^6 ifu/calf resulted in mild clinical signs, doses of 10^7 to 10^8 ifu/calf induced a moderate respiratory disease, and 10^9 ifu/calf a severe clinical illness. Also, severity of respiratory and clinical signs, extent and quality of pneumonia, and systemic inflammation increased with increasing challenge doses and resulted in gradually reduced efficacy of pulmonary gas exchange. After inoculation of the finally defined dose of 10^8 ifu/calf, clinical signs peaked 2 – 3 days post inoculation (dpi). Signs and markers of acute disease subsided considerably, but not completely, within 10 dpi after acute illness. Sentinels acquired the infection but did not develop visible signs of an apparent disease. Thus, two different facets of *C. psittaci* infection in bovines could be distinguished by the present model: (i) acute clinical disease after experimental challenge and (ii) clinically inconspicuous persistent infection after natural exposure to the pathogen. In both groups, systemic spread and ongoing host-pathogen interactions were detected by chlamydaemia, faecal shedding of the pathogen, and slightly increased levels of monocytes and lipopolysaccharide-binding protein (LBP) in blood, indicating that neither group eliminated the chlamydiae within 5 weeks after exposure.

Pathophysiologically, inflammatory cells, mainly neutrophil granulocytes, were recruited into the lung during the acute phase of infection. Pulmonary inflammation resulted in tissue damage, accumulation of detritus and protein rich fluid causing reduced gas exchange, airway obstructions and pulmonary restrictions. Attempts to compensate for alveolar hypoventilation and hypoxaemia included the elevation of both respiratory rate and minute ventilation. Consequences for the acid-base equilibrium were not only determined by pulmonary dysfunctions, compensatory mechanisms or anaerobe metabolism, which are classically assessable by the Hendersen-Hasselbalch approach. A strong ion model revealed mixed acid-base disorders, mediated by metabolic and immunologic influences, i.e. by the reduction of

chloride and sodium (assessed by the strong ion difference, SID) and by hypo-albuminaemia and hyper-gammaglobulinaemia (assessed by the sum of non-volatile weak acids, A_{tot}).

After experimental challenge, the pathogen was not only detected in lung tissue and blood, but it was also excreted via faeces, nasal excretions and exhaled breath. Despite complete daily cleaning of the animal rooms, contamination sufficed to transmit the infection to naïve sentinels. Although these naturally exposed animals did not develop obvious clinical signs, they became frequent faecal shedders, suggesting a risk of spreading the pathogen under natural conditions.

Humoral immune response was generally weak. Only two thirds of experimentally challenged calves developed specific antibodies against *C. psittaci* detected by immunoblotting. In sentinels, no humoral immune response was observed.

In conclusion, the newly introduced large animal model provides a valuable support in elucidating the pathophysiology and complex interactions during pathogenesis of *C. psittaci* infection in the mammalian lung. In comparative medicine, it can be regarded as a translational model. Pulmonary function tests derived from human medicine and applicable to calves provided comparable data between bovines and humans about pulmonary dysfunction involved in the pathogenesis of this respiratory infection. In veterinary medicine, this biologically relevant model may serve as a suitable basis for studies focusing on vaccine development and treatment evaluation.

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5 Zusammenfassung

Evaluierung und pathophysiologische Charakterisierung eines bovinen Modells der respiratorischen *Chlamydia psittaci* Infektion

Einleitung: *Chlamydia* (*C.*) *psittaci* ist ein gram-negatives, obligat intrazelluläres Bakterium, welches in der Lage ist, respiratorische Erkrankungen und persistente Infektionen bei Menschen und Vögeln zu induzieren, und dessen zoonotisches Potential schon lange bekannt ist. Das Wirtsspektrum erweiterte sich mit der Verbesserung molekularbiologischer Nachweismethoden auf diverse Wild- und Haussäugetiere. Bislang sind jedoch die pathogenetische Bedeutung und die funktionellen Konsequenzen der *C. psittaci* Infektion für die Säugetierlunge nur unzureichend erfasst.

Ziele und Projektdesign: Um die Pathogenese der respiratorischen *C. psittaci* Infektion in der Säugetierlunge besser zu verstehen und um das pathogenetische und epidemiologische Potential von *C. psittaci* bei Rindern bewerten zu können, bestand das Ziel des vorgestellten Projekts in der Etablierung und Charakterisierung eines bovinen Modells der experimentell induzierten respiratorischen *C. psittaci* Infektion.

Hierfür war zunächst zu klären, ob mit einem *C. psittaci*-Stamm boviner Herkunft unter experimentellen Bedingungen eine in ihrem Schwergrad von der Inokulationsdosis abhängige, klinisch manifeste Erkrankung im Kalb induzierbar ist (STUDIE 1). Darauffolgend waren die dosisabhängigen Effekte dieser *C. psittaci*-Infektion auf die Gasaustauschfunktion der Lunge, die angeborenen Immunabwehrmechanismen und die Akute-Phase-Reaktion pathophysiologisch zu charakterisieren (STUDIE 2). Des Weiteren galt es zu eruieren, ob bzw. wie der Erreger sich im Wirtsorganismus ausbreitet, wie lange und über welche Wege er ausgeschieden wird, ob die Infektion spontan von Tier zu Tier übertragbar ist, und wie sich eine spontan erworbene Infektion von einer experimentell induzierte Infektion im Hinblick auf das klinische Bild und die pathophysiologischen Mechanismen unterscheidet (STUDIE 3). Die Evaluierung der durch *C. psittaci* induzierten pulmonalen Dysfunktionen und Störungen des Säuren-Basen-Gleichgewichts waren Inhalt von STUDIE 4 und dienten dem Ziel, die wesentlichsten Funktionsstörungen auf Organebene aufzuklären, die dem klinischen Bild der induzierten akuten respiratorischen Erkrankung zugrunde liegen.

Tiere, Material und Methoden: Insgesamt wurden 69 Holstein-Friesian Kälber im Alter von 6 bis 9 Wochen in das Gesamtprojekt eingeschlossen und wie folgt experimentell belastet:

- intrabronchiale Applikation von lebensfähigen *C. psittaci*, Stamm DC 15 (n = 35)
- intrabronchiale Applikation von UV-inaktivierten *C. psittaci*, Stamm DC 15 (n = 6)
- intrabronchiale Applikation von Zellkultur-Medium (n = 25)
- naive Sentinel-Kälber, die mit akut erkrankten Kälbern (nach experimenteller Applikation lebensfähiger *C. psittaci*) vergesellschaftet wurden (n = 3).

Alle intrabronchialen Applikationen erfolgten nach einem standardisierten Inokulationsschema, wonach je Tier 6 ml Inokulat in definierten Portionen an 8 definierten Lokalisationen abgesetzt wurden.

STUDIEN 1 & 2: Zur Beurteilung der Dosis-Wirkungs-Beziehung erhielten 14 Tiere Dosen von 10^6 bis 10^9 einschlussbildenden Einheiten (EBE) lebensfähiger *C. psittaci* pro Kalb. Der Vergleich zu zwei Kontrollgruppen, denen entweder Zellkultur-Medium (n = 4) oder 10^8 UV-inaktivierte EBE/Kalb (n = 6) inokuliert wurden, ermöglichte die Bewertung von unspezifischen Effekten.

STUDIEN 3 & 4: Auf der Basis einer standardisierte Inokulation von 10^8 EBE/Kalb (n = 21) wurden die pathophysiologischen Langzeit-Auswirkungen der akuten respiratorischen *C. psittaci* Infektion sowohl im intra-individuellen Verlauf als auch im Vergleich zu einer mit Zellkultur-Medium inokulierten Kontrollgruppe (n = 21) erfasst (Studienende 35 Tage nach Inokulation (dpi)). Des Weiteren wurden die Ausscheidungs- und Transmissionswege des inokulierten Erregers evaluiert. Die Einbeziehung von naiven Sentinel-Kälbern (n = 3) diente der Dokumentation des Krankheitsverlaufes nach spontan erworbener Infektion.

Die Beurteilung der systemischen Wirtsantwort auf den Erreger basierte auf folgenden Kenngrößen: klinischer Score, Parameter der zellulären und humorale Immunantwort, Akute-Phase-Proteine, Parameter des Säuren-Basen-Status sowie Konzentrationen von Elektrolyten und Metaboliten im peripheren Blut. Lokale Entzündungsmechanismen innerhalb der Lunge wurden sowohl durch die Analyse von inflammatorischen Markern in der broncho-alveolären Lavageflüssigkeit (Konzentrationen von Eicosanoiden und Gesamtprotein, Differentialzellbild) als auch mittels pathologischer und histologischer Untersuchungen charakterisiert. Die Erfassung der pulmonalen Dysfunktionen erfolgte quantitativ und qualitativ auf der Basis von arteriellen Blutgasen und Parametern der Hämoxymetrie sowie Parametern der Atmungsmechanik, des Atmungsmusters und der alveolären Ventilation. Für die Lungenfunktionstests standen nicht-invasive Methoden aus der Humanmedizin zur Verfügung (Impuls-Oszilloresistometrie, volumetrische Kapnographie, Helium-Dilution), die zuvor für den Einsatz am wachen Kalb unter Spontanatmung validiert worden waren.
Für die Detektion des Erregers im Blut, im Lungengewebe, in Kot-, Nasen- und Augentupfern, sowie in der Ausatem- und Raumluft kamen Polymerase-Kettenreaktion (PCR)-basierte Methoden zum Einsatz (real-time PCR, Microarray).

Ergebnisse:

Dosis-Wirkungs-Beziehung und Infektionsverlauf

Aus der experimentellen Inokulation des vitalen *C. psittaci*-Stammes resultierten reproduzierbare, von der Erregerdosis abhängige, klinische Bilder einer milden (10^6 EBE/Kalb), klinisch manifesten (10^7 bis 10^8 EBE/Kalb) oder schweren (10^9 EBE/Kalb) respiratorischen Erkrankung. Mit ansteigenden Erreger-Dosen stiegen zugleich die respiratorischen und

klinischen Scorewerte, das Ausmaß der systemischen Entzündungsreaktionen, die Quantität und Qualität der pneumonischen Veränderungen und die Einschränkungen im pulmonalen Gasaustausch.

Nach Inokulation des Erregers erreichte die akute klinische Krankheits-Phase 2 – 3 dpi ihren Höhepunkt und klang innerhalb von 7 – 10 dpi wieder ab. Sentinel-Kälber akquirierten die Infektion, bildeten aber keine vergleichbaren klinisch manifesten Symptome aus. Interessanterweise entwickelten sich in beiden Gruppen – also nach dem Abklingen der akuten Krankheitsphase bzw. nach der auf natürlichem Wege erworbenen Infektion – klinisch unauffällige, persistente Infektionsverläufe ohne *restitutio ad integrum* und ohne komplette Erreger-Elimination. Indikatoren hierfür waren eine bis ca. 5 Wochen nach Erregeraufnahme andauernde Bakteriämie, eine fortwährende Erregerausscheidung im Kot sowie leicht erhöhte Gehalte von Monozyten und Lipopolysaccharid-bindenden Protein im Blut.

Pathogenese und Pathophysiologie

Während der akuten Erkrankungsphase wurden Entzündungszellen, vor allem neutrophile Granulozyten, in die Lunge rekrutiert. Infektion und Entzündung der Lunge verursachten Gewebeschäden, Anreicherung von Detritus und proteinreicher Flüssigkeit, welche sowohl Obstruktionen der Atemwege als auch eine verminderte Dehnbarkeit des Lungengewebes (bis 11 dpi) zur Folge hatten. Die pathophysiologischen Konsequenzen dieser inflammatorischen Prozesse waren: alveolären Hypoventilation, ein reduzierter pulmonaler Gasaustausch sowie Hypoxämie, in deren Folge kompensatorisch die Atmungsfrequenz und das Atemminuten-volumen anstiegen. Imbalancen des Säuren-Basen-Gleichgewichts resultierten nicht nur aus pulmonalen Dysfunktionen, kompensatorischen Mechanismen oder anaerobem Metabolismus, sondern auch aus der Reduktion von Chlorid- und Natriumkonzentrationen im Blut sowie einer Hypoalbuminämie und Hypergammaglobulinämie.

Nach experimenteller Infektion war der Erreger nicht nur im Lungengewebe, sondern auch in Blut, Kot, Nasensekret und in der Ausatemluft nachweisbar. Trotz kompletter täglicher Säuberung der Tierräume reichte diese Kontamination aus, um die Infektion auf naive Sentinel-Kälber zu übertragen. Obwohl diese Tiere nur milde klinische Krankheitszeichen entwickelten schieden sie den Erreger häufig aus, was auch auf ein Verbreitungsrisiko unter natürlichen Haltungsbedingungen schließen lässt.

Die humorale Immunantwort war insgesamt schwach ausgeprägt. Nur zwei Drittel der experimentell inokulierten Kälber entwickelten gegen *C. psittaci* gerichtete Antikörper. Im Gegensatz dazu war bei keinem der 3 Sentinel-Kälber eine humorale Wirtsreaktion nachweisbar.

Schlussfolgerungen: Das etablierte Modell leistet einen wertvollen Beitrag zur Aufklärung der Pathophysiologie und der komplexen pathogenetischen Interaktionen der *C. psittaci* Infektion in der Säugetierlunge. In der vergleichenden respiratorischen Medizin ist es von translationalem Nutzen, insbesondere da die Lungenfunktionstests eine gute Vergleichbarkeit der Daten

zwischen Kalb und Mensch erlauben. In der Veterinärmedizin bietet dieses biologisch relevante Tiermodell eine solide Grundlage für weiterführende Studien zur Wirksamkeit von Vakzinen oder medikamentöser Behandlungen.

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7   List of abbreviations

a	arterial
AA	arachidonic acid
A-aO$_2$	alveolar arterial oxygen partial pressure difference
ABs	aberrant bodies
ACE	angiotensin-converting enzyme
AG	anion gap
a.i.	*ante inoculationem* (latin for before inoculation)
Alb	albumin
ANOVA	multifactorial analysis of variance
APPs	acute phase proteins
A$_{tot}$	acid total (represents the sum of non-volatile weak acids)
BALF	broncho-alveolar lavage fluid
BALT	bronchus-associated lymphoid tissue
BGM	buffalo green monkey kidney
BGP	bisphosphoglycerate
BHV-1	bovine herpes virus 1
BMBF	Bundesministerium für Bildung und Forschung (German Federal Ministry of Education and Research)
BRSV	bovine respiratory syncytial virus
$_{BT}$	mathematically corrected for individual body temperature measured immediately before blood sampling
BT	body temperature
BVDV	bovine virus diarrhoea/mucosal disease virus
b.w.	body weight
c	concentration
C.	*Chlamydia*
Ca^{2+}	calcium
CAP	community-acquired pneumonia
cBase	actual base excess
cBase (Ecf)	standard base excess
Cl$^-$	chloride
CO	carbon monoxide
CO$_2$	carbon dioxide
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
Cp	*Chlamydia psittaci*
CRP	C-reactive protein
ctHb	concentration of total haemoglobin
dpc	days post contact
dpi	days post inoculation
EBs	elementary bodies
e.g.	example giving
ELISA	enzyme-linked immunosorbent assay
Eq	equivalent

F_c	fragment crystallisable
FRC	functional residual capacity
h	hour
Hb	haemoglobin
HCO_3^-	bicarbonate
$HCO_3^-(st)$	standard bicarbonate
Hct	haematocrit
He	Helium
HETE	hydroxyeicosatetraenoic acid
HHb	percentage of deoxy- haemoglobin in total haemoglobin
Hp	haptoglobin
HPLC	high-performance liquid chromatography
HPV	hypoxic pulmonary vasoconstriction
HRP	horseradish peroxidase
HSP60	heat shock protein 60
Hz	Hertz
g	earth's standard acceleration due to gravity
IBs	intermediate bodies
i.e.	*id est* (latin that is)
IFN	interferon
ifu	inclusion-forming units
Ig	immunoglobulin
IL	interleukin
IOS	impulse oscillometry system
IU	international units
k	kilo
K^+	potassium
K_a	acid dissociation constant
Kdo	3-deoxy-alpha-d-manno-oct-2-ulosonic acid
LBP	lipopolysaccharide-binding protein
LC-MS	liquid Chromathography – Mass Spectrometry
LC-MS-MS	liquid Chromathography – Tandem Mass Spectrometry
LPS	lipopolysaccharide
LSD	least significant difference
m	number of strong ions measured in plasma
MALT	mucosal-associated lymphoid tissue
max	maximum
min	minimum
MOMP	major outer membrane protein
mRNA	messenger ribonucleic acid
n	sample size
N.	*nervus* (latin for nerve)

N_2	nitrogen
Na^+	sodium
NALT	nasal-associated lymphoid tissue
n.d.	not defined
NO	nitric oxide
O_2	oxygen
O_2Hb	percentage of oxy-haemoglobin in total haemoglobin
OD	optical density
OEA	ovine enzootic abortion
OIE	World Organisation for Animal Health
ompA gene	MOMP encoding gene
P	probability level
p	partial pressure
p50	partial pressure of oxygen at 50% haemoglobin saturation
Pa	Pascal
$p(a)O_2$	partial pressure of oxygen in arterial blood
$p(a)CO_2$	partial pressure of carbon dioxide in arterial blood
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFTs	pulmonary function tests
PG	prostaglandin
PGE2	prostaglandin E2
pH	negative logarithm of the activity of the (solvated) hydronium ions
PI-3 virus	parainfluenza 3 virus
pK_a	$-\log_{10}$ of the acid dissociation constant (K_a)
Prt	protein total
PVDF	polyvinylidene difluoride membranes
RBs	reticular bodies
Rdist	distal airway resistance
resp.	respiratory
RNA	ribonucleic acid
Rprox	proximal airway resistance
RR	respiratory rate
rRNA	ribosomal ribonucleic acid
Rrs	respiratory resistance
rt – PCR	real-time polymerase chain reaction
S	subunit of the bacterial ribosome
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SID	strong ion difference
$SID_{m+number}$	calculation of SID based on the number of strong ions measured in plasma
SIG	strong ion gap
sO_2	percentage of haemoglobin saturated with oxygen
SO_4^{2-}	
SPGA	stabilising medium (containing saccharose, phosphatile substances, glucose and bovine albumin)

ssAB   strain specific antibodies

Tab.   table
TX   thromboxane
TXB2   thromboxane B2
Tex   time of expiration
Tin   time of inspiration
TLR   toll-like receptor

UA   unmeasured anions
UC   unmeasured cations

v   venous
V'   airflow
V'ex   airflow during expiration
V'in   airflow during inspiration
VC   volumetric capnography
Vd   dead space volume
Vd_{alv} alveolar dead space volume
Vd_{anat} anatomical dead space volume
Vd/Vt   ratio between dead space volume and tidal volume
Vd:Vt   ratio between dead space volume and tidal volume
Vmin   volume of minute ventilation
Vt   tidal volume

WBC   white blood cell count
w/v   weight to volume ratio

Xrs   respiratory reactance

8   List of figures

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Introduction

Publications

**STUDY 1: A Bovine Model of Respiratory *Chlamydia psittaci* Infection:
        Challenge Dose Titration**

**STUDY 2: Dose-dependent effects of *Chlamydia psittaci* infection on pulmonary gas
        exchange, innate immunity and acute-phase reaction in a bovine respiratory
        model**

**STUDY 3: Infection, Disease, and Transmission Dynamics in Calves after Experimental
        and Natural Challenge with a Bovine *Chlamydia psittaci* Isolate**

**STUDY 4: Evaluation of pulmonary dysfunctions and acid base imbalances induced by
        Chlamydia psittaci in a bovine model of respiratory infection**

Discussion

10   List of publications

10.1   Publications in peer-reviewed journals

Reinhold, P.; **Ostermann, C**. ; Liebler-Tenorio ,E.; Berndt, A.; Vogel, A.; Lambertz,, J.; Rothe, M.; Rüttger, A.; Schubert, E.; Sachse, K. (2012):
A Bovine Model of Respiratory *Chlamydia psittaci* Infection: Challenge Dose Titration.
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Dose-dependent effects of *Chlamydia psittaci* infection on pulmonary gas exchange, innate immunity and acute-phase reaction in a bovine respiratory model.
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Infection, Disease, and Transmission Dynamics in Calves after Experimental and Natural Challenge with a Bovine *Chlamydia psittaci* Isolate.
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Ostermann, C.; Linde, S.; Siegling-Vlitakis, C.; & Reinhold, P. (2014):
Evaluation of pulmonary dysfunctions and acid–base imbalances induced by *Chlamydia psittaci* in a bovine model of respiratory infection.
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11   Danksagung

12   Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Jena, den 15.10.2013   Carola Ostermann