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Evaluation of antimicrobial treatment strategies against *Chlamydia psittaci* using a bovine respiratory infection model

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Meiner Familie

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1 Introduction

1.1 Background/Objective

The facultative intracellular *Chlamydiae* cause a variety of infections in humans and animals. Their successful antimicrobial treatment is still an unresolved issue. The aim of this project was to evaluate the efficacy of common treatment regimens of chlamydial infections in an experimental setting, and to compare them to newly proposed treatment regimens that had been evaluated *in vitro* before. The treatment studies were to be carried out in a previously established bovine model of respiratory *Chlamydia psittaci* infection (Reinhold*, et al.* 2012; Ostermann*, et al.* 2013a; Ostermann*, et al.* 2013b; Ostermann*, et al.* 2014). The model is well characterized in terms of inoculation dose and scheme as well as kinetics of clinical course, inflammatory parameters and pathological changes in inoculated animals. To monitor the host's reaction to treatment during the study, bronchoscopical sampling methods had to be adapted for the use in calves at the age of 6-10 weeks. The outcome of the study was expected to give valuable insights into the treatment of chlamydial infections in both, human and veterinary medicine.

The following introduction will familiarize the reader with important characteristics of *Chlamydiae* and their relevance in the human and bovine host. It will also provide background information on the bovine model, on the current treatment options for chlamydial infections in human and veterinary medicine, and on the antimicrobial substances used in the present treatment studies. Finally, aim and design of the project are explained in detail to exemplify the logical context of the three studies included in this thesis.

1.2 The pathogen - Chlamydiae

1.2.1 History and taxonomic classification

Chlamydiae are nonmotile, obligate intracellular, gram negative bacteria with a characteristic biphasic developmental cycle. The name originates from their detection in intracytoplasmatic vacouoles in scrapings from trachoma cases in 1907 by Halberstaedter and von Prowazek, who thought to have discovered "mantled protozoans". They named their findings "*Chlamydozoa*", originating from the Greek word "Chlamys/Khlamus", meaning mantle (Halberstaedter and von Prowazek 1907; Halberstaedter and von Prowazek 1909). It took another 60 years until *Chlamydiae* were finally classified as bacteria (Moulder 1966).

The Phylum *Chlamydiae* contains one Class, *Chlamydiia*, which includes only one Order, the *Chlamydiales,* currently consisting of eight families. Throughout the decades, taxonomy of chlamydiae was subject to numerous changes. Page introduced the genus *Chlamydia* within the family *Chlamydiaceae* in 1966 (Page 1966). First, the genus *Chlamydia* included only the two species *C. trachomatis* (strains from human sources) and *C. psittaci* (strains from animal sources) (Page 1968; Storz and Page 1971). *C. pneumoniae* (Campbell*, et al.* 1989) and *C. pecorum* (Fukushi and Hirai 1992) were first described more than 20 years later. With the availability of

molecular methods, the diversity of *Chlamydiaceae* became obvious, and the family was divided into two genera (*Chlamydia* and *Chlamydophila*) with nine species (Everett*, et al.* 1999). Therefore, literature published before 1999 must be regarded critically. Currently, the genus *Chlamydophila* is not well accepted and is therefore not used by most researchers in the field (Sachse*, et al.* 2015). Recently published genomic data and insights on shared unique biological properties have led to the proposal to reunite all species into one genus, *Chlamydia* (Schachter*, et al.* 2001; Stephens*, et al.* 2009; Greub 2010; Bavoil*, et al.* 2013; Sachse*, et al.* 2015). The single genus *Chlamydia* is already used by the current edition of Bergey's Manual (Kuo*, et al.* 2011b), even though it is not yet formally revised according to the requirements of the Bacterial Code (Lapage*, et al.* 1992).

1.2.2 Species

Assuming only one genus, *Chlamydia* (Kuo*, et al.* 2011a), there are currently 11 species. *Chlamydia (C.) trachomatis, C. muridarum, C. pneumoniae, C. pecorum, C. suis, C. abortus, C. felis, C. caviae*, and *C. psittaci* were described in 1999 (Everett*, et al.* 1999). Very recently, two new species, *C. avium* and *C. gallinacea,* have been introduced (Sachse*, et al.* 2014) and another one*, 'Candidatus* C. ibidis' (Vorimore*, et al.* 2013), has been proposed. Hosts and most common infection sites of the current species are given in Table 1.

Table 1. Hosts and most common infection sites of *Chlamydia* spp.

species	Natural host	Other hosts	Site of infection
*C. abortus**	Sheep, goat	Cattle, swine, horse, birds, human	Genital, respiratory
C. avium	Pigeon, parrots		Respiratory
C. caviae	Guinea pig	Horse, cat, rabbit, dog, human	Ocular, genital
*C. felis**	Cat	Dog, human	Ocular, respiratory
C. gallinacea	Chicken	Other poultry	Respiratory
C. muridarum	Rodents		Gastro-intestinal
C. pecorum	Cattle, koala	Sheep, goat, swine, birds	Gastro-intestinal, respiratory, urogenital
C. pneumonia	Human, horse, koala	Cat, amphibians, reptiles	Respiratory, cardiovascular
*C. psittaci**	Birds	Mammals	Respiratory
C. suis	Swine	Ruminants, horse	Gastro-interstinal, genital
C. trachomatis	Human	Swine, birds	Ocular, urogenital

* species with known zoonotic potential

modified from (Reinhold*, et al.* 2013; Sachse*, et al.* 2015).

1.2.3 Developmental cycle

Members of the family *Chlamydiaceae* undergo a characteristic biphasic developmental cycle. It comprises of the small (0.3 µm diameter) elementary bodies (EB), which are located extracellularly and are the infectious form, and the larger (0.5-1.6 µm diameter) metabolically active reticulate bodies (RB), which are located intracellularly and are noninfectious.

EB are small, round, electron dense and metabolically inert. Their rigid cell wall enables them to survive in an extracellular environment, representing a "spore-like" form of the pathogen. The rigidity of their cell wall is due to a large amount of cystine bonds between the cystine rich proteins of the outer envelope (outer membrane protein A, outer membrane complex protein (Omc) B, OmcA) (Hatch 1999). The high density of the EB is caused by the condensation of nuclear material by the bacterial histone-like proteins HctA and HctB (Barry*, et al.* 1992; Brickman*, et al.* 1993). EB bind to their target cells in a two-step process. Electrostatic interactions between host cell and RB lead to a reversible initial attachment (Su*, et al.* 1996; Su and Caldwell 1998; Stephens*, et al.* 2001; Taraktchoglou*, et al.* 2001). The following irreversible binding to the host cell is further secured by ligands, such as heatshock protein 70, OmcB, and the major outer membrane protein (Su*, et al.* 1990; Raulston*, et al.* 1993; Ting*, et al.* 1995; Carabeo and Hackstadt 2001; Stephens*, et al.* 2001). Immediately after the irreversible binding step, chlamydial proteins are exported into the host cell by the Type III secretion system (TTSS) of the EB (Clifton*, et al.* 2004). Now, the EB are internalized into the host cell by phagocytosis. *Chlamydiae* have the ability to prevent fusion of phagosome and lysosome, thus avoiding destruction by the host cell. The required energy for the whole processes is provided by the host cell, making *Chlamydiae* so called energy parasites. Their membrane is permeable to host adenosine triphosphate (ATP) and their intrinsic ATPase can generate energy by hydrolization of ATP (Peeling*, et al.* 1989). In the process of differentiation from EB to RB, the chromosomes are decondensed and the genome becomes transcriptionally active. This is resembled by the granular appearance of the cytoplasm and the presence of diffuse fibrillary nucleic acids (Rockey and Matsumoto 2000). This stage between EB and RB is referred to as intermediate body by some authors. The function of the so called early genes, which are transcribed in the beginning of the developmental cycle, are (i) nutrient acquisition from the host cell, (ii) modifying of the parasitophorous vacuole, and (iii) to prevent lysosomal fusion. Once internalized, the chlamydial inclusions are moved close to the Golgi apparatus, where they fuse with sphingomyelin containing host vesicles (Abdelrahman and Belland 2005). After 6-8 h post inoculation (pi) the RB are highly transcriptionally active, and the most intense transcriptional activity at 16-24 h pi, the middle part of the circle, correlates with rapid growth and division by binary fission of the RB (Belland*, et al.* 2003; Nicholson*, et al.* 2003). With a size of 1 - 2.4 Megabases, the chlamydial genome is minimal in size, and virtually every gene is expressed at some point of the developmental cycle (Belland*, et al.* 2003; Tan and Bavoil 2012). For the differentiation from RB to EB, late cycle genes are expressed. Amongst other things, they encode for components of the outer membrane complex (e.g., OmcA and B), proteins necessary for chromosome condensation (e.g., HctA and B), and proteins needed for the early stage of the next infectious cycle (e.g., parts of the TTSS) (Hatch 1999; Abdelrahman and Belland 2005). The release of infectious EB

follows by host cell lysis or extrusion after 30-72 hours, depending on host species and strain (Hybiske and Stephens 2007). The developmental cycle of chlamydiae is not synchronized, the phases overlap, and RB can be present in the same inclusion as EB. Cells first invaded by chlamydiae in the living host are mucosal epithelial cells, although many other cell types can be successfully infected in cell culture (Moulder 1991; Rasmussen, *et al.* 1997; Stephens 2003).

1.2.4 Chlamydial persistence

Under certain environmental conditions, chlamydiae can interrupt their productive developmental and enter a state termed persistence. Persistence in chlamydiae is defined as a developmental stage in which the chlamydiae are viable but non-infectious (Schoborg 2011). This definition is different from the commonly used microbiological term persistence, meaning a long-term infection of a host. Persistent chlamydiae occur in the form of so called aberrant bodies (AB), morphologically characterized by larger size and less electron density than RB, and irregular shape (Matsumoto and Manire 1970). In cell culture, AB can be induced by exposure to interferon-γ (IFNγ) or penicillin, chlamydiaphage infection, heat shock, and deprivation of amino acids, iron and glucose (reviewed in Hogan, *et al.* 2004). Newer cell culture models of chlamydial persistence include cigarette smoke exposure (Wiedeman, *et al.* 2004) and co-infection with viruses (Deka, *et al.* 2006; Deka, *et al.* 2007; Borel, *et al.* 2010). After removal of the inducer, chlamydiae continue reproduction. Persistence seems to allow them to withstand hostile conditions within the host cell to maintain the infection. *In vivo*, aberrant bodies were for example detected in the gut of *C. suis* infected pigs (Pospischil, *et al.* 2009) and in human atherosclerotic tissue (Borel, *et al.* 2008), and could be experimentally induced in mice (Phillips Campbell, *et al.* 2012). Nevertheless, it has not yet been proven that chlamydiae enter persistence to establish chronic infections, although this is often proposed as a strategy to outlast antibiotic treatment and as an explanation for limited success in the treatment of chlamydial infections.

1.2.5 Common chlamydial infections in humans

Infections with chlamydiae can lead to a variety of diseases in humans. Urogenital infections with *C. trachomatis* are the most common sexually transmitted genital infection worldwide (Kucinskiene, *et al.* 2006). In women, up to 70% of the infections remain asymptomatic, but pelvic inflammatory disease, tubal factor infertility, and ectopic pregnancy are well documented complications. In men, approximately half of the infected individuals remain without symptoms. The most common clinical manifestation in males is nongonococcal urethritis, which can be accompanied by epididymitis and orchitis (reviewed in (Ljubin-Sternak and Mestrovic 2014)). Among men who have sex with men, lymphogranuloma venereum has become endemic in industrialized countries in the last decade (White and Ison 2008). Occular *C. trachomatis* infections are the most common infective cause of blindness and are wide spread in developmental countries. They cause trachoma, an intense inflammation of the conjunctivae, leading to scarring, entropium, trichiasis, and finally blindness (Taylor, *et al.* 2014).

C. pneumoniae is widespread in the human population with seroprevalence of over 50% among adults in different countries (Kanamoto, *et al.* 1991; Marton, *et al.* 1992). Infections with *C.*

pneumoniae mainly affect the respiratory tract and may lead to persistent upper respiratory tract infections and community acquired pneumonia. Also, there is cumulating evidence for a causative role of *C. pneumoniae* in the pathogenesis of asthma in a subgroup of patients (Atkinson 2013). A study in children with therapy-refractory bronchitis or pneumonia could associate bronchial *C. pneumoniae* infection with a more severe disease in case of several, mostly bacterial co-infections. Furthermore, pulmonary function has been demonstrated to improve after adequate antibiotic therapy for *C. pneumoniae* infection (Schmidt, *et al.* 2005).
Contribution of *C. pneumoniae* to the pathogenesis of atherosclerosis is strongly suggested as viable organisms could be isolated from atherosclerotic plaques, and numerous clinical studies and animal models support this hypothesis (Rosenfeld and Campbell 2011).

As a complication, patients may develop reactive and chronic arthritis after infection with *C. trachomatis* or *C. pneumoniae* (Zeidler and Hudson 2014).

1.2.6 Common chlamydial infections in cattle

C. pecorum and *C. abortus* have been detected in cattle regularly (Jee, *et al.* 2004; Twomey, *et al.* 2006; Kauffold, *et al.* 2007; Kemmerling, *et al.* 2009; Pantchev, *et al.* 2009). The seroprevalence of chlamydial infections in cattle is up to 90% in randomly selected herds (El-Rahim 2002) and up to 100% in pre-selected herds with a history of possibly chlamydia-associated problems (Kaltenboeck, *et al.* 1997; Wang, *et al.* 2001; Jee, *et al.* 2004). Mixed infections with more than one chlamydial species are often seen (Jaeger, *et al.* 2007; Reinhold, *et al.* 2008).

Clinical manifestation of chlamydial infections in cattle can be obvious and cause abortion (Pospischil, *et al.* 2002; Borel, *et al.* 2006), polyarthritis (Storz, *et al.* 1966; Twomey, *et al.* 2003), keratoconjunctivitis (Otter, *et al.* 2003), pneumonia (Wilson and Thomson 1968), enteritis (Doughri, *et al.* 1974), hepatitis (Reggiardo, *et al.* 1989), encephalomyelitis with systemic infection (Jelocnik, *et al.* 2014), vaginitis and endometritis (Wittenbrink, *et al.* 1993), infertility (DeGraves, *et al.* 2004; Wehrend, *et al.* 2005) and chronic mastitis (Biesenkamp-Uhe, *et al.* 2007). The pathogenetic mechanisms of chlamydiae in cattle are far from being fully understood. One research group could isolate *C. pecorum* with identical sequence from severely diseased and clinically unsuspicious animals (Jelocnik, *et al.* 2013; Jelocnik, *et al.* 2014). In contrast, another group could associate certain genetic polymorphisms with either high or low virulence of *C. pecorum* isolates (Mohamad, *et al.* 2014). Clinically obvious signs of chlamydial infections in cattle are only considered the ‘tip of the iceberg’. Considerable economic losses are caused by the impact that subclinical chlamydial infections have on herd health, fertility and milk-production in dairy cows (reviewed in Reinhold, *et al.* 2011b). Also, severe growth retardations due to multiple organ alterations due to chlamydial infections in calves have been identified (Poudel, *et al.* 2012).

1.2.7 Infections with *C. psittaci* in humans and cattle

Infections with *C. psittaci* in humans are called psittacosis or ornithosis. Typically, infected individuals suffer from respiratory and flu like symptoms which may be anything from mild (Moroney, *et al.* 1998) to life-threatening (Kovacova, *et al.* 2007). Dissemination to other organs is also possible, leading to complications such as myocarditis, endocarditis, encephalitis, icterus,

adult respiratory distress syndrome, and multiorgan failure. The incubation period is usually 5-14 days, but may also be longer. The actual prevalence of psittacosis is currently underestimated due to the limited awareness of physicians of the widespread occurrence and the zoonotic nature of avian chlamydiosis (Beeckman and Vanrompay 2009; Harkinezhad*, et al.* 2009). An anamnestic hint is the contact to birds, as psittacosis is frequently acquired by poultry breeders, veterinarians, slaughterhouse workers and pet bird owners (Hinton*, et al.* 1993; Vanrompay*, et al.* 2007; Gaede*, et al.* 2008; Verminnen*, et al.* 2008; Dickx and Vanrompay 2011).

The role of *C. psittaci* as the causative agent of ocular adnexal lymphoma is controversially discussed as evidence is conflicting (Zucca and Bertoni 2006).

In cattle, *C. psittaci* can be frequently found (Kauffold*, et al.* 2007; Teankum*, et al.* 2007; Kemmerling*, et al.* 2009). It has been associated with abortion (Pospischil*, et al.* 2002; Borel*, et al.* 2006) and reduced milk yield in combination with respiratory symptoms (Van Loo*, et al.* 2014). Experimental inoculation of calves with *C. psittaci* has been shown to lead to acute respiratory disease associated with fever, pulmonary dysfunction and systemic consequences in acid-base status (Reinhold*, et al.* 2012; Ostermann*, et al.* 2013a; Ostermann*, et al.* 2013b; Ostermann*, et al.* 2014).

Transmission of *C. psittaci* from cattle to humans is not yet proven, but there is a recent study from Egypt which could find DNA of *C. psittaci* in conjunctival swabs from both cattle and farmers with and without clinical symptoms (Osman*, et al.* 2013). However, co-housing of infected animals with naïve sentinel animals showed that direct transmission of *C. psittaci* between the animals is possible, as the sentinels acquired the infection. Sentinel animals developed only mild to subclinical disease (Ostermann*, et al.* 2013a). These facts strongly suggest that transmission to humans is possible, and the zoonotic potential of chlamydial infections in cattle should not be underestimated.

Cluster analysis of several avian, one bovine and two human isolates revealed an association of *C. psittaci* genotype and host species. The strain isolated from a bovine sample did not cluster with the groups of avian isolates, while the two strains isolated from humans belonged to the groups of pigeon- and duck-associated strains (Pannekoek*, et al.* 2010).

1.3 The host – Bovines as infection models

1.3.1 Bovine models of chlamydial infections

Bovines are a natural host to *Chlamydiae* and have therefore been widely used to study the impact of this pathogen on the host. Both, natural and experimental infections have been subject to intense research.

Inoculation of *C. abortus* and *C. psittaci* via the teat canal was described to produce a severe acute mastitis of the inoculated mammary glands accompanied by fever and anorexia (Corner*, et al.* 1968; Ronsholt and Basse 1981). Uterine deposition of *C. abortus* in pre-exposed cows reduced the fertility rate in these animals (DeGraves*, et al.* 2004). Application of *C. psittaci* in the

lung of calves was described to induce respiratory symptoms (Bednarek and Niemczuk 2005) and a few years later, a bovine model of respiratory *C. psittaci* infection was developed and the effects of the pathogen on the calves were further characterized (Reinhold*, et al.* 2012; Ostermann*, et al.* 2013a; Ostermann*, et al.* 2013b; Ostermann*, et al.* 2014).
Naturally occurring chlamydial infections of the bovine mammary gland have been used to study the effects of vaccination on udder health and pathogen shedding (Biesenkamp-Uhe*, et al.* 2007). The negative impacts of subclinical chlamydial infections on calf health and economic parameters was described by studying naturally infected calves under standardized conditions in an experimental setting (Jaeger*, et al.* 2007; Reinhold*, et al.* 2008).

1.3.2 Bovines as model species for respiratory disease

1.3.2.1 Peculiarities of the bovine lung and resulting suitability as a model for human respiratory diseases

In bovines, the right lung consists of four lobes (*Lobus cranialis*, *Lobus medius, Lobus caudalis* and *lobus accessorius*), while the left lung consists of two lobes (*Lobus cranialis*, which is divided into two segments, and *lobus caudalis*). Unlike the lung anatomy of most other mammals, there is a *Bronchus trachealis* branching directly from the right lateral side of the trachea leading to the *Lobus cranialis dexter*. Subgross anatomy of the bovine lung is characterized by a high degree of lobulation and a high percentage of interstitial tissue (McLaughlin*, et al.* 1961; Robinson 1982), which lead to a relatively low specific lung compliance and a higher pulmonic tissue resistance (Lekeux*, et al.* 1984). As a consequence, the required breathing activity in bovines is rather high compared to other species (Veit and Farrell 1978; Gallivan*, et al.* 1989). Strong independence of the segments is caused by the high degree of lobulation through connective tissue septa, limiting inflammatory processes by sharp demarcation (Reinhold 1997). Therefore, diseased and healthy segments often lie within the same lobe. Lacking collateral airways, the bovine lung is particularly suitable to mirror obstructive pulmonary dysfunctions (Kirschvink and Reinhold 2008). Regarding the vasculature in the bovine lung, the small pulmonary arteries show very prominent smooth muscle layers. Utilizing this peculiarity, the calf also serves as a well-established animal model of pulmonary hypertension or vascular remodeling (Stenmark*, et al.* 1987; Hunter*, et al.* 2010; Tian*, et al.* 2012).

Regarding respiratory infections, there are numerous naturally occurring diseases in livestock sharing similarities with the comparable disease in man. Typical examples are bovine tuberculosis (Van Rhijn*, et al.* 2008), respiratory syncytial virus infections in calves (Otto*, et al.* 1996; Gershwin*, et al.* 1998; Gershwin 2012), or naturally acquired *Chlamydia* infections (Jaeger*, et al.* 2007). This means that large animal models closely resemble the situation in the natural host, making them most useful for studying host-pathogen interactions and the complex pathophysiology of the corresponding disease in humans (Martinez-Olondris*, et al.* 2010; Sadowitz*, et al.* 2011).

Within the first 3-4 months of age, calves have approximately the same body weight (50 - 100 kg) and lung size as adult humans, leading to comparable functional characteristics (i.e.

lung volumes, airflows and respiratory mechanics) in the two species (Kirschvink and Reinhold 2008). This and the fact that they represent a natural host of *C. psittaci* make them a biologically relevant model of respiratory infection with this pathogen. The work with a large animal model brings the possibility of sampling every animal multiple times during the observation period, which was absolutely necessary for the present study. A further advantage is that the results obtained in studies with this model may be of benefit for both, veterinary and human medicine.

1.3.2.2 Methodological aspects

Different methods are available for experimental challenge of large animals with respiratory pathogens. Common examples are aerosol inhalation (McBride*, et al.* 1999; Herrmann-Hoesing*, et al.* 2012), intranasal (Ganheim*, et al.* 2003; Molina*, et al.* 2014), intratracheal (Ganheim*, et al.* 2003) or intrabronchial (Reinhold*, et al.* 2012) application. The bronchoscopic administration of the pathogen under visual control offers the possibility of exact definition of the application site and therefore increases the standardization of the inoculation process.

Bronchoscopy is generally considered a safe procedure. It has routinely been performed in humans since the 1960s (Dionisio 2012), and experimental bronchoscopy in calves was first described in 1968 (Hilding 1968). Intrabronchial application of pathogens is a widespread method in bovine research, as it is a reliable method to produce lower respiratory tract disease (Potgieter*, et al.* 1984; Ackermann*, et al.* 1996; Malazdrewich*, et al.* 2001; Reinhold*, et al.* 2012). The selective placement of the infectious agent in the lung under videoendoscopic control leads to consistent clinical and pathological findings in all animals (Reinhold*, et al.* 2012). In order to perform treatment studies in a bovine model of respiratory *C. psittaci* infection, there was the need to establish a bronchoscopic sampling protocol that would allow targeted sampling of lung regions that were expected to be altered due to pathogen exposure.
The methods that were to be utilized in the present project were bronchoalveolar lavage (BAL), bronchial brushings, and transbronchial lung biopsy. Presence and severity of lung inflammation can easily be evaluated by analysis of BAL fluid (BALF). The BAL is a standard procedure in humans (Wells 2010) and in cattle, where it has diagnostic (Franz 2013) and experimental value (Wilkie and Markham 1979; Pringle*, et al.* 1988; Silflow*, et al.* 2005; Mitchell*, et al.* 2007; Mitchell*, et al.* 2008). For the applicability of BAL in our studies, a suitable localization and amount of fluid for the procedure had to be determined.
Bronchial brushings are often obtained from human patients to sample neoplastic lesions or for microbiological analysis (Dionisio 2012). Their use in cattle has been described to characterize the microbial environment of the lung (Pringle*, et al.* 1988), therefore they were thought to be useful in detecting differences in the amount of viable pathogen present in the lungs of treated and untreated animals. This method is barely used in bovines, therefore suitable instruments had to be evaluated and the procedure itself had to be tested with regard to applicability and compatibility.
Lung tissue samples can be provided by transbronchial lung biopsy, which is a valuable diagnostic tool for diffuse lung diseases in humans. Due to the high cost of the equipment required and the time needed to obtain biopsies, this method is not suitable for routine use in

cattle. Instead, transcutaneous lung biopsies are more convenient under field conditions (Braun, *et al.* 1999; Sydler, *et al.* 2004; Burgess, *et al.* 2011). For our studies, we chose transbronchial lung biopsies, since they are less invasive than the transcutaneous ones. A limitation of transbronchial lung biopsies in bovines is the length of the available endoscope and biopsy forceps, therefore a suitable part of the lung had to be determined for sampling. Additionally, the transbronchial lung biopsy was the method expected to lead to most visible alterations of the lung, stressing the importance to limit the sampled regions in order to not interfere with the pathological assessment of lung lesions induced by the pathogen.
Not only the single sampling methods had to be evaluated and standardized, but the whole sampling protocol needed to define the exact order and localization of the samples to prevent artefacts or contamination of samples due to bleeding.

1.3.3 The bovine model of acute respiratory *C. psittaci*-infection

Previously, a bovine model of intrabronchial inoculation with *C. psittaci* was developed and characterized, providing the basis for the present thesis evaluating treatment options for respiratory chlamydial infections.
The intrabronchial inoculation was performed endoscopically, allowing the deposition of defined amounts of bacterial suspension at the same anatomical sites in each animal (Reinhold, *et al.* 2012).

Inoculation of calves with *C. psittaci* was found to result in a reproducible, dose dependent outcome in all animals. Inoculation with 10^6 inclusion forming units (ifu) led to mild respiratory infection, whereas animals inoculated with 10^7 and 10^8 ifu developed fever, tachypnea, cough, and tachycardia 2–3 days post inoculation (dpi). Symptoms lasted for about one week. When inoculated with 10^9 ifu *C. psittaci*, calves suffered from respiratory disease accompanied by severe systemic illness (apathy, tremor, markedly reduced appetite). For further studies, 10^8 ifu *C. psittaci* was identified as suitable inoculation dose and the outcome of inoculation with this dose will be described from hereon.

Animals developed obstructive and restrictive pulmonary disorders resulting in transient hypoxemia. Disturbances in acid-base equilibrium resulted from the respiratory and the metabolic site. The course of clinical signs was accompanied by a marked increase in the concentration of the acute phase protein lipopolysaccaride binding protein (LBP) in peripheral blood. Changes in the peripheral blood count were characterized by initial leukocytosis, followed by a phase of leukopenia. This was mainly driven by an increase in neutrophilic granulocytes with a regenerative left shift, while numbers and percentages of lymphocytes initially droped below baseline level and returned to pre-inoculational values 5 dpi. Numbers and percentages of monocytes in the blood slightly increased after inoculation. In the BALF, raised numbers and percentages of neutrophilic granulocytes were observed from 2 to 9 dpi. Also, concentration of total protein was elevated in the BALF supernatant of infected animals, indicating a loss of integrity of the alveolo-capillary barrier. After 10 days, all animals had clinically recovered, and

blood and BALF cell counts as well as LPB-levels returned to pre-inoculation values (Reinhold, *et al.* 2012; Ostermann, *et al.* 2013a; Ostermann, *et al.* 2013b; Ostermann, *et al.* 2014).

Bronchopneumonia at lung areas corresponding to the previous inoculation sites was seen in all *C. psittaci* inoculated calves at necropsy. In animals inoculated with 10^8 ifu, about 15% of total lung volume was altered 3 dpi and lesions were histopathologically characterized as fibrinopurulent bronchopneumonia with multifocal areas of necrosis and pleuritis. First signs of regeneration were visible 7 dpi (Reinhold, *et al.* 2012).

1.4 The treatment – Antibiotics and chlamydial infections

1.4.1 Humans

The treatment of choice for human patients suffering from chlamydial infections is based on tetracyclines, followed by macrolides and quinolones (Smith, *et al.* 2010). These antimicrobial substances reach high intracellular concentrations and are therefore considered as suitable drugs against the obligate intracellular chlamydiae. There are countless clinical studies on antimicrobial treatment of various chlamydial infections in humans (Lipsky, *et al.* 1990; Whatley, *et al.* 1991; Block, *et al.* 1995; Thorpe, *et al.* 1996; File, *et al.* 1997; Harris, *et al.* 1998; Moola, *et al.* 1999; Hammerschlag and Roblin 2000; Patel, *et al.* 2000; Finch, *et al.* 2002; Lau and Qureshi 2002; Carter, *et al.* 2010), most of them reporting very successful treatment with the above mentioned drugs. For example, two recent studies in *C. trachomatis*-infected patients reported cure rates of 90% or higher after doxycycline treatment (Schwebke, *et al.* 2011; Manhart, *et al.* 2013). However, many studies are based on serological diagnosis only, and the success of treatment is associated to the improvement of clinical signs rather than elimination of the pathogen. Another fact limiting the validity of these clinical studies is that they are usually not placebo controlled. Studies including pathogen detection after treatment revealed that doxycycline failed to eliminate *C. trachomatis* from the urinary tract of treated patients (Manhart, *et al.* 2013; Pitt, *et al.* 2013). Even if studies include screening for chlamydial DNA or RNA, they must be interpreted very carefully, since a single follow-up is not suitable for determining the actual infection state (Dukers-Muijrers, *et al.* 2012).

In fact, there is strong evidence of recurring chlamydial infections after the end of antibiotic treatment in humans (Bragina, *et al.* 2001; de Vries, *et al.* 2009).

Taken together, this leads to the conclusion that there is still research needed in the field of treating chlamydial infections, even though the problem is commonly underestimated.

1.4.2 Animals

As in human medicine, drugs used for the treatment of chlamydial infections are tetracyclines, macrolides and quinolones.

Evidence in the veterinary field is conflicting, as successful doxycycline treatment of experimental and naturally occurring infections with *C. psittaci* has been reported in birds

(Guzman, *et al.* 2010; Krautwald-Junghanns, *et al.* 2013). At the same time there are reported limitations of chlamydial treatment. Treatment with doxycycline or azithromycin in combination with rifampicin after experimental infection of mice with *C. trachomatis* showed that even though culture-positive results were reduced in treated animals, there was still chlamydial DNA in the tissue (Malinverni, *et al.* 1995; Wolf and Malinverni 1999). In pigs, shedding of chlamydiae was suppressed by enrofloxacin application, but it reoccurred after the end of treatment (Reinhold, *et al.* 2011a). The treatment of *C. felis* infected cats with doxycycline or enrofloxacin lead to remission of clinical signs, but in both groups there were still animals shedding the antigen (Gerhardt, *et al.* 2006).

Additionally, the veterinary field is confronted with chlamydiae resistant to antibiotics, as tetracycline resistant *C. suis* strains are an emerging problem in pigs (Lenart, *et al.* 2001; Di Francesco, *et al.* 2008; Borel, *et al.* 2012).

1.4.3 Antimicrobial resistance in *Chlamydiae*

Right now, antimicrobial resistance is not considered a problem in C*hlamydiae* although evidence of emerging antimicrobial resistance was reported. For practical reasons, testing for antimicrobial susceptibility is rarely carried out in clinical practice; therefore the actual situation in clinical relevant isolates is not known (Senn, *et al.* 2005). In *C. trachomatis,* antimicrobial resistance has been shown *in vitro* against rifampicin and fluorchinolones (Dessus-Babus, *et al.* 1998; Dreses-Werringloer, *et al.* 2001; Kutlin, *et al.* 2005; Schautteet, *et al.* 2013) and also in clinical isolates against tetracyclines (Lefevre and Lepargneur 1998) and even multiple antibiotics (Jones, *et al.* 1990; Somani, *et al.* 2000). *In vivo*, tetracycline resistant *C. suis* strains are a known problem in pigs (Lenart, *et al.* 2001; Di Francesco, *et al.* 2008; Borel, *et al.* 2012). For *C. psittaci* strains from ducks, multidrug resistance was described (Johnson and Spencer 1983), but this case seems to be a very rare exception. *In vitro* experiments could show that the development of antibiotic resistance in *C. psittaci* is possible (Binet and Maurelli 2005). Since the use of tetracyclines is widespread in the poultry and pet bird industry, there is the possibility of emerging resistances in avian strains of *C. psittaci* (Vanrompay, *et al.* 2007). Most human cases of psittacosis are due to zoonotic transmission from pet birds and poultry, so the infection with tetracycline-resistant *C. psittaci* strains would be a serious public health issue. In this context, the search for new therapeutic approaches in the treatment of *C. psittaci* infections is an important field of research (Knittler, *et al.* 2014).
The *C. psittaci* strain DC15 used in the present study was confirmed to be sensitive against the antimicrobial substances we intended to use (Wolf, *et al.* 2010).

1.4.4 Open questions and new strategies

It was recently shown in a cell culture model of an acute and an IFNγ-mediated persistent C. *psittaci* infection that adding rifampicin increased the antichlamydial effect of tetracyclines, macrolides and quinolones (Wolf, *et al.* 2010). Numbers of chlamydial inclusions were lower in epithelial cells treated with combination therapy than in cells treated with only one drug. These results led to the hypothesis that the treatment of *C. psittaci*-infected calves with combination

therapy would reduce numbers of detectable pathogen and time needed for disease remission in comparison to animals treated with monotherapy and untreated animals. In order to verify this hypothesis, promising combination therapies identified *in vitro* by Wolf *et al.* (2010) were now to be tested *in vivo* and compared to the respective monotherapies (i.e., doxycycline + rifampicin, erythromycin + rifampicin, azithromycin + rifampicin, enrofloxacin + rifampicin).

1.4.5 Substances to be tested *in vivo*

1.4.5.1 Doxycycline

Doxycycline belongs to the class of tetracyclines. Substances of this group are produced by bacteria of the genus *Streptomyces*. According to their name, tetracyclines comprise of four hexagonal rings with different substituents. They have bactericidal activity against many gram-positive and gram-negative aerobe and anaerobe bacteria. Synthesis of bacterial proteins is inhibited by binding to the 30S ribosomal messenger RNA (Kroker*, et al.* 2010). Doxycycline easily enters tissues and cells and is therefore suitable for the treatment of intracellularly located pathogens (Riond*, et al.* 1989). Resistance to tetracyclines is widespread; therefore their therapeutic value in human and veterinary medicine is limited and they should only be used against bacteria which are known to be sensitive (Kroker*, et al.* 2010). When applying tetracyclines orally, the manufacturer's instructions must be followed, since they build chelates with cations (e.g. Ca^{2+}-ions) in the food and this could limit the drug uptake. The drug used in the present study was approved for the oral application with milk substitute in calves.

1.4.5.2 Erythromycin and azithromycin

Erythromycin and azithromycin belong to the class of macrolides, which are characterized by a large macrocyclic lactone ring to which one or more sugars may be attached. The source of macrolides used to be *Streptomyces* spp.; now they can be produced synthetically. This class of antimicrobial substances inhibits bacterial protein synthesis by covalently binding to the 50S ribosomal subunit and hindering elongation (Burrows 1980).

Erythromycin is the oldest member of the macrolide family and is frequently used to treat a variety of bacterial diseases in veterinary medicine. It is also available for the use in human patients. Depending on its concentration and on the sensitivity of the microorganism, erythromycin can either act bactericidal or bacteriostatic (Haight and Finland 1952). Due to its lipid solubility it is easily distributed to peripheral tissues, where it reaches higher concentrations than in blood serum (Burrows 1980; Lakritz*, et al.* 1997). Erythromycin is enriched in leukocytes, which enables its transportation to the infection site (Prokesch and Hand 1982). In addition to its antimicrobial properties, erythromycin can act anti-inflammatory by reducing the chemotaxis of neutrophilic granulocytes and their adhesion to the lung (Lakritz*, et al.* 1997).

Azithromycin belongs to the azalides, a synthetic subgroup of the macrolide family. Due to changes in their chemical structure, azalides are superior to older macrolides, such as erythromycin, in terms of pharmacokinetic profile, gastric acid stability, half-life, and bactericidal activity (Girard*, et al.* 1987; Shepard and Falkner 1990). Tissue concentrations of azithromycin

exceed serum concentrations by far and its amphiphilic properties allow it to enter cells and tissue rapidly by diffusion (Donowitz 1994). As a weak base it is mainly concentrated in sour cell compartments such as lysosomes of host defence cells, which is the place where chlamydiae replicate. Phagocytes transport azithromycin to the inflammation site, where it reaches particularly high concentrations (McDonald and Pruul 1992; Girard*, et al.* 1996; Lode*, et al.* 1996). High concentrations are also reached in lung (mucosa, cells and bronchoalveolar lining fluid), tonsils and lymphnodes of humans and horses up to 96 h after the last application (Baldwin*, et al.* 1990; Lalak and Morris 1993; Jacks*, et al.* 2001). Azithromycin has, depending on concentration and susceptibility of the pathogen, either bacteriostatic or bactericidal properties and is a valuable tool in the treatment of a variety of bacterial infections in humans. The long half-life and slow excretion of this drug allow long interdose intervals and short term treatment (Girard*, et al.* 1987), therefore in most cases a single dose is sufficient for therapy of genital tract infections with *C. trachomatis* (reviewed in (Lode 1991)). A recent meta-analysis reported similar cure rates for azithromycin and doxycycline in treatment of human *C. trachomatis* infections (Kong*, et al.* 2014). Currently, the use of azithromycin in food-producing animals is banned in the European Union.

1.4.5.3 Enrofloxacin

Enrofloxacin belongs to the class of fluoroquinolones, which is characterized by a remarkably broad spectrum of activity. They act bactericidal by inhibiting bacterial DNA-gyrase (topoisomerase II) and also topoisomerase IV, which are necessary for DNA replication and metabolism (Elsheikh*, et al.* 2002). DNA-gyrase is responsible for DNA supercoiling, which is needed to ensure proper spatial arrangement of the DNA within the bacterial cell (Lopez-Cadenas*, et al.* 2013). Fluoroquinolones show a large volume of distribution. They reach high intracellular concentrations and accumulate in bronchial secretions, making them particularly suitable for the treatment of respiratory infections (McKellar*, et al.* 1999). Enrofloxacin was exclusively developed for the use in veterinary medicine (Elsheikh*, et al.* 2002). It can achieve therapeutic concentrations in most tissues of the body with exception of cerebrospinal fluid (Lopez-Cadenas*, et al.* 2013). Excretion of enrofloxacin includes unchanged renal excretion and hepatic metabolism (Martinez*, et al.* 2006). In cattle, enrofloxacin is partly metabolized to its active metabolite ciprofloxacin by de-ethylation in the liver. Ciprofloxacin itself is a potent antimicrobial agent (Küng*, et al.* 1993; Kaartinen*, et al.* 1995) and is widely used as a drug in human medicine. Both, enrofloxacin and ciprofloxacin have a broad antimicrobial activity against gram-positive and gram-negative bacteria, except for anaerobic microorganisms (Davis*, et al.* 1996).

1.4.5.4 Rifampicin

Rifampicin is a half synthetic hydracine derivative of rifamycin B, which itself is produced by the bacterium *Amycolatopsis rifamycinica* (formerly *Streptomyces mediterranei*) and belongs to the group of ansamycins (Furesz 1970). By inhibiting bacterial protein synthesis through inhibition of the bacterial DNA-dependent RNA polymerase, rifampicin is bactericidal against many gram-negative and most gram-positive microorganisms (Thornsberry*, et al.* 1983; Wehrli 1983). Its

lipophilic character enables rifampicin to enter tissues and cells very well, thus making it effective against intra- and extracellular pathogens (Acocella 1978; Prokesch and Hand 1982; Acocella 1983). Concentrations of rifampicin in lung, liver, bile and urine even exceed blood concentrations (Furesz 1970).
When rifampicin is applied over a longer time period, there is a potential of quickly developing resistance, therefore it should always be administered in combination with other antimicrobial substances (Farr and Mandell 1982).
Due to its intense red color, the application of rifampicin can lead to a harmless reddening of body fluids (e.g. urine, saliva, tears), but otherwise it is well tolerated over longer time periods.
Rifampicin was introduced for the use in human patients in the late sixties and is now widely used to treat mycobacterial infections and brucellosis (Baronti and Lukinovich 1968). In ruminants, it has been successfully used for treatment of tuberculosis and paratuberculosis under experimental conditions (St-Jean and Jernigan 1991), but its application in the field would contravene current legislation banning the use of rifampicin in food-producing animals in the European Union.
Rifampicin is mainly metabolized in the liver by deacetylation and excreted via urine and bile, where it undergoes enterohepatic circulation (Acocella 1978). Rifampicin is a potent inducer of cytochrome P450 enzymes in the liver, and thereby increasing its own metabolization rate (Adachi*, et al.* 1985; Rodriguez-Antona*, et al.* 2000; Williamson*, et al.* 2013). The fact that the peak serum concentration of rifampicin was lower after repeated oral administration in calves than after the first dose was already described by Sweeney et al. (Sweeney*, et al.* 1988).

1.5 Aim of the project

The aim of the project was to verify generally accepted and alternative therapeutic options for the elimination of pulmonary *C. psittaci* infections using a previously established bovine model. The lung as the primary site of infection and inflammation was of special interest in the evaluation of the different treatment regimens and should therefore be sampled repeatedly *in vivo* during the duration of the study to compare local reactions and pathogen load. In order to achieve this, bronchoscopical sampling methods had to be adapted for the use in calves, and a sampling protocol suitable for the use under experimental conditions had to be developed. Results were expected to increase the value and applicability of the bovine model through newly introduced standardized sampling procedures on the one hand and to broaden the knowledge about treatment of chlamydial infections on the other hand. The latter aspect is of interest in both veterinary and human medicine.

1.6 Design of the project

In the beginning of the project, the bronchoscopical sampling methods BAL, bronchial brushing and transbronchial lung biopsy needed to be adapted for the use in calves and evaluated for their practicability under experimental conditions in a small number of healthy animals (n=4). A sampling protocol was developed, which included order, location and amount of samples to be

obtained during the following studies. The methods were subject of a videopublication (STUDY 1).

In the following controlled and partially blinded prospective treatment study, 80 conventionally raised male Holstein-Friesian calves were inoculated with *C. psittaci* and assigned to either the untreated control group (n=13) or to one of the following 11 treatment groups:

Tetracyclines:
- low dose doxycycline (n=6)
- low dose doxycycline + rifampicin(n=6)
- high dose doxycycline (n=6)
- high dose doxycycline + rifampicin(n=6)

Quinolones:
- enrofloxacin (n=6)
- enrofloxacin + rifampicin (n=6)

Macrolides
- azithromycin (n=7)
- azithromycin + rifampicin (n=6)
- erythromycin (n=6)
- erythromycin + rifampicin (n=6)

- rifampicin (n=6).

To assess success and pathophysiological effects of the 11 different antimicrobial treatment regimens, clinical outcome, pathogen shedding and dissemination, reisolation, pathogen detection in affected tissue, as well as host response in terms of local and systemic inflammation were used as read-out parameters. The duration of the study was 14 days after inoculation of the calves, and bronchoscopic samples were obtained according to the previously established protocol on 4 and 9 dpi from each animal.

Due to the capacity of the institute's animal housing facility, the trials were carried out in groups of 10 animals at a time, distributed to two housing units of 5 calves each. To rule out effects of environmental conditions, different treatment regimens were assigned to the animals within one housing unit. The first three groups of 10 animals included 6 untreated control animals; the remaining 24 were treated with doxycycline alone or in combination with rifampicin. The time period for this phase was from January 2012 until June 2012. Results were published in STUDY 2, which was designed to evaluate the effects of doxycycline treatment alone or in combination with rifampicin. In order to obtain results of value for both, human and veterinary medicine, two dosages of doxycycline were tested: The dose commonly used for the treatment of human patients and the dose described for the use in calves (Papich 2011). From January 2013 until December 2013, the trial was continued with another 5 groups of 10 animals to evaluate the

remaining promising treatment regimens (i.e. macrolides and quinolones and/or rifampicin) and to include another 7 untreated control animals. The results of this second part of the treatment study were published in STUDY 3. In this study, the antichlamydial effects of the recently widely used macrolides and quinolones were to be tested alone and in combination with rifampicin. Furthermore, a group of animals was treated with rifampicin alone, to identify effects of this treatment as a monotherapy.

2 Publications and disclosure of personal contributions

STUDY 1

Prohl A, Ostermann C, Lohr M, Reinhold P (2014):
The bovine lung in biomedical research: visually guided bronchoscopy, intrabronchial inoculation and *in vivo* sampling techniques.
Journal of Visualized Experiments 89: e51557.

STUDY 2

Prohl A, Lohr M, Ostermann C, Liebler-Tenorio, E., Berndt, A., Schroedl, W., Rothe, M., Schubert, E., Sachse, K., Reinhold, P. (2015):
Evaluation of antimicrobial treatment in a bovine model of acute *Chlamydia psittaci* infection: tetracycline versus tetracycline plus rifampicin.
Pathogens and disease 73, 1-12.

STUDY 3

Prohl A, Lohr M, Ostermann C, Liebler-Tenorio, E., Berndt, A., Schroedl, W., Rothe, M., Schubert, E., Sachse, K., Reinhold, P. (2015):
Enrofloxacin and macrolides alone or in combination with rifampicin as antimicrobial treatment in a bovine model of acute *Chlamydia psittaci* infection.
PLoS ONE 10(3): e0119736.

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

2.1 STUDY 1: The bovine lung in biomedical research: visually guided bronchoscopy, intrabronchial inoculation and *in vivo* sampling techniques

Personal contributions:

- establishing the sampling protocols (bronchoalveolar lavage, bronchial brushing, transbronchial lung biopsies)
- *in vivo* sampling (bronchoalveolar lavage, bronchial brushing, transbronchial lung biopsies)
- further sample preparation
    - Sedimentation preparations of BALF cells
    - Modified Giemsa staining of BALF cells, immunohistochemistry (IHC) of BALF cells
    - Preparation of frozen sections of transbronchial lung biopsies and IHC
- drafting the manuscript
- Preparation of all figures and of video material filmed with the camera of the endoscope
- revision of the script for the video
- demonstrating the procedure in the video
- revision of the video

The article with the video is available under http://dx.doi.org/10.3791/51557.

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Video Article

The Bovine Lung in Biomedical Research: Visually Guided Bronchoscopy, Intrabronchial Inoculation and *In Vivo* Sampling Techniques

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Abstract

There is an ongoing search for alternative animal models in research of respiratory medicine. Depending on the goal of the research, large animals as models of pulmonary disease often resemble the situation of the human lung much better than mice do. Working with large animals also offers the opportunity to sample the same animal repeatedly over a certain course of time, which allows long-term studies without sacrificing the animals.

The aim was to establish *in vivo* sampling methods for the use in a bovine model of a respiratory *Chlamydia psittaci* infection. Sampling should be performed at various time points in each animal during the study, and the samples should be suitable to study the host response, as well as the pathogen under experimental conditions.

Bronchoscopy is a valuable diagnostic tool in human and veterinary medicine. It is a safe and minimally invasive procedure. This article describes the intrabronchial inoculation of calves as well as sampling methods for the lower respiratory tract. Videoendoscopic, intrabronchial inoculation leads to very consistent clinical and pathological findings in all inoculated animals and is, therefore, well-suited for use in models of infectious lung disease. The sampling methods described are bronchoalveolar lavage, bronchial brushing and transbronchial lung biopsy. All of these are valuable diagnostic tools in human medicine and could be adapted for experimental purposes to calves aged 6-8 weeks. The samples obtained were suitable for both pathogen detection and characterization of the severity of lung inflammation in the host.

Video Link

The video component of this article can be found at http://www.jove.com/video/51557/

Introduction

The Values of Large Animal Models in Biomedical Research

In modern interdisciplinary biomedical research, animal models are still indispensable to elucidate complex interactions — related to health or a disease status — within mammalian organisms. Despite 17 Nobel prizes being awarded to scientists that studied cattle, horses, sheep, or poultry as models for biomedical research[1], nowadays the vast majority of animal experiments are undertaken with rodents, while less than 1% of the studies are working with domestic animals or livestock.

Small animals offer many practical advantages (*i.e.* low cost, genetic malleability, high throughput, availability of numerous genetic, and immunologic tools and kits), and genetically modified murine models are generally accepted to perform mechanistic studies discovering particular molecular pathways. In biomedical research of complex systems, the biological relevance and clinical usefulness of mice models is becoming more and more questionable. They could be misleading and bear the risk of oversimplification of biological complexity[2-9].

Due to inter-species peculiarities, no single animal species will completely mirror the human situation, and the use of more than one model seems to be beneficial in an interdisciplinary biomedical research approach. In the context of translational medicine, large animals offer the opportunity to serve as comparative models providing results with high biological relevance of dual use for both human and animal health[1]. Remarkably, the human genome is more closely resembled by the bovine genome than by the genome of laboratory rodents. It also has been confirmed recently that, compared to other taxa, the genome of mice is much more rearranged[10-12].

In a complex study design, use of livestock offers the unique opportunity of intra-individual, long-term studies by repeated collection of a variety of samples *in vivo* from one-and-the-same individuum without sacrificing the animal. Therefore, functional, inflammatory and morphological changes can be monitored in the same subject over a certain period of time[13].

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The Bovine Lung as a Suitable Respiratory Model

Due to the high number of significant differences in lung anatomy, respiratory physiology, and pulmonary immunology, mice do not reproduce many important pathophysiological aspects of human pulmonary disease. This must be taken into account when using them as animal models of respiratory disease[2,9,14-16]. Although peculiarities of anatomy and structure do exist for each mammalian lung, functional characteristics (*i.e.* lung volumes, airflows and respiratory mechanics) are better comparable between adult humans and calves due to similar body weights (50-100 kg).

The species-specific characteristics of the bovine lung are summarized as follows: the left lung consists of two lobes (*lobus cranialis*, which is divided into two segments, and *lobus caudalis*), while the right lung consists of four lobes (*lobus cranialis*, *lobus medius, lobus caudalis*, and *lobus accessorius*). Unlike the lung anatomy of most other mammals, the bronchus of the right cranial lobe branches directly from the right lateral side of the trachea. With respect to subgross anatomy, the bovine lung presents a high degree of lobulation and a high percentage of interstitial tissue[17,18] leading to a relatively low specific lung compliance and a higher pulmonic tissue resistance[19]. Therefore, the required breathing activity is rather high compared to other species[20,21]. The high degree of lobulation leads to strong independence of the segments. Thus, inflammatory processes are limited by connective tissue septa, and diseased and healthy segments often lie within the same lobe. Due to the lack of collateral airways, the bovine lung is particularly suited to mirror obstructive pulmonary dysfunctions[13]. Regarding the vasculature in the bovine lung, the small pulmonary arteries show very prominent smooth muscle layers. Therefore, the calf may also serve as a well-established animal model of pulmonary hypertension or vascular remodeling[22-24].

With respect to respiratory infections, naturally occurring diseases exist in livestock that share many similarities with the comparable disease in man. Typical examples are bovine tuberculosis[25], respiratory syncytial virus (RSV) infections in calves[26-28], or naturally acquired *Chlamydia* infections[29]. Thus, large animal models do closely resemble the situation in the natural host. Therefore, they are most useful for studying host-pathogen interactions and the complex pathophysiology of the corresponding disease in human beings[30,31].

As a biologically relevant model of respiratory *Chlamydia psittaci* infection, calves were chosen since bovines represent natural hosts for this pathogen[32-35]. Information obtained from this model, with respect to pathogenesis of the disease or possible transmission routes between animals and humans, will help to broaden our knowledge with impact for both cattle and man. The model can also help to verify generally accepted and alternative therapeutic options for the elimination of pulmonary *C. psittaci* infections, which is, again, of interest in both veterinary and human medicine.

Techniques Applied to and Specimens Obtainable from the Bovine Respiratory System

This paper describes and illustrates the techniques and diagnostic methods applicable to the bovine lung and used in our model to evaluate both the effects of the pathogen on the mammalian lung and the efficacy of therapeutic intervention.

Bronchoscopy has been performed in human medicine since the 1960s and is considered a safe procedure[36]. In calves, experimental bronchoscopy was described in 1968 for the first time[37]. The intrabronchial application of pathogens was suggested by Potgieter *et al.* as a reliable method to produce lower respiratory tract disease in calves[38] and is now a widespread method in bovine research[34,39,40]. Intrabronchial inoculation of a defined amount of the pathogen under videoendoscopic control allows for selective placement of the infectious agent in the lung. This leads to consistent clinical and pathological findings in all animals[34] and allows targeted sampling of lung regions that are expected to be altered due to pathogen exposure.

Bronchoalveolar lavage fluid (BALF) is a well-described indicator for the presence and severity of lung inflammation. The bronchoalveolar lavage (BAL) is a standard procedure in human medicine for the diagnosis of a variety of respiratory diseases[41]. In live cattle, BAL was introduced by Wilkie and Markham in the late seventies of the last century[42]. It was considered a safe and repeatable technique to study the lower respiratory tract of cattle. Due to the lack of sufficient data on BALF parameters in healthy animals, in 1988 Pringle *et al.* performed BAL on healthy calves with a flexible fiberoptic bronchoscope. The authors also pointed out the need to standardize BAL protocols under experimental conditions to acquire comparable results[43]. BAL is still used as an *in vivo* sampling method in calves[44-46].

Bronchial brushing is commonly used in human medicine as a diagnostic tool to sample neoplastic lesions or for microbiological analysis[36]. For research purposes, primary cell cultures of epithelial cells harvested by cytological brushing can be obtained[47]. In cattle, the use of bronchial brushings for microbiological analysis has been described to characterize the microbial environment of the lung[43].

Transbronchial lung biopsy provides lung tissue samples and is a valuable diagnostic tool for diffuse lung diseases in humans. Iatrogenic pneumothorax and procedure-related hemorrhage are complications associated with this technique. Their incidence is reported to be less than one percent in human patients[48]. Transbronchial lung biopsy is not a common method for the use in cattle, due to the high cost of the equipment required and the time needed to obtain biopsies. Instead, transcutaneous lung biopsies are more convenient under field conditions[49-51].

Protocol

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 in the State of Thuringia, Germany (Permit Number: 04-004/11). All experiments were performed under supervision of the authorized institutional Agent for Animal Protection. Bronchoscopy was strictly performed under general anesthesia. During the study, every effort was made to minimize discomfort or suffering.

General remarks
The described techniques have been developed for calves of approximately 6-8 weeks of age, weighing about 60-80 kg. For the use in other large animal species or calves different in age and bodyweight, the techniques must be adapted to fit the size and weight and take into account the lung anatomy of the particular animal species. All equipment used must be sterile. *Chlamydia psittaci* is a zoonotic bacterium which can cause respiratory and general disease in humans. The non-avian *C. psittaci* strain DC15 used in the present protocol must be handled under biosafety level 2. All work with the pathogen and with infected animals must be performed wearing personal protective equipment, such as

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a respirator, a splash water proof jumpsuit, rubber boots and gloves. Wearing a suitable respirator is of great importance, since the natural route of infection for *C. psittaci* is aerogen. Packaging and labeling of samples must be disinfectant proof since all things must be treated with a disinfectant effective against chlamydiae according to the manufacturer's instructions before leaving the animal housing unit.

1. Preparing the Animal for Bronchoscopy

1. Determine the weight of the calf for dosage of anesthetics.
2. Place an intravenous (IV) access in the left jugular vein.
3. First, slowly inject xylazine (0.2 mg/kg bodyweight) over approximately 30 sec, then, after sedation occurs, inject ketamine (2.0 mg/kg bodyweight).
4. Lift the animal onto the table and place it in right lateral recumbency. Once the animal is adequately positioned on the table, check if the IV access is still in place and readjust it if necessary. During anesthesia, regularly check the eyelid reflex to determine the depth of anesthesia.
5. Once the animal is breathing steadily, have someone pull the tongue out and stretch the neck. Place a metal tube speculum in the animal's mouth, using slight rotating movements. Push the speculum forward under sight control, using a flashlight, until the larynx is visible.
6. Maintain anesthesia throughout the whole endoscopic procedure by injecting a bolus of 7 mg xylazine and 70 mg ketamine as needed.

2. Inoculation (Inoculation Sites: Figure 1)

1. Prepare 3 syringes with inoculum, containing 1, 2, and 5 ml of the inoculum.
2. Insert a Teflon tube into the endoscope's working channel. The tube should not protrude from the endoscope's tip.
3. Insert the endoscope through the metal speculum. Slight readjustments of the speculum might be necessary to enable the passing of the larynx. The *Bronchus trachealis*, which branches off to the right side, helps to align the picture on the monitor.
4. Attach the syringe with 5 ml inoculum to the end of the Teflon tube. Navigate the tube into the branches where the inoculum shall be deposited and apply the desired amount (Right lung: *Lobus medius*: 0.5 ml, *Lobus accessorius*: 0.5 ml, *Lobus caudalis*: 0.5 ml and 1.0 ml; Left lung: *Lobus cranialis*, *Pars cranialis*: 0.5 ml, *Pars caudalis*: 0.5 ml, *Lobus caudalis*: 1.5 ml). Attach the syringe with 1 ml inoculum to the tube, then navigate to the *Bronchus trachealis* and deposit the inoculum (*Lobus cranialis, Pars caudalis*: 1.0 ml). It is helpful to always approach the localizations in the same order.
5. Remove the endoscope and the speculum.
6. Spray 1 ml of the inoculum into each nostril with an actuator.
7. Bring the animal back to the stable and place it in prone position for waking up. Do not leave the animal unattended or in the company of other animals until it has regained sufficient consciousness to maintain sternal recumbency. The recovery stable should be air-conditioned, since the animal's ability for thermoregulation is decreased under general anesthesia.
   NOTE: First clinical signs should occur about 24-36 hr after inoculation, depending on the pathogen used.

3. Sampling Procedures (Sampling Sites: Figure 2)

1. Prepare the animal as described in steps 1.1-1.6.
2. Bronchoalveolar lavage
   1. Place 5 syringes each containing 20 ml of sterile isotonic saline, in a water-bath and allow them to warm up to approximately 38 °C.
   2. Insert a lavage catheter into the endoscope's working channel, then insert the endoscope into the metal speculum and navigate forward into the main bronchus of the left lung until the "wedge position" is reached where the endoscope cannot be pushed any further ahead.
   3. One after another, attach the syringes with the warm NaCl solution to the lavage catheter, instill the fluid and aspirate it directly. The bronchoalveolar lavage fluid must be stored in siliconized glass bottles and put on ice immediately after recovery to prevent the alveolar macrophages from attaching to the glass surface. Note both the amount of instilled saline and the amount of recovered fluid.
   4. Remove the lavage catheter from the working channel.
3. Bronchial brushing
   1. Navigate the endoscope to the desired sampling location, in the described protocol this is the *Bifurcatio tracheae*.
   2. Cover the brush with the tube before inserting it into the endoscope's working channel until the brush's tip appears on the monitor.
   3. Push the brush with the plastic tube forward about 5 cm and uncover it from the plastic tube by pushing the handle, then navigate it to the location that is to be brushed.
   4. Rub off epithelial calls by gently pushing and pulling the brush back and forth while navigating the endoscope to ensure contact between the brush and the wall of the bronchus. Stop rubbing when bleeding occurs. Cover the brush with the tube before pulling it out of the working channel.
   5. Prepare up to five smears on microscope slides by gently rolling the brush over the slide. Fixate the smears in cold methanol for 10 min, air dry and store at -20 °C.
   6. The brush can be rinsed in various media, depending on the purpose of the sampled cells. If taking multiple brushings with the same brush, be sure to only rinse it in media which do not irritate the mucous membrane.
4. Transbronchial lung biopsy
   1. Navigate the endoscope to the desired sampling location, in the described protocol this is the *Pars caudalis* of the *Lobus cranialis*. Before inserting the biopsy forceps into the working channel open and close it a couple of times to ensure that it is working smoothly.
   2. Push the biopsy forceps into the caudal branch of the *Bronchus trachealis* until a slight resistance occurs. Pull back 2-3 cm, open the forceps, push forward about 2 cm, close the forceps, pull back and remove the forceps from the working channel. This requires some practice.

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3. Carefully remove the tissue from the biopsy forceps, using a needle or small forceps. Depending on the further use of the tissue, store it in liquid nitrogen or a suitable fixation medium. This should happen right after removal to prevent autolytic processes.

5. Post-procedural treatment
    1. Bring the animal back to the stable and place it in prone position for waking up. Do not leave the animal unattended or in the company of other animals until it has regained sufficient consciousness to maintain sternal recumbency. The recovery stable should be air-conditioned, since the animal's ability for thermoregulation is decreased under general anesthesia.
    2. Monitor the animal closely for signs of pneumothorax for the next 24 hr. Provide feed and fresh water when the animal has regained full consciousness.

Representative Results

Course of Disease

The effect of the pathogen on the animals' health can be assessed by clinical examination. In our respiratory infection models, animals were examined twice daily and clinical observations were recorded using a scoring system. Additional information was captured by performing other *in vivo* sampling methods, *e.g.* ,collection of blood and swabs or lung function measurement. Pathological examinations were carried out at different time points after inoculation to describe the progress of the infection[32-34].

BALF Recovery Rate

The recovery rate of the instilled fluid was 83.05 ± 4.58% (mean ± SD).

Pathogen Detection

Recultivation of the pathogen can be performed from bronchial brushings. Also, PCR screening of various samples is possible to detect the pathogen, *e.g.* tissue biopsy, cytology brush sample, BALF-cells[52] or pharyngeal swab. Visualization of the pathogen is possible by performing immunohistochemistry of frozen sections of the lung biopsies and sedimentation preparations of the BALF-cells (**Figure 3**). In previous experiments, PCR of blood samples and swabs (conjunctival, fecal, nasal) were performed to characterize the spreading and shedding of the pathogen[32].

Markers of Local Inflammation of Lung Tissue

In the BALF, various parameters of lung inflammation can be studied. The total cell count and the proportion of neutrophils usually increase when lung inflammation is present. For cell differentiation, sedimentation preparations of BALF-cells can be stained according to Giemsa and differentiated using oil immersion (**Figure 4**). Cellular and liquid proportions of the BALF are separated by centrifugation (300 x g; 20 min). The BALF-supernatant contains various markers that change during inflammatory processes in the lung and can be studied under experimental conditions. Examples are total protein and eicosanoids[29,34].

A schematic overview of the potential further use of the described samples is shown in **Figure 5**.

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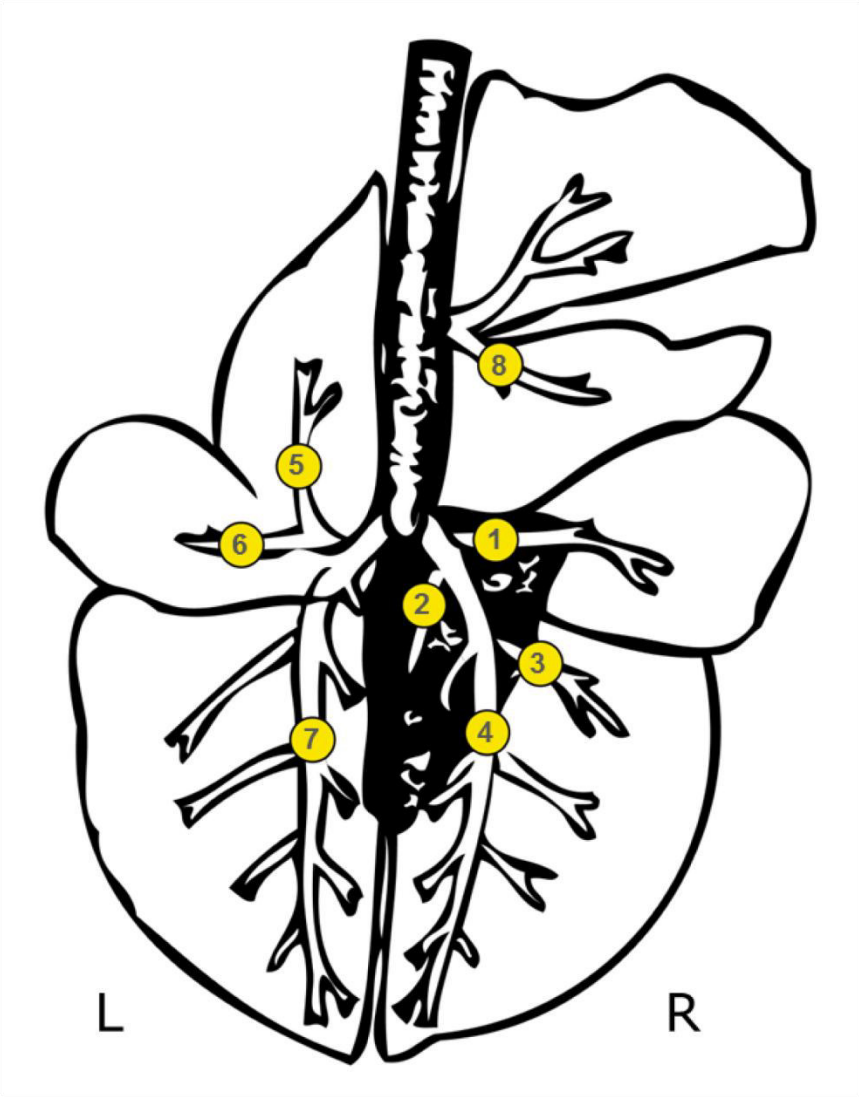

Figure 1. Scheme of the bovine lung with inoculation sites (yellow). The numbers indicate the order in which the inoculum is administered into the different bronchi. R: right; L: left. Right lung: **1** *Lobus medius*: 0.5 ml, **2** *Lobus accessorius*: 0.5 ml, **3** *Lobus caudalis*: 0.5 ml and **4** 1.0 ml; Left lung: *Lobus cranialis,* **5** *Pars cranialis*: 0.5 ml, **6** *Pars caudalis*: 0.5 ml, **7** *Lobus caudalis*: 1.5 ml, **8** *Lobus cranialis, Pars caudalis*: 1.0 ml.

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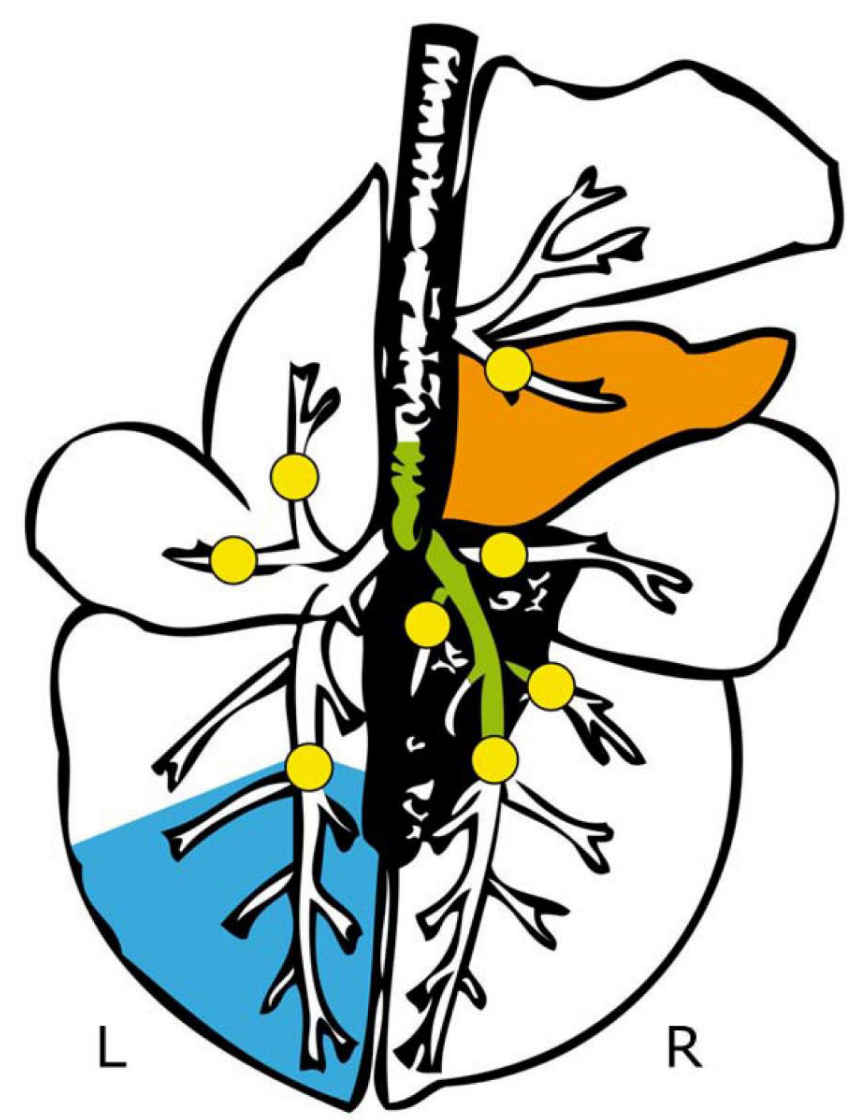

Figure 2. Scheme of the bovine lung with inoculation sites (yellow) and sampling sites: bronchoalveolar lavage (blue), bronchial brushing (green), and lung biopsy (orange). Note that all samples are obtained from regions where the pathogen was deposited before. R: right, L: left.

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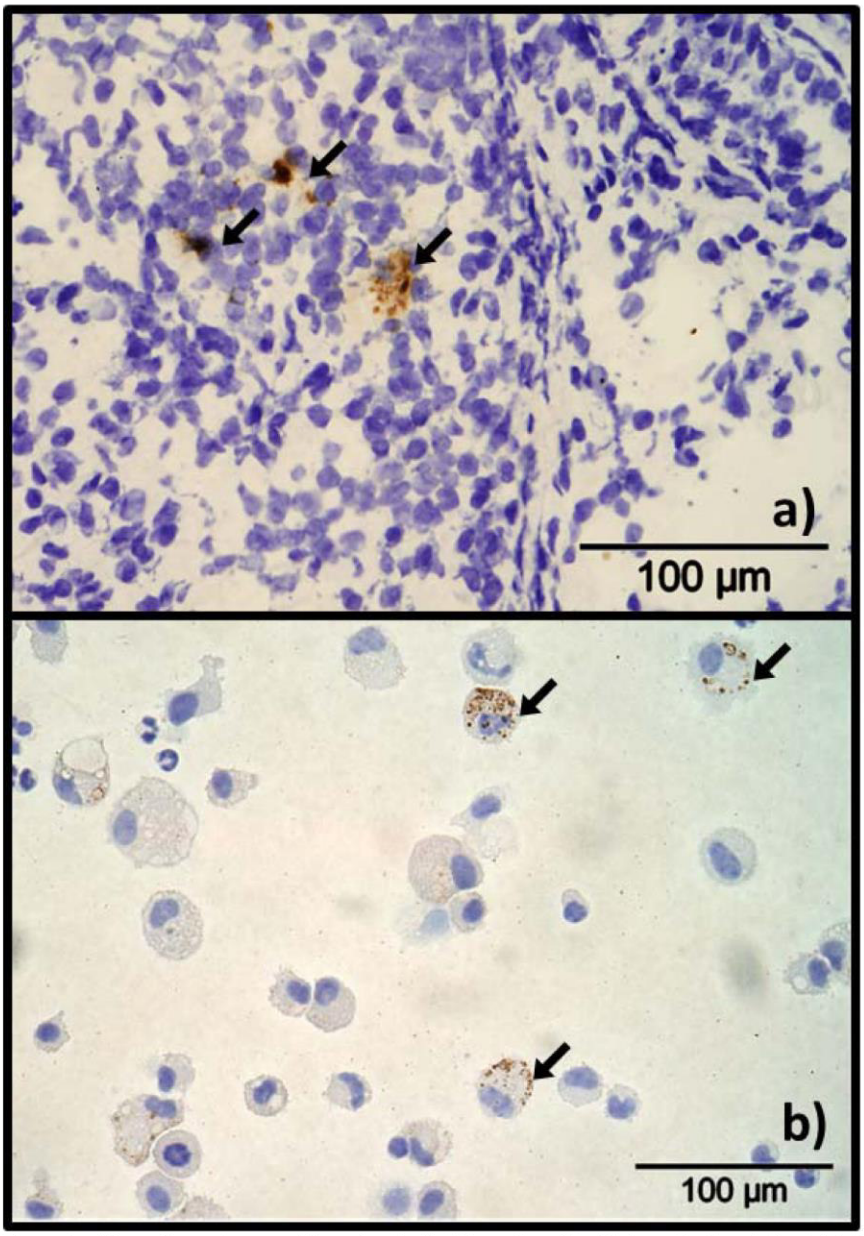

Figure 3. **a**) Lung biopsy from a calf inoculated with *Chlamydia psittaci* 4 days after inoculation (dpi), **b**) cellular sediment of BALF from a calf inoculated with *C. psittaci* 9 dpi. Immunohistochemical labeling for chlamydiae. Chlamydial inclusions (arrows) are present in the lung (**a**) and in alveolar macrophages (**b**). Hematoxylin counterstain.

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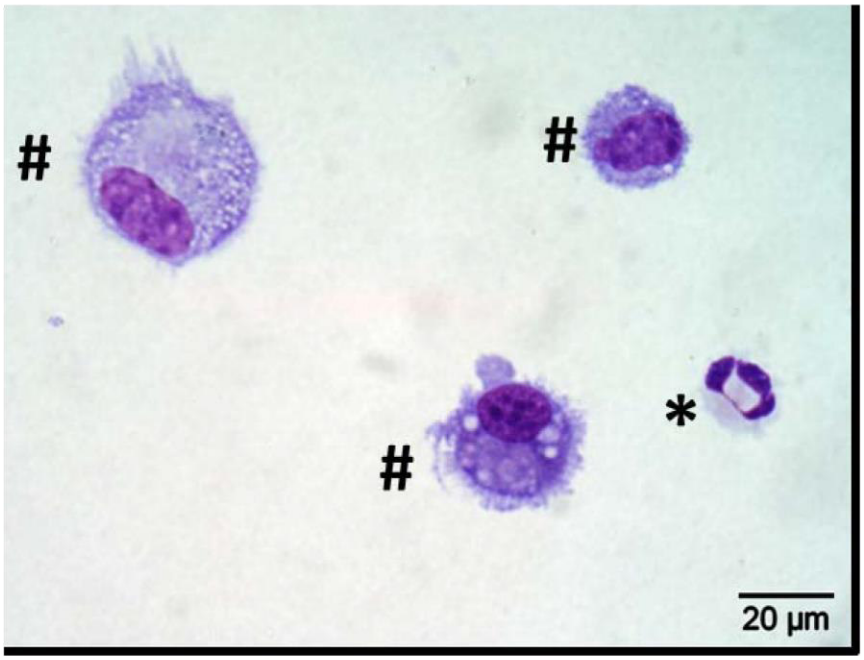

Figure 4. Cellular sediment of BALF from a calf inoculated with *C. psittaci* 9 dpi. Alveolar macrophages (#) are the predominant cell type in the BALF. The amount of neutrophil granulocytes (*) increases when inflammatory processes are present. Modified Pappenheim staining.

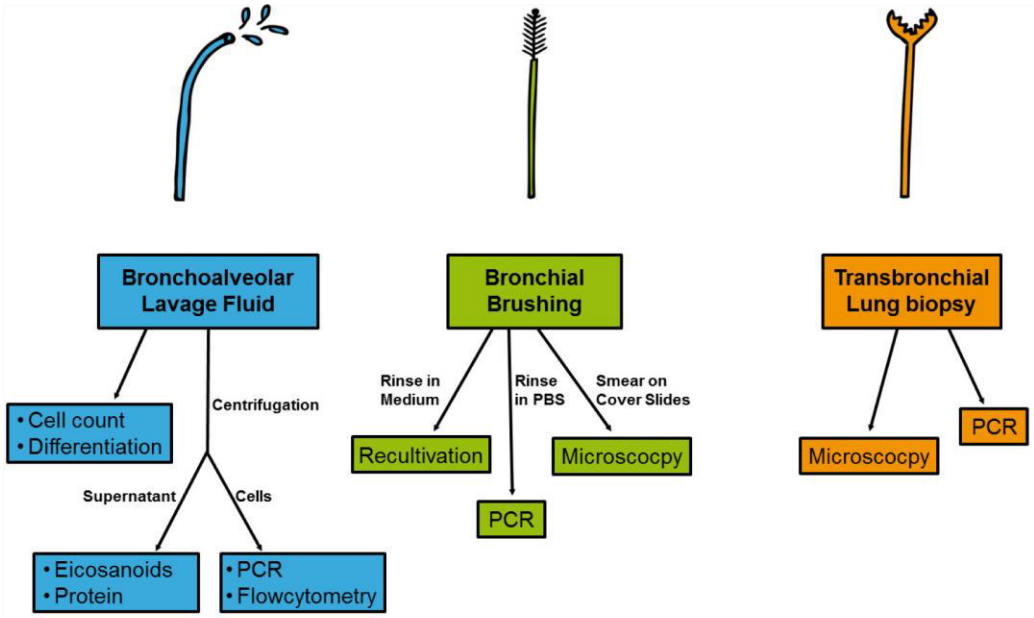

Figure 5. Possible methods for sample preparation. Please click here to view a larger version of this figure.

Discussion

A bronchoscopic method of inoculation was developed and various bronchoscopic sampling methods were adapted to be used in large animals under experimental conditions. The described techniques are easy to learn, even for examiners with little experience in endoscopy. The process of bronchoscopy is minimally invasive and no adverse effects associated with the methods of the inoculation, as well as the described sampling methods (BAL, transbronchial lung biopsy, bronchial brushing), were ever seen in any of the animals. The complications associated with transbronchial lung biopsies in humans are bleeding and pneumothorax[48], none of these were seen in the calves that underwent this procedure.

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The transbronchial lung biopsy is more time consuming and requires more equipment than the transcutaneous method, but it is less invasive and does not bear the risk of wound infection.

The visually controlled, endoscopic method of inoculation allows the deposition of a defined amount of pathogen at specific sites of the lung. Thus, it results in very consistent clinical and pathological findings in all inoculated animals[32-34]. However, it does not resemble all features of natural infection in calves. In a model of a respiratory *C. psittaci* infection, the described technique of inoculation led to lung lesions associated with the sites of pathogen placement[34], whereas, in naturally acquired infections calves usually develop pneumonia of the apical lobes. This fact has to be taken into account when interpreting the relevance of experimental findings in the context of natural acquired lung infections in bovines.

Videoendoscopic BAL allows sampling a defined area of the lung. For experimental purposes, this is an advantage compared to the use of a nasal catheter under blind conditions. Due to the anatomy of the bovine lung, the blindly inserted catheter would be pushed to the right diaphragmatic lobe in most cases[53,54] and the examiner has no influence on the area of the lung that is lavaged. Another advantage of the endoscopic BAL in anesthetized calves in lateral recumbency is the high average recovery rate of instilled fluid of more than 80%. A comparison with other studies reveals that, in standing, sedated calves, a recovery of 133.3 ± 1.6 ml [46] and 127.13 ± 3.53 ml [45] after instillation of 240 ml fluid into the caudal lobe is reported. In sedated calves in sternal recumbency 51% of the instilled fluid could be recovered from the cranial lobe and 62% from the caudal lobe[43]. This means that approximately half of the instilled fluid could be recovered in upright position of the calf. Depending on the amount of BALF needed for further sample preparation, this might not leave enough material to carry out all desired experiments.
BAL in cattle has been used by many research groups and many different parameters have been examined under various conditions. Most authors performed lavage of the basal lobes[43,45,46], but the amount of fluid used for lavage differs between the research groups. This leads to inconsistency in dilution of the recovered cells, proteins and other substances, making it difficult to compare the findings from different publications. Therefore, for the use in cattle it is recommended to lavage with five fractions of 20 ml (*i.e.* 100 ml in total) body warm, isotonic saline, which are recovered immediately after instillation. When using a lavage catheter with a large diameter (*i.e.* >2 mm), the volume of each fraction needs to be slightly increased, depending on the amount of fluid that will remain in the catheter.

The highly segmented anatomy of the bovine lung leads to a methodical limitation; results obtained from one part of the lung may not be true for the rest of the lung. Since there is no sight control of the whole lung area probed by transbronchial biopsy and lavage, the examiner cannot know whether the sampled areas were healthy or diseased. Therefore, it is very important to sample locations where the pathogen was inoculated before in order to have a higher recovery rate of the pathogen and to have a higher possibility of sampling diseased lung areas. Another limitation is the increased anesthetic risk in animals of poor clinical condition. The described methods should only be used in models of mild to moderate disease to keep the burden for the animals as low as possible. General anesthesia in ruminants should always be kept as short as possible, as the gas development in the rumen increases the anesthetic risk in these species. Animals must be placed in prone position immediately after bronchoscopy to allow the efflux of the developed gas and must be monitored closely until they are completely recovered from anesthesia. Also, the described techniques are not suitable for sampling intervals of less than 24 hr.

The described protocol can be adapted to other infectious agents. Endoscopic inoculation of various pathogens has been described, such as *C. psittaci*[32-34], *Pasteurella haemolytica*[38-40,42], *Haemophilus somni*[55], and bovine viral diarrhea virus[44]. Also, the sites of pathogen deposit in the lung can be adapted to the desired model. When choosing the sampling sites, some important facts have to be taken into account: (i) Sampling sites should be chosen based on the locations of inoculation and on the expected pathological findings. (ii) When necropsy is to be performed care must be taken to leave enough unsampled lung areas for *ex vivo* sampling. (iii) Sampling site locations must be chosen so they can be reached with the equipment. Especially for transbronchial lung biopsy, there are limitations due to the length of biopsy forceps. (iv) The order of sampling is important, bronchial brushing and transbronchial lung biopsy might lead to minor bleeding, which would contaminate the BALF. Therefore, BALF must always be obtained first. When using the protocol in other species, the species-specific lung anatomy must be taken into account.

Disclosures

The authors have nothing to disclose.

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Materials List for:

The Bovine Lung in Biomedical Research: Visually Guided Bronchoscopy, Intrabronchial Inoculation and *In Vivo* Sampling Techniques

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URL: http://www.jove.com/video/51557
DOI: doi:10.3791/51557

Materials

Name	Company	Catalog Number	Comments
Veterinary Video Endoscope	Karl Storz GmbH & Co. KG, Tuttlingen, Germany	PV-SG 22–140	diameter: 9 mm, working channel: 2.2 mm, working length 140 cm
Lavage catheter	Karl Storz GmbH & Co. KG, Tuttlingen, Germany		diameter: 2 mm; length: 180 cm, Luer-lock-adapter
Actuator	WEPA Apothekenbedarf GmbH & Co KG, Hillscheid, Germany	32660	length: 60 mm
Biopsy forceps	Karl Storz GmbH & Co. KG, Tuttlingen, Germany	REF 60180LT	1.8 mm, serrated, oval
Omnifix 20 ml, Luer-Lock	B. Braun Melsungen AG, Melsungen, Germany	4617207V	
Cytology brush	mtp GmbH, Neuhausen ob Eck, Germany	110240-10	working length 180 cm, brush length: 15 mm, diameter 1.8 mm
iv acess	Henry Schein Vet GmbH, Hamburg, Germany	370-211	diameter: 1.2 mm; length: 43 mm
Rompun 2% (xylazin)	Bayer Vital GmbH, Leverkusen, Germany		0.2 mg/kg bodyweight
Ketamin 10% (ketamine)	bela-pharm GmbH & Co. KG, Vechta, Germany		2.0 mg/kg bodyweight
Isotonic saline solution	B. Braun Melsungen AG, Melsungen, Germany	3200950	
SUB 6 waterbath	CLF analytische Laborgeräte GmbH, Emersacker, Germany	n/a	
Metal tube speculum	n/a	n/a	diameter: 3.5 cm; length: 35 cm
Flashlight	n/a	n/a	
Siliconized glass bottles	n/a	n/a	siliconize with Sigmacote (Sigma-Aldrich Co. LLC)
Omnifix Luer 3 ml	B. Braun Melsungen AG, Melsungen, Germany	4616025V	
Omnifix Luer 5 ml	B. Braun Melsungen AG, Melsungen, Germany	4616057V	
Sealing plugs	Henry Schein Vet GmbH, Hamburg, Germany	900-3057	
Inoculum		n/a	dilute pathogen in 8 ml buffer

2.2 STUDY 2
Evaluation of antimicrobial treatment in a bovine model of acute *Chlamydia psittaci* infection: tetracycline versus tetracycline plus rifampicin

Personal contributions:

- Animal experiments:
  - bronchoscopy (inoculation and sampling of BALF, bronchial brushings and transbronchial lung biopsies)
  - participation in the clinical examination of the calves
  - participation in the collection of blood and swab samples, preparing and counting blood cells
  - participation in the antibiotic treatment of the animals
  - anesthesia prior to necropsies, sample collection during necropsies
  - preparation of BALF, counting and differentiating BALF cells
- Analysis of all data
- Drafting of the manuscript
- Preparation of all figures and tables

The article is available under http://dx.doi.org/10.1111/2049-632X.12212.

2.3 STUDY 3
Enrofloxacin and Macrolides Alone or in Combination with Rifampicin as Antimicrobial Treatment in a Bovine Model of Acute *Chlamydia psittaci* Infection

Personal contributions:

- Animal experiments:
  - bronchoscopy (inoculation and sampling of BALF, bronchial brushings and transbronchial lung biopsies)
  - participation in the clinical examination of the calves
  - participation in the collection of blood and swab samples, preparing and counting blood cells
  - participation in the antibiotic treatment of the animals
  - anesthesia prior to necropsies, sample collection during necropsies
  - preparation of BALF, counting and differentiating BALF cells
- Analysis of all data
- Drafting of the manuscript
- Preparation of all figures and tables

The article is available under http://dx.doi.org/10.1371/journal.pone.0119736.

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RESEARCH ARTICLE

Enrofloxacin and Macrolides Alone or in Combination with Rifampicin as Antimicrobial Treatment in a Bovine Model of Acute *Chlamydia psittaci* Infection

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Abstract

Chlamydia psittaci is a zoonotic bacterium with a wide host range that can cause respiratory disease in humans and cattle. In the present study, effects of treatment with macrolides and quinolones applied alone or in combination with rifampicin were tested in a previously established bovine model of respiratory *C. psittaci* infection. Fifty animals were inoculated intrabronchially at the age of 6–8 weeks. Seven served as untreated controls, the others were assigned to seven treatment groups: (i) rifampicin, (ii) enrofloxacin, (iii) enrofloxacin + rifampicin, (iv) azithromycin, (v) azithromycin + rifampicin, (vi) erythromycin, and (vii) erythromycin + rifampicin. Treatment started 30 hours after inoculation and continued until 14 days after inoculation (dpi), when all animals were necropsied. The infection was successful in all animals and sufficient antibiotic levels were detected in blood plasma and tissue of the treated animals. Reisolation of the pathogen was achieved more often from untreated animals than from other groups. Nevertheless, pathogen detection by PCR was possible to the same extent in all animals and there were no significant differences between treated and untreated animals in terms of local (i.e. cell count and differentiation of BALF-cells) and systemic inflammation (i.e. white blood cells and concentration of acute phase protein LBP), clinical signs, and pathological findings at necropsy. Regardless of the reduced reisolation rate in treated animals, the treatment of experimentally induced respiratory *C. psittaci* infection with enrofloxacin, azithromycin or erythromycin alone or in combination with rifampicin was without obvious benefit for the host, since no significant differences in clinical and pathological findings or inflammatory parameters were detected and all animals recovered clinically within two weeks.

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Introduction

Infections with the zoonotic bacterium *Chlamydia (C.) psittaci* can cause severe respiratory and systemic disease in humans. The original hosts of *C. psittaci* are birds, but it has also been detected in several mammalian species, including cattle, horses, sheep, and pigs, where it is often associated with respiratory and reproductive disease [1–4]. In cattle, *C. psittaci* is by far more widespread than formerly assumed and is now known to have a significant impact on livestock productivity [2,5–7]. A study detecting DNA of *C. psittaci* in conjunctival swabs of human and bovine samples from the same farms suggested that transmission from cattle to human or vice versa is possible [8]. Therefore, chlamydiosis in cattle is not only an economic problem, but might also become a public health issue.

The pathogenesis of chlamydiosis has been subject to intense research in the last years (reviewed by Knittler and colleagues [9]), but reasons for the failure of successful treatment of chlamydial infections in both humans and animals are only suspected in most cases.

When it comes to treatment of chlamydial infections in various species, the drugs of choice are tetracyclines in most cases [10]. In birds, successful doxycycline treatment of experimental and naturally occurring infections with *C. psittaci* has been reported [11,12], whereas no sufficient impact of doxycycline treatment on the clinical outcome or severity of inflammation in an experimentally induced respiratory *C. psittaci* infection in calves could be shown [13]. Recent studies in *C. trachomatis*-infected humans reported cure rates of 90% or higher after doxycycline treatment [14,15]. However, most studies in humans should be interpreted very carefully, since a single follow-up screening for chlamydial DNA or RNA is not suitable for determining the actual infection state [16].

In pigs, some *C. suis* strains have developed tetracycline resistance [17–19] and it has been shown *in vitro* that the development of drug-resistant *C. psittaci* strains is possible [20,21]. Right now, antimicrobial resistance is not assumed to be a problem in chlamydiae other than *C. suis*, but field isolates are only very rarely screened for antimicrobial resistance in routine diagnostic procedures. Therefore, the actual situation can only be roughly assessed [22].

Alternative treatments include macrolides and quinolones, which are also frequently used in human [23,24] and veterinary medicine [25,26] to treat different chlamydial infections. Both are able to reach sufficiently high intracellular levels necessary for elimination of the obligate intracellular chlamydiae. It was shown in a cell culture model of an acute and a persistent C. psittaci infection that adding rifampicin increases the antichlamydial effect of macrolides and quinolones [27]. This new therapeutic approach was now intended to be applied to the situation in a living host. For this, we chose a previously established and well-characterized bovine model of acute respiratory infection with *C. psittaci* [28–31]. Calves often acquire chlamydial infections in field settings and therefore represent a frequent host to chlamydiae and, at the age of 6–8 weeks, they resemble adult humans in size and lung volumes. Also, in contrast to laboratory rodents, they offer the possibility of sampling in one and the same individual repeatedly to monitor the course of disease. After inoculation, the clinical picture resembles the course of acute human psittacosis, thus making calves a valuable model animal for this disease and allowing results to be beneficial for both human and veterinary medicine.

The present controlled and partially blinded prospective study was conceived to answer two questions: (i) What are the effects of treatment with macrolides or quinolones on the course of an acute respiratory *C. psittaci* infection in calves and (ii) can the results of the cell culture model, i.e. increased antichlamydial activity of macrolides and quinolones when combined with rifampicin, be transferred to the situation in the living frequent host and if so, is this clinically relevant?

Results

Antimicrobial levels

No significant levels of azithromycin, erythromycin, rifampicin, enrofloxacin and its active metabolite ciprofloxacin could be detected in blood plasma sampled 1 h prior to inoculation. Median and range of antibiotic levels in blood plasma and tissue for the different treatment groups from 3 days post inoculation (dpi) until 14 dpi are given in Table 1.

Plasma levels of enrofloxacin, ciprofloxacin, erythromycin, and rifampicin reached their maximum shortly after the beginning of the treatment on 3 and 5 dpi. Azithromycin reached maximal plasma levels on 10 dpi.

Enrofloxacin concentrations in tissues (lung, liver, and muscle sampled 14 dpi) and in plasma (sampled 3, 5, 7, 10, and 14 dpi) were clearly lower in animals treated with enrofloxacin and rifampicin in combination than in animals treated with enrofloxacin alone, although this difference could not be statistically secured (Mann-Whitney U-test, $P > 0.05$). In four animals treated with both enrofloxacin and rifampicin, no measurable enrofloxacin concentrations were detected in the lung. In lung tissue of three out of these four animals, ciprofloxacin was detectable, only in one animal neither enrofloxacin nor ciprofloxacin could be detected. Concentrations of ciprofloxacin in tissue and plasma were comparably high in both groups treated with enrofloxacin.

Plasma levels of azithromycin were higher in animals treated with both azithromycin and rifampicin than in animals treated with azithromycin alone. This difference was only significant for 5 dpi (Mann-Whitney U-test, 5 dpi: $P = 0.02$; all other days: $P > 0.05$). Tissue levels of azithromycin were comparable in both groups treated with this drug.

In animals treated with erythromycin and rifampicin in combination, levels of erythromycin in plasma were slightly higher than in animals treated with erythromycin alone. Tissue concentrations of erythromycin were higher in animals receiving combination therapy than in animals receiving only erythromycin injections. For the concentration of erythromycin in lung tissue this was statistically significant (Mann-Whitney U-test, $P = 0.01$).

Detection of the pathogen

Reisolation. In animals of all groups, reisolation of *C. psittaci* was possible from bronchial brushings obtained 4 dpi. Untreated controls and rifampicin-treated animals had most culture-positive specimens at that time point. From samples obtained 9 dpi, reisolation was possible in 4 untreated control animals and in one animal treated with erythromycin and rifampicin, and 14 dpi in one lung tissue sample from an untreated animal. Passaging of selected specimens of treated and untreated animals revealed that the pathogen was viable. It could be confirmed by DNA sequencing that the cultivated strain was the same as the strain DC15 used for inoculation of the animals. Numbers of culture-positive animals per group at the different time points are given in Table 2.

Swabs, Tissue. Chlamydial DNA was detected by qrt-PCR in normal and altered lung tissue and in pieces of the mediastinal lymph node sampled at necropsy 14 dpi in animals of all groups. The amount of detected genome copies did not differ statistically significant between treated and untreated animals (many-to-one comparisons by Gao *et al.* [32] with Hochberg-adjustment, $P > 0.25$) (Fig. 1).

DNA of *C. psittaci* was detected in pharyngeal swabs obtained 4 dpi in three animals treated with enrofloxacin, two animals treated with rifampicin and one animal treated with enrofloxacin and rifampicin. At 9 dpi, DNA of *C. psittaci* was detected in a pharyngeal swab of an animal treated with enrofloxacin and rifampicin and 14 dpi in the fecal swab of an untreated animal.

DNA of *C. psittaci* could not be detected in any other swabs.

Table 1. Concentrations of antimicrobial substances in blood plasma and tissue samples.

		Rifampicin	Enrofloxacin		Enrofloxacin in combination with Rifampicin			Azithromycin	Azithromycin in combination with Rifampicin		Erythromycin	Erythromycin in combination with Rifampicin	
		Rifampicin	Enrofloxacin	Ciprofloxacin	Enrofloxacin	Ciprofloxacin	Rifampicin	Azithromycin	Azithromycin	Rifampicin	Erythromycin	Erythromycin	Rifampicin
plasma	time, dpi	[ng/mL]	[ng/mL]	[ng/mL]	[ng/mL]	[ng/mL]	[ng/mL]	[ng/mL]	[ng/mL]	[ng/mL]	[ng/mL]	[ng/mL]	[ng/mL]
	3	3053.0 (2728.0; 3907.0)	766.5 (461.0; 885.0)	141.0 (114.0; 195.0)	770.0 (445.0; 2363.0)	174.5 (105.0; 210.0)	3839.5 (1592.0; 5044.0)	68.0 (42.1; 145.3)	127.6 (71.0; 199.6)	2654.0 (1649.0; 4027.0)	268.3 (13.4; 852.0)	502.3 (245.9; 733.3)	2389.0 (1657.0; 4842.0)
	5	3220.5 (1902.0; 5140.0)	490.5 (327.0; 1321.0)	103.5 (61.3; 348.0)	364.0 (236.0; 847.0)	145.0 (89.0; 166.0)	3669.0 (1354.0; 5211.0)	103.7 (69.0; 154.9)[a]	151.9 (103.8; 245.1)[b]	3740.5 (1329.0; 6993.0)	431.6 (235.4; 585.3)	316.7 (144.7; 620.7)	3057.5 (582.0; 6773.0)
	7	1172.5 (827.0; 1983.0)	315.5 (104.0; 371.0)	86.65 (57.7; 310.0)	153.5 (105.0; 306.0)	82.45 (72.6; 87.4)	2074.0 (828.0; 4309.0)	154.6 (102.4; 225.1)	201.0 (151.1; 289.4)	2115.5 (389.0; 6211.0)	248.4 (134.4; 465.5)	323.3 (20.2; 596.3)	1246.5 (182.6 6643.0)
	10	1472.0 (977.0; 1968.0)	223.5 (151.0; 354.0)	75.0 (60.4; 216.0)	160.5 (72.9; 235.0)	88.25 (57.6; 140.0)	1275.5 (760.0; 2150.0)	192.5 (148.8; 355.4)	203.2 (173.4; 307.8)	1813.0 (420.0; 3437.0)	253.1 (95.6; 609.0)	315.2 (235.1; 478.9)	1056.5 (192.0; 2563.0)
	14	488.0 (315.0; 1457.0)	107.5 (45.0; 247.0)	54.05 (27.6; 78.1)	76 (28.9; 188.0)	47.75 (0.0; 81.4)	433.5 (133.0; 1298.0)	167.8 (141.8; 312.2)	202.2 (165.4; 399.5)	386.0 (117.0; 1516.0)	231.1 (9.2; 341.3)	271.0 (136.2; 420.3)	297.0 (77.5; 552.0)
tissue		[ng/g]	[ng/g]	[ng/g]	[ng/g]	[ng/g]	[ng/g]	[ng/g]	[ng/g]	[ng/g]	[ng/g]	[ng/g]	[ng/g]
lung	14	272.5 (122.0; 635.0)	119.0 (102.0; 326.0)	113.5 (0.0; 226.0)	0.0 (0.0; 272.0)	123.0 (0.0; 230.0)	165.0 (13.4; 851.0)	15994.9 (3584.3; 31123.6)	14427.0 (9823.2; 25318.6)	292.5 (62.1; 708.9)	324.2 (65.1; 793.6)[a]	821.1 (457.3; 1205.6)[b]	247.3 (83.0; 913.1)
muscle	14	222.5 (75.9; 365.0)	203.0 (0.0; 270.0)	129.0 (0.0; 239.0)	119.0 (0.0; 239.0)	125.0 (0.0; 159.0)	175.0 (52.9; 734.0)	625.3 (489.9; 923.8)	611.6 (395.7; 978.7)	230.4 (41.2; 376.5)	124.5 (23.1; 336.6)	353.0 (100.8; 557.5)	178.9; (66.4 462.0)
liver	14	10802.0 (7587.0; 13090.0)	301.5 (178.0; 764.0)	401.0 (272.0; 635.0)	219.5 (100.0; 515.0)	410.5 (173.0; 751.0)	10679.5 (5153.0; 16838.0)	23115.8 (12239.9; 53693.3)	14747.8 (6051.0; 25731.5)	16726.9 (7855.3; 35993.0)	702.3 (42.1; 1233.0)	1030.2 (573.5; 1842.3)	19778.8 (13172.7; 49231.1)

Values are given as median and range. Plasma levels of enrofloxacin, its active metabolite ciprofloxacin, erythromycin, and rifampicin were highest 3 and 5 dpi, whereas levels of azithromycin reached their maximum 10 dpi. Different letters (a, b) indicate statistically significant differences in antibiotic concentration in plasma and tissue between groups treated with or without rifampicin on the same day ($P < 0.05$, Mann-Whitney U-test).

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Table 2. Reisolation of Chlamydia psittaci from bronchial brushings.

	C (n = 7)	R (n = 6)	En (n = 6)	En + R (n = 6)	Az (n = 7)	Az + R (n = 6)	Ery (n = 6)	Ery + R (n = 6)
4 dpi	4	4	2	2	1	1	2	2
9 dpi	4	0	0	0	0	0	0	1
14 dpi	1	0	0	0	0	0	0	0
total	9	4	2	2	1	1	2	3

Number of animals with positive reisolation results. Reisolation of *C. psittaci* was possible more often in untreated animals than in treated animals. C: untreated controls, R: rifampicin, En: enrofloxacin, En + R: enrofloxacin + rifampicin, Az: azithromycin, Az + R: azithromycin + rifampicin, Ery: erythromycin, Ery + R: erythromycin + rifampicin. dpi: days post inoculation.

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Blood. None of the blood samples obtained during the study tested positive for chlamydial DNA.

Clinical Score

Daily examination revealed the development of clinical signs of respiratory and general disease after inoculation with *C. psittaci* in all animals. The calves showed cough, raised body

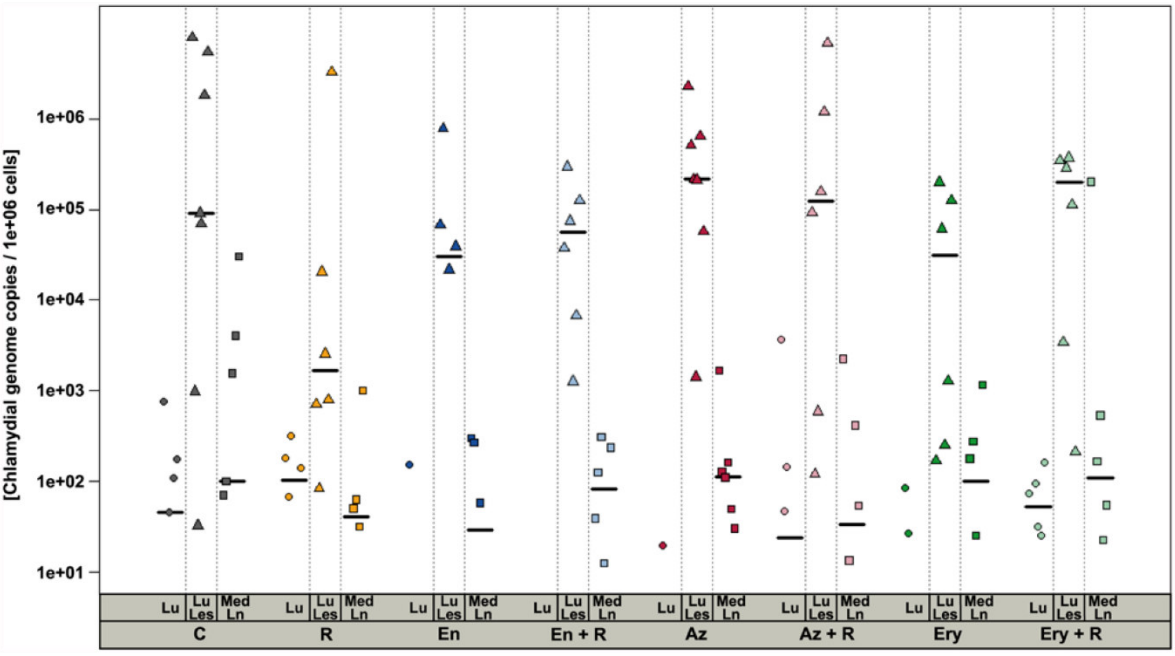

Fig 1. Chlamydial genome copies in tissue 14 days after inoculation. The number of chlamydial genome copies per one million cells determined with qrtPCR was higher in altered lung tissue (Lu Les, triangles) than it was in macroscopically normal lung tissue (Lu, circles) and in mediastinal lymph node (Med Ln, squares). Chlamydial DNA was detected in altered lung tissue of all animals except for two enrofloxacin-treated animals, whereas in some animals of all treatment groups no chlamydial DNA was detected in normal lung tissue and in the mediastinal lymph node. There was a high degree of variation in chlamydial genome copy numbers within the treatment groups and no statistically significant differences between treated and untreated groups for the amount of chlamydial DNA in the tissues sampled 14 dpi (many-to-one comparisons by Gao *et al*. (2008) with Hochberg-adjustment, $P > 0.25$). Black lines: medians, C: untreated controls, R: rifampicin, En: enrofloxacin, En + R: enrofloxacin + rifampicin, Az: azithromycin, Az + R: azithromycin + rifampicin, Ery: erythromycin, Ery + R: erythromycin + rifampicin. dpi: days post inoculation.

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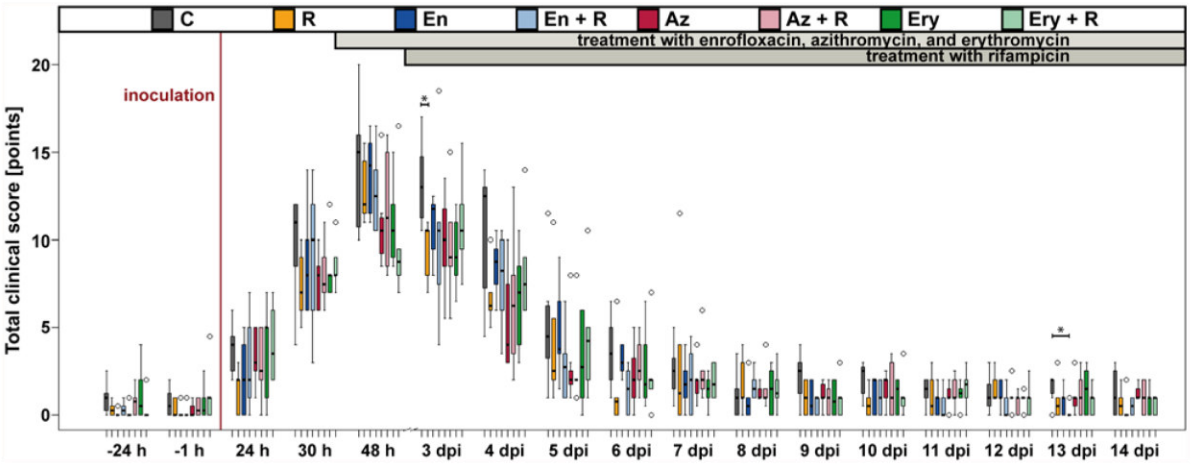

Fig 2. Total clinical score. Following inoculation with *Chlamydia psittaci*, all animals developed signs of an acute respiratory illness, which were maximal 48 hours to 3 days after inoculation. Regardless of treatment, the total clinical score dropped back to baseline level within seven days the latest. Only on two occasions (3 dpi and 13 dpi) there were differences between treated groups and untreated controls that could be statistically secured. Significant differences to the untreated control group at the same day are indicated with asterisks (many-to-one comparisons by Gao *et al*. (2008) with Hochberg-adjustment, $P \leq 0.05$).

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temperature, heart rate and respiratory rate, forced breathing, reduced appetite, swollen lymph nodes, and hyperemia of mucous membranes. Almost half of the clinical score was contributed by respiratory signs. The maximum of the clinical score was reached at 2 and 3 dpi. From there on, clinical health started to improve in all animals until 7 dpi, when the clinical score almost reached baseline level again. The rectal body temperature was maximal from 30 to 48 hours after inoculation (41.0°C; 39.9–42.0; median, range) and returned to physiological values until 6 dpi in all animals (38.7°C; 38.3–39.3).

In untreated controls, the total clinical score was slightly higher than in all other groups, but this was already visible before the beginning of antimicrobial treatment. Differences in the total clinical score between untreated controls and other groups were only statistically significant for the rifampicin treated group at 3 dpi (many-to-one comparisons by Gao *et al.* [32] with Hochberg-adjustment, $P = 0.03$) and the enrofloxacin + rifampicin treated group on 13 dpi ($P = 0.02$) (Fig. 2).

The daily weight gain during the study was highest in azithromycin treated groups, but the difference was not statistically significant when compared to untreated controls (many-to-one comparisons by Gao *et al.* [32] with Hochberg-adjustment, $P > 0.47$) (Fig. 3).

Side effects of treatment

Animals that had received subcutaneous injections of erythromycin during the study developed palm-sized, painful swellings at the injection sites (thorax and neck) that lasted for several days. Injections had to be applied after feeding, since the animals refused milk intake after the injections due to pain. At the injection sites, extensive coagulation necrosis of connective tissue and musculature was seen at necropsy, which was demarcated by granulation tissue and surrounded by edema.

The other treatment regimens were without side effects, no adverse effects on gut microbiome were observed since treated animals did not develop diarrhea or other digestive disorders.

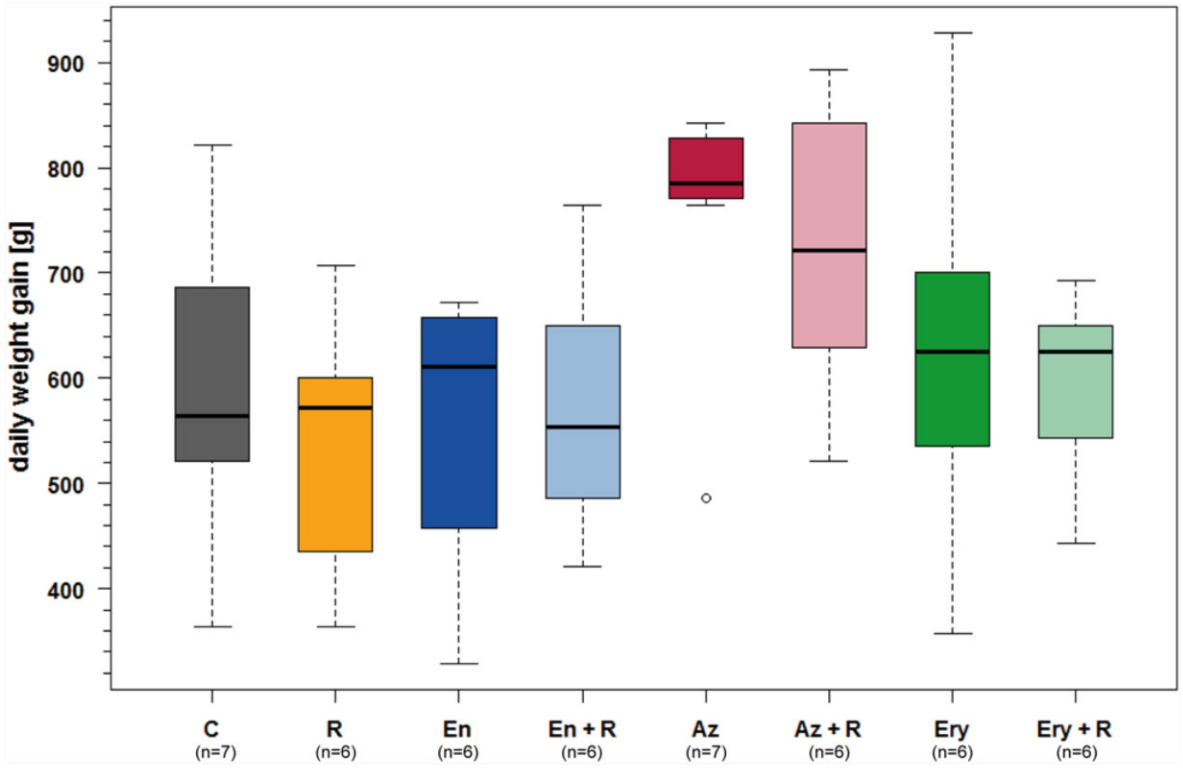

Fig 3. Daily weight gain. Daily weight gain was highest in azithromycin treated animals, but the difference compared to untreated controls was not statistically significant (many-to-one comparisons by Gao *et al*. (2008) with Hochberg-adjustment, $P > 0.47$). C: untreated controls, R: rifampicin, En: enrofloxacin, En + R: enrofloxacin + rifampicin, Az: azithromycin, Az + R: azithromycin + rifampicin, Ery: erythromycin, Ery + R: erythromycin + rifampicin.

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Systemic inflammation

White blood cells. The total number of blood leukocytes was increased 1.7-fold within two days after inoculation in all calves. Values dropped to 80% of baseline level the next day, 3 dpi, and baseline values were reached again at 14 dpi. Statistically significant differences between treated and untreated animals were only present for the enrofloxacin + rifampicin treated group on 3 dpi (many-to-one comparisons by Gao *et al.* [32] with Hochberg-adjustment, $P = 0.009$). On 2 dpi, the rifampicin treated group showed higher numbers of total blood leukocytes and neutrophilic granulocytes than other groups (not statistically different when compared to untreated control group), but these values were measured before the first application of rifampicin, therefore a treatment effect as an explanation for this phenomenon can be ruled out (Fig. 4a).

Percentage of neutrophilic granulocytes (segmented and banded forms) increased 2-fold until 2 dpi while total numbers increased 4-fold. Both percentage and total numbers declined to 80% of initial values until 5 dpi and then increased again to be about 1.4-fold of baseline level from 7–14 dpi (Fig. 4b). The percentage of lymphocytes decreased by half within two days after inoculation, leading to a reduction of total lymphocyte numbers to 75% of the initial values. Both, absolute numbers and percentages of lymphocytes were at baseline level at 5 dpi, were they remained until the end of the study. Monocytes increased in both, absolute numbers

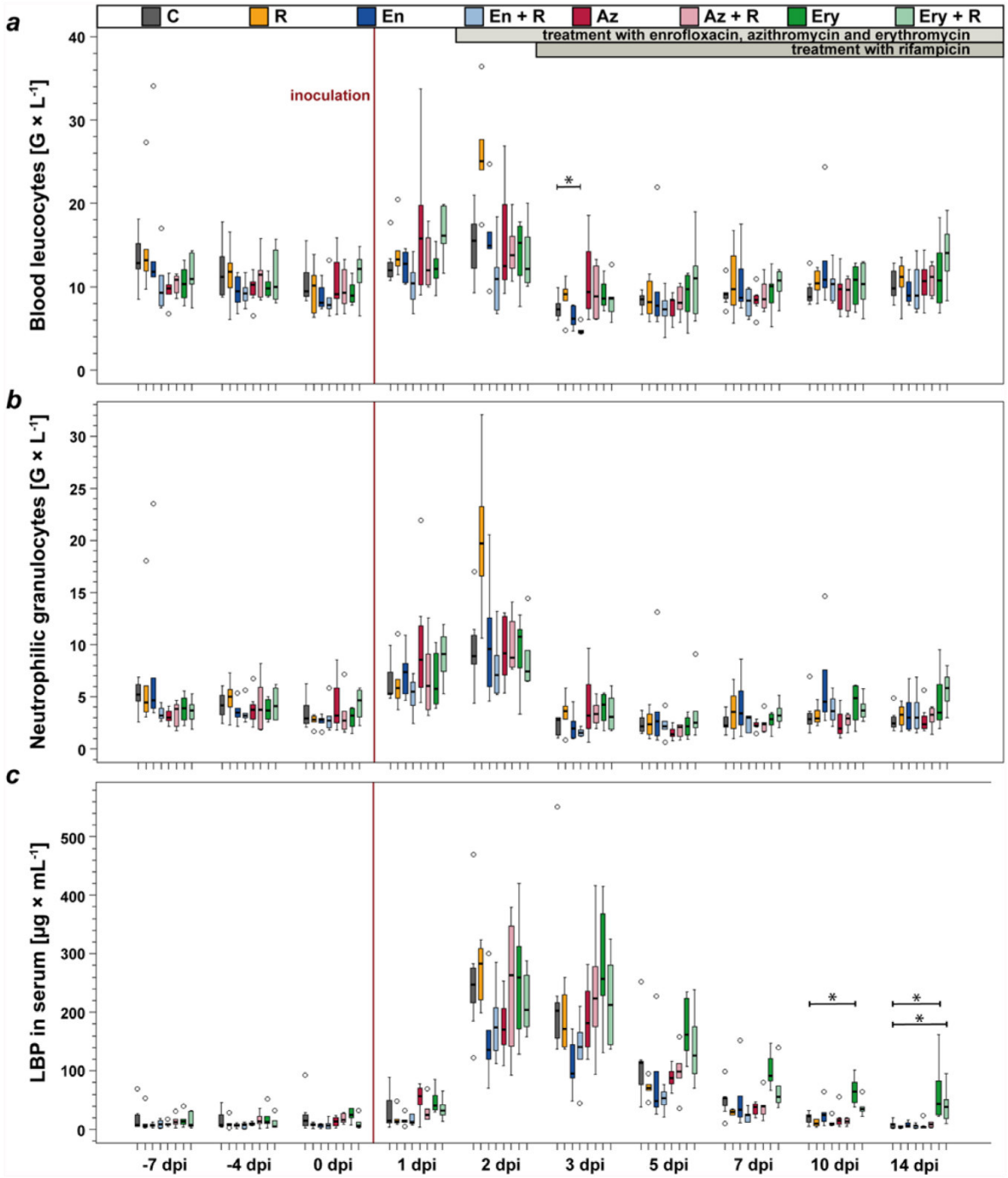

Fig 4. Blood parameters. The day after inoculation with *Chlamydia psittaci*, total blood leucocyte numbers (a) were already markedly increased and numbers stayed above baseline level 2 dpi. The initial increase was followed by a drop slightly below baseline level in all groups. Neutrophilic granulocytes (b) were the cell population being mainly responsible for the changes in absolute leukocyte numbers after inoculation. Other than in the group treated with enrofloxacin + rifampicin on 3 dpi, total leukocyte numbers did not differ significantly between treated and untreated animals, nor did numbers of neutrophilic granulocytes.

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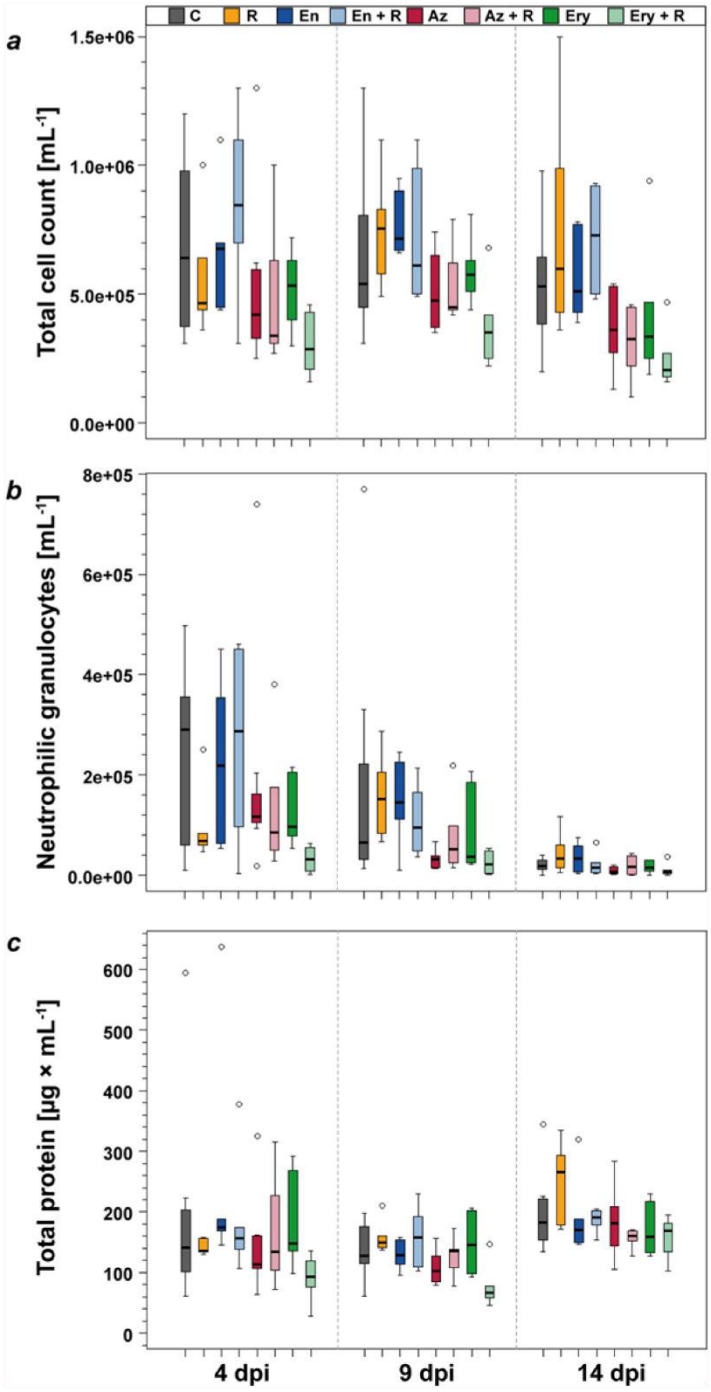

Fig 5. Bronchoalveolar Lavage Fluid (BALF). In the BALF, total cell count (a) remained within comparable limits at all examined time points in all groups. Numbers of neutrophilic granulocytes per mL (b) were highest 4 dpi and dropped continually until the last day of the study. Total protein concentration in BALF-supernatant remained constant throughout the whole study. Statistically significant differences between treated and untreated animals could neither be detected for protein levels nor for numbers of cells or neutrophils per mL in

the BALF (many-to-one comparisons by Gao *et al*. (2008) with Hochberg-adjustment, $P > 0.05$). C: untreated controls, R: rifampicin, En: enrofloxacin, En + R: enrofloxacin + rifampicin, Az: azithromycin, Az + R: azithromycin + rifampicin, Ery: erythromycin, Ery + R: erythromycin + rifampicin. dpi: days post inoculation.

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and percentage of peripheral blood leukocytes, reaching a maximum of 2-fold increase at 5 dpi and levels remaining increased until 14 dpi.

Acute phase reaction. Inoculation with *C. psittaci* led to an increase in lipopolysaccharide binding protein (LBP) concentration in the peripheral blood of all animals. LBP-levels were maximal at 2 dpi, ranging from 2-fold to 166-fold (median: 19-fold) compared to the levels measured directly before inoculation. In animals of all groups except for the two erythromycin treated groups, LBP concentration in the blood returned to pre-inoculation values by 10 dpi. In animals receiving subcutaneous injections of erythromycin, LBP levels where higher than in the untreated control group from 5 dpi on. For the group treated with only erythromycin, this increase was significant 10 dpi (many-to-one comparisons by Gao *et al.* [32] with Hochberg-adjustment, $P = 0.03$) and 14 dpi ($P = 0.03$) and for animals treated with erythromycin and rifampicin on 14 dpi ($P = 0.04$) (Fig. 4c).

Levels of lipopolysaccharide binding protein (LBP) as a marker of inflammation (c) reached maximal numbers 2 and 3 dpi in all animals, meaning a delay of 24 hours compared to numbers of blood leukocytes and neutrophilic granulocytes. Kinetics of LBP-levels were the same as of the clinical score, baseline level were reached at 10 dpi. In enrofloxacin treated animals, levels of LBP showed a lesser increase than in the untreated control group, but this difference could not be statistically secured. In erythromycin treated animals, LBP levels remained increased until the end of the study; this was statistically significant in comparison to the untreated control group on 10 and 14 dpi.

Significant differences to the untreated control group at the same day are indicated with asterisks (many-to-one comparisons by Gao *et al.* (2008) with Hochberg-adjustment, $P \leq 0.05$).

C: untreated controls, R: rifampicin, En: enrofloxacin, En + R: enrofloxacin + rifampicin, Az: azithromycin, Az + R: azithromycin + rifampicin, Ery: erythromycin, Ery + R: erythromycin + rifampicin. dpi: days post inoculation.

Pulmonary inflammation

BALF cytology. Cell count per mL BALF remained relatively constant throughout the study from 4 dpi (5.8e+05; 1.6e+05–1.3e+06) to 9 dpi (6.1e+05; 2.2e+04–1.3e+06) and 14 dpi (4.9e+05; 1.0+05–1.5e+06) (Fig. 5a). Neutrophil numbers per mL BALF (Fig. 5b) and percentage of neutrophils (data not shown) were maximal at 4 dpi (1.7e+05; 2.2e+03–7.4e+05) and decreased continually until 14 dpi (2.2e+04; 0.0e+00–1.2e+05). Neither cell count per mL BALF nor number of neutrophilic granulocytes per mL BALF nor percentage of neutrophils in the total amount of BALF cells differed significantly between treated animals and the untreated control group at any time. However, as shown in Fig. 5a and b, in macrolide treated animals absolute cell count and neutrophilic granulocytes were slightly reduced compared to animals of all other groups, but not significantly lower than in untreated controls (many-to-one comparisons by Gao *et al.* [32] with Hochberg-adjustment, $P > 0.14$).

Concentrations of total protein in BALF. The concentration of total protein in the BALF supernatant remained constant from 4 dpi (170 µg/mL; 28–638) until 14 dpi (187 µg/mL; 103–345). There was no significant difference in BALF protein concentration between treated animals and the untreated control group (many-to-one comparisons by Gao *et al.* [32] with Hochberg-adjustment, $P > 0.09$) (Fig. 5c).

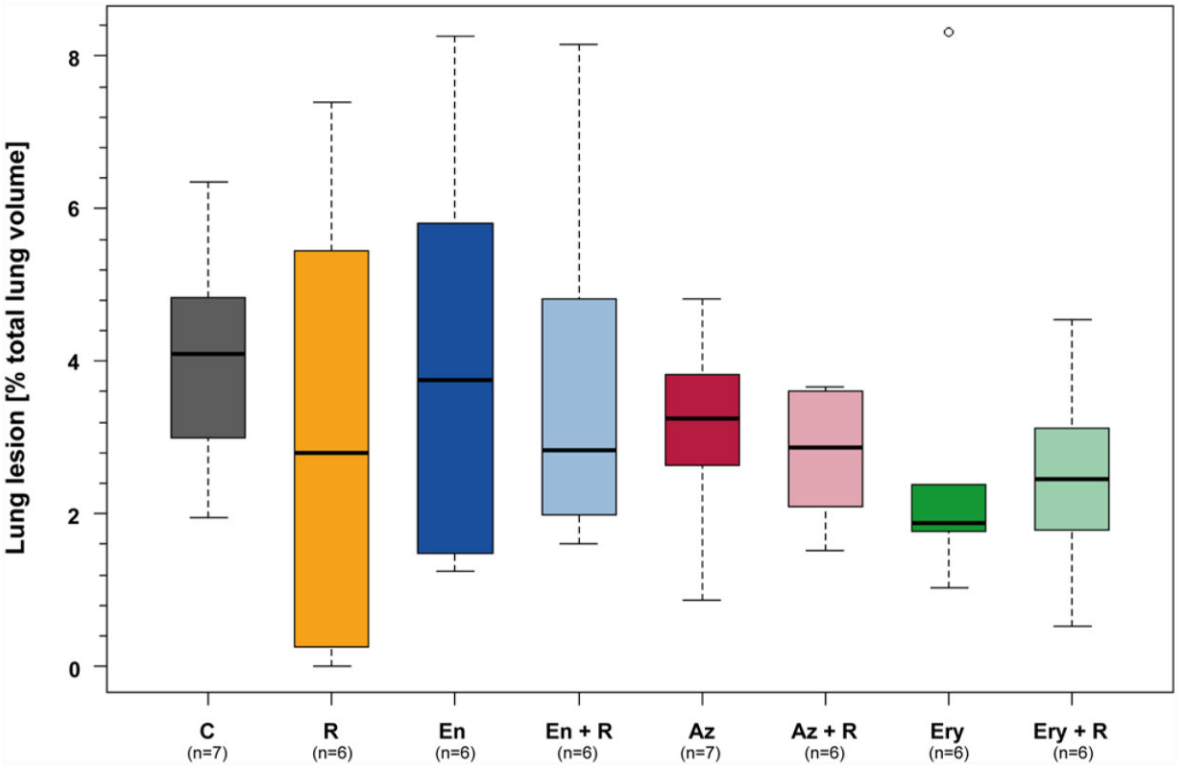

Fig 6. Percentage of altered lung tissue on total lung volume assessed 14 dpi at necropsy. The proportion of altered lung tissue 14 days after inoculation with *C. psittaci* did not differ between treated and untreated animals (many-to-one comparisons by Gao *et al*. (2008) with Hochberg-adjustment, $P > 0.58$). C: untreated controls, R: rifampicin, En: enrofloxacin, En + R: enrofloxacin + rifampicin, Az: azithromycin, Az + R: azithromycin + rifampicin, Ery: erythromycin, Ery + R: erythromycin + rifampicin. dpi: days post inoculation.

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Pulmonary lesions

At necropsy 14 dpi, foci of bronchopneumonia and pleuritis were seen in all calves except for one rifampicin treated animal which showed no lesions. Lung alterations were corresponding to the sites where the inoculum had been applied, i.e. in the caudal part of the left and right apical lobes, the medial lobe and the basal lobes. Necrotic centers and demarcation by fibrous connective tissue were characteristic for the circumscribed pulmonary lesions. The extent of lesions ranged between 0.0% and 8.3% (median: 3.0%) of pulmonary tissue in all animals without significant differences between the treated and untreated animals (many-to-one comparisons by Gao *et al.* [32] with Hochberg-adjustment, $P > 0.58$) (Fig. 6).

Histological examination revealed fibrinous bronchopneumonia in variable degrees of organization characterized by multifocal necrosis, infiltration with lympho-plasma-histiocytic inflammatory cells, hyperplasia of alveolar type 2 epithelial cells, bronchiolitis obliterans, perivascular fibrosis, fibrosis of interlobular septae and ectopic lymphoid follicles. In the majority of calves regeneration predominated. The ratio between zones of necrosis and regeneration varied, however, between individual animals and was not related to treatment groups. In treated animals, chlamydial inclusions were detected by immunohistochemistry (IHC) in 40 calves as small granular labeling in areas with necrosis and few large inclusions in macrophages

in zones of regeneration. One of the three calves where chlamydial inclusions were not found by IHC had no pulmonary lesions and the other two calves had small zones of progressed pulmonary regeneration. Two of these calves had been treated with rifampicin only and one with azithromycin and rifampicin.

Discussion

Study design

Enrofloxacin, erythromycin, and azithromycin have been used to treat chlamydial infections for years. To address both aspects of veterinary and human medicine, different antimicrobial substances from the family of quinolones and macrolides were chosen: Enrofloxacin is a quinolone commonly used in the field of veterinary medicine, whereas its active metabolite ciprofloxacin is frequently used to treat bacterial infections in human patients. In the class of macrolides, we chose one substance available for the use in animals, i.e. erythromycin and a corresponding drug currently used for treatment of chlamydial infections in humans, i.e. azithromycin. In this study, all drugs were administered alone to assess their effect in treatment of an acute respiratory *C. psittaci* infection, and in combination with rifampicin to evaluate whether the increased antichlamydial activity found in the cell culture model by Wolf and colleagues [27] could be reproduced in the living host. One group of animals was treated with rifampicin alone to reveal effects induced only by rifampicin. The calves used in this study had approximately the same weight as adult humans; therefore the rifampicin dose commonly used to treat adult humans was used (600 mg/day per animal).

Treatment was initiated at 30 hours after inoculation. At this time point, clinical signs were beginning to become severe. In a clinical setting, this would be the point to start antimicrobial treatment.

Antibiotics

Enrofloxacin is a fluorquinolone antibiotic with bactericidal activity by inhibiting bacterial DNA gyrase and thereby preventing DNA supercoiling and DNA synthesis. Fluorquinolones reach high intracellular concentrations and accumulate in bronchial secretions [33]. Enrofloxacin is metabolized to its active metabolite ciprofloxacin, which itself is available as a drug in human medicine, whereas enrofloxacin is only available for veterinary use. Both, enrofloxacin and ciprofloxacin have a broad antimicrobial activity against gram-positive and gram-negative bacteria, except for anaerobic microorganisms and are considered reserve antibiotics [34].

Azithromycin and erythromycin belong to the class of macrolides. This class of antimicrobial substances inhibits bacterial protein synthesis by covalently binding to the 50S ribosomal subunit and is effective mainly against gram-positive microorganisms [35].

Erythromycin is the oldest member of the macrolide family and is frequently used to treat a variety of bacterial diseases in veterinary medicine. It is also available for the use in human patients. Depending on its concentration and on the sensitivity of the microorganism, erythromycin is either bactericidal or bacteriostatic [36].

Azithromycin is superior to erythromycin in terms of pharmacokinetic profile, gastric acid stability, half-life, and bactericidal activity. Tissue concentrations of azithromycin exceed serum concentrations by far and its amphiphilic properties allow it to enter cells and tissue rapidly. It is concentrated in lysosomes of host defence cells, which is the place where chlamydiae replicate. Azithromycin is a valuable tool in the treatment a variety of bacterial infections in humans, including genital tract infections with *C. trachomatis*, since in most cases a single dose is sufficient for therapy (reviewed in [37]). A recent meta-analysis reported similar cure rates for

azithromycin and doxycycline in treatment of human *C. trachomatis* infections. Currently, the use of azithromycin in food-producing animals is banned in the European Union.

Rifampicin, a semi-synthetic antimicrobial drug, enters leukocytes and tissues very well due to its lipid solubility [38,39]. It exhibits bactericidal activity against many gram-negative and most gram-positive bacteria through inhibiting DNA-dependent RNA polymerase [40,41]. In human patients, rifampicin is widely used for the treatment of brucellosis and mycobacterial infections. Yet there is a potential of quickly developing resistance, therefore rifampicin must always be administered in combination with another antimicrobial drug. In experimental settings, rifampicin has been found useful for the treatment of mycobacterial infections in ruminants [42], but in practical settings this would contravene current legislation on authorized substances for the use in food-producing animals in the European Union.

The plasma concentration of ciprofloxacin measured in this study was about 25 to 50% of the plasma concentration of enrofloxacin. This is as expected and reported previously [43]. The MIC of 0.25 μg/mL described for *C. psittaci* isolates from turkeys [44] was reached in plasma from 3 dpi to 5 dpi in all calves treated with enrofloxacin. Afterwards, plasma levels of enrofloxacin in some animals were below 0.25 μg/mL. In the lung of animals treated with enrofloxacin, tissue levels of enrofloxacin alone were often lower than 0.25 μg/mL, but this does not take into account the antichlamydial activity of ciprofloxacin. Here, animals with lower lung tissue levels did not show higher clinical scores or more positive reisolation results than animals with higher lung tissue levels. Nevertheless, studies on enrofloxacin treatment of chlamydiae in koalas, cats and pigs revealed that therapeutic levels of the drug are often not reached [25] and that shedding of the pathogen can still occur during [26] or after the end of treatment [45]. Chlamydiae can develop resistance against fluorquinolones when exposed to subtherapeutic dosages over a longer time period [46,47].

The MIC for the *C. psittaci* strain DC15 for macrolides and rifampicin reported from *in vitro* studies were between 0.016 and 0.08 μg/mL [27]. These values were reached in plasma and lung tissue of animals treated with macrolides and/or rifampicin with only very few exceptions on single days. As expected, azithromycin levels in lung tissue were by far higher than plasmatic levels.

Macrolides do not only possess antimicrobial, but also anti-inflammatory properties that have been known for decades [48]. They suppress lung defence mechanisms by decreasing neutrophil adhesion and increasing apoptosis of neutrophils, this is why they are successfully used in the maintenance treatment of a variety of chronic airway diseases in humans (reviewed in [49] and [50]). The same effect was shown in foals; erythromycin treatment reduced neutrophil influx into the lung after lavage [51].

In the present study in *C. psittaci* infected calves, absolute cell count and numbers of neutrophils in BALF were slightly reduced in macrolide-treated animals as compared to the untreated control group, but this difference could not be secured statistically. Possibly this is due to the fact that macrolide treatment started 30 h post inoculation, leaving enough time for migration of macrophages and neutrophils to inflamed lung areas. Regarding the parameters observed we could not show a significant impact on lung defence mechanisms by erythromycin at a dose of 12 mg/kg bw/day and by azithromycin at a dose of 6 mg/kg bw/day in calves.

Chlamydiae + antibiotics

There are numerous clinical studies on antimicrobial treatment of chlamydial infections in humans [52–60], but many of them are not placebo-controlled and success of treatment is defined by the eradication of clinical signs rather than the elimination of the pathogen. In studies performing pathogen detection after azithromycin treatment, DNA of *C. trachomatis* was still

detectable in the urinary tracts of human patients [14,61] and in tissue of experimentally infected mice [62,63]. In the present study, despite considerably high antibiotic levels in the plasma measured at 3 and 5 dpi, reisolation of living *C. psittaci* was possible from samples obtained 4 dpi in animals of all groups. The fact that living chlamydiae could be isolated 9 and 14 dpi from control animals, but not from treated animals (except for one animal from the Ery + R group 9 dpi) indicates that the applied substances inhibit the growth of *C. psittaci* in the living host, but only after more than 3 days of treatment, thus confirming the used strain DC15 to be sensitive against macrolides, quinolones and rifampicin, as reported by Wolf and colleagues [27].

Even though we could show that treatment with macrolides and quinolones alone or in combination with rifampicin inhibited proliferation of *C. psittaci* in the living host, we could not detect any impact on parameters of clinical illness, severity of local and systemic inflammation, or on the number of genome copies detectable in tissue at 14 dpi. Statistically significant differences between treated and untreated groups in the clinical score, numbers of blood leukocytes and neutrophilic granulocytes were not assumed to be of biological relevance, since they occurred only sporadically.

From these facts, one can assume that, in this particular model, it did not matter to the host whether the pathogen was able to replicate or not, because host immune defense mechanisms alone were sufficient to cope with the infection.

There are plenty of reports on mild or subclinical infections with *C. psittaci* in humans [64–68] and of asymptomatic *C. trachomatis* infections in women [69,70] that resolved without antimicrobial treatment.

Similar findings were reported from studies on subclinical pulmonary abscesses caused by Rhodococcus equi in foals, where spontaneous healing could not be accelerated by adequate antimicrobial treatment [71,72].

These reports are very similar to the results of the present study as neither clinical outcome nor extent of lung lesions differed between treated and untreated animals. Increased LBP response in erythromycin-treated animals in comparison with controls is thought to be due to the inflammatory reaction at the injection site. Apart from this side effect, the LBP response did not differ significantly between treated and untreated animals, thus indicating a comparable severity of inflammation regardless of antimicrobial treatment. Taking all findings together, this leads to the conclusion that the beginning of the treatment in this study was too late to have a significant influence on the course of inflammatory lung disease in the animals, i.e. clinical signs, elevated LBP levels, increased total cell count and neutrophils in blood and BALF. The severity of lesions due to inoculation was already determined at that time point, and everything observed from thereon was the course of remission. As the course of disease in control animals showed, the immune response of the animal sufficed to restore the absence of clinical and inflammatory signs by the end of the study, since clinical score, LBP-levels and blood count reached baseline levels at 10 dpi at the latest. The fact that viable chlamydiae could be reisolated more often and at later time points from control animals than from treated animals did obviously not influence any other parameters that we analysed.

The question whether macrolides or quinolones are adequate for treatment of chlamydial infections in calves cannot be answered by this study. For further studies it would be interesting to screen animals for shedding of chlamydiae after the end of treatment to find out whether treated or untreated animals could clear the infection. The problem with this approach is that shedding of chlamydiae does not occur on a regular basis in infected individuals as could be shown in this and former studies with the used bovine model [13,29] and in a study with *C. trachomatis* infected human patients [16].

The fact that chlamydial infections can only be detected when the pathogen is actually shed by the host or when the host becomes seropositive very much limits research in this field, since both are not necessarily true for all individuals infected.

Having in mind that antimicrobial treatment of *Chlamydia*-infected animals is very likely to be without benefit to the host, development of vaccines could be an alternative strategy to limit chlamydial infections in cattle. Currently, available vaccines against chlamydial components of diseases in animals are: abortion in small ruminants (*C. abortus*), respiratory disease complex in turkeys (*C. psittaci*), and conjunctivitis and respiratory infections in cats (*C. felis*). Sterile immunity cannot be expected, a study on *Chlamydia*-associated subclinical mastitis in dairy cows revealed that although bovine mastitis was decreased after vaccination, chlamydiae were still shed into the milk [73].

Recommendations

Due to the very painful side effects at the injection site, subcutaneous application of erythromycin cannot be suggested for long-term treatment. These adverse reactions at the injection site are known side effects and are reported to last for no longer than 6 days [74]. When using enrofloxacin for treatment of chlamydial infections, the dose must be adapted to reach plasma and tissue levels that are higher than the MIC of the pathogen to enable eradication of chlamydiae and avoid the development of antimicrobial resistance.

Combination with rifampicin

It has not been possible to extrapolate the findings from the cell culture model, i.e. increased antichlamydial activity of macrolides and quinolones when applied together with rifampicin, to the situation in the living host. This is mainly due to the fact that the host alone was able to cope with the induced infection and treatment itself did not have any measurable influence in terms of host reaction. Reisolation of the living pathogen was possible to the same extent in animals treated with a single drug or the same drug in combination with rifampicin, indicating that the additional application of rifampicin in the living host did not increase the antichlamydial effect of enrofloxacin, erythromycin or azithromycin. On the contrary, the only treated animal in which reisolation was possible 9 dpi had received erythromycin in combination of rifampicin, which does not support the thesis that the combination of erythromycin and rifampicin is particularly effective against chlamydiae in the living host.

Conclusion

Our results show that treatment of *Chlamydia*-infected calves with enrofloxacin, erythromycin and azithromycin alone or in combination with rifampicin could reduce the number of viable pathogens to the same extent, thus rejecting the hypothesis that combined treatment with rifampicin is superior to single-drug therapy with macrolides or quinolones in the living host. Nevertheless, these findings had no influence on severity of disease and time course of its resolution, as assessed by clinical and pathological findings as well as inflammatory parameters. We can conclude that in this study, a carefully selected and effective antimicrobial treatment in an acute infection was without benefit, since the host's immune defense mechanisms were sufficient to cope with the infection and all animals, regardless if treated or not, regained clinical health by the end of the study.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the German Animal Welfare Act. 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: 04–004/11). All experiments were conducted in a containment of biosafety level 2 under supervision of the authorized institutional Agent for Animal Protection. Bronchoscopy was strictly performed under general anesthesia. During the entire study, every effort was made to minimize suffering.

Animals

Fifty male conventionally raised calves (Holstein-Friesian) were included in this prospective and controlled study. The farm the animals were purchased from did not have any history of *Chlamydia*-associated health problems. In advance, the herd of origin was randomly checked for chlamydial antigen and antibodies by *Chlamydiaceae*-specific PCR and indirect ELISA, respectively, at the National Reference Laboratory for Chlamydiosis. Calves were purchased at the age of 12 to 30 days weighing between 43.6 and 77.6 kg (58.9 ± 8.9; mean ± SD). Animals were included in the study after a quarantine period of at least 21 days and confirmation of a clinically healthy status.

Animals were reared under standardized conditions (room climate: 18–20°C, relative humidity: 60–65%) throughout the entire study. Animal housing was in accordance with the guidelines for animal welfare set forth by the European Community. Nutrition included commercial milk replacers and coarse meal. Water and hay were supplied *ad libitum*. None of the given feed contained antibiotics.

Study design

All animals were inoculated with 10^8 inclusion forming units (ifu) of *C. psittaci* strain DC15 at the age of 33–53 days. Preparation of the challenge strain was described elsewhere [31]. Eight mL of the *Chlamydia*-containing inoculum were applied intrabronchially and intranasally as described previously [75]. The treatment groups were age matched, and weight of the animals at the time of inoculation (71.7 kg ± 8.6 kg, mean ± SD) did not differ significantly between the groups (Kruskal-Wallis test, $P = 0.99$).

Forty-three calves underwent antimicrobial treatment, whereas 7 calves served as untreated controls (C). The seven treatment groups were treated according to the following regimens: Rifampicin (n = 6): 600 mg rifampicin/day (Eremfat i.v. 600 mg, Riemser Arzneimittel AG, Greifswald, Germany);

Enrofloxacin (n = 6): initially (first treatment) 7 mg/kg bw/day, then (second treatment and following treatments) 5 mg/kg bw/day enrofloxacin (Baytril 10%; Bayer Vital GmbH, Leverkusen, Germany);

Enrofloxacin + Rifampicin (n = 6): initially 7 mg/kg bw/day, then 5 mg/kg bw/day enrofloxacin plus 600 mg rifampicin/day;

Azithromycin (n = 7): initially 10 mg/kg bw/day, then 6 mg/kg bw/day azithromycin (Zithromax Trockensaft 1500 mg, PFIZER PHARMA GmbH, Berlin, Germany);

Azithromycin + Rifampicin (n = 6): initially 10 mg/kg bw/day, then 6mg/kg bw/day azithromycin plus 600 mg rifampicin/day;

Erythromycin (n = 6): 12 mg/kg bw/day erythromycin (Erythrocin Vet. 200mg/ml, CEVA Tiergesundheit GmbH, Düsseldorf, Germany).

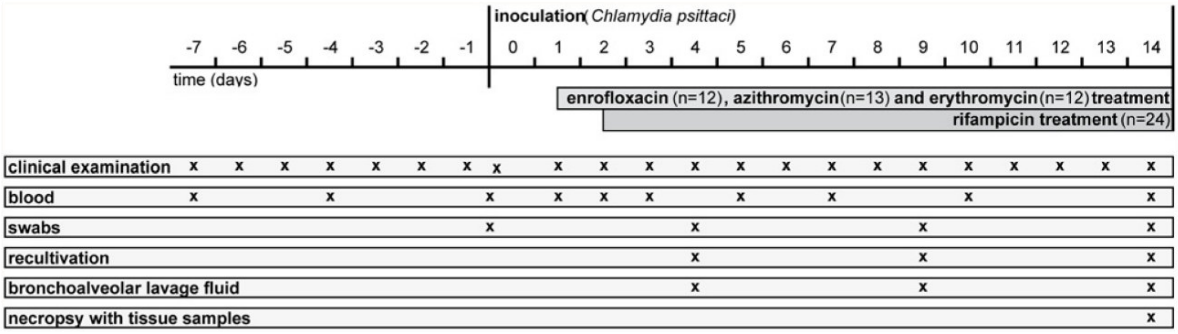

Fig 7. Study design.

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Erythromycin + Rifampicin (n = 6): 12 mg/kg bw/day erythromycin plus 600 mg rifampicin/day.

Azithromycin was applied orally with the milk replacer once daily starting 36 hours after inoculation (pi). Rifampicin was administered intravenously in 500 mL isotonic saline solution once daily over 30 minutes starting 48 hours pi. Enrofloxacin and erythromycin were injected subcutaneously once daily, starting 30 hours pi. Treatment continued until 13 days pi (dpi), and all animals were euthanized and necropsied 14 dpi. Sampling throughout the study is illustrated in Fig. 7.

The two veterinarians performing the treatments and monitoring the animals were the only persons who knew the assignment of the animals to the different treatment groups. Variables assessed *in vivo* (body temperature, respiratory rate, heart rate) can be regarded as 'objective' since they are measured values. An individual ID according to the rules of our institute's quality management system was given to all specimens examined *ex vivo*. Lab investigators were therefore disabled to identify individual animals or treatment regimens.

Clinical Scoring and daily weight gain

Clinical examination of all animals was performed twice daily, starting 4 days before challenge. A previously described scoring system was used to summarize the observations [31]. For determination of daily weight gain, each animal was weighed directly before inoculation and directly before necropsy. The difference was calculated and divided by the number of days in between.

Collection of swabs and blood and blood analyses

Nasal and fecal swabs were taken one hour before inoculation and immediately before necropsy. Pharyngeal and fecal swabs were obtained 4 dpi and 9 dpi. Swabs were sampled as described previously [13].

Venous blood of all animals was drawn from the *Vena jugularis* 7 days, 4 days and 1 hour prior to inoculation and 1 dpi, 2 dpi, 3 dpi, 5 dpi, 7 dpi, 10 dpi, and 14 dpi (Fig. 7). After the beginning of antimicrobial treatment, blood was always drawn 18 hours after treatment with enrofloxacin, azithromycin or erythromycin and 21 hours after treatment with rifampicin.

Collection of blood, white blood cell count, differentiation of white blood cells, serum preparation, and quantitative detection of LBP with an enzyme-linked immunosorbent assay (ELISA) were described previously [29,30].

Necropsy, pathological evaluation, collection of tissue samples, histology, and immunohistology

Euthanasia of the calves and exenteration of the lung was performed as described before [31]. Pathological evaluation of lung lesions and tissue sampling as well as histology and immunohistology has been described in detail [13].

Collection of bronchoalveolar lavage fluid (*in vivo*, *post mortem*) and BALF analyses

Endoscopic sampling of BALF in the anesthetized animal was performed 4 and 9 dpi as described previously [75]. At 14 dpi, BALF was obtained from freshly exenterated lungs immediately after exsanguination as described in [13]. Isotonic, sterile, body-warm saline was always used as flushing liquid. The obtained BALF was immediately collected in siliconized glass bottles and stored on ice until further preparation.

BALF recovery *in vivo* was 79.7 ± 4.5% (mean ± SD); *post mortem* it was 63.1 ± 7.2% (mean ± SD). Both values did not differ significantly between the treatment groups (Kruskal-Wallis test, $P > 0.13$).

Preparation of BALF, BALF cell count and differentiation were done as described previously [13]. Analysis of total protein in BALF supernatant was also described elsewhere [31].

Collection of bronchial brushings

Bronchial brushings for the reisolation of the chlamydiae were obtained 4 and 9 dpi during bronchoscopy from the right lung, just caudal of the *Bifurcatio tracheae* as described previously [75]. The brush was rinsed thoroughly in stabilizing SPGA medium (containing sucrose, phosphatile substances, glucose and bovine albumin [76]). Samples obtained at 4 dpi were processed immediately, whereas samples obtained at 9 dpi were stored at -20°C until further use.

Reisolation

Isolation of *C. psittaci* in buffalo green monkey kidney cells was performed according to standard procedures. To confirm viability of the pathogen, five randomly selected culture positive samples were subjected to further passaging and 11 samples were subjected to DNA-sequencing. Samples were bronchial brushings obtained 4 and 9 dpi and altered lung tissue collected at necropsy (14 dpi).

Detection of chlamydial DNA using quantitative real-time PCR (qrt-PCR) and sequencing

Samples of macroscopically altered and macroscopically normal lung tissue, mediastinal lymph node, swabs (pharyngeal, nasal, faecal), venous blood, and selected samples of reisolated and passaged chlamydiae were examined for chlamydial DNA. Extraction of DNA was performed using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. In qrt-PCR testing for the family *Chlamydiaceae*, two 1.0 µl aliquots of the final eluate were used as templates [77]. Ct values of less than 38.0 in both replicates measured were considered positive.

Additional testing for the species of *C. psittaci* was performed for the swabs and blood samples [78]. Passaged chlamydiae that were reisolated in cell culture from bronchial brushings and lung tissue were subjected to sequencing of the complete *ompA* gene to confirm the identity to challenge strain DC15 [79].

Antibiotic levels

Antibiotic levels of azithromycin, erythromycin and rifampicin were determined in plasma obtained 1 h prior to inoculation as well as 3, 5, 7, 10, and 14 dpi and in tissue samples collected at necropsy 14 dpi (unaltered lung, liver and muscle). The samples were all stored at -80°C until analysis and were tested for the presence of the respective drugs that had been administered. Preparation of samples and analysis was performed as follows:

Levels of erythromycin, azithromycin and rifampicin in animals treated with macrolide alone or in combination with rifampicin. Two hundred μL of each plasma sample were extracted with 600 μL acetonitrile containing sulfaphenazole as internal standard. After vortex and centrifugation, the supernatant was evaporated in a nitrogen stream until dryness. The extract was resuspended in 500 μL acetonitrile/milliQwater 20/80 and then filtered on polyvinylidene fluoride (PVDF) 0.45 μm.

Pieces of 0.5 g of each tissue sample were shaken with 250 μL milliQwater containing sulfaphenazole as internal standard, and then extracted with 2 mL acetonitrile for 10 min.

After centrifugation, the supernatant was evaporated in a nitrogen stream until dryness. The extract was resuspended in 500 μL acetonitrile/milliQwater 20/80 and filtered on PVDF 0.45 μm.

LC-MS-MS analysis of all samples was done using an Agilent1290 HPLC (Agilent Technologies) coupled with MS-MS detector 6460 (Agilent Technologies). The chromatographic column was Waters BEH C18 (150mm x 2.1mm x 1.7 μm). Five μL of each sample were injected and separated using a gradient from 80% water 0.05% formic acid and 20% acetonitrile to 100% acetonitrile and a flow rate of 0.4 mL/min for 8 min.

For plasma quantification, the calibration was prepared in matrix with spiked plasma from 1 to 200 ng/mL with sulfaphenazole as internal standard.

For tissue quantification, the calibration was prepared in matrix with spiked tissue from 10 to 500 ng/mL with sulfaphenazole as internal standard.

The detection was performed using positive electrospray ionization (ESI+) with the MRM transitions: azithromycin 749/83.2 and 749/158, erythromycin 734/158 and 734/83, rifampicin: 823/791 and 823/399 sulfaphenazole (internal standard): 315/158 and 315/92.

The threshold was set to 3.5 ng/mL for all analytes.

Levels of enrofloxacin and rifampicin in animals treated with rifampicin and/or enrofloxacin. Plasma samples were mixed 1 + 9 (v/v) with water. After addition of internal standard of enrofloxacin and ciprofloxacin an aliquot was injected directly into an online solid phase extraction chromatography (OSPE) system while it was in the loading position. Matrix components contained in the injected sample were separated from the retained analytes on an extraction column suited for pre-treatment of samples at high flow rates. After switching to the elution position, the analytes were transferred to an analytical column. The quantitative determination was performed in a tandem mass spectrometric detector (MS/MS).

The limit of quantitation was 25 μg/L for each analyte.

For analysis of tissue samples, the entire sample material (between 0.4 and 1.4 g) was cut into small pieces using a scalpel and then filled into an Ultra-Turrax Tube Drive tube (IKA-Werke GmbH & Co. KG, Staufen, Germany). Enrofloxacin and ciprofloxacin were extracted simultaneously by homogenisation with 15 mL of a mixture of acetonitrile, water and formic acid (500/500/0.1, v/v/v) and subsequent centrifugation.

For the determination of enrofloxacin and ciprofloxacin the extract was diluted 1 + 1 (v/v) with water and internal standard was added. Measurement was performed as described above by OSPE-LC-MS/MS.

For rifampicin analysis the extract was diluted 1+1 (v/v), for liver 1 + 9 (v/v), with acidic water by HPLC with tandem mass spectrometric detection.

The limit of quantification was 100 μg/kg for enrofloxacin and ciprofloxacin and 10 μg/kg for rifampicin for all sample materials.

A more detailed description of the analysis of enrofloxacin and ciprofloxacin levels has been published earlier [80].

Antibiotic levels above threshold (animals treated with macrolide alone or in combination rifampicin) or detection level (animals treated with rifampicin and/or enrofloxacin) were considered significant.

Exclusion of co-infections

The herd the calves originated from was known to be free of bovine herpes virus 1 (BHV-1) and bovine virus diarrhea/mucosal disease virus (BVDV). Immunohistochemical examination of ear biopsies for BVDV antigen [81] was negative indicating that no calves were immunocompromised by persistent BVDV infection.

Routine microbiological screening on the day after purchase revealed that all animals were negative for *Salmonella* infections (fecal swabs). During the quarantine period, all 50 calves included were checked serologically for antibodies against *C. abortus* and *C. psittaci* (ID SCREEN Chlamydophila abortus indirect multi-species ELISA Kit; ID vet, Grabels, France). All animals were serologically negative prior to inoculation.

The presence of the relevant respiratory co-pathogens *Mycoplasma*, *Pasteurella* or *Mannheimia* spp. was evaluated in nasal swabs taken immediately before challenge and before necropsy, and in samples of lung tissue obtained during necropsy. Neither *Mannheimia haemolytica* nor *Mycoplasma bovis* nor *Pasteurella multocida* were detected in any swab or lung sample. Serology at the beginning of the quarantine period and at 14 dpi was used to check for viral co-pathogens i.e. bovine respiratory syncytial virus (BRSV), parainfluenza 3 virus (PI3), adenovirus type 3, BHV1 and BVDV (Bio-X respiratory penta ELISA Kit, Bio-X-Diagnostics, Jemelle, Belgium). Maternal antibodies were seen against BRSV (42/50), PI3 (46/50), BVDV (26/50), BHV1 (20/50) and adenovirus type 3 (47/50).

To eliminate any unknown bacterial infections, all animals received antibiotic treatment in the week after purchase according to our animal facility's regulations for the entry of new animals. Calves to be treated with enrofloxacin, enrofloxacin and rifampicin or rifampicin alone and the two co-housed untreated control animals were treated with subcutaneous injections of 10 mg/kg bw/day erythromycin on three consecutive days in the week after purchase. All other animals received subcutaneous injections of 5 mg/kg bw/day enrofloxacin on three consecutive days, beginning two days after purchase.

It has been reported that seven days after subcutaneous injection of enrofloxacin in cattle, residue levels in liver, kidney, muscle, and fat are <10 μg/kg [82]. In calves that had received intramuscular injections of erythromycin, antimicrobial activity in tissue was detected no longer than 3 days after the last injection [74], thus ruling out an influence of pre-study treatment on any results.

Statistical methods

R [83] was used for statistical evaluation of the data. In addition, the package Plotrix [84] was used for computing the graphs. Due to the small group size of n = 6 and n = 7, data was assumed not to be normally distributed. Kruskal-Wallis test was carried out to compare values of more than two different groups. To compare values of treated groups against values of the untreated control group, the nonparametric multiple tests for many-to-one comparisons by Gao

et al. [32] with Hochberg-adjustment of the p-value from the package nparcomp [85] was performed. Only significant differences between control group and treated groups are given, differences between treated groups were of no interest in this study. For comparison of two groups, Mann-Whitney U test from the package coin [86] was used. Unless stated differently, data are given as median and range. In 'Box and Whiskers plots', outlier values (circles) were more than 1.5 times the length of a box away from the median. Values of $P \leq 0.05$ were considered significant.

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Author Contributions

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

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3 Discussion

The aim of the present study was to evaluate standard antimicrobial therapies of chlamydial infections and to compare them to newly proposed treatment regimens. The treatment studies were to be performed in a previously established bovine model of respiratory *C. psittaci* infection. In order to repeatedly obtain samples from the lung as the primary site of infection from each animal, bronchoscopical sampling methods had to be adapted for the use in calves aged 6-10 weeks under experimental conditions.

3.1 Suitability of samples for monitoring the treatment studies

The following sections are focused on the practical value of the obtained samples for STUDY 2 and STUDY 3, i.e. BALF, bronchial brushings and transbronchial lung biopsies. For a more general discussion of the applicability of the sampling procedures, the reader is referred to the discussion section of STUDY 1.

3.1.1 Overall sampling procedure

Under the given conditions in the animal experiments, the developed sampling protocol proved to be practicable especially for experienced examiners, requiring approximately 30 minutes per animal. The experiments were carried out under biosafety level 2, meaning that all staff in contact with the animals needed to wear protective gear, including respirators. Five animals had to be sampled consecutively via bronchoscopy, and the samples had to be further processed in the laboratory on the same day by the same examiner. In the treatment studies we could acquire valuable results on severity and time course of local inflammation as well as on the viability of the pathogen in treated and untreated animals with the newly established methods of bronchoscopical sampling in the bovine lung. The sampling process and associated general anesthesia were well tolerated by all animals as no adverse reactions were observed. Strictly following the protocol defining precise localization of the sampling sites and of the order in which the samples were obtained provided defined samples of comparable quality from all animals, and it ensured that the different sampling procedures would not influence each other. This aspect is discussed in detail in STUDY 1.

3.1.2 BALF

BALF samples proved suitable for the monitoring of the animals under the given circumstances. By lavaging bigger areas of the lung are sampled than by biopsy or bronchial brushing, thus including both altered and unaltered parts of the lung. Previous studies in this model showed BALF sampled *ex vivo* as a valuable diagnostic material to assess the inflammatory status of the lung (Reinhold*, et al.* 2012). Read-out parameters were total cell number, differential cell count, and concentrations of protein. We could confirm these findings *in vivo*. With respect to antimicrobial treatment, the analysis of BALF samples in STUDY 2 and STUDY 3 revealed convincingly that there were no differences in the course of local inflammation in the lung between treated and untreated animals.

The differential and total BALF cell count was comparable between *in vivo* samples from the present studies and samples obtained from exenterated lungs at comparable time points after inoculation in previous studies in the same model (Reinhold*, et al.* 2012; Ostermann*, et al.* 2013a). However, in this study it was noticeable that BALF sampled bronchoscopically was macroscopically different from BALF sampled from the exenterated lung 14 dpi. BALF sampled *ex vivo* had less foam on the surface, which develops from the sphingolipid-containing surfactant covering the inside of the alveoli. It was also more often contaminated with blood, resulting in a pale reddish color. The contamination with blood is a possible source of errors for measured parameters. An example is the cell composition: The stained smears contain cells from the lung and, to a lesser extent, cells originated from the blood, which may influence the differential cell count. The quality of the stained smears also markedly differed between *in vivo* and *ex vivo* samples: Cells from *in vivo* samples were in a much better condition and easier to differentiate than cells from *ex vivo* samples. These differences were very obvious to the examiner, therefore it is imaginable that other parameters, such as protein concentration, may as well be influenced by the sampling method. With the samples at hand, evaluation of such influences however was not possible since in former studies (Reinhold*, et al.* 2012; Ostermann*, et al.* 2013a), most changes for these parameters were seen very early after inoculation (i.e., 2 and 3 dpi), and we did not have *in vivo* samples available from this time points. For some, not yet published parameters we could detect differences between *in vivo* and *ex vivo* samples, but we cannot definitely tell whether this is due to the time course of the disease or due to the different sampling methods. Methods of sampling BALF differ considerably depending on the animal model used. To increase comparability between different data, we therefore point out the urge to clarify the influence of the sampling method on measurable parameters in the BALF in future experiments.

3.1.3 Bronchial brushings

Bronchial brushings were used for the first time in this model, and provided excellent samples for recultivation of *Chlamydiae* from the bovine lung. It was possible to obtain samples precisely from the sites of inoculation. These were the lung sites with the highest chance of recovering the pathogen, as they were previously identified to correspond with the localization of inflammatory alterations (Reinhold*, et al.* 2012). The prepared smears of bronchial brushings were not suitable for immunohistochemical staining as planned beforehand. The high number of erythrocytes in the samples led to distinct artefacts, and correct interpretation was therefore not possible. Yet this did not impair the study, since the significance of the recultivation results was of higher importance for the given question, as it could differentiate between viable and not viable *Chlamydiae*. With the bronchial brushings we could prove that recultivation of *Chlamydiae* was more often possible from untreated than from treated animals, which was a very important contribution to the informative value of the studies.

3.1.4 Transbronchial lung biopsies

Obtaining transbronchial lung biopsies was the most time consuming of all sampling procedures and the one requiring the most practice and dexterity of the examiner. Biopsies of animals from STUDY 2 were examined for the presence of *Chlamydiae* by polymerase chain reaction (PCR)

and IHC. However, the results did not provide insights of additional value for the study, since there were big differences in the amount of *Chlamydiae* that could be detected even in control animals without antimicrobial treatment. In some of the samples, no pathogen could be detected. Therefore, the results from examination of the lung biopsies were not included in the publication, and the number of biopsies sampled from the animals included in STUDY 3 was reduced for the sake of time. A reason for the described problem is the focal character of the chlamydia-induced lesions in the bovine lung, resulting from the demarkation by connective tissue, which is typical for bovine respiratory disease (Reinhold 1997). The lesions could not be sampled in a targeted manner with the equipment we had at hand. Theoretically, there are devices available which allow targeted sampling of lung lesions by combination of endoscopy with imaging methods such as ultrasound, fluoroscopy or computer tomography. However, these devices are designed for bronchoscopy in humans and are therefore not suitable for the use in calves due to their great length of nasopharynx and neck in comparison to humans. Despite of those limitations, numerous transbronchial lung biopsies of all animals were conserved in glutaraldehyde to be scanned by electron microscopy for aberrant bodies, the morphological resemblance of chlamydial persistence. The samples were set back for later investigation, their analysis was not included in the aims of the present project.

3.2 Design of the treatment studies

3.2.1 Choice of antimicrobial substances

The goal was to find the best suited options for treatment of chlamydial infections in humans and in cattle. The classes of tetracyclines, macrolides and quinolones were to be evaluated alone or in combination with rifampicin for their suitability of treating respiratory *C. psittaci* infections in the bovine host. From the class of tetracyclines, doxycycline was chosen as it is available for the use in humans and calves. Doxycycline was administered at two different concentrations, i.e., 5 mg/kg/day as the dose commonly used for the treatment of human patients and 10 mg/kg/day as a dose previously described for the use in calves (Papich 2011). From the class of macrolides, erythromycin was chosen, which is available for the use in humans and animals. Additionally, azithromycin was tested, as it is often used in human patients to treat chlamydial infections due to its very good pharmacokinetic properties (Lode 1991; Kong*, et al.* 2014). A series of case reports revealed that for patients with therapy resistant asthma, tetsted positive for *C. pneumonia*, a six month azithromycin treatment led to remission of the symptoms (Hahn 2013). This is a clue of the suitability of azithromycin for the treatment of chlamydial infections. Enrofloxacin was chosen from the class of quinolones as it is very widely used in veterinary medicine, and its active metabolite ciprofloxacin represents a drug often used in the treatment of human patients. Where possible, preparations for oral application were chosen (doxycycline, azithromycin). For erythromycin and enrofloxacin this was not possible because we had to choose preparations that were approved for the use in cattle. Both were applied subcutaneously.
Rifampicin had been shown to significantly increase the antichlamydial effect of tetracyclines, quinolones and macrolides *in vitro* (Wolf*, et al.* 2010). Therefore it was applied together with the respective substances or alone, to identify any effects of monotherapy with rifampicin and to

compare them to the effects of the combination therapies. Since calves at the age of 6-10 weeks have body weights comparable to those of adult humans, the dosage used to treat human patients was chosen. Rifampicin was dissolved in isotonic saline solution and applied intravenously.

3.2.2 Start of treatment

Our treatment studies were designed to mimic the scenario of an acute respiratory infection. The time point to start treatment was therefore chosen at 30-36 h pi, when pronounced clinical symptoms became present in all animals. In a clinical setting, this would be an appropriate time point to initiate antimicrobial treatment. Intravenous rifampicin treatment was always started 48 h pi, since it was expected to have a faster onset of effect than substances applied orally or subcutaneously.

3.2.3 Duration of the study and samples taken

The duration of the study needed to be long enough to ensure adequate duration of treatment, but at the same time, marked pathological findings at necropsy had to be present to evaluate the effect of treatment. From former studies it was known that later than 14 dpi lesions had completely resolved in parts of the animals (Reinhold*, et al.* 2012). Since the extent of lung lesions and their histopathological characteristics were important parameters to be compared between treated and untreated animals, 14 dpi was chosen as the termination of the study.
To evaluate the treatment regimens, parameters of local and systemic inflammation and pathogen detection were to be compared among the groups. Time points for sampling were identical in STUDY 2 and STUDY 3 and are specified in the corresponding 'material and methods' sections. Briefly, blood was sampled 8 times during the study period of 14 days, and bronchoscopic sampling was performed on 4 and 9 dpi. For ethical reasons, the first time point for bronchoscopic sampling under general anesthesia was chosen at 4 dpi. Earlier time points would have meant a higher burden for the animals due to the increased anesthetic risk in individuals with impaired breathing and reduced general condition. As a second time point, 9 dpi was chosen to have samples from the lung available at consistent intervals throughout the study. 4 dpi represents the acute phase of the disease, whereas 9 and 14 dpi represent the phase of remission. Examined markers of local inflammation were total and differential cell count of BALF cells and total protein concentration in the BALF supernatant.To compare systemic inflammatory parameters between the treatment groups, total and differential blood count were determined, and LBP-concentrations in the peripheral blood were measured, as in previous studies these parameters showed consistent changes in animals inoculated with 10^8 ifu *C. psittaci* (Reinhold*, et al.* 2012; Ostermann*, et al.* 2013a; Ostermann*, et al.* 2013b).
To monitor the impact of the different treatment regimens on the presence and viability of the inoculated pathogen, additional samples were taken:

- bronchial brushings (recultivation of viable *Chlamydiae*),
- swabs (qrtPCR, shedding of *Chlamydiae*),
- blood (qrtPCR, spreading through bacteraemia), and
- tissue samples at necropsy (qrtPCR, pathogen load in the tissue).

The pathological outcome of the intrabronchial inoculation with *C. psittaci* was described previously (Reinhold*, et al.* 2012). It was therefore thought to be a good parameter to describe the effect of treatment. At necropsy 14 dpi, the proportion of inflammatory altered lung tissue on total lung volume was recorded and samples from every animal were later examined histopathologically to further characterize the lesions. Immunohistological detection of *Chlamydiae* was also performed.
To verify sufficient levels of the applied antimicrobial substances in all treated animals, blood serum was sampled repeatedly throughout the study and tissues (muscle, liver, lung) were sampled at necropsy.

The chosen samples provided a good overview on intensity and duration of local and systemic inflammatory processes in the animals with enough detail to evaluate the value of the different treatment regimens for the host. The quantification of the pathogen load and shedding in the individual animals showed a very high degree of variation. However, we believe that this is not due to the quality of the chosen samples, but to the properties of *Chlamydiae*. In previous studies it was shown that this intracellular pathogen is not continuously shed by infected individuals, and the significance of a sample tested negative for chlamydial DNA is rather low (Dukers-Muijrers*, et al.* 2012; Ostermann*, et al.* 2013a). Therefore, the determination of the infection state in one individuum is rather difficult.

3.2.4 Group composition and blinding

Animals were assigned to the different treatment groups at 30 h pi by an experienced researcher. To ensure the best possible quality of the study, groups were matched by age, weight, and clinical score at 30 h pi. For practical reasons, randomization was not always possible: To facilitate appropriate treatment, only animals that tolerated handling and venipuncture could be included in the rifampicin treated groups which included daily intravenous infusion over 30 min, and animals that were known to be “picky eaters” could not be included in the groups receiving oral medication.
The study can be regarded as partly blinded: The two veterinarians performing the treatments and monitoring the animals were the only persons who knew the assignment of the animals to the different treatment groups. Variables assessed *in vivo* (body temperature, respiratory rate, heart rate) can be regarded as ‘objective’ since they are measured values. An individual ID according to the rules of our institute’s quality management system was given to all specimens examined *ex vivo*. Lab investigators were therefore disabled to identify individual animals or treatment regimens.
Under the given circumstances, we believe that the compromises with regard to randomization (i.e., risk of problems with drug application and resulting subtherapeutic levels of antimicrobials) and complete blinding (i.e., increasing the number of people with access to the infection unit) in this particular study justify the decisions made regarding the group compositions and the degree of blinding.

3.3 Overall outcome of the treatment studies

3.3.1 Pathogen detection

Recultivation of *Chlamydiae* from material obtained by bronchial brushings showed convincingly that the chosen treatment regimens inhibited chlamydial growth, as more viable *Chlamydiae* were detected in samples from untreated than in samples from treated animals. Also, in STUDY 3 recultivation was possible in untreated animals more often at later time points, indicating a minimal duration of 3 days is necessary for the effectivity of antichlamydial treatment. However, we could not convincingly confirm an increased antichlamydial effect by the addition of rifampicin in all combination therapies. Reisolation of the living pathogen was possible to the same extent in animals treated with a single drug or the same drug in combination with rifampicin in STUDY 3. In STUDY 2 however, no living pathogen could be reisolated from animals receiving combination therapy, but from two animals in each of the doxycycline treated groups. This might suggest the conclusion that of all tested substances, only the antichlamydial effect of doxycycline can be enhanced by rifampicin *in vivo*, but both treatment regimens, monotherapy and combination therapy, led to negative recultivation results in all animals at later time points (i.e. 9 and 14 dpi). As we learned from STUDY 3, a minimal time of 3 days is necessary to reliably inhibit chlamydial growth from bronchial brushings, therefore the results from STUDY 2 on 4 dpi should not be overinterpreted.
Even though antimicrobial treatment could reduce the numbers of viable *Chlamydiae* isolated from the animals, in both STUDY 2 and STUDY 3, it did not have an influence on the numbers of genome copies detectable in tissue sampled at necropsy 14 dpi. The shedding and spreading of the pathogen as assessed by the presence of chlamydial DNA in various swabs and in venous blood was only detected very sporadically with no obvious differences between the different groups.

3.3.2 Host response

As described in detail in STUDY 2 and STUDY 3, none of evaluated treatment regimens had a measurable impact on the course and severity of the disease induced by inoculation of *C. psittaci*, as all 80 animals regained clinical health by the end of the study.
Furthermore, no differences between the treatment groups were detectable with respect to severity of local (i.e. blood count and LBP-levels in the blood) and systemic inflammation (i.e. BALF cell count and protein concentration in the BALF). Also, time needed for their remission did not indicate any beneficial effect of the applied antichlamydial treatment for the animals. The pathological outcome was assessed by the amount and histopathological characterization of altered lung tissue and did not differ between the groups. Statistically significant differences between the different groups in the clinical score, numbers of blood leukocytes and neutrophilic granulocytes occurred only very sporadically and were therefore not assumed to be of biological relevance. Possible explanations for this phenomenon are discussed in section 3.4.

3.3.3 Side effects and applicability of treatment

Subcutaneous injection of erythromycin resulted in painful swellings at the injection site. The swellings were palm sized and would last for several days. A more detailed description can be found in STUDY 3. Animals treated with erythromycin showed higher levels of LBP, which was believed to be due to the local inflammation caused by the treatment. We therefore do not recommend the subcutaneous application of erythromycin over longer time periods in calves. Application of rifampicin had to be strictly intravenous, even small amounts accidentally applied paravenously would lead to local swelling of the perivenous tissue.
Other possible side effects of antimicrobial treatment, such as diarrhea due to alterations of the intestinal microflora, were not observed during the studies.
Oral medication was the most gentle treatment form for the animals. Yet one has to keep in mind that under experimental settings there was enough personnel available to ensure intake of milk and medication in sick animals with reduced appetite, which might not be the case in a farm setting. If intake of the required amount of medication cannot be secured, the danger of subinhibitory antimicrobial concentrations arises, which can lead to antimicrobial resistance of bacteria. This would justify parenteral application to not jeopardize the success of treatment.

3.4 Reasons for the lacking impact of the treatment on the host

Concentrations of applied antimicrobial substances were determined in blood and tissue of all animals and were shown to reach sufficient concentrations to inhibit growth of the *Chlamydiae*. Furthermore it was known from previous *in vitro* studies that the used strain DC15 was sensitive to the substances utilized in the present studies (Wolf*, et al.* 2010). Antimicrobial resistance and subinhibitory concentrations of the applied antimicrobial substances could therefore be ruled out in the present treatment studies. The results from recultivation of the pathogen in STUDY 2 and STUDY 3 clearly showed that chlamydial growth was impaired in treated animals. Taken together, this leads to the conclusion that the chosen treatment regimens were sufficient for the treatment of the *C. psittaci*-infection. Nevertheless, we could not find differences between treated and untreated animals in any of the observed clinical and inflammatory parameters (see section 3.3.2). As discussed in detail in STUDY 2 and in STUDY 3, we assumed that the presence of clinical signs and changes in local and systemic inflammatory parameters were induced by the host's reaction to the inoculation with *C. psittaci,* and the extent of lung lesions was already determined when the treatment started. Every host reaction observed during the studies could therefore be attributed to the healing of the *Chlamydia*-induced lesions, without being significantly influenced by antimicrobial treatment. These results go in hand with reports on mild or subclinical human infections with *C. psittaci* (Heddema*, et al.* 2006; Harkinezhad*, et al.* 2007; Vanrompay*, et al.* 2007; Branley*, et al.* 2008; Verminnen*, et al.* 2008) and of asymptomatic *C. trachomatis* infections in women (Morre*, et al.* 2002; Molano*, et al.* 2005) that resolved clinically without antimicrobial treatment.

3.5 Persistence of *Chlamydiae*

Although so far it has not been proven, it is often hypothesized that antibiotic treatment induces persistence in *Chlamydiae* and allows them to survive in their host. This could explain reoccurring chlamydial infections after the end of treatment. However, in a clinical setting it is almost impossible to exclude reinfection as a reason for the presence of *Chlamydiae* after treatment. Nevertheless there is evidence for the presence of aberrant bodies - the morphologic correlate of persistence - *in vivo* (Borel*, et al.* 2008; Pospischil*, et al.* 2009), and recently a mouse model of an amoxicillin induced persistent *C. trachomatis* infection has been published (Phillips Campbell*, et al.* 2012). This led to the question whether or not antibiotic treatment of the *C. psittaci* infected calves could cause the *Chlamydiae* to enter the state of persistence. To address this aspect, lung samples of all 80 calves inoculated with *C. psittaci* were taken throughout the study and conserved in either glutaraldehyde or RNA stabilizing media. This allows the electronmicroscopical search for aberrant bodies as well as the analysis of transcriptional patterns typical for persistence (Goellner*, et al.* 2006). However, the investigation of this interesting aspect of antimicrobial treatment of chlamydial infections had to be deferred due to restricted availability of personnel. This remains to be subject of future research.

3.6 Limitations and chances of the use of the present model in treatment studies

A different approach would be the application of antibiotics before inoculation of the animals. This type of study design does play a role in the research on treatment options for bacterial infections in bovines (Sgoifo Rossi*, et al.* 2010; Baggott*, et al.* 2011; Forbes*, et al.* 2011). However, the rational is a different one than intended in the studies included in this thesis. Treatment prior to pathogen exposure mimics metaphylactic treatment, which is a common instrument in intensive lifestock farming to prevent healthy animals from infectious diseases present on the farm. This concept makes sense when dealing with pathogens that cause severe clinical illness, such as bovine respiratory disease associated with the bacterial pathogens *Mannheimia haemolytica* or *Pasteurella multocida* and/or *Mycoplasma bovis*. For chlamydial infections in cattle, this does not apply, as they primarily cause subclinical disease. Our treatment studies were designed to mimic the clinical setting of an acute infection with *C. psittaci*, as it may be present in human patients. In this scenario, the patient would seek medical advice when suffering from severe clinical settings, and at that point, a treatment would be initiated. That is the reason we chose 30-36 h pi as the start of antimicrobial treatment, at that time point obvious clinical signs became visible in all animals. We believe to have mimicked a very realistic scenario: an otherwise healthy individual, no matter if human or animal, suffers from an uncomplicated bacterial infection and receives antimicrobial treatment. A few days later, clinical symptoms resolve, which is of course attributed to the treatment.

Also, the housing conditions of the animals were very good (thorough daily cleaning of the stables, small group sizes, much effort that sick animals would drink their milk replacer, little external stress, careful handling) and not comparable to the situation in conventional farms. Co-infections were excluded, the animals were healthy prior to inoculation and the housing conditions prevented secondary infections, which represent a common source for complications in a natural setting. All these factors facilitate fast remission of the *C. psittaci*-induced disease. This valuable result points out the importance of good housing environmental conditions in the prevention and management of bacterial infections to maximize the hosts immune competence.

A possible alteration of the model would be to add immunosuppression to the animals, either by altering the environmental conditions or by application of glucocorticoids. Administration of dexamethasone to cattle is frequently used in experimental settings to induce immunosuppression (Anderson*, et al.* 1999; Narita*, et al.* 2003; Lomborg*, et al.* 2007; Sreerama*, et al.* 2008). Induced immunosuppression would impair the hosts ability to fight off the chlamydial infection as a study in birds could demonstrate (Takashima*, et al.* 1996). This approach could possibly show a difference between treated and untreated animals, and mimic conditions where individuals are especially susceptible to disease.

As previously discussed, the bovine lung is especially suited to deal with infectious processes by demarcating them and thereby preventing further dissemination of the pathogen in the lung (Reinhold 1997). In the present studies this was visible upon necropsy: lesions showed a focal character and affected only minor parts of the lung by 14 dpi. Perhaps in animal species with less connective tissue in the lung and collateral airways, the pathogen would spread more widely, better mimicking the situation in human patients. Examples would be horses, cats or dogs (McLaughlin*, et al.* 1961; Robinson 1982). However, the pathogenetic role and potential of *C. psittaci* in these species is not yet fully understood, and the lung dimensions differ significantly from the ones of the human lung. Foals or small ponies might be an exclusion in terms of lung dimensions, but they would bring significant disadvantages in handling compared to calves due to the peculiarities of their behavior.

4 Conclusions and recommendations for further research

The present study once more points out the relevance of large animal models and their advantages compared to wide spread murine models. We were able to study treatment options in a natural host of *C. psittaci* and could at the same time benefit from the possibility of sampling the animals repeatedly throughout the study, making intraindividual comparisons available for parameters of local and systemic inflammation, pathogen detection, and antibiotic levels.
With the introduction of the bronchoscopic sampling methods, the value and applicability of the bovine model of acute respiratory *C. psittaci* infection could be further increased. Now the lung as the primary infection site can be closely monitored and sampled repeatedly in the living animal. This model could be used in future projects to study the pathogenic potential of emerging pathogens, and to study host-pathogen interactions of known pathogens.
The present treatment studies have broadened our knowledge on the transferability of results from *in vitro* studies to the situation in the living host. The infection of cells *in vitro* obviously represents a very simplified model, disregarding the complexity of the natural host's reaction to the infection with the pathogen. The effectiveness of the calves' immune response masked the influence of the antibiotic treatment on the chlamydial growth. Possibly, the increase of the antichlamydial effect by the application of rifampicin observed *in vitro* was too small to play a visible role in the living host. However, due to the short length of the study, we could not draw any conclusions on the influence of the antimicrobial treatment on the total elimination of the pathogen. It would be an interesting question for further research to follow the calves for a longer time period after the end of treatment to see whether there are differences in long term presence of the *Chlamydiae.* Another burning question for further research in this particular model is the nature of the underlying mechanisms making the immune response of the calves to the inoculation with *C. psittaci* thus effective.

5 Summary

Background

Chlamydiae are obligate intracellular pathogens that can cause a variety of diseases in many different mammalian and avian hosts. The successful antimicrobial treatment of chlamydial infections is still an unresolved issue in both, human and veterinary medicine. The development of new antichlamydial treatment regimens is a subject of intensive research. Recently, it was found in a cell culture model of an acute and a persistent *Chlamydia (C.) psittaci* infection that the addition of rifampicin enhanced the antichlamydial effect of the widely used tetracyclines, macrolides and quinolones.

Aim and design of the project

The project was designed to evaluate regimens commonly used for the treatment of chlamydial infections (i.e., tetracyclines, macrolides and quinolones), and to compare them to the most promising treatment regimens that were identified *in vitro* (i.e., combination of the antimicrobial substances with rifampicin). As animal model we chose a recently developed bovine model of an acute respiratory *C. psittaci* infection, using the same strain (i.e., DC15) as in the cell culture studies. This particular animal model was chosen for the present project since it was well characterized in terms of clinical and pathological outcome and kinetics of various local and systemic inflammatory parameters had been well described previously. Furthermore, calves represent a natural host to *C. psittaci* and, up to 3-4 months of age, are comparable to adult humans in lung volume and body weight. This translational approach was expected to provide valuable results on the treatment of chlamydial infections in both, human and veterinary medicine.

In order to perform the treatment studies in the afore mentioned animal model, bronchoscopic sampling methods had to be adapted for the use in calves aged 6-10 weeks and for the use under experimental conditions in a biosafety level 2. The accurately defined methods of intrabronchial inoculation and endoscopic sampling of the animals during the studies was subject of STUDY 1. The treatment studies were divided into two separate trials, evaluating the treatment effects of (i) tetracyclines alone or in combination with rifampicin (STUDY 2), and (ii) of macrolides and quinolones alone or in combination with rifampicin (STUDY 3).

Animals, material and methods

For STUDY 1, four male Holstein-Friesian calves were repeatedly bronchoscoped under general anesthesia beginning at the age of 6 weeks. The following methods were evaluated: bronchial brushing, broncholaveolar lavage and transbronchial lung biopsy.

For STUDY 2 and STUDY 3, a total of 80 conventionally raised, male Holstein-Friesian calves were intrabronchially inoculated with 10^8 inclusion forming units *C. psittaci* strain DC15. At 30 h post inoculation (pi), the animals were assigned either to the untreated control groups (n=6 in STUDY 2 and n=7 in STUDY 3) or to one of the 11 different treatment groups:

Tetracyclines:

» 5 mg/kg/day **doxycycline** (n=6)
» 5 mg/kg/day **doxycycline** + 600 mg/day **rifampicin** (n=6)
» 10 mg/kg/day **doxycycline** (n=6)
» 10 mg/kg/day **doxycycline** + 600 mg/day **rifampicin** (n=6)

Quinolones:

» initially 7 mg/kg bw/day, then 5 mg/kg bw/day **enrofloxacin** (n=6)
» initially 7 mg/kg bw/day, then 5 mg/kg bw/day **enrofloxacin** + 600 mg/day **rifampicin** (n=6)

Macrolides

» initially 10 mg/kg bw/day, then 6mg/kg bw/day **azithromycin** (n=7)
» initially 10 mg/kg bw/day, then 6mg/kg bw/day **azithromycin** + 600 mg/day **rifampicin** (n=6)
» 12 mg/kg bw/day **erythromycin** (n=6)
» 12 mg/kg bw/day **erythromycin** + 600 mg/day **rifampicin** (n=6)

» 600 mg/day **rifampicin** (n=6).

Treatment with doxycycline (orally), enrofloxacin (subcutaneously) azithromycin (orally), and erythromycin (subcutaneously) was initiated with the first presence of obvious clinical signs at 30-36 h pi, whereas treatment with rifampicin (intravenously) started 48 h pi. All animals were clinically examined on a daily basis starting one week before inoculation until the end of the study. Results were summarized using a clinical scoring system. Venous blood was sampled 7 and 4 days and one hour before inoculation as well as 1, 2, 3, 5, 7, 10, and 14 days pi (dpi). On 4 and 9 dpi, bronchial brushings, bronchoalveolar lavage fluid (BALF) and pharyngeal swabs were sampled under general anesthesia. At the end of the study on 14 dpi, all animals were euthanized and necropsied and extent and character of lung lesions were assessed. BALF was sampled from the exenterated lung and tissue samples of lung, liver, muscle and mediastinal lymph node were obtained.
Levels of antimicrobial substances were determined in blood, lung, liver and muscle of all treated animals. The presence of the inoculated pathogen in treated and untreated animals was assessed by recultivation from bronchial brushings and by quantitative real-time PCR testing of blood, lung and mediastinal lymph node and swabs (i.e., nasal, conjunctival, pharyngeal, and rectal swabs). The hosts' reaction was characterized by the clinical scores and signs of local (i.e., BALF-cell count and protein concentration) and systemic inflammation (i.e., white blood cell count and concentrations of lipopolysaccharide-binding protein (LBP) in the blood).

Results

The intrabronchial inoculation with *C. psittaci* led to acute respiratory disease with fever in all animals that resolved until 10 dpi. The established protocol of obtaining bronchial brushings,

BALF and transbronchial lung biopsies proved suitable for the use under experimental conditions. It was possible to sample the required amount of 5 animals per day in an adequate time frame. All animals tolerated the sampling procedure very well.
Sufficient antibiotic levels were detected in in blood and tissue samples of all treated animals. Recultivation results revealed that viable *Chlamydiae* could more often be isolated from untreated than from treated animals. Single drug therapy inhibited chlamydial growth in the same extent as combination therapy with rifampicin. However, numbers of chlamydial transcripts in mediastinal lymph node, lung, blood, and swabs did not differ between treated and untreated animals. Clinical score, white blood cell count, LBP concentration in the blood, and BALF cell count revealed acute respiratory and systemic disease in all animals, but again, no differences were visible between treated and untreated animals. All 80 infected animals included in the project regained clinical health by the end of the study, regardless if they were treated or not.

Conclusion

The overall value and applicability of the bovine model of respiratory *C. psittaci* infection could be increased by the bronchoscopic sampling methods introduced in STUDY 1, since the *in vivo* monitoring of the lung as the infection site during the following treatment studies became possible. The results from STUDY 2 and STUDY 3 proved that the treatment of *C. psittaci*-infected calves with doxycycline, enrofloxacin, azithromycin and erythromycin alone or in combination with rifampicin was sufficient to reduce the number of viable pathogens isolated from the animals. Yet there was no obvious difference in this parameter between animals treated with monotherapy and animals receiving combination therapy with rifampicin. The results from the previous *in vitro* study that combined treatment with rifampicin was superior to single treatment with tetracyclines, macrolides and quinolones, could not be confirmed in the living host.
Our finding that chlamydial growth was inhibited by antimicrobial treatment was without any detectable consequence for the bovine host: There was no influence of any treatment regimen on severity of disease and time course of its resolution, as assessed by clinical and pathological findings as well as local and systemic inflammatory parameters. We can therefore conclude that in the present studies, a carefully selected and effective antimicrobial treatment of an acute bacterial infection was not superior compared to the host's immune defense mechanisms that were sufficient to cope with the infection. The present treatment studies show the need to properly evaluate the necessity for the antimicrobial treatment of bacterial infections in each case. In times of emotional discussions on the antimicrobial treatment of food producing animals this is a very valuable result.

6 Zusammenfassung

Evaluierung antimikrobieller Behandlungsstrategien gegen *Chlamydia psittaci* in einem bovinen respiratorischen Infektionsmodell

Einleitung

Chlamydien sind gram-negative Bakterien mit einer obligat intrazellulären Lebensweise. Als Pathogene können sie in eine Vielzahl unterschiedlichster Erkrankungen bei Säugern und Vögeln involviert sein. Antimikrobiellen Behandlungen chlamydialer Infektionen sind mit Tetrazyklinen, Chinolonen oder Makroliden beschrieben, allerdings sind die dokumentierten Effekte dieser antichlamydialen Therapieversuche oft nicht zufriedenstellend. Die Entwicklung neuer Behandlungsstrategien gegen Chlamydien-Infektionen ist demzufolge aktueller Gegenstand von intensiven Forschungsarbeiten. *In vitro* etablierte Zellkulturmodelle von akuten und persistenten Infektionen mit *Chlamydia (C.) psittaci* führten zu dem hoffnungsvollen Ergebnis, dass der Zusatz von Rifampicin (zur Stoffgruppe der Ansamycine gehörend) die antichlamydialen Effekte der zuvor genannten konventionell eingesetzten Wirkstoffgruppen steigert. Hieraus wurde folgende These abgeleitet:
Die Kombinationstherapie, bestehend aus einem der o.g. herkömmlichen Antibiotika und Rifampicin, ist zur effektiven Bekämpfung von chlamydialen Infektionen besser geeignet als die entsprechende Monotherapie.

Ziel und Projektdesign

Das Ziel dieses Projektes war es, die aus *In vitro*-Experimenten abgeleitete Hypothese zur Kombinationstherapie chlamydialer Infektionen in einem geeigneten Tiermodell auf ihre Validität *in vivo* zu testen. Hierfür kam das zuvor etablierte bovine Modell der akuten respiratorischen chlamydialen Infektion zum Einsatz. Die Relevanz dieses Modells begründet sich daraus, dass (i) Kälber natürliche Wirte von *C. psittaci* darstellen und (ii) derselbe *C. psittaci*-Infektionsstamm (DC15) verwendet wurde wie in den eingangs erwähnten *In vitro*-Studien. Das Tiermodell war hinsichtlich des klinischen und pathologischen Bildes sowie des zeitlichen Verlaufs der induzierten Erkrankung hinreichend charakterisiert und bildete somit eine solide Basis für die Durchführung von Therapiestudien. Um die zur Evaluierung der geplanten Therapiestudien als notwendig erachteten Proben *in vivo* aus den Lungen der zuvor infizierten Kälber gewinnen zu können, mussten zunächst diverse bronchoskopische Methoden an 6-10 Wochen alte Kälber unter S2-Bedingungen adaptiert werden (STUDIE 1). Die sich anschließenden Therapiestudien erfolgten in zwei separaten Abschnitten. In STUDIE 2 wurden die Behandlungseffekte von Tetrazyklinen, entweder allein oder in Kombination mit Rifampicin, untersucht. STUDIE 3 diente zur Dokumentation der Behandlungseffekte von Chinolonen und Makroliden, entweder als Monotherapie oder in jeweiliger Kombination mit Rifampicin.

Tiere, Studiendesign, Material und Methoden

Für STUDIE 1 wurden vier Kälber (männlich, Rasse: Holstein-Friesian, Alter: 6 Wochen) innerhalb von zwei Monaten bis zu viermal je Tier unter Allgemeinanästhesie bronchoskopiert,

um die Gewinnung von broncho-alveolärer Lavageflüssigkeit (BALF), bronchialen Bürstenabstrichen und transbronchialen Lungenbiopsien einzuarbeiten und zu evaluieren.

Für STUDIE 2 und STUDIE 3 wurden insgesamt 80 konventionell aufgezogene Kälber (männlich, Rasse: Holstein-Friesian) intrabronchial mit 10^8 einschlussbildenden Einheiten von *C. psittaci* (Stamm DC15) pro Tier inokuliert. Dreißig Stunden nach Inokulation (pi) wurden die Tiere entweder der unbehandelten Kontrollgruppe (n=6 in STUDIE 2 und n=7 in STUDIE 3) oder einer der folgenden elf Behandlungsgruppen zugeteilt:

Tetrazykline:

» 5 mg/kg/Tag **Doxyzyklin** (n=6)
» 5 mg/kg/Tag **Doxyzyklin** + 600 mg/Tag **Rifampicin**(n=6)
» 10 mg/kg/Tag **Doxyzyklin** (n=6)
» 10 mg/kg/Tag **Doxyzyklin** + 600 mg/Tag **Rifampicin** (n=6)

Chinolone:

» initial 7 mg/kg/Tag, dann 5 mg/kg bw/Tag **Enrofloxacin** (n=6)
» initial 7 mg/kg/Tag, dann 5 mg/kg bw/Tag **Enrofloxacin** + 600 mg/Tag **Rifampicin** (n=6)

Makrolide

» initial 10 mg/kg/Tag, dann 6 mg/kg bw/Tag **Azithromycin** (n=7)
» initial 10 mg/kg/Tag, dann 6 mg/kg bw/Tag **Azithromycin** + 600 mg/Tag **Rifampicin** (n=6)
» 12 mg/kg/Tag **Erythromycin** (n=6)
» 12 mg/kg/Tag **Erythromycin** + 600 mg/Tag **Rifampicin** (n=6)
» 600 mg/Tag **Rifampicin** (n=6).

Die Behandlung mit Doxyzyklin (oral), Enrofloxacin (subkutan), Azithromycin (oral) bzw. Erythromycin (subkutan) begann jeweils zum Zeitpunkt 30-36 Stunden pi, d.h. als erste klinische Symptome bei allen Tieren sichtbar wurden. Die intravenöse Behandlung mit Rifampicin begann 48 Stunden pi. Alle antimikrobiellen Behandlungen wurden bis 13 Tage pi (dpi) fortgesetzt. Zu Versuchsende (14 dpi) wurden alle Tiere der Sektion zugeführt.

Als Read-out Parameter zur Dokumentation der Behandlungserfolge dienten:

» Befunde des täglich durchgeführten klinischen Untersuchungsganges,
» Anzucht des Erregers aus bronchialen Bürstenabstrichen (4 und 9 dpi) und Lungengewebe (14 dpi),
» Nachweis von chlamydialer DNA mittels quantitativer real time PCR in Rachentupfern (4 und 9 dpi), Kottupfern (4, 9 und 14 dpi) sowie Lungen- und Lymphknotengewebe (14 dpi),

- » Leukozytenzahl, Differentialblutbild und Konzentration von Lipopolysaccharid bindendem Protein (LBP) im venösen Blut (7 und 4 Tage und eine Stunde vor Inokulation sowie 1, 2, 3, 5, 7, 10 und 14 dpi),
- » Gesamtzellzahl, Differentialzellbild und Proteinkonzentration in der BALF (4, 9 und14 dpi),
- » Ausmaß und Charakter der Lungenveränderungen (14 dpi) und
- » Konzentration der applizierten antimikrobiellen Wirkstoffe im Blut (vor Inokulation sowie 3, 5, 7, 10 und 14 dpi) und im Lungen-, Muskel- und Lebergewebe (14 dpi).

Ergebnisse

STUDIE 1: Das erarbeitete Protokoll zur Gewinnung von BALF, bronchialen Bürstenabstrichen und transbronchialen Lungenbiopsien unter Allgemeinanästhesie erwies sich unter den gegebenen experimentellen Umständen als praktikabel. Im Versuchsdesign der STUDIEN 2 & 3 war es problemlos möglich, 5 Kälber pro Tag in einem angemessenen Zeitrahmen von ca. 30 min pro Tier zu beproben. Die Prozedur wurde von allen Tieren gut toleriert. Unerwünschte Neben- oder Nachwirkungen wurden nicht beobachtet. Die BALF-Proben erwiesen sich als geeignet, um lokale Entzündungsreaktionen in der Lunge zu charakterisieren. Bronchiale Bürstenbiopsien dienten der kulturellen Anzucht von *C. psittaci* im Sinne der Re-Isolation des Erregers. Die Lungenbiopsien wurden für spätere Untersuchungen im Rahmen eines weiterführenden Projektes zurückgestellt.

STUDIEN 2 & 3: Die intrabronchiale Inokulation mit *C. psittaci* führte bei allen Tieren zu Symptomen einer fieberhaften akuten respiratorischen Erkrankung, welche ihren Höhepunkt um den Tag 3 dpi erreichte und innerhalb von 10 Tagen abklang. Bei den behandelten Tieren wurden ausnahmslos therapeutisch wirksame Konzentrationen der entsprechenden Wirkstoffe in Blut und Gewebe nachgewiesen. Die Re-Isolation des inokulierten Pathogens *C. psittaci* gelang häufiger aus unbehandelten Tieren als aus behandelten – jedoch ohne signifikanten Einfluss des angewandten Therapieschemas. Die Anzahl chlamydialer Transkripte in Gewebe- und Tupferproben unterschied sich zu keinem Zeitpunkt signifikant zwischen behandelten und unbehandelten Tieren. Parallel zum klinischen Verlauf der Erkrankung dokumentierte Veränderungen im Differentialzellbild der Blut- und der BALF-Proben belegten das Vorhandensein einer akuten inflammatorischen Reaktion unter maßgeblicher Beteiligung neutrophiler Granulozyten. Weder diese zytologischen Marker noch die während der akuten Erkrankung signifikant erhöhten LBP-Konzentrationen im Blut der Kälber unterschieden sich bezüglich ihrer absoluten Veränderungen oder ihres zeitlichen Verlaufes zwischen den verschiedenen Tier- bzw. Behandlungsgruppen. Die mit Versuchsende dokumentierten Lungenveränderungen zeigten weder in ihrem Charakter noch in ihrem Ausmaß eindeutige Unterschiede zwischen den antimikrobiell behandelten Tieren und den unbehandelten Kontrolltieren.

Schlussfolgerungen

Die in STUDIE 1 evaluierten bronchoskopischen Techniken zur Probengewinnung aus der bovinen Lunge haben die Anwendungsmöglichkeiten des Modells der respiratorischen *C. psittaci* Infektion im Sinne der translationalen Forschung erheblich erweitert.

STUDIEN 2 & 3 zeigten, dass eine Behandlung von *C. psittaci* infizierten Kälbern mit Tetrazyklinen, Chinolonen oder Makroliden allein oder in Kombination mit Rifampicin zwar geeignet war, die Anzahl isolierbarer lebender Erreger im Wirt zu reduzieren, jedoch gab es hierbei keinen signifikanten Unterschied zwischen Mono- und Kombinationstherapie. Demzufolge konnte die aus *In vitro*-Studien abgeleitete Hypothese, dass eine Kombinationstherapie mit Rifampicin der jeweiligen Monotherapie überlegen sei, *in vivo* nicht bestätigt werden.

Keines der antimikrobiellen Behandlungsregimes hatte einen signifikanten Einfluss auf den klinischen Schweregrad oder die Dauer der Erkrankung. Die Tatsache, dass auch die unbehandelten Kontrolltiere bis 10-14 dpi wieder klinisch unauffällig waren und zu Versuchsende keine höhere Anzahl an chlamydialen Transkripten im Lungengewebe aufwiesen als die antimikrobiell behandelten Kälber, spricht dafür, dass das Immunsystem des Wirtes in der Lage war, die experimentell induzierte akute Infektion in geeigneter Weise zu bekämpfen. Im Kontext der aktuellen Diskussionen über den Antibiotika-Einsatz bei lebensmittelliefernden Tieren verdeutlicht diese Beobachtung, dass die Notwendigkeit einer antimikrobiellen Behandlung in jedem Fall sorgfältig abzuwägen ist.

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8 List of abbreviations

AB .....abberant bodies
ATP .....adenosine triphosphate
Az .....Azithromycin
BAL .....bronchoalveolar lavage
BALF .....bronchoalveolar lavege fluid
BGM .....buffalo green monkey kidney
BHV-1 .....bovine herpes virus 1
BMBF .....Bundesministerium für Bildung und Forschung
BRSV ..... bovine respiratory syncytial virus
BVDV .....bovine virus diarrhea/mucosal disease virus
C. .....*Chlamydia*
D .....doxycycline
D_{10} .....10 mg/kg bodyweight/day doxycycline
D_{10}R .....10 mg/kg bodyweight/day doxycycline + 600 mg rifampicin/day
D_5 .....5 mg/kg bodyweight/day doxycycline
D_5R .....5 mg/kg bodyweight/day doxycycline + 600 mg rifampicin/day
dpi .....days past inoculation
EB .....elementary bodies
En .....Enrofloxacin
Ery .....Erythromycin
ESI+ .....positive electrospray ionization
HctA .....histone like protein A
HctB .....histone like protein B
IFNγ .....interferon γ
ifu .....inclusion forming units
IHC .....immunohistochemistry
LBP ..... lipopolysaccharide-binding protein
Lu .....macroscopically unaltered lung
LuLes ..... inflamed lung
Med Ln .....mediastinal lymph node
MIC .....minimal inhibitory concentration
Omc .....outer membrane complex protein
OSPE .....online solid phase extraction
PCR .....polymerase chain reaction
pi .....past inoculation
PI-3 .....parainfluenza 3 virus
PVDF .....polyvinylidene fluoride
qrtPCR.....quantitative real-time PCR
R .....Rifampicin
RB .....reticulate bodies
SD .....standard deviation
SPGA .....containing sucrose, phosphatile substances, glucose, and bovine albumin
TTSS .....Type III secretion system

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11.1 Publications in peer reviewed journals

Prohl A, Ostermann C, Lohr M & Reinhold P (2014) The bovine lung in biomedical research: visually guided bronchoscopy, intrabronchial inoculation and in vivo sampling techniques. Journal of Visualized Experiments 89: e51557.

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11.2 Published abstracts

Annette Prohl, Carola Ostermann, Markus Lohr, Angela Berndt, Konrad Sachse, Petra Reinhold ***Introduction of in vivo techniques to evaluate the effects of antimicrobial treatment of Chlamydia psittaci infections in calves.*** (Abstract)
Proceedings of the National Symposium on Zoonoses Research 2012, October 11-12 2012, Berlin, 193

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Bedeutung von ‚Chlamydia-like organisms' als Erreger – Bovines Modell für Parachlamydia acanthamoebae. (Abstract)
6. Arbeitstagung des Nationalen Referenzlabors für Chlamydiose, In: Proceedings of the 2nd European Meeting on Animal Chlamydioses and Zoonotic Implications (EMAC-2), Jena (Germany), 13.-14. June 2013, 101

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Eur Respir J 2014; 44: Suppl. 58, P2539

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13 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 28.4.2015 Annette Prohl